Chapter 1

Introduction and Literature Review
1.1 INTRODUCTION

* Duboisia myoporoides * R. Br. is a medicinal plant of the Solanaceae family. It contains different groups of alkaloids; for example, the pyridine and tropane alkaloids (Endo and Yamada, 1985; Kitamura et al., 1985 a; Gritsanapan and Griffin, 1991), the α-alkylpiperidine and the quinolizidine alkaloids (Bachmann et al., 1989). Among these, the tropane alkaloids; hyoscyamine and scopolamine are medicinally important (Evans, 1990; Hashimoto and Yamada, 1992). In commercial production, the leaves of the mature plants are harvested continually throughout the year, dried, coarsely powdered and exported to different countries (Kitamura et al., 1991).

To overcome the dependency of commercial production on the natural plant, plant tissue culture (PTC) has been tried for large-scale production of tropane alkaloids. Tropane alkaloid production by plant cell and organ culture of different plant species such as *Atropa belladonna* (Subroto et al., 1995), *Hyoscyamus niger* (Strauss, 1989), *H. muticus* L (Basu and Chand, 1998) and *Duboisia myoporoides* (Endo and Yamada, 1985) has been reported but not yet commercialized.

Using plant cell and tissue culture techniques, production of medicinally important alkaloids depends upon the knowledge of their biosynthesis in the whole plant. Investigation of different *Duboisia* species provided information on biosynthesis, translocation and accumulation of alkaloids. In the complete plant, biosynthesis of the alkaloids takes place mainly in the root cells as initially suggested by reciprocal grafting experiments between alkaloid-producing and non-producing plants (Waller and Nowacki, 1978). The established tropane alkaloid biosynthetic pathway showed that nicotine forms in the early stages of biosynthesis while hyoscyamine and scopolamine formation take place in the last two steps. These two steps are catalysed by the enzyme, hyoscyamine 6β-hydroxylase (H6H) (Hashimoto and Yamada, 1994). From immunohistochemical studies using monoclonal antibody and immunogold-silver enhancement Hashimoto et al., (1991) localized H6H in the...
pericycle cells of the young root in several scopolamine-producing plants. Studies on the cultured tissues clarified that conversion of hyoscyamine to scopolamine takes place in the shoots as well as in the roots (Yamada and Endo, 1984).

After biosynthesis in the root, hyoscyamine is translocated and accumulated in the aerial parts (James, 1950; Wink, 1987; Hashimoto and Yamada, 1992). Translocation of tropane alkaloids takes place via xylem cells (James, 1950; Wink, 1987; Hashimoto and Yamada, 1992). For pyridine alkaloids, translocation is similar to that of tropane alkaloids (Kitamura et al., 1993). However, tropane alkaloids have been found in the roots as well as in the aerial parts whereas pyridine alkaloids are mainly found in the roots (Yamada and Endo, 1984). Different analytical techniques have been used for analysing tropane and pyridine alkaloids present in the different plant organs (Tabata et al., 1972; Eapen et al., 1978; Yukimune et al., 1994 d; Mahagamasekera and Doran, 1998; Naqvi et al., 1998).

Because of its medicinal importance, a number of research studies have been conducted on D. myoporoides. Tissue culture techniques have been used for regenerating complete plants (Kitamura et al., 1980; Kukreja et al., 1986), untransformed root culture (Endo and Yamada, 1985); transformed shoot (Subroto et al., 1995) and root culture (Deno et al., 1987) and co-culture of genetically transformed roots and shoots (Subroto et al., 1996 a). These techniques were mainly tested for large-scale biomass production as well as to elevate levels of scopolamine. These techniques however, are more complex and time consuming than untransformed shoot culture technique, which is the focus of my study. If it were possible to produce alkaloids efficiently and easily from shoot culture, the commercial tropane alkaloid production could be revolutionized.

In tropane alkaloid-producing plant species, organization of root structure is necessary for biosynthesis of alkaloids (Bhandary et al., 1969). Nicotine biosynthesis is possible in cultured shoots but shoots are not suitable for tropane alkaloid biosynthesis without root initiation (Kitamura et al., 1985 a). However, untransformed and transformed shoot cultures of different Duboisia species have the ability to transform hyoscyamine to scopolamine without root formation (Hashimoto and Yamada, 1989; Mahagamasekera and Doran, 1998). These reported results were
mainly explained on the basis of gene expression for the enzymes of the tropane alkaloid biosynthetic pathway (Yamada and Endo, 1984; Yun et al., 1992). No explanation on the basis of the cell organization in the tissues and organs has previously been reported. A literature survey was carried out to study organogenesis, differentiation and alkaloid localization in *D. myoporoides* R. Br.
1.2 LITERATURE REVIEW

1.2.1 Plants As a Source of Chemicals

Plant life is essential for human life. It maintains the delicate balance of pure air supply and supplies food, cloth and timber. Together with these, it has also been used as a source of chemicals for centuries. Among these the source of chemicals is a major field of interest for scientists. A wide variety of chemicals, which are important in healthcare and used as medicinals, agrochemicals and fine chemicals are only obtained from plants. Stimulants, narcotics and poisons are considered as medicinals.

At present there are approximately 2x10^4 known plant-derived chemicals with an annual rate of about 1.6x10^3 new additions (Fowler and Stepan-Sarkissian, 1983). About 10% of an estimated 7.5x10^5 species of higher plants have been surveyed for biological activity (Floers, 1987). In spite of advances in synthetic organic chemistry, plants are the major source of these chemicals. Table 1.1 lists some secondary metabolites from different plants, their main products and the use of those chemicals for humankind (Fransworth, 1988; Hamil and Rhodes, 1993).

These plant-derived chemicals represent multibillion dollar industries; for example, an insecticide derived from *Azadirachta indica* the antitumor alkaloids vinblastine and vincristine found in the periwinkle (*Catharanthus roseus*) (Flores, 1987). The latter are the most effective chemical therapeutic agents for various forms of leukaemia and have a retail price of over $ 6000 g^-1 (Flores, 1987). Although the amount of synthetically produced drugs has increased during the past 10 decades; more than 25% of all prescriptions dispensed from pharmacies still contain one or more active ingredients of natural origin as the main compound. The plant-derived drugs and intermediates account for approximately US $ 9-11 billion annually in the US market alone (Heinstein 1986; Principe, 1989). According to an
### TABLE 1.1 Secondary metabolites from plants (source in text)

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Plant source (Family and Genus)</th>
<th>Application</th>
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<tbody>
<tr>
<td><strong>Pharmaceuticals</strong></td>
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<tr>
<td>Ajmalicine</td>
<td><em>Rauwolfia serpentina</em> (L.) Benth. Ex Kurz (Apocynaceae) (Indian snakeroot)</td>
<td>Circulatory stimulant</td>
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<tr>
<td>Bromelain*</td>
<td><em>Ananas comosus</em> (L.) Merrill (Bromeliaceae) (Pineapple)</td>
<td>Antiinflammatory;</td>
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<tr>
<td>Camphor*</td>
<td><em>Cinnamomum camphora</em> (L.) Nees &amp; Eberm (Lauraceae) (Camphor tree)</td>
<td>Protolytic;</td>
</tr>
<tr>
<td>Castanospermine</td>
<td><em>Castanospermum</em></td>
<td>Rubefacient</td>
</tr>
<tr>
<td>Cocaine*</td>
<td><em>Erythroxylum coca</em> Lam.</td>
<td>Antiviral, Anti-HIV**</td>
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<td></td>
<td>(Erythroxylaceae) (Coca)</td>
<td>Local anesthetic</td>
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<tr>
<td>Hyoscyamine*</td>
<td><em>Daboisla myoporoides</em> R.Br. (Solanaceae) (Australian cork tree)</td>
<td>Anticholinergic</td>
</tr>
<tr>
<td>Reserpine*</td>
<td><em>Rauwolfia serpentina</em> (L.) Benth. Ex Kurz (Apocynaceae) (Indian snakeroot)</td>
<td>Antihypertensive;</td>
</tr>
<tr>
<td>Saponin, Polyacetylenes</td>
<td><em>Panax</em></td>
<td>Tranquilizer</td>
</tr>
<tr>
<td>Scopolamine*</td>
<td><em>Datura metel</em> L. (Solanaceae) (Recurved thorapple)</td>
<td>Circulatory stimulants</td>
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<td></td>
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<td>Sedative</td>
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<tr>
<td>Vinblastine*</td>
<td><em>Catharanthus roseus</em> (L.) G. Don (Apocynaceae) (Madagascaran perwinkli)</td>
<td>Antitumor agent</td>
</tr>
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<td><strong>Agrochemicals</strong></td>
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<td>Thioiphenes</td>
<td><em>Tagetes</em></td>
<td>Biocides, Antinematode Insecticides</td>
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<td>Pyrethrins</td>
<td><em>Pyrethrum</em></td>
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<td><strong>Foods, Flavours, Fragrances and Dyes</strong></td>
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<td>Betacyanine</td>
<td><em>Beta</em></td>
<td>Food colorant</td>
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<tr>
<td>Quinine, Quassin</td>
<td><em>Cinchona, Quassia</em></td>
<td>Food bittering agent</td>
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<td>Essential oils (Mint, Lavender, Rose, Jasmine)</td>
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<td>Perfumes and Food Flavouring</td>
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<tr>
<td>Vanillin, Capsaicin</td>
<td><em>Jasmin</em></td>
<td>Food Flavouring</td>
</tr>
<tr>
<td>Shikonin</td>
<td><em>Vanilla, Capsicum</em></td>
<td>Dye</td>
</tr>
<tr>
<td><strong>Stimulants and Hallucinogens</strong></td>
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<tr>
<td>Caffeine, Nicotine, Theophtline</td>
<td>*Coffee, Nicotiana, Thea</td>
<td>Stimulants</td>
</tr>
<tr>
<td>Cocaine, Cannabinol</td>
<td>*Erythroxylum, Cannabis</td>
<td>Hallucinogens</td>
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*: Currently used in the United States; **: Trials being undertaken.
OECD report, the amount of pharmaceuticals based on natural products will increase to 40% by the end of this century (Principe, 1989). The total value of current plant-derived pharmaceuticals in OECD countries up to the year 2000 will be in the range of US $ 400-600 billion (Oksman-Caldentey and Hiltunen, 1996).

Now-a-days, these plant-derived chemicals are obtained by direct extraction from plants growing in a plantation or from their original habitats (Griffin, 1985). Several factors alter the yield of these economically important products. The quantity of the raw materials varies widely due to the following reasons; 1) some plants need several years to grow before they are ready for harvesting, 2) lack of proper post-harvest procedures which are essential to preserve the active compounds until their extraction, 3) limitation in the control of pests and diseases of these plants (Loyola-Vargas and Miranda-Ham, 1995) and 4) the availability of raw materials in tropical or subtropical, geographically remote areas. Therefore, supply depends largely on the political stability and local government regulations of the country of origin. Whatever the factors are, it is necessary to study and understand the chemical potential of these plants before they lost forever.

In plants, these chemicals are usually present in small amounts. To get a few grams of the desired compound, a large volume of raw material is necessary. To fulfil the high demand of these plant-derived chemicals, it is necessary to harvest the raw materials throughout the year, which may result in the extinction of the plant species and finally to loss of the chemical forever. To overcome this problem and to get a continuous supply of these plant-derived products, development of an alternative method to the whole plant extraction was found to be important. Plant cell and tissue culture systems may provide a competitive plant-derived chemical production system.

Using plant cell and tissue culture technique, production of plant-derived chemicals depends upon the knowledge of their biosynthesis in the whole plant. In the whole plant, biosynthesis of these chemicals takes place by 'secondary metabolism'.
1.2.2 Secondary Metabolism

Two different types of metabolic pathways are available in higher plants. Well organised enzyme-catalysed reactions form metabolic pathways, which include a number involved in synthesis (anabolism) and utilisation (catabolism) of large molecules including sugars, amino acids, common fatty acids, nucleotides etc. This catabolic and anabolic process are known as 'primary metabolism' and the compounds involved which are essential for the plant are called 'primary metabolites'. Plants also utilise other metabolic pathways producing different types of compounds; these compounds are secondary metabolites and the pathways of synthesis and utilization of these compounds constitute 'secondary metabolism' (Paech, 1950) (cited in Luckner et al., 1977). A few key intermediates in primary metabolism supply the precursors for most secondary metabolites. Fig. 1.1 shows some key intermediates from which primary and secondary metabolite biosynthesis take place.

Text-Figure 1.1 Key intermediates from which primary and secondary metabolite biosynthesis take place (Payne et al., 1991)
In plants, secondary metabolic pathways are often restricted to an individual species or genus and might only be activated during particular stages of growth and development or during periods of stress caused by the attack of microorganisms or the limitation of nutrients. It has also been suggested that the lack of mechanism for true excretion in higher plants may result in the accumulation of waste products in the vacuoles, cell walls or in special excretory cells or spaces (Luckner et al., 1976).

Plants produce more than $8 \times 10^4$ different classes of compounds through their secondary metabolic pathways (Loyola-Vargas and Miranda-Ham, 1995). About 20% of all known secondary metabolites are classified as alkaloids (Verpoorte et al. 1993). This is the most important class of compound that exert major pharmacological effects in the human body (Papadoyannis, 1994); for example, morphine, codeine, atropine, scopolamine, quinine etc.

1.2.3 Alkaloids

Alkaloids are natural plant compounds containing at least one nitrogen atom in a heterocyclic ring. Although alkaloids have been found in marine organisms (Fenical, 1986), in a number of arthropods (Jones and Blum, 1983) and in many brightly coloured neotropical frogs (Daley and Spande, 1986), they are considered mainly to be plant products (Luckner, 1972). In the early 19th century, the first alkaloid was isolated in Europe. About 50 years later, in a German laboratory, Zeyer (1861) (cited in Bick, 1996) isolated the first alkaloid, atherospermine from an Australian plant, the Tasmanian sassafras *Atherosperma moschatum* of the Monimiaceae family (Bick et al. 1956) (cited in Bick, 1996).

At present the chemical structures of about $10^5$ alkaloids are known (Drager et al. 1993). Their structures have been characterised by elemental analysis, NMR and IR (Papadoyannis, 1994). All of these alkaloids are not yet tested for their useful pharmacological properties. For example, *Apocyaneae* contains a significant amount of alkaloids but they have not been studied for their pharmacological properties (Bick, 1996). About 30 alkaloids are used therapeutically and are of high
value. They cover a broad spectrum of pharmacological effects. Table 1.2 lists some alkaloids with their pharmacological activities. Only a few of these 30 alkaloids are now produced by chemical synthesis; for example, quinine.

Alkaloids have been isolated from different plant families (Bick, 1996). In a particular plant family, the same alkaloid is sometimes found in different plant species but in varying amounts. For example, the tropane alkaloids; hyoscyamine and scopolamine are present in different plant species such as *Atropa belladon* (Deadly nightshade) (Bhandary et al., 1969), *Datura stramonium* (Jimsonweed) (Maldonado-Mendoza et al., 1993), *Hyoscyamus niger* (Blake henbane) (Dhoot and Henshaw, 1977) and *Duboisia* (Kitamura et al., 1985 a) of the *Solanaceae* plant family. *D. myoporoides* is cultivated in Australia for its high scopolamine content (Griffin, 1985). On the other hand, different alkaloids may be present in the same plant species. For example, nicotine, a pyridine alkaloid, and hyoscyamine, a tropane alkaloid, are present in *D. myoporoides* (Kitamura et al., 1985 a).

### 1.2.4 *Duboisia myoporoides* R. Br.

*Duboisia myoporoides* R. Br. is indigenous to Australia. It belongs to the order Tubiflorae, family *Solanaceae* (Cromwell, 1955). There are 3 species of *Duboisia*; *D. myoporoides* R.Br., *D. hopwoodii* F. Muell. and *D. leichhardtii* F. Muell. In recent years, a hybrid of *D. leichhardtii* x *D. myoporoides* has been made. Because of its high scopolamine content, *D. myoporoides* has been used as a major commercial source of tropane alkaloids for over 40 years but now has been replaced by the *Duboisia* hybrid (Griffin, 1985). The *Duboisia* hybrid contains more than 2% scopolamine on a dry weight basis (Griffin, 1985). A plantation of *D. myoporoides* and *Duboisia* hybrid in south-east Queensland has more than 4.5x10⁴ trees on a 6x10³ acres. The leaves are harvested more or less continually over the year. The dried, coarsely powdered leaves are exported to Japan, Germany and Switzerland (Kitamura et al. 1991).

*D. myoporoides* also known as “Corkwood tree”, is a tree with broad-lanceolate to obovate glabrous leaves 6-9cm long. The bark of the trunk is usually
<table>
<thead>
<tr>
<th>Pharmacological Activity</th>
<th>Alkaloid</th>
</tr>
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<tbody>
<tr>
<td><strong>Central Nervous System</strong></td>
<td>Reserpine, Caffeine</td>
</tr>
<tr>
<td><strong>Autonomic Nervous System</strong></td>
<td></td>
</tr>
<tr>
<td>Cholinergic</td>
<td>Phystostigmine, Pilocarpine</td>
</tr>
<tr>
<td>Cholinergic blocking</td>
<td>Atropine</td>
</tr>
<tr>
<td>Adrenergic</td>
<td>Ephedrine</td>
</tr>
<tr>
<td>Ganglion blocking</td>
<td>Nicotine</td>
</tr>
<tr>
<td><strong>Chemotherapeutic</strong></td>
<td></td>
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<tr>
<td>Anticancer</td>
<td>Vinblastine, Vincristine, Harringtonine</td>
</tr>
<tr>
<td>Antiamoebic</td>
<td>Emetine</td>
</tr>
<tr>
<td>Antimalarial</td>
<td>Quinine</td>
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<tr>
<td>Antibacterial</td>
<td>Berberine</td>
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<tr>
<td><strong>Cardiovascular</strong></td>
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<tr>
<td>Vasodilator</td>
<td>Papaverine, Theophylline, Vincamine</td>
</tr>
<tr>
<td>Hypotensive</td>
<td>Reserpine, Rescinnamine</td>
</tr>
<tr>
<td>Antiarrythmic</td>
<td>Quinidine, Ajmaline</td>
</tr>
<tr>
<td><strong>Analgesic</strong></td>
<td>Morphine, Codeine</td>
</tr>
<tr>
<td><strong>Antitussive</strong></td>
<td>Glaucine, Noscapine</td>
</tr>
<tr>
<td><strong>Anti-inflammatory</strong></td>
<td>Colchicine</td>
</tr>
<tr>
<td><strong>Muscle Relaxant</strong></td>
<td>Tubocurarine</td>
</tr>
<tr>
<td><strong>Local Anaesthetic</strong></td>
<td>Cocaine</td>
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</table>
very corky and the wood extremely light. It flowers profusely in spring; the blossoms are small, white and bell-shaped with occasional mauve streaking in the throat of the corolla, and are borne in cymose panicles. The fruit is a small black globular berry about 5mm in diameter (Hurst, 1942).

* D. myoporoides * occurs along the coast of Australia from latitude 35° in the south to at least latitude 15° in the north. In the extreme south, it is restricted to the coastal plain, but in the more northern districts, it extends into the valleys of the Great Dividing Range (Fig. 1.2) (Barnard, 1952). Early investigations revealed that different populations of * D. myoporoides * vary in their alkaloid properties. Scopolamine is the main alkaloid in samples from the north of Gosford, N. S. W., and hyoscyamine is the major alkaloid in samples from the south of Gosford. A third variety, collected from Acacia plateau near Killarney, Queensland, contains nicotine and anabasine as the major alkaloids (Griffin, 1985). Figure 1.3 shows the occurrence of chemical varieties of * D. myoporoides * and their major alkaloids.

During the 1940’s some investigations on * D. myoporoides * were carried out. Hills *et al.*, (1946) conducted grafting experiments in which tobacco and tomato scions were grafted to a rootstock of * D. myoporoides * and they concluded that the alkaloids or their precursors were formed in the root. Trautner and Roberts (1948) reported improved method for assay of the alkaloids present in this plant.

1.2.4.1 Early History

In 1802, Robert Brown, a naturalist to the Flinders expedition discovered * D. myoporoides * and he named the genus after the English botanist Charles du Bois. The specific name was given because of the morphological similarity to * Myoporum * species (Griffin, 1985). During the first quarter of the 19th century, C. Fraser, the first colonial Botanist and Superintendent of the Sydney Botanical Garden collected it at Pt. Macquarie (Barnard, 1952). The only * Duboisia * known outside Australia is a form of * D. myoporoides * found in New Caledonia and possibly occurs in New Guinea (Barnard, 1952).
Text-Figure 1.2 Distribution of *Duboisia* in Australia. *D. myoporoides* is restricted to the eastern coastline; *D. Leichhardtii* to a small area in southeastern Queensland and *D. Hopwoodii* is sparsely scattered over a wide area of the interior (Barnard, 1952).

Text-Figure 1.3 Major alkaloids of *Duboisia myoporoides* R. Br.
(Gritsanapan and Griffin, 1991)
Wills (1861) (cited in Barnard, 1952), first reported 'pituri' which was used by Aborigines. To prepare 'pituri' a fine powder of one tablespoonful of dried, half-pulverised leaves and stems of *D. hopwoodii* was mixed with alkaline wood ash. The quid thus prepared was chewed or maintained in the check pouch. It was used to alleviate physical stress (Griffin 1985).

The earliest information about the physiological activity of *D. myoporoides* was made by Rev. W. Woolls (1867) (cited in Barnard, 1952), a keen amateur botanist and clergyman in the Richmond parish near Sydney. He reported that the Aborigines used *D. myoporoides* for its intoxicant properties. They used to make holes in the trunks and put some fluid in them, which they latter drank and on the following morning it produced a stupor. They also put the branches of the *D. myoporoides* tree into pools to intoxicate the eels and to bring them to the surface.

Dr. Joseph Bancroft (1872) (cited in Barnard, 1952), of Brisbane investigated the physiological action of watery extract of the leaves of *D. myoporoides*. Later the active principles of *D. myoporoides* were investigated and identified.

### 1.2.4.2 Alkaloids of *Duboisia myoporoides* R. Br.

A number of analytical chemical investigations of *D. myoporoides* have been carried out in this century and the plant is reported to contain a large number of alkaloids. According to different authors, the leaf samples were found to contain the following alkaloids; Bottomley and Mortimer (1954) reported the presence of hyoscine, hyoscyamine, norhyoscyamine, valeroidine, tigloidine, isoporoidine, poroidine, apoatropine, nicotine, nornicotine; Coulson and Griffin (1967) reported the presence of scopolamine, valtropine, hyoscymine, butropine, poroidine, valeroidine, acetyl tropine, noratropine, isoporoidine, apohyoscine, tropyli butyrate and tropine; Gritsanapan and Griffin (1991) also reported the presence of tetramethylputrescine together with the other alkaloids. The old stems of *D. myoporoides* were found to contain valtropine, scopolamine, atropine, tropine, valeroidine, norhyoscine, apohyoscine, noratropine, butropine and hyoscyamine
(Coulson and Griffin, 1967). Presence of the α-alkylpiperidine alkaloid pelletierine, isopelletierine the quinolizidine alkaloid myrline and the β-phenylethylamine alkaloids tyramine and 3-methoxytyramine were identified in D. myoporoides roots and suspension cultures (Bachmann, et al., 1989). In addition to these alkaloids, Yukimune et al. (1994 d) reported tropinone, acetyltropine, acetyltropine, 6-hydroxyapoatropine, hygrine, acetoxytropine, propionylxotropine, butyryloxotropine, methylbutyryloxotropine, cuscohgrine, 6β-hydroxyhyoscyamine in the cultured roots. According to the chemical analysis results, the main alkaloids were found to be the pyridine alkaloid nicotine and tropane alkaloids hyoscyamine and scopolamine (Fig. 1.4 a, b and c).

![Fig. 1.4 a Nicotine (Gross, 1996)](image1)
![Fig. 1.4 b Hyoscyamine (Strauss, 1989)](image2)
![Fig. 1.4 c Scopolamine (Strauss, 1989)](image3)

### 1.2.5 Tropane Alkaloids

Tropane alkaloids occur mainly in the Solanaceae plant family and are also found in the Convolvulaceae, Erythroxylaceae, Proteaceae, Rhizophoraceae, Euphorbiaceae and Cruciferae families (Lounasmaa, 1988). The genera Atropa, Datura, Duboisia, Hyoscyamus, and Scopolia (Kato et al., 1997) and Mandragora, Physochlaina, Przewalskia (Evans, 1979) of the Solanaceae are especially rich sources of hyoscyamine, scopolamine, or both.
The bicyclic heterocyclic compound 8-azabicyclo[3.2.1] octane is present in all the tropane alkaloids. Tropane is its 8-methyl derivative. Over 150 tropane alkaloids have been isolated from plants or chemically synthesised and are usually found as esters between organic acids with either 3α or 3β-hydroxytropane derivatives (Lounasmaa, 1988). The 3α-hydroxytropane esters, also known as belladonna alkaloids (Hashimoto and Yamada, 1992). Hyoscyamine and scopolamine are responsible for the pharmacological properties of *D. myoporoides* (Evans, 1990).

### 1.2.6 Importance and Poisoning Syndrome of Tropane Alkaloids

The racemic mixture of [S]- and [R]-hyoscyamine is called atropine, of which only the [S]-isomer is synthesised in plants and is pharmacologically active (Hashimoto and Yamada, 1992). During processing of the plant material, racemisation takes place. Hyoscyamine and scopolamine act as competitive antagonists by binding to the muscarinic sites of the cholinergic neuroreceptors present in nerve cells (Hashimoto and Yamada, 1992). At present, the tropane alkaloids have a wide range of pharmaceutical applications, including uses as mydriatics, spasmyotics, analgesics, narcotics, sedatives, antiperspirants, antidotes to intoxications or for their beneficial effects in cases of asthma, Parkinson's disease, ulcers and motion sickness. L-scopolamine is more active than L-hyoscyamine and L-hyoscyamine is more active than its DL-racemate atropine, with respect to their peripheral effects (Cordell, 1978). Since hyoscyamine has a stimulant action on the central nervous system, scopolamine as the N-butylbromide derivative is the preferred parasympathomimetic agent (Hashimoto and Yamada, 1992).

Accidental poisoning by this plant material is by absorption through the intact mucous membranes of the conjunctiva, nose and upper oropharynx. The poisoning affects brain, eyes and skin. Severe symptoms are impaired vision, nausea, headache, dehydration of mucous membranes, loss of balance, depression, cramps and mental confusion (Askari and Ahmed, 1987).
1.2.7 Biosynthesis of Tropane alkaloids

In last few decades, alkaloid biosynthesis has been studied by a number of investigators by using radioactive tracers (Spenser, 1968; Saxton, 1971-1975; Grundon, 1976-1983; Dalton, 1979; Robinson, 1981). Basically, the alkaloids are derived from 4 amino acids, such as ornithine, lysine, phenylalanine and tryptophan (Dalton, 1979; Moths et al. 1985). Biosynthesis of tropane alkaloids has been reviewed recently by Leete (1990) and Hashimoto and Yamada (1992). The proposed biosynthetic pathways of pyridine and tropane alkaloids (Hashimoto and Yamada, 1992; Hashimoto and Yamada, 1994) are given in Fig. 1.5.

The common biosynthetic pathway for nicotine and tropane alkaloids originates from ornithine and/or arginine by the actions of ornithine decarboxylase and/or arginine decarboxylase for biosynthesis of the tropane ring (Hashimoto and Yamada, 1994). At this stage, a diamine putrescine formation takes place. After formation of N-methyl putrescine, it is oxidatively deaminated by a diamine oxide and spontaneously cyclised to give 1-methyl-Δ¹-pyrrolinium salt and the nicotine and tropane diverge at this point (see Fig. 1.5) (Leete, 1979). Conversion of the iminium salt to tropinone, the first compound with the tropane ring system, proceeds by way of hygrine.

Tropic acid, the acid moiety of hyoscyamine, is derived from phenylalanine by an intramolecular rearrangement of the side chain of phenyllactic acid. Tropic acid and tropine then condense to form hyoscyamine (Hashomoto and Yamada, 1992).

Conversion of hyoscyamine to its epoxide, scopolamine, proceeds in two oxidative steps; hydroxylation at the 6β-position of the tropane ring followed by intramolecular epoxide formation by removal of the 7β-hydrogen. Both of these steps are catalysed by hyoscyamine 6β-hydroxylase (H6H), and the hydroxylase activity is about 40-fold stronger than the epoxidase activity. This enzyme requires 2-oxoglutarate, ferrous ions, ascorbate and molecular oxygen for catalysis and belongs to the 20 oxoacid dependent oxygenase family (Hashimoto and Yamada, 1992).
1. arginine
2. ornithine
3. N-methylputrescine
4. N-methylpyrrolinium salt
5. acetoacetic acid
6. hygrine
7. tropinone
8. tropine
9. trropic acid
10. hyoscyamine
11. 6β-hydroxyhyoscyamine
12. scopolamine
13. nicotinic acid
14. nicotine
15. nornicotine
16. cuscohygrine
17. acetyltropic acid
18. propionylxypotropine
19. butrylyxypotropine
20. methylbutrylyxypotropine
21. apoafratropine
22. acetylatropine
23. 6-hydroxyapoafratropine
24. aposcopolamine

Text-Figure 1.5 The proposed biosynthetic pathways of nicotine, hyoscyamine and scopolamine (Yukimune et al., 1994 d)
Not all enzymes of this biosynthetic pathway have so far been investigated. For example, the enzyme that condenses the tropic acid derivative and tropine to form hyoscymine has not yet been discovered (Hashimoto and Yamada, 1992). So the regulation of tropane alkaloid biosynthesis is not clear. Furthermore, biosynthesis of tropane alkaloids in the roots is the main problem for investigation of the enzymes. However, there is some evidence that the expression of some of the enzymes is possible in the plant cell culture. Therefore, plant cell culture may overcome the problem of root biosynthesis by allowing the complete biosynthetic pathways to be investigated in the laboratory.

Not only the final products, but also the intermediate products of this biosynthetic pathway are identified in the field-grown or cultured plant material extracts. For example, hygrine, tropinone, tropine, the intermediate products have also been identified in the *D. myoporoides* cultured root extracts (Yukimune et al. 1994 d).

The regulation of nicotine biosynthesis is now relatively well understood (Hashimoto *et al.*, 1993; Kutchan, 1995). The lack of knowledge of the regulation of tropane alkaloid biosynthesis in the whole plant as well as cultured cells is the main constraint for controlling and improving the yield of tropane alkaloids in culture. A clear idea about the tropane alkaloid biosynthetic pathway and related enzymology of a the particular products especially the limiting steps in the pathway will help to apply gene technology for producing consistent and high levels of tropane alkaloids in culture.

### 1.2.8 Biosynthetic and Accumulation Sites for Tropane Alkaloids

Secondary metabolites are usually not distributed uniformly within the whole plant (Wiermann, 1981; Strack *et al.*, 1985). Biosynthetic and accumulation sites for secondary metabolites in different plant species are different. Biosynthesis and accumulation sites of some secondary metabolites are summarised in Table 1.3. A
### TABLE 1.3 Organ-specific biosynthesis and accumulation of alkaloids and other secondary metabolites (modified from Wink, 1987)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plant species</th>
<th>Main sites of biosynthesis</th>
<th>Accumulation site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropane</td>
<td><em>Atropa, Datura</em></td>
<td>Roots (shoots ?)</td>
<td>Whole plant</td>
</tr>
<tr>
<td>Cocaine</td>
<td><em>Erythroxylon</em></td>
<td>Green parts</td>
<td>Green parts</td>
</tr>
<tr>
<td>Coniine</td>
<td><em>Conium</em></td>
<td>Green parts</td>
<td>Whole plant</td>
</tr>
<tr>
<td>Quinolizidine</td>
<td><em>Lupinus</em></td>
<td>Green parts</td>
<td>Whole plant</td>
</tr>
<tr>
<td>Pyrolizidine</td>
<td><em>Senecio</em></td>
<td>Roots, green parts</td>
<td>Whole plant</td>
</tr>
<tr>
<td>Morphine</td>
<td><em>Papaver</em></td>
<td>Leaves, latex</td>
<td>Latex</td>
</tr>
<tr>
<td>Berberine</td>
<td><em>Bberberts</em></td>
<td>Rhizomes</td>
<td>Rhizomes, stems</td>
</tr>
<tr>
<td>Quinine</td>
<td><em>Cinchona</em></td>
<td>Aerial parts</td>
<td>Aerial parts</td>
</tr>
<tr>
<td>Caffeine</td>
<td><em>Caffea</em></td>
<td>Green parts</td>
<td>Fruits, leaves</td>
</tr>
<tr>
<td>Betalaines</td>
<td><em>Beta</em></td>
<td>Roots (stems)</td>
<td>Roots (stems)</td>
</tr>
<tr>
<td>Terpenes</td>
<td></td>
<td>Aerial parts</td>
<td></td>
</tr>
<tr>
<td>Monoterpines</td>
<td><em>Labiateae, etc.</em></td>
<td>Aerial parts</td>
<td>Aerial parts</td>
</tr>
<tr>
<td>Phenolics</td>
<td></td>
<td>Aerial parts</td>
<td>Aerial parts</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Many species</td>
<td>Aerial parts</td>
<td>Aerial parts</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>Many species</td>
<td>Aerial parts</td>
<td>Aerial parts</td>
</tr>
<tr>
<td>Glocosinolates</td>
<td><em>Brassica</em></td>
<td>Whole plant</td>
<td>Whole plant</td>
</tr>
</tbody>
</table>
number of secondary metabolites has been found to be localised in the vacuoles (Marty et al., 1980).

By chemical analysis, presence of tropane alkaloids has been identified in the aerial parts (stem, leaves) and the roots of different plant species. In a particular organ, the alkaloids are found to be stored in the specific storage cells and in definite cell layers and it varies from species to species. Their localization was identified by histochemical methods (James, 1950; Ferreira et al., 1998). Histochemical results were then supported by modern analytical techniques such as GC (Hashimoto et al., 1993), HPLC (Subroto et al. 1996 a). Accumulation of alkaloids in cell cultures has also been observed in various alkaloid producing species.

Accumulation of alkaloid takes by different mechanisms. Diffusion or ion-trap mechanism is responsible for alkaloid accumulation in lupine vacuoles (Mende and Wink, 1987). Presence of carrier proteins for the transport of some secondary metabolites such as; coumaryl glucosides (Werner and Matile, 1985), flavonoides (Matern et al., 1986) has been observed. Specific carrier-mediated transport of alkaloids to the vacuoles has been observed (Deus-Newmann and Zenk, 1986).

1.2.9 Degradation of Alkaloids in the Plant Tissues

After formation, alkaloids are further metabolised in the plants. In some plant species, alkaloids are accumulated in seeds and during germination, they are metabolised and their nitrogen is used for the seedling’s metabolism (Wink and Witte, 1985). During growing season, the alkaloids are also metabolised (James, 1950; Waller and Nowacki, 1978). Cultured cells are also able to degrade exogenously added alkaloids (Robinson, 1974; Wink and Witte, 1985).
1.2.10 Plant Tissue Culture: Organogenesis, Differentiation and Secondary Metabolites with Special Reference to Tropane Alkaloid Production

1.2.10.1 Plant Tissue Culture in Production of Secondary Metabolites

For the production of plant secondary metabolites, plant tissue culture (PTC) is an alternative method to growing the whole plant. Several advantages arise using this technique; 1) useful compounds could be obtained throughout the year, without depending on climatic changes or political problems, 2) cultured cells, being free of microbes, may produce higher amounts of the desired compound and 3) by changing culture conditions, production of the compound could be increased.

Some of the plant-derived chemicals such as medicinals (scopolamine, vincristine, digoxin); sweeteners (thaumatin); flavours and fragrances (strawberry, vanilla, mint); food colours (anthocyanin) have been investigated using PTC technique. Over 30 cell culture systems, which are better producers than the respective plants, have been identified (Tabata, 1977; Zenk, 1978; Berlin, 1984; Staba, 1985; Wink, 1987; Matsubara et al., 1989; Takahashi and Fujita, 1991). A few of them are used for large-scale production using PTC technique. Table 1.4 lists some examples of secondary metabolites produced by PTC.

Commercial production of the plant-derived chemicals via plant cell and tissue culture systems becomes viable when the product commands a price exceeding about $1000 kg⁻¹ (Staba, 1980). Mitsui Petrochemical Industry Company in Japan, was the first to produce a pharmaceutical and dyestuff shikonin on commercial scale, valued at $4,500 kg⁻¹. By using PTC technology, the company also produced the pharmaceutical berberine at a yield of 3.5g L⁻¹ of culture fluid (Discosmo and Misawa, 1995). Nitto Denko Company in Japan also cultivated Panax ginseng cells in 20m³ bioreactors for biomass production (DisCosmo and Misawa, 1995). These
**TABLE 1.4** Secondary metabolite produced by plant tissue culture (modified from Constabel, 1987)

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Secondary metabolite</th>
<th>Culture method</th>
<th>Contents in culture (% dwt)</th>
<th>Contents in plant (% dwt)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Coffea arabica</em></td>
<td>Caffeine</td>
<td>Agar</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td><em>Coptis japonica</em></td>
<td>Berberine</td>
<td>Agar</td>
<td>7.4</td>
<td>1</td>
</tr>
<tr>
<td><em>Coptis japonica</em></td>
<td>Berberine</td>
<td>Liquid</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td><em>Catharanthus roseus</em></td>
<td>Ajmalicine</td>
<td>Liquid</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>Ubiquinone-10</td>
<td>Liquid</td>
<td>0.18</td>
<td>0.003</td>
</tr>
<tr>
<td><em>Catharanthus roseus</em></td>
<td>Catharanthine</td>
<td>Liquid</td>
<td>0.24</td>
<td>0.002</td>
</tr>
<tr>
<td><em>Coleus blumei</em></td>
<td>Rosmarinic acid</td>
<td>Liquid</td>
<td>15</td>
<td>3.6</td>
</tr>
<tr>
<td><em>Dioscorea deltoidea</em></td>
<td>Diosgenin</td>
<td>Liquid</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>Panax ginseng</em></td>
<td>Ginsengoside</td>
<td>Agar</td>
<td>27</td>
<td>4.5</td>
</tr>
<tr>
<td><em>Lithospermum erythrorhizon</em></td>
<td>Shikonin</td>
<td>Liquid</td>
<td>14</td>
<td>1.5</td>
</tr>
<tr>
<td><em>Lithospermum erythrorhizon</em></td>
<td>Shikonin</td>
<td>Agar</td>
<td>12</td>
<td>-</td>
</tr>
</tbody>
</table>

dwt: dry weight
results indicated a promising future for the PTC technique in the production of plant-derived chemicals.

1.2.10.2 Plant Tissue Culture

In 1838, Schwann and Schleiden first said that plant cells are ‘totipotent’, that is a complete plant could be regenerated from a single cell. Totipotency of a plant cell is the basis of PTC (Sharp et al., 1995). In this technique, different plant organs or ‘differentiated’ tissues are ‘de-differentiated’ to single cells. Depending on the medium constituents, the de-differentiated cells either continue to grow as an unorganised mass of tissue or ‘re-differentiate’ into organs.

1.2.10.2.1 Medium Constituents

Five different components such as; inorganic macro and micro nutrients, energy and carbon source, vitamins, organic nitrogen and plant growth regulators (PGRs) are required to make a single cell grow into an unorganised mass and produce organs (Thorpe, 1980). The macro nutrients (e.g., K⁺, NH₄⁺, Ca²⁺, PO₄³⁻), micro nutrients (e.g., Fe⁺⁺, Zn⁺⁺, Cu⁺⁺, BO₃³⁻), carbon source (e.g., sucrose), nitrogen source (NO₃⁻), vitamins (e.g. pyridoxine, thiamine) and PGRs (auxin, cytokinin) are used as the medium constituents. Depending on the experiment, some complex organics (e.g., yeast extract), amino acids or other chemicals are also added to the medium. The cultures are maintained in semi-solid or in liquid medium. For semi-solid medium, a gelling agent (e.g., agar) is added. Different basal media have been formulated for culturing plant tissues; for example, Murashige and Skoog (1962) or MS, Gamborg et al., (1968) or B5, White (1954), Nitsch and Nitsch (1969), Blaydes (1966), which vary mainly in the amounts of macro-nutrients, micro-nutrients or vitamins.
1.2.10.2.2 Plant Growth Regulators

It was not until 1926, F. W. Went in Holland initiated research into a group of PGRs now known as auxins which was the first type of PGR to be identified (Tomic et al., 1998). A PGR is generally regarded as an organic compound which is produced naturally in plants and is active in minute amounts in controlling growth and other functions.

Currently, 6 principal classes of PGRs, cytokinin, auxin, gibberellin (GA), abscisic acid (ABA), ethylene and brassinosteroids (BRs) are recognised (Hooley, 1998). Some PGRs and their uses in PTC are listed in Table 1.5. Among them cytokinins and auxins are widely used in PTC work. The terms cytokinin and auxin are derived from cytokinesis (the cellular effect first associated with cytokinins), auxin (Greek, to increase) respectively (Letham, 1969). In addition to the naturally occurring cytokinins and auxins, many synthetic cytokinins and auxins are also known.

Although cytokinin and auxin receptors have not been characterised, recently it has been suggested that cytokinins and auxins may be perceived by membrane-located receptors. Cytokinins may be perceived by a transmembrane protein receptor (Kakimoto, 1996) and auxins are thought to act at the cell surface (Venis and Napier, 1995). This membrane-located signalling systems are involved in the perception and transduction of the PGRs.

The biosynthetic site and distribution of these PGRs are different in the intact plant (Matthysse and Scott, 1984). The biosynthetic site and transport of some PGRs in the whole plant are presented in Fig. 1.6. Cytokinin is produced in the root tips and promotes branching in the roots. Some stimulation and inhibition effects of cytokinin are presented in Fig. 1.7. Auxin is produced in the shoot meristem and expanding leaves (Matthysse and Scott, 1984). It promotes branching in the shoots.
### TABLE 1.5 Commonly used plant growth regulators (Franklin and Dixon, 1994)

<table>
<thead>
<tr>
<th>Class</th>
<th>Abbreviation*</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auxin</td>
<td>IAA</td>
<td>Callus induction, Stimulate organogenesis</td>
</tr>
<tr>
<td></td>
<td>IBA</td>
<td>Rooting regenerated shoots.</td>
</tr>
<tr>
<td></td>
<td>2,4-D</td>
<td>Induction and maintaining callus and suspension cells.</td>
</tr>
<tr>
<td></td>
<td>pCPA</td>
<td>Similar to 2,4-D; less commonly used</td>
</tr>
<tr>
<td></td>
<td>NAA</td>
<td>Synthetic analogue of IAA Commonly used for callus induction and growth of callus and suspension cultures</td>
</tr>
<tr>
<td>Cytokinin</td>
<td>Kin (Kinetin)</td>
<td>Callus induction, growth of callus and cell suspensions, induction of morphogenesis, shoot bud or meristem induction</td>
</tr>
<tr>
<td></td>
<td>BAP (BA)</td>
<td>Callus induction, growth of callus and cell suspensions. Shoot bud, meristem induction</td>
</tr>
<tr>
<td></td>
<td>2iP</td>
<td>Less commonly used for callus Induction and growth. Induction of morphogenesis, shoot buds and meristems</td>
</tr>
<tr>
<td></td>
<td>Zea (Zeatin)</td>
<td>Induction of morphogenesis</td>
</tr>
<tr>
<td>Gibberellin</td>
<td>GA₃</td>
<td>Seldom used in callus and suspension culture</td>
</tr>
<tr>
<td>Abscisic</td>
<td>Abscisic acid</td>
<td>Promote normal development of somatic embryos</td>
</tr>
</tbody>
</table>

*: Indolyl-3-acetic acid
IAA
  *Indolyl-3-butyric acid
IBA
  *2,4-Dichlorophenoxyacetic acid
2,4-D
  *Para-chlorophenoxy acetic acid
pCPA
  *1-Naphthaleneacetic acid
NAA
  *6-Furfurylaminopurine
Kin
  *6-Benzyleaminopurine or benzyladenine
BAP
  *N⁶- (2-Isopentyl) adenine
2-iP
  *(5-Hydroxy-3-methylbut-2-enyl)purine
Zea
  *3-Oxo-2-(2'-pentenyl)-cyclopenteneacetic acid
GA₃
  *Abscisic acid trans-cinnamic acid
Text-Figure 1.6 Sites of plant growth regulator synthesis and transport in a whole plant (Matthysse and Scott, 1984)
(Matthysse and Scott, 1984). Some stimulation and inhibition effects of auxin (IAA) in the whole plant are presented in Fig. 1.8.

PGRs are chemically diverse. Cytokinins (Fig. 1.9) are \( N^6 \)-substituted adenine derivatives (Schmulling et al., 1997) and discovered more than 40 years ago (Miller et al., 1955). In PTC, naturally occurring Zeatin and synthetic compounds Kinetin and BAP are most commonly used as cytokinins (Fig. 1.9). Coconut milk (liquid endosperm) at 10-15% can also serve as a source of cytokinins (Sharp et al., 1995).

A large number of developmental and physiological processes can be influenced by cytokinin or are correlated with changes in the endogenous cytokinin concentration (Mok, 1994). They modulate gene expression in a wide variety of plant tissues and cell types over a variety of response time and involved in cell division, branching of shoots, leaf senescence, chloroplast development and nutrient metabolism (Schmulling et al., 1997).

The most characteristic effect of cytokinins on cultured plant cells is their influence on cell proliferation (Szweykowska, 1974). Cytokinins are necessary for mitosis as well as for cytokinesis (Das et al., 1956) and are applied at concentrations of \((1-10) \times 10^{-6}\)M (Schmulling et al., 1997). For callus induction, continued callus culture or cell suspension culture, 0.1mg L\(^{-1}\) of cytokinin is generally used in combination with 1-5mg L\(^{-1}\) of auxins (Sharp et al., 1995). Cytokinin alone or a high ratio of exogenous cytokinin to exogenous auxin induce adventitious shoot formation (Profumo et al., 1985). Very low concentrations of cytokinin (e.g., 0.01mg L\(^{-1}\)) used with auxin (1-5mg L\(^{-1}\)) can promote root formation (Sharp et al., 1995).

Auxins are indole derivatives (Fig. 1.9). The most commonly used auxins are naturally occurring IAA and synthetic compounds 2,4-D; NAA; IBA. The effects of an auxin on cultured plant tissues vary from species to species and depend on the concentration and the presence of other plant PGRs. Generally, auxins promote cell elongation (Sharp et al., 1995). In combination with other PGRs, auxin effects long-term callus growth (Moon and Stomp, 1997). Other major effects are the initiation or enhancement of root organogenesis (Profumo et al., 1985; Teo et al., 1997) and
**BAP (BA)**
6-benzyleaminopurine
or
benzyladenine

**Kinetin (Kin)**
6-furfurylaminopurine

**Zeatin**
6-(4-hydroxy-3-methyl-but-2-enylamino) purine

**IAA**
Indolyl-3-acetic acid

**IBA**
Indolyl-3-butyric acid

**2,4-D**
2,4-Dichlorophenoxyacetic acid

**NAA**
1-Naphthaleneacetic acid

**FIGURE 1.9** Some cytokinins and auxins used in plant tissue culture
(modified from Gaspar et al., 1996)
rooting of shoots obtained through micropropagation (Murashige, 1974; Hussey, 1980). Different auxins are used in different concentrations; for example, IAA is used at 1-31mg L\(^{-1}\), NAA and 2,4-D are used 0.1-2.0mg L\(^{-1}\) (Sharp et al., 1995).

Skoog and Miller (1957) reported that both cytokinin and auxin are required to induce cell division and growth in PTC. Subsequent studies on whole plants and excised tissues have demonstrated the existence of synergistic, antagonistic and additive interactions between these 2 PGRs. They regulate cell division synergistically and control lateral bud or root outgrowth antagonistically (Coenen and Lomax, 1997). These findings suggest that there may be multiple underlying mechanisms of interactions. Auxin may influence cytokinin levels in plant cells by down regulation of cytokinin biosynthesis and/or by promotion of cytokinin degradation (Kaminek et al., 1997). Dominov et al. (1992) suggested that cytokinin enhances the auxin response by sensitising the cells or by blocking the feedback inhibition of the auxin response.

Genetic evidence suggests that cytokinin can interact with at least two independent auxin signal-transducing pathways (Hobbie and Estelle, 1994; Timpte et al., 1995). In cultured tissues, cytokinin and auxin regulate cell division synergistically in callus cells and protoplasts and antagonistically during the formation of lateral root primordia (Coenen and Lomax, 1997).

Except the mentioned PGRs, there are some other chemicals which are used in PTC work; for example, paclobutrazol is effective in inhibiting extension growth in a wide range of plant species (Davies et al., 1986), dikegulac-sodium is used as a growth retardant (Bhattacharjee et al., 1986), thidiazuron (TDZ) is used for shoot organogenesis in different plant species (Nielsen et al., 1993; Castillo and Jordan, 1997).

Thidiazuron is one of several substituted phenyl ureas. The most active of these compounds are TDZ, DPU (Mok et al., 1980) and CPPU (Fellman et al., 1987). TDZ is registered as a cotton defoliants under the trade name DROPP(R) (NOR-AM Technical Bulletin, 1987) (cited in Huetteman and Preece, 1993).
TDZ may be the most potent of the diphenyl ureas that have been evaluated for use in PTC (Mok et al., 1982) and now emerges as an effective bioregulant of morphogenesis in tissue culture of many plant species (Johanne et al., 1991; Hutchinson et al., 1997; Murthy et al., 1998). TDZ is highly soluble in DMSO and slightly soluble in water. Most published reports described DMSO as the solvent for TDZ. It can be incorporated into the tissue culture medium and autoclaved and still retain its activity (Huettemann and Preece, 1993).

A number of physiological and biochemical events in cells are influenced by TDZ, but these may or may not be directly related to the induction of morphogenic response and hence the mode of action of TDZ is unknown (Murthy et al., 1998). Application of TDZ induces various cultural responses ranging from induction of callus to formation of somatic embryos and exhibits the properties of both cytokinin and auxin on growth and differentiation (Murthy et al., 1998).

In last couple of years, the effect of TDZ alone or in combination with auxins has been investigated for organogenesis in different plant species. Hutchinson et al., (1996) provided an evidence of the role of TDZ in somatic embryogenesis in hypocotyl culture of geranium. According to their investigations, TDZ modulates endogenous auxin metabolism during embryo development.

Kapaun and Cheng (1997) used TDZ at various concentrations and found organogenesis on the leaf tissues of the greenhouse-grown seedlings of Siberian elm. Hutchinson et al., (1996) studied the physiological and morphological changes in the complete development of somatic embryos from hypocotyl explants of geranium cultured on media supplemented with TDZ, IAA or BA alone. They reported that TDZ may be a more potent PGR in inducing physiological and morphological changes than combined cytokinin and auxin during the process of somatic embryogenesis in geranium.

Cousineau and Donnelly (1991) reported a higher rate of shoot regeneration using leaf-petiole explant of raspberry incubated in modified MS basal medium supplemented with various concentrations of TDZ in combination with IBA. From
these reports, it is clear that TDZ has an effect on organogenesis, however, it has not yet been tested for organogenesis in *D. myoporoides*. Table 1.6 lists some effects of TDZ + IBA on organogenesis and differentiation on different plant species.

### 1.2.10.2.3 Initiation and Maintenance of Plant Tissues in Culture

For initiation of PTC, different surface sterilised plant organs (e.g., leaf, stem, shoot tip) are used as explants. The explants are put on semi-solid culture medium and incubated in controlled environment. The cultures are grown in sterile conditions in culture vials made of glass or other materials. On incubation, an unorganised tissue or specialised organ (shoot-bud, root) forms on the explants. The unorganised tissue which is a collection of de-differentiated new cells known as ‘callus’, continues to grow without any specialised organ formation.

Although the callus is maintained in controlled conditions, a heterogeneity both between the calli and within the callus itself is observed. This heterogeneity is seen in differences in colour, morphology, structure, growth and metabolism (Torrey, 1966). As the callus grows, cells are pushed upwards and outwards from the surface of the medium so that nutrient gradients are established between the cells and the constituents of the medium. This gradient affects growth patterns by promoting and inhibiting cell expansion and division.

Two different physical forms of culture medium i.e., semi-solid and liquid media are used in PTC. Semi-solid medium is used for organ formation, somatic embryo formation and complete plant regeneration. Cell suspension is preferred for large-scale production of secondary plant products (Fu, 1998). Suspension culture is established by transferring a friable callus to liquid medium and can be maintained for long periods by regularly transferring ca. 20% of the established suspension to the fresh medium. The cultures are grown continuously by agitated medium.

The growth rates of the cultured tissues and organs are found to be higher in the liquid medium than in the semi-solid medium. The metabolic heterogeneity of
<table>
<thead>
<tr>
<th>Reference</th>
<th>Some sterile embryos</th>
<th>Hypocotyl</th>
<th>Pseudomonas haloctonus B</th>
<th>TDCZ (10^{-6}) x 10^{-10} x 10^{-14}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hutchinson et al.</td>
<td>1996</td>
<td></td>
<td></td>
<td>TDCZ (10^{-6}) x 10^{-10} x 10^{-14}</td>
</tr>
<tr>
<td>Ayub et al.</td>
<td>1998</td>
<td></td>
<td></td>
<td>TDCZ (10^{-6}) x 10^{-10} x 10^{-14}</td>
</tr>
<tr>
<td>Cusimano and Donnelly</td>
<td>1999</td>
<td></td>
<td></td>
<td>TDCZ (4.5-9.1) x 10^{-10} x 10^{-10} x 10^{-10}</td>
</tr>
<tr>
<td>Ahrol et al.</td>
<td>1997</td>
<td></td>
<td></td>
<td>TDCZ (3.6 x 10^{-10} x 10^{-10} x 10^{-10} x 10^{-10})</td>
</tr>
</tbody>
</table>

**Table 1.6:** Effects of TDCZ onogenous response on various plant species using different explants included in the Murashige and Skoog (1962) basal medium supplemented with different concentrations of auxins.
callus in the semi-solid medium is reduced in the suspension. Although a single-cell suspension culture has been reported, it is not long lasting and eventually aggregate forms.

1.2.10.2.4 Vitrification or Hyperhydricity

A common problem found mainly during plantlet regeneration is the irreversible swelling and distortion of the regenerating tissues accompanied by a water-soaked and translucent appearance and sometimes leading to tissue death (Kevers et al., 1984). This process is known as vitrification or hyperhydricity (Warren, 1991). Though it is mainly encountered in micropropagation, it is a potential problem for secondary metabolite producing shoot cultures. Completely submerged shoots may be more susceptible to vitrification than shoots exposed to air (Payne et al., 1991). For this reason, semi-solid media is used for plant regeneration (Warren, 1991).

Factors implicated include the type and concentration of cytokinin in the medium, water potential, concentration of ammonium ions, relative humidity in air space (which cause low levels of wax formation in the cuticles of regenerated plant tissues), buildup of ethylene (Warren, 1991; Payne et al., 1991; Ziv, 1991a, b). Some of these problems were solved by using vessels with loose fitting tops which allowed a certain degree of gas exchange without contamination (Warren, 1991).

1.2.10.3 Organogenesis and Differentiation

Depending on the component of the nutrient medium, the callus either continues to grow as an unorganised mass or differentiates and forms shoot bud primordium, root primordium or flowers. This process of shoot-bud, root primordium and flower formation is collectively known as ‘organogenesis’ (Thorpe, 1980; Warren, 1991). The primordia formed then develop into the respective organs and
this acquisition of a cell’s specific structural and functional feature is known as ‘differentiation’. *In vitro* organ formation was first reported by White (1939) who obtained shoots in a tobacco hybrid, and by Nobecourt (1939) (cited in Thorpe, 1980) who obtained roots in carrot callus.

In the early studies of the 40s and 50s, organogenesis and differentiation have been achieved in numerous plant species by culturing a variety of explants, callus derived from explants and cell suspensions on defined medium. Successful organ formation by PTC, is achieved by manipulating explants, culture media and the culture environment.

**1.2.10.3.1 Explant**

Various factors play important roles in the selection of explants for PTC. These include; the organ which serves as the tissue source, the age of the organ, the season in which the explant was collected, the size of the explant and the quality of the plant from which the explants are collected (Narayanaswamy, 1977; Murashige, 1979). Different plant parts can be used as an explant to initiate the callus which is maintained in culture on semi-solid medium or in suspension. The explants include; stem segments (Ahroni *et al.*, 1997), leaf sections (Kukreja *et al.*, 1986; Aljuboori *et al.*, 1998), root sections (Lucchesini and Mensualisodi, 1996), shoot tips (Chandra *et al.*, 1998), seed (Lin and Griffin, 1992a), tubers (Profumo *et al.*, 1985), cotyledons (Choi *et al.*, 1998), hypocotyls (Soh *et al.*, 1998), bulb scales (Van-aatrjik and Blom-barnhoorn, 1983), thin cell-layers, the 3-6 epidermal and sub-epidermal cell layers (Klimaszewesa and Keller, 1985). Shoot-buds can be regenerated from different explants (Kukreja *et al.*, 1986; Badaoui *et al.*, 1996; Teo *et al.*, 1997) as well as from sub-cultured callus tissues (Lin and Griffin, 1992a; Badaoui *et al.*, 1996). Similarly, adventitious roots can be regenerated from various explants (Habaguchi, 1977; Carswell and Loci, 1984; Jasik and Deklerk, 1997) and also from sub-cultured callus tissues (Soh *et al.*, 1998).
Depending on the type of the explant used for callus induction, the shoot-bud regeneration on the induced calli may vary (Badaoui et al., 1996).

1.2.10.3.2 Light

Organogenesis and differentiation in various plant species can vary depending on the presence, quantity or quality of light. Light incubation was necessary for meristematic tissue formation as well as shoot formation in radiata pine (Villalobos et al., 1984). A 12-hr daily photoperiod at an intensity of 5000 lux was optimal for root initiation in Helianthus tuber sections (Gautheret, 1969) (cited in Thorpe, 1980). In tobacco callus, root and shoot formation took place in a 16-h photoperiod of intensity 1000 lux (Murashige, 1977). Efficiency of shoot production on the shoot apex of Brazilian indica rice was influenced by light treatments (Padua et al., 1998).

There are numerous reports of interactions between light and PGRs. However, the exact nature of this interaction is not well understood (Kraepiel and Miginiac, 1997).

1.2.10.3.3 Physical form of the Medium

The physical form of the medium can change the pattern of differentiation. A root explant of Chichorium intybus, cultured on the liquid nutrient medium and supported by a filter paper bridge, produced vegetative buds, whereas on the semi-solid medium containing a high concentration of agar, it produced floral buds (Bouniols, 1974) (cited in Thorpe, 1980).
1.2.10.3.4 Plant Growth Regulators

In manipulating organogenesis and differentiation in vitro, PGRs, many growth active substances or other types of compounds play an important role. With a suitable cytokinin/auxin balance, a large number of plant species form an unorganised callus which needs an altered cytokinin/auxin balance for organ formation to occur. For organogenesis and differentiation on callus, cytokinin/auxin combinations in the right quantities, in the right sequences and under optimal culture conditions are necessary. Cytokinin alone can produce shoot-buds on callus (Endo and Yamada, 1985; Lin and Griffin, 1992a; Lakshmanan et al., 1997). When Kinetin or BA used in the range of 0.05 to 46μM, generally, 75% of the plant species form shoots (Evans et al., 1981).

Optimal cytokinin/auxin combinations can regenerate shoot-buds on the explant (Kukreja et al., 1986; Badaoui et al., 1996). Sometimes only selective cytokinin and auxin combinations induce shoot regeneration on the callus (Jethwani and Kothari, 1996).

Thomas and Street (1970) found that omission of auxin from the medium stimulated rooting in cell aggregates of Atropa belladonna. Cytokinins had stimulating effects on root formation on callus induced on Cowpea hypocotyl which did not show rhizogenic activity on medium with auxins alone (Soh et al., 1998). Cheng (1975) showed that multiple cytokinins and auxins were more effective in shoot-bud induction in Douglas fir explants, than if the PGRs were applied separately.

A variety of substituted purines, pyrimidines and ureas have been shown to possess cytokinin activity and some are capable of replacing cytokinins for shoot formation. These include 6-benzylxoxypurine (Wilcox and Wain, 1976) and thidiazuron, a urea derivative (Ahroni et al., 1997; Aljuboory et al., 1998). Thidiazuron showed the unique property of mimicking both cytokinin and auxin effects on growth and differentiation of cultured explants (Murthy et al., 1998).
Besides PGRs, some other compounds also play important roles in stimulating organogenesis and differentiation. Combinations of glycerol and 2,4-D and/or the polyamines putrescine, spermidine and spermine resulted in a bigger size of the cell masses of in vitro cultures of Grateloupia doryphor (Garciajimenez et al., 1998).

PGRs are one of the most important factors in shoot elongation. However, no generally-applicable formula has been found for secondary metabolite producing plants. By varying the cytokinin and auxin combinations and concentrations, elongation of the regenerated shoot-buds can be obtained (Lin and Griffin, 1992a; Badaoui et al., 1996; Ferreira and Janick, 1996). Stem elongation in the regenerated shoot-buds incubated in the same incubation condition may vary (Flygh et al., 1998). For D. myoporoides, different cytokinin and auxin combinations have been suggested for organogenesis, differentiation and shoot elongation (Kitamura et al., 1980; Kukreja et al., 1986).

Some auxins showed significant effects on root induction and development in the cultured tissues or regenerated shoots. In various plant species, IBA is used for root formation on the regenerated shoots (Flygh et al., 1998). For root induction and development in the regenerated shoots of Brassica rapa, NAA was found to be the most effective auxin (Teo et al., 1997). On the other hand, IAA induced root formation on the regenerated Hackelia venusta (Boraginaceae) shoots (Edson et al., 1996). Number of root primordia formed on the cuttings derived from seedlings of Pinus strobus L. was found to be related to the amount of NAA used (Goldfarb et al., 1998).

1.2.10.4 Effects of Environmental Factors on Secondary Metabolite Biosynthesis

To express a stable secondary metabolite biosynthesis or to increase productivity by regulating secondary metabolism in culture, efforts have focused on the stimulation of the biosynthetic activities of the cultured cells. Several
environmental factors affecting secondary metabolism in callus and suspension culture have been tested in various plant species. These factors include media components, extra-factors, pH, temperature, pO$_2$, pCO$_2$, agitation, light, PGRs, cell line selection, precursors.

1.2.10.4.1 Plant Growth Regulators

Cytokinins also showed different effects on secondary metabolite biosynthesis. Berberine content in the callus cultures of *Coptis japonica* was reduced when 2,4-D was removed from the culture medium and decreased when kinetin in combination with 2,4-D was used (Ikuta et al., 1975). In suspension culture, kinetin enhanced anthocyanin accumulation in *Haplopappus gracilis* (Constable et al., 1971) but inhibited it in *Populus* (Matsumoto et al., 1973). The effects of kinetin also vary depending on the secondary metabolites or on the plant species. Carotenoid biosynthesis in carrot cultures was inhibited by kinetin (Mok et al., 1976) while berberine production in suspension cultures of *Thalictrum minus* was enhanced by BA and kinetin (Nakagawa et al., 1984).

The effect of auxins on biosynthesis of secondary metabolites in culture is contradictory. Biosynthesis of nicotine in *Nicotiana tabacum* cultures (Furuya et al., 1971), Shikonin derivatives in *Lithospermum erythrorhizon* cultures (Tabata et al., 1974) and anthraquinones in *Morinda citrifolia* cultures (Zenk et al., 1975) are inhibited by 2,4-D. In contrast, biosynthesis of carotenoid in *Daucus carota* cultures (Mok et al., 1976) and berberine in suspension culture of *Thalictrum minus* are stimulated by 2,4-D and NAA (Nakagawa et al., 1986).

At lower concentration of 2,4-D, conversion rate of hyoscyamine to scopolamine was higher in the transformed calli of *Datura stramonium* (Palazon et al., 1995). Auxins in the presence of low levels of kinetin induced disorganisation of transformed roots of *Nicotiana rustica* and ultimately to form suspension cultures of transformed cells and this process was associated with a decrease in nicotine content of the cells (Rhodes et al., 1994).
Auxins (NAA, IAA, 2,4-D) inhibited alkaloid formation in the cultured roots of *Hyoscyamus albus*. Generally, higher auxin concentration decreased alkaloid content, but 2,4-D at a concentration of $10^{-6}$M to $10^{-4}$M, hyoscyamine content was recovered to 2/3 of the alkaloid contents produced in auxin free medium (Hashimoto and Yamada, 1986). Grewal *et al.*, (1979) reported various effects of different auxins used in untransformed root culture of *Hyoscyamus muticus*. They found that alkaloid content was inhibited when IAA was used alone whereas alkaloid content was doubled when NAA was used. Kinetin in combination with IAA promoted alkaloid promotion in the early stages and in combination with NAA during later stages of root growth. Transformed root cultures of *Hyoscyamus muticus* are usually cultivated in PGR free medium and produce significant amount of hyoscyamine. Alkaloid accumulation in those roots was found to be doubled in the presence of exogenous auxins (Vanhalaa *et al.*, 1998).

### 1.2.10.4.2 Precursors

Addition of precursors to the culture medium either increases the amount or expresses the biosynthesis of secondary metabolites; for example, when phenylalanine was added to *Salvia officiatis* suspension cultures, production of rosamarinic acid was increased (Ellis and Towers, 1970). Addition of berberine in the culture medium increased alkaloid content in *Coptis japonica* (Yamamoto, 1980). Biosynthesis of scopolamine from hyoscyamine was expressed in the cultured shoots (without root formation) of *D. myoporoides*, when atropine was added to the culture medium (Hashiomoto and Yamada, 1989).

### 1.2.10.5 Secondary Metabolite Biosynthesis Using Different Plant Tissue Culture Techniques

Generally, plant cell cultures lose the ability to produce secondary metabolites that are characteristic of the intact plant (DiCosmo and Towers, 1984). In order to increase the amount of the secondary plant products or to express secondary metabolism at different stages of culture, various PTC techniques have been trialed.
Those techniques are as follows; callus culture, suspension culture, biotransformation, elicitation, immobilisation of plant cells, organ culture, transformed organ culture, genetic engineering technology.

1.2.10.5.1 Callus Culture

Callus cultures of different plant species showed that secondary metabolites can be produced in the unorganised state; for example, caffeine in Coffea arabica (Frischknecht et al., 1977), berberine in Coptis japonica (Fukui et al., 1982), ginsengoside in Panax ginseng (Furuya et al., 1983).

Tropane alkaloid biosynthesis in the unorganised state of different plant species is contradictory. Different plant species such as; Scopolia perviflora (Tabata et al., 1972), Atropa belladonna (Hamilton et al., 1986), Atropa belladonna, L. and Atropa belladonna Cultivar lutea Doll (Bhandary et al., 1969), Duboisia hybrid (Lin and Griffin, 1992b), indicated the absence of tropane alkaloids in the undifferentiated state. On the other hand, the presence of tropane alkaloids in the callus culture of D. myoporoides (Sipply and Friedrich, 1975; Kagii et al., 1980), Atropa belladonna L. (Eapen et al., 1978), Hyoscyamus muticus L (Basu and Chand, 1998), has been reported. Callus cultures of Scopolia japonica and several species of Datura contain small amount of tropane alkaloids (0.002-0.02 %). The presence of tropane alkaloids in the callus showed that the genes responsible for the biosynthesis of secondary metabolites are active in the unproductive cell cultures (Hirotani and Furuya, 1977). But the reason for unreproducible biosynthesis of tropane alkaloids in the callus culture of D. myoporoides is not yet clear and it needs further investigation.

1.2.10.5.2 Suspension Culture

Since the growth rate of the callus is higher in the suspension than in the semi-solid medium (Street et al., 1965; Hakman and von Arnold, 1988; Sharp et al., 1995), production of secondary metabolites from different plant species has been
tested in suspension; for example, anthraquinones in *Morinda citrifolia* (Zenk *et al*.,1975), ajmalicine in *Catharanthus roseus* (Zenk *et al*.,1977), anthocyanin in *Vitis vinifera* (Yamakawa *et al*.,1983), berberine in *Coptis japonica* (Sato and Yamada,1984), shikonin in *Lithospermum erythrorhizon* (Fujita and Tabata,1986), catharanthine in *Catharanthus roseus* (Smith *et al*.,1987). Some species such as; *Datura innoxia* (Kibler and Newmann,1979) (cited in Guern *et al*.,1987); *Hyoscyamus niger* (Hashimoto and Yamada,1983) showed the presence of tropane alkaloids in the suspension culture but it is not yet used for commercial production. Tropane alkaloids; hyoscyamine and scopolamine have not been detected in the cell suspension culture of *D. myoporoides* (Bachmann *et al*.,1989; Betry *et al*.,1995; Fliniaux *et al*.,1997). These results show that by changing the physical form of the medium, a stable biosynthesis of tropane alkaloids did not take place in the unorganised state.

### 1.2.10.5.3 Organ Culture

Some plant species need morphological differentiation such as shoot and root for secondary metabolite biosynthesis; for example, *Daucus carota* (Komamine *et al*.,1989), *Atropa, Datura, Senecio, Cephaelis, Berberis* (Mothes,1955; Moths *et al*. 1985). Some secondary metabolites are biosynthesised in the shoots of different plant species. Table 1.7 lists some secondary metabolites produced by shoot culture. Tropane alkaloid biosynthesis in the cultured shoots depends on plant species. Regenerated shoots of *Hyoscyamus naticus* L can biosynthesise tropane alkaloids before root formation (Basu and Chand,1998). Cultured shoots of *D. leichhardtii* have the ability to convert hyoscyamine to scopolamine (Yamada and Endo,1984). Presence of nicotine and anabasine (Kitamura *et al*.,1985 a) in the cultured shoots of *D. myoporoides* indicated that biosynthesis of alkaloids in this species could be possible without root formation. However, presence of tropane alkaloids in the cultured shoots without root formation has not yet been reported in this species.
**TABLE 1.7** Example of cases in which shoot differentiation is necessary for enhanced secondary metabolism (modified from Payne *et al.*, 1991)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Catharanthus roseus</em></td>
<td>Vindoline, vinblastine</td>
</tr>
<tr>
<td><em>Chrysanthemum spp.</em></td>
<td>Pyrethrins</td>
</tr>
<tr>
<td><em>Digitalis spp.</em></td>
<td>Cardenolides</td>
</tr>
<tr>
<td><em>Heimia salicifolia</em></td>
<td>Quinolizidine alkaloids</td>
</tr>
<tr>
<td><em>Lupinus spp.</em></td>
<td>Quinolizidine alkaloids</td>
</tr>
<tr>
<td><em>Papaver somniferum</em></td>
<td>Morphinan alkaloids</td>
</tr>
<tr>
<td><em>Withania somnifera</em></td>
<td>Withanolides</td>
</tr>
<tr>
<td><em>Pelargonium spp.</em></td>
<td>Essential oils</td>
</tr>
<tr>
<td><em>Mentha spp.</em></td>
<td>Essential oils</td>
</tr>
<tr>
<td><em>Citrus spp.</em></td>
<td>Limonin, naringin</td>
</tr>
</tbody>
</table>
Since partial biosynthesis (conversion of hyoscyamine to scopolamine) of tropane alkaloids takes place in the aerial parts of *D. myoporoides*, a detectable activity of H6H has been found in the aerial parts of *Datura ferox* and *D. myoporoides*. Other enzymes such as (DAO and TRII) are also present in the aerial parts of *Datura* and *Atropa* (Hashimoto *et al.*, 1992). Although all enzymes of tropane alkaloid biosynthetic pathway have not been detected in the aerial parts, the presence of the mentioned enzymes indicated that the aerial parts have the ability to form those enzymes. A manipulation of environment, may trigger the expression of other enzyme activities.

Since regenerated shoots are not suitable as an organ for tropane alkaloid biosynthesis, root organ culture has been tested for production of a higher amount of tropane alkaloids in last few decades. A stable biosynthesis of tropane alkaloids in the cultured roots of the 3 *Duboisia* species was observed throughout the culture period. A high alkaloid producing cell line of *D. Leichhardtii* root culture also released scopolamine in the culture medium (Endo and Yamada, 1985).

Addition of different alkaloid precursors to the root culture medium showed different effects on alkaloid production. Addition of putrescine to culture medium increased the hyoscyamine and scopolamine contents of the cultured roots without affecting growth, while a little effect was observed on alkaloid production, on adding ornithine, arginine and tropine to the culture medium (Yoshioka *et al.*, 1989). No enhancement of scopolamine content was observed in the *D. myoporoides* root culture obtained by repeated selection (Yukimune *et al.*, 1994 a). A two-stage culture method for scopolamine production was investigated and a circulation system for the high density culture of *D. myoporoides* roots was established (Yukimune *et al.*, 1994 c). The effects of vessel shape, dissolve oxygen concentration, inoculum size on scopolamine production were investigated in a circulation culture system of *D. myoporoides* root culture and were found to be useful for obtaining high-density root cultures (Yukimune *et al.*, 1994 b). However, slow growth of the untransformed root is not suitable for large-scale production.
1.2.10.5.4 Transformed Organ Culture

Some of the problems of normal organ culture have been overcome using the recombinant DNA (genetic engineering) where plasmids of *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* are used as gene vectors. Members of the genus Agrobacterium are soil-borne Gram-negative bacteria which belong to the family Rhizobium (Grierson and Covey, 1988). In this process, certain genes from the virulence plasmid are inserted into the host cell genome. The inserted T-DNA includes genes that modulate the cytokinin and auxin levels in the host or the efficiency of the cellular perception of these PGRs and resulted in the formation of transformed shoots or roots. One of the characteristic features of *Agrobacterium* transformed cells is PGR independent growth in culture.

The infection can be carried out in field-grown or *in vitro* grown plants, on detached leaves, leaf discs or stem segments from greenhouse grown plants. The surface sterilised leaf-midrib or the stems are scratched with an hypodermic containing a thick bacterial suspension and a small droplets (about 5-10μL) are inoculated. An alternative way for transformation is to co-cultivate plant protoplasts with the bacteria. After formation the transferred organs are separated and placed in a semi-solid medium with antibiotics to eliminate bacteria (Grierson and Covey, 1988).

Transformation by wild type *A. tumefaciens* induces tumor (crown gall) formation that consists of outgrowths of undifferentiated callus-like tissue, which do not form any root or shoot. The virulence is known as Ti for tumor induction. When auxin associated loci are deleted, the plant does not produce gall tissue but induces growth of teratomas, which are tumors containing abnormal differentiated cells and this treatment showed a tendency to form shoots. These transformed shoots are cultured *in vitro* and grow in the absence of PGRs. Similarly, when cytokinin locus is deleted, root formation occurs. Formation of transformed organs suggests that genes at the loci are involved in regulating PGR levels in the tumor (Weiler and Schrodes, 1987).
Shooty teratoma formation as well as the production of secondary metabolites in the transformed shoots has been investigated in some plant species; for example, tropane alkaloids in *Atropa belladonna* (Saito et al., 1991; Subroto et al., 1996a), nicotine in *Nicotiana tabacum* (Saito et al., 1991), steroidal alkaloids (Saito et al., 1991; Subroto et al., 1996a). Presence of steroidal alkaloids in the transferred shoots of *Solanum eleagnifolium* (Alvarez et al., 1994) and *Solanum aviculare* (Subroto et al., 1996a) has been reported recently. Presence of tropane alkaloids in the transformed shoots has not yet been reported. However, co-culture of *Atropa belladonna* transformed roots and *Duboisia* hybrid shooty teratomas in dual shake flasks and bioreactors showed that, hyoscyamine released from the crushed roots can be translocated and transformed to produce significant levels of scopolamine in the shoots (Subroto et al., 1996b; Mahagamasekera and Doran, 1998). These results indicated that, a stable tropane alkaloid biosynthesis did not occur in the genetically transformed shoots.

Fast growing adventitious root or tumorous ‘hairy root’ disease is associated with infection by *Agrobacterium rhizogenes*. On infection, the Ri (root-inducing) plasmid is transferred into the plant genome and causes alteration in auxin metabolism. Plants or callus cultures infected with the bacterium produce a proliferation of ‘hairy root’ which grow in the absence of PGRs (Cresswell, 1991).

Transformed roots grew faster than untransformed root culture. In some tropane alkaloid producing plant species, the transformed roots produced similar concentration per unit dry weight of hyoscyamine and remained stable for long time; for example, root culture of *Hyoscyamus nicaeas* was stable for 8 years (Flores and Filners, 1985a and b), *Datura stramonium* for 6 years (Flores and Filner, 1985a and b; Maldonado-Mendoza et al., 1993).

Early studies on the production of secondary metabolites by hairy root cultures were concentrated on plant species of the Solanaceae family (Deno et al., 1987; Sharp and Doran, 1990). These culture systems are now used in 26 families such as; Araliaceae, Boraginaceae, Cruciferae, Leguminosae etc. and 78 species of higher plants (Loyola-vargas and Miranda-Ham, 1995). Although this procedure
gave a good result in better biomass production, it has not yet been used for large-scale production.

1.2.10.5.5 Plant Regeneration

For increased secondary metabolite production in vivo, PTC techniques have been tested by regenerating complete plant of different plant species. Plant regeneration by using PTC occurs through 2 methods such as; 1) Somatic embryogenesis and 2) organogenesis. Somatic embryogenesis is the development of embryo structures from plant tissue of non-sexual origin. Embryos can be directly produced from the source plant tissue (Backs-Husemann and Reinert, 1970) (cited in Rao, 1987). Indirect somatic embryogenesis refers to the differentiation of embryos from undifferentiated callus or suspension cultures (Sharp et al., 1980; Vasil and Vasil, 1986). Embryogenesis has been reported in more than 100 species of various plant families (Rao, 1987).

Plant regeneration by organogenesis has been achieved either through direct shoot-bud formation on explant or via callus phase. The regenerated shoot-buds then elongated to a complete shoot. The complete shoot is then transferred to rooting medium to induce root formation on the cut end of the differentiated shoot. After formation of the roots, the regenerated complete plant is transferred to a suitable sterile potting mix in small pots and incubated for few weeks in the same incubation temperature and light used during the plant regeneration procedure. The properly developed regenerated plant is then transferred to ambient environmental conditions.

Secondary metabolite contents of the in vitro regenerated plants have been investigated in a few plant species. Regenerated plants of Catharanthus roseus contained as much serpentine as the parent plant (Abou-Mandour et al., 1979) (cited in Rao, 1987). Physalin profile of the regenerated Physalis minima plant was similar to that of the complete plant (Sipahimalani et al., 1981). The regenerated flowering plants of Atropa belladonna contained the principal belladonna alkaloids in quantities comparable to plants raised from seeds (Eapen et al., 1978). Solamargine
content of the seeds from the regenerated plants of *Solanum paludosum* has been found to be the same as in the wild fruits (Badaoui *et al.*, 1996). Tropane alkaloid content of *Datura innoxia* regenerated plant has been found to be comparable to that of the normal plant (Hiraoka and Tabata, 1974). Three androgenic *Datura innoxia* Mill micropropagated plants contained higher levels of alkaloids than plants grown from seeds (Gontier *et al.*, 1993). In metabolically engineered *Atropa belladonna* plants, biosynthesis of an improved level of tropane alkaloids has been reported (Yun *et al.*, 1992).

*Duhoisia hybrid* regenerated plants contained low level of tropane alkaloids than the parent plant (Lin and Griffin, 1992b). *D. myoporoides* plants were regenerated from stem derived callus (Kitamura *et al.*, 1980) and shoot-buds regenerated on the leaf (Kukreja *et al.*, 1986). The alkaloid contents of these regenerated plants have not been reported. In the regenerated plant, normal tropane alkaloid patterns are obtained after development into the mature plant (Kitamura *et al.*, 1985b; Lin and Griffin, 1992b). These results indicated that, production of high yielding strains for tropane alkaloid production in *D. myoporoides* has not yet been established.

### 1.2.11 Some Considerations Regarding this Research Strategies

Different environmental factors can affect organogenesis and differentiation in different secondary metabolite producing plant species. No general optimal formulation of environmental factors were found in the literature for organogenesis and differentiation in secondary metabolite producing plant species. However, for a specific plant species, an organogenic response and differentiation can be obtained by manipulating the culture environment. It is apparently necessary to select optimal conditions for organogenesis and differentiation while working with a particular tropane alkaloid producing plant species. The optimal condition may help to express tropane alkaloid biosynthesis at an early stage culture or to regenerate a plant with higher biosynthetic ability.
Different PTC techniques were tested for large-scale production of secondary metabolites. If the secondary metabolite biosynthesis is stable in the unorganised state, then it can be produced on a large-scale using suspension culture. On the other hand, secondary metabolite biosynthesis which requires differentiation of tissues can be produced by organ culture. However, the problem of tropane alkaloid biosynthesis in the unorganised state and by shoot culture is not yet solved.

Tropane alkaloid biosynthesis either by manipulating environmental factors or by using different PTC techniques showed similar results. The biosynthesis is unstable in the unorganised state and stable in the cultured roots. For cultured shoots, the biosynthesis takes place depending on the plant species. In the mature plant, organization of cells in the aerial parts is different from that in the roots. During shoot or root regeneration on callus, cell organization takes place step by step. For tropane alkaloid-producing plant species, it is not clear whether organization of cells in the cultured shoots is similar to that in the complete plant. It is also not known whether cell organization in the cultured shoots takes place earlier than in the cultured roots.

The literature gave no information, at what level of tissue differentiation in the cultured roots does biosynthesis of tropane alkaloids takes place. There are no reports on the level of tissue differentiation related to the ability for transforming hyoscyamine to scopolamine in the regenerated shoots, the presence of nicotine and the absence of hyoscyamine in the regenerated shoots without root formation. The same situation also has been found in the genetically transformed shoots. These reports indicate that, application of different PTC techniques alone is not enough to get a solution to obtain stable biosynthesis of tropane alkaloids. Before manipulating the environment or applying PTC techniques, it is essential to identify the difference in cellular organization between the cultured and the mature plant materials. A suitable solution to this problem will help further investigation. Later by manipulating environment or by applying special technological procedures, it will be possible to convert the lower expression to a stable biosynthesis of tropane alkaloids in the unorganised state or in the cultured shoots.
It has been found that the environmental factors required for organogenesis, differentiation and alkaloid production in culture are the same. Among these factors, PGRs are the most influential. For investigation of cellular organization in different stages of development, PGR combinations and concentrations can be varied to affect organogenesis, differentiation and tropane alkaloid production in cultured plant materials.

From the literature, it is possible to use PTC for the production of secondary metabolites. Some secondary metabolites are now being produced by using cell suspension culture. For tropane alkaloid production by using PTC, some basic questions are yet to be answered.

1.2.12 Histochemical Analysis: Organogenesis, Differentiation and Alkaloid Localization

1.2.12.1 Histochemical Technique

In 1826, the French botanist Francois-Vincent Raspail first introduced the idea of combining chemistry with the vision of the microscope. Plant histochemical analysis is a combination of histology, analytical chemistry and biochemistry. By using this technique study of the anatomical structure of the tissue component; identification, localization and quantification of the specific substances, reactive groups and enzyme catalysed activities in the cells and tissues can be possible.

The early stages of the development of the histological technique (also known as microtechnique) were mainly concerned about the morphological structure of the tissues. At the turn of this century, the technique was expanded which includes tissue fixation, dehydration, paraffin infiltration and embedding, sectioning, staining and mounting. Sections of fixed, dehydrated and embedded tissues retain only the colourless macromolecular constituents of the living tissues. The colourless sections
gave useful information when examined by phase contrast or interference contrast optics (O'Brien and McCully, 1981).

Histological techniques were developed with the improvement of microscope, use of different dyes and staining methods. By 1900, only natural dyes were used in this technique. Recently, different synthetic dyes have been developed. Among the dyes, most commonly used in botanical microtechnique are safranin, fast green, hematoxylin, toluidine blue, orange G, toluidine blue O and aniline blue (Jensen, 1962).

1.2.12.2 Stains (dyes) and Staining of Plant Tissue Sections

A dye is an organic molecule that contains one or more specific groups called chromophores, usually aromatic rings bonded in such a way that the molecule absorbs electromagnetic radiation in the visible part of the spectrum, appearing coloured to our eyes. The 2 most important chromophore groups are quinonoid rings and azo linkages. In the dye, there also exist groups that allow it to bind to a substrate. Depending on the type of this group, the dye may form cations, anions or zwitterions. Cationic dyes contain basic groups and are known as basic dyes, (e.g., safranin). Anionic dyes contain acidic groups and are known as acidic dyes, (e.g., fast green). Both acidic and basic dyes are manufactured as salts. Depending on the degree of dissociation of the salts, their net charge in solution may vary. A solution of toluidine blue O, a basic dye, has a pH of about 2.2 (O'Brine and McCully, 1981). The sections of the tissues also contain groups that can ionise in water.

When tissues are stained with certain cationic dyes, some tissue structures stain a colour that is different from that of the dye in solution and such dyes are known as metachromatic. Toluidine blue O is a metachromatic dye and depending on the pH of the dye solution, it shows a variation in staining of the macromolecules present in the plant tissues. Hand-cut sections of fresh plant materials stained with 0.05% toluidine blue O (in water) mounted in water stained cell walls impregnated
with lignin or other phenols and tannin-rich vacuoles green, turquoise or bright blue. Polysaccharides rich in carboxyl groups (e.g., pectic acid, alginic acid) or sulphate groups stain pink or reddish purple, DNA stains green or purplish blue and RNA stains purple or purplish blue (O’Brien and McCully, 1981).

Toluidine blue O 0.05% solution in benzoate buffer at pH 4.4 stains polyphosphates, polysulphates and polycarboxylic acids including alginic acid and pectic acid, red or reddish purple, RNA purple, DNA blue or purple, polyphenols and lignin, green or blue-green (Sidman et al., 1961; O’Brien et al., 1964; Feder and Wolf, 1965). A 0.5% solution of toluidine blue O in 0.1% sodium carbonate at pH 11.1 stains lignified walls and tannins, green; other components stained various shades of purple and some cell walls may stain metachromatically (Trump et al., 1961). In spite of this type of variation in staining, toluidine blue O is considered as an excellent general stain for survey work although not all organelles always stain (O’Brien and McCully, 1981).

1.2.12.3 Organogenesis

Histochemical analysis on organogenesis in callus and cell suspension cultures of several plant species have been reported; for example, carrot (Steward et al., 1958), endive (Vasil and Hildebrandt, 1966), tobacco (Thorpe and Murashige, 1970; Maeda and Thorpe, 1979). From these studies, it has been found that, the callus tissue consists of parenchyma cells which are highly vacuolated. In some tissues, scattered lignified elements was also found (Thorpe, 1980).

Depending on the culture period, cell division occurred in some regions in the callus tissue. These scattered cell division regions gave rise to regions of preferential cell division activity. Such regions of high mitotic activity led to the formation of meristematic centres of meristemoids, which are located on the surface or embedded in the tissues. The meristemoid consists of a spherical mass of small isodiametric meristematic cells. Depending on the culture conditions and the plant species used, those meristemoid regions gave rise to shoot primordia or root primordia (Bonnet
and Torrey, 1966). In *Atropa belladonna* both superficial and inner meristemoids formed roots (Thomas and Street, 1972). Formation of meristemoid cells from callus cells was examined in different plant species. In shoot-forming tobacco callus, the starch deposits decreased during meristemoid formation (Thorpe and Murashige, 1970; Ross and Thorpe, 1973). The main feature of these meristemoid cells was their small size. These cells are similar to the dividing cells of the meristem, which are most active mitotically in the plant and give rise to the bulk of the cells that make up the primary plant body (Gemmell, 1969). From the previous studies, it is not clear, whether meristemoid region in the calli of different secondary metabolite producing plant species is different from that of the non-producing plant species. For *D. myoporoides*, the effects of different PGRs on the meristemoid regions and the presence of different macromolecules are not yet reported.

### 1.2.12.4 Differentiation

Histochemical studies of the differentiated plant organs have also been investigated in last few decades. In a mature plant, the cells are organised into 3 tissue systems; the dermal, vascular and ground tissues. Each tissue system is continuous throughout the plant body. The dermal tissues or epidermis are generally a single layer of highly packed cells that cover and protect all young parts of the plant.

The continuum of xylem and phloem throughout the plant forms the vascular tissue system which functions in transport and support. The water-conducting elements of xylem are elongated cells of 2 types; tracheids and vessel elements. Both types of cells are dead at functional maturity but they produce secondary walls before the protoplast dies. Tracheids are long, thin cells with tapered ends and functions in support as well as water transport. Vessel elements are generally wider, shorter, thinner walled and less tapered than tracheids. They are aligned end to end forming long micropipes, the xylem vessels. The ground tissue system makes up the bulk of a young plant filling the space between the dermal and vascular tissue systems.
Taking xylem cells as a model system, histochemical studies of differentiated plant organs can be divided into 3 different types; 1) regeneration of vascular tissue in wounded stems and leaves; 2) induction of differentiation in plant tissue cultures and 3) formation of secondary xylem elements following cambial reactivation.

Plant growth regulators play an important role in tissue differentiation. Auxin has been found to be a limiting factor in wound xylem differentiation (Jacobs, 1952; 1954), primary vascular differentiation (Wangermann, 1967), secondary xylem differentiation (Digby and Wareing, 1966; Wareing et al., 1964) and in tracheary element formation in cultured callus tissues (Jeffs and Northcote, 1966; Wetmore and Sorokin, 1955; Wetmore and Rier, 1963).

Sorokin et al., (1962) investigated the effects of auxins and kinetin on the development of the xylem vessels in the stem and bud of the pea plant during the inhibition of lateral bud development. After auxin treatment, they observed cambium activation and abnormal growth; hyperplastic tissue and imperfectly formed xylem in the stem sections. Kinetin addition resulted normal growth and regular xylem development.

Jacobs (1998) investigated the effect of IAA and cytokinin on the control of the normal differentiation and regeneration of tracheary cells and sieve-tube in the Coleus blume. They found that IAA from the leaf blade controlled the timing of leaf abscission and the ability of the petiole to transport IAA from the blade of the abscission zone declines with leaf age. Using cytokinins and auxins, tracheary elements were induced in the mesophyll cells freshly isolated from the leaves of Zinnia. It was proved to be an useful model system for studying differentiation (McCann, 1997).

Tuominen et al., (1997) investigated the radial distribution pattern of IAA across the developing tissues of the cambial region in the stem of hybrid aspen (Populus tremula L. x Populus tremuloides Michx). They reported that IAA has a role in regulating the rate of physiological process such as cell division, the duration of developmental process such as xylem fibre expansion. The authors suggested that
IAA functions as a morphogen, carrying positional information during xylem development. Histological investigation on tracheid differentiation in *Larix laricina* cambium cultured on a defined medium containing various concentrations of NAA revealed that auxin was essential for cambial growth and tracheid differentiation (Leitch and Savidge, 1995).

Kalev and Aloni (1998) studied the hormonal mechanisms which control tracheid differentiation in the hypocotyl of young *Pinus pinea* L. seedlings. They found that the tracheids were redifferentiated from parenchyma cells in the hypocotyl. The experimentally induced tracheids have unique shapes and patterns and were different from the primary and secondary tracheids formed before the experiments. Auxin NAA caused the redifferentiation of short tracheids and showed a discontinuous pattern across the hypocotyl.

An abnormal pattern of secondary vascular development in the tomato mutant diageotropica was observed in absence of ethylene (Zobel, 1974). Depending on the combinations and concentrations of cytokinin and auxins, different patterns of xylem formation can take place in the shoots (Dalessandro and Roberts, 1971). However, there has been no investigation done on the effect of different cytokinins and auxins combinations and concentrations on the xylem formation in the regenerated shoots of *D. myoporoides*. Whether the pattern of xylem formed in the cultured shoots remains same or any change takes place after formation of root, is not clear. Investigation on the cell organisation, particularly on xylem formation in the regenerated shoots may indicate a relationship between cytokinin/auxin combinations and cell organisation in the cultured shoots before root formation.

1.2.12.5 Alkaloid Localization

Except cell constituents of the tissue component, different chemicals such as; alkaloids, pigments etc. are present in the plant tissues, which are identified by using 'microchemical methods' (Johansen, 1940). Microchemical methods include microchemistry in which qualitative reactions are employed to identify and localize
different chemicals present in the plant tissues. For microchemical tests, fresh untreated plant materials are used. This helps to keep the chemical nature of the desired compounds unchanged. By using different reagents, presence of lignin, cellulose, proteins, alkaloids, fats and oils in the fresh plant materials are carried out.

Localization of alkaloids in the fresh plant materials is carried out by using different colour reagents. Alkaloid colour reagents are generally used as qualitative test for the presence of alkaloids. One of the most characteristic properties of alkaloids is that of forming complex double salts with certain metallic halides. These double salts are nearly insoluble in water, so that a trace amount can be detected by their formation (Henry, 1924; Johansen, 1940). The best of such reagents are 5% aqueous solutions of either gold or platinic chloride (Johansen, 1940). The others are; mercuric chloride, ferric chloride, lead tetra chloride, telluric chloride, thallic chloride, iodoplatinate (Henry, 1924; Cromwell, 1955; Stevens, 1986).

Some double metallic halides are used as alkaloid precipitants and most commonly used for detecting their presence; for example, potassium mercuric iodide or Mayer’s reagent, potassium bismuth iodide or Dragendorff’s reagent, potassium cadmium iodide or Marme’s reagent, iodine in potassium iodide or Wagner reagent (Henry, 1924; Cromwell, 1955; Jensen, 1962; Stevens, 1986). A number of acids form insoluble complex double salts with alkaloids; for example, picric acid or Hager’s reagent, phosphomolybdic acid or Sonnenschein’s reagent, phosphotungstic acid or Scheibler’s reagent (Henry, 1924; Cromwell, 1955). Specific reagents are used for a particular type of alkaloid; for example, cyanogen bromide is used to identify nicotine (Stevens, 1986). For identification of hyoscyamine and scopolamine using by TLC, Dragendorff’s (Bhandary et al., 1969; Tabata et al., 1972; Lindsey and Yeomen, 1983) and iodoplatinate reagent (Lindsey and Yeomen, 1983) were used as spray.

In some alkaloid producing plant species, colour reagents have been used for localizing different alkaloids in different plant organs. Yoder and Mahlberg (1976) localized alkaloids in the various tissues of different plant organs of Cataranthis roseus using Jeffrey (Johansen, 1940), Dragendorff, CAS (Farnsworth et al., 1964) and IKI as alkaloid indicators. Corsi and Biasci (1998) localized the alkaloids in
both root and shoot tips in the seedlings of *Conium maculatum* L by using Wagner, Dragendorff's reagent, 1% picric acid, phosphomolybdic acid and IKI as alkaloid indicators and identified the possible biosynthetic site for the alkaloids. Tropane alkaloids were also localized in the different plant tissues of various plant species (James, 1950; Ferreira *et al.*, 1998). However, colour reagents have not yet been used for localizing tropane alkaloids in different plant organs of *D. myoporoides*.

In the cultured plant materials, colour reagents are also used for localizing different alkaloids. Sanguinarine alkaloid in the cell culture of *Macleaya* species has been localized by hexachloroplatinic (iv) reagent (Newman, unpublished data) (cited in Luckner *et al.*, 1977). By using colour reagents, the alkaloids have not yet been localized in the cultured tissues and organs of *D. myoporoides*.

In conclusion, by using colour reagents, localization of alkaloids in the cultured tissues and organs will indicate a probable relationship between cell organization in the different stages of plant regeneration and alkaloid biosynthesis in the cultured plant materials of *D. myoporoides*. 
1.2.13 Analysis of Plant Materials for Nicotine, Hyoscyamine and Scopolamine

*Duoboisia myoporoides* R. Br contains both tropane and pyridine alkaloids (Endo and Yamada, 1985; Kitamura *et al.*, 1985a). Though this study was aimed at analysing hyoscyamine, scopolamine and nicotine present in the cultured plant materials, the literature review also covers other related alkaloids for completeness. Because of their medicinal importance, extraction and analysis of tropane alkaloids are a major subject of interest.

Analysis of tropane alkaloids can be divided into 1) extraction and 2) identification and quantification. For extraction of alkaloid bases present in the plant material, either of the two procedures is followed; 1) the alkaloid is extracted overnight with EtOH-NH₄OH and the extract evaporated to dryness. The residue is dissolved in acid solution, make alkaline and then extracted with chloroform. The chloroform phase is evaporated to dryness and the residue analysed for the tropane alkaloids and 2) the alkaloid is extracted with acid, filtered and the resultant solution make alkaline before being extracted by organic solvents. The organic extract of the tropane alkaloid is then analysed.

For extraction, different acids, e.g., HCl (Lin and Griffin, 1992b), H₂SO₄ (Yukimune *et al.*, 1994 d), different organic solvents e.g., chloroform (Yukimune *et al.*, 1994 d) and different clean up procedures (Tabata *et al.*, 1972; Yukimune *et al.*, 1994 d) have been reported. Nitric acid is not used since it is an oxidising agent even when dilute.

Analyses of the mature plant materials, cultured tissues and organs showed a difference in the number and the amount of alkaloids. These alkaloids were identified by using paper chromatography (Bhandary *et al.*, 1969) and TLC (Kitamura *et al.*, 1985a). Identification as well as estimation of alkaloids are generally carried out by using spectrophotometry or IR (Tabata *et al.*, 1972), HPLC (Subroto *et al.*, 1995), mixed column high-performance liquid chromatography (Mandal *et al.*, 1991), direct photodensitometry of thin-layer chromatogram (Chu *et
al., 1969), GC-FID (Sharp and Doran, 1990; Yukimune et al., 1994 b), GC-MS (Endo and Yamada, 1985; Hashimoto and Yamada, 1989; Sharp and Doran, 1990; Yukimune et al., 1994 d), NMR (Naqvi et al., 1998).

1.2.13.1 Analysis of Field-Grown Plant Materials

In 1880, Gerard isolated tropane alkaloids from *D. myoporoides* R. Br. leaves as a crystalline substance named "duboisine." In 1887, Ladenberg identified "duboisine" as scopolamine. Later in 1890, both scopolamine and hyoscyamine were isolated from the same leaf sample (Schering and Co., 1890) (cited in Coulson and Griffin, 1967). In 1941, Barnard and Finnemore (1945) were the first to undertake a systematic analysis of *D. myoporoides* R. Br. plant materials obtained from a region between Queensland and New South Wales. They made assays of leaves from 60 different locations and found that the alkaloid content varied between 0.6% and 3.1% on a dry leaf weight basis. It has been found that, as with other classes of alkaloids, hyoscyamine and scopolamine occur as free bases (Trautner et al., 1948). These 2 alkaloids were determined as total alkaloids (Trautner and Roberts, 1948). Because of their structural similarity, their analytical separation was found to be difficult at that time (Bottomley and Mortimer, 1954).

Trautner and Shaw (1945) described a method for separation of alkaloids by preparing their picrate salts. The plant materials were acidified to make it free from other compounds and then made alkaline with ammonia. After removal of ammonia and volatile amines, the alkaloid solution was transferred to either chloroform or phenol-chloroform solution for titration with dimethyl-amino-azo-benzene. The products of titration were fractionated by preparing their picrate salts. Trautner and Roberts (1948) developed a method for separating hyoscyamine and scopolamine. The powdered plant materials were diluted with sodium carbonate and percolated with benzene. The extract was then separated on a silica column. This method was found to be applicable to alkaloid mixtures above 6 to 10 mg.

In 1948, Evans and Partridge, described the separation of hyoscyamine and scopolamine by column chromatography using an aqueous buffer. Bottomly and
Mortimer (1954) modified this method by using phosphate buffers and chloroform. By varying the pH of the buffers and the eluent composition, the alkaloids were successfully separated. After equilibration, the alkaloids in the organic layer were quantified by titration with p-toluenesulphonic acid in chloroform using dimethyl yellow indicator. The alkaloids were identified by their distribution coefficient.

Bottomly and Mortimer (1954) used a Chapman extractor for alkaloid extraction. The sample was allowed to stand overnight in ammoniacal chloroform and then percolated for five hours with 1M sulphuric acid. After clean up with sulphuric acid and water, the chloroform extract was evaporated to dryness under reduced pressure. The residue was dissolved in chloroform and titrated with p-toluenesulphonic acid using dimethyl yellow indicator to determine the total alkaloid bases.

The alkaloids were identified by using partition chromatography. A chromatography column was prepared from kieselguhr and pH 7.8 potassium phosphate buffer. The chromatogram was run by collecting 5mL fractions using a series of eluents from ether to chloroform. Each fraction was titrated with p-toluenesulphonic acid to obtain the percentage of that alkaloid compared to the total bases. The identity of each alkaloid was confirmed by the melting points of their picrate salts. This method was unable to extract nicotine.

Coulson and Griffin (1967) used Soxhlet extractor for alkaloid extraction and the sample was made alkaline with calcium hydroxide. The extract was cleaned up using a kieselguhr column and rechromatographed on another kieselguhr column where the eluate fraction containing the alkaloid bases were collected and titrated with sulphuric acid. The titrated bases were recovered and further purified by chromatography. The alkaloids were identified by their melting points, C, H, N composition and IR spectra. This method extracted only tropane alkaloids.

Luanratana and Griffin (1982) used a different method for extraction of nicotine and nornicotine from Duboisia hopwoodii sample. The sample was extracted with ethanol and made acidic. The pigment was removed from the filtrate by shaking with diethyl ether. The filtrate was then made alkaline and the alkaloids extracted with chloroform. Nicotine and nornicotine were separated by adding
NaOH, followed by CHCl₃ extraction of the crude extract. The products were identified by their melting points, C, H, N compositions and GC-MS spectra. Scopolamine and nicotine present in the root sample were separated by using different chromatography columns and diethyl ether-ethanol eluents.

Gritsanapan and Griffin (1991) analysed leaf samples collected from Mt. Glorious, Brisbane; Platapus Rock, Tinaroo Dam, North Queensland; Brooklyn Holdings, North Queensland; Acacia Plateau, near Killarney, South Queensland and the University of Queensland Agricultural Science Farm at Redland Bay. A crude extract of total alkaloids was obtained from dry powdered leaves (0.4 to 1.7 kg) after extraction with ethanol and evaporation. The residue was extracted with sulphuric acid, filtered, made alkaline with ammonia and extracted with chloroform. After neutralisation with sulphuric acid and subsequent dilution with water, the crude oily mixture was fractionated by the addition of sodium hydroxide followed by extraction with chloroform. The fractions were resolved into their respective alkaloids on a kieselguhr column containing pH 6.5 Pi buffer. The column was successively eluted with diethylether, chloroform and ammoniacal chloroform. For a few samples further chromatography was done on an alumina column and eluted with different composition of ethanol/diethylether. A silica column using chloroform-methanol-ammonia (85:14:1) as the eluent was also used. The alkaloids were analysed by GC and the contents of hyoscymamine and scopolamine determined.

1.2.13.2 Analysis of Cultured Plant Materials

In the past 10 years, there has been increasing interest in the extraction and quantification of tropane alkaloids in the cultured plant materials. This literature review covers different Duboisia species as well as some other tropane alkaloid-producing plant species.
1.2.13.2.1 Different *Duboisia* Species

Yamada and Endo (1984) analysed *D. leichhardtii* cultured plant materials by using a simplified extraction. The freeze-dried powdered samples (ca. 200mg) were immersed in 5mL of ethanol-28% ammonia (19 : 1 v/v) overnight. The macerated samples were then centrifuged and then extracted with basic alcohol and evaporated to dryness. The residue was dissolved in 1.6 mL of 0.1M HCl and the solution was made alkaline to pH 10 with dilute KOH. After extracting with 6 mL of CHCl₃, the CHCl₃ phase was evaporated to dryness. The dry residue was dissolved in 0.4mL of 1,4-dioxane-BSA (bis-TMS-acetamide) (10 : 1) solution and analysed for alkaloids.

The alkaloids were determined by GC-FID using a 2.5m x 3.4mm glass tube packed with 2% silicon OV-1. A temperature program of 150-280°C was used. Using two different columns, GC-MS analysis of tropane and pyridine alkaloids were carried out. The column used for hyoscyamine and scopolamine was a 1m x 3.4mm glass tube packed with 3% silicon OV-1. For nicotine, 1% silicon OV-101 was used.

Lin and Griffin (1992 b) used GC and radioimmunoassay (RIA) to determine scopolamine levels in *Duboisia* hybrid callus. The freeze-dried sample was extracted with ethanol-ammonia, the extract was evaporated to dryness and redissolved in chloroform. The organic layer was extracted with 0.5M HCl. The acid extract was made alkaline and reextracted with chloroform. After evaporation, the alkaloid residue was derivatised using hexamethyldisilizane before GC analysis with a FID using an OV-101 capillary column at 210°C. Detection and quantification were reported for hyoscyamine and scopolamine but not for nicotine.

For radioimmunoassay, the antiserum of a single alkaloid scopolamine was prepared from goat. The alkaloids from plants extracted by a normal procedure and a series of standards were mixed with the tracer tritium scopolamine and the antiserum. The bound fraction was precipitated with 25% PEG 6000 solution and centrifuged. The supernatant was decanted and the precipitate dissolved in tissue stabiliser and mixed with Dimilume-30. After 2 hours, the radioactivity was measured by scintillation counting. Comparison of RIA and GC assay for scopolamine did not
demonstrate significant difference in the results. However, the RIA method is tedious and the antiserum is specific to one alkaloid, making simultaneous analysis of several alkaloids difficult.

Kitamura et al., (1985 b) used a shorter extraction method for the analysis of regenerated plants of *D. myoporoides* during development. The freeze-dried, powdered samples were extracted 3 times overnight with 20mL of 80% methanol at 26°C. The combined extracts were evaporated to dryness under reduced pressure below 50°C and the residue dissolved in 20mL of distilled water before filtration. The filtrate was extracted 3 times with 10mL of chloroform. The aqueous layer was adjusted to pH 9.0 with 0.1M NaOH and extracted 5 times with 10mL of chloroform. The combined chloroform layer was analysed for alkaloids after drying with sodium sulphate.

The alkaloids were identified and quantified by TLC and GC. The GC was fitted with FID and 2.1m x 3mm glass column. For pyridine alkaloids, the column used was 10% DC550. The column temperature was 160°C. For tropane alkaloids, the column was packed with acid-washed, silanised chromosorb W containing 1.5% SE-30. The column temperature was set at 200°C. The alkaloids were derivatised with N,O-bis-(trimethylsilyl) acetamide before analysis. In this method, the extraction was short but the analytical step was long and tedious. It required different columns and chromatographic conditions for pyridine and tropane alkaloids. For the tropane alkaloids, derivatisation was required which was a time consuming step. The author did not report the recovery efficiency for the method nor any other validation parameters.

For dual wave length densitometry of the TLC (Silica gel GF_{254}) plates, the alkaloids were separated using the solvent mixture of chloroform-ethanol-28%ammonia (85 : 14 : 1). Pyridine alkaloids were determined at 260nm (\(\lambda_s\)) and 285nm (\(\lambda_R\)). Tropane alkaloids coloured by Dragendorff's reagent were determined at 505nm (\(\lambda_s\)) and 560nm (\(\lambda_R\)).
Yukimune et al. (1994 d) used a short extraction procedure for analysing cultured root samples of *D. myoporoides*. About 50mg of powdered sample was sonicated for 30 min in 5mL of 0.1M sulphuric acid. The solution was then made alkaline with 28% ammonia and passed through an Extrelut-1 column. After 5-10min, 6mL of chloroform was run through the column and the chloroform elute evaporated to dryness at 30°C and analysed for alkaloids.

Alkaloid contents were analysed by GC with FID using a CBP-1 capillary column. The temperature program of 120-260°C was used. GC-MS of the extract was run using a DB-1 capillary column. The temperature program of 120-260°C was used. Identities of 17 different alkaloids were confirmed by their mass spectra. No retention time was reported for nicotine, hyoscyamine and scopolamine.

### 1.2.13.2.2 Plant Species Other than *Duboisia*

Bhandary et al., (1969) used a different extraction procedure for extracting tropane alkaloids from cultured tissues and organs of *Atropa belladonna* L. and *Atropa belladonna*, Cultivar *lutea* Doll. Calli, cell suspensions and regenerated plantlets were powdered and treated with 0.1mL of 10% ammonia solution and soxhlet extracted with 20mL chloroform for 24h. The extract was made up to 40mL with additional chloroform and extracted with 25mL acetic acid-ethanol-water (6:5:89). 1-5mL of the upper layer was used for alkaloid analysis. The concentrated liquid culture medium was made alkaline with 10% ammonia solution and extracted with 3x40mL chloroform. The chloroform extract was evaporated to dryness, dissolved in 6% acetic acid and used for analysis.

The cultured roots (50mg) were ground with 2 drops of 1% sodium carbonate and extracted with 5x1mL chloroform. The combined chloroform extracts were shaken with 5mL acetic acid-ethanol-water and the upper layer used for alkaloid analysis. Alkaloids were identified by paper chromatography. Hyoscyamine and scopolamine present in all the extracts were estimated by colorimetry using the Vitali-Morin reaction.
Eapen et al., (1978) used a different extraction procedure for extracting tropane alkaloids from the cultured tissues of *Atropa belladonna* L. Dry and powdered tissues were macerated overnight with ethanol-28%ammonia (9:1) and refluxed with methanol. The methanol extract was evaporated to dryness and the residue dissolved in 0.1M hydrochloric acid and extracted with chloroform. The chloroform layer was shaken with 0.1M hydrochloric acid. The acidic extract was then made alkaline with 1M sodium hydroxide and extracted with chloroform. The chloroform extract was dried over sodium sulphate and evaporated to dryness. This fraction was used for colorimetry and GC analysis.

Estimation of total alkaloid was done by the Vitali-Morin method and calculated by using the atropine calibration curve. GC analysis was carried out on different glass columns packed with 2% OV-17 and 3% SE-30 on Chromosorb W 80 (100 mesh)\(^{-1}\). A FID detector was used. For the SE-30 column a temperature program of 150-210\(^{0}\)C was used and for the OV-17 column a temperature 230\(^{0}\)C, temperature program160-250\(^{0}\)C was used.

Collline and Yeoman (1986) analysed *Atropa belladonna* and *Hyoscyamus muticus* cultured plant materials as follows; the tissues were macerated with 5% ammonia in methanol and left overnight. The filtered extract was concentrated under reduced pressure and taken up in 15mL 0.1M hydrochloric acid. The acid extract was filtered and made alkaline with a buffer prepared from 10mL 0.2M ammonium chloride and 9.6mL 25% ammonia and made up to 20mL with water. The extract was added to an 'Extrelut' column. After 20min, the column was eluted with 40mL chloroform. The eluate was evaporated to dryness and stored in methanol before analysis. HPLC analysis was carried out by using Spherisorb S5 Octyl 25cmx46mm column with UV detector at 254nm. The column was eluted with an isocratic mobile phase consisting of 22.5% acetonitrile and a 50mM pH phosphate buffer.

Tabata et al., (1972) analysed callus and suspension cultured plant materials of *Scopolia parviflora*. The dry powdered samples were macerated overnight with a mixture of ethanol and 28% ammonia (9:1) and then soxhlet-extracted with chloroform for 6hr. After evaporating the extract to dryness under reduced pressure, the residue was dissolved in 20mL 0.1M hydrochloric acid and extracted with 3
aliquots of chloroform. The aqueous layer was made alkaline with 1M sodium hydroxide and extracted with chloroform thrice. After drying over potassium carbonate, the chloroform was evaporated and the residue was dissolved in 20mL of 0.05M hydrochloric acid before spectrophotometric analysis by using the modified Vitali-Morin method. Identification of hyoscyamine and scopolamine was by preparing their picrate salts and by direct comparison with authentic materials as well as by mixed m.p., co-chromatography (two solvents, TLC) and IR analysis.

Woo et al., (1995) analysed cultured root samples of Hyoscyamus niger using a shorter extraction method. Root samples (25-100mg) were ground in 5mL of the mobile phase, phosphate buffer [1(15mM)]', pH 2.5] -acetonitrile (65:35) containing 17.5mM SDS and centrifuged at 2000g, 5mL of mobile phase was added to the residue and left to stand at 50°C for 1hr before being centrifuged. After filtration the supernatant was used for HPLC analysis. Tropane alkaloids were analysed using a µ-Bondpack C-18 Column (3.9x300mm) with UV detector at 210nm.

Mahagamasekera and Doran (1998) analysed Atropa belladonna transformed roots and Duboisia hybrid shooty teratoma co-cultured samples for hyoscyamine and scopolamine. The dried ground tissues were extracted twice by using ethanol-ammonia (19:1) with sonication. The supernatants were pooled and evaporated to dryness. The residue was redissolved in HPLC eluent and analysed. The HPLC analysis was carried out with an Alltima C8 column (5µm, 250mmx4.6mm) at ambient temperature with UV detection at 205nm. The mobile phase was methanol-water (21:29) containing 0.005M low-UV PIC-B7 (heptane sulphonic acid; pH 4.1).

From literature, it has been found that different techniques have been used for analysing cultured plant materials. Paper chromatography is now not favoured because of its poor quantification ability. TLC analysis is mainly used for qualitative purpose. Quantitative analysis by TLC is performed by using scanning densitometers. Though scanning densitometers can give accurate and fairly reproducible results, TLC is rather time consuming in the development of the TLC plates.
There are various examples of using HPLC for the determination of tropane alkaloids from cultured plant materials. This technique generally allows good separation and sensitivity but the extracts have to be purified prior to injection onto the HPLC column. These purification steps can result in long assay times which are problematic where large number of samples are involved.

GC has been found to be a suitable technique for separating mixtures of alkaloids from cultured plant materials. Since this project was aimed at analysing nicotine, hyoscyamine and scopolamine, GC was selected for analysing the cultured samples obtained in this work. It has been found that the GC column determines the efficiency of the separation and the correct choice of both support material and stationary phase is of prime importance (Clark, 1978; Briggs and Simons, 1983; Pham et al., 1986). From the literature, it has been found that for analysing tropane alkaloids from cultured plant materials either a non-polar (OV-1; OV-101; SE-30) or an intermediate polar column (OV-17) column has been used. Some investigations used 2 different GC columns for analysing tropane and pyridine alkaloids in the same cultured sample (Yamada and Endo, 1984; Kitamura et al., 1985 b). Since this project was concerned with analysing cultured tissues and organs (shoot, root), BP-1 column was selected based on its polarity.

For quantification of alkaloids, the internal standard method is more desirable than direct calibration. Some investigators have used quinoline as an internal standard for nicotine (Kitamura et al., 1985 b). The internal standard santonin (Kitamura et al., 1985 b), homatropine bromide (Yamada and Endo, 1984) and homatropine (Yukimune et al., 1994 d) have been used as an internal standard for the quantitation of both pyridine and tropane alkaloids. For this work, homatropine was selected as an internal standard for the quantification of both pyridine and tropane alkaloids.

The available analytical techniques for analysing nicotine, hyoscyamine and scopolamine using by TLC, GC and HPLC are numerous. However, none of them has been method validated.
1.2.14 Mycorrhizal Status of the Secondary Metabolite Producing Plants

The mycorrhizae are universal symbionts of plants grown under field conditions (Smith and Read, 1997). The most common type of mycorrhiza is the arbuscular mycorrhizae (AM) which usually increases the growth of plants by enhancing nutrient uptake (Rhodes and Gerdemann, 1975; Marschner and Dell, 1994; Smith et al., 1994). AM fungi also have a beneficial effect on biological control of root pathogens (MacGuidwin et al., 1985; Garica-Garrido and Ocampo, 1989).

Allen (1982, 1992) reported an influence of AM fungi on plant growth through the production of hormonal compounds such as auxins, cytokinins, gibberellins and vitamins. Mathur and Vyas (1996) found an increased amino acid contents in AM fungal infected Prospis cineraria plant. An increased phenol contents in Terminalia arjuna and Jackfruit also have been reported when they were infected with the AM fungi (Kanakadurga and Rao, 1995; Sivaprasad et al., 1995).

Many recent workers found the presence of mycorrhizae in various medicinal plants. AM fungal colonization has been reported in various species of medicinal plants in India (Laksman and Raghavendra, 1990; Selvaraj and Subramanian, 1990; Sharma and Roy, 1991). Burni et al. (1994) reported AM fungal infection in Posralea corylifolia, a medicinal plant in Pakistan.

The AM fungal infection levels varied from one plant species to another (Sullia and Sampath, 1990). Ueda et al. (1992) found AM fungal colonization with infection ranging from 8% to 90% of the root length in 26 species of the medicinal plants in Japan. The authors also reported a variation of infection levels with the location. Some workers found a correlation between AM fungal infection and secondary metabolite production in various plant species (Volpin et al., 1994; Peipp et al., 1996). No such study has been conducted regarding the occurrence, biodiversity and taxonomy of AM in the Australian medicinal plants.
1.2.15 Results of the Literature Search

From the literature search concerning *D. myoporoides* R. Br., no published information was found about:

1) The effects of various cytokinin/auxin combinations used at the induction stage of the calli, on shoot differentiation
2) The effects of cytokinin/auxin combinations used at the induction stage of the calli, on cell organization in the differentiated shoots before root formation
3) Localization of alkaloids in different cells of mature plant organs
4) Relationship between cell organization and alkaloid localization in the cultured shoots without root formation
5) The effects of various concentrations of TDZ+IBA on shoot-bud induction
6) The effects of environmental factors on shoot growth
7) A validated methodology for the simultaneous extraction and quantification of nicotine, hyoscyamine and scopolamine
8) Occurrence of AM fungi in the roots of the field-grown trees.
1.3 AIM AND OBJECTIVES

AIM

This project was aimed to investigate the relationship between organogenesis, differentiation and histolocalization of selected alkaloids in *Duboisia myoporoides* R.Br.

OBJECTIVES

The objectives were:
1. To investigate the organogenic response of the mature leaf explant to various cytokinin/auxin combinations
2. To regenerate shoots and roots separately from the calli tissues in semi-solid medium
3. To study the effects of thidiazuron (TDZ+IBA) on shoot-bud induction on the leaf explant
4. To study the morphological difference between seedling and shoot differentiated from the calli induced in the BM supplemented with various cytokinin / auxin combinations
5. To regenerate shoots and roots separately in suspension culture
6. To study the effects of environmental factors on shoot growth without any root formation
7. To investigate the relationship between cell organization and histolocalization of selected alkaloids in the cultured plant materials
8. To analyse different cultured tissues and organs as well as mature plant organs for identification and quantification of nicotine, hyoscyamine and scopolamine by using GC-MS
9. To study the mycorrhizal status of the roots and alkaloid contents in the leaves of the field-grown trees.
Production of Cultured Tissues and Organs of
Duboisia myoporoides R. Br.
in Semi-solid Medium
2.1 INTRODUCTION

Plant tissue culture is a technique that can be used as a possible source of secondary metabolites. This technique is particularly applicable for those plant species which synthesise products of medicinal importance but are difficult to cultivate by conventional plantation methods due to problems of sexual or vegetative propagation. *Dabois* *ia myoporoides* R. Br. is one of those plant species which has medicinal importance and is difficult to cultivate (Griffin, 1985).

To obtain a higher yield of alkaloid, different PTC techniques have been tested. One of these techniques is the complete plant regeneration using different explants. Treatment of an explant with different cytokinin and auxin combinations can result in the formation of different types of tissues such as: 1) non-organogenic callus; 2) organogenic callus (Canteno et al., 1996; Kevers et al., 1996) [some authors used the terms ‘highly regenerative’ (Kukreja et al., 1986;) or ‘organogenous’ (Badaoui et al., 1996) to describe organogenic callus]; 3) shoot-bud and 4) root. Shoot-bud or root regeneration from the non-organogenic callus needs cytokinin and auxin combinations different from that used at the callus induction stage.

Two different types of organogenic calli are induced: 1) shoot-bud regenerating and 2) root regenerating calli. Shoot-bud or root can be regenerated from the organogenic calli using the same cytokinin and auxin combinations used at the callus induction stage. Shoot-bud induction on an explant results in a quicker plant regeneration procedure than via callus culture (Kukreja et al., 1986). In the different stages of the plant regeneration, combinations of cytokinins and auxins, which regulate plant growth, induce callus and cause organogenesis, must be identified by trial and error. These combinations vary from species to species.

Kitamura et al. (1980) reported the first plant regeneration of *D. myoporoides* R. Br. via organogenesis. Kukreja et al. (1986) reported the plant regeneration via shoot-bud induction on the leaf explant. However, they did not report the alkaloid biosynthetic ability of the regenerated plants. Kitamura et al., (1985 b) studied the biosynthetic ability of a regenerated and seed germinated *Dabois* *ia myoporoides* R.
Br. plant. They reported qualitative and quantitative differences between the alkaloid distributions in the regenerated plantlets and the seedlings. According to their observations, alkaloid content was highest in the leaves of the seedlings and roots in the 1-month-old regenerated plants. The leaves of the seedlings always contained the main alkaloids throughout development whereas the leaves of the regenerated plants contained no or few alkaloids at the early stages of development. After development, the leaves of the regenerated plant contained the main alkaloids. Whether these variations in alkaloid pattern are identical to the plants regenerated with other cytokinin and auxin combinations are not yet reported.

There are several studies on root culture, because alkaloids are synthesised in the complete plant roots (Waller and Nowacki, 1978) or cultured roots (Endo and Yamada, 1985). Chemical analysis of the cultured roots showed the presence of nicotine, hyoscyamine and scopolamine, which are found in the mature plant roots. Presence of alkaloids in the roots has been explained on the basis of the localization of the enzymes of alkaloid biosynthesis in the root cells.

In contrast to root culture, shoot culture has not yet been widely investigated in different D. duboisia species. This is probably due to the reported absence of tropane alkaloids in the shoots before root formation (Lin and Griffin, 1992b). In the previous reports, the absence of alkaloids in the cultured shoots before root formation has been explained on the basis of the gene expression (Yamada and Endo, 1984). Other factors have not yet been considered. For example, in the regenerated shoot, cells may not be sufficiently differentiated for alkaloid biosynthesis. Consequently, by varying cytokinin and auxin combinations, cells in the regenerated shoots may become differentiated.

The effect of cytokinin and auxin combinations on cell organization in the regenerated shoots before root formation has not yet been investigated in D. myoporoides. For this investigation, separated shoot and root culture from the non-organogenic and organogenic calli is necessary. Induction of the non-organogenic and organogenic calli using different cytokinin and auxin combinations and subsequent shoot and root culture from the induced calli will show a relationship between cytokinin and auxin combinations and cell organization and thereby alkaloid formation in the cultured shoots and roots.
For this study, it is necessary to determine the morphological differences between a cultured shoot (in vitro) and a seedling (natural). The morphological and subsequent histological study of the shoots regenerated from the non-organogenic and organogenic calli induced in the BM supplemented with various cytokinin and auxin combinations will show the probable relationship between shoot morphology and cell organization in the cultured shoots.

Generally, cytokinins and auxins are used for investigating the morphogenetic responses on different explants, organogenesis in non-organogenic and organogenic calli and in plant regeneration. The effects of other PGRs or compounds having plant growth regulating properties have not been studied in D. myoporoides for organogenesis to occur. Thidiazuron (TDZ) is one of the several substituted ureas that has been used for organogenesis in different plant species (Cousineau and Donnelly, 1991; Kapaun and Cheng, 1997; Aljuboori et al., 1998). The effects of TDZ on organogenesis in D. myoporoides have not yet been reported.

The present study has the following objectives;
1) To produce plants in the greenhouse by using the cuttings collected from the field-grown plant
2) To observe morphogenetic response on the leaf explant using different cytokinins/auxins combinations
3) To regenerate complete shoots from the non-organogenic calli
4) To regenerate complete shoots from the organogenic calli
5) To regenerate roots from the non-organogenic calli
6) To regenerate roots from the organogenic calli
7) To compare the organogenic ability of a selected cytokinin and auxin combination on the in vitro grown leaf explant with that of the leaf explant collected from greenhouse-grown cuttings
8) To examine the effects of TDZ on the shoot-bud induction on the leaf explant
9) To produce young seedlings by using seeds collected from greenhouse-grown cuttings
10) To observe the morphological differences in the shoot part of a seedling and a cultured shoot
2.2 MATERIALS AND METHODS

2.2.1 Chemicals and Tissue Culture Vials

All chemicals of macro and micro nutrients, PGRs, thidiazuron (TDZ), vitamins, sodium hydroxide, sodium hypochlorite, Tween 80 solution and sucrose were obtained from Sigma Chemical Company (USA). Agar was obtained from Bacto Laboratories. AR grade hydrochloric acid was obtained from Ajax Chemicals (Australia). The household bleach ZIXO (premium grade) was obtained from grocery stores. Milli-Q water (Millipore Australia) was used for making up stock solutions and media preparation.

Plastic Petri dishes (90x14mm), used at the callus induction stage, phytatrays used at the initial stage of shoot-bud development and for all other stages used glass and plastic vials [5.5cm x 5.0cm; 7.0cm x 6.5cm; (10.5cm x 8.5cm, catalog number p-4928)] fitted with lids having a 0.2μm pore, were supplied by Sigma Chemical Company (USA).

2.2.2 Source of Plant Materials

Duboisia myoporoides R. Br. plant materials were collected from Mount Annan Botanic Gardens (Figures 2.1- 2.3), where it was grown from cuttings (Appendix 1). For the work reported in this thesis, small branches were collected from Mount Annan Botanic Gardens and cuttings were prepared and grown in the controlled environmental conditions at the Cobbitty Plant Breeding Institute, University of Sydney. After 6 months, those cuttings were transferred to the greenhouse in UWS Macarthur. Leaves from both Mount Annan Botanic Gardens and greenhouse-grown cuttings were used for tissue culture work.
Text-Figure 2.1 Location of Mount Annan Botanic Gardens, at Mount Annan, near Campbelltown NSW, Australia.

Text-Figure 2.2 The bed area in Mount Annan Botanic Gardens where *Duboisia myoporoides* R. Br. plants, prepared from cuttings, were planted in the botanic garden natural habitat. The arrow indicates the particular bed (no. 23) in the figure.
Text-Figure 2.3 *Duboisia myoporoides* R. Br. plant (arrow) in the natural habitat of Mount Annan Botanic Gardens. The plant was prepared from cuttings, planted in November, 1993 and about 3 to 4 meter high.
2.2.3 Preparation of Cuttings

Stem sections, about 2.5 cm long were cut from the apical region of the well grown branches of a mature tree (Section 2.2.2). The leaves on the stem were reduced with a sharp knife. The lower portion of the stem was cut without injuring the tissues and soaked in a mixture of IBA + NAA (1000ppm in 50% ethanol) for 5 sec. The stems were then put in small pots (90mm inside diameter and 90mm height) containing potting mix consisting of 2 parts coarse sand (all purpose sand, Rocks Galore, N.S.W. Pty Ltd.), 1 part coarse compost and 1 part perlite (Perlite/Vermiculite media, R&D Aquaponics, chemicals Pty. Ltd.). The pot mix was moistened with tap water. About 10 cuttings were planted per pot which were placed in a chamber of controlled conditions of temperature and humidity (D-33 unit, inner temperature 28°C bottom heat and upper mist), at the Cobbity Plant Breeding Institute, University of Sydney (Fig. 2.4).

After 4 months of growth in the controlled chamber, about 10 to 15cm long, rooted cuttings were transferred to single pots (195mm inside diameter and 190mm height) and kept in the greenhouse of UWS Macarthur until flowering and fruiting (Fig. 2.5). The greenhouse was shaded with green shade cloth (50μm) and subjected to 16/8 hr light/dark cycle, a photosynthetic photon flux density of 500μmol s⁻¹m⁻² (OSRAM HQI-T 250W/D). During summer (December-February), the inside temperature of the greenhouse was maintained at (25-27)°C by a BRAEMAR (SE A 200 GY) air cooler. The pots were watered once a week.

2.2.4 Surface Sterilization of Explants

Mature, green and healthy leaves were used as explants in this experiment. The leaves were collected and washed thoroughly under running tap water without
Text-Figure 2.4 *Duboisia myoporoides* R. Br. cuttings (C) in a chamber of controlled conditions of temperature and humidity (bottom heat and upper mist) at the Cobbity Plant Breeding Institute, University of Sydney, NSW, Australia.

Text-Figure 2.5 Two-year-old *Duboisia myoporoides* R. Br. cuttings in the greenhouse-conditions of UWS Macarthur. Note the fruit (F) production on the cuttings arrowed.
damaging the tissues and then fixed in 70% ethanol for 30 sec. The leaves were then washed twice with sterile water. The excess water present on the explant was soaked in sterile tissue paper. Leaves were then transferred to a McCartney bottle containing 1% hypochlorite solution. After 10 min, the solution was replaced with fresh 1% hypochlorite solution and surface sterilization was continued with occasional shaking for another 10 min. The surface sterilized leaves were transferred to a sterile beaker containing sterile water. The time period was maintained by using a stopwatch. The leaves were washed 3 times using fresh sterile water and left in sterile water till the next step. The excess water on the leaves was removed by soaking on sterile tissue paper before use.

The surface sterilized leaves were transferred to a Petri dish with sterile forceps and the edge of the whole leaf removed with a sterile scalpel blade (Swann-Morton, B. S. 2982, I. S. O. 7740). The leaves were then cut into 1 cm pieces and transferred to the semi-solid culture medium by placing the leaf-petiole adaxial surface in contact with the culture medium. All steps of surface sterilization and arrangement of sterile explants were conducted in aseptic conditions in a laminar flow cabinet.

2.2.5 Preparation of Tissue Culture Medium

The plant tissue culture medium used in this experiment was based on the basal salt and vitamin amounts as formulated by Murashige and Skoog (1962) (Appendix 2). The medium was semi-solid with Bacto agar (0.9%) containing 3% sucrose. Individual stock solutions of macro and micro nutrients, vitamins, cytokinins and auxins were prepared at higher concentrations in Milli-Q water. Macro and micro nutrient solutions were stored in the cold room (4°C) and used within 1 year. Vitamin, cytokinin and auxin solutions were freshly prepared each month.
For preparation of medium, required volumes of the individual stock solutions of the micro and macro nutrients (Solution A) were mixed together, the required amount of Milli-Q water added and the pH of the solution was adjusted at 5.7 (T.P.S. Digital pH meter, Model no. 1852mV) by adding either 0.1M HCl or 0.1M NaOH. After agar and sucrose were added, the medium was sterilised at 121°C for 15min at 103.4Kpa pressure. The required volume of vitamins, cytokinins and auxins were mixed together (Solution B) and filter-sterilized by using 0.2µm filter membrane (sterile, MILLEX-GS, filter unit, non pyrogenic, MILLIPORE). After mixing sterile solutions A and B together with a sterile magnetic stirrer, the final medium was poured into sterile vials.

2.2.6 Incubation Conditions for Cultured Tissues and Regenerants

The cultures were incubated in an incubation chamber (Fig. 2.7, page 97) (2.2m x 2.3m x 3.0m provided with 120mm insulation and automatic air supply) at 25 ± 2°C by placing on a Zinc plated steel wire rack (25mm spacing). For light incubation, the culture plates or vials were placed on the rack at a distance 40cm from the light source (Fig. 2.7, page 97). A 14h photo-period of cool-white light from a 35W (OSRAM) fluorescent tube was arranged and the light intensity 15.2µmol s⁻¹m⁻² was measured by a light meter (Appendix 3). The mentioned light intensity was used in all experiments unless otherwise stated. Dark incubation was arranged by wrapping culture plates with aluminium foil.
2.2.7 Standardization of Complete Plant Regeneration

The complete plant was regenerated by using the procedure as described by Lin and Griffin (1992 a). The procedure includes the following steps.

2.2.7.1 Callus Induction

For callus induction, mature healthy green leaves collected from a greenhouse-grown cuttings (Section 2.2.3) were used as explant. Surface sterilised 1cm leaf segments were placed on the semi-solid MS basal medium supplemented with BA10^{-6}M + NAA54x10^{-5}M and incubated in the dark at 25 ± 2°C for 2 weeks. The induced calli were incubated in the dark for another 10 weeks.

2.2.7.2 Production of Green Callus

About 500mg (used throughout the experiment) (Kitamura et al.,1980) 11-week-old above mentioned callus pieces were transferred to the BM supplemented with BA0.1x10^{-6}M + NAA27x10^{-6}M and incubated in the light (Section 2.2.6) at 25 ± 2°C for 2 weeks.

2.2.7.3 Shoot-bud Induction

The green calli thus produced were transferred to the BM supplemented with BA22x10^{-6}M and incubated in the light (Section 2.2.6) at 25 ± 2°C for 8 weeks. Some of the 1 to 2-week-old shoot-buds were harvested for alkaloid analysis (Chapter 6).

2.2.7.4 Shoot Elongation

The shoot-buds formed were transferred to the BM supplemented with BA5x10^{-6}M + NAA0.5x10^{-6}M and incubated in the dark at 25 ± 2°C for 2 weeks and
in the light for 4 weeks. At the end of the incubation period, the differentiated shoots were harvested for alkaloid analysis (Chapter 6).

2.2.7.5 Root Induction

The regenerated shoots were transferred to the BM supplemented with IBA25x10^{-6}M and incubated in the dark at 25 ± 2°C for 2 weeks. The shoots were then transferred to fresh BM devoid of any PGR and incubated in the light for another 4 weeks.

The cytokinin / auxin combinations and growth conditions used in this study were also used in the shoot culture from the non-organogenic calli. By using these cytokinin / auxin combinations, it was possible to keep the cytokinin and auxin combination (BA+NAA) (only concentrations were varied at different stages) constant for greening of the callus, shoot-bud induction and shoot elongation to study the effects of cytokinins / auxins (used at the callus induction stage) on cell layer organization and alkaloid localization in the shoots differentiated from the non-organogenic calli.

2.2.8 Some Considerations Regarding Experimental Design

All the 3 alkaloids i.e, nicotine, hyoscyamine and scopolamine were detected by GC-FID in the differentiated shoots (from the standardization of plant regeneration) before root initiation. Based on the above results, different stages of the separated organ cultures from the non-organogenic and organogenic calli were selected for histochemical and GC-MS analyses. Since no alkaloids were detected in the shoot-buds, it was decided to analyse shoot-buds only by GC-MS and the differentiated shoots both by histochemical and chemical analyses. Figure 2.6 represents a flow chart for the histochemical and the chemical analyses of different tissues obtained at different stages of the separated organ cultures.
STAGES OF ORGAN CULTURE

Fig. 2.6 Flow chart for analysis of tissues and organs at different
2.2.9 Induction and Subculture of the Calli and the Regenerants

The surface sterilized (Section 2.2.4) leaf explant pieces were placed on approximately 15ml of semi-solid callus induction medium contained in Petri dishes (Section 2.2.1) sealed with para-film to prevent moisture loss. The callus induction medium composed of MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and cytokinins / auxins. Sixty five treatments consisted of a control (without cytokinin, auxin ) and cytokinins (BA and Kinetin), auxins (IAA, IBA, NAA, 2,4-D) individually or in combination in the range (0.10^{-5}, 10^{-6})M and (0.10^{-5}, 10^{-6}, 10^{-7})M respectively. The cultures were incubated in the dark (Section 2.2.6) at 25 ± 2°C and subcultured at every 3 week intervals in the fresh semi-solid media supplemented with the cytokinin / auxin combinations used for callus induction.

Six weeks after calli induction, the number of explants with calli was counted and the calli were classified into 2 different types such as, non-organogenic and organogenic with respect to appearance. The non-organogenic calli were transferred to 120mL glass tissue culture vials containing 50mL semi-solid media and incubated in the dark for 12 weeks (from the date of culture initiation). The average fresh weight of the 11-week-old non-organogenic calli was determined at the end of the incubation period. Callus formation, colour and compactness of the calli were judged visually. For shoot and root culture, the fresh weight and appearance of the 11-week-old calli were considered. From each type of cytokinin and auxin combination (BA + NAA, BA + 2,4-D, Kin + IAA, Kin + IBA, Kin + NAA and Kin +2,4-D), the callus with the highest fresh weight was selected for shoot and root culture.

At the end of the incubation period, some of the fresh 11-week-old calli induced in the BM supplemented with the selected cytokinin and auxin combinations were used for the histochemical analysis (Chapter 5) and some were harvested for the chemical analysis (Chapter 6). The rest of the calli were used for shoot (Section 2.2.10) and root (Section 2.2.12) culture.
Some of the fresh shoot-bud producing organogenic calli (within 1-2 weeks after induction) were used for the histochemical analysis (Chapter 5) and some were harvested for the chemical analysis (Chapter 6). The shoot-buds (healthy) formed on the organogenic calli attached with the explant were judged visually, counted and their number per explant was determined after 4 weeks. After formation of the shoot-buds, the cultures were grown in 120mL glass and plastic tissue culture vials and incubated in the light (Section 2.2.6). From these shoot-buds, shoots were differentiated in the semi-solid medium (Section 2.2.11). The calli with the highest number of shoot-buds were selected for shoot culture. Some of the 3-week-old shoot-buds were harvested for chemical analysis (Chapter 6).

Some of the fresh root-producing calli (2-week-old) were used for histochemical analysis (Chapter 5). The roots formed on the organogenic calli attached with the explant were judged visually, counted and their number per explant was determined after 4 weeks. After formation of the roots, the cultures were grown in 120mL glass and plastic tissue culture vials and incubated in the dark. The roots formed were used for root culture in the semi-solid medium (Section 2.2.13).

2.2.10 Shoot Culture from the Non-organogenic Calli

Eleven-week-old non-organogenic calli grown on the MS basal medium supplemented with the following cytokinin/auxin combinations: 1) 2,4-D10⁻⁷M; 2) BA10⁻⁵M + NAA10⁻⁶M; 3) BA10⁻⁵M + 2,4-D10⁻⁷M; 4) Kin10⁻⁵M + IAA10⁻⁶M; Kin10⁻⁵M + IBA10⁻⁵M; 6) Kin10⁻⁵M + NAA10⁻⁵M; 7) Kin10⁻⁵M + 2,4-D10⁻⁷M were used for shoot culture. The following stages as explained in the standardization of complete plant regeneration procedures were followed for shoot culture: 1) Production of green callus from the 11-week-old callus; 2) Induction of shoot-buds on the 2-week-old green callus and 3) Elongation of the shoot-buds to differentiate shoot.
2.2.10.1 Production of Green Callus

Eleven-week-old non-organogenic calli induced in the BM supplemented with the selected cytokinin and auxin combinations were transferred to the BM supplemented with BA 0.1 x 10^{-6} M + NAA 27 x 10^{-6} M and incubated in the light (Section 2.2.6) at 25 ± 2°C for 2 weeks. At the end of the incubation period, some of the green calli were harvested for chemical analysis (Chapter 6) and the rest were used for the shoot-bud induction.

2.2.10.2 Shoot-bud Induction

The green calli thus produced were transferred to the BM supplemented with BA 22 x 10^{-6} M and incubated in the light (Section 2.2.6) at 25 ± 2°C for 8 weeks. At the end of the incubation period, the number of shoot-buds produced was counted. The shoot-buds were incubated for 1 more week in the same medium and same incubation conditions to allow the shoot-buds to grow enough for transferring to the shoot elongation medium. Some of the 1-2-week-old shoot-buds attached with the calli were harvested for chemical analysis (Chapter 6).

2.2.10.3 Shoot Elongation

The healthy shoot-buds were separated from the calli and transferred to the BM supplemented with BA 5 x 10^{-6} M + NAA 0.5 x 10^{-6} M and incubated in the dark at 25 ± 2°C for 2 weeks to promote shoot growth. After 2 weeks the elongated shoots were transferred to the fresh medium containing the same cytokinin and auxin combination and incubated in the light (Section 2.2.6) at 25 ± 2°C for 4 weeks.

At the end of the incubation period, the number of leaves produced per shoot was counted (Kitamura et al., 1980) and the length of the leaves and the shoots (from the base to the upper end of the smallest leaf) were measured (Nielsen et al., 1993; Badaoui et al., 1996). Some of the basal stems of the differentiated fresh shoots were
used for the histochemical analysis (Chapter 5) and the rest of the shoots (leaves + basal stem) were harvested for the chemical analysis (Chapter 6).

2.2.11 Shoot Culture from the Organogenic Calli

Of the 3 concentrations of BA + IAA used, the callus induced in the BM supplemented with BA$10^{-5}$M + IAA$10^{-6}$M with highest number of shoot-buds was selected for shoot culture. The only shoot-bud producing callus induced in the BM supplemented with BA$10^{-5}$M + IBA$10^{-7}$M was also selected for shoot culture. The healthy shoot-buds were separated from the calli (as many as possible) and transferred to the fresh BM supplemented with the selected cytokinin/auxin combinations. The shoot-buds were then incubated in the light and at 25 ± 2°C (Section 2.2.6). At 3 to 4 week intervals, the cultures were transferred to the fresh BM supplemented with the selected cytokinin/auxin combinations used at the calli induction stage.

Some of the 4-week-old elongated shoots were harvested for alkaloid analysis (Chapter 6). The other shoots were transferred to 10.5cm x 8.5cm vials containing fresh media and incubated in the above mentioned incubation conditions for another 5 weeks. At the end of the incubation period, the length of the shoots (from the base to the upper end of the smallest leaf) and leaves (Nielsen et al., 1993; Badaoui et al., 1996), were measured and the number of the leaves counted (Kitamura et al., 1980). The fresh 9-week-old shoots were divided into 2 parts to give the shoot and the base of the shoot (the tissue to which the shoot attached) and analyzed independently by histochemical (Chapter 5) and chemical analysis (Chapter 6). About 20 regenerated shoots were incubated for another 6 to 7 weeks to observe the effect of a longer incubation period.
2.2.12 Root Culture from the Non-organogenic Calli

For root culture, 11-week-old non-organogenic calli induced in the BM supplemented with the following cytokinin and auxin combinations were selected: 1) BA10⁻⁵M + NAA10⁻⁶M; 2) BA10⁻⁵M + 2,4-D10⁻⁷M; 3) Kin10⁻⁵M + IAA10⁻⁶M; 4) Kin10⁻⁵M + IBA10⁻⁵M; 5) Kin10⁻⁵M + NAA10⁻⁵M; 6) Kin10⁻⁵M + 2,4-D10⁻⁷M. The calli were transferred to the media containing the following medium additives:

1) 1/2 MS salt + full vitamin and no PGR  
2) Full MS salt + full vitamin and BA10⁻⁶M + IBA10⁻⁵M  
3) 1/2 MS salt + 10mg L⁻¹ tropic acid  
4) 1/2 MS salt + full vitamin and BA10⁻⁶M + IBA10⁻⁵M  
5) Full MS salt + full vitamin + IBA25x10⁻⁶M

The cultures were incubated in the dark at 25 ± 2°C for 6 weeks and subcultured every 3 weeks in the fresh semi-solid media containing the same medium additives. After formation of roots, incubation was continued for 4 more weeks. At the end of the incubation period, the regenerated roots were harvested for alkaloid analysis (Chapter 6).

2.2.13 Root Culture from the Organogenic Callus

Organogenic callus with small roots was subcultured in the dark at 25 ± 2°C at every 3 weeks in the fresh BM supplemented with the same cytokinin/auxin combinations used at the callus induction stage. Some of the fresh 4-week-old roots were used for histochemical analysis (Chapter 5). The rest were harvested for chemical analysis (Chapter 6).
2.2.14 Selected Cytokinin and Auxin Combination Studied

The effects of BA$10^{-5}$M + IBA$10^{-7}$M on the organogenesis and differentiation on the leaf explants from the greenhouse-grown plant as well as from the shoot differentiated from the callus induced in the BM supplemented with 2,4-D$10^{-7}$M were observed. *In vitro* grown leaves of the shoots differentiated from the callus induced in the BM supplemented with 2,4-D$10^{-7}$M were cut into 1cm pieces and transferred to Petri dishes containing semi-solid BM supplemented with BA$10^{-5}$M + IBA$10^{-7}$M. All cultures were subcultured at every 3 weeks in the fresh media containing cytokinin and auxin combination used at the callus induction stage. The edge of the Petri dishes were sealed with para film and the cultures were incubated in the dark at 25 ± 2°C for 4 weeks. The cultures where roots formed were incubated in the dark for another 2 weeks. Shoot-buds induced on the organogenic calli were transferred to the culture vials (Section 2.2.1) containing 50mL semi-solid BM supplemented with the cytokinin and auxin combinations used at the callus induction stage and incubated in the above mentioned incubation conditions. The shoot-buds were incubated for another 4 weeks.

2.2.15 Effects of TDZ on Shoot-bud Induction

The effects of TDZ + IBA on shoot-bud induction were studied. Surface sterilized leaf explants from a mature greenhouse-grown cuttings (Section 2.2.3) were transferred to Petri dishes containing semi-solid MS basal media supplemented with various concentrations of TDZ and IBA and the edge of the Petri dishes were sealed with para film. Thirteen treatments consisted of a control (without TDZ or IBA) and combinations of IBA and TDZ in the range (0,10$^{-5}$,10$^{-6}$,10$^{-7}$)M and (5,10,15)x10$^{-6}$M respectively. The cultures were incubated in the dark at 25 ± 2°C and transferred to the fresh BM supplemented with the same TDZ and IBA combinations used at the callus induction stage at every 3 week intervals. About 500mg of 3-week-old calli, were transferred to 50mL culture vials to observe the effects of TDZ+IBA on shoot-bud induction.
All cultures were incubated for 12 weeks in total (counted from the date of the callus induction). At the end of the incubation period, the shoot-buds induced on the calli incubated in the BM supplemented with different combinations of TDZ and IBA were observed.

### 2.2.16 Seed Germination

The fruits collected from the greenhouse-grown cuttings (Section 2.2.3) were de-coated with sand paper, soaked in Gibberellic acid 50mg L\(^{-1}\) for 24 hours and washed with Tween 80 [2 drops (200ml water)]\(^{-1}\) solution (Sharp and Doran, 1990). The seeds were then surface sterilised for 10min in 4% hypochlorite solution and washed 3 times with sterile water and placed on the semi-solid UNE #B medium (Appendix 4). The plates were incubated in the dark at 25 ± 2\(^\circ\)C. After germination, the seedlings were transferred to the tissue culture vials and incubated in the above mentioned incubation conditions for 2 weeks. The morphological characteristics of the seedlings were observed. The number of leaves and roots formed were counted and the length of the seedlings (from the base of the shoot to the upper end of the leaf) was measured.

### 2.2.17 Data Collection and Statistical Analyses

For calli induction, 30-40 replicate plates each containing 1 segment of leaf explant were prepared per treatment (Section 2.2.7.1 and 2.2.9). For the production of green calli (Section 2.2.7.2 and 2.2.10.1) and shoot-bud induction (Section 2.2.7.3 and 2.2.10.2), 30 replicate plates each containing 500mg of the non-organogenic and green calli respectively were prepared per treatment. Shoot cultures (from non-organogenic and organogenic calli) were carried out by preparing 15 replicate vials each containing 4-5 different healthy shoot-buds per treatment (Section 2.2.7.4, 2.2.10.3 and 2.2.11). Root initiation in the differentiated shoots was carried out by preparing 15 replicate vials (Section 2.2.7.5). For root cultures (from non-organogenic and organogenic calli), 15 replicate vials were prepared per treatment.
(Section 2.2.12 and 2.2.13). To study the effects of the selected cytokinin and auxin combination and the effects of TDZ on shoot-bud induction, 15 replicate vials were prepared per treatment (Section 2.2.14 and 2.2.15). It was preferable to have an excess because of probable contamination. All experiments were repeated twice.

For the calli induction, production of green calli, shoot-bud induction, shoot cultures and root culture from the organogenic callus, 10 samples were selected at random from each repeated experiment treatment. The results are expressed as average response of 20 replicates. For root culture from the non-organogenic calli, effects of the selected cytokinin and auxin combination and the effects of TDZ on shoot-bud induction, 5 samples were selected at random from each repeated experiment treatment. The results are expressed as average response of 10 replicates.

For calculating the mean and standard deviation of the fresh weight of the non-organogenic calli, only the number of culture vials where calli growth continued for 11 weeks was used.

The results for callus induction and the production of green callus were calculated in percentages (mean ± standard error) (Liskova et al., 1994). The mathematical expression for calculation of standard error from proportion is in Appendix 5.

The calli induction percentage and the production of green calli were calculated as:
Callus induction percentage = (number of explants with calli / total number of explants) x 100.

The percentage of each callus type was calculated as:
Callus type percentage = (number of specific callus type / total number of calli) x 100 (Holme and Petersen, 1996).

Callus growth was evaluated on a fresh weight basis after 11 weeks (Ferreira and Janick, 1996).
For the selection of the non-organogenic calli for organ culture and organogenic callus for shoot culture, results were analyzed using analysis of variance (Anova). The results for the effects of the selected cytokinin/auxin combinations on the shoot-bud induction and shoot differentiation from the non-organogenic calli, results were also analyzed using analysis of variance (Anova). Analysis of variance showed the differences between the treatment means. Exactly which means differ could not be determined by the Anova. The Least Significant Difference (LSD) method (Appendix 6) was used to determine which of the treatment means differed significantly from one another.

For the effects of cytokinin and auxin combinations on shoot-bud induction and the morphological characteristics of the shoots cultured from the organogenic calli and for the effects of TDZ + IBA on shoot-bud induction, results were compared using one-tailed two-sample t test. All statistical tests and mean ± standard deviation calculations were conducted using Microsoft Excel '97.
2.3 RESULTS

2.3.1 Standardization of Plant Regeneration

A complete plant was regenerated from the callus induced on the leaf explant incubated in the MS basal medium supplemented with BA \(10^{-6}\)M + NAA \(5\times10^{-5}\)M. The responses obtained, the cytokinin/auxin combinations and incubation period and conditions (light and temperature) used at the different stages of plant regeneration are summarised in Table 2.1.

Non-organogenic calli were induced on 100% of the leaf explant (Table 2.1) (Fig. 2.8). Green calli were formed (100%) when 11-week-old non-organogenic calli were incubated in the light for 2 weeks (Table 2.1) (Fig. 2.9). About 7 to 8 shoot-buds were induced per 500mg callus (Table 2.1) (Fig. 2.10). When the shoot-buds were transferred to the shoot elongation medium and incubated in the dark for 2 weeks, they were elongated to 3 to 4cm long shoots with 10-12 leaves/shoot (Table 2.1) (Fig 2.11). After incubation in the light for 4 weeks, the basal stem and the leaves became dark green similar to the parent plant (Fig. 2.12).

When the differentiated shoots were transferred to the root inducing medium supplemented with IBA \(25\times10^{-6}\)M and incubated in the dark for 2 weeks, 2-3 small roots were formed at the cut end of each shoot. On transferring to the medium devoid of PGR and incubated in the light for 4 weeks, the small roots were elongated (1 to 2cm) and the number of roots formed on each differentiated shoot increased (5 to 6) (Table 2.1) (Fig 2.13).
<table>
<thead>
<tr>
<th>Solution concentration in μM</th>
<th>μM</th>
<th>Mean ± standard deviation</th>
<th>4.15 ± 0.98</th>
<th>7.98 ± 2.04</th>
</tr>
</thead>
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<tr>
<td>without PGR</td>
<td>D0</td>
<td>Number of roots formed</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>IB42x10^6</td>
<td>Length of the differentiated</td>
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<td></td>
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<td>D0</td>
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<td>2 weeks</td>
<td>NB42x10^6</td>
<td>Number of shoot buds</td>
<td></td>
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</tr>
</tbody>
</table>

**Table 2.1** Response obtained at different stages of the standardisation of complete plant regeneration from the non-organogenic callus induced on the leaf explant of *Duchesnia myoporoides* R. Br. by differentiation.
Text-Figure 2.7 Incubation chamber for culturing tissues and regenerants. The culture vials were arranged on the zinc plated steel rack (r) at a distance 40cm from the light source (L). Note the light arrangement for providing 15.2 \( \mu \text{mol s}^{-1}\text{m}^{2}\) light intensity for a 14hr photoperiod.

FIGURES 2.8 – 2.13 Different stages of standardization of plant regeneration from the non-organogenic callus induced on the leaf explant of *Duboisia myoporoides* R. Br. incubated in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various cytokinin/auxin combinations (mentioned in the text-figure).

Text-Figure 2.8 Eleven-week-old non-organogenic callus (c) induced in the BM supplemented with BA 10^{-5} M + NAA 5x10^{-5} M.

Text-Figure 2.9 Two-week-old green callus (gc) produced in the BM supplemented with BA 0.1x10^{-5} M + NAA 27x10^{-5} M incubated in the light for 2 weeks.
**Text-Figure 2.10** Shoot-buds (sb) induced on the green callus (gc) incubated in the BM supplemented with BA2x10^{-6} M in the light for 8 weeks.

**Text-Figure 2.11** Elongation of the shoot-buds incubated in the BM supplemented with BA5x10^{-6} M+NAA0.5x10^{-6} M for 2 weeks in the dark. Note the elongated basal stem (bs) of the shoot. Bar size 1.1 cm

**Text-Figure 2.12** Six-week-old differentiated shoot incubated in the BM supplemented with BA5x10^{-6} M+NAA0.5x10^{-6} M for 4-weeks in the light. Note the dark green elongated basal stem (bs). Bar size 1.6cm

**Text-Figure 2.13** Root (r) induction in the differentiated shoot incubated in the BM supplemented with IBA25x10^{-6} M for 2 weeks in the dark and then in the BM devoid of PGR for 4 weeks.
2.3.2 Morphogenetic Response on the Leaf Explant

Leaf explants cultured on cytokinin and auxin free BM did not produce any callus, turned brown and ultimately died after a few weeks incubation. Supplementation of cytokinin BA, kinetin and auxin IAA, IBA, NAA alone to the BM also did not show any morphogenetic response after 6 weeks incubation. Two different types i.e, non-organogenic and organogenic calli were induced on the cut end of the leaf explant incubated in the BM supplemented with only 2,4-D or different cytokinin and auxin combinations. The frequency (%) of non-organogenic and organogenic calli formation on the leaf explant is summarised in Table 2.2a. Mainly non-organogenic calli were induced in the BM supplemented with various cytokinin and auxin combinations (Table 2.2a) (Fig. 2.14). Non-organogenic calli were formed on 90.19% and organogenic calli were formed on 9.80% of the leaf explant. Non-organogenic calli formation took place after 7 to 10 days of dark incubation. Organogenic calli formed within 3 to 4 weeks of dark incubation.

Analysis of variance shows a significant (P < 0.001) difference between the effects of cytokinin/auxin combinations on the frequency (%) of callus induction on the leaf explant (Table 2.2b).

Shoot-buds were regenerated on the organogenic calli after 1 more week (Fig. 2.15) and root regeneration started after 2 to 3 more weeks of dark incubation (Fig. 2.16). The morphogenetic responses on the leaf explant to various cytokinin/auxin combinations after 6 weeks dark incubation are summarised in Table 2.3.
TABLE 2.2a Effects of various cytokinin/auxin combinations and concentrations on the frequency (%) of callus induction on the leaf explant of *Duboisia myoporoides* R. Br. The frequency of callus induction was observed on 20 leaf segments after 4 weeks of culture.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytokinins/auxins (M)</th>
<th>Non-organogenic calli formation (%)</th>
<th>Organogenic calli formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>2</td>
<td>BA10^{-5}M</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>3</td>
<td>BA10^{-6}M</td>
<td>x</td>
<td>x</td>
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<td>4</td>
<td>Kin10^{-3}M</td>
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<tr>
<td>15</td>
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<td>36.8 ± 0.10</td>
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<td>70.5 ± 0.10</td>
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<td>Solution Concentration (M)</td>
<td>Percentage (± Standard Error)</td>
<td>Response</td>
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<td>BA10⁻⁷M + IBA10⁻⁷M</td>
<td>x</td>
<td>90 ± 0.06</td>
</tr>
<tr>
<td>34</td>
<td>BA10⁻⁵M + IBA10⁻⁸M</td>
<td>95 ± 0.04</td>
<td>x</td>
</tr>
<tr>
<td>35</td>
<td>BA10⁻⁸M + IBA10⁻⁸M</td>
<td>95 ± 0.04</td>
<td>x</td>
</tr>
<tr>
<td>36</td>
<td>BA10⁻⁸M + NAA10⁻⁸M</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td>37</td>
<td>BA10⁻⁸M + NAA10⁻⁵M</td>
<td>95 ± 0.04</td>
<td>x</td>
</tr>
<tr>
<td>38</td>
<td>BA10⁻⁸M + NAA10⁻⁷M</td>
<td>78.9 ± 0.09</td>
<td>x</td>
</tr>
<tr>
<td>39</td>
<td>BA10⁻⁸M + 2,4-D10⁻⁴M</td>
<td>57.9 ± 0.11</td>
<td>x</td>
</tr>
<tr>
<td>40</td>
<td>BA10⁻⁸M + 2,4-D10⁻⁶M</td>
<td>80 ± 0.08</td>
<td>x</td>
</tr>
<tr>
<td>41</td>
<td>BA10⁻⁸M + 2,4-D10⁻⁷M</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td>42</td>
<td>Kin10⁻⁵M + IAA10⁻⁹M</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td>43</td>
<td>Kin10⁻³M + IAA10⁻⁹M</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td>44</td>
<td>Kin10⁻³M + IAA10⁻⁷M</td>
<td>94.4 ± 0.05</td>
<td>x</td>
</tr>
<tr>
<td>45</td>
<td>Kin10⁻³M + IBA10⁻⁹M</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td>46</td>
<td>Kin10⁻³M + IBA10⁻⁶M</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td>47</td>
<td>Kin10⁻³M + IBA10⁻⁷M</td>
<td>95 ± 0.04</td>
<td>x</td>
</tr>
<tr>
<td>48</td>
<td>Kin10⁻³M + NAA10⁻⁸M</td>
<td>94.7 ± 0.05</td>
<td>x</td>
</tr>
<tr>
<td>49</td>
<td>Kin10⁻³M + NAA10⁻⁴M</td>
<td>83.3 ± 0.08</td>
<td>x</td>
</tr>
<tr>
<td>50</td>
<td>Kin10⁻⁵M + NAA10⁻⁷M</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td>51</td>
<td>Kin10⁻⁵M + 2,4-D10⁻⁴M</td>
<td>88.2 ± 0.07</td>
<td>x</td>
</tr>
<tr>
<td>52</td>
<td>Kin10⁻⁵M + 2,4-D10⁻⁶M</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td>53</td>
<td>Kin10⁻⁵M + 2,4-D10⁻⁷M</td>
<td>80 ± 0.08</td>
<td>x</td>
</tr>
<tr>
<td>54</td>
<td>Kin10⁻⁵M + IAA10⁻⁹M</td>
<td>53.4 ± 0.11</td>
<td>x</td>
</tr>
<tr>
<td>55</td>
<td>Kin10⁻⁶M + IAA10⁻⁶M</td>
<td>65.3 ± 0.10</td>
<td>x</td>
</tr>
<tr>
<td>56</td>
<td>Kin10⁻⁶M + IAA10⁻⁷M</td>
<td>50.5 ± 0.11</td>
<td>x</td>
</tr>
<tr>
<td>57</td>
<td>Kin10⁻⁶M + IBA10⁻⁹M</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td>58</td>
<td>Kin10⁻⁶M + IBA10⁻⁶M</td>
<td>88.2 ± 0.07</td>
<td>x</td>
</tr>
<tr>
<td>59</td>
<td>Kin10⁻⁶M + IBA10⁻⁷M</td>
<td>94.7 ± 0.05</td>
<td>x</td>
</tr>
<tr>
<td>60</td>
<td>Kin10⁻⁶M + NAA10⁻⁴M</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td>61</td>
<td>Kin10⁻⁶M + NAA10⁻⁸M</td>
<td>100</td>
<td>x</td>
</tr>
<tr>
<td>62</td>
<td>Kin10⁻⁶M + NAA10⁻⁷M</td>
<td>85.7 ± 0.07</td>
<td>x</td>
</tr>
<tr>
<td>63</td>
<td>Kin10⁻⁶M + 2,4-D10⁻⁵M</td>
<td>62.5 ± 0.10</td>
<td>x</td>
</tr>
<tr>
<td>64</td>
<td>Kin10⁻⁶M + 2,4-D10⁻⁹M</td>
<td>78.3 ± 0.09</td>
<td>x</td>
</tr>
<tr>
<td>65</td>
<td>Kin10⁻⁶M + 2,4-D10⁻⁷M</td>
<td>95.6 ± 0.04</td>
<td>x</td>
</tr>
</tbody>
</table>

x : No response
M: Solution concentration in molarity
**TABLE 2.2 b** Summary analysis of variance of effects of various cytokinin/auxin combinations and concentrations on the frequency (%) of callus induction on the leaf explant of *Duboisia myoporoides* R. Br.

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean squares</th>
<th>F ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>2560.38</td>
<td>46</td>
<td>55.66042</td>
<td>20.50994</td>
</tr>
<tr>
<td>Within groups</td>
<td>2423.447</td>
<td>893</td>
<td>2.713827</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4983.827</td>
<td>939</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* : $P < 0.001$
FIGURES 2.14 – 2.16 Morphogenetic responses on the leaf explant of *Daboisla arvaporoides* R. Br. incubated in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various cytokinin and auxin combinations (mentioned in the text-figure) after 6 weeks.

**Text-Figure 2.14** Non-organogenic callus (c) induced in the BM supplemented with BA$10^{-5}$M+2,4-D$10^{-5}$M.

**Fig. 2.14**

**Text-Figure 2.15** Shoot-buds (sb) induction on the organogenic callus induced in the BM supplemented with BA$10^{-5}$M+IBA$10^{-5}$M.

**Fig. 2.15**

**Text-Figure 2.16** Root (r) induction on the organogenic callus (white callus wc) induced in the BM supplemented with BA$10^{-5}$M+IBA$10^{-5}$M.

**Fig. 2.16**
<table>
<thead>
<tr>
<th></th>
<th>BM</th>
<th>RN</th>
<th>HA</th>
<th>NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Con.</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>2% 4D</td>
<td>N.A</td>
<td>N.A</td>
<td>N.A</td>
<td>N.A</td>
</tr>
<tr>
<td>IBA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2.3**

| Inhibition of Juraca pyriformis L. BR. incubated in the MS-CMS medium and Shok’s salts supplemented with various cytokinins/auxins combinations and vitamins, 1962. |
2.3.3 Interactions of Cytokinins / Auxins on The Induction and Growth of the Non-organogenic Calli

Fresh weight of the 11-week-old non-organogenic calli induced in the BM supplemented with various cytokinin/auxin combinations is summarised in Table 2.4a. After 7 to 10 days of dark incubation, the calli were formed at the cut end of the leaf explants incubated in the BM supplemented with 2,4-D (10^5-10^-7)M. The calli induced in the BM supplemented with 2,4-D (10^-2-10^-6)M became brown after 6 weeks and did not continue for 11 weeks (Table 2.4a). However, the callus induced in the BM supplemented with 2,4-D10^-7M grew for 11 weeks (Table 2.4a).

Depending on the individual treatment, 3 different types of calli were distinguished after 11 weeks of incubation: (1) A compact white calli were induced on 80-100% of explants incubated in the BM supplemented with BA10^-5M + NAA or 2,4-D(10^-5-10^-7)M, Kin10^-5M + IAA, IBA or 2,4-D (10^-5-10^-7)M or NAA(10^-5-10^-6)M, (Table 2.2a). The fresh weight of those calli increased considerably and growth continued for 11 weeks without any browning in the calli (Table 2.4a).

(2) A compact and brownish white calli were induced on 50.5-100% of explants in the BM supplemented with BA10^-5M + IBA10^-6M, BA10^-6M + NAA or 2,4-D (10^-5-10^-7)M, Kin10^-6M + IAA, NAA or 2,4-D (10^-5-10^-7)M (Table 2.2a). The calli growth was slow and continued for 11-weeks (Table 2.4a).

(3) A light brown callus induced on 60-100% of explants in the BM supplemented with BA10^-5M + IBA10^-5M, BA10^-6M + IAA (10^-5-10^-7)M or IBA (10^-6-10^-7)M, Kin10^-5M + NAA10^-7M, Kin10^-6M + IBA (10^-5-10^-7)M, which grew for 2 to 3 weeks and then became dark brown (Table 2.2a) (Table 2.4a).

Analysis of variance shows a significant (P < 0.001) difference between the effects of various cytokinin / auxin combinations on the fresh weight of the non-organogenic calli (Table 2.4b).
TABLE 2.4a  Effects of cytokinins and auxins on the fresh weight and appearance of the 11-week-old non-organogenic calli induced on the leaf explant of *Duboisia myoporoides* R. Br. incubated in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various cytokinin and auxin combinations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytokinin / auxin</th>
<th>Appearance</th>
<th>Fresh weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>2,4-D10⁻⁶M</td>
<td>Compact, white</td>
<td>x</td>
</tr>
<tr>
<td>16</td>
<td>2,4-D10⁻⁶M</td>
<td>Compact, white</td>
<td>x</td>
</tr>
<tr>
<td>17</td>
<td>2,4-D10⁻⁶M</td>
<td>Compact, white</td>
<td>0.46 ± 0.36</td>
</tr>
<tr>
<td>21</td>
<td>BA10⁻⁶M + IBA10⁻⁶M</td>
<td>Compact, light brown</td>
<td>x</td>
</tr>
<tr>
<td>22</td>
<td>BA10⁻⁶M + IBA10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>0.67 ± 0.48</td>
</tr>
<tr>
<td>24</td>
<td>BA10⁻⁶M + NAA10⁻⁶M</td>
<td>Compact, white</td>
<td>2.60 ± 1.53</td>
</tr>
<tr>
<td>25</td>
<td>BA10⁻⁶M + NAA10⁻⁶M</td>
<td>Compact, white</td>
<td>3.63 ± 1.41</td>
</tr>
<tr>
<td>26</td>
<td>BA10⁻⁶M + NAA10⁻⁶M</td>
<td>Compact, white</td>
<td>1.57 ± 0.63</td>
</tr>
<tr>
<td>27</td>
<td>BA10⁻⁶M + 2,4-D10⁻⁶M</td>
<td>Compact, white</td>
<td>0.32 ± 0.16</td>
</tr>
<tr>
<td>28</td>
<td>BA10⁻⁶M + 2,4-D10⁻⁶M</td>
<td>Compact, white</td>
<td>3.12 ± 0.81</td>
</tr>
<tr>
<td>29</td>
<td>BA10⁻⁶M + 2,4-D10⁻⁶M</td>
<td>Compact, white</td>
<td>3.13 ± 0.95</td>
</tr>
<tr>
<td>30</td>
<td>BA10⁻⁶M + IAA10⁻⁷M</td>
<td>Compact, light brown</td>
<td>x</td>
</tr>
<tr>
<td>31</td>
<td>BA10⁻⁶M + IAA10⁻⁷M</td>
<td>Compact, light brown</td>
<td>x</td>
</tr>
<tr>
<td>32</td>
<td>BA10⁻⁶M + IAA10⁻⁷M</td>
<td>Compact, light brown</td>
<td>x</td>
</tr>
<tr>
<td>33</td>
<td>BA10⁻⁶M + IBA10⁻⁶M</td>
<td>Compact, light brown</td>
<td>x</td>
</tr>
<tr>
<td>34</td>
<td>BA10⁻⁶M + IBA10⁻⁶M</td>
<td>Compact, light brown</td>
<td>x</td>
</tr>
<tr>
<td>35</td>
<td>BA10⁻⁶M + IBA10⁻⁶M</td>
<td>Compact, light brown</td>
<td>x</td>
</tr>
<tr>
<td>36</td>
<td>BA10⁻⁶M + NAA10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>1.08 ± 0.38</td>
</tr>
<tr>
<td>37</td>
<td>BA10⁻⁶M + NAA10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>1.21 ± 0.81</td>
</tr>
<tr>
<td>38</td>
<td>BA10⁻⁶M + NAA10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>0.23 ± 0.16</td>
</tr>
<tr>
<td>39</td>
<td>BA10⁻⁶M + 2,4-D10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>1.17 ± 0.47</td>
</tr>
<tr>
<td>40</td>
<td>BA10⁻⁶M + 2,4-D10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>0.98 ± 0.22</td>
</tr>
<tr>
<td>41</td>
<td>BA10⁻⁶M + 2,4-D10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>0.97 ± 0.37</td>
</tr>
<tr>
<td>42</td>
<td>Kin10⁻⁶M + IAA10⁻⁷M</td>
<td>Compact, white</td>
<td>1.20 ± 0.51</td>
</tr>
<tr>
<td>43</td>
<td>Kin10⁻⁶M + IAA10⁻⁷M</td>
<td>Compact, white</td>
<td>1.93 ± 0.72</td>
</tr>
<tr>
<td>44</td>
<td>Kin10⁻⁶M + IAA10⁻⁷M</td>
<td>Compact, white</td>
<td>0.80 ± 0.19</td>
</tr>
<tr>
<td>45</td>
<td>Kin10⁻⁶M + IBA10⁻⁶M</td>
<td>Compact, white</td>
<td>1.55 ± 0.83</td>
</tr>
<tr>
<td>46</td>
<td>Kin10⁻⁶M + IBA10⁻⁶M</td>
<td>Compact, white</td>
<td>1.33 ± 0.087</td>
</tr>
<tr>
<td>47</td>
<td>Kin10⁻⁶M + IBA10⁻⁶M</td>
<td>Compact, white</td>
<td>1.09 ± 0.63</td>
</tr>
<tr>
<td>48</td>
<td>Kin10⁻⁶M + NAA10⁻⁶M</td>
<td>Compact, white</td>
<td>1.64 ± 0.71</td>
</tr>
<tr>
<td>49</td>
<td>Kin10⁻⁶M + NAA10⁻⁶M</td>
<td>Compact, white</td>
<td>0.87 ± 0.58</td>
</tr>
<tr>
<td>50</td>
<td>Kin10⁻⁶M + NAA10⁻⁶M</td>
<td>Compact, light brown</td>
<td>x</td>
</tr>
<tr>
<td>51</td>
<td>Kin10⁻⁶M + 2,4-D10⁻⁶M</td>
<td>Compact, white</td>
<td>1.47 ± 0.59</td>
</tr>
<tr>
<td>52</td>
<td>Kin10⁻⁶M + 2,4-D10⁻⁶M</td>
<td>Compact, white</td>
<td>1.70 ± 0.71</td>
</tr>
<tr>
<td>53</td>
<td>Kin10⁻⁶M + 2,4-D10⁻⁶M</td>
<td>Compact, white</td>
<td>1.75 ± 0.64</td>
</tr>
</tbody>
</table>

(Contd.)
### TABLE 2.4a Cont.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytokinins / auxins</th>
<th>Appearance</th>
<th>Fresh weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>Kin10⁻⁶M + IAA10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>1.18 ± 1.03</td>
</tr>
<tr>
<td>55</td>
<td>Kin10⁻⁶M + IAA10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>1.53 ± 0.82</td>
</tr>
<tr>
<td>56</td>
<td>Kin10⁻⁶M + IAA10⁻³M</td>
<td>Compact, brownish white</td>
<td>1.28 ± 0.72</td>
</tr>
<tr>
<td>57</td>
<td>Kin10⁻⁶M + IBA10⁻⁶M</td>
<td>Compact, light brown</td>
<td>x</td>
</tr>
<tr>
<td>58</td>
<td>Kin10⁻⁶M + IBA10⁻⁶M</td>
<td>Compact, light brown</td>
<td>x</td>
</tr>
<tr>
<td>59</td>
<td>Kin10⁻⁶M + IBA10⁻³M</td>
<td>Compact, light brown</td>
<td>x</td>
</tr>
<tr>
<td>60</td>
<td>Kin10⁻⁶M + NAA10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>1.59 ± 0.63</td>
</tr>
<tr>
<td>61</td>
<td>Kin10⁻⁶M + NAA10⁻³M</td>
<td>Compact, brownish white</td>
<td>1.38 ± 0.67</td>
</tr>
<tr>
<td>62</td>
<td>Kin10⁻³M + NAA10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>1.51 ± 0.54</td>
</tr>
<tr>
<td>63</td>
<td>Kin10⁻⁶M + 2,4-D10⁻⁶M</td>
<td>Compact, brownish white</td>
<td>0.85 ± 0.65</td>
</tr>
<tr>
<td>64</td>
<td>Kin10⁻⁶M + 2,4-D10⁻³M</td>
<td>Compact, brownish white</td>
<td>1.00 ± 0.51</td>
</tr>
<tr>
<td>65</td>
<td>Kin10⁻⁶M + 2,4-D10⁻³M</td>
<td>Compact, brownish white</td>
<td>0.86 ± 0.47</td>
</tr>
</tbody>
</table>

* : Results given as means of 20 replicates
** : Initial fresh weight of the callus used was 500mg

M : Solution concentration in molarity
x : Did not measure the fresh weight as the callus turned dark brown and was not used for further study

### TABLE 2.4b Summary analysis of variance of effects of various cytokinin / auxin combinations on the fresh weight of the 11-week-old non-organogenic calli induced on the leaf explant of *Duboisia myoporoides* R. Br.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean squares</th>
<th>F ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>317.7007</td>
<td>32</td>
<td>9.928148</td>
<td>17.46137</td>
</tr>
<tr>
<td>Within groups</td>
<td>246.1942</td>
<td>433</td>
<td>0.568578</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>563.8949</td>
<td>465</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* : P < 0.001
In the tested range of concentrations, kinetin produced non-organogenic callus when used in combination with any one of the auxins whereas BA produced a variety of effects. A strong interaction between BA and NAA or 2,4-D on induction and growth of the non-organogenic callus was observed. Fresh weight of the calli increased considerably on the BM supplemented with \( BA10^{-5}\text{M} + \text{NAA}10^{-6}\text{M} \) (average 3.6 g) or \( BA10^{-5}\text{M} + \text{2,4-D}10^{-7}\text{M} \) (average 3.12 g) (Table 2.4a). These calli were white and had the highest fresh weight as compared to the calli induced in the BM supplemented with various concentrations of Kin + NAA or 2,4-D.

The callus induced in the BM supplemented with only auxin 2,4-D\(10^{-7}\text{M} \) was selected for shoot culture because this auxin alone and without any supplementation with cytokinin gave positive results for continued growth for 11 weeks (Table 2.4a).

Fresh weights of the non-organogenic calli induced in the BM supplemented with different concentrations of each type of the 6 cytokinin and auxin combinations (BA+NAA, BA+2,4-D, Kin+IAA, Kin+IBA, Kin+NAA, Kin+2,4-D) showed differential effects (Table 2.5a) (Fig. 2.17).

Of the 6 different concentrations of BA + NAA, the highest fresh weight of the calli was obtained when the callus induction medium was supplemented with \( BA10^{-5}\text{M} + \text{NAA}10^{-6}\text{M} \) (Table 2.5a) (Fig. 2.17). The callus induction medium supplemented with \( BA10^{-6}\text{M} + \text{NAA}10^{-7}\text{M} \) produced the least amount of callus (Table 2.5a) (Fig. 2.17). The fresh weight of the callus induced in the BM supplemented with \( BA10^{-5}\text{M} + \text{NAA}10^{-6}\text{M} \) was significantly \((P < 0.001)\) higher than those induced in the BM supplemented with the other BA + NAA concentrations (Table 2.5b) (Fig. 2.17). Based on these results, the callus induced in the BM supplemented with \( BA10^{-5}\text{M} + \text{NAA}10^{-6}\text{M} \) was selected for organ culture.

The same fresh weight of the calli was obtained when the calli induction media were supplemented with \( BA10^{-5}\text{M} + 2,4\text{-D}10^{-6}\text{M} \) or \( BA10^{-5}\text{M} + 2,4\text{-D}10^{-7}\text{M} \) (Table 2.5a) (Fig. 2.17). The least fresh weight of callus was produced when the callus induction medium was supplemented with \( BA10^{-5}\text{M}+2,4\text{-D}10^{-5}\text{M} \) (Table 2.5a)
TABLE 2.5a Effects of various cytokinin and auxin concentrations on the fresh weight of the 11-week-old non-organogenic calli induced on the leaf explant of 
* Duboisia myoporoides * R. Br. incubated in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various cytokinin and auxin combinations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytokinins &amp; auxins</th>
<th>Concentration (M)</th>
<th>Fresh weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>BA + NAA</td>
<td>BA10⁻²M + NAA10⁻⁶M</td>
<td>2.60 ± 1.53</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>BA10⁻²M + NAA10⁻⁶M</td>
<td>3.63 ± 1.41</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>BA10⁻²M + NAA10⁻⁶M</td>
<td>1.57 ± 0.63</td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>BA10⁻⁶M + NAA10⁻⁵M</td>
<td>1.08 ± 0.38</td>
</tr>
<tr>
<td>37</td>
<td></td>
<td>BA10⁻⁶M + NAA10⁻⁶M</td>
<td>1.21 ± 0.81</td>
</tr>
<tr>
<td>38</td>
<td></td>
<td>BA10⁻⁶M + NAA10⁻⁷M</td>
<td>0.23 ± 0.16</td>
</tr>
<tr>
<td>27</td>
<td>BA + 2,4-D</td>
<td>BA10⁻²M + 2,4-D10⁻⁷M</td>
<td>0.32 ± 0.16</td>
</tr>
<tr>
<td>28</td>
<td></td>
<td>BA10⁻²M + 2,4-D10⁻⁷M</td>
<td>3.12 ± 0.81</td>
</tr>
<tr>
<td>29</td>
<td></td>
<td>BA10⁻²M + 2,4-D10⁻⁷M</td>
<td>3.13 ± 0.95</td>
</tr>
<tr>
<td>39</td>
<td></td>
<td>BA10⁻⁶M + 2,4-D10⁻⁷M</td>
<td>1.17 ± 0.47</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>BA10⁻⁶M + 2,4-D10⁻⁷M</td>
<td>0.98 ± 0.22</td>
</tr>
<tr>
<td>41</td>
<td></td>
<td>BA10⁻⁶M + 2,4-D10⁻⁷M</td>
<td>0.97 ± 0.37</td>
</tr>
<tr>
<td>42</td>
<td>Kin + IAA</td>
<td>Kin10⁻⁴M + IAA10⁻⁶M</td>
<td>1.20 ± 0.51</td>
</tr>
<tr>
<td>43</td>
<td></td>
<td>Kin10⁻⁴M + IAA10⁻⁶M</td>
<td>1.93 ± 0.72</td>
</tr>
<tr>
<td>44</td>
<td></td>
<td>Kin10⁻⁴M + IAA10⁻⁶M</td>
<td>0.80 ± 0.19</td>
</tr>
<tr>
<td>54</td>
<td></td>
<td>Kin10⁻⁴M + IAA10⁻⁷M</td>
<td>1.18 ± 1.03</td>
</tr>
<tr>
<td>55</td>
<td></td>
<td>Kin10⁻⁴M + IAA10⁻⁷M</td>
<td>1.53 ± 0.82</td>
</tr>
<tr>
<td>56</td>
<td></td>
<td>Kin10⁻⁴M + IAA10⁻⁷M</td>
<td>1.28 ± 0.72</td>
</tr>
<tr>
<td>45</td>
<td>Kin + IBA</td>
<td>Kin10⁻⁴M + IBA10⁻⁸M</td>
<td>1.55 ± 0.83</td>
</tr>
<tr>
<td>46</td>
<td></td>
<td>Kin10⁻⁴M + IBA10⁻¹⁰M</td>
<td>1.33 ± 0.87</td>
</tr>
<tr>
<td>47</td>
<td></td>
<td>Kin10⁻⁴M + IBA10⁻¹⁰M</td>
<td>1.29 ± 0.63</td>
</tr>
<tr>
<td>57</td>
<td></td>
<td>Kin10⁻⁴M + IBA10⁻⁸M</td>
<td>x</td>
</tr>
<tr>
<td>58</td>
<td></td>
<td>Kin10⁻⁴M + IBA10⁻¹⁰M</td>
<td>x</td>
</tr>
<tr>
<td>59</td>
<td></td>
<td>Kin10⁻⁴M + IBA10⁻¹⁰M</td>
<td>x</td>
</tr>
<tr>
<td>48</td>
<td>Kin + NAA</td>
<td>Kin10⁻⁷M + NAA10⁻⁷M</td>
<td>1.64 ± 0.71</td>
</tr>
<tr>
<td>49</td>
<td></td>
<td>Kin10⁻⁷M + NAA10⁻⁷M</td>
<td>0.87 ± 0.58</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>Kin10⁻⁷M + NAA10⁻⁷M</td>
<td>x</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>Kin10⁻⁷M + NAA10⁻⁷M</td>
<td>1.59 ± 0.63</td>
</tr>
<tr>
<td>61</td>
<td></td>
<td>Kin10⁻⁷M + NAA10⁻⁷M</td>
<td>1.38 ± 0.67</td>
</tr>
<tr>
<td>62</td>
<td></td>
<td>Kin10⁻⁷M + NAA10⁻⁷M</td>
<td>1.51 ± 0.54</td>
</tr>
<tr>
<td>51</td>
<td>Kin + 2,4-D</td>
<td>Kin10⁻⁷M + 2,4-D10⁻⁸M</td>
<td>1.47 ± 0.59</td>
</tr>
<tr>
<td>52</td>
<td></td>
<td>Kin10⁻⁷M + 2,4-D10⁻⁸M</td>
<td>1.70 ± 0.71</td>
</tr>
<tr>
<td>53</td>
<td></td>
<td>Kin10⁻⁷M + 2,4-D10⁻⁸M</td>
<td>1.75 ± 0.64</td>
</tr>
<tr>
<td>63</td>
<td></td>
<td>Kin10⁻⁷M + 2,4-D10⁻⁸M</td>
<td>0.85 ± 0.65</td>
</tr>
<tr>
<td>64</td>
<td></td>
<td>Kin10⁻⁷M + 2,4-D10⁻⁸M</td>
<td>1.00 ± 0.51</td>
</tr>
<tr>
<td>65</td>
<td></td>
<td>Kin10⁻⁷M + 2,4-D10⁻⁸M</td>
<td>0.86 ± 0.47</td>
</tr>
</tbody>
</table>

* : Results given as means of 20 replicates
** : Initial fresh weight of the callus used was 500mg
M : Solution concentration in molarity
x : Did not measure the fresh weight as the callus turned dark brown and was not used for further study
TABLE 2.5b Summary analysis of variance of effects of various cytokinin and auxin concentrations on the fresh weight of the 11-week-old non-organogenic calli induced on the leaf explant of Duboisia myoporoides R. Br.

<table>
<thead>
<tr>
<th>Cytokinins &amp; auxins</th>
<th>Source of variance</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean squares</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA + NAA</td>
<td>Between groups</td>
<td>99.47886</td>
<td>5</td>
<td>19.89577</td>
<td>15.28022 *</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>87.23809</td>
<td>67</td>
<td>1.302061</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>318.983</td>
<td>72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA + 2,4-D</td>
<td>Between groups</td>
<td>108.6509</td>
<td>5</td>
<td>21.73018</td>
<td>49.43025 *</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>33.8502</td>
<td>77</td>
<td>0.439613</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>142.5011</td>
<td>82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kin+ IAA</td>
<td>Between groups</td>
<td>10.87039</td>
<td>5</td>
<td>2.174078</td>
<td>4.667597 *</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>38.19405</td>
<td>82</td>
<td>0.465781</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>49.06444</td>
<td>87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kin + IBA</td>
<td>Between groups</td>
<td>0.891303</td>
<td>2</td>
<td>0.445652</td>
<td>0.628622 ns</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>26.23057</td>
<td>37</td>
<td>0.708934</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>27.12188</td>
<td>39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kin + NAA</td>
<td>Between groups</td>
<td>5.923389</td>
<td>4</td>
<td>1.480847</td>
<td>3.557346 **</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>29.13951</td>
<td>70</td>
<td>0.416279</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>35.0629</td>
<td>74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kin + 2,4-D</td>
<td>Between groups</td>
<td>13.01868</td>
<td>5</td>
<td>2.603736</td>
<td>7.131289 *</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>29.93938</td>
<td>82</td>
<td>0.365114</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>42.95806</td>
<td>87</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* : P < 0.001  
** : P < 0.05  
ns : Not significant
Text-Figure 2.17  Effects of cytokinin and auxin concentrations on the fresh weight of the 11-week-old non-organogenic calli induced on the leaf explant of *Duboisia myoporoides* R.Br. incubated in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various cytokinin and auxin combinations; the vertical bar at the top of each column shows ± standard deviation.

1: Cytokinin $10^{-5}$M + auxin $10^{-5}$M  
2: Cytokinin $10^{-5}$M + auxin $10^{-6}$M  
3: Cytokinin $10^{-5}$M + auxin $10^{-7}$M  
4: Cytokinin $10^{-6}$M + auxin $10^{-5}$M  
5: Cytokinin $10^{-6}$M + auxin $10^{-6}$M  
6: Cytokinin $10^{-6}$M + auxin $10^{-7}$M
The fresh weight of the callus induced in the BM supplemented with BA$10^{-5}$M+2,4-D$10^{-7}$M did not differ significantly than that induced in the BM supplemented with BA$10^{-5}$M + 2,4-D$10^{-6}$M. However, the BM supplemented with BA$10^{-5}$M + 2,4-D$10^{-7}$M produced the highest fresh weight of the callus (Table 2.5a) (Fig. 2.17). Based on these results, the callus induced in the BM supplemented with BA$10^{-5}$M + 2,4-D$10^{-7}$M was selected for organ culture.

The highest fresh weight of the callus was produced when the callus induction medium was supplemented with Kin$10^{-5}$M + IAA$10^{-6}$M (Table 2.5a) (Fig. 2.17). The least amount of callus was produced in the BM supplemented with Kin$10^{-5}$M + IAA$10^{-7}$M (Table 2.5a) (Fig. 2.17). Almost same amount of the calli were produced in the BM supplemented with Kin$10^{-6}$M + IAA (10$^{-5}$ or 10$^{-7}$)M (Table 2.5a) (Fig. 2.17). The fresh weight of the callus induced in the BM supplemented with Kin$10^{-5}$M+IAA$10^{-6}$M was significantly (P < 0.001) higher than those induced in the BM supplemented with other Kin+IAA concentrations (Table 2.5a, b). Depending on these results, the callus induced in the BM supplemented with Kin$10^{-5}$M+IAA$10^{-6}$M, was selected for organ culture.

The calli induced in the BM supplemented with Kin$10^{-6}$M + IBA (10$^{-5}$-10$^{-7}$)M did not grow for 11 weeks. Of the 3 concentrations of Kin+IBA, the BM supplemented with Kin$10^{-5}$M + IBA$10^{-5}$M produced highest fresh weight of the callus (Table 2.5a) (Fig 2.17). However, the fresh weight of callus did not differ significantly (P > 0.1) between the treatments (Table 2.5b). Based on these results, the callus induced in the BM supplemented with Kin$10^{-5}$M + IBA$10^{-5}$M was selected for organ culture.

The highest fresh weight of the callus was produced in the BM supplemented with Kin$10^{-5}$M + NAA$10^{-5}$M (Table 2.5a) (Fig. 2.17). The callus induced in the BM supplemented with Kin$10^{-5}$M + NAA$10^{-7}$M did not grow for 11 weeks (Table 2.5a) (Fig. 2.17). The least fresh weight of the callus was produced in the BM supplemented with Kin$10^{-5}$M + NAA$10^{-6}$M (Table 2.5a) (Fig. 2.17). The fresh weight of the callus induced in the BM supplemented with Kin$10^{-5}$M + NAA$10^{-5}$M was significantly (P < 0.05) higher than those induced in the BM supplemented with other Kin + NAA concentrations (Table 2.5b) (Fig. 2.17). According to these results
the callus induced in the BM supplemented with Kin$10^{-5}$M + NAA$10^{-5}$M was selected for organ culture.

The callus induced in the BM supplemented with Kin$10^{-5}$M + 2,4-D$10^{-7}$M produced the highest fresh weight (Table 2.5a) (Fig. 2.17). The fresh weights of the calli induced in the BM supplemented with Kin$10^{-6}$M + 2,4-D$(10^{-5}-10^{-7})$M were lower than those induced in the BM supplemented with Kin$10^{-5}$M+2,4-D$(10^{-5}-10^{-7})$M (Table 2.5a) (Fig. 2.17). The calli induced in the BM supplemented with Kin$10^{-5}$M + 2,4-D$10^{-7}$M was significantly (P < 0.001) higher than those induced in the BM supplemented with other Kin+2,4-D concentrations (Table 2.5b). Depending on these results, the callus induced in the BM supplemented with Kin$10^{-5}$M+2,4-D$10^{-7}$M was selected for organ culture.

The results for the frequency (%) of the non-organogenic calli induced on the leaf explant incubated in the BM supplemented with the cytokinin / auxin combinations which gave the best growth values are presented in Fig. 2.18. The same frequency (%) of callus induction was observed when the callus induction media were supplemented with BA$10^{-5}$M+NAA$10^{-5}$M, BA$10^{-5}$M+2,4-D$10^{-7}$M, Kin$10^{-5}$M+IAA$10^{-5}$M or Kin$10^{-5}$M+IBA$10^{-5}$M (Table 2.2a) (Fig. 2.18). The frequency of callus induction was lowest when the callus induction medium was supplemented with Kin$10^{-5}$M+2,4-D$10^{-7}$M (Fig. 2.18).

### 2.3.4 Interactions of Cytokinins and Auxins on the Induction of the Regenerants on the Organogenic Calli

The frequency (%) of organogenic callus induction is summarised in Table 2.2a (page 100). Organogenic calli were mainly induced in the BM supplemented with BA in combination with either IAA or IBA. After 4 weeks of dark incubation, compact, small green calli were induced on 90-100% of explants incubated in the BM supplemented with BA$10^{-5}$M + IAA$(10^{-6}-10^{-7})$M or BA$10^{-5}$M + IBA$10^{-7}$M. After 5 to 6 weeks, organogenic calli were induced on 80% of explants incubated in the BM supplemented with BA$10^{-5}$M+ IAA$10^{-5}$M. With all combinations, calli growth was slow and within 1 to 2 weeks of light incubation (after callus induction), shoot-buds appeared on the green calli (Fig. 2.15 page 103).
Text-Figure 2.18 Effects of cytokinin / auxin combinations on the frequency (%) of non-organogenic calli induction on the leaf explant of *Duboisia myoporoides* R. Br. incubated in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and the selected cytokinin / auxin combinations

1 : 2,4-D10⁻⁷M  
2 : BA10⁻⁵M + NAA10⁻⁶M  
3 : BA10⁻⁵M + 2,4-D10⁻⁷M  
4 : Kin10⁻⁵M + IAA10⁻⁶M  
5 : Kin10⁻⁵M + IBA10⁻⁵M  
6 : Kin10⁻⁵M + NAA10⁻⁵M  
7 : Kin10⁻⁵M + 2,4-D10⁻⁷M
The number of shoot-buds formed on each explant incubated in the BM supplemented with various cytokinin and auxin combinations is summarised in Table 2.6a and presented in Fig. 2.19. Within the tested range of concentrations, BA favoured organogenic callus induction when used in combination with either IAA or IBA. A few shoot-buds (< 4 healthy shoot-buds per explant) were induced in the BM supplemented with BA $10^{-5}$ M + IAA ($10^{-5}$ or $10^{-7}$) M (Table 2.6a) (Fig. 2.19). The organogenic capacity of the calli induced in the BM supplemented with BA $10^{-5}$ M + IAA ($10^{-5}$ or $10^{-7}$)M decreased significantly as the calli were subcultured, whereas shoot-bud induction in the BM supplemented with BA $10^{-5}$ M + IAA $10^{-6}$M was continued throughout the incubation period. A significantly (P < 0.001) higher number of shoot-buds (< 6 healthy shoot-buds per explant) were induced in the BM supplemented with BA $10^{-5}$M+IAA$10^{-6}$M (Table 2.6a and b) (Fig. 2.19). According to these results, shoot-buds formed on the calli induced in the BM supplemented with BA $10^{-5}$ M + IAA $10^{-6}$M was selected for shoot culture.

Of the 3 concentrations of BA + IBA, shoot-bud producing organogenic calli were only induced in the BM supplemented with BA $10^{-5}$M + IBA $10^{-7}$M. Extensive shoot-bud induction (>14 healthy shoot-buds per explant) was evident in the BM supplemented with BA $10^{-5}$M + IBA $10^{-7}$M (Table 2.6a). With a longer incubation period (> 15 weeks) the organogenic capacity of the calli decreased but never disappeared entirely as they were still able to regenerate shoot-buds. A higher number of shoot-buds were induced in the BM supplemented with BA $10^{-5}$M + IBA $10^{-7}$M than that in the BM supplemented with BA $10^{-5}$M + IAA $10^{-6}$M (Table 2.6a) (Fig. 2.19). The shoot-buds formed on the calli induced in the BM supplemented with BA $10^{-5}$ M + IBA $10^{-7}$M were also selected for shoot culture.

After 6 to 7 weeks of dark incubation, callus formation started on the leaf explant incubated in the BM supplemented with BA $10^{-6}$M + IBA $10^{-5}$M. The calli were compact and brownish white (Fig. 2.16, page 103). At the edge of the brown calli, slow growing white calli formed and growth continued over a period of 11 weeks. After 6 weeks of incubation (starting from the callus induction), small roots were appeared on the calli (Fig. 2.16, page 103). About 4 roots were formed per explant (Table 2.6a). The
TABLE 2.6a Effects of cytokinin and auxin combinations* on the number of shoot-bud and root induction on the 2-week-old organogenic calli induced on the leaf explant of *Duboisia myoporoides* R. Br. incubated in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various cytokinin and auxin combinations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytokinin/auxin concentrations (M)</th>
<th>Number of regenerants/explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>BA10⁻³M+IAA10⁻³M</td>
<td>3.72 ± 0.33</td>
</tr>
<tr>
<td>19</td>
<td>BA10⁻⁵M+IAA10⁻³M</td>
<td>5.35 ± 1.4</td>
</tr>
<tr>
<td>20</td>
<td>BA10⁻³M+IAA10⁻⁷M</td>
<td>3.80 ± 0.50</td>
</tr>
<tr>
<td>23</td>
<td>BA10⁻³M+IBA10⁻⁷M</td>
<td>14.80 ± 3.87</td>
</tr>
<tr>
<td>33</td>
<td>BA10⁻⁶M+IBA10⁻⁷M**</td>
<td>4 ± 1.23</td>
</tr>
</tbody>
</table>

*: Results given as means of 20 replicates

**: Root regenerated

M : Solution concentration in molarity

Column means sharing a common letter within the row are not significant at α = 0.05 level

Note : LSD calculation was carried out between treatments 18, 19 and 20

TABLE 2.6b Summary analysis of variance of effects of various BA+IAA concentrations on shoot-bud induction on the 2-week-old organogenic calli

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean squares</th>
<th>F ratio *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Shoot-buds</td>
<td>Between groups</td>
<td>32.82177</td>
<td>2</td>
<td>16.41089</td>
<td>18.88037</td>
</tr>
<tr>
<td>/ leaf explant</td>
<td>Within groups</td>
<td>49.54459</td>
<td>57</td>
<td>0.869203</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>82.36637</td>
<td>59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: P < 0.001
Text-Figure 2.19 Effects of cytokinin and auxin concentrations on shoot-bud induction on the organogenic calli induced on the leaf explant of *Duboisia myoporoides* R. Br. incubated in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various cytokinin and auxin combinations; the vertical bar at the top of each column shows ± standard deviation.

Concentration 1: cytokinin $10^{-5}$M + auxin $10^{-5}$M  
2: cytokinin $10^{-5}$M + auxin$10^{-6}$M  
3: cytokinin $10^{-5}$M + auxin$10^{-7}$M
organogenic callus induced in the BM supplemented with BA10^{-6}M + IBA10^{-5}M were used for root culture in the semi-solid medium.

2.3.5 Interactions of Cytokinins and Auxins on Shoot Culture from the Non-organogenic Calli

The effects of the selected cytokinin/auxin combinations (used at the callus induction stage as identified previously) on the green callus formation, shoot-bud induction and morphological characteristics of the shoots differentiated from the non-organogenic calli are summarised below.

2.3.5.1 Production of Green Calli

The percentage of the green calli formation from the 11-week-old non-organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations are summarised in Table 2.7a and presented in Fig. 2.20. The frequency (%) of green calli formation from the 11-week-old non-organogenic calli was highest when the callus induction media were supplemented with 2,4-D10^{-7}M, BA10^{-5}M + NAA10^{-6}M, BA10^{-5}M + 2,4-D10^{-7}M, Kin10^{-5}M + NAA10^{-5}M or Kin10^{-5}M + 2,4-D10^{-7}M (Table 2.7a) (Fig.2.20). Overall, a culture period of 2 weeks was sufficient to turn the non-organogenic calli to green calli when incubated in the BM supplemented with BA0.1x10^{-6}M + NAA27x10^{-6}M. However, the calli induced in the BM supplemented with BA in combination with either NAA or 2,4-D turned green within 1 week. The calli induced in the BM supplemented with Kin in combination with either NAA or 2,4-D turned green within the mentioned incubation period. The lowest frequency (%) of green callus formation was observed in the BM supplemented with Kin10^{-5}M + IAA10^{-6}M (Table 2.7a) (Fig. 2.20). Approximately 3 weeks was required for greening of the calli induced in the BM supplemented with Kin in combination with either IAA or IBA.
For the length of the leaves and the shoots, column means sharing a common letter within the row are not significantly different at α = 0.05 level. For the number of shoot buds and the number of leaves/shoot, column means sharing a common letter within the row are not significantly different at α = 0.05 level.

Solution concentration in medium

and incubated for 2 weeks in the dark and further 4 weeks in the light.

Complete shoot differentiation from the shoot buds grown in the BM supplemented with BA = 1.0 × 10⁻⁶ M and NAA = 1.0 × 10⁻⁴ M and grown for further 8 weeks in the light.

Results given as mean of 20 replicates.

<table>
<thead>
<tr>
<th>Phenol (mg/ml)</th>
<th>Len of Shoots (cm)</th>
<th>No. of Leaves/shoot</th>
<th>Shoot Inhibition (%</th>
<th>Green Callus Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.23 ± 0.039</td>
<td>1.19 ± 0.012</td>
<td>1.15 ± 0.013</td>
<td>1.17 ± 0.014</td>
<td>1.18 ± 0.013</td>
</tr>
<tr>
<td>1.32 ± 0.041</td>
<td>1.30 ± 0.014</td>
<td>1.29 ± 0.015</td>
<td>1.28 ± 0.016</td>
<td>1.27 ± 0.017</td>
</tr>
<tr>
<td>0.69 ± 0.027</td>
<td>0.67 ± 0.019</td>
<td>0.65 ± 0.018</td>
<td>0.64 ± 0.016</td>
<td>0.63 ± 0.017</td>
</tr>
<tr>
<td>0.59 ± 0.032</td>
<td>0.57 ± 0.024</td>
<td>0.56 ± 0.023</td>
<td>0.55 ± 0.022</td>
<td>0.54 ± 0.021</td>
</tr>
<tr>
<td>0.49 ± 0.036</td>
<td>0.47 ± 0.028</td>
<td>0.46 ± 0.027</td>
<td>0.45 ± 0.026</td>
<td>0.44 ± 0.025</td>
</tr>
<tr>
<td>0.39 ± 0.040</td>
<td>0.37 ± 0.032</td>
<td>0.36 ± 0.031</td>
<td>0.35 ± 0.030</td>
<td>0.34 ± 0.029</td>
</tr>
<tr>
<td>0.29 ± 0.044</td>
<td>0.27 ± 0.036</td>
<td>0.26 ± 0.035</td>
<td>0.25 ± 0.034</td>
<td>0.24 ± 0.033</td>
</tr>
</tbody>
</table>

* Results shown in parentheses indicate < 20 replicates.

** Results given as mean of 20 replicates.

Effects of cytokinin/auxin combinations supplemented with the selected cytokinin/auxin combinations

**The combination of BA (1.0 × 10⁻⁶ M) and NAA (1.0 × 10⁻⁴ M) is the most effective in inducing shoot differentiation and shoot bud induction.**
### TABLE 2.7b
Summary analysis of variance of effects of the selected cytokinin/auxin combinations on shoot-bud induction and morphological characteristics of the *Duboisia myoporoides* R. Br. shoot

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean squares</th>
<th>F ratio *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of shoot buds/500mg callus</td>
<td>Between groups Within groups</td>
<td>297.5047</td>
<td>6</td>
<td>49.58412</td>
<td>15.48341</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>425.9195</td>
<td>133</td>
<td>3.202402</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>723.4242</td>
<td>139</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of leaves/shoot</td>
<td>Between groups Within groups</td>
<td>439.8975</td>
<td>6</td>
<td>73.31624</td>
<td>21.17389</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>460.5229</td>
<td>133</td>
<td>3.462578</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>900.4203</td>
<td>139</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of the leaves</td>
<td>Between groups Within groups</td>
<td>26.69325</td>
<td>6</td>
<td>4.448875</td>
<td>73.11387</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>8.09286</td>
<td>133</td>
<td>0.060849</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>34.78611</td>
<td>139</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of the shoots</td>
<td>Between groups Within groups</td>
<td>282.9036</td>
<td>6</td>
<td>47.1506</td>
<td>266.237</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>23.55431</td>
<td>133</td>
<td>0.1771</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>306.4579</td>
<td>139</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* : P < 0.001
Text-Figure 2.20  Effects of cytokinin / auxin combinations (used at the calli induction stage) on the frequency (%) of green calli formation from the 11-week-old non-organogenic calli induced on the leaf explant of *Daboisia myoporoides* R. Br. in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and the selected cytokinin / auxin combinations

1: 2,4-D \(10^{-7}\)M  
2: BA \(10^{-5}\)M + NAA \(10^{-6}\)M  
3: BA \(10^{-5}\)M + 2,4-D \(10^{-7}\)M  
4: Kin \(10^{-5}\)M + IAA \(10^{-6}\)M  
5: Kin \(10^{-5}\)M + IBA \(10^{-5}\)M  
6: Kin \(10^{-5}\)M + NAA \(10^{-5}\)M  
7: Kin \(10^{-5}\)M + 2,4-D \(10^{-7}\)M
2.3.5.2 Shoot-bud Induction on the Green Calli

The number of shoot-buds induced on the green calli produced from the 11-week-old non-organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations are summarised in Table 2.7a and presented in Fig. 2.21.

The least number of shoot-buds was produced on the green callus when the callus induction medium was supplemented with only auxin 2.4-D\(10^{-7}\)M (Table 2.7a) (Fig. 2.21). A culture period of more than 8 weeks was required to induce the maximum number of shoot-buds. The number of shoot-buds was significantly (\(P < 0.001\)) lower than those induced in the BM supplemented with the other selected cytokinin and auxin combinations (Table 2.7a and b) (Fig. 2.21).

The highest number of shoot-buds was produced on the green callus when the callus induction medium was supplemented with BA\(10^{-5}\)M + NAA\(10^{-6}\)M (Table 2.7a) (Fig. 2.21). A culture period of 6 weeks was sufficient to yield the maximum number of shoot-buds under the culture conditions used in this study. The number of shoot-buds produced on the callus induced in the BM supplemented with BA\(10^{-5}\)M + NAA\(10^{-6}\)M was significantly (\(P < 0.001\)) higher than those induced in the BM supplemented with the other selected cytokinin/auxin combinations (Table 2.7a and b) (Fig. 2.21).

Overall, 4.65-5.95 shoot-buds were produced per 500mg green callus when the calli induction media were supplemented with Kin in combination with different auxins used (Table 2.7a).

A culture period of more than 8 weeks was required to induce the maximum number of shoot-buds on the calli induced in the BM supplemented with 2,4-D in combination with either BA or Kin. The number of shoot-buds did not differ
Text-Figure 2.21  Effects of cytokinin / auxin combinations on shoot-bud induction and the morphological characteristics of the 6-week-old shoot differentiated from the non-organogenic calli induced in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and the selected cytokinin / auxin combinations; the vertical bar at the top of each column shows ± standard deviation

Cytokinin / auxin combination

1: 2,4-D10^{-7}M
2: BA10^{-5} + NAA10^{-6}M
3: BA10^{-5}M + 2,4-D10^{-7}M
4: Kin10^{-5}M + IAA10^{-6}M
5: Kin10^{-5}M + IBA10^{-5}M
6: Kin10^{-5}M + NAA10^{-5}M
7: Kin10^{-5}M + 2,4-D10^{-7}M
significantly when the callus induction medium was supplemented with 2,4-D in combination with either BA or Kin (Table 2.7a and b, page 119-120) (Fig. 2.21, page 123). The number of shoot-bud induction was dependent on the type of auxin (IAA, IBA, NAA and 2,4-D) when the calli induction media was supplemented with Kin $10^{-5}$M (Table 2.7a, page 119) (Fig. 2.21, page 123).

The number of shoot-buds did not differ significantly when the calli induction media were supplemented with 2,4-D$10^{-7}$M alone or in combination with BA or kinetin (Table 2.7a and b, page 119-120) (Fig. 2.21, page 123).

### 2.3.5.3 Shoot Differentiation

The morphological characteristics of the shoots differentiated from the non-organogenic calli induced in the BM supplemented with the selected cytokinin and auxin combinations are summarised in Table 2.7a (page 119) and presented in Fig. 2.21 (page 123).

Shoots differentiated from the calli induced in the BM supplemented with 2,4-D$10^{-7}$M were unlike the parent plant. Two different types of basal stem, 1) elongated, thick (>1cm diameter) and pale green in 60% of shoots and 2) small (0.6-1.0cm), erect, narrow and dark green in 40% of shoots were observed. Shoots with elongated stem (type 1) (Fig. 2.22) continued throughout the incubation period and attained an average height of 2.09cm. The leaves (average 5.95 per shoot) were pale green, flat and some of them were partially attached with the stem (Fig. 2.22). Shoots with small stem (type 2) were healthy at the initial stage but after 1 to 2 weeks of light incubation, they failed to grow any further and were discarded.

The number of leaves formed per shoot was lower than that on the BM supplemented with BA$10^{-5}$M + NAA$10^{-6}$M or Kin$10^{-5}$M + NAA$10^{-5}$M, higher than that on the BM supplemented with BA$10^{-5}$M + 2,4-D$10^{-7}$M, Kin$10^{-5}$M + IAA$10^{-6}$M or Kin$10^{-5}$M + 2,4-D$10^{-7}$M and similar to that on the BM supplemented with Kin$10^{-5}$M + IBA$10^{-5}$M (Table 2.7a, page 119) (Fig. 2.21, page 123). The length of the
**Text-Figure 2.22** Six-week-old shoot differentiated from the non-organogenic callus induced in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and 2,4-D $10^{-5}$M. Note the thick basal stem (ts) and the flat leaves (fl) partially attached to the basal stem. Bar size 1.08 cm

**Text-Figure 2.23** Six-week-old shoot differentiated from the non-organogenic callus induced in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA $10^{-5}$M + NAA $10^{-6}$M. Note the erect stem (s) and dark green leaves (l). Bar size 2.8cm

**Text-Figure 2.24** Six-week-old shoot differentiated from the non-organogenic callus induced in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and Kin $10^{-5}$M + NAA $10^{-6}$M. Note the non-elongated basal stem (bs) of the shoot. Bar size 1.32 cm
leaves formed on the shoot differentiated from the calli induced in the BM supplemented with 2,4-D10^{-7}M was shorter than that in the BM supplemented with BA10^{-5}M + NAA10^{-6}M, Kin10^{-5}M + IAA10^{-6}M, Kin10^{-5}M + NAA10^{-5}M or Kin10^{-5}M + 2,4-D10^{-7}M, longer than that in the BM supplemented with BA10^{-5}M + 2,4-D10^{-7}M or Kin10^{-5}M + IBA10^{-5}M (Table 2.7a, page 119) (Fig. 2.21, page 123). The length of the shoot differentiated from the calli induced in the BM supplemented with 2,4-D10^{-7}M was shorter than that in the BM supplemented with BA10^{-5}M + NAA10^{-6}M, longer than that in the BM supplemented with the other selected cytokinin and auxin combinations (Table 2.7a, page 119) (Fig. 2.21, page 123). The number of leaves, the length of the leaves and the shoots differentiated from the calli induced in the BM supplemented with 2,4-D10^{-7}M were significantly (P < 0.001) lower than those differentiated from the calli induced in the BM supplemented with BA10^{-5}M + NAA10^{-6}M (Table 2.7a and b, page 119-120) (Fig. 2.21, page 123).

The highest number of leaves, the longest leaves and shoots were produced from the callus induced in the BM supplemented with BA10^{-5}M + NAA10^{-6}M (Table 2.7a, page 119) (Fig. 2.21, page 123). The shoots were like the parent plant and attained an average height of 5.1cm within the mentioned incubation period (2+4 weeks). The shoots were well grown with an erect dark green stem and a maximum number (>10) of dark green healthy leaves per shoot. No axillary bud or branching was observed in those shoots (Fig. 2.23, page 125). The number of leaves, the length of the leaves and the shoots were significantly (P < 0.001) higher than those produced in the BM supplemented with the other selected cytokinin/auxin combinations (Table 2.7a and b, page 119-120) (Fig. 2.21, page 123).

The morphological characteristics of the shoots differentiated from the calli induced in the BM supplemented with the other selected cytokinin and auxin combinations were similar (Table 2.7a, page 119) (Fig. 2.21, page 123). The basal stem and the leaves were pale green. They did not elongate properly during the 6 weeks incubation period. Figure 2.24 shows the shoot differentiated from the callus induced in the BM supplemented with Kin10^{-5}M + NAA10^{-5}M. The number of leaves produced per shoot differentiated from the calli induced in the BM supplemented with Kin10^{-5}M + NAA10^{-5}M was significantly (P < 0.001) higher than those produced per shoot differentiated from the calli induced in the BM.
supplemented with BA10^{-5}M + 2,4-D10^{-7}M, Kin10^{-5}M + IAA10^{-6}M, Kin10^{-5}M + IBA10^{-5}M or Kin10^{-5}M + 2,4-D10^{-7}M (Table 2.7a and b, page 119-120) (Fig. 2.21, page 123). The length of the leaves produced on the shoot differentiated from the calli induced in the BM supplemented with 2,4-D10^{-7}M, Kin10^{-5}M + IAA10^{-6}M or Kin10^{-5}M + 2,4-D10^{-7}M were not significant (Table 2.7a, page 119) (Fig. 2.21, page 123). The length of the shoots differentiated from the calli induced in the BM supplemented with BA10^{-5}M + 2,4-D10^{-7}M or Kin10^{-5}M + IBA10^{-5}M, Kin10^{-5}M + IAA10^{-6}M or Kin10^{-5}M + NAA10^{-5}M, Kin10^{-5}M + NAA10^{-5}M or Kin10^{-5}M + 2,4-D10^{-7}M and Kin10^{-5}M + IBA10^{-5}M or Kin10^{-5}M + 2,4-D10^{-7}M was not significantly different (Table 2.7a, page 119) (Fig. 2.21, page 123).

Of the 4 parameters studied for the morphological characteristics of the differentiated shoots, the highest significant value was obtained for the length of the shoots at \(\alpha = 0.05\) level (Table 2.7b, page 120).

### 2.3.6 Interactions of Cytokinins and Auxins on Shoot Culture from the Organogenic Calli

The effects of cytokinins and auxins (used at the calli induction stage) on the shoot-bud induction and the morphological characteristics of the shoots differentiated from the organogenic calli are summarised in Table 2.8a and presented in Fig. 2.25. Subsequent growth and development of the regenerated shoot-buds took place in the BM supplemented with the cytokinin and auxin combinations used at the callus induction stage.

Shoots differentiated from the organogenic calli induced in the BM supplemented with BA10^{-5}M + IAA10^{-6}M attained a height of 2.7cm with 1.63cm long and 6.76 dark green leaves per shoot (average values), during 9 weeks of incubation (Table 2.8a). The basal stems of the shoots were pale green and no nodes or axillary buds were observed (Fig. 2.26). The number of leaves formed per shoot, the length of the leaves and the shoots produced from the calli induced in the BM supplemented with BA10^{-5}M + IAA10^{-6}M were lower than those produced from the
TABLE 2.8a  Effects of cytokinin and auxin combinations* (used at the callus induction stage) on the shoot-bud induction and morphological characteristics of the shoots differentiated from the organogenic calli induced on the leaf explant of *Duboisa myoporoides* R. Br. incubated in the semi-solid MS (Murashige and Skoog's salts and vitamins,1962) basal medium.

<table>
<thead>
<tr>
<th>Cytokinin and Auxin</th>
<th>No of shoot-buds / leaf explant **</th>
<th>Morphology of the differentiated shoots **</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of leaves / shoot</td>
<td>Length of the leaves (cm)</td>
</tr>
<tr>
<td>BA10⁻⁷M+IAA10⁻⁹M</td>
<td>5.35 ± 1.4</td>
<td>6.76 ± 1.26</td>
</tr>
<tr>
<td>BA10⁻⁷M+IBA10⁻⁷M</td>
<td>14.80 ± 3.87</td>
<td>8.90 ± 1.97</td>
</tr>
</tbody>
</table>

Note: The cultures were incubated in the same media supplemented with the cytokinin and auxin combinations used at the calli induction stage. The number of shoot-buds formed per leaf explant were counted over a period of 4 weeks.

* : Results given as means of 20 replicates
** : Mean ± standard deviation

TABLE 2.8b  Summary *t* test of effects of cytokinin and auxin combinations on the shoot-bud induction and morphological characteristics of the shoots differentiated from the organogenic calli

<table>
<thead>
<tr>
<th>Sample</th>
<th>Degrees of freedom</th>
<th><em>t</em> Stat *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of shoot-buds/ explant</td>
<td>38</td>
<td>10.1754</td>
</tr>
<tr>
<td>Number of leaves/shoot</td>
<td>38</td>
<td>4.08497</td>
</tr>
<tr>
<td>Length of the leaves</td>
<td>38</td>
<td>6.78635</td>
</tr>
<tr>
<td>Length of the shoots</td>
<td>38</td>
<td>11.7512</td>
</tr>
</tbody>
</table>

* : *P* < 0.001
Text-Figure 2.25 Effects of cytokinin and auxin combinations on shoot-bud induction and morphological characteristics of the 9-week-old shoot differentiated from the organogenic calli induced on the leaf explant of *Daboissia myoporoides* R. Br. incubated in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and the selected cytokinin and auxin combinations; the vertical bar at the top of each column shows ± standard deviation.

Cytokinin and auxin combination

1 : BA $10^{-5}$M + IAA $10^{-6}$M
2 : BA $10^{-5}$M + IBA $10^{-7}$M
Text-Figure 2.26 Nine-week-old shoot differentiated from the organogenic callus induced in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA10^{-5} M + IAA10^{-6}M. Note the non-elongated basal stem (bs) without any nodes. Bar size 2.7 cm

Text-Figure 2.27 Nine-week-old shoot differentiated from the organogenic callus induced in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA10^{-5}M+IBA10^{-7}M. Note the erect dark green basal stem (bs) with nodes and dark green leaves (l). Bar size 2.36 cm

Text-Figure 2.28 Four-week-old root (r) regenerated from the non-organogenic callus induced in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA10^{-5} M+NAA10^{-6}M incubated in the MS basal medium supplemented with IBA25 x 10^{-7}M.
calli induced in the BM supplemented with BA10⁻⁵M + IBA10⁻⁷M (Table 2.8a) (Fig. 2.25).

Approximately 70% (results presented for these shoots) of the shoots elongated in the BM supplemented with BA10⁻⁵M + IBA10⁻⁷M attained 9.45 cm height with 3.64 cm long and 8.90 dark green leaves per shoot (average value) (Table 2.8a). The shoots were like the parent plant with an erect, dark green basal stem and 6 to 10 nodes (Fig. 2.27). In 70% of these shoots, 0.31 axillary buds / shoot (average values) and in 30%, 3 to 4 branching were observed. About 20% shoots were able to form roots directly on the same medium (only un-rooted shoots were used for alkaloid analysis) (Fig. 2.33, page 135). On prolonged incubation (more than 15 weeks), no morphological change in the leaves or stems was found.

The remainder of the 30% shoots elongated in the medium supplemented with BA10⁻⁵M + IBA10⁻⁷M attained 2-3 cm height with dark green leaves. The basal stems of these shoots were erect, dark green and without node, axillary bud or branching.

The number of shoot-buds formed / leaf explant, number of leaves / shoot, the length of the leaves and shoots were significantly higher (P < 0.001) in the medium supplemented with BA10⁻⁵M + IBA10⁻⁷M than those in the BM supplemented with BA10⁻⁵M + IAA10⁻⁶M when t test was applied (Table 2.8b, page 128).

2.3.7 Interactions of Cytokinins and Auxins on Root Culture from the Non-organogenic Calli

Table 2.9 shows the results of root regeneration from the non-organogenic calli induced in the medium supplemented with different medium additives. Of the 5 different medium additives used for root regeneration, roots were only induced on the calli incubated in the MS basal medium supplemented with IBA25x10⁻⁶M. No root hairs were found on the regenerated roots and they did not elongate (< 0.5 cm) during the incubation period (Fig. 2.28, page 130). No roots were regenerated on the
calli incubated in the media supplemented with other medium additives (Table 2.9). Calli growth continued for 2 to 3 weeks, then the calli turned brown and ultimately died.

**TABLE 2.9** Effects of different medium additives on root regeneration from the non-organogenic calli, induced on the leaf explant of *Duboisia myoporoides* R. Br. incubated in the MS basal medium supplemented with the selected cytokinin and auxin combinations.

<table>
<thead>
<tr>
<th>Cytokinin &amp; auxin used for callus induction</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA10^{-5}M+NAA10^{-6}M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BA10^{-5}M+2.4-D10^{-7}M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kin10^{-5}M+IAA10^{-6}M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kin10^{-5}M+IBA10^{-5}M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kin10^{-5}M+NAA10^{-5}M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kin10^{-5}M+2.4-D10^{-6}M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ : Root formation  
- : No root formation  
* : Results given as means of 10 replicates after 6 weeks of culture  
** : Medium additives for root regeneration:

1) 1/2 MS salt + full vitamin and no PGR  
2) Full MS salt + full vitamin and BA10^{-6}M+IBA10^{-5}M  
3) 1/2 MS salt + 10mg/L tropic acid  
4) 1/2 MS salt + full vitamin and BA10^{-6}M+IBA10^{-5}M  
5) Full MS salt + full vitamin + IBA25x10^{-6}M
2.3.8 **Interactions of Cytokinins and Auxins on Root Culture from the Organogenic Callus**

The roots induced on the organogenic calli did not grow well. The roots had many hairs and elongated to 0.4 - 0.6cm during the incubation period (Fig. 2.16, page 103). At the end of the incubation period, the root regenerating calli turned brown.

2.3.9 **Selected Cytokinin and Auxin Combination Studied**

The effects of different concentrations of BA + IBA on the morphogenetic response (Table 2.3, page 104) on the greenhouse-grown leaf explant of *D. myoporoides* are presented in Fig. 2.29a. Depending upon the concentrations of BA + IBA, 3 different types of morphogenetic responses were observed on the leaf explant. Shoots and roots were formed in the BM when BA : IBA ratio was 100 : 1 and 1 : 10 respectively. Whereas, BA : IBA 10 : 1 caused continued growth of the non-organogenic callus. The shoots differentiated from the organogenic calli induced on the leaf explant from the greenhouse-grown cuttings were similar to the parent plant (Fig. 2.30). Roots were initiated in 20% of the (9-11 week-old) differentiated shoots (Fig. 2.33).

After 4 weeks, compact green calli were induced on the leaf explant obtained from the *in vitro* grown shoot differentiated from the non-organogenic calli induced in the BM supplemented with 2.4-D10⁻⁷M, which regenerated shoot-buds after 1 week. Roots were induced on the calli after 1 more week. The morphogenetic response on the *in vitro* grown leaf explant is presented in Fig. 2.29b. Shoots and roots were formed in the BM when BA : IBA ratio was 100 : 1. The shoot-buds were similar to those induced on the leaf explant from the greenhouse-grown cuttings (Fig. 2.31). The shoot-buds were elongated to 5cm long shoots with green basal stems. About 1 to 2.5cm long and 5 to 6 dark green leaves were formed on each shoot. No nodes or axillary buds were observed in the shoots. Roots were regenerated on 10% of explants. The roots were healthy and devoid of root hair (Fig. 2.32, page 135).
Text-Figure 2.29a Effects of BA + IBA concentrations on the morphogenetic response on the leaf explant of *Duboisia myoporoides* R. Br. collected from greenhouse-grown cuttings.

Text-Figure 2.29b Effects of BA + IBA concentrations on morphogenetic response on the leaf explant of *in vitro* grown *Duboisia myoporoides* shoot differentiated from the non-organogenic calli induced on the leaf explant incubated in the BM supplemented with 2,4-D10^{-7} M.
FIGURES 2.30 – 2.33 Effects of BA $10^{-5}$M + IBA $10^{-7}$M on organogenesis and differentiation on the leaf explant of *Daboisia myoporoides* R. Br. incubated in the MS basal medium supplemented with Bacto agar (0.9%) and sucrose (3%).

**Text-Figure 2.30** Nine-week-old shoot (without root initiation) similar to the parent plant shoot differentiated from the organogenic callus induced on the leaf explant collected from the greenhouse-grown cuttings.

**Text-Figure 2.31** Shoot-bud (sb) induction on the organogenic calli induced on the leaf explant collected from the 6-week-old shoot (without root initiation) differentiated from the non-organogenic callus induced in the BM supplemented with 2,4-D $10^{-5}$M.

**Text-Figure 2.32** Root (r) induction on the organogenic calli induced on the leaf explant of 6-week-old (without root initiation) shoot differentiated from the non-organogenic callus induced in the BM supplemented with 2,4-D $10^{-5}$M.

**Text-Figure 2.33** Root (r) initiation on the 9-week-old shoot differentiated from the organogenic calli induced on the leaf explant of mature greenhouse-grown cuttings.
2.3.10 Effects of TDZ on the Shoot-bud Induction

No calli were induced in the BM supplemented with different concentrations of TDZ only. Within 7 to 10 days of dark incubation, callus induction started on the cut edge of the leaf explants incubated in the BM supplemented with TDZ (5, 10, 15) x 10^{-6}M in combination with IBA (10^{-5}, 10^{-6}, 10^{-7})M. The frequency (%) of calli induction on the leaf explant is summarised in Table 2.10.

**TABLE 2.10** Effects* of various TDZ +IBA combinations on frequency (%) of callus induction on the leaf explant of *Duboisia myoporoides* R. Br. incubated in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various concentrations of TDZ and IBA for 2 weeks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TDZ + IBA (M)</th>
<th>Callus induction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>control</td>
<td>x</td>
</tr>
<tr>
<td>2</td>
<td>TDZ 5x10^{-6}M</td>
<td>x</td>
</tr>
<tr>
<td>3</td>
<td>TDZ 10x10^{-6}M</td>
<td>x</td>
</tr>
<tr>
<td>4</td>
<td>TDZ 15x10^{-6}M</td>
<td>x</td>
</tr>
<tr>
<td>5</td>
<td>TDZ 5x10^{-6}M + IBA10^{-5}M</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>TDZ 5x10^{-6}M + IBA10^{-6}M</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>TDZ 5x10^{-6}M + IBA10^{-7}M</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>TDZ 10x10^{-6}M + IBA10^{-5}M</td>
<td>95 ± 0.04</td>
</tr>
<tr>
<td>9</td>
<td>TDZ 10x10^{-6}M + IBA10^{-6}M</td>
<td>95 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>TDZ 10x10^{-6}M + IBA10^{-7}M</td>
<td>95 ± 0.04</td>
</tr>
<tr>
<td>11</td>
<td>TDZ 15x10^{-6}M + IBA10^{-5}M</td>
<td>95 ± 0.04</td>
</tr>
<tr>
<td>12</td>
<td>TDZ 15x10^{-6}M + IBA10^{-6}M</td>
<td>95 ± 0.04</td>
</tr>
<tr>
<td>13</td>
<td>TDZ 15x10^{-6}M + IBA10^{-7}M</td>
<td>95 ± 0.04</td>
</tr>
</tbody>
</table>

* : Results given as means of 10 replicates
M : Solution concentration in molarity
x : No response
The frequency (%) of callus induction was mainly dependent on the amount of TDZ present in the callus induction media and was higher when the media were supplemented with lower concentrations of TDZ (5x10^{-6}M) (Table 2.10).

Table 2.11 shows the effects of various concentrations of TDZ in combination with IBA on the morphogenetic responses on the leaf explant of *D. myoporoides* after 6 weeks incubation. After transferring to the light, the induced white calli were nearly doubled in size (visual observation) during the first week of culture. These white calli turned yellowish-white after 1 week and then green (Fig. 2.34). After 5 to 6 weeks of incubation, shoot-buds were induced on the green calli incubated in the BM supplemented with TDZ5x10^{-6} M + IBA(10^{-6},10^{-7}) M (Table 2.11) (Fig. 235). The calli induced in the BM supplemented with other TDZ and IBA combinations grew as non-organogenic calli. Morphogenetic response for BA+IBA combination (Table 2.3, page 104) are presented in Table 2.11 for comparison.

**TABLE 2.11** Effects of various concentrations of TDZ in combination with IBA on the morphogenetic responses on the leaf explant of *Duoboisia myoporoides* R. Br. incubated in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various concentrations of TDZ+IBA for 6 weeks

<table>
<thead>
<tr>
<th>IBA</th>
<th>TDZ</th>
<th>BA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0M</td>
<td>5x10^{-6}M</td>
</tr>
<tr>
<td>0M</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>10^{-5}M</td>
<td>x</td>
<td>c</td>
</tr>
<tr>
<td>10^{-6}M</td>
<td>x</td>
<td>s</td>
</tr>
<tr>
<td>10^{-7}M</td>
<td>x</td>
<td>s</td>
</tr>
</tbody>
</table>

*: Results given as means of 10 replicates for TDZ+IBA combinations

x: No response

c: Callus formation

s: Shoot-bud formation on the organogenic calli
FIGURES 2.34 – 2.37 Effects of TDZ on shoot-bud induction on the leaf explant of *Daboisia Myoporoides* R. Br. incubated in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and different concentrations of TDZ + IBA.

Text-Figure 2.34 Two-week-old callus (c) induced in the BM supplemented with TDZ5x10^{-6} M + IBA10^{-5} M.

Text-Figure 2.35 Shoot-bud (s) induction on the 6-week-old organogenic calli induced in the BM supplemented with TDZ 5x10^{-6} M + IBA 10^{-5} M.

Text-Figure 2.36 Four-week-old shoot-buds grown in the BM supplemented with TDZ 5x10^{-6} M + IBA10^{-5} M. Note the shoot-buds (sb) partially attached with the calli.

Text-Figure 2.37 Four-week-old shoot-buds grown in the BM supplemented with TDZ 5x10^{-6} M + IBA10^{-5} M. Note the thin shoot-buds (sb) separated from the calli.
Mainly non-organogenic calli were induced in the BM supplemented with various concentrations of TDZ + IBA (Table 2.11). A lower TDZ concentration (5 x 10^{-6} M) caused shoot-bud induction on the calli (Table 2.11), whereas a higher concentration of BA (10^{-5} M) was necessary for shoot-bud induction on the calli (Table 2.11). Comparatively, a lower concentrations of TDZ in combination with lower concentrations of IBA was optimal for shoot-bud induction (Table 2.11).

Table 2.12a shows the effects of various concentrations of TDZ + IBA on shoot-bud induction on the calli. Shoot-buds induced in the BM supplemented with TDZ 5x10^{-6} M + IBA10^{-6} M were partially attached with the calli and they did not grow well during 4 (age of the shoot-buds) weeks incubation (Fig 2.36, page 138). More than 3 shoot-buds / 500mg calli were induced in the BM supplemented with TDZ 5x10^{-6} M + IBA10^{-7} M (Table 2.12a). The shoot-buds were thin but separated from the callus (Fig. 2.37, page 138). No marked growth was observed during the incubation period. The number of shoot-buds induced in the BM supplemented with TDZ 5x10^{-6} M + IBA10^{-7} M was significantly (P < 0.001) higher than those in the BM supplemented with TDZ 5x10^{-6} M + IBA10^{-6} M (Table 2.12b).

**TABLE 2.12a** Effects* of various TDZ + IBA concentrations on the shoot-bud induction on the calli incubated in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various concentrations of TDZ + IBZ for 8 weeks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TDZ +IBA</th>
<th>Number of shoot-buds / 500mg calli **</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>TDZ 5x10^{-6}M + IBA10^{-5} M</td>
<td>x</td>
</tr>
<tr>
<td>6</td>
<td>TDZ 5x10^{-6}M + IBA10^{-6} M</td>
<td>1.9 ± 0.73</td>
</tr>
<tr>
<td>7</td>
<td>TDZ 5x10^{-5}M + IBA10^{-7} M</td>
<td>3.6 ± 0.966</td>
</tr>
<tr>
<td>8</td>
<td>TDZ 10x10^{-6}M + IBA10^{-5} M</td>
<td>x</td>
</tr>
<tr>
<td>9</td>
<td>TDZ 10x10^{-6}M + IBA10^{-6} M</td>
<td>x</td>
</tr>
<tr>
<td>10</td>
<td>TDZ 10x10^{-6}M + IBA10^{-5} M</td>
<td>x</td>
</tr>
<tr>
<td>11</td>
<td>TDZ 15x10^{-6}M + IBA10^{-7} M</td>
<td>x</td>
</tr>
<tr>
<td>12</td>
<td>TDZ 15x10^{-6}M + IBA10^{-6} M</td>
<td>x</td>
</tr>
<tr>
<td>13</td>
<td>TDZ 15x10^{-6}M + IBA10^{-5} M</td>
<td>x</td>
</tr>
</tbody>
</table>

* : Results given as means of 10 replicates
** : Mean ± standard deviation
M : Solution concentration in molarity
x : No response
TABLE 2.12b  Summary t test of effects of TDZ + IBA concentrations on shoot-bud induction

<table>
<thead>
<tr>
<th>Sample</th>
<th>Degrees of freedom</th>
<th>t Stat *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Shoot-buds</td>
<td>18</td>
<td>4.422261</td>
</tr>
</tbody>
</table>

* : P < 0.001

2.3.11  Morphology of the Seed Germinated Shoot

After 7 days of dark incubation, 6% of seeds (Fig. 2.38) were germinated (Fig. 2.39). The seedlings had 3 to 4cm long basal stems, 4 to 5 roots and 3 to 4 dark green leaves. At the end of the incubation period, no nodes or axillary-buds were observed in those seedlings.
Text-Figure 2.38 *Daboisias myoporoides* R. Br. seeds collected from the 2-year-old cuttings grown in the greenhouse-conditions of UWS, Macarthur.

Text-Figure 2.39 *Daboisias myoporoides* R. Br. seeds germinated on the semi-solid UNE # B medium incubated in the dark at 25 ± 2°C for 2 weeks. Note the elongated hypocotyl (h). r: root.
2.4 DISCUSSION

2.4.1 Plant Regeneration

In this study, complete plants were regenerated by inducing shoot-buds on the non-organogenic callus induced on the leaf explant of *Duboisia myoporoides* R. Br. Incubation periods and cytokinin/auxin combinations used at different stages such as, callus induction, greening of the induced callus, shoot-bud induction on the green callus, shoot elongation and root induction in the differentiated shoots have been specified. These results are consistent with those of Lin and Griffin (1992a) who also reported complete plant regeneration of a *Duboisia* hybrid using the same cytokinin/auxin combinations and incubation conditions. Kitamura et al., (1980) however, reported a complete plant regeneration of *D. myoporoides* from the non-organogenic callus. They used kinetin as a cytokinin in combination with IAA or NAA as auxins for callus induction on the leaf explant were used. My results showed that a complete *D. myoporoides* plant can be regenerated from the non-organogenic callus induced in the BM supplemented with cytokinin and auxins other than Kin+ IAA or NAA.

My results also indicated that, BA in combination with NAA (at different concentrations) could be used for greening of the non-organogenic calli, shoot-bud induction and shoot elongation. By using this plant regeneration procedure, it was possible to keep the auxin and cytokinin combination (BA+NAA) constant for greening of the callus, shoot-bud induction on the green calli and shoot elongation. This constancy helped to study the effects of cytokinins/auxins (used at the callus induction stage) on cell layer organization and alkaloid localization in the organs (shoots and roots) of *D. myoporoides* regenerated separately.
2.4.2 Induction and Growth of the Non-organogenic and Organogenic Calli

Various morphogenetic responses on the leaf explant of *Duboisia myoporoides* were obtained by varying cytokinin and auxin combinations in the present study. These observations are consistent with those of Kukreja *et al.*, (1986) and Badaoui *et al.*, (1996) Non-organogenic callus formation was readily achieved with various cytokinin/auxin combinations in my studies. Similar results were obtained in different *Duboisia* species (Kitamura *et al.*, 1980; Yamada and Endo, 1984; Kukreja *et al.*, 1986; Kitamura *et al.*, 1986; Lin and Griffin, 1992a) and non-secondary metabolite producing plant species (Kamo, 1994).

Auxins and combinations of cytokinins and auxins increased non-organogenic callus induction on the leaf explant. The auxins 2,4-D and NAA were significantly more effective than IAA or IBA for non-organogenic callus induction in the concentration range used in this study. Remotti and Loffler (1995) also reported better non-organogenic callus induction on cormlets and cormels of gladiolus in MS basal medium supplemented with various concentrations of 2,4-D and NAA. It appears that auxins play an important role in callus induction and certain combination of auxins with cytokinins is required for non-organogenic calli induction.

The observed growth of the non-organogenic calli over a period of 11 weeks was dependent on the cytokinin and auxin combinations. The growth and the browning of the calli induced with various concentrations of kinetin in combination with IAA is consistent with the results of Kitamura *et al.*, (1980) while effects of BA+NAA are similar to those reported by other workers (Yamada and Endo, 1984; Lin and Griffin, 1992a). My results indicate that irrespective of the cytokinin and auxin combinations and concentrations used for induction of the non-organogenic calli, it is preferable to use the calli before their browning i.e., within 12 weeks for organogenesis to occur.
Organogenic calli formation with BA10⁻⁵M in combination with IAA (10⁻⁵-10⁻⁷) M or IBA10⁻⁷M observed in this study is consistent with the results reported for *D. myoporoides* (Kukreja *et al*.,1986) and other secondary metabolite producing plant species (Badaou *et al*.,1996; Nin *et al*.,1996). These results indicate that BA supports induction of a highly regenerative callus in different plant species. Since regeneration ability in culture depends on the plant species (Nin *et al*.,1996), the possibility of improving organogenesis efficiency in *D. myoporoides* by employing other PGR combinations and concentrations may exist.

Induction of the shoot-bud producing organogenic calli observed in this study was found to be related to the concentrations of the auxins used. BA10⁻⁵M in combination with IAA in the concentrations range (10⁻⁵10⁻⁷)M was effective for inducing the shoot-bud producing organogenic calli whereas IBA10⁻⁷M was optimal for the induction of the shoot-bud producing organogenic calli in my studies. This is consistent with the results of Badaou *et al*.,(1996) who also observed the induction of the shoot-bud producing organogenic calli on various explants of *Solanum paludosum* (a steroidal alkaloid producing plant species) in semi-solid medium containing Murashige and Skoog’s mineral nutrients (1962), Gamborg’s vitamins (1968) and BA10⁻⁷M in combination with IAA(10⁻⁵-10⁻⁷)M or IBA10⁻⁷M. These authors also reported the induction of organogenic calli on various explants of *Solanum paludosum* with IAA only. It appears that the natural auxin IAA is more effective than synthetic auxin IBA for the induction of the shoot-bud producing organogenic calli on the leaf explants of some secondary metabolite producing plant species including *D. myoporoides*.

Induction of the non-organogenic and organogenic calli depended on the type of cytokinin used. BA favoured organogenic calli, whereas kinetin favoured non-organogenic calli induction when they were used in combination with IAA or IBA. This is consistent with the previous results reported by different researchers. Benjamin *et al*.,(1990) reported the induction of shoot-bud producing organogenic calli in *Artemisia pallens* in a medium supplemented with BA+IAA. Badaou *et al*.,(1996) reported non-organogenic calli induction on the basal medium mentioned earlier supplemented with various concentrations of kinetin and IAA or kinetin and
IBA and the organogenic callus induction with BA and IBA. Kukreja et al., (1986) reported non-organogenic callus induction in Murashige and Skoog (1962) basal medium supplemented with kinetin + IAA, kinetin + IBA and organogenic callus induction with BA + IAA, BA + IBA. It appears that induction of the non-organogenic and organogenic calli on the leaf explant depends on the interaction between the cytokinins and auxins as well as their concentrations.

My results from the selection of the non-organogenic and organogenic calli for shoot regeneration showed that for more effective non-organogenic callus induction or a higher number of shoot-buds formed on the organogenic calli, a particular BA or kinetin concentration (10^{-3} \text{M}) was optimal, whereas IAA, IBA, NAA and 2,4-D concentrations varied from (10^{-5}-10^{-7}) \text{M}. Improving organogenesis appears to depend on formulating the appropriate concentrations of auxin to match a generalised cytokinin concentration for *D. myoporoides*.

### 2.4.3 Shoot culture from the non-organogenic calli

The percentage of green calli formation from the non-organogenic calli showed that cytokinin/auxin combinations used at the calli induction stage had some effects on the greening of the calli. The calli induced with BA as cytokinin in combination with auxin NAA or 2,4-D, turned green within 1 week as compared to other combinations which took longer to become green. These results are partially consistent with those of Idrisova and Berezneg (1978) where the combination of nutrients in the culture medium used for *Datura innoxia* callus was related to the chlorophyll accumulation. Physical factors (light) as well as the chemical constituents of the medium may enhance greening of the calli.

A significant difference was observed in the shoot-bud producing ability of the non-organogenic calli i.e., highest number of shoot-buds induced in the medium supplemented with BA+NAA and least when no cytokinin was added to the callus.
induction medium (Table 2.7a). These results indicate an effect of cytokinin/auxin combinations used at the callus induction stage on shoot-bud induction. As suggested previously for different *Duboisia* as well as other plant species (Kitamura *et al.*, 1980; Lin and Griffin, 1992a; Nin *et al.*, 1996; Lucchesini and Mensualisodi, 1996; Panizza *et al.*, 1997) BA alone can cause shoot-bud initiation on the non-organogenic calli induced in the medium supplemented with various cytokinin and auxin combinations. In plant cells, cytokinin levels depend on cytokinin biosynthesis and/or uptake from extracellular sources, metabolic interconversion, inactivation and degradation and auxins may influence cytokinin levels by affecting cytokinin biosynthesis and/or by promoting cytokinin degradation (Kaminek *et al.*, 1997). In this study, different cytokinin/auxin combinations used for callus induction are more likely to affect the level of the exogenous BA used for shoot-bud induction. Since only one BA concentration (BA22x10^{-6}M) was used for shoot-bud induction on the non-organogenic calli induced in the medium supplemented with the selected cytokinin/auxin combinations in the present study, it may be that by varying BA concentrations, a higher percentage of shoot-bud regeneration on the non-organogenic calli may be achieved.

The morphological differences noted for the basal stem of the shoots differentiated from the callus induced in the BM supplemented with 2,4-D10^{-7}M observed in this study indicate probable vitrification. However, the growth of these shoots during the incubation period and the presence of other shoots with erect stems showed that the vitrification may be reduced by manipulating the culture environment (Debergh *et al.*, 1981; Kevers *et al.*, 1984; Ziv, 1991a, b).

Analysis of the morphological characteristics of the shoots differentiated from the non-organogenic calli showed that longest shoot elongation was obtained when the initial callus induction medium contained BA10^{-5}M+NAA10^{-6}M. These results are consistent with those of Lin and Griffin (1992a) who reported a favourable elongation of *Duboisia* hybrid shoots differentiated from the calli induced in the BM supplemented with BA10^{-6}M + NAA54x10^{-6}M. My results also showed a longer shoot elongation when the initial callus induction media contained NAA in combination with either BA or kinetin. The interaction between BA10^{-5}M and
NAA10^{-6} M or Kin10^{-5} M and NAA10^{-5} M may increase shoot elongation more efficiently than the other cytokinin and auxin combinations used in this study.

Longer shoots with higher number of leaves and longer leaf length were obtained when the callus induction medium was supplemented with BA10^{-5} M+ NAA10^{-6} M. Moreover, shoot regeneration was optimal when the initial callus induction medium contained lower amounts of NAA. A similar effect of cytokinin/auxin used in the callus induction medium on shoot regeneration of *Aeschynomene americana*, *A. falcata*, *A. fluminensis*, *A. sensitava*, *A. villosa* was reported by Rey and Mrogrinski (1996). They reported that shoot regeneration was dependent on the cytokinin and auxin combinations used in the callus induction medium and the regeneration was highest when the callus induction medium contained relatively low concentration of NAA. Although cytokinin and auxin interaction play a role in the shoot regeneration from the non-organogenic calli, my work indicates an effect of auxin used at the callus induction stage on shoot elongation and NAA was found to be the most effective among all auxins used in this study.

The frequency of leaf formation (5.49-6.06) on the shoots differentiated from the non-organogenic calli induced in the BM supplemented with Kin10^{-5} M in combination with IAA10^{-6} M, IBA10^{-5} M or 2,4-D10^{-6} M is consistent with the results of Kitamura et al. (1980) who reported formation of 5 to 6 leaves on the shoots regenerated form the calli induced in Murashige and Skoog (1962) basal medium supplemented with Kin 4.65x10^{-8} M+2,4-D 4.5x10^{-6} M. Similar cytokinin and auxin effects on leaf formation might be obtained by varying the auxin concentrations.

### 2.4.4 Shoot culture from the organogenic calli

Shoot-buds were regenerated on the organogenic calli induced in the medium supplemented with the selected cytokinin and auxin combinations. These results are consistent with the results reported for *D. myoporoides* R. Br. (Kukreja et al., 1986), other alkaloid-producing plant species (Badaoui et al., 1996), some medicinal (Nin et al., 1996) and non-medicinal plant species (Laprara et al., 1997; Lakshmanan et
Although regeneration ability depends on the plant species (Nin *et al.*, 1996), a particular cytokinin and auxin combination can be optimal for shoot-bud regeneration in the secondary metabolite producing or non-producing plant species.

The efficiency of shoot-bud induction on the organogenic calli was related to the concentration of the auxins used in this study. Although IAA in a wide range of concentrations was able to induce shoot-buds on the organogenic calli, a higher number of shoot-buds were induced with IBA10⁻⁷M. It may be that in combination with BA10⁻⁵M, the synthetic auxin IBA10⁻⁷M was more effective than the natural auxin IAA10⁻⁷M for the shoot-bud induction on the organogenic calli induced on the leaf explant.

The morphological characteristics of the regenerated shoots showed a clear effect of the type of the auxin on shoot differentiation from the organogenic calli. Elongation of shoot and leaf, formation of axillary buds and occurrence of branching were higher in the shoots differentiated from the calli induced in the BM supplemented with BA10⁻⁵M+IBA10⁻⁷M than that with BA10⁻⁵ M+IAA10⁻⁶M. As suggested previously, IBA alone can elongate leaf (Kukulezanka and Poturala, 1994) (cited in Galek and Kukulezanka, 1994) and inhibits the development of axillary buds in the leaf axils at the base of the shoots in the seedlings of *Guzmania monostachya* (Galek and Kukulezanka, 1996). However, BA can increase stem diameter through activation of the axillary buds in the seedlings of *Tillandsia coronata* and *Guzmania monostachya* (Galek and Kukulezanka, 1996). My results indicate that BA10⁻⁵M interaction with the synthetic auxin IBA10⁻⁷M is more effective than with the natural auxin IAA10⁻⁶M for the formation of the elongated shoots, leaves and axillary buds in the cultured shoots of *D. myoporoides* R. Br.

In my study, best results were obtained for inducing shoot-buds on the organogenic calli, when the callus induction medium was supplemented with different combinations of BA(10⁻⁵, 10⁻⁶)M+IAA(10⁻⁵-10⁻⁷)M. Kukreja *et al.*, (1986) reported direct shoot-bud regeneration on the leaf explant of *D. myoporoides* incubated in the MS basal regeneration with Kin13.9x10⁻⁶M +IAA(2.8-5.7)x10⁻⁶M. Therefore, IAA appears to be a suitable auxin for quicker plant
regeneration in *D. myoporoides* in cultures. However, the secondary metabolite producing ability of the regenerated plant must also be considered.

A longer incubation period (more than 15 weeks) did not affect the growth and morphological characteristics of the shoots cultured in the MS basal medium supplemented with BA10⁻⁵M+IBA10⁻⁷M. Miura *et al.* (1988) reported the stable maintenance of a relatively fast-growing *Catharanthus roseus* shoot culture for 30 months with little change in the growth rates and morphological characteristics. It appears that shoot cultures of *D. myoporoides* may also be maintained for a longer incubation period. However, alkaloid productivity of the shoots without root formation should be considered.

The organogenic ability of the BM supplemented with BA10⁻⁵M+IBA10⁻⁷M on regeneration and differentiation observed in this study varied. Although occasional, root differentiation in the regenerated shoots indicated that the complete plant could be regenerated from the leaf explant using a single cytokinin and auxin combination. Badaou *et al.* (1996) reported rooting of the *Solanum paludosum* cultured shoots i.e., regeneration of a complete plant using a single cytokinin and auxin combination. Matthisse and Scott (1984) reported that auxin can induce adventitious rooting of the stems. My results showed that after a longer incubation period, adventitious rooting of the cultured shoots of *D. myoporoides* may be possible in the BM supplemented with BA10⁻⁵M+IBA10⁻⁷M. However, supplementation of the type and concentration of the cytokinin in combination with IBA10⁻⁷M should be considered.

My results showed the synergistic effect of cytokinin and auxin (used at the callus induction stage) on shoot-bud induction and shoot differentiation from the non-organogenic and organogenic calli. The results also indicated that the cytokinin BA10⁻⁵M was optimal for shoot-bud induction and shoot differentiation from the non-organogenic and organogenic calli. However, shoot-bud induction and shoot differentiation were varied depending on the type and concentration of the auxins used in this study. Thus, the results clearly demonstrated that the auxins played an important role in shoot-bud induction and shoot differentiation from the non-organogenic and organogenic calli.
2.4.5 Root culture from the non-organogenic and organogenic calli

The non-organogenic calli investigated for root regeneration by using different medium additives showed that the roots were only regenerated in the medium supplemented with full MS salt + full vitamin + IBA25×10⁻⁶M. The tested medium additives (1,3,5; section 2.3.7) are generally used for root differentiation on the calli or rooting in the regenerated shoots (Kitamura et al., 1980; Hamilton et al., 1986; Lin and Griffin, 1992a; Choi et al., 1998). In my study, these medium additives (1,3,5; section 2.3.7) gave negative results with all except the calli induced in the BM supplemented with BA10⁻⁵ M +NAA10⁻⁶ M. This indicates the importance of an auxin in the root induction medium as well as the cytokinin and auxin combination used for callus induction for root differentiation.

Although the tested combination BA10⁻⁶M+IBA10⁻⁵M induced roots on the organogenic calli, it did not produce root on the non-organogenic calli (medium additive 2 and 4). This indicates that the mentioned cytokinin and auxin combination is not optimal for root regeneration on the non-organogenic calli of D. myoporoides induced in the BM supplemented with cytokinin and auxin combinations used in this study.

Root regeneration on the organogenic calli is consistent with the results of Kukreja et al., (1986) who reported root regeneration on the leaf explant of D. myoporoides R. Br. incubated in the MS basal media supplemented with various cytokinins and auxins. As observed for various plant species (Endo and Yamada, 1985; Yukimune et al., 1994d; Basu and Chand, 1998) roots are generally regenerated in a medium supplemented with cytokinin and auxin combinations different from that used at the callus induction stage. My results showed that roots can be regenerated in the induction medium of the calli. However, an investigation using various BA+IBA concentrations is necessary to overcome the problem of the slow growth of the roots.
2.4.6 Selected Cytokinin and Auxin Combination Studied

The selected cytokinin and auxin combination BA10^{-5}M + IBA10^{-7}M showed different organogenic effects on the field grown as well as in vitro grown leaf explant of *D. myoporoides* in the present study. It showed 1) direct root formation on the leaf explant; 2) root formation on the induced organogenic callus; 3) complete shoot differentiation starting form the callus induction stage and 4) plantlet formation. This combination has not previously been reported for regenerating *D. myoporoides* R. Br. plants. However, plantlet regeneration was reported for *Solamum paludosum*, a steroidal alkaloid-producing plant species using BA10^{-7}M+IBA10^{-7}M (Badaoui et al., 1996).

2.4.7 Effects of TDZ on Shoot-bud Induction

Shoot-buds were initiated on the calli induced on the leaf explant incubated in the MS basal medium supplemented with TDZ5x10^{-6}M+IBA(10^{-6} and 10^{-7})M. Cousineau and Donnelly (1991) reported shoot-bud induction on the leaf-derived calli of *Rubus idaeus* incubated in the BM supplemented with TDZ (4.5-9.1)x10^{-6}M+IBA(2.5-4.9)x10^{-6}M. My results indicate that, in combination with IBA, TDZ can be used as a cytokinin for shoot-bud induction in *D. myoporoides* R. Br.

The number of shoot-buds induced in the BM supplemented with BA10^{-5}M+IBA10^{-7}M was higher than that in the BM supplemented with TDZ5x10^{-6}M+IBA(10^{-6} and 10^{-7})M. These results indicate that, in the tested range of concentrations, IBA, in combination with the urea compound TDZ, is less effective than the purine compound BA for shoot-bud induction.

However, the cytokinin like activity of TDZ observed in this study could be exploited for shoot-bud regeneration on the leaf explant of *D. myoporoides* R. Br.
Although the concentrations of TDZ tested in this experiment were not optimal for shoot-bud regeneration, efficiency may be improved by employing other TDZ concentrations in combination with other auxins.

2.4.8 Shoot Morphology: Germinated Seed vs Cultured

My results showed that some of the shoots differentiated from the non-organogenic and organogenic calli were morphologically different from those produced by germinated seeds. A seed germinated shoot always had an elongated basal stem whereas such elongation occurred in only 40-60% of the cultured shoots. A similar comparative study of the Pinus contorata shoots regenerated in the BM supplemented with BA250x10^-5M was carried out by Flygh et al.,(1998). They reported a variation in the basal stem elongation in the regenerated plantlets and the importance of the size of the plantlets for their survival when potted. My results indicate that elongation of the basal stem similar to that of seedling can be obtained in the cultured shoots by varying the cytokinin and auxin combinations.

In conclusion, my results demonstrate that shoots can be regenerated from the non-organogenic or organogenic calli using different cytokinin and auxin combinations. However, regeneration of a shoot similar to the parent plant can only be obtained with some selected cytokinin and auxin combinations. It seems that elongation of the basal stem in the regenerated shoot is related to cell differentiation during the regeneration process. The histochemical analyses (Chapter 5) and the chemical analyses (Chapter 6) of the differentiated shoots will provide the information required to understand the relationship between the alkaloid-producing abilities of the differentiated shoots and the cytokinin/auxin combinations used at the callus induction stage in this study.
Production of Organs (Shoot, Root) in Suspension Cell Cultures of *Duboisia myoporoides* R. Br.
3.1 INTRODUCTION

One of the most important applications of plant cell suspension culture is the production of secondary metabolites (Payne et al., 1991). In the last few decades, plant cell suspension cultures have been tested for some secondary metabolite producing plant species, but without much success. Production of Shikonin by plant cell suspension culture indicated a good future of this technology (Tabata and Fujita, 1985).

For the production of alkaloids by using plant tissue culture (PTC), stable alkaloid biosynthesis in the callus is preferable (Basu and Chand, 1998). In Duboisia myoporoides R. Br., alkaloid biosynthesis takes place in the plant root cells (Luanratana and Griffin, 1982; Endo and Yamada, 1985). Suspension culture of D. myoporoides can accumulate traces of tropine, nicotine, α-acetoxypeptropane and significant amounts of the β-phenylethylamine alkaloids tyramine and 3-methoxytyramine (Bachmann et al., 1989) indicating that biosynthesis and accumulation of tropane alkaloids i.e., hyoscyamine and scopolamine in suspension culture may also be possible.

Plant cells remain in the unorganized state in suspension but they have a tendency to form clumps. Lindsey and Yeoman (1983) found a low level of alkaloid in the fast growing friable callus of different alkaloid-producing plant species cultured in suspension. They also detected a higher amounts of alkaloid in the slow growing calli, where cells were more compactly associated or organized into a morphologically recognisable structure. However, tropane alkaloids i.e., hyoscyamine and scopolamine accumulation in relation to cell organization in suspension have not been investigated for D. myoporoides.

Since tropane alkaloids are normally biosynthesised in the root cells, it is important to investigate whether tropane alkaloids are produced in a less organised state than root tissue. To demonstrate at what differentiated state alkaloid
biosynthesis and accumulation take place, it is necessary to regenerate shoots and roots independently from the non-organogenic callus cultured in suspension. The shoots and roots produced from suspension culture will be analysed for alkaloid contents (Chapter 6).

Therefore, the aim of the present study was to produce shoots and roots in suspension from the established suspension culture and to analyse the suspension culture samples for the presence of nicotine, hyoscyamine and scopolamine.

The objectives of this study were to:

1) produce friable callus on the semi-solid MS basal medium from the 11-week-old non-organogenic calli induced on the semi-solid MS basal medium supplemented with BA10^{-5}M+NAA10^{-6}M for shoot regeneration
2) establish suspension culture in the liquid MS basal medium supplemented with BA0.1x10^{-6}M+NAA27x10^{-6}M using friable callus for shoot culture
3) regenerate shoot-buds from the established suspension culture using MS basal suspension medium supplemented with BA22x10^{-6}M and BA10^{-5}M+IBA10^{-7}M independently (as these combinations produced highest number of shoot-buds from the non-organogenic and organogenic calli respectively as per Chapter 2)
4) produce friable calli from the 11-week-old non-organogenic calli induced on the semi-solid MS basal medium supplemented with BA10^{-5}M+NAA10^{-6}M for root regeneration
5) establish suspension culture in the medium supplemented with 1/2MS basal salt and IBA25x10^{-6}M using friable calli for root regeneration
6) regenerate roots in suspension culture from the established suspension using B5 basal medium supplemented with IBA10^{-2}M
7) analyse suspension cultured samples (shoot, root and culture media) for nicotine, hyoscyamine and scopolamine contents (Chapter 6).
3.2 MATERIALS AND METHODS

3.2.1 Chemicals and Equipments

All chemicals for tissue culture media, test tubes and conical flasks were obtained from Sigma Chemicals (USA). A multi-shaker (EYELA multi-shaker MMS) was used for suspension culture.

3.2.2 Preparation and Storage of Media

Required volumes (i.e., volume necessary for the amounts of the MS or B5 medium to be prepared) of the individual stock solutions of macro and micro nutrients, vitamins, PGRs were mixed together and the required volume of milli-Q water was added. The pH of the solution was adjusted at 5.7 by adding either 0.1M HCl or 0.1M NaOH. The required amount of sucrose was added and dissolved by stirring the solution. The liquid media were filter sterilized by using a Sartorius cellulose acetate filter membrane (pore size 0.2μm, filter diameter-47mm) in a Sartorius polycarbonate filter holder (16510,16511). The sterile medium was then transferred to a sterile Schott bottle, stored in a deep freezer (Westinghouse Silhouette 393) at \(-20^\circ\text{C}\) and used within 4 weeks after preparation.
3.2.3 Establishment and Subculture of Suspension Culture for Shoot Culture

Suspension culture was established by using the procedure as described by Sharp et al., (1995). Eleven-week-old calli induced on the semi-solid MS basal medium (Chapter 2) supplemented with BA10⁻⁵M+NAA10⁻⁶M were aseptically transferred to the semi-solid MS basal medium supplemented with BA0.1x10⁻⁶M+NAA27x10⁻⁶M and incubated in the light (Section 2.2.6) for 2 weeks. Small pieces of the green calli so produced were used for establishment of suspension culture.

The callus pieces were transferred to 50mL sterile volumetric flasks containing sterile liquid medium (enough to cover the lower portion of the callus without completely submersing it). The liquid MS basal medium (Appendix 2) used for establishment for suspension culture was supplemented with BA0.1x10⁻⁶ M+NAA27x10⁻⁶M. The volumetric flasks were plugged with sterile cotton wool (Non-absorbent) wrapped in gauze, covered with aluminium foil. Six flasks thus prepared were placed on a multi shaker (Section 3.2.1) in the light (Section 2.2.6) at 25±2⁰C. The flasks were kept at 60rpm for 2 days, then at 90rpm for 3 days and finally shaken at 120rpm for 8 weeks. The cultures were transferred weekly to the fresh media. When callus growth was established in suspension, about 10g (fresh weight) of the vigorously grown calli were transferred to 250mL volumetric flasks containing 50mL of the same fresh liquid medium and incubated in the same conditions. The cultures were subcultured weekly.

3.2.4 Shoot Culture in Suspension

About 10g (fresh weight) of the calli collected from the second subculture from above were transferred to 250mL volumetric flasks containing 50mL sterile liquid MS basal medium supplemented with BA22x10⁻⁶M and BA10⁻⁵M+IBA10⁻⁷M independently. Six replicate flasks were prepared per treatment and incubated in the light (Section 2.2.6) at 25 ± 2⁰C and shaken at 120rpm. The cultures were transferred weekly to the fresh media and incubated for 3 weeks. The small clumps
of cells formed during the mentioned incubation period were collected for alkaloid analysis (Chapter 6).

After formation of shoot-buds on the small clumps of suspension cells, the shaker speed was reduced to 90rpm and the cultures were further incubated for another 4 weeks. The callus pieces with the regenerated shoot-buds were transferred weekly to the fresh medium. At the conclusion of the experiment, the length of the shoots were measured, the number of leaves per shoot was counted and the differentiated small shoots were collected for alkaloid analysis (Chapter 6). The turbid liquid medium in which the shoots were grown was also collected from the culture flasks at the time of medium transfer. This was done over a period of 3 weeks and the composite medium was then analysed for alkaloid content (Chapter 6).

3.2.5 **Production of Friable Callus for Root Culture in Suspension**

The calli induced on the leaf explant incubated in the semi-solid MS basal medium supplemented with BA$10^{-5}$M+NAA$10^{-6}$M were used for the production of friable callus. The 11-week-old calli were transferred to the test tubes (Sigmaware™ Culture tubes, glass 25x150mm) containing semi-solid medium supplemented with 1/2 MS basal salt without any PGR. The cultures were incubated in the dark at $25 \pm 2^0$C for 3 weeks. The friable calli thus produced were transferred to the Petri dishes containing semi-solid medium supplemented with 1/2 MS basal salts and IBA25 x $10^{-6}$M and incubated in the dark. The cultures were maintained in the same medium, incubated in the dark at $25 \pm 2^0$C and transferred to the fresh medium at 3-4 weekly intervals.
3.2.6 Establishment and Subculture of Suspension Culture for Root Culture

The suspension culture was established by using the procedure as described by Sharp et al.,(1995). Friable calli collected from the 3rd subculture were aseptically transferred to the sterile 50mL conical flasks and enough suspension medium supplemented with 1/2MS basal salts and IBA25x10^{-6}M was added to the flasks to cover the bottom of the calli. The flasks were plugged with sterile cotton wool (non-absorbent) wrapped in gauze, covered with aluminium foil. The culture flasks were placed on an multi-shaker (Section 3.2.1) initially set at 60rpm for 2 days and then at 90rpm and incubated in the dark at 25 ± 2\(^\circ\)C for the rest of the incubation period. The cultures were transferred to the fresh medium weekly. Within 1 week, the callus growth was established in suspension culture medium.

About 10g fresh weight of the calli collected from the established suspension culture were transferred to 250mL sterile volumetric flasks containing 50mL fresh suspension medium supplemented with B5 basal salt (Appendix 7) and IBA10^{-5}M and subcultured weekly. Root formation was noted when the cultures were 1-2 week-old. The cultures were further incubated for another 4 weeks. At the end of the incubation period, the elongated roots were harvested for alkaloid analysis (Chapter 6).
3.3 RESULTS

3.3.1 Shoot Culture in Suspension

On transferring to the shake flasks containing liquid medium, the callus pieces became pale green and formed small clumps. A fast growing callus suspension culture was established after 2-3 weeks of incubation. The culture medium remained clear, indicating the absence of suspended cells in the medium. After 3rd subculture, the calli started to become brown. So, the calli pieces collected from the second subculture were used for shoot-bud induction.

The effects of cytokinin/auxin combinations on shoot-bud induction in suspension culture are summarised in Table 3.1 and explained below.

**Table 3.1** Effects of cytokinin/auxin combinations on *Duboisia myoporoides* R. Br. shoot-bud induction on the calli incubated in the light in an agitated suspension culture medium for 3 weeks

<table>
<thead>
<tr>
<th>Cytokinins/auxins</th>
<th>Greening of the calli</th>
<th>Formation of cell aggregates</th>
<th>Shoot-bud induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA22x10^{-6}M</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BA10^{-5}M+IBA10^{-7}M</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: Experiments were conducted in 6 replicate flasks
+  : Observed
-  : Not observed

On transferring the callus pieces to the shoot-bud induction medium supplemented with BA22x10^{-6}M, they became pale green after 1 week incubation
and aggregated to small masses after 1 more week. However, during the incubation period, no shoot-bud formation was observed.

The small calli pieces collected from the established suspension when transferred to the shoot-bud induction medium supplemented with BA10^{-5}M+IBA10^{-7}M, the cells aggregated into a larger mass than the initial calli (visually observed). The larger mass of the calli became dark green and 4-5 shoots-buds were regenerated within 2-3 weeks. The shoot-buds were elongated to small shoots with 0.4 to 0.6cm long basal stem and 3-4 healthy green leaves within 1 week (Fig 3.1).

Although leaves were elongated, no further stem elongation was observed during the incubation period. Since the small shoots were grown under continuous shaking, they were separated from the calli pieces and submersed into the medium. After formation of the shoot-buds, the culture medium became turbid. Plating of this turbid medium on the PCA plates did not reveal any contamination indicating that some metabolites have been discharged into the liquid medium (see Chapter 6). After 4 weeks, the submersed small shoots started to become light brown. The light brown shoots, green shoots together with the calli pieces were also analysed for alkaloids (Chapter 6).

### 3.3.2 Root Culture in Suspension

A fast growing friable callus was produced in the semi-solid medium supplemented with 1/2MS basal salts devoid of cytokinin and auxin (Fig 3.2). Friable calli were vigorously growing for about 2 years in the semi-solid medium supplemented with 1/2MS basal salt and IBA25x10^{-6}M. When the friable calli were transferred to the suspension medium supplemented with 1/2MS basal salt and IBA25x10^{-6}M, a vigorously growing suspension was established within 1 week.

On transferring to the root induction suspension medium supplemented with B5 basal salt and root inducing auxin IBA10^{-5}M, the friable calli grew faster (doubled in mass within 2-3 days) and formed small clumps after 2 weeks. After 1
**Text-Figure 3.1** Shoot regenerated on the friable callus incubated in the MS basal suspension medium supplemented with sucrose (3%) and BA $10^{-3}$M + IBA $10^{-3}$M in the light at 25 ± 2°C for 4 weeks. Note the small basal stem (bs). Bar size 0.33 cm

**Text-Figure 3.2** Friable callus (fc) (for root culture) produced in the semi-solid MS basal medium supplemented with 1/2MS basal salt, sucrose (3%) and devoid of plant growth regulator when non-organogenic callus induced in the MS basal medium supplemented with BA $10^{-5}$ M+NAA $10^{-6}$M was incubated in the dark at 25 ± 2°C for 3 weeks.

**Text-Figure 3.3** Root (r) regenerated on the friable callus cultured in the suspension medium supplemented with B5 basal salt, sucrose (3%) and IBA $10^{-5}$M incubated in the dark at 25 ± 2°C for 4 weeks.
more week, small roots were regenerated on the callus. About 4 to 5 roots were formed per callus piece. The roots were 1-1.5cm long, thin and no root hairs were found during the 4 weeks incubation period (Fig 3.3). The alkaloid contents of the cultured roots are presented in Chapter 6.
3.4 DISCUSSION

The initial leaf-derived callus cultured in the semi-solid MS basal medium supplemented with BA10^{-5}M+NAA10^{-6}M resulted in the formation of friable callus when incubated in the suspension medium supplemented with BA0.1x10^{-6} M+NAA27x10^{-6}M. However, no shoot-bud formation was observed in suspension culture as happened in the semi-solid medium (Chapter 2). These results indicate a similar organogenic property of the callus grown on the semi-solid and suspension media supplemented with MS basal salt and BA0.1x10^{-6}M+ NAA27x10^{-6}M.

The observed small clump and aggregate formation of the friable callus in the MS basal suspension medium supplemented with BA10^{-5}M+IBA10^{-7}M indicated the initial stage of differentiation which resulted in the formation of shoot-buds. These results are partially consistent with those of Koul et al., (1983) who also reported the formation of small aggregates and clumps with shoot-buds in suspension culture of *Hyoscyamus niger* maintained in the RT medium supplemented with NAA10^{-5}M. It appears that for different plant species, organogenesis and differentiation in suspension culture depend on the basal salt as well as the cytokinins/auxins.

Although greening of the calli and formation of small aggregates took place, no shoot-buds were induced in the suspension medium supplemented with MS basal salts and BA22x10^{-6}M although shoot-buds were induced when the calli were incubated in the semi-solid BM supplemented with BA22x10^{-6}M for 8 weeks (Chapter 2). However, shoot-buds were regenerated in the suspension medium supplemented with MS basal salts and BA10^{-5}M+IBA10^{-7}M. This result indicates a better shoot-bud regenerating ability of BA10^{-5}M+IBA10^{-7}M than BA22x10^{-6}M in suspension. It may be that, 1) the presence of both cytokinins and auxins is necessary for shoot-bud induction in suspension culture or, 2) the change in the physical form of the culture medium affected the organogenic ability of the BA22x10^{-6}M and 3) further incubation will be necessary for organogenesis to occur in the suspension medium supplemented with BA22x10^{-6}M as I terminated my experiment before 8 weeks.
Browning of the shoots cultured in suspension indicated vitrification. However, the erect, green stem and leaf morphology of the shoots indicate that the diminished vitrification problem of the present suspension culture can be reduced by manipulating the culture environment (Debergh et al., 1981; Kevers et al., 1984; Ziv, 1991a, b).

The observed turbidity of the culture medium is an indication of the formation and discharge of biochemicals after shoot-bud induction. Endo and Yamada, (1985) reported that alkaloids can be discharged in the root culture suspension medium. Chemical analysis of the suspension culture medium will be performed (Chapter 6) to confirm the presence of alkaloid in the culture medium, discharged from the shoots cultured in suspension. However, other macromolecules, besides alkaloid may be released during shoot culture in suspension.

Friable calli were formed when the compact calli induced in the MS basal medium supplemented with BA10^{-5}M+NAA10^{-6}M were incubated in the semi-solid medium supplemented with 1/2 MS basal salt without any PGR. However, 1/2MS basal salt formulation is generally used for root induction at the cut end of the regenerated shoots of different plant species (Badaoui et al., 1996; Choi et al., 1998). My results indicate that 1/2MS basal salt formulation can also be used for friable callus formation from the non-organogenic compact callus. It appears that the level of the basal salts in a cytokinin and auxin free medium caused the formation of the friable callus from the compact non-organogenic callus.

The observed root induction in the B5 basal medium supplemented with IBA10^{-5}M is consistent with that of the previous results observed in D. myoporoides R. Br. (Endo and Yamada, 1985). Yukimune et al., (1994 d) reported hormonally induced root cultures of D. myoporoides in suspension using NN basal medium supplemented with IBA10x10^{-6} M. It appears that D. myoporoides root culture in suspension can be established in different basal media supplemented with various IBA concentrations.
Based on the previous reports, IBA is a suitable auxin for root induction in the shoots regenerated via organogenesis in different plant species (Franklin and Dixon, 1994). My results showed that auxin IBA can be used for root organogenesis and differentiation in suspension culture of *D. myoporoides*. However, only selected cytokinin and auxin combinations can cause shoot organogenesis and differentiation in suspension cultures of *D. myoporoides*. 
Chapter 4

Effects of Environmental Factors on Shoot Growth in *Duboisia myoporoides* R.Br.
4.1 INTRODUCTION

In the production of secondary metabolites by using PTC, environmental factors play an important role (Payne, 1991). These factors include physical ones such as light and temperature and chemical factors such as the energy source (sugar), vitamins and basal media. By changing one or more factors, biosynthesis of secondary metabolites in cultured tissues and organs can be altered (Corduan and Reinhard, 1972; Tabata et al., 1974; Zenk et al., 1975). Generally, by changing the amounts of different environmental factors, an optimal environment can be created to optimise differentiation of the cultured tissues or organs and consequent biosynthesis of the secondary metabolites.

In tropane alkaloid-producing plant species, alkaloid biosynthesis takes place in the plant root cells (Waller and Nowacki, 1978). Accordingly, untransformed and transformed root cultures of different tropane alkaloid-producing plant species have been established (Tabata et al., 1972; Endo and Yamada, 1985; Yukimune et al., 1994 a, d; Alvarez et al., 1994; Subroto et al., 1996 b). In some of these investigations, the effects of environmental factors like basal media, extra-factors, alkaloid precursors on root culture were studied (Tabata et al., 1972; Endo and Yamada, 1985). The effects of various environmental factors on shoot growth of D. myoporoides R. Br. have not been tested.

One of the major factors of the chemical environment in the tissue culture technique is the composition of the basal medium. Different basal media have been formulated for tissue culture work, for example, MS (1962), B5 (1968), White (1954), NN(1969). These media mainly vary in macro and micro nutrients and vitamin composition, for example, MS and White’s media are higher and lower salt containing media respectively and B5 is a lower salt and higher vitamin containing medium. In culturing tissues and organs or producing secondary metabolites, different plant species respond differently to various basal media (Badaoui et al., 1996; Moon and Stomp, 1997). For D. myoporoides, plant regeneration and other
tissue culture studies have been carried out using MS and NN basal media (Kitamura et al., 1980; Yukimune et al., 1994 d) and B5 for root culture (Endo and Yamada, 1985). The effects of various basal media on shoot growth of *D. myoporoides* have not yet been reported.

Effects of different extra factors and alkaloid precursors have been tested mainly on alkaloid production in different tropane alkaloid-producing plant species. Tabata *et al.*, (1972) reported the effects of extra-factors and alkaloid precursors on root growth and alkaloid production in the root culture of *Scopolia parviflora*. The effects of these factors have not yet been tested on *in vitro* shoot growth of *D. myoporoides*. Similarly, the effects of various alkaloid precursors on *in vitro* shoot growth of *D. myoporoides* have not yet been investigated.

In tissue culture work, generally, a standard light intensity has been used at the different stages of plant regeneration (Murashige, 1977; Lin and Griffin, 1992 a). However, for different tissue culture studies, different quantities as well as qualities of light have been used (Furuya and Torrey, 1964; Padua et al., 1998). The effects of different light regimes on shoot growth of *D. myoporoides* have not yet been reported.

Therefore, the aim of the present study was to observe the effects of various chemical factors and different light regimes on shoot growth of *D. myoporoides* R. Br.

Specific objectives for the study of shoot growth were to:

1) induce shoot-buds in the semi-solid B5 basal medium supplemented with BA $10^{-5}M$ + IBA $10^{-7}M$ (as this cytokinin and auxin combination produced highest number of shoot-buds from the organogenic calli induced in the MS basal medium as per Chapter 2)
2) compare the effects of MS and B5 basal media supplemented with $BA10^{-5}M + IBA10^{-7}M$ on shoot growth

3) induce shoot-buds in the MS basal medium supplemented with $BA10^{-5}M + IBA10^{-7}M$ to observe the effects of extra-factors, alkaloid precursors and different light regimes on shoot growth

a) observe the effects of extra-factors such as, yeast extract, peptone and casamino acid independently added to the MS basal medium supplemented with $BA10^{-5}M + IBA10^{-7}M$ on shoot growth

b) observe the effects of alkaloid precursors, L-ornithine, L-phenylalanine and DL-tropic acid independently added to the MS basal medium supplemented with $BA10^{-5}M + IBA10^{-7}M$ on shoot growth

c) observe the effects of different light regimes, i.e., to study the effects of different light intensities, exposure of culture to light from different sides and under shaded conditions, on shoot growth in the MS basal medium supplemented with $BA10^{-5}M + IBA10^{-7}M$ exposed to constant photoperiod (using light intensity $15.2 \mu$mol s$^{-1}$m$^{-2}$ as a standard because highest shoot elongation was observed when the shoot-buds were incubated under the mentioned light intensity as per Chapter 2).
4.2 MATERIALS AND METHODS

4.2.1 Chemicals and Culture Vials

Peptone, yeast extract and casamino acid were obtained from Bacto Laboratories, L-phenyl alanine, L-ornithine, tropic acid and other chemicals and tissue culture vials (5.5cm x 5.0cm; 7.0cm x 6.5cm) used in this experiment were obtained from Sigma Chemical Company (USA).

4.2.2 Shoot Culture in the B5 Basal Medium

Leaves collected from the vigorously grown cuttings maintained in the greenhouse-conditions (Section 2.2.3) were used as explant for this work. Surface sterilised (Section 2.2.4) 1 cm segment leaf pieces were placed on the semi-solid B5 basal medium (Appendix 7) supplemented with BA10^{-5}M+IBA10^{-7}M and incubated in the dark at 25\pm2^\circ C for 4 weeks. The shoot-buds induced on the organogenic calli were transferred to the same fresh medium and incubated in the light (Section 2.2.6) for 1 more week to allow elongation of the shoot-buds enough to separate from the calli.

One-week-old shoot-buds induced on the organogenic calli were aseptically transferred to 5.5cm x 5.0cm culture vials containing semi-solid B5 basal medium supplemented with BA10^{-5}M+IBA10^{-7}M and incubated in the light (Section 2.2.6) at 25\pm2^\circ C. All cultures were transferred to 50mL of the same fresh medium at 4 to 5 week intervals and the 4-week-old shoots were transferred to 7.0cm x 6.5cm culture vials. When the shoots were 9-week-old, the number of leaves formed on each of the shoot was then counted and the length of the leaves and shoots measured.
4.2.3 Shoot-bud Induction for Investigating the Effects of Extra-factors, Alkaloid Precursors and Different Light Regimes on Shoot Growth

Leaves collected from the vigorously grown cuttings maintained in the greenhouse-conditions (Section 2.2.3) were used as explant for this work. Surface sterilised (Section 2.2.4) 1 cm segment leaf pieces were placed on the semi-solid MS basal medium supplemented with BA$10^{-5}$M+IBA$10^{-7}$M and incubated in the dark at $25\pm 2^\circ$C for 4 weeks. The shoot-buds induced on the organogenic calli were transferred to the same fresh medium and incubated in the light (Section 2.2.6) for 1 more week to allow elongation of the shoot-buds enough to separate from the calli.

4.2.3.1 Shoot Culture in the BM Supplemented with Various Extra-factors

The regenerated shoot-buds were transferred to the semi-solid MS basal medium supplemented with BA$10^{-5}$M+IBA$10^{-7}$M and 2g L$^{-1}$ of either yeast extract, casamino acid or peptone (Tabata et al., 1972). The cultures were incubated in the light (Section 2.2.6) at $25\pm 2^\circ$ C. All cultures were transferred to 50mL of the same fresh media at 4 to 5 week intervals. Incubation was continued for 10 weeks.

4.2.3.2 Shoot Culture in the BM Supplemented with Various Alkaloid Precursors

The shoot-buds were transferred to 5.5cm x 5.0cm culture vials containing semi-solid MS basal medium supplemented with BA$10^{-5}$M+IBA$10^{-7}$M and 1000 x $10^{-6}$M of either L-ornithine, L-phenylalanine or tropic acid (Tabata et al., 1972). The cultures were incubated in the light (Section 2.2.6) at $25\pm 2^\circ$C. All cultures were transferred to 50mL same fresh semi-solid media at 4 to 5 week intervals and the 4-week-old shoots were transferred to 7.0cm x 6.5cm culture vials. When the shoots
were 9-week-old, the number of leaves formed on each shoot was then counted and the length of the leaves and shoots measured.

4.2.3.3 Shoot Culture in Different Light Regimes

The shoot-buds were transferred to 5.5cm x 5.0cm culture vials containing semi-solid MS basal medium supplemented with BA$10^{-5}$M+IBA$10^{-7}$M. The cultures were incubated under 4 different light conditions (light arrangement in the incubation room is presented in Fig. 4.1) at 25±2°C. All cultures were transferred to 50mL of the same fresh semi-solid media at 4 to 5 week intervals and the 4-week-old shoots were transferred to 7.0cm x 6.5cm culture vials. When the shoots were 9-week-old, the number of leaves formed on each shoot was then counted and the length of the leaves and shoots measured.

All cultures were incubated in 14 hr photo-period of cool-white light from 35W (OSRAM) fluorescent tubes under the following 4 different light intensities:
1) 65.3 μmol s$^{-1}$ m$^{-2}$ (L1);
2) 19.7 μmol s$^{-1}$ m$^{-2}$ (L2);
3) 11.0 μmol s$^{-1}$ m$^{-2}$ (L3);
4) 5.8 μmol s$^{-1}$ m$^{-2}$ (L4).

The light intensity was measured by a light meter (LI-COR:Terrestrial Radiation Sensors, Type-SA, Model No: LI-200Sa, Pyranometer sensor, Serial BO: PY 13321; LI-COR, inc/LI-COR.LTD) (Appendix 3). The different cultures were produced by placing the cultures on zinc-plated-steel-racks at different distances from the light source and by shading one group as follows:

Fig. 4.1 shows 1) the culture vials were arranged at a distance 13cm, 170cm, 170cm and 120cm from the light source L, x1, x2 and x3 respectively and the light intensity on rack no.1 was 65.3 μmol s$^{-1}$ m$^{-2}$ (L1); 2) the culture vials were arranged at a distance 40cm, 180cm, 180cm and 110cm from the light source L, x1, x2, and x3.
Text-Figure 4.1 Light arrangement for incubation of cultured shoots under different light regimes. The intensity of light was 1) 65.3 μ mol s⁻¹ m⁻² (L1) on rack no.1 at a distance 13cm, 170cm, 170cm and 120cm from the light sources L, x1, x2 and x3 respectively. 2) 19.7 μ mol s⁻¹ m⁻² (L2) on rack no.2 at a distance 40cm, 180cm, 180cm and 110cm from the light source L, x1, x2 and x3 respectively. 3) 11.0 μ mol s⁻¹ m⁻² (L3) on rack no.3 at a distance 66.5cm, 190cm, 190cm and 107cm from the light source L, x1, x2 and x3 respectively and 4) 5.8 μ mol s⁻¹ m⁻² (L4) on rack no.4 at a distance 90cm, 200cm, 200cm and 110cm from the light source L, x1, x2 and x3 respectively. All distances were measured from the light source to the middle of the each rack. All sides of rack no.4 were covered with aluminium foil so that the cultures were exposed to light from the upper side only.
respectively and the light intensity on rack no.2 was 19.7 \( \mu \text{mol s}^{-1} \text{m}^{-2} \) (L2); 3) the culture vials were arranged at a distance 66.5cm, 190cm, 190cm and 107cm from the light source L, x1, x2 and x3 respectively and the light intensity on rack no.3 was 11.0 \( \mu \text{mol s}^{-1} \text{m}^{-2} \) (L3) and 4) the culture vials were set at a distance 90cm, 200cm, 200cm and 110cm from the light source L, x1, x2 and x3 respectively and the light intensity on rack no. 4 was 5.8 \( \mu \text{mol s}^{-1} \text{m}^{-2} \) (L4). All distances were measured from the light source to the middle of the each rack. All sides of rack no.4 were covered with aluminium foil so that the culture vials were exposed to light from the upper side only.

### 4.2.4 Data Collection and Statistical Analyses

Twenty replicate vials each containing 4-5 different shoots were prepared per treatment. It was preferable to have an excess because of probable contamination. The experiment was repeated twice (Section 4.2.2, 4.2.3.1 - 4.3.3). Ten samples were selected at random from each repeated experiment treatment. The results are therefore, expressed as the average response of 20 different shoots.

For the effects of MS and B5 basal media on shoot-bud induction and shoot growth, the results were compared using a one-tailed two-sample \( t \) test. For the effects of alkaloid precursors and different light regimes on shoot growth, the results were analysed by using analysis of variance (Anova). Dunnett’s test (Appendix 8) was used to test the statistical significance between each treatment and the standard. The Least Significant Difference (LSD) method (Appendix 6) was used to test mean values of treatments which were significantly different from each other, if the Anova indicated a significant difference.

The correlation coefficient (r) was calculated between different light intensities and the number of leaves formed / shoot, length of the leaves and the shoots. Test for correlation coefficient significance (Appendix 9) was applied to determine whether there was any significant correlation between the light intensity and the number of leaves formed / shoot, the length of the leaves and the shoots. All statistical tests and mean ± standard deviation calculations were conducted using Microsoft Excel ‘97.
4.3 RESULTS

4.3.1 Effects of Basal Media on Shoot-bud Induction and Shoot Growth

The number of shoot-buds induced and the morphological characteristics of the 9-week-old shoots differentiated on the MS and B5 basal media are summarised in Table 4.1a and illustrated in Fig. 4.2. Data for MS basal medium were collected from Chapter 2.

TABLE 4.1a Effects of basal media on shoot-bud induction and morphological characteristics* of the 9-week-old Duboisia myoporoides R. Br. shoots grown in MS and B5 basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA10⁻⁵M+IBA10⁻⁷M

<table>
<thead>
<tr>
<th>Basal Medium</th>
<th>No. of shoot-buds / leaf explant **</th>
<th>Number of Leaves / shoot **</th>
<th>Length of the leaves (cm) **</th>
<th>Length of the shoots (cm) **</th>
</tr>
</thead>
<tbody>
<tr>
<td>B5</td>
<td>8.80 ± 3.23</td>
<td>6.43 ± 1.40</td>
<td>1.04 ± 0.27</td>
<td>1.06 ± 0.23</td>
</tr>
<tr>
<td>MS</td>
<td>14.80 ± 3.87</td>
<td>8.90 ± 1.97</td>
<td>3.64 ± 1.26</td>
<td>9.45 ± 2.40</td>
</tr>
</tbody>
</table>

* : Results given as means of 20 replicates

** : Mean ± standard deviation
**TABLE 4.1b** Summary of $t$ - test of effects of B5 and MS basal media on shoot-bud induction and morphological characteristics of the 9-week-old *Duboisia myoporoides* R. Br. shoot

<table>
<thead>
<tr>
<th>Sample</th>
<th>Degrees of freedom</th>
<th>$t$ Stat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of shoot-buds / leaf explant</td>
<td>38</td>
<td>5.314238 *</td>
</tr>
<tr>
<td>Number of leaves / shoot</td>
<td>38</td>
<td>4.55911 *</td>
</tr>
<tr>
<td>Length of the leaves</td>
<td>38</td>
<td>9.005337 *</td>
</tr>
<tr>
<td>Length of the shoots</td>
<td>38</td>
<td>15.52445 *</td>
</tr>
</tbody>
</table>

* $P < 0.001$

A lower number of shoot-buds were produced on the organogenic calli induced on the leaf explant incubated in the B5 basal medium than those incubated in the MS basal medium (Table 4.1a) (Fig. 4.2). Shoot-buds induced in the B5 basal medium elongated to small shoots with green basal stem (Fig. 4.3). Shoots grown in the B5 basal medium produced a lower number of leaves than those grown in the MS basal medium (Table 4.1a) (Fig. 4.2). The leaves were also small and green (Fig. 4.3). The length of the leaves and the shoots were also shorter in the B5 basal medium than those in the MS basal medium (Table 4.1a) (Fig. 4.2). In the B5 basal medium, the shoot growth continued during the incubation period but the basal stem did not elongate like that incubated in the MS basal medium.

The number of shoot-buds induced / leaf explant, the number of leaves formed / shoot and the length of the leaves and the shoots were found to be significantly ($P < 0.001$) higher in the MS basal medium than those in the B5 basal medium when $t$ - test was applied (Table 4.1b). Of the 4 parameters studied to observe the effects of basal media on shoot growth, the highest significant difference was observed for the length of shoots (Table 4.1b). The lowest significant difference was observed for the number of leaves formed per shoot (Table 4.1b).
Text-Figure 4.2 Effects of B5 and MS basal media on shoot-bud induction and morphological characteristics of the 9-week-old *Duroisia myoporoides* R. Br. shoot differentiated from the organogenic calli induced in the semi-solid medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA10⁻⁵M+IBA10⁻⁷M; the vertical bar at the top of each column shows ± standard deviation.
**Text-Figure 4.3** Nine-week-old shoot grown on the B5 basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA10^{-5}M + IBA10^{-5}M. Note the non-elongated basal stem (bs). Bar size 0.53 cm

**Text-Figure 4.5** Shoot-buds grown in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%), BA10^{-5}M + IBA10^{-5}M and 1000 x 10^{-6}M L-ornithine. Note the formation of axillary buds (ab) on the shoot-buds before the shoot-buds were separated from the leaf explant (le).
4.3.2 Effects of Extra-factors on Shoot Growth

The regenerated shoot-buds when transferred to the MS basal media supplemented with \( \text{BA}10^{-5}\text{M} + \text{IBA}10^{-7}\text{M} \), containing either yeast extract, peptone or casamino acid, grew for 4 weeks, then turned pale and ultimately died. Attempts to revive them by transfer to the fresh medium were unsuccessful. These results show that yeast extract, peptone and casamino acid in the tested range of concentration inhibited \textit{in vitro} shoot growth.

4.3.3 Effects of Alkaloid Precursors on Shoot Growth

The morphological characteristics of the 9-week-old shoots grown in the MS basal medium supplemented with either phenylalanine, tropic acid or L-ornithine are summarised in Table 4.2a and presented in Fig. 4.4. The morphological characteristics of the 9-week-old shoots differentiated in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and \( \text{BA}10^{-5}\text{M} + \text{IBA}10^{-7}\text{M} \) (Chapter 2) were used as a standard in this experiment.

Of the 3 different alkaloid precursors used, the highest number of leaves per shoot was produced in the BM supplemented with tropic acid (Table 4.2a) (Fig. 4.4). The shoots grown in the BM supplemented with phenylalanine or tropic acid produced a higher number of leaves than those grown in the BM without any alkaloid precursors (Table 4.2a) (Fig. 4.4). The number of leaves per shoot was lowest when the shoots were grown in the BM supplemented with L-ornithine (Table 4.2a) (Fig. 4.4).

The least elongated leaves were produced when the shoots were grown in the BM supplemented with phenylalanine (Table 4.2a) (Fig. 4.4). Of the 3 alkaloid precursors used, the longest leaves were produced on the shoots grown in the BM supplemented with L-ornithine (Table 4.2a) (Fig. 4.4). However, the length of the
TABLE 4.2a Effects of various alkaloid precursors on the morphological characteristics of the 9-week-old *D*uboisa *myoporoides* R. Br. shoots grown in MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA $10^{-3}$ M+IBA $10^{-7}$ M.

<table>
<thead>
<tr>
<th>Alkaloid precursors</th>
<th>Number of leaves/shoot**</th>
<th>Length of the leaves (cm)**</th>
<th>Number of axillary-buds / shoot **</th>
<th>Length of the Shoots (cm)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>9.33± ± 1.70</td>
<td>2.07 ± 0.30</td>
<td>0.40± ± 1.39</td>
<td>2.83± ± 0.54</td>
</tr>
<tr>
<td>Tropic acid</td>
<td>9.98± ± 1.46</td>
<td>2.35± ± 0.57</td>
<td>1.20± ± 2.48</td>
<td>3.20± ± 1.02</td>
</tr>
<tr>
<td>L-ornithine</td>
<td>8.05± ± 2.98</td>
<td>2.73± ± 1.05</td>
<td>0.95± ± 1.53</td>
<td>2.70± ± 0.82</td>
</tr>
<tr>
<td>Standard (without alkaloid precursor)</td>
<td>8.90 ± 1.97</td>
<td>3.64 ± 1.26</td>
<td>0.30 ± 0.97</td>
<td>9.45 ± 2.40</td>
</tr>
</tbody>
</table>

* : Results given as means of 20 replicates  
** : Mean ± standard deviation

Column means sharing letter x within the row are not significantly different but y significantly different from the standard at $\alpha = 0.05$ level.  
Column means sharing a common letter within the row are significantly different at $\alpha = 0.05$ level.

TABLE 4.2b Summary analysis of variance (treatment + standard) of effects of various alkaloid precursors on shoot growth of *D*uboisa *myoporoides* R. Br.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean Squares</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of leaves/shoot</td>
<td>Between groups</td>
<td>39.298</td>
<td>3</td>
<td>13.09933</td>
<td>2.940332**</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>338.584</td>
<td>76</td>
<td>4.455053</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>377.882</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of the leaves</td>
<td>Between groups</td>
<td>29.908</td>
<td>3</td>
<td>9.969333</td>
<td>12.77258**</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>59.32</td>
<td>76</td>
<td>0.780526</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>89.228</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of axillary-buds</td>
<td>Between groups</td>
<td>11.2375</td>
<td>3</td>
<td>3.745833</td>
<td>1.310999 ns</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>217.15</td>
<td>76</td>
<td>2.857237</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>228.3875</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of the shoots</td>
<td>Between groups</td>
<td>643.6545</td>
<td>3</td>
<td>214.5515</td>
<td>109.8152**</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>148.485</td>
<td>76</td>
<td>1.95375</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>792.1395</td>
<td>79</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* : P < 0.05  
** : P < 0.001  
ns : not significant
**TABLE 4.2c** Summary analysis of variance (treatments) of effects of various alkaloid precursors on shoot growth of *Duboisia myoporoides* R. Br.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean Squares</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of leaves/shoot</td>
<td>Between groups</td>
<td>38.572</td>
<td>2</td>
<td>19.286</td>
<td>4.151693*</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>264.784</td>
<td>57</td>
<td>4.645333</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>303.356</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of the leaves</td>
<td>Between groups</td>
<td>5.332</td>
<td>2</td>
<td>2.666</td>
<td>5.223498**</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>29.092</td>
<td>57</td>
<td>0.510386</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>34.424</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of axillary-buds</td>
<td>Between groups</td>
<td>6.7</td>
<td>2</td>
<td>3.35</td>
<td>0.959789 ns</td>
</tr>
<tr>
<td>shoot/shoot</td>
<td>Within groups</td>
<td>198.95</td>
<td>57</td>
<td>3.490351</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>205.65</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of the shoots</td>
<td>Between groups</td>
<td>2.734333</td>
<td>2</td>
<td>1.367167</td>
<td>2.016001 ns</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>38.655</td>
<td>57</td>
<td>0.678158</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>41.38933</td>
<td>59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* : $P < 0.05$

** : $P < 0.01$

ns : not significant
Text-Figure 4.4  Effects of alkaloid precursors on *Duboisia myoporoides* R. Br. shoots grown in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA $10^{-5}$ M+IBA $10^{-3}$M; the vertical bar at the top of each column shows ± standard deviation

Alkaloid precursor 1 : phenylalanine; 2 : tropic acid; 3 : L-ornithine
4 : standard (without any alkaloid precursor)
leaves produced on the shoots grown in the BM supplemented with alkaloid precursors were shorter than those grown in the BM without any alkaloid precursors (Table 4.2a) (Fig. 4.4). All the leaves produced on the shoots grown in the BM supplemented with various alkaloid precursors were green but the basal stems were pale green.

The shoots grown in the BM supplemented with different alkaloid precursors produced a higher number of axillary-buds than those grown in the BM without any alkaloid precursors (Table 4.2a) (Fig. 4.4). The highest number of axillary-buds were produced in the shoots grown in the BM supplemented with tropic acid (Table 4.2a) (Fig. 4.4) and some of the shoot-buds produced axillary-buds before the shoot-buds were separated from the leaf explant (Fig. 4.5, page 179).

The length of the shoots grown in the BM supplemented with tropic acid was higher than those grown in the BM supplemented with phenylalanine and L-ornithine (Table 4.2a) (Fig. 4.4). Of the 3 alkaloid precursors used, the length of the shoots was shortest when the shoots were grown in the BM supplemented with L-ornithine (Table 4.2a) (Fig. 4.4). However, the shoots were longer when grown in the BM without any alkaloid precursors than those grown in the BM supplemented with various alkaloid precursors (Table 4.2a) (Fig. 4.4). The basal stem of the shoots grown in the BM supplemented with various alkaloid precursors was pale green and was not like the basal stem of the shoots grown in the BM without any alkaloid precursor.

The number of leaves produced per shoot grown in the BM supplemented with different alkaloid precursors was not significantly different from those grown in the BM without any alkaloid precursor (Table 4.2a). The length of the leaves produced on the shoots grown in the BM supplemented with either phenylalanine, tropic acid or L-ornithine differed significantly (P < 0.001) from those grown in the BM without any alkaloid precursor (Table 4.2a and b). No significant difference was observed between the number of axillary-buds formed on the shoots grown in the BM supplemented with alkaloid precursors and those grown in the BM without any alkaloid precursor (Table 4.2a and b). The length of the shoots grown in the BM
supplemented with alkaloid precursors was significantly ($P < 0.001$) different from those grown in the BM without any alkaloid precursor (Table 4.2a and b).

The number of leaves produced per shoot ($P < 0.05$) and the length of the leaves ($P < 0.01$) produced on the shoots grown in the BM supplemented with different alkaloid precursors differed significantly between the treatments (Table 4.2a and c). No significant difference ($P > 0.1$) was found between the number of axillary-buds formed on the shoot and the length of the shoots grown in the BM supplemented with different alkaloid-precursors (Table 4.2a and c).

### 4.3.4 Effects of Different Light Regimes on Shoot Growth

The morphological characteristics of the 9-week-old shoots grown under different light regimes are summarised in Table 4.3a and presented in Fig. 4.6.

The shoots incubated under L1 (65.3 µmol s$^{-1}$m$^{-2}$) produced a higher number of leaves than those incubated under L3 and lower number of leaves than those incubated under L2, L4 and the standard (Table 4.3a) (Fig. 4.6). The leaves remained green throughout the incubation period. The leaves on the shoots incubated under L1 were longer than those incubated under L3 but shorter than those incubated under L2, L4 and the standard (Table 4.3a) (Fig. 4.6). The least elongated shoots were produced when the shoot-buds were incubated under L1 (Table 4.3a) (Fig. 4.6). The number of leaves produced on the shoots incubated under L1 and the standard (15.2µmol s$^{-1}$m$^{-2}$) was not significantly different (Dunnett’s test) (Table 4.3a). However, the length of the leaves and the shoots incubated under the L1 and the standard differed significantly ($P < 0.001$) (Table 4.3a and b). Branching in the basal stem was observed in 20% of the shoots. Average leaf breadth under L1 condition was 0.4cm.
TABLE 4.3a Effects of different light regimes on the morphological characteristics* of 9-week-old Duboisia myoporoides R. Br. shoots grown in MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA10^{-5}M + IBA10^{-7}M

<table>
<thead>
<tr>
<th>Light intensity ((\mu) mol s(^{-1}) m(^{-2}))</th>
<th>Number of leaves/shoot **</th>
<th>Length of the leaves (cm) **</th>
<th>Length of the shoots (cm) **</th>
</tr>
</thead>
<tbody>
<tr>
<td>65.3 (without shade) (L1)</td>
<td>7.40(^{\text{aabc}}) ± 1.53</td>
<td>1.89(^{\text{aabc}}) ± 0.45</td>
<td>1.51(^{\text{aabc}}) ± 0.52</td>
</tr>
<tr>
<td>19.7 (without shade) (L2)</td>
<td>9.10(^{\text{abc}}) ± 3.41</td>
<td>2.42(^{\text{abc}}) ± 0.60</td>
<td>2.00(^{\text{abc}}) ± 0.80</td>
</tr>
<tr>
<td>11.0 (without shade) (L3)</td>
<td>5.10(^{\text{abcd}}) ± 0.85</td>
<td>1.40(^{\text{abcd}}) ± 0.71</td>
<td>1.70(^{\text{abcd}}) ± 0.48</td>
</tr>
<tr>
<td>5.8 (with shade) (L4)</td>
<td>12.05(^{\text{abc}}) ± 2.98</td>
<td>3.07(^{\text{abc}}) ± 0.65</td>
<td>2.96(^{\text{ab}}) ± 0.88</td>
</tr>
<tr>
<td>15.2 (standard)</td>
<td>8.90 ± 1.97</td>
<td>3.64 ± 1.26</td>
<td>9.45 ± 2.40</td>
</tr>
</tbody>
</table>

* : Results given as means of 20 replicates  
**: Mean ± standard deviation  
Column means sharing letter x within the row are not significantly different but y significantly different from the standard at \(\alpha = 0.05\) level  
Column means sharing a common letter within the row are significantly different between the treatments at \(\alpha = 0.05\) level

TABLE 4.3b Summary analysis of variance (treatment + standard) of effects of different light regimes on shoot growth of Duboisia myoporoides R. Br.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean squares</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of leaves/shoot</td>
<td>Between groups</td>
<td>517.84</td>
<td>4</td>
<td>129.46</td>
<td>23.50894</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>523.15</td>
<td>95</td>
<td>5.506842</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1040.99</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of the leaves</td>
<td>Between groups</td>
<td>63.8994</td>
<td>4</td>
<td>15.97485</td>
<td>25.78296</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>58.861</td>
<td>95</td>
<td>0.619589</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>122.7604</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of the shoot</td>
<td>Between groups</td>
<td>902.2716</td>
<td>4</td>
<td>225.5679</td>
<td>145.9083</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>146.8659</td>
<td>95</td>
<td>1.545957</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1049.137</td>
<td>99</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* \(P < 0.001\)
**TABLE 4.3c** Summary analysis of variance (treatments) of effects of different light regimes on shoot growth of *Duboisia myoporoides* R. Br.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean squares</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of leaves/shoot</td>
<td>Between groups</td>
<td>514.0375</td>
<td>3</td>
<td>171.3458</td>
<td>28.98027*</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>449.35</td>
<td>76</td>
<td>5.9125</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>963.3875</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of the leaves</td>
<td>Between groups</td>
<td>30.6065</td>
<td>3</td>
<td>10.20217</td>
<td>27.07941*</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>28.633</td>
<td>76</td>
<td>0.37675</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>59.2395</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of the shoot</td>
<td>Between groups</td>
<td>24.77909</td>
<td>3</td>
<td>8.259698</td>
<td>16.94943*</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>37.03588</td>
<td>76</td>
<td>0.487314</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>61.81497</td>
<td>79</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.001

**TABLE 4.4** The correlation coefficient (r) between the light intensity and the morphological characteristics of the 9-week-old shoot differentiated from the organogenic calli induced on the leaf explant of *Duboisia myoporoides* R. Br. grown in the MS basal medium supplemented with Bacto agar (0.9%), sucrose and BA10⁻⁵ M + IBA10⁻⁷ M

<table>
<thead>
<tr>
<th>Variables</th>
<th>Correlation coefficient (r) (treatment + standard)</th>
<th>Correlation coefficient (r) (treatments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of leaves / shoot</td>
<td>-0.20783</td>
<td>-0.20786</td>
</tr>
<tr>
<td>Length of the leaves (cm)</td>
<td>-0.25969</td>
<td>-0.23485</td>
</tr>
<tr>
<td>Length of the shoots (cm)</td>
<td>-0.27234</td>
<td>-0.40927</td>
</tr>
</tbody>
</table>
Text-Figure 4.6 Effects of different light regimes on the 9-week-old Duboisia myoporoides shoots grown in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA10⁻³ M+IBA10⁻⁷ M; the vertical bar at the top of each column shows ± standard deviation.

Light intensity (μ mol s⁻¹ m⁻²):
1: 65.3 (without shade)
2: 19.7 (without shade)
3: 11.0 (without shade)
4: 5.8 (with shade)
5: 15.2 (standard)
The shoot-buds incubated under L2 (19.7\mu mol s^{-1} m^{-2}) exposed to the light from all sides and without any shade elongated to 2.0cm long leafy shoots (Table 4.3a). The shoots incubated under L2 produced a higher number of leaves than those incubated under L1, L3 and the standard and a lower number of leaves than those incubated under L4 (Table 4.3a) (Fig. 4.6). The leaves remained green up to the end of the incubation period. The length of the leaves and shoots under L2 were more elongated than those incubated under L1 and L3 and less elongated than those incubated under L4 and the standard (Table 4.3a) (Fig. 4.6). The basal stems of the shoots were less green than those incubated under L4 and the standard. The number of the leaves formed on the shoots grown under L2 did not differ significantly but the length of the leaves and shoots differed significantly from those of the standard (Table 4.3a). Branching of the basal stem was observed in 25% of the shoots. Average leaf breadth was 0.5cm under L2 condition.

The shoot-buds incubated under L3 (11.0 \mu mol s^{-1} m^{-2}) exposed to the light from all sides and without any shade elongated to 1.7cm long shoots (Table 4.3a). Of the 4 different treatments and the standard, the shoots incubated under L3 produced least number and elongated leaves (Table 4.3a) (Fig. 4.6). The leaves remained green up to the end of the incubation period. The length of the shoots incubated under L3 was longer than those incubated under L1 but shorter than those incubated under L2, L4 and the standard (Table 4.3a) (Fig. 4.6). The basal stem of the shoots was less green than those incubated under the standard. The number of leaves and the length of the leaves and shoots grown under L3 differed significantly (P < 0.001) from those of the standard (Table 4.3a and b). No branching was observed in the basal stem of the shoots. The average leaf breadth was 0.5cm under L3 condition.

Of the 4 different light regimes used, the shoot-buds incubated under L4 (5.8\mu mol s^{-1} m^{-2}) exposed to light from upper side only produced most elongated leafy shoots (Table 4.3a) (Fig. 4.6). The number of leaves and the length of the leaves under L4 were also higher than those produced under L1, L2 and L3 (Table 4.3a) (Fig. 4.6). Shoot growth was rapid and continued for more than 9 weeks. The
basal stem and leaves were dark green. The shoots incubated under L4 produced a higher number of leaves than those incubated under the standard (Table 4.3a) (Fig. 4.6). However, the length of the leaves and the shoots incubated under L4 were shorter than those incubated under the standard (Table 4.3a) (Fig. 4.6). The number of leaves and the length of the shoots differed significantly (P < 0.001) but the length of the leaves did not differ significantly from the standard (Table 4.3a). No branching was observed in the basal stem of the shoots. The average leaf breadth was 1.3 cm under L4 condition.

In the shoots exposed to light from upper side only (L4), the number of leaves, the length and breadth of the leaves and the length of the shoots were greater than in those exposed to light from all sides (Table 4.3a). However, the light intensity used in the standard was optimal for the elongation of the leaves and the basal stem of the shoots.

Of the 3 different parameters i.e., number of leaves, length of the leaves and the shoots studied, the analysis of variance indicated the highest significant (P < 0.001) difference for the length of the shoots incubated under different light regimes and the standard (Table 4.3b).

The number of leaves and the length of the leaves and the shoots differed significantly between treatments (P < 0.001) (Table 4.3c, page 187). However, the highest significant difference was observed for the number of leaves produced in the shoots incubated under different treatments (Table 4.3c, page 187).

The results of correlation coefficient analyses between the light intensity (treatment + standard and between treatments) and the morphological characteristics of the differentiated shoots are summarised in Table 4.4 (page 187). A significantly negative correlation was found between the light intensity (treatment + standard and treatments) and the number of leaves / shoot, length of the leaves and the shoots produced under different light regimes (Table 4.4, page 187).
4.4 DISCUSSION

My results showed that various morphological parameters such as number of shoot-buds produced on the organogenic calli, number of leaves formed per shoot, the length of the leaves and basal stem elongation of the shoot were significantly higher in the MS basal medium than those in the B5 basal medium. These results partially support previous observations. Zimmerman (1982) reported that in different stages of plant regeneration, MS basal medium is effective for a wide variety of plant species. Fujita and Tabata (1987) reported that LS medium is more suitable for Lithospermum erythrorhizon cell growth than White’s medium. It appears that the higher amounts of macro and micro (except Na₂ MoO₄,2H₂O, CuSO₄,6H₂O and CoCl₂,6H₂O) nutrients present in the MS basal medium are more suitable for shoot growth of D. myoporoides than those in the B5 basal medium.

Although B5 basal medium contains higher amounts of vitamin than MS basal medium, basal stem elongation of the shoot of D. myoporoides was higher in the MS basal medium than that in the B5 basal medium in the present study. These results indicate that the higher amounts of vitamin in the B5 basal medium may inhibit shoot growth and also that the higher salt containing MS basal medium is preferable for shoot growth in D. myoporoides.

My results indicate that the 3 extra-factors peptone, casamino acid and yeast extract (each 2g L⁻¹) inhibited in vitro shoot growth. However, Tabata et al. (1972) reported enhanced root growth of Scopilia parviflora in the BM supplemented with the 3 above mentioned extra-factors (each 2g L⁻¹). My literature search failed to reveal such studies in D. myoporoides. It may be that the amounts of the extra-factors used in this study were not optimal for in vitro shoot growth of D. myoporoides.

In comparison to the standard, different alkaloid precursors in the tested range of concentration used in this study, negatively affected shoot growth of D.
myoporoides. The observed negative effect of alkaloid precursors on the shoot growth is consistent with the results of Benazmin et al. (1987) who reported a negative effect of phenylalanine+L-ornithine on shoot growth of Atropa belladonna. Chung and Staba (1988) reported a negative effect of tryptophan and secologanin on shoot growth of Cinchona ledgeriana. Despite the negative effect of alkaloid precursors on shoot growth, a higher number of axillary-buds per shoot grown on the BM supplemented with various alkaloid precursors than that on the standard as observed in my studies suggest that increasing the number of adventitious shoots may result in the production of higher amounts of tropane alkaloids by shoot culture.

My results show a differential effect of different light regimes on the shoot growth of D. myoporoides. Most research studies did not critically evaluate the light requirement for optimal shoot growth of D. myoporoides in culture. However, Thorpe (1980) commented that light requirements for differentiation involve a combination of several components, including intensity, quantity and quality of light. Rajagopal et al. (1986) showed that the influence of light on the cytokinin and auxin status of the cultured shoots may also affect the morphological characteristics of the shoots.

The morphological characteristics of the shoots grown under different light regimes observed in my study indicate that auxin IBA or cytokinin BA supplemented in the culture medium may influence the shoot growth of D. myoporoides grown in culture. According to Kraepiel and Miginiac (1997), cytokinin and light affect growth and development of the leaves.

Cotton et al. (1990) reported that BA inhibits the transcription of the Phy A gene in plants, as does light. Similarly, the amounts of BA or light intensity used in this study may affect transcription of Phy A gene and thereby morphological characteristics. However, the sequence of events that occurs after light perception and the mechanisms transducing the initial signal into a physiological effect are largely undetermined (Furuya, 1993). My results indicate a clear effect of different light regimes on in vitro shoot growth of D. myoporoides. However, further study on in vitro shoot growth in a controlled light environment is necessary.
My results indicate that various environmental factors affect in vitro shoot growth of *D. myoporoides*. Previous studies (Yamada and Hashimoto, 1982; Oksman-Caldentey *et al.*, 1987) have also reported differential effects of various environmental factors on the growth of plant tissues in culture. These environmental factors are also known to influence the secondary metabolite biosynthesis in tissue culture (Tabata *et al.*, 1972; Payne *et al.*, 1991; Subroto *et al.*, 1996b). The alkaloid contents in the *D. myoporoides* shoots produced under various environmental conditions in the present study were not analysed but a differential effect is possible.
Organogenesis, Differentiation and Localization of Alkaloids in *Duboisia myoporoides* R.Br.
5.1 INTRODUCTION

Tropane alkaloid biosynthesis in cultured tissues and organs such as shoot, root and transformed root cultures of *Duboisia myoporoides* R. Br. has been investigated in the last few decades. Sippy and Friedrich (1975) reported tropane alkaloids in the non-organogenic callus while Endo and Yamada (1985) found no alkaloids in the callus. Kitamura *et al.* (1985 a) detected the small amounts of nicotine in the regenerated shoots without root formation. However, they did not report the localization and biosynthetic site of nicotine in the cultured shoots without root formation. Endo and Yamada (1985) detected nicotine, hyoscyamine and scopolamine in the regenerated roots but their accumulation and localization sites have not been compared with those of the mature plant roots.

Evidence of tropane alkaloid biosynthesis in the cultured tissues and organs of different plant species is contradictory. Callus cultures of *Atropa belladonna* (West and Mika, 1957; Hartmann *et al.*, 1986) and *Duboisia hybrid* (Lin and Griffin, 1992 b) did not contain tropane alkaloid. In *D. leichhardtii*, tropane alkaloid was not detected in the callus culture. However, regenerated shoots and roots can convert hyoscyamine to scopolamine (Yamada and Endo, 1984). Basu and Chand (1998) detected tropane alkaloids in the non-organogenic callus, regenerated shoots and roots of *Hyoscyamus muticus* L. In none of these studies has the cell arrangement in different stages of cell differentiation been reported.

Contradictory results regarding the presence of alkaloids in the callus and regenerated shoots were indicated by chemical analysis. An investigation on the arrangement of cells in the unorganised state as well as in the differentiated stages might reveal the cause of the presence or absence of alkaloids in different stages of the cultured plant materials.

Cell arrangement in the cultured organs starts at the initial stage of culture (Fosket, 1968). Like the control of organogenesis by the relative levels of cytokinin and auxin in culture (Skoog and Miller, 1957), PGR interactions are also important in the initiation of specific types of cellular differentiation (Torrey, 1968). An
investigation on the cell arrangement in the differentiated organs might reveal a relationship between PGR combinations and cell arrangement and thereby alkaloid localization in the cultured organs.

Presence of tropane alkaloids in the mature plants has been identified by chemical analysis. For identifying the site of alkaloid biosynthesis, a series of reciprocal grafting experiments between alkaloid-producing and non-producing plant species were set up and the aerial parts and the roots of the graft were analysed for the presence of alkaloids (James, 1950; Waller and Nowacki, 1978). These experiments showed that roots are the main site for tropane alkaloid biosynthesis in various tropane alkaloid-producing plant species.

In the mature *D. myoporoides* plants, alkaloid biosynthesis also takes place in the root cells (Kitamura *et al.*, 1985 b; Hashimoto and Yamada, 1992). Hashimoto *et al.*, (1991) localized the enzyme H6H (Section 1.2.7) in the pericycle cells of the cultured roots and of relatively young roots of *Duboisia* and *Hyoscyamus* species. They concluded that root pericycle cells are the probable sites for alkaloid biosynthesis. Although the presence of the enzyme H6H in the pericycle cells of the roots indicates those cells as the biosynthetic site for alkaloids, localization of alkaloids in those cells is not yet established.

Since roots have been identified as the biosynthetic site for alkaloids, translocation of those alkaloids to the aerial parts has been investigated. In some tropane alkaloid-producing plant species such as *Datura* and *Atropa*, root to shoot translocation was found to take place via xylem cells (Waller and Nowacki, 1978). Kitamura *et al.*, (1991) found that both pyridine and tropane alkaloids in *D. myoporoides* were transported from root to leaf through the xylem cells. The EDTA treatment method used by Kitamura *et al.*, (1993) for phloem sap collection revealed tropane and pyridine alkaloids in the phloem cells which according to the authors are used for translocation of alkaloid from the aerial parts to the root cells.

In a particular organ, the alkaloids are found to be stored in the specific storage cells. James (1950) reported the presence of tropane alkaloids in the different cells of the aerial organs of *Atropa belladonna*. Ferreira *et al.*, (1998)
localized tropane alkaloids within the photosynthetic tissues and inside the vacuoles of *Erythroxylum coca* var. *coca* and *E. novogranatense* var. *novogranatense*. In *D. myoporoides*, however, alkaloids have not yet been localized. Whether the xylem and phloem translocating vessels are used as the storage or accumulation site for alkaloids is not known.

In some alkaloid-producing plant species, specialized tissues are used for alkaloid accumulation and storage. Some specialized lecithinators and parenchyma cells of *Catharanthus roseus* have been identified as the accumulation site for the indole alkaloids vincristine and vinblastine in *Apocynaceae* (Yoder and Mahlberg, 1976). Presence of such vittae, latex or special ducts has not been demonstrated for storage, accumulation or biosynthesis of alkaloids in *D. myoporoides*.

For analysing tropane alkaloids present in the cultured and mature plant materials, various analytical techniques have been used (Tabata *et al.*, 1972; Sharp and Doran, 1990; Yukimune *et al.*, 1994; Subroto *et al.*, 1995). These techniques require destruction of the plant tissues during alkaloid extraction. On the other hand, the use of colour reagents (Yoder and Mahlberg, 1976; Corsi and Biasci, 1998) allowed the localization of different alkaloids to specific cells and organs without damaging the tissues.

Although alkaloid colour reagents have previously been used for localization of tropane alkaloids in *Atropa belladonna* (James, 1950) and *Erythroxylum coca* and *E. novogranatense* (Ferreira *et al.*, 1998), these colour reagents have not been used to localize tropane alkaloids in *D. myoporoides*.

The aim, therefore, of the present study was to investigate the cell organization and localization of alkaloids in the cultured tissues and organs of *D. myoporoides* and their relationship.

There are 3 main objectives of this study:

1) To study the cell organization and macromolecular constituents in the cultured tissues
2) To study the presence or absence and arrangement of differentiated cells in the calli and cultured organs (shoot, root)

3) To localize nicotine, hyoscyamine and scopolamine in the mature and cultured plant materials

The achievement of objective (1) includes the following phases:

a) To select a general purpose histochemical staining reagent with a differential staining property

b) To localize and identify different macromolecules and observe the cell organization in the non-organogenic and organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations

c) To examine the effects of the selected cytokinin/auxin combinations on cell organization in the non-organogenic and organogenic calli

The achievement of objective (2) includes the following phases:

a) To identify the presence of differentiated cells in the cultured tissues

b) To determine the cell organization in the cultured roots and basal stem sections of the shoots differentiated from the non-organogenic and organogenic calli

The achievement of objective (3) includes the following phases:

a) To select the appropriate alkaloid colour reagents and then use them to identify the presence or absence of nicotine, hyoscyamine and scopolamine in the fresh sections of the plant organs from the mature greenhouse-grown plants of *D. myoporoides*

b) To investigate the probable presence of special ducts for storage or accumulation of the alkaloids in the different plant organs of *D. myoporoides*

c) To determine the presence or absence of the alkaloids in the (i) non-organogenic and organogenic calli; (ii) shoot-bearing organogenic calli; (iii) basal stem sections of the differentiated shoots (without any root formation), using the selected alkaloid colour reagents

d) To compare the cell organization and localization of alkaloids in the cultured roots with that of the mature plant root
5.2 MATERIALS AND METHODS

5.2.1 Chemicals and Reagents

Nicotine (95%) was obtained from BDH (UK) and other chemicals used in this work were obtained from Sigma Chemical Company (USA). The reagents were prepared in Milli-Q water (Millipore Australia). Paraffin wax (m.p. 52-58°C) was obtained from Aldrich Chemical Company, Inc.

5.2.2 Source of Plant Materials

Plant materials from 4 vigorously growing cuttings maintained in UWS Macarthur greenhouse-conditions (Section 2.2.3) and 2 prepared cuttings supplied by Mount Annan Botanic Garden (No. 950458) were used for selecting a general purpose histochemical staining reagent and investigating the histolocalization of alkaloids in the different plant organs. The tissues and differentiated organs cultured in the different chemical conditions (Chapter 2) were used to investigate the macromolecular constituents, cell organization and localization of alkaloids.

5.2.3 Preparation of Sections

Microtome and free-hand sections of the plant materials were used for histochemical investigations.
5.2.3.1 Microtome Sections

Microtome sections of different plant organs from a mature greenhouse-grown cuttings (Chapter 2) were prepared as follows:

5.2.3.1.1 Microtome

A hand microtome (model no. Leitz 1512, Serial no. ERNST LEITZ WETZLAR GMBH 530586/548, made in Germany) was used in this study.

5.2.3.1.2 Fixation of Plant Materials

The fresh plant materials were sliced into 3–5mm thick pieces with a scalpel blade (Swann-Morton, B.S.2982, I.S.O.7740) and transferred to a vial containing FAA fixative (Appendix 10). The pieces were dipped into the fixative solution to remove air. Materials were used within 3–4 weeks after fixation.

5.2.3.1.3 Dehydration

The plant materials were removed from the fixative and washed 3 times in ethanol of the same concentration as present in the FAA fixative. These were then completely dehydrated by passing through a series of ascending concentration of alcohol in the TBA series (Appendix 11).

5.2.3.1.4 Paraffin Infiltration

The dehydrated tissues were transferred into small vials filled with solid paraffin wax (m. p. 52-58°C) and just covered with TBA. The vials were placed in an oven, set at a temperature 65°C. When the paraffin melted, the liquid was poured
off and replaced with fresh molten paraffin (65°C). The vials were returned to the heated oven. This procedure was repeated once more allowing 1-2 hr between changes.

5.2.3.1.5 Embedding

The molten paraffin containing plant materials was poured off into a paper boat (Appendix 12). The plant materials were arranged and oriented with a warm needle. The block was allowed to harden completely before removal from the paper boat.

5.2.3.1.6 Section Cutting

The paraffin blocks containing the plant tissues were mounted on the microtome. The microtome scale was first adjusted at 20μm and then changed to 10μm thickness. After releasing the safety catch, the section cutting was started. When the ribbon was formed, it was removed from the knife edge with a hair brush and placed on a clean black paper.

5.2.3.1.7 Mounting the Ribbon on the Glass Slides

With a scalpel blade, the ribbon was cut into pieces containing 2–3 sections and placed on a clean microscope slide coated with a few drops of Mayers fixative (Appendix 13) and flooded with distilled water. The slide was put on a warm glass slab (Appendix 14) to remove the wrinkles in the wax ribbon and to expand the sections. The excess fluid was drained off and the slide was transferred to an incubator and left overnight at 30°C to fix the sections firmly onto the glass slide.
5.2.3.1.8 Hydration and Staining

The paraffin was removed by putting the slides containing the sections in xylene for 10 min. This step was repeated once more and then the sections were hydrated gradually by passing through a series of ethyl alcohol of decreasing concentrations i.e., absolute, 95%, 70%, 50%, and 30% in distilled water. The slides were allowed to remain in each solution for 2-5 min to bring the sections to the medium similar to that of the staining solution. These sections were stained with different general purpose staining reagents (Appendix 15).

5.2.3.2 Free-hand Sections

Free-hand sections of mature plant organs as well as cultured tissues and organs were prepared as follows. Plant materials were held directly between the thumb and forefinger and sliced by passing the scalpel blade (Swann-Morton, B.S.2982, I.S.O.7740) through the material. About 5-6 sections were cut with each blade. The cut sections were placed in distilled water in a watch glass and handled individually with a hair brush.

5.2.4 Histochemical Investigation for Organogenesis

Microtome sections of the roots and stems from a mature greenhouse-grown cuttings (Section 5.2.2) were stained with safranin, methyl green and toluidine blue O (for preparation details see Appendix 16). On the basis of the staining results (Table 5.1), one general purpose histochemical staining reagent i.e., toluidine blue O was selected. Free-hand fresh sections of the 11-week-old non-organogenic and 2-week-old organogenic calli (Chapter 2) were then stained with the selected reagent for investigating cell arrangement and macromolecular constituents.
5.2.4.1 Staining

Microtome sections of the mature plant organs were stained by transferring the slide into a coplin jar containing staining reagent. With Safranin and Methyl green, the sections were allowed to stain for 5 min. With toluidine blue O, the sections were stained for 1-2 min.

Free-hand fresh sections of the 11-week-old non-organogenic and 2-week-old organogenic calli were stained with toluidine blue O for 1-2 min and then de-stained with water. The stained sections were examined microscopically by using an inverted microscope fitted with a camera (Section 5.2.7).

5.2.5 Histological Investigation for Differentiation

Free-hand fresh sections (Section 5.2.3.2) of the 11-week-old non-organogenic and 2-week-old organogenic calli, differentiated roots and the basal stem sections of the shoots differentiated from the non-organogenic and organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations (Chapter 2) were examined under light microscope for the arrangement of different types of cells.

5.2.6 Histolocalization of Alkaloids in the Mature and Cultured Plant Materials

Free-hand fresh sections of the mature plant organs (root, stem, leaf mid-rib) were stained with 8 different alkaloid colour reagents to localize the alkaloids present in the different types of plant cells. On the basis of those staining results (Table 5.6), reagents for alkaloid localization were selected. The selected reagents were then used to localize alkaloids in the free-hand fresh sections of the cultured tissues and
organs. For the parallel test (Johansen, 1940), alkaloid-free sections of the mature and cultured plant materials were also stained with the selected reagents.

5.2.6.1 Preparation of Alkaloid-free Sections

Free-hand fresh sections of the greenhouse-grown plant organs (Section 5.2.4.1) as well as the cultured tissues and organs (Chapter 2) were kept in alcoholic tartaric acid solution (Appendix 17) for 4 to 5 weeks to free the sections from alkaloid (Johansen, 1940). After this period, the sections were washed thoroughly 3 times in fresh water. These sections were then stained with the selected alkaloid colour reagents.

5.2.6.2 Alkaloid Colour Reagents Used

Free-hand fresh sections of the mature plant organs were stained with the following alkaloid colour reagents: platinic chloride (5% aqueous solution), iodoplatinate, ferric chloride, gold chloride, Mayer’s reagent, Dragendorff’s reagent and Wagner’s reagent (Henry, 1924; James, 1950; Cromwell, 1955; Stevens, 1986). Cyanogen bromide (Stevens, 1986) was used for localization of nicotine (for preparation details See Appendix 18).

5.2.6.3 Staining of the Fresh and Alkaloid-free Sections

Fresh and alkaloid-free sections were stained by transferring them directly in the reagent solutions. About 3 to 5 sections were placed in 1 ml of the reagent taken in a watch glass and allowed to stain for 5 to 10 min. Some of the stained sections were then de-stained by washing with water for 15 min. Stained and de-stained sections were examined under the light microscope.
For comparison, a small amount of atropine, scopolamine and nicotine (Section 5.2.1) was taken separately on a microscopic glass slide and a few ml of the staining reagents added individually. The colour so produced on the slide was then photographed through the microscope for use as a standard.

5.2.7 Light Microscopy and Photography

Microtome and free-hand sections prepared for histochemical investigation of organogenesis (Section 5.2.4), differentiation (Section 5.2.5) and alkaloid localization (Section 5.2.6) were examined under an inverted microscope (Olympus CK2, ULWCD 0.30, JAPAN) fitted with a 35mm camera (SC35, type 12) and photographed at different magnifications using Kodak 400 film.

5.2.8 Sectioning Protocols for Histochemical and Histological Investigations

To select a general purpose histochemical reagent, 10 pieces of roots and stems were collected from a mature plant. All root pieces were mixed together. From these root pieces about 40 to 50 sections were prepared to test for each of the 3 staining reagents (Section 5.2.4) and examined under the light microscope (l. m). The results are presented as average response of 25 randomly selected sections. The same procedures were followed for the stem.

To examine cell arrangement and macromolecular constituents in the selected non-organogenic and organogenic calli, tissues were collected from 5 different culture vials (the tissues from the culture vials were collected in aseptic conditions) per cytokinin/auxin treatment. About 50 to 60 sections were prepared from different sides of the calli pieces per treatment, stained with toluidine blue O and examined
under l. m. The results are presented as average response of 30 randomly selected sections.

To examine differentiation in the cultured tissues (non-organogenic, organogenic calli) and organs (shoot, root), tissues were collected from 5 different culture vials (the tissues from the culture vials were collected in aseptic conditions) per selected cytokinin/auxin treatment. About 50 to 60 sections were prepared from, 1) different sides of the calli pieces, 2) basal stem of the shoot per treatment and 3) root and examined under l. m. The results are presented as average response of 30 randomly selected sections.

To select alkaloid colour reagents, 10 pieces of roots, stems and leaves collected from each of the 6 cuttings (Section 2.2.3) were collected. All root pieces were mixed together. From these root pieces, about 60 to 70 sections were prepared to test for each of the 8 different alkaloid colour reagents (Section 5.2.6) and examined under l. m. The results are presented as average response of 30 randomly selected sections per each of the 8 different alkaloid colour reagents. The same procedures were followed for the stem and leaf pieces.

To localize alkaloids in the cultured tissues (non-organogenic, organogenic calli) and organs (shoot, root), tissues were collected from 5 different culture vials (the tissues from the culture vials were collected in aseptic conditions) per selected cytokinin/auxin treatment. About 50 to 60 sections were prepared from, 1) different sides of the calli pieces, 2) basal stem of the shoot per treatment and 3) cultured root, stained with the selected alkaloid colour reagents independently and examined under l. m. The results are presented as average response of 30 randomly selected sections.
5.3 RESULTS

5.3.1 Selection of a General Purpose Histochemical Staining Reagent

Table 5.1 summarises reactions of different macromolecules to histochemical staining reagents. Of the 3 staining reagents used, only toluidine blue O stained different macromolecules. These results were interpreted according to O’Brien et al., (1964) and Feder and Wolf (1965). The fully differentiated cortical cells were stained pink and purple indicating the presence of polysaccharides, whereas small partially differentiated cells were stained green showing the presence of DNA. The cells around vascular regions stained purple showing the presence of RNA. Some cells in the vascular region stained blue indicating the presence of lignin or other phenols. From these results, it was decided to select toluidine blue O as the staining reagent for staining the cultured tissues. Safranin and methyl green stained the lignified cell wall only and were not used further in this study.

TABLE 5.1 Staining reactions for the presence or absence of different macromolecules in the root sections of a mature greenhouse-grown cuttings of *Duboisia myoporoides* R. Br. using different staining reagents.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Polysaccharide</th>
<th>DNA</th>
<th>RNA</th>
<th>Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl green</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>D</td>
</tr>
<tr>
<td>Safranin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>D</td>
</tr>
<tr>
<td>Toluidine blue O</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

D: Detected  
ND: Not detected
5.3.2. Cell Arrangement and Macromolecular Constituents of the Non-organogenic Calli

The 11-week-old calli grown on the semi-solid MS basal medium supplemented with the selected cytokinin/auxin combinations contained small areas of meristem-like cells or meristemoides. Typical effects of different cytokinin/auxin combinations on the meristemoid region, cell arrangement and the presence of different macromolecules in the 11-week-old non-organogenic calli are tabulated in Table 5.2 and described below:

5.3.2.1 Callus induced in the BM supplemented with 2,4-D10⁻⁷M

A compact and large meristemoid region (Fig. 5.1) was observed in the callus. The cells in the meristemoid region were small and stained dark blue whereas those around that region were round and stained pink, indicating the presence of polysaccharides (Table 5.2).

5.3.2.2 Callus induced in the BM supplemented with BA10⁻⁶M+NAA10⁻⁶M

The meristemoid region in the 11-week-old callus was moderately large and compact (Fig. 5.2). The cells in the meristemoid region were small and stained dark blue while those around that region were round and relatively large. The contents in some cells around meristemoid region were stained light purple or pink, indicating the presence of polysaccharides (Table 5.2).

5.3.2.3 Callus induced in the BM supplemented with BA10⁻⁶M+2,4-D10⁻⁷M

A moderately compact and large meristemoid region was found in the 11-week-old callus (Fig. 5.3). The cells in the meristemoid region were small and stained dark blue. In the outer area of the callus, the cells were relatively large and
<table>
<thead>
<tr>
<th>ND</th>
<th>ND</th>
<th>ND</th>
<th>ND</th>
<th>ND</th>
</tr>
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<tbody>
<tr>
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<td>ND</td>
<td>D</td>
<td>ND</td>
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<tr>
<td>ND</td>
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<td>D</td>
<td>D</td>
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</tr>
<tr>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
<td>D</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phenol</th>
<th>Macromolecules</th>
<th>DNA/RNA</th>
<th>Differentiation Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>Macromolecules</td>
<td>DNA/RNA</td>
<td>Differentiation Parameters</td>
</tr>
</tbody>
</table>

Presence of different macromolecules and stem cells in the culture medium is explained in Table 5.2. Differentiation and stem cells were cultured in the semi-solid MS basal medium supplemented with the selected cytokinins/auxins combinations. The stem cells were induced to differentiate at different concentrations of cytokinins/auxins combinations.
FIGURES 5.1 - 5.7 Free-hand fresh sections of the 11-week-old non-organogenic calli induced on the leaf explant of *Duboisia myoporoides* R. Br. incubated in the semi-solid Murashige and Skoog (1962) basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various cytokinin and auxin combinations (mentioned in the text-figure), stained with toluidine blue O pH 4.4 mounted in aqueous mountant. Presence of different macromolecules are summarised on the basis of toluidine blue O staining as explained by Feder and Wolf (1965) and O’Brine *et al.*, (1964).

**Text-Figure 5.1** Callus induced in the BM supplemented with 2,4-D $10^{-7}$ M. Note the compact and large meristemoid region (m). x 100

**Fig. 5.1**

**Text-Figure 5.2** Callus induced in the BM supplemented with BA $10^{-5}$ M + NAA $10^{-5}$M. Note the moderately large compact meristemoid region (m). x 100

**Fig. 5.2**

**Text-Figure 5.3** Callus induced in the BM supplemented with BA $10^{-5}$ M + 2,4-D $10^{-5}$ M. Note the compact meristemoid region (m). x 100

**Fig. 5.3**
stained light pink, indicating the presence of polysaccharides. Some of the cells were stained green, indicating the presence of DNA (Table 5.2).

5.3.2.4 Callus induced in the BM supplemented with Kin10⁻⁵M+IAA10⁻⁶M

The meristemoid region in the 11-week-old callus was moderately large and compact (Fig. 5.4). The cells in the meristemoid region were small and stained dark blue. Around that region, the cells were round and their contents were stained blue, indicating the presence of phenols (Table 5.2).

5.3.2.5 Callus induced in the BM supplemented with Kin10⁻⁵M+IBA10⁻⁵M

A moderately large and compact meristemoid region was found in the 11-week-old callus (Fig. 5.5). The cells in the meristemoid region were small and stained dark blue. Around the meristemoid region, the cells were round and the contents stained blue, indicating the presence of phenols (Table 5.2). In the periphery of the callus, the cells were round and relatively large.

5.3.2.6 Callus induced in the BM supplemented with Kin10⁻⁵M+NAA10⁻⁵M

A small, elongated and compact meristemoid region was found in the 11-week-old callus (Fig. 5.6). The cells in the meristemoid region were small and stained dark blue whereas those around that region were elongated and mainly stained pink, indicating the presence of polysaccharides (Table 5.2).
**Text-Figure 5.4** Callus induced in the BM supplemented with Kin$10^{-5}$M + IAA$10^{-6}$M. Note the moderately large compact meristemoid region (m) x 100

**Text-Figure 5.5** Callus induced in the BM supplemented with Kin$10^{-5}$M + IBA$10^{-5}$M. Note the moderately large compact meristemoid region (m) x 100

**Text-Figure 5.6** Callus induced in the BM supplemented with Kin$10^{-5}$M + NAA$10^{-5}$M. Note the elongated meristemoid region (m) x 100

**Text-Figure 5.7** Callus induced in the BM supplemented with Kin$10^{-5}$M + 2,4-D$10^{-3}$M. Note the small compact meristemoid region (m) x 100
5.3.2.7 Callus induced in the BM supplemented with Kin10⁻⁵M+2,4-D10⁻⁷M

A small and compact meristemoid region was found in the 11-week-old callus (Fig. 5.7). The cells in the meristemoid region were small and stained dark blue, whereas those around that region were relatively large, round and stained blue violet, indicating the presence of phenols (Table 5.2).

5.3.3 Cell Arrangement and Macromolecular Constituents of the Organogenic Calli

Typical effects of the selected cytokinin and auxin combinations on the arrangement of cells and the macromolecular constituents of the 2-week-old organogenic calli are summarised in Table 5.3.

5.3.3.1 Shoot-bud producing callus induced in the BM supplemented with BA10⁻⁵M+IAA10⁻⁶M

The 2-week-old calli showed the organization of shoot-bud meristem. In the meristematic region and around that region, the cells were elongated and stained pink, indicating the presence of polysaccharides (Table 5.3).

5.3.3.2 Shoot-bud producing callus induced in the BM supplemented with BA10⁻⁵M+IBA10⁻⁷M

The 2-week-old calli showed the organization of shoot-bud meristem (Fig. 5.8). The cells in that region were elongated whereas those around that region were round. At the centre, the cell walls were stained blue, indicating the presence of lignin or other phenols. The area with the blue pigmented cells ended with a sharp change to an area with lightly pink cells, indicating the presence of polysaccharides in those cells.
TABLE 5.3  Cell arrangement and macromolecular constituents of the 2-week-old organogenic calli induced on the leaf explant of *Duboisia myoporoides* R. Br. incubated in the semi-solid MS basal medium supplemented with various cytokinin and auxin combinations. Presence of different macromolecules is summarised on the basis of toluidine blue O staining as explained by O'Brine *et al.*, (1964) and Feder and Wolf (1965)

<table>
<thead>
<tr>
<th>Cytokinin and auxin combinations</th>
<th>Region of organised cell</th>
<th>Polysaccharide</th>
<th>DNA/RNA</th>
<th>Phenol</th>
<th>Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA10⁻⁵M+1AA10⁻⁶M⁺</td>
<td>+</td>
<td>D</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BA10⁻⁵M+1BA10⁻⁷M⁺</td>
<td>+</td>
<td>D</td>
<td>ND</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>BA10⁻⁵M+1BA10⁻⁷M⁺</td>
<td>+</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>BA10⁻⁶M+1BA10⁻⁸M⁺</td>
<td>+</td>
<td>D</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a: 2-week-old shoot-bud producing calli  
b: 14-week-old calli bearing differentiated shoot  
c: 2-week-old root producing calli  

+: Region of organised cells present  
D: Detected  
ND: Not detected
FIGURES 5.8 – 5.10 Free-hand fresh sections of the organogenic calli induced on the leaf explant of *Daboisit myoporoides* R. Br. incubated in the semi-solid Murashige and Skoog (1962) basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various cytokinin and auxin combinations (mentioned in the text-figure), stained with toluidine blue O pH 4.4 mounted in aqueous mountant. Presence of different macromolecules are summarised on the basis of toluidine blue O staining as explained by Feder and Wolf (1965) and O'Brine et al., (1964)

**Text-Figure 5.8** Shoot-bud producing 2-week-old callus induced in the BM supplemented with BA$10^{-5}$M + IBA$10^{-3}$M. Note the organization of shoot-bud meristem (*sm*). x 400

**Text-Figure 5.9** Fourteen-week-old organogenic callus induced in the BM supplemented with BA$10^{-5}$M + IBA$10^{-3}$M collected from the base of the 9-week-old differentiated shoot without root initiation. Note the variable staining in the elongated cells of the callus. x 400

**Text-Figure 5.10** Root-producing 2-week-old callus induced in the BM supplemented with BA$10^{-5}$M + IBA$10^{-3}$M. Note the organization of root meristem (*rm*). x 400
The sections of the 14-week-old calli (Fig. 5.9) bearing shoots without roots had elongated cells with variable stain e.g., pink showing the presence of polysaccharides; blue indicating the presence of phenols; and green indicating the presence of DNA (Table 5.3).

5.3.3.3 Root producing callus induced in the BM supplemented with BA10⁶M+IBA10⁵M

In the 2-week-old calli, organization of meristem cells was observed. The cells in that region were elongated and outside that region were round (Fig. 5.10). Polysaccharide grains were found in the cells (Table 5.3).

5.3.4 Differentiation in the Tissues and Organs Cultured from the Non-organogenic Calli

The results regarding the differentiation of vascular tissues in the non-organogenic calli are described below. The arrangement of different types cell and differentiation of vascular tissues in the basal stem sections of the 6-week-old shoot differentiated from the non-organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations are summarised in Table 5.4.

5.3.4.1 Calli induced in the BM supplemented with the selected cytokinin/auxin combinations

No differentiated xylem or phloem cells and tracheary elements were observed in the 11-week-old non-organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations.
<table>
<thead>
<tr>
<th>Location of alkaldols</th>
<th>Cortex</th>
<th>Vascular Region</th>
<th>Fresh Section</th>
<th>All smaller vessels</th>
<th>All smaller vessels</th>
<th>Large &amp; smaller vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
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<td>ND</td>
<td>+</td>
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<td>ND</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
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<tr>
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<td>ND</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

**Table S4: Cell layer organization and histolocalization of alkaldols in the basal stem sections of the 6-week-old shoots of Duboisia myoporoda**

The alkaldols were localized in the free-hand fresh basal stem sections using plasma chloride (5%) and iodophorine alkaldol color.

R. Pr. different from the non-organogenic cell induced in the semi-solid MS basal media supplemented with the selected cytokinins/auxins.

Reagents

- **ND**: Not detected
- **+**: Detected
- **+++**: Detected
- **++++**: Detected
- **+++++++**: Detected
- **+++++++**: Detected
5.3.4.2 Shoot differentiated from the callus induced in the BM supplemented with 2,4-D10^{-7}M

The basal stem section of the 6-week-old shoot had a broad cortex and pith region (Table 5.4). The vascular region was narrow with small cells (Fig. 5.11). No large vessel in the secondary xylem was found.

5.3.4.3 Shoot differentiated form the callus induced in the BM supplemented with BA10^{-5}M+NAA10^{-6}M

Cortex and pith region in the basal stem sections of the 6-week-old shoot were similar to the parent plant. A well developed vascular region with large vessels in the secondary xylem was observed (Table 5.4).

5.3.4.4 Shoot differentiated from the calli induced in the BM supplemented with the other selected cytokinin and auxin combinations

The arrangement of cells in the basal stem section of the 6-week-old shoot differentiated from the calli induced in the BM supplemented with Kin10^{-5}M+NAA10^{-5}M is presented in Fig. 5.12. A similar arrangement of different cell layers was also found in the basal stem sections of the shoot differentiated from the calli induced in the BM supplemented with the other selected cytokinin and auxin combinations (Table 5.4). Cortex and pith regions in the basal stem sections of the shoot were comparable to the parent plant. The vascular region was narrow consisting of small cells and no large vessels in the secondary xylem were found (Table 5.4).
5.3.5 Differentiation in the Tissues and Organs Cultured from the Organogenic Calli

The results regarding the differentiation of vascular tissues in the organogenic calli are described below. The arrangement of different cell layers and the differentiation of vascular tissues in the 4-week-old root and the basal stem sections of the 9-week-old shoot differentiated from the selected organogenic calli are summarised in Table 5.5.

5.3.5.1 Calli induced in the BM supplemented with the selected cytokinin and auxin combinations

No differentiated xylem or phloem cells and tracheary elements were observed in the 2-week-old organogenic calli induced with the selected cytokinin and auxin combinations.

5.3.5.2 Shoot-bearing organogenic callus induced in the BM supplemented with BA$10^{-6}$M + IBA$10^{-6}$M

The cell arrangement in the 14-week-old callus collected from the base of the differentiated shoot was different from that of the stem sections i.e., the cells at the base were undifferentiated and arranged in concentric rings (Table 5.5) (Fig. 5.37, page 235).

5.3.5.3 Shoot differentiated from the callus induced in the BM supplemented with BA$10^{-6}$M + IAA$10^{-6}$M

Cortex and pith regions in the basal stem sections of the 9-week-old shoot were found to be comparable to the parent plant. A broad vascular region of small xylem cells was observed (Fig. 5.13). No large vessels were found in the secondary xylem (Table 5.5).
<table>
<thead>
<tr>
<th>Location of Alkaloids</th>
<th>Cortex</th>
<th>Fresh Section</th>
<th>Vascular Region</th>
<th>Combination and section</th>
<th>Phan organ section</th>
<th>Root section</th>
<th>Stem section</th>
<th>Plane organ section</th>
<th>Main section</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>Xylem x phloem</td>
<td>D</td>
<td>+++</td>
<td>NA</td>
<td>++++</td>
<td>15 &lt; 10 cell layers</td>
<td>+++</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
</tr>
<tr>
<td>ND</td>
<td>Xylem</td>
<td>D</td>
<td>++</td>
<td>NA</td>
<td>++++</td>
<td>7 &lt; 10 cell layers</td>
<td>+++</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
</tr>
<tr>
<td>ND</td>
<td>Xylem x phloem</td>
<td>D</td>
<td>+</td>
<td>NA</td>
<td>+++</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
</tr>
<tr>
<td>ND</td>
<td>Xylem</td>
<td>D</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
</tr>
<tr>
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<td>D</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
</tr>
<tr>
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<td>Xylem</td>
<td>D</td>
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<td>NA</td>
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<td>BAV10aM, aBAV10aM, P</td>
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<td>Xylem</td>
<td>D</td>
<td>NA</td>
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<td>NA</td>
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<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
</tr>
<tr>
<td>ND</td>
<td>Xylem</td>
<td>D</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
<td>BAV10aM, aBAV10aM, P</td>
</tr>
</tbody>
</table>

**TABLE S.5** Cell organization and histolocation of alkaloids in the tissue, root and basal stem sections of the shoot of Dypsis lutescens.
FIGURES 5.11–5.14 Free-hand fresh transverse sections of the basal stem sections of the shoot differentiated from the non-organogenic and organogenic calli induced on the leaf explant of *Dolichoa myoporoides* R. Br. incubated in the semi-solid MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various cytokinin and auxin combinations (mentioned in the text-figure).

**Text-Figure 5.11** Basal stem section of the 6-week-old shoot differentiated from the non-organogenic callus induced in the BM supplemented with 2.4-D10⁻⁷M. Note the narrow vascular region (vr) without any large vessel in the secondary xylem. x 100

**Text-Figure 5.12** Basal stem section of the 6-week-old shoot differentiated from the non-organogenic callus induced in the BM supplemented with Kin10⁻⁵M + NAA10⁻⁵M. Note the vascular region (vr) without any large vessel in the secondary xylem. x 100.

**Text-Figure 5.13** Basal stem section of the 9-week-old shoot differentiated from the organogenic callus induced in the BM supplemented with BA10⁻⁴M + IAA10⁻⁴M. Note the broad vascular region (vr) without any large vessel in the secondary xylem. x 100.

**Text-Figure 5.14** Basal stem section of the 9-week-old shoot differentiated from the organogenic callus induced in the BM supplemented with BA10⁻⁴M + IBA10⁻⁷M. Note the vascular region (vr) with small and large vessels (l) in the secondary xylem. x 100.
5.3.5.4 Shoot differentiated from the callus induced in the BM supplemented with BA10⁶M+IBA10⁷M

The basal stem sections of the 9-week-old shoot contained a cortex and a pith region similar to that of the parent plant (Table 5.5). A well developed vascular region with small and large vessels in the secondary xylem was found (Fig. 5.14, page 221).

5.3.5.5 Root differentiated from the callus induced in the BM supplemented with BA10⁶M+IBA10⁷M

Differentiated xylem and phloem cells were found in the 4-week-old regenerated roots (Fig. 5.40, page 235). The vascular region was smaller than that of the mature plant roots (Table 5.5).

5.3.6 Histolocalization of Alkaloid in the Mature and Cultured Plant Materials

The results for the histochemical staining for alkaloid localization in the mature and cultured plant materials are summarised below:

5.3.6.1 Histolocalization of alkaloid in the mature plant materials

Staining reactions for alkaloid localization in the stained sections of the root, stem and leaf mid-rib of the mature plant are summarised in Table 5.6.

On treatment with ferric chloride, gold chloride, Mayer's reagent, Wagner's reagent and cyanogen bromide (Fig. 5.15 and 5.16), no precipitation or typical
| - | - | - | - | - | - | + | + |
| - | - | - | - | - | - | + | + |
| - | - | - | - | - | - | + | + |
| Cyanogen bromide | Regent’s reagent | Wagneur’s | Dragendorff’s | Mayer’s reagent | Cold chloretic | Ferric chloretic | Iodoplatinate | Phlorin chloretic | Iodine used | Plant organs | Plant organs |

TABLE 5.6 Shining reactions for localization of alkaloids in the free-hand fresh sections of the main plant organs of Duboisia myoporoides.
FIGURES 5.15 – 5.22 Free-hand fresh transverse sections of the root collected from a mature greenhouse-grown cuttings of Daboisya myoporoides R. Br. stained for histolocalization of alkaloid.

**Text-Figure 5.15** The section was stained with cyanogen bromide for histolocalization of nicotine in different cells. xy: xylem cell. x 100.

**Text-Figure 5.16** The section was stained with cyanogen bromide for histolocalization of nicotine in different cells and then de-stained with water. Note the absence of stained xylem cells (xy) indicating the absence of nicotine in those cells. x 100.

**Text-Figure 5.17** The section was stained with Dragendorff’s reagent. Note the orange red staining in the intercellular spaces (s) and the absence of staining in different cells. x 100.

**Text-Figure 5.18** The section was stained with Dragendorff’s reagent and then de-stained with water. Note the absence of orange red colour in the intercellular spaces (s) indicating the absence of alkaloid in those spaces. x 100.
colours were produced in the different cells or intercellular spaces (Table 5.6), indicating negative results for localization of the alkaloids.

On staining with Dragendorff’s reagent, the intercellular spaces were stained orange-red (Fig. 5.17) whereas the cells remain colourless. On de-staining with water, the stained intercellular spaces became colourless (Fig. 5.18), indicating a negative results for the alkaloids (Table 5.6).

With iodoplatinate, all the xylem cells in the root, stem, leaf mid-rib and medullary rays of the stem sections were stained grey-violet (Figs. 5.19, 5.23, 5.27). The commercial samples of the atropine also stained grey-violet with iodoplatinate reagent (Fig. 5.30). Platinic chloride stained all the xylem cells in the root, stem and leaf mid-rib sections a greenish yellow (Figs. 5.21, 5.24, 5.28). The commercial samples of the atropine also stained greenish yellow with platinic chloride reagent. When iodoplatinate and platinic chloride stained sections were de-stained with water, the xylem cells in different plant organs (Table 5.6) and the medullary rays of the stem sections (Fig. 5.25) remained stained, indicating the presence of alkaloids in those cells. The alkaloid-free sections of different organs remained colourless when stained with the above reagents (Figs. 5.20, 5.22, 5.26, 5.29).

Cyanogen bromide reagent, used for localization of nicotine in the mature plant organs, gave negative results (Table 5.6).

The staining reagents i.e., platinic chloride (5%) and iodoplatinate which gave positive reactions for the alkaloids in the mature plant organs, were selected for further study to localize alkaloids in the cultured plant materials.

In the mature plant organs, no special ducts for storage or accumulation of the alkaloids were found.
Text-Figure 5.19 The free-hand fresh section was stained with iodoplatinate alkaloid colour reagent and then de-stained with water. Note the grey-violet stained xylem cells (xy) indicating the presence of alkaloid in those cells. x 100

Text-Figure 5.20 The free-hand alkaloid-free section was stained with iodoplatinate alkaloid colour reagent. Note the absence of stained xylem (xy) and phloem (ph) cells indicating the absence of alkaloid in those cells. x 100

Text-Figure 5.21 Fresh section was stained with platinic chloride (5%) alkaloid colour reagent and de-stained with water. Note the stained xylem cells (xy) indicating the presence of alkaloid in those cells. x 100

Text-Figure 5.22 Alkaloid-free section was stained with platinic chloride (5%) alkaloid colour reagent. Note the absence of stained xylem cells (xy) indicating the absence of alkaloid in those cells. x 100
FIGURES 5.23 – 5.26 Free-hand fresh transverse sections of stem collected from a mature greenhouse-grown cuttings of *Duboisia myoporoides* R. Br. stained for histolocalization of alkaloid.

**Text-Figure 5.23** Fresh section was stained with iodoplatinate alkaloid colour reagent and then de-stained with water. Note the stained xylem cells in the vascular region (vr) indicating the presence of alkaloid in those cells. x 40

**Text-Figure 5.24** Fresh section was stained with platinic chloride (5%) alkaloid colour reagent and then de-stained with water. Note the stained xylem cells in the vascular region (vr) indicating the presence of alkaloid in those cells. x 100

**Text-Figure 5.25** Fresh section was stained with iodoplatinate alkaloid colour reagent and then de-stained with water. Note the stained medullary rays (mr) indicating the presence of alkaloid in those cells. x 200

**Text-Figure 5.26** Alkaloid-free section was stained with iodoplatinate alkaloid colour reagent. Note the absence of stained cells in the vascular region (vr) indicating the absence of alkaloid in those cells. x 40
FIGURES 5.27 – 5.30 Free-hand fresh transverse sections of leaf mid-rib collected from a mature greenhouse-grown cuttings of *Dabeisia myoporoides* R. Br. stained for histolocalization of alkaloid

Text-Figure 5.27 Fresh section was stained with iodoplatisate alkaloid colour reagent and then de-stained with water. Note the stained xylem cells (xy) indicating the presence of alkaloid in those cells. x 100

Text-Figure 5.28 Fresh section was stained with platinic chloride (5%) alkaloid colour reagent and then de-stained with water. Note the stained xylem cells (xy) indicating the presence of alkaloid in those cells. x 100

Text-Figure 5.29 Alkaloid-free section was stained with iodoplatisate alkaloid colour reagent and then de-stained with water. Note the absence of stained xylem cells (xy) indicating the absence of alkaloid in those cells. x 100

Text-Figure 5.30 Commercial atropine sample was stained with iodoplatisate alkaloid colour reagent. Note the grey-violet colour produced after staining. x 100
5.3.6.2 Histolocalization of alkaloid in the tissues and organs cultured from the non-organogenic calli

The staining results for localization of alkaloid in the non-organogenic calli are described below. Histolocalization of alkaloid in the root and stem sections of the shoot (without any root formation) differentiated from the non-organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations are summarised in Table 5.4 (page 217).

5.3.6.2.1 Non-organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations

When 11-week-old non-organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations were stained with the selected alkaloid colour reagents, no cells were found to be stained positively, indicating the absence of alkaloids in those cells.

5.3.6.2.2 Shoot differentiated from the callus induced in the BM supplemented with 2,4-D10⁻⁷M

The vascular region in the basal stem sections of the 6-week-old shoot was found to be narrower in comparison to the cortical or pith region (Table 5.4, page 217). The xylem tissues in the vascular region were composed of small cells with no large vessels in the secondary xylem (Fig. 5.31). When the fresh basal stem sections of the shoots were stained with the selected alkaloid colour reagents, no cells in the vascular bundle showed a positive reaction for the alkaloids (Table 5.4, page 217). No difference was observed between the stained fresh and alkaloid free sections, indicating the absence of alkaloid in the cells.
FIGURE 5.31 - 5.33 Free-hand fresh transverse sections of the basal stem of the shoot differentiated from the non-organogenic and organogenic calli induced on the leaf explant of *Duboisia myoporoides* R. Br. incubated in the Murashige and Skoog (1962) basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and various cytokinin and auxin combinations (mentioned in the text-figure).

Text-Figure 5.31 Fresh basal stem section of the 6-week-old shoot differentiated from the non-organogenic callus induced in the BM supplemented with 2,4-D $10^{-7}$ M was stained with iodoplatinate alkaloid colour reagent. Note the absence of stained xylem cells in the vascular region (vr) indicating the absence of alkaloid in those cells. x 100

Text-Figure 5.32 Fresh basal stem section of the 6-week-old shoot differentiated from the non-organogenic callus induced in the BM supplemented with Kin $10^{-7}$M + IAA $10^{-7}$M was stained with iodoplatinate alkaloid colour reagent. Note the absence of stained xylem cells in the vascular region (vr) indicating the absence of alkaloid in those cells. x 100

Text-Figure 5.33 Fresh basal stem section of the 9-week-old shoot differentiated from the organogenic callus induced in the BM supplemented with BA $10^{-7}$M + IAA $10^{-7}$M was stained with iodoplatinate alkaloid colour reagent. Note the absence of stained xylem cells in the broad vascular region (vr) indicating the absence of alkaloid in those cells. x 100
5.3.6.2.3 Shoot differentiated from the callus induced in the BM supplemented with BA$10^{-9}$M+NAA$10^{-6}$M

The cells in the vascular region were well developed, with large vessels in the secondary xylem (Table 5.4, page 217). In the fresh basal stem sections of the 6-week-old shoot, the xylem cells in the vascular region were stained grey-violet with iodonplatinate and greenish yellow with platinic chloride. In the alkaloid-free sections, the cells remained colourless indicating the presence of alkaloids in the fresh sections (Table 5.4, page 217).

5.3.6.2.4 Shoot differentiated from the calli induced in the BM supplemented with the other selected cytokinin and auxin combinations

The results of iodonplatinate alkaloid colour reagent staining of the basal stem section of the 6-week-old shoot differentiated from the callus induced in the BM supplemented with Kin$10^{-5}$M+IAA$10^{-6}$M are presented in Fig. 5.32 (page 230). A narrow vascular region with small xylem cells was found in the basal stem sections of the shoots differentiated from the non-organogenic calli induced in the BM supplemented with the other selected cytokinin and auxin combinations (Table 5.4, page 217). No large vessels in the secondary xylem were observed in the sections studied. All the cells in the fresh and alkaloid-free sections of the shoots when stained with the selected alkaloid colour reagents remained colourless indicating the absence of alkaloids in the fresh stem sections of the differentiated shoots (Table 5.4, page 217).

5.3.6.3 Histolocalization of alkaloid in the tissues and organs cultured from the organogenic calli

The staining results for localization of alkaloid in the organogenic calli are described below. Histolocalization of alkaloid in the root and the basal stem sections
of the shoot differentiated from the organogenic calli are summarised in Table 5.5 (page 220).

5.3.6.3.1 Organogenic calli induced in the BM supplemented with the selected cytokinin and auxin combinations

When 2-week-old organogenic calli induced in the BM supplemented with the selected cytokinin and auxin combinations were stained with the selected alkaloid colour reagents, no cells were found to be stained positively, indicating the absence of alkaloid in those cells.

5.3.6.3.2 Shoot-bearing organogenic callus induced in the BM supplemented with the selected cytokinin and auxin combinations

No alkaloids were localized in the undifferentiated cells of the 14-week-old callus (bearing the differentiated shoot) induced in the BM supplemented with BA $10^{-5}$M+IAA$10^{-5}$M (Table 5.5, page 220). When the fresh sections of the 14-week-old callus bearing the differentiated shoot were stained with iodoplatinate, the cells organized in the concentric rings stained grey-violet (Table 5.5, page 220) (Fig. 5.37), indicating the presence of alkaloids in the fresh sections. The alkaloid-free sections remained colourless indicating the absence of alkaloid in those sections (Table 5.5, page 220) (Fig. 5.38).

5.3.6.3.3 Shoot differentiated from the callus induced in the BM supplemented with BA$10^{-6}$M + IAA$10^{-6}$M

No large vessels were found in the secondary xylem of the broad vascular region (Fig. 5.33, page 230) in the basal stem sections of the 9-week-old shoot (Table 5.5, page 220). All the cells in the vascular region of the fresh and alkaloid-free sections remained colourless when stained with the selected colour reagents (Table 5.5, page 220), indicating the absence of alkaloid in the differentiated shoots.
5.3.6.3.4  Shoot differentiated from the callus induced in the BM supplemented with BA10⁻⁵M + IBA10⁻⁷M

Well developed vascular region with well organized large vessels in the secondary xylem was observed in the basal stem sections of the 9-week-old shoot (Table 5.5, page 220). When the fresh basal stem sections were stained with the selected alkaloid colour reagents, the xylem cells of the vascular region stained greenish yellow with platinic chloride (Fig. 5.34) and grey-violet with iodoplatinate (Fig. 5.35), indicating the presence of alkaloids in the cells. However, in the alkaloid free sections stained with the alkaloid colour reagents, all the cells in the vascular region remained colourless, indicating the absence of alkaloid in those cells (Fig. 5.36), (Table 5.5, page 220).

5.3.6.3.5  Root differentiated from the callus induced in the BM supplemented with BA10⁻⁵M + IBA10⁻⁷M

The cell organization in the 4-week-old cultured roots was similar to that of the complete plant roots. However, the amount of xylem vessels was less in cultured roots (Fig. 5.40) than that in the mature plant roots (Fig. 5.20, page 226). When the fresh sections of cultured roots were stained with the selected alkaloid colour reagents, the xylem cells became stained (Fig. 5.39), indicating the presence of alkaloids in the cells of the fresh sections (Table 5.5, page 220). Whereas in the alkaloid-free sections, the xylem cells remained colourless (Fig. 5.40), indicating the absence of alkaloids in those cells (Table 5.5, page 220).
FIGURES 5.34 – 5.36 Free-hand fresh transverse sections of the basal stem of the 9-week-old shoot differentiated from the organogenic callus induced on the leaf explant of *Duboisia myoporoides* R. Br, incubated in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA $10^{-7}$M + IBA $10^{-7}$M

Text-Figure 5.34 The fresh section was stained with platinic chloride (5%) alkaloid colour reagent. Note the stained xylem cells (xy) indicating the presence of alkaloid in those cells. x 100

Text-Figure 5.35 The fresh section was stained with iodoxplatinate alkaloid colour reagent. Note the stained xylem cells (xy) indicating the presence of alkaloid in those cells. x 100

Text-Figure 5.36 The alkaloid-free section was stained with iodoxplatinate alkaloid colour reagent. Note the absence of stained small and large xylem cells (lx) in the vascular region (vr) indicating the absence of alkaloid in those cells. x 100
Text-Figure 5.37 Free-hand fresh section of the 14-week-old callus collected from the base of the differentiated shoot was stained with iodoplatinate alkaloid colour reagent. Note the grey-violet staining of the undifferentiated cells arranged in concentric ring (cr). \( x \times 100 \)

Text-Figure 5.38 Alkaloid-free section of the 14-week-old callus collected from the base of the differentiated shoot was stained with iodoplatinate alkaloid colour reagent. Note the absence of stained undifferentiated cells in the concentric ring (cr). \( x \times 400 \)

Text-Figure 5.39 Free-hand fresh transverse section of the 4-week-old root differentiated from the organogenic callus induced on the leaf explant of Daboisia myoporoides R. Br. incubated in the MS basal medium supplemented with Bacto agar (0.9%), sucrose (3%) and BA10^{-7}M+IBA10^{-3}M was stained with iodoplatinate alkaloid colour reagent. Note the stained xylem cells (xy) indicating the presence of alkaloid in those cells. \( x \times 100 \)

Text-Figure 5.40 Free-hand alkaloid-free transverse section of the 4-week-old root (as fig. 5.39) was stained with iodoplatinate alkaloid colour reagent and then de-stained with water. Note the small number of xylem cells in the vascular region (vr) and the absence of stained xylem cells (xy) indicating the absence of alkaloid in those cells. \( x \times 400 \)
5.4 DISCUSSION

5.4.1 Cell Arrangement and Macromolecular Constituents of the Non-organogenic and Organogenic Calli

Of the 3 reagents used in this study, only toluidine blue O stained different macromolecules in the root sections of mature Duboisia myoporoides R. Br. plant. This result is consistent with previous results reported by other researchers. O’Brien and McCully (1981) reported the staining of various macromolecules in hand-cut sections of fresh plant materials using toluidine blue O and mentioned this reagent as an excellent general purpose histochemical reagent. In my studies, toluidine blue O localized DNA and RNA in some of the root cells only. It appears that DNA and RNA present in other cells of the fresh root sections were lost from free-hand sections as also noted by O’Brine and McCully (1981).

Histochemical studies of the 11-week-old non-organogenic calli induced in the BM supplemented with the selected cytokinin and auxin combinations showed the presence of meristemoid regions with small cells. These results are consistent with those of Thorpe (1980) who also reported the presence of meristemoid regions and the small size of the cells in the meristemoid regions in the callus tissues of various plants. After 11 weeks of incubation, although organogenesis did not occur in the non-organogenic calli, the presence of the meristemoid regions indicated active cell division in the calli. It appears that the cytokinin and auxin combinations used at the induction stage were not suitable for organogenesis to occur.

A variation in the size of the meristemoid regions in the 11-week-old non-organogenic calli induced in the BM supplemented with the selected cytokinin and auxin combinations was observed indicating that, depending on the cytokinin/auxin combinations, the area of the active cell division may vary. This may be due to the
interaction between the cytokinins and auxins used at the callus induction stage. The difference between the tested cytokinins is that kinetin tended to reduce the active cell division area more than did BA. This result indicated that the size of the meristemoid region depends on the effective concentration of the cytokinin/auxin used.

In my study, cells around the meristemoid regions in the 11-week-old non-organogenic calli induced in the BM supplemented with Kin10^{-5}M+NAA10^{-5}M were elongated (Section 5.3.2.6), whereas those in the non-organogenic calli induced in the BM supplemented with other cytokinin and auxin combinations were round. Yeung et al. (1981) reported that variation in cell size could arise by unequal rates of cell enlargement. Similar effects of Kin+NAA on the variation of cell size in the calli were also observed by other researchers. Anzidei et al. (1996) noticed a variation in the size of the non-organogenic calli cells of *Foeniculum vulgare* Millner induced in the BM supplemented with Kin2.3x10^{-6}M+NAA2.6x10^{-6}M. Dudits et al. (1991) recorded an induction of tumorous growth without organogenesis in alfalfa. My results showed that the Kin10^{-5}M+NAA10^{-5}M caused the formation of elongated cells in the non-organogenic calli.

Results from the toluidine blue O staining showed the presence of polysaccharides and scattered phenols in the 11-week-old non-organogenic calli cells. This observation is consistent with that of Thorpe (1980) who reported the presence of starch in tobacco callus and scattered lignified elements in the callus and cell colonies from the various tissues of different plant species. This result indicates that lignified elements can be produced in the calli cells before organogenesis occurs.

An organized cell arrangement was observed in the 2-week-old shoot-bud and root producing organogenic calli indicating the initial stage of shoot-bud and root formation respectively in the calli. Since the organogenic calli were induced in the BM supplemented with cytokinin BA in combination with different auxins (IAA, IBA), it appears that BA has some effect on induction of organogenic calli. This is consistent with the findings of Patel and Thorpe (1984), who found that BA mediated organized cell division leads to shoot formation in radiata pine.
Eleven-week-old non-organogenic and 2-week-old shoot-bud producing organogenic calli differed in the degree of polysaccharide accumulation in the tissues, particularly in the shoot-forming regions. The accumulation preceded the initiation of organized development, i.e., the formation of the zones of preferential cell division activity. These observations indicated a higher energy requirement during shoot-bud formation. According to Thorpe (1980), accumulation of reserve storage material may be a general feature of the organogenic cells. Later it was well demonstrated in various plant species that starch accumulation is required for shoot induction in callus (Ho and Vasil, 1983; Stamp, 1987; Mangat et al., 1990).

A significant feature of this study is the difference between the macromolecular constituents of the 2-week-old shoot-bud producing calli and 14-week-old calli bearing differentiated shoots. Staining intensity indicates larger amount of macromolecules in the shoot bearing calli. With a longer incubation period, the shoot-bearing calli became able to produce more macromolecules.

5.4.2 Differentiation in the Cultured Tissues and Organs

No differentiated tracheary elements or xylem cells were found in the 11-week-old non-organogenic and 2-week-old organogenic calli induced in the BM supplemented with the selected cytokinin and auxin combinations. Geissbuhler and Skoog (1957) reported that with each subculture, callus tissues derived from the cambium of jack pine lost the ability for tracheary element formation. On the other hand, Torrey (1967) reported that tracheary element formation continued in pea callus with prolonged culture. My results showed that the incubation period is not a factor for tracheary element formation or xylem differentiation in the callus tissues. However, it may be that the cytokinin/auxin combinations used at the calli induction stage are not optimal for tracheary element formation or xylem differentiation in the calli cells.
My results showed that the different patterns of xylem formed in the stem of the shoots regenerated from the non-organogenic and organogenic calli were related to the cytokinin/auxin combinations used at the callus induction stage. These observations are partially consistent with those of Dalessandro (1973) who studied the effect of different cytokinin and auxin combinations on different patterns of xylem differentiation in the pith parenchyma explants of Jerusalem artichoke tubers (Helianthus tuberosus L.). Cell division, which is dependent upon the cytokinin and auxin combination, may be related to the pattern of xylem formation.

The pattern of xylem formation in the stem segments of the differentiated shoots of D. myoporoides in the present study showed a probable relationship between the cell division and the xylem formation in the cultured organs. Previous studies indicated a relationship between the xylem differentiation and the cell division in the different plant tissues. Torrey (1966) proposed that xylem differentiation in the intact plant usually followed a recent cell division. Fosket (1968) showed that xylem differentiation in the cultured Coleus stem segments is initiated during some phase of the mitotic cycle. My results indicate that a particular cytokinin and auxin combination is necessary for cell division leading to xylem differentiation in the cultured shoots.

Presence or absence of large vessels in the secondary xylem is the main difference observed in different patterns of xylem formed in the basal stems of the differentiated shoot in the present study. Hormonal interactions are important for organogenesis in the cultured tissues (Skoog and Miller, 1957) and specific types of cellular differentiation (Stockdale and Topper, 1966). My results substantiate this idea with respect to xylem differentiation in the stems of the differentiated shoots. Of all the selected cytokinin and auxin combinations used, 2 combinations i.e., BA10^{-5}M+NAA10^{-6}M and BA10^{-5}M+IBA10^{-7}M were found to be effective for formation of large vessels in the secondary xylem. These combinations were later found to be the only 2 combinations in which the xylem vessels contained the alkaloids (see later).

Although basal stems of the shoots differentiated from the non-organogenic callus induced in the BM supplemented with 2,4-D10^{-7}M showed formation of xylem
cells, lack of large vessels in the secondary xylem and many layered pith and cortex indicate that cytokinin and auxin are both necessary at the callus induction stage for different cell layer organization in the differentiated shoots.

The xylem formed in the basal stems of the shoots differentiated from the organogenetic callus induced in the BM supplemented with BA10^{-5}M+IAA10^{-6}M showed a larger xylem element population. Fosket and Torrey (1969) reported that during secondary xylem formation, active cell division by the cambium is directly involved in the increase of the xylem element population. It appears that active cell division influenced by the interaction of the mentioned cytokinin and auxin combination caused the increase in the xylem element population in the basal stem of the differentiated shoots.

Formation of different patterns of xylem in the basal stems of the differentiated shoots can be explained from the genetic point of view. Schmulling et al. (1997) reported that the cytokinin target genes are regulated by auxins and the regulated genes play a role in cell division. Some of the selected cytokinin and auxin combinations used at the callus induction stage of the present study caused the xylem cell arrangement in the differentiated stems similar to that of the parent plant while other combinations failed to do so. These cells may need a longer period to arrange the cells in the parental pattern. This indicates that the cytokinin and auxin combination used at the callus induction stage influences the final arrangement and differentiation of the xylem cells in the basal stem of the differentiated shoot.

Considering the results of the cell arrangement and xylem differentiation in relation to the cytokinin and auxin combinations used in this study, it may be concluded that 1) absence of cytokinin in the callus induction stage causes a significant increase in the number of the cell layers in the pith and cortex; 2) the induction medium should be supplemented at the callus induction stage with the appropriate combination and concentration of cytokinin and auxin to regenerate a shoot with organized cells as in the intact plant and 3) formation of large vessels in the secondary xylem in the basal stems of the differentiated shoot needs the appropriate combination and concentration of cytokinin and auxin at the callus induction stage.
5.4.3 Histolocalization of Alkaloids in the Mature Plant Organs

My results indicate that alkaloid colour reagents can be used to detect alkaloids in the cells of the different plant organs of *D. myoporoides*. Platinic chloride (5%) and iodoplatinate staining of the contents of the cell were similar to those when the reagents were allowed to react with the commercial alkaloid samples, indicating the presence of alkaloid in the cells. Platinic chloride staining is consistent with that of Newman (unpublished data, cited in Luckner et al., 1977), who detected sanguinarine alkaloid in the vacuoles of the alkaloid-accumulating cells in cell cultures of *Macleaya* species. Localization of alkaloids in the different cells of the fresh tissues is further supported by the loss of reactivity when the sections were pretreated with alkaloid extractants.

Platininc chloride staining helped to avoid a probable confusing result of simultaneous alkaloid and starchy material staining of the fresh tissues using an iodine based reagent (iodoplatinate). However, Corsi and Biaisci (1998) reported simultaneous staining of starchy materials present in the fresh plant organs and they obtained their results by using Lugol and Wagner reagents for the localization of hemlock alkaloids in *Conium maculatum* L (Apiaceae). A positive reaction for alkaloid localization using these 2 reagents in the present study suggests that these colour reagents are reacting only with the alkaloids present in the fresh sections of *D. myoporoides*.

My results showed the presence of alkaloids in the xylem and the phloem cells of different organs of *D. myoporoides*. James (1950) reported the presence of tropane alkaloids in the xylem and phloem parenchyma, medullary rays, endodermis and epidermis and cortex of the mature plant stems of *Atropa belladonna* and the presence of alkaloid in the different cells also depends on seasonal variation as well as the age of the plant. In addition, Ferreira et al., (1998) found tropane alkaloids in the mesophyll, including palisade, spongy, and vascular parenchyma cell layers, and in some cells of the collenchyma of leaves, stems and fruits of *Erythroxylum coca* var. *coca* L. and *E. novogranatense* var. *novogranatense* M. Absence of alkaloids in
epidermal, cortical or pericycle cells, as observed in this study, may be due to 1) alkaloids present in other cells but the amount was not identifiable by the selected colour reagents; 2) alkaloid absent in other cells or exhibiting seasonal variation; 3) alkaloids are absent due to the age of the plant.

Xylem cells are reportedly used for root to shoot translocation of alkaloids (James,1950; Wink,1987; Kitamura et al.,1993) and phloem cells for shoot to root translocation (Kitamura et al.,1993) in different alkaloid-producing plant species. My results indicate that the xylem and the phloem cells in the mature plant roots and stems of *D. myoporoides* may be involved in the accumulation of the alkaloids. However, Gritsanapan and Griffin (1992) explained the presence of alkaloids in the xylem cells as a seasonal change in the rate of transpiration.

No alkaloid was detected in the pericycle cells of the root sections in my study. Hashimoto et al.,(1991) identified the pericycle cells of the roots as the probable biosynthetic site for alkaloids. It appears that, after being biosynthesised in the pericycle cells of the root, the alkaloids are transferred completely to the xylem cells for accumulation and translocation. An identifiable quantity of alkaloid may not be left in the pericycle cells in the present study to be detected by the alkaloid colour reagents used.

My results showed that the aerial parts of the mature plant contained the alkaloids in the xylem cells of the stems and leaf mid-ribs. This indicates that either the biosynthesis of the alkaloids takes place in the aerial parts independent of the root, or those cells are used as the accumulation and storage site for the alkaloids biosynthesised and translocated from the root cells. In alkaloid-producing plant species such as *Datura, Atropa* (Waller and Nowacki,1978) and *D. leichhardtii* (Yamada and Endo,1984), alkaloids are translocated to the aerial organs after biosynthesis in the root cells where partial biosynthesis, i.e., conversion of hyoscyamine to scopolamine takes place. However, in *D. leichhardtii* both root and aerial organs can convert hyoscyamine to scopolamine (Yamada and Endo, 1984). If the rate of root to shoot translocation is slow, the conversion may also take place in the translocating xylem cells (Yun et al.,1992). Since the selected alkaloid reagents did not differentiate between hyoscyamine and scopolamine in this study, it cannot
be concluded with certainty that the alkaloids are biosynthesised in the aerial parts and/or converted during their translocation from root to aerial parts of the plant. My literature search failed to find a specific histochemical reagent which could differentiate between hyoscyamine and scopolamine alkaloids in the sections of the fresh plant materials. Further investigations are needed in this field.

5.4.4 Cell differentiation and histolocalization of alkaloids in the Cultured Tissues and Organs

The undifferentiated cells in the 11-week-old non-organogenic and 2-week-old organogenic calli did not reveal tropane alkaloids when the cells were stained with the selected alkaloid colour reagents. However, Sipply and Friedrich (1975) reported tropane alkaloids in the 24-week-old calli cells of *D. myoporoides* R. Br. using TLC analysis. It appears that in this study, either the alkaloids were biosynthesised in the calli but the amount was not identifiable by the selected alkaloid colour reagents used or no alkaloid biosynthesis took place in the calli.

Basal stem sections of the shoots (without root formation) differentiated from the non-organogenic and organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations responded to the alkaloid colour reagents according to the cell differentiation in the vascular region. Vascular regions with large vessels in the secondary xylem gave a positive alkaloid test, whereas no alkaloid was detected in the vascular region with only small vessels. This result indicates that presence of large vessels in the secondary xylem may be related to either alkaloid biosynthesis or accumulation in the cultured shoots before root formation.

In some alkaloid-producing plant species, alkaloid transport is mediated by carrier proteins (Warner and Matile, 1985; Matern et al., 1986; Wink, 1987). Wink (1987) reported that a significant alkaloid production occurred when gene expression for alkaloid biosynthesis and transport (which is related to accumulation) took place at the same time. Guern et al., (1987) reported that gene expression for both alkaloid
biosynthesis and transport takes place in the presence of a suitable storage site where accumulation without degradation occurs. Since large xylem vessels are dead cells, biosynthesis of enzyme is not possible in those cells. It appears that biosynthesis of all the enzymes of the alkaloid biosynthetic pathway and carrier proteins of alkaloid transport takes place in the cells other than the large xylem vessels. It may be that the gene expression for the alkaloid biosynthesis and the carrier proteins takes place after the formation of the large vessels in the secondary xylem.

My results showed the presence of alkaloid in the sections of 14-week-old callus (callus attached with the 9-week-old differentiated shoot without any root initiation) induced in the BM supplemented with BA10^{-5}M+IBA10^{-7}M. This result indicates that either alkaloid biosynthesis takes place in the callus cells or the alkaloids formed in the shoots are returning to the callus for accumulation. Endo and Yamada (1985) reported biosynthesis of alkaloid in the cultured roots (without aerial organs) only but not in the cultured shoots (without root). However, Kitamura et al., (1996) reported the recycling of alkaloid from the shoots to the roots in D. myoporoides. It appears that in my study, the callus similarly may be used for accumulation and storage of the alkaloids biosynthesised in the shoots without root formation.

Four-week-old roots differentiated from the organogenic callus induced in the MS basal medium supplemented with BA10^{-6}M+IBA10^{-5}M had a vascular region with fewer xylem cells than in the mature plant roots and they stained positively for alkaloids similar to that in the mature plant roots. Contrary to this, it took 6 and 9 weeks (age of the shoot differentiated from the non-organogenic and organogenic calli respectively) for xylem differentiation similar to that of the mature plant, in the basal stem of the cultured shoot. Comparing the incubation period for differentiation in the cultured root and the shoot, it appears that the formation of the xylem vessels in the differentiated roots takes place earlier than in the basal stems of the differentiated shoots. Earlier formation of xylem vessels in the regenerated roots than in the regenerated shoots may account for the earlier presence of alkaloids in the former tissue.
In summary, my studies of cell differentiation and tissue localization of alkaloids indicate that, 1) differentiation of the cells in the basal stems of the cultured shoots depends on the cytokinin/auxin combinations used at the callus induction stage; 2) tropane alkaloid can be localized in the cultured shoots without root initiation and 3) the formation of large vessels in the secondary xylem is necessary for the accumulation of the alkaloids in the cultured shoot.

This model can be further tested by investigating xylem differentiation for a stable tropane alkaloid biosynthesis in the cultured shoots using various combinations and concentrations of different PGRs. Further investigations of xylem differentiation in the calli may help to express a stable tropane alkaloid biosynthesis without the need to regenerate differentiated organs. That is, this finding may allow commercial alkaloid production from the calli with the differentiated xylem but not requiring organ development.
Identification and Quantification of the Selected Alkaloids of *Duboisia myoporoides* R. Br. Plant Materials
6.1 INTRODUCTION

*Duboisia myoporoides* R. Br. contains different groups of alkaloids such as the tropane alkaloids hyoscyamine and scopolamine, the pyridine alkaloid nicotine (Endo and Yamada, 1985; Kitamura *et al.*, 1985a, b; Gritsanapan and Griffin, 1991), the α-alkylpiperidine alkaloid pelletierine, the quinolizidine alkaloid myrtine and the β-phenylethylamine alkaloids tyramine and 3-methoxytyramine (Bachmann *et al.* 1989). Tropane alkaloids are medicinally important. In commercial production, the leaves of the mature plants are harvested continually throughout the year, dried, coarsely powdered and exported to different countries (Kitamura *et al.*, 1991).

Field-grown mature plants or cultured plant materials are analysed by extracting the alkaloids. Generally, field-grown mature plants produce more alkaloids than do cultured plant materials. For example, intact plants normally contain from 100-1000mg (100g dry weight)$^{-1}$ total tropane alkaloids and callus cultures contain approximately 1-35mg (100g)$^{-1}$ according to some workers (Hiraoka *et al.*, 1973; Konoshima *et al.*, 1970) and 50-100mg (100g dry weight)$^{-1}$ according to others (Chan and Staba, 1965; West and Mika, 1957) while root organ cultures contain from 500-1000mg (100g dry weight)$^{-1}$ total alkaloids (Mitra, 1972). From high alkaloid-producing root lines of the *Duboisia* species, hyoscyamine and scopolamine contents were found to be 0.53 and 1.16% respectively on a dry weight basis (Endo and Yamada, 1985). Analysis of cultured plant materials needs a sensitive and selective analytical procedure as the analyte may be present in small quantities in a complicated matrix.

For analysing the extracts, different analytical techniques such as TLC (Kitamura *et al.*, 1985a), GC (Yukimune *et al.*, 1994 b) and HPLC Subroto *et al.*, 1995) have been used. Identification and quantification of the alkaloids are mainly carried out by using GC-FID (Sharp and Doran, 1990), GC-MS (Yukimune *et al.*, 1994 d) and HPLC (Subroto *et al.*, 1995). *D. myoporoides*, plant materials, cultured under different experimental conditions have been widely analysed by GC-
MS (Endo and Yamada, 1985; Yukimune et al., 1994 d). Strangely, none of the analytical methods used have been put through a systematic validation process.

For this work, nicotine, hyoscyamine and scopolamine present in the cultured as well as mature plant materials were analyzed. Although the main desirable alkaloids are hyoscyamine and scopolamine, the undesirable alkaloid nicotine was also analyzed to reveal the biosynthetic history of alkaloids in the cultured plant materials.

Therefore, the aim of the present study was to analyze nicotine, hyoscyamine and scopolamine in the cultured tissues and organs and the mature plant materials of *D. myoporoides* R. Br.

The objectives were:
1. To conduct systematic validation of the analytical method
2. To analyse nicotine, hyoscyamine and scopolamine in the following plant materials by using the validated method
   a) Eleven-week-old calli, 2-week-old green calli, 1-2-week-old shoot-buds and 6-week-old shoots cultured from the non-organogenic calli incubated on the semi-solid MS basal medium supplemented with the selected cytokinin and auxin combinations
   b) Two-week-old shoot-bud producing calli, 14-week-old calli collected from the base of the differentiated shoot, 3-week-old shoot-buds, 4-week-old shoot and 9-week-old shoots cultured from the organogenic calli induced on the semi-solid MS basal medium supplemented with various cytokinin and auxin combinations
   c) Roots regenerated from the non-organogenic calli incubated on the semi-solid MS basal medium supplemented with IBA25*10^{-6}M
   d) Roots regenerated from the organogenic calli induced in the BM supplemented with BA10^{-6}M+IBA10^{-5}M
   e) Cell aggregates without shoot initiation, cell aggregates with shoot and suspension culture medium
   f) Roots regenerated in suspension culture and the suspension culture medium
   g) Leaves from the mature plant collected from the greenhouse-grown cuttings
   h) Roots and leaves from the mature plant collected from Mount Annan Botanic Gardens
6.2 MATERIALS AND METHODS

6.2.1 Chemicals, Stock Solutions and Analytical Equipments

Atropine, scopolamine hydrochloride and homatropine (all 99%) purity were obtained from Sigma Chemicals (USA). Nicotine (95%) was obtained from BDH (UK). HPLC grade methanol and dichloromethane were obtained from Merck (Germany) and isopropanol from EM Science (USA). AR grade hydrochloric acid and ammonia solutions were obtained from Ajax Chemicals (Australia).

The solid phase extraction (SPE) column “Extrelut 20” was from Merck (Germany). 500 ppm, 100 ppm, 100 ppm and 200 ppm stock solutions of atropine, scopolamine hydrochloride, homatropine and nicotine respectively were prepared daily using methanol solvent along with the dilute working solutions.

6.2.2 Systematic Validation of the Analytical Method Used

A method for the simultaneous analysis of nicotine, atropine and scopolamine present in the *D. myoporoides* leaf samples has been developed and the performance of the analytical method characterized by method validation procedures (Nou, 1997). The optimal extracting solvent and gas chromatographic conditions for analysis were determined. The best result was obtained with a dichloromethane : isopropanol (4 : 1 v/v) eluting solvent in combination with ‘Extrelut’ solid phase extraction. The method was found to be applicable for analysing nicotine, atropine and scopolamine in the range of 20 to 600μg g⁻¹, 30 to 1800μg g⁻¹ and 60 to 2400μg g⁻¹ respectively.
of the dry samples. The recoveries for nicotine, atropine and scopolamine were between 87% and 100% with a precision between 1% and 7%. The method was also validated for root and berry samples. This analytical technique was validated by testing for precision, accuracy, recovery, linearity, sample and standard stability, limits of detection and quantification (Nou, 1997).

6.2.3 Plant Materials Analyzed

The following samples were analysed for nicotine, hyoscyamine and scopolamine: (1) the cultured tissues and organs produced under different conditions (Chapter 2 and 3); (2) the leaves and soft stems collected from the greenhouse-grown cuttings (Chapter 2) and (3) the leaves and root samples from a mature plant collected from Mount Annan Botanic Gardens (Chapter 2).

6.2.4 Extraction of Alkaloids from Plant Materials

The alkaloids were extracted from the cultured tissues and the organs collected from the semi-solid and suspension media and samples collected from the mature plants by using the validated analytical method (Section 6.2.2). The samples were air dried to constant weight at room temperature. For each extraction 0.1 to 0.5g of dry sample was used. Liquid media from the suspension culture were collected over a period of 3 weeks, combined, freeze dried, extracted and tested for alkaloid content.

The samples were powdered in a mortar with a small quantity of acid-washed sand. To the powdered sample, 1mL of 10ppm homatropine and 10mL of 0.5M HCl were added. After 15min, the solution was vacuum filtered through a No.1 Whatman filter paper. The filtrate was made alkaline with ammonia solution and passed through an Extrelut column. After 20 to 30min, 40mL of the eluting solvent (dichloromethane : isopropanol 4:1 v/v) was added to the column. For all samples, 2
to 4 extracts were combined after separate Extrelut extractions. The combined extracts were then evaporated to dryness in a rotary evaporator. The dry residue was dissolved in 1mL of methanol for GC-MS analysis. In the analysis, the racemic mixture atropine, which consists of (S) and (R) hyoscyamine, is not resolved and appears as a single chromatographic peak.

6.2.5 Gas Chromatography

The GC-FID was used for screening purposes. Positive samples were then analysed by GC-MS. The confirmation of alkaloid identity in different samples was achieved by the GC-FID or GC-MS runs of the pure standards, standard mixture and sample extracts on the same day. For GC-MS analysis, further identity confirmation of each alkaloid was achieved by comparing the characteristic ions of the sample’s alkaloids to those of the pure standard, literature reported and from the NIST 62 library ions of the alkaloid in question and their relative abundances.

6.2.5.1 GC-FID

Individual standards, mixed standards and tissue extracts were analyzed on a DANI 8500 GC with FID using a SGE BP1 capillary column (25m x 0.53mm, 1μm film thickness). This column has 100% bonded dimethylpolysiloxane on fused silica. An oven temperature program of 83°C maintained for 3.5min, rising to 220°C at a rate of 30°C min⁻¹ was used. The injector and detector temperatures were set at 240°C. Nitrogen was used as the carrier gas.

6.2.5.2 GC-MS

The GC-MS analysis of the sample extracts was performed by the Australian Government Analytical Laboratories (AGAL) at Pymble, Sydney. A SGE BPX5
(30m x 22mm, 0.25μm film thickness) column with helium carrier gas was used. Initial oven temperature was set at 85°C for 3min, rising at a rate of 12°C min⁻¹ to 280°C. The injector temperature was 220°C and detector temperature 280°C. The mass spectrometer was run in the electron impact mode. The mass spectra of the detected alkaloids are given in the results section (Section 6.3).

6.2.5.3 Quantification

The synthetic alkaloid homatropine was used as an internal standard for the quantification of both pyridine and tropane alkaloids. Quantification was achieved by taking the ratio of the abundance of the analyte to that of the internal standard homatropine. Identity of sample peaks was obtained by matching their retention times (Rₜ) with that of the standard peak. Identity confirmation of the peaks was achieved by matching the mass spectra (MS) of the sample peaks with at least 4 m/z ions of the standard peak and their relative abundances. Freshly prepared individual standard and mixed standard samples were analyzed on the same day as the sample analysis. The detection limits for nicotine, hyoscyamine and scopolamine in dry leaves were estimated to be 20, 30 and 60 μg g⁻¹ respectively (Nou, 1997).

6.2.6 Statistical Analyses

Nicotine contents in the 6-week-old shoot differentiated from the non-organogenic calli induced in the BM supplemented with the selected 7 cytokinin / auxin combinations were analyzed by using analysis of variance (Anova). Nicotine, hyoscyamine and scopolamine contents in the 6-week-old shoot differentiated from the non-organogenic calli induced in the BM supplemented with BA10⁻⁵M + NAA10⁻⁶ M and in the 9-week-old shoot differentiated from the organogenic calli induced in the BM supplemented with BA10⁻⁵M + IBA10⁻⁷M were also analyzed by using analysis of variance (Anova). The Least Significant Difference (LSD) method (Appendix 6) was used to test mean values of the alkaloid contents which were significantly different from each other, if Anova indicated a significant difference.
Nicotine, hyoscymamine and scopolamine contents in the 9-week-old shoot differentiated from the organogenic callus induced in the BM supplemented with BA10^{-5} M+IBA10^{-7}M and those in the 4-week-old roots regenerated from the organogenic callus induced in the BM supplemented with BA10^{-6}M+IBA10^{-5}M were compared using one-tailed two-sample $t$ test.

All statistical tests and mean ± standard deviation calculations were conducted using Microsoft Excel '97.
6.3 RESULTS

6.3.1 Alkaloid Production in the Semi-solid Medium

Presence, absence and contents of nicotine, hyoscyamine and scopolamine in the plant materials cultured in the semi-solid medium (Chapter 2) are summarised bellow.

6.3.1.1 Standardization of Plant Regeneration

No alkaloids were detected by GC-MS in the 2-week-old shoot-buds induced on the non-organogenic calli grown in the BM supplemented with BA22x10⁻⁶M. All the 3 alkaloids i.e., nicotine, hyoscyamine and scopolamine were detected by GC-FID and GC-MS in the 6-week-old shoots (without root formation) differentiated from the calli induced in the BM supplemented with BA10⁻⁶M + NAA54x10⁻⁵M. The retention times (Rₜ) for nicotine, hyoscyamine and scopolamine were compared well with those of the mixed standards analyzed on the same day. The GC-MS total ion chromatogram and mass spectra for nicotine and hyoscyamine are presented in Fig. 6.3 a-c (page 273). The m/z values for the alkaloids present in the sample are given in Section 6.3.4.2.3 a (page 272).

6.3.1.2 Shoot Culture from the Non-organogenic Calli

Nicotine, hyoscyamine and scopolamine contents in the cultured samples grown on the semi-solid MS basal medium supplemented with the selected cytokinins / auxins were determined at different stages of the organ (shoot and root) development. The results obtained for 11-week-old calli, 2-week-old green calli, 1-
2-week-old shoot-buds and 6-week-old differentiated shoots are summarised in Table 6.1.

Nicotine, hyoscyamine and scopolamine were found in the 11-week-old non-organogenic callus induced in the BM supplemented with BA10^{-5}M + NAA10^{-6}M. The m/z values for the alkaloids present in the sample are given in Section 6.3.4.2.3. b (page 272). These alkaloids were absent in the 11-week-old non-organogenic calli induced in the BM supplemented with the other selected cytokinin/auxin combinations (Table 6.1).

No alkaloids were detected in the 2-week-old green calli obtained from the 11-week-old non-organogenic calli induced in the BM supplemented with the selected cytokinin / auxin combinations (Table 6.1). The alkaloids were absent in the 1-2-week-old shoot-buds regenerated from the 11-week-old non-organogenic calli induced in the BM supplemented with the selected cytokinin / auxin combinations (Table 6.1).

Alkaloid contents of the 6-week-old shoots (without root initiation) differentiated from the non-organogenic calli induced in the BM supplemented with the selected cytokinin / auxin combinations and grown on the semi-solid MS basal medium supplemented with BA5x10^{-6}M + NAA0.5x10^{-6}M are also summarised in Table 6.1. Only nicotine was present in the shoots differentiated from the non-organogenic calli induced in the BM supplemented with 2,4-D10^{-7}M (Table 6.1). A typical GC-MS total ion chromatogram and mass spectrum are presented in Fig. 6.4 a, b (page 274) and m/z values are presented in Section 6.3.4.2.3. c (page 272).

All the 3 alkaloids i.e., nicotine, hyoscyamine and scopolamine were detected in the 6-week-old shoots differentiated from the non-organogenic calli induced in the BM supplemented with BA10^{-5}M + NAA10^{-6}M (Table 6.1). A typical GC-MS total ion chromatogram and mass spectrum for hyoscyamine detected in the 6-week-old (from the first of the duplicate experiment) shoot are presented in Fig. 6.5.1a and b respectively (page 276). A typical GC-MS total ion chromatogram and mass spectra for nicotine and scopolamine detected in the 6-week-old shoot (from the second of the duplicate experiment) are presented in Fig. 6.5.2 a-c respectively (page 277). The m/z values obtained for
Column means sharing a common letter within the row are significant at α = 0.05 level.

<table>
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<th>Sample</th>
<th>6-week-old Shoots</th>
<th>11-week-old Shoots</th>
<th>2-week-old Shoots</th>
<th>11-week-old Non-organogenic Calli</th>
<th>Combination used</th>
<th>Cell induction stage</th>
<th>Mean ± Standard Deviation</th>
<th>Average of determinations</th>
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TABLE 6.1 Alkaloid content* (mg/g dry weight) in different stages of shoot differentiation from the non-organogenic calli induced on the basal MS medium supplemented with the selected cytokinin and auxin.
nicotine, hyoscyamine and scopolamine are given in Section 6.3.4.2.3 d (page 275). The results from these duplicate experiments show the presence of nicotine, hyoscyamine and scopolamine in the 6-week-old shoot (without root formation) differentiated from the non-organogenic calli.

Only nicotine was also present in the 6-week-old shoots differentiated from the non-organogenic calli induced in the BM supplemented with BA10⁻⁵M + 2.4-D10⁻⁷M or Kin10⁻⁵M + 2.4-D10⁻⁷M (Table 6.1). The m/z values, total ion chromatogram and mass spectra of these two samples are presented in Section 6.3.4.2.3 e (page 275), f (page 279) and Fig. 6.6 a, b (page 278) and 6.7 a, b (page 280) respectively.

No alkaloids were detected in the 6-week-old shoot differentiated from the non-organogenic calli induced in the BM supplemented with the other selected cytokinin and auxin combinations (Table 6.1).

Six-week-old shoots differentiated from the non-organogenic calli induced in the BM supplemented with 2,4-D10⁻⁷M produced significantly higher (P < 0.001) [Table 6.1 and 6.3 (page 260)] amounts of nicotine than the other treatments.

Nicotine, hyoscyamine and scopolamine contents of the 6-week-old shoot differentiated from the non-organogenic callus induced in the BM supplemented with BA10⁻⁵M + NAA10⁻⁶M differed significantly within the treatment (P < 0.001) (Table 6.3, page 260).

**6.3.1.3 Shoot Culture from the Organogenic Calli**

The results obtained for (i) 2-week-old calli, (ii) 3-week-old shoot-buds, (iii) 4-week-old shoots, (iv) 9-week-old shoots and (v) 14-week-old calli collected from the base of the shoots are summarised in Table 6.2.

No alkaloids were detected in the 2-week-old calli induced in the BM supplemented with the selected cytokinin and auxin combinations (Table 6.2).

The alkaloids were absent in the 3-week-old shoot-buds regenerated from the organogenic calli induced in the BM supplemented with the selected cytokinin and auxin combinations (Table 6.2).

No alkaloids were detected in the 4-week-old and 9-week-old shoots differentiated from the organogenic calli induced in the BM supplemented with BA$10^{-5}$M+IAA$10^{-6}$M (Table 6.2). The alkaloid contents of the shoots (without root formation) differentiated from the organogenic calli induced in the BM supplemented with BA$10^{-5}$M+IBA$10^{-7}$M grown on the same medium varied with the age of the shoot. No alkaloid was detected in the 4-week-old shoots, whereas the 9-week-old shoots were able to produce nicotine, hyoscyamine and scopolamine without root formation (Table 6.2). A typical GC-MS total ion chromatogram and mass spectra for nicotine, hyoscyamine and scopolamine detected in the 9-week-old shoot are presented in Fig. 6.8 a–d respectively (page 281-282). The m/z values obtained for nicotine, hyoscyamine and scopolamine are presented in Section 6.3.4.2.3. g (page 279).

Nicotine, hyoscyamine and scopolamine contents of the 9-week-old shoot differentiated from the organogenic calli induced in the BM supplemented with BA$10^{-5}$M + IBA$10^{-7}$M differed significantly within the treatment ($P < 0.001$) (Table 6.3).

No alkaloids were detected in the 14-week-old callus (collected from the base of the differentiated shoot) induced in the BM supplemented with BA$10^{-5}$M + IAA$10^{-6}$M (Table 6.2). The 14-week-old callus (collected from the base of the 9-week-old shoots) induced in the BM supplemented with BA$10^{-5}$M+IBA$10^{-7}$M contained hyoscyamine only. No nicotine and scopolamine were detected (Table 6.2). A typical GC-MS total-ion chromatogram and mass spectrum for hyoscyamine are presented in Fig. 6.9 a, b (page 283) and the m/z values obtained are given in Section 6.3.4.2.3. h (page 279).
### TABLE 6.3 Summary analysis of variance of effects of the selected cytokinin / auxin combinations on alkaloid contents of the shoots differentiated from the non-organogenic and organogenic calli

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean squares</th>
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</thead>
<tbody>
<tr>
<td>Nicotine contents of the 6-week-old shoot a</td>
<td>Between groups Within groups</td>
<td>5.246282 0.023266</td>
<td>6</td>
<td>6.5138</td>
<td>789.206</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>5.269548</td>
<td>21</td>
<td>0.87438</td>
<td></td>
</tr>
<tr>
<td>Nicotine, hyoscyamine and scopolamine contents of the 6-week-old shoot b</td>
<td>Between groups Within groups</td>
<td>8.33E-06 6.57E-07</td>
<td>2</td>
<td>2.93E-06</td>
<td>57.03311</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>8.98E-06</td>
<td>9</td>
<td>4.16E-06</td>
<td></td>
</tr>
<tr>
<td>Nicotine, hyoscyamine and scopolamine contents of the 9-week-old shoot c</td>
<td>Between groups Within groups</td>
<td>0.003062 4.11E-07</td>
<td>2</td>
<td>0.001531</td>
<td>33556.5</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0.003062</td>
<td>9</td>
<td>4.56E-08</td>
<td></td>
</tr>
</tbody>
</table>

* : P < 0.001

a : Shoots were differentiated from the non-organogenic calli induced in the MS basal medium supplemented with 7 selected cytokinin / auxin combinations (see Table 6.1)

b : Shoots were differentiated from the non-organogenic calli induced in the MS basal medium supplemented with BA10⁻⁵M + NAA10⁻⁵M

c : Shoots were differentiated from the organogenic calli induced in the MS basal medium supplemented with BA10⁻⁵M + IBA10⁻⁵M

### 6.3.1.4 Root Culture from the Non-organogenic and Organogenic calli

Alkaloid contents of the roots cultured from the non-organogenic and organogenic calli grown on the BM supplemented with the selected cytokinin and auxin combinations are summarised in Table 6.4. All the 3 alkaloids i.e., nicotine, hyoscyamine and scopolamine were detected in the roots regenerated from both the non-organogenic as well as organogenic calli. Nicotine, hyoscyamine and scopolamine contents of the roots regenerated from the organogenic calli were higher than in those regenerated from the non-organogenic calli (Table 6.4).
The m/z values obtained are given in Sections 6.3.4.2.3 i and j respectively (page 284). Figure 6.10 a-d (page 285-286) shows a typical GC-MS total ion chromatogram and mass spectra for nicotine, hyoscyamine and scopolamine respectively detected in the 4-week-old roots regenerated from the organogenic calli induced in the BM supplemented with BA10^{-6} M + IBA10^{-5} M.

**TABLE 6.4** Alkaloid contents* of the 4-week-old roots regenerated from the non-organogenic and organogenic calli induced on the leaf explant of *Dubreisia myoporoides* R. Br. incubated on the semi-solid MS basal medium supplemented with the selected cytokinin and auxin combinations

<table>
<thead>
<tr>
<th>Types of calli and cytokinin and auxin combinations used at the induction stage of the calli</th>
<th>Alkaloid contents (mg g dry weight)^{-1} **</th>
<th>Nicotine</th>
<th>Hyoscyamine</th>
<th>Scopolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-organogenic calli</td>
<td>0.086 ± 0.004</td>
<td>0.079 ± 0.001</td>
<td>0.0373 ± 0.0003</td>
<td></td>
</tr>
<tr>
<td>BA10^{-7}M+NAA10^{-6}M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organogenic calli</td>
<td>0.091 ± 0.003</td>
<td>0.082 ± 0.003</td>
<td>0.0482 ± 0.0006</td>
<td></td>
</tr>
<tr>
<td>BA10^{-6}M+IBA10^{-5} M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: Average of 4 determinations

**: Mean ± Standard deviation

A significantly higher (P < 0.001) amounts of nicotine, hyoscyamine and scopolamine were produced in the 4-week-old roots cultured in the BM supplemented with BA10^{-6} M + IBA10^{-5} M than in the 9-week-old shoot differentiated from the organogenic calli induced in the BM supplemented with BA10^{-5} M+IBA10^{-7} M (Table 6.5).
TABLE 6.5  Summary analysis of $t$ test of alkaloid contents in the 4-week-old root cultured in the BM supplemented with BA10$^{-6}$M + IBA10$^{-5}$M and 9-week-old shoot differentiated from the organogenic calli induced in the BM supplemented with BA10$^{-5}$M + IBA10$^{-7}$M

<table>
<thead>
<tr>
<th>Sample</th>
<th>Degrees of freedom</th>
<th>$t$ Stat $^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>6</td>
<td>49.24626</td>
</tr>
<tr>
<td>Hyoscyamine</td>
<td>6</td>
<td>23.74704</td>
</tr>
<tr>
<td>Scopolamine</td>
<td>6</td>
<td>128.1202</td>
</tr>
</tbody>
</table>

$^*$: $P < 0.001$

6.3.2  Alkaloid Production in the Suspension Culture

Nicotine, hyoscyamine and scopolamine contents in the cultured organs grown in suspension culture are summarised below.

6.3.2.1 Shoot Culture in Suspension

Alkaloid analysis results of different shoot culture samples i.e., cell aggregates with or without shoot initiation and suspension culture medium are summarised in Table 6.6. Only nicotine was detected in the cell aggregates with shoots cultured in suspension. Typical GC-MS total ion chromatogram and mass spectrum for nicotine are presented in Fig. 6.11 a and b respectively (page 288). The m/z values obtained are given in Section 6.3.4.2.3 k (page 287). GC-MS analysis showed the absence of tropane alkaloids i.e., hyoscyamine and scopolamine in the cell aggregates with shoots cultured in the suspension medium. No alkaloids were detected in the cell aggregates without any shoot initiation and in the suspension culture medium (Table 6.6).
**TABLE 6.6** Alkaloid contents* in cell aggregates, suspension medium and shoots of *Duboisia myoporoides* R. Br. cultured in the MS basal suspension medium supplemented with BA$10^{-5}$M +IBA$10^{-7}$M incubated for 3 weeks

<table>
<thead>
<tr>
<th>Sample analyzed</th>
<th>Alkaloid contents (mg (g dry weight)$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nicotine</td>
</tr>
<tr>
<td>Cell aggregates without any shoot initiation</td>
<td>ND</td>
</tr>
<tr>
<td>Cell aggregates with shoot</td>
<td>0.062 ± 0.002**</td>
</tr>
<tr>
<td>Suspension medium</td>
<td>ND</td>
</tr>
</tbody>
</table>

*: Average of 4 determinations;

**: Mean ± Standard deviation;

ND: Not detected.

### 6.3.2.2 Root Culture in Suspension

Alkaloid analyses results for the different root culture samples are summarised in Table 6.7. All the 3 alkaloids i.e., nicotine, hyoscyamine and scopolamine were detected in the 4-week-old roots cultured in the B5 suspension medium supplemented with IBA25 x $10^{-6}$M. The m/z values obtained are given in Section 6.3.4.2.3 1 (page 287).
TABLE 6.7  Alkaloid contents* in the root culture medium and roots of *D*ubo*isia* *myoporoides* R. Br. cultured in the B5 basal suspension medium supplemented with IBA25 x 10^-6 M and incubated for 4 weeks

<table>
<thead>
<tr>
<th>Sample analyzed</th>
<th>Alkaloid contents mg (g dry weight)^-1 **</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nicotine</td>
</tr>
<tr>
<td>Cell aggregates with root initiation</td>
<td>0.054 ± 0.003</td>
</tr>
<tr>
<td>Suspension medium</td>
<td>ND</td>
</tr>
</tbody>
</table>

* : Average of 4 determinations;
**: Mean ± Standard deviation;
ND: Not detected.

6.3.3  **Alkaloid Contents in the Mature Plant Materials**

Alkaloid analyses results of the mature plant materials are summarised in Table 6.8. Alkaloid contents of the differentiated organs regenerated from the non-organogenic and organogenic calli cultured in the semi-solid media as well as in the organs cultured in the suspension media were compared with those of the mature plant.
<table>
<thead>
<tr>
<th>ND</th>
<th>Root</th>
<th>Suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.038 ± 0.002</td>
<td>0.034 ± 0.003</td>
</tr>
<tr>
<td>NDF</td>
<td>0.062 ± 0.002</td>
<td>0.062 ± 0.002</td>
</tr>
</tbody>
</table>

**Table 6.8**  
Alkaloid contents in the mature plant materials and in the organs differentiated from the non-organogenic and organogenic calli.  

ND: Not detected  
Sample collected from Mount Aman Botanic Gardens  
Two-year-old plant prepared from cuttings (Section 7.3)  
Avrage of 4 determinations = *
The amount of nicotine in the leaves collected from the greenhouse-grown plants and the Mount Annan Botanic Gardens was lower than that in the roots. On the other hand, the amounts of tropane alkaloids i.e., hyoscyamine and scopolamine were higher in the leaves than that in the roots (Table 6.8). Nicotine and tropane alkaloid contents of the shoots (without any root formation) differentiated from the non-organogenic and organogenic calli cultured in the semi-solid media were lower than those in the roots (without any aerial organs) (Table 6.8). Nicotine content of the shoots cultured in suspension was higher than that in the roots. The amount of nicotine in the roots cultured in suspension was lower than that in the roots cultured in the semi-solid medium (Table 6.8).

Hyoscyamine content of the roots cultured in suspension was higher than that in the roots cultured in the semi-solid medium (Table 6.8). Scopolamine content of the roots cultured in suspension was higher than that in the roots regenerated from the non-organogenic calli, but lower than that in the roots regenerated from the organogenic calli (Table 6.8).

6.3.4 Gas Chromatography

Typical GC-FID and GC-MS chromatograms obtained for mixed standard and different samples are presented below.

6.3.4.1 Screening of the Alkaloids

Typical chromatograms obtained for the mixed standard alkaloids (nicotine, hyoscyamine, scopolamine and homatropine) and Daboistia myoporoides leaf (from Mount Annan Botanic Gardens) extracts are shown in Fig. 6.1a and b respectively. The retention times (R_t) are shown above the peaks. The retention times (R_t) for hyoscyamine and scopolamine found in the leaf extract collected from Mount Annan
**Text-Figure 6.1.** Typical chromatograms of alkaloid mixture analysed by using GC-FID method described in Section 6.2.5.1, using a BP1 column (SGE BP1 capillary column : 25m x 0.53mm, 1μm film thickness) with N₂ carrier gas. Temperature program: 83°C for 3.5 min rising to 220°C at 30°C/ min. Detector and injector temperature set at 240°C. 

a) Mixed standard solution of nicotine (Rₜ = 5.34 min), atropine (Rₜ = 9.25 min), scopolamine (Rₜ = 10.19 min) and internal standard homatropine (Rₜ = 8.59 min). 

b) leaf extract of mature *Duboisia myoporoides* R. Br. plant collected from Mount Annan Botanic Gardens. Atropine (Rₜ = 9.16 min) and scopolamine (Rₜ = 10.02 min).
Botanic Gardens (Fig. 6.1b) compare well with those of the mixed standard (Fig. 6.1a) indicating their presence. A sample peak with retention time matching that of nicotine was observed but its presence could not be confirmed because it was too small.

6.3.4.2 Confirmation of the Alkaloids

Confirmation of the presence of nicotine, hyoscyamine and scopolamine in the samples was achieved by comparing with the retention times (Rt) of the standards and their mass spectra. The following characteristic ions and m/z’s for nicotine: 162, 161 133, 119, 92 85 and 84, for hyoscyamine (or atropine): 289, 140, 124, 96, 94, 92, 83, 82 and 67, and for scopolamine: 303, 207, 154, 138, 108 and 94.

6.3.4.2.1 The ions and relative abundance values from literature and NIST 62 library

a) Literature

The ions and their relative abundance m/z (rel. int.) values for nicotine, hyoscyamine and scopolamine obtained from the analysis of *D. myoporoides* cultured roots (Yukimune *et al.*, 1994d) are;

Nicotine: 162 [M⁺] (21), 84 (100), 133 (42), 161 (19)
Hyoscyamine: 289 [M⁺] (14), 124 (100), 140 (7), 96 (8), 94 (24)
Scopolamine: 303 [M⁺] (30), 94 (100), 154 (33), 138 (77), 137 (42), 108 (49)

The ions and their relative abundance m/z (rel. int.) values for nicotine, hyoscyamine and scopolamine obtained from the analysis of the *D. myoporoides* and *D. leichhardtii* cultured roots (Kitamura *et al.*, 1991) are;

Nicotine: 162 [M⁺] (23), 161 (14), 133 (22), 119 (6), 85 (6), 84 (100)
Atropine: 289 [M⁺] (23), 140 (9), 124 (100), 94 (17), 83 (25), 82 (24)
Scopolamine: 303 [M⁺] (55), 154 (42), 138 (86), 136 (34), 108 (49), 94 (100)
b) NIST 62 library

The ions and their relative abundance values for nicotine, hyoscyamine and scopolamine obtained from the NIST 62 library are m/z (rel. int.)
Nicotine : 162 [M⁺] (28), 84 (100), 161 (19), 133 (32), 19 (8), 85 (8)
Hyoscyamine : 289 [M⁺] (6), 124 (100), 140 (5), 96 (13), 94 (21), 83 (16), 82 (11), 67 (16)
Scopolamine : 303 [M⁺] (40), 94 (100), 154 (43), 138 (73), 108 (49)

6.3.4.2.2 GC-MS total ion chromatogram and mass spectra of the mixed standards

The retention times (Rš) for nicotine, hyoscyamine, scopolamine and homatropine were 10.79 min, 19.66 min, 20.85 min and 18.37 min respectively (Fig. 6.2a). A typical GC-MS total ion chromatogram and mass spectra for the mixed standard containing nicotine, hyoscyamine, scopolamine and homatropine are shown in Fig. 6.2. a-e respectively. Typical results obtained are m/z (rel. int) ;
Nicotine : 162 [M⁺] (14), 84 (100), 161 (15), 133 (29), 119 (7), 92 (6), 85 (7)
Hyoscyamine : 289 [M⁺] (7), 124 (100), 140 (8), 96 (10), 94 (21), 92 (21), 83 (23), 82 (25), 67 (11)
Scopolamine : 303 [M⁺] (21), 94 (100), 207 (13), 154 (30), 138 (74), 108 (47)
Homatropine : 271 [M⁺] (14), 124 (100), 83 (29), 82 (38), 67 (16), 36 (40)

6.3.4.2.3 GC-MS total ion chromatogram and mass spectra of the cultured samples

The total ion chromatogram and MS spectra of the alkaloids present in the different analysed samples are presented as follows:
Text-Figure 6.2a Typical GC-MS total ion chromatogram obtained from analysis of the mixed standard alkaloid mixture. Column: SGE BPX5 (30m x 22mm, 0.25μm film thickness) (for analysis details see Section 6.2.5.2). Nicotine (Rt = 10.79 min), hyoscymine (Rt = 19.66 min), scopolamine (Rt = 20.85 min) and homatropine (Rt = 18.37 min).

Text-Figure 6.2b Mass spectrum of nicotine
Text-Figure 6.2c  Mass spectrum of hyoscymine

Text-Figure 6.2d  Mass spectrum of scopolamine

Text-Figure 6.2e  Mass spectrum of homatropine
a) Standardization of plant regeneration

The retention times ($R_t$) for nicotine (10.80 min) and hyoscyamine (19.62 min) in the sample (Fig. 6.3 a) were within ± 0.02 min of those in the mixed standard analyzed on the same day. The m/z values and their relative abundances were comparable to those of the literature and NIST 62 library (Section 6.3.4.2.1 a and b). A typical GC-MS total ion chromatogram and mass spectra for the alkaloids present in the sample are presented in Fig. 6.3. a - c. Typical results obtained are: MS m/z (rel.int):

Nicotine: 162 [M$^+$] (15), 84 (100), 161 (12), 133 (20), 92 (6)
Hyoscyamine: 289 [M$^+$] (15), 124 (100), 140 (7), 83 (10), 82 (20), 67 (11)

b) Eleven-week-old non-organogenic calli induced in the BM supplemented with BA10$^{-4}$M + NAA10$^{-4}$M

The retention times ($R_t$) for nicotine (10.82 min), hyoscyamine (19.68 min) and scopolamine (20.84 min) in the sample were within ± 0.01 min of those in the mixed standard analyzed on the same day. The m/z values and their relative abundances were comparable to those of the literature and NIST 62 library (Section 6.3.4.2.1 a and b). Typical results obtained are: MS m/z (rel. int)

Nicotine: 162 [M$^+$] (16), 84 (100), 161 (17), 133 (26), 92 (8)
Hyoscyamine: 289 [M$^+$] (7), 124 (100), 82 (23), 67 (12)
Scopolamine: 303 [M$^+$] (26), 94 (100), 154 (43), 138 (83), 108 (62)

c) Six-week-old shoots differentiated from the non-organogenic calli induced in the BM supplemented with 2,4-D10$^{-7}$M

The retention time ($R_t$) for nicotine (10.81 min) in the sample was within ± 0.01 min of that present in the mixed standard analysed on the same day. The m/z values and the relative abundances were comparable to those of the literature and NIST 62 library (Section 6.3.4.2.1 a and b). A typical GC-MS total ion
**Text-Figure 6.3a** Typical GC-MS total ion chromatogram obtained from analysis of the 6-week-old shoot (without any root initiation) differentiated for the standardization of plant regeneration. Column: SGE BPX5 (30m x 22mm, 0.25μm film thickness) (for analysis details see Section 6.2.5.2). Nicotine ($R_t = 10.8$ min), hyoscyamine ($R_t = 19.62$ min).

**Text-Figure 6.3b** Mass spectrum of nicotine

**Text-Figure 6.3c** Mass spectrum of hyoscyamine
Text-Figure 6.4a  Typical GC-MS total ion chromatogram obtained from analysis of the 6-week-old shoot (without any root initiation) differentiated from the non-organogenic callus induced in the semi-solid MS basal medium supplemented with 2,4-D10^{-7}M. Column: SGE BPX5 (30m x 22mm, 0.25μm film thickness) (for analysis details see Section 6.2.5.2). Nicotine (Rt = 10.81 min).

Text-Figure 6.4b  Mass spectrum of nicotine
chromatogram and mass spectrum for nicotine present in the sample are shown in
Fig. 6.4 a and b (page 274). Typical results obtained are: MS m/z (rel.int)
Nicotine: 162 [M⁺] (15), 84 (100), 161 (14), 133 (27), 92 (7).

d) Six-week-old shoots differentiated from the non-organogenic calli induced in
the BM supplemented with BA10⁻⁶M + NAA10⁻⁶M

The retention times (Rₜ) for nicotine (10.81 min) (Fig. 6.5.2a), hyoscyamine
(15.58 min) (Fig. 6.5.1a) and scopolamine (20.86 min) (Fig. 6.5.2a) in the sample
were within ± 0.16 min of those in the mixed standards analysed on the same days.
The m/z values and their relative abundances were comparable to those of the
literature and the NIST 62 library (Section 6.3.4.2.1 a and b). A typical GC-MS total
ion chromatogram and mass spectrum for hyoscyamine are presented in Fig. 6.5.1a
and b respectively. A typical GC-MS total ion chromatogram and mass spectra for
nicotine and scopolamine present in the sample are presented in Fig. 6.5.2a-c
respectively. Typical results obtained are: MS m/z (rel.int)
Nicotine: 162 [M⁺] (21), 84 (100), 161 (18), 133 (38), 92 (7)
Hyoscyamine: 289 [M⁺] (9), 124 (100), 140 (10), 83 (24), 82 (32), 67 (12)
Scopolamine: 303 [M⁺] (17), 94 (100), 207 (6), 154 (30), 138 (76), 108 (45)

e) Six-week-old shoots differentiated from the non-organogenic calli induced in
the BM supplemented with BA10⁻⁶M + 2,4-D10⁻⁷M

The retention time (Rₜ) for nicotine present in the sample was 10.82 min (Fig.
6.6a) and was within ± 0.01 min of that in the mixed standard analysed on the same
day. The m/z values and their relative abundances were comparable to those of the
literature and NIST 62 library. A typical GC-MS total ion chromatogram and mass
spectra for nicotine are presented in Fig. 6.6a and b respectively. Typical results
obtained are: MS m/z (rel.int)
Nicotine: 162 [M⁺] (14), 84 (100), 161 (9), 133 (23), 92 (6)
Text-Figure 6.5.1a Typical GC-MS total ion chromatogram obtained from analysis of the 6-week-old shoot (without any root initiation) differentiated from the non-organogenic callus induced in the MS basal medium supplemented with BA10^{-5}M + NAA10^{-5}M. Column: SGE BPX5 (30m x 22mm, 0.25 μm film thickness) (for analysis details see Section 6.2.5.2). Hyoscyamine (Rt = 15.58 min).

Text-Figure 6.5.1b Mass spectrum of hyoscyamine
Text-Figure 6.5.2a Typical GC-MS total ion chromatogram obtained from analysis of the 6-week-old shoot (without any root initiation) differentiated from the non-organogenic callus induced in the MS basal medium supplemented with BA10^{-5}M + NAA10^{-5}M. Column: SGE BPX5 (30m x 22mm, 0.25 µm film thickness) (for analysis details see Section 6.2.5.2). Nicotine (R_t = 10.81 min) and scopolamine (R_t = 20.86 min).

Text-Figure 6.5.2b Mass spectrum of nicotine

Text-Figure 6.5.2c Mass spectrum of scopolamine
Text-Figure 6.6a  Typical GC-MS total ion chromatogram obtained from analysis of the 6-week-old shoot (without any root initiation) differentiated from the non-organogenic callus induced in the semi-solid MS basal medium supplemented with BA $10^{-5}$M + 2,4-D $10^{-7}$M. Column: SGE BPX5 (30m x 22mm, 0.25μm film thickness) (for analysis details see Section 6.2.5.2). Nicotine ($R_t = 10.82$ min).

Text-Figure 6.6b  Mass spectrum of nicotine.
f) Six-week-old shoots differentiated from the non-organogenic calli induced in the BM supplemented with Kin10⁻⁵ M + 2,4-D10⁻⁷ M

The retention time (Rₜ) for nicotine (8.97 min) in the sample was within ± 0.01 min which was similar to that in the mixed standard. The m/z values and their relative abundances were similar to those of the literature and NIST 62 library (Section 6.3.4.2.1 a and b). A typical total ion chromatogram and mass spectrum for nicotine present in the sample are presented in Fig. 6.7 a and b. Typical results obtained are: MS m/z (rel. int)
Nicotine: 162 [M⁺]^+ (13), 84 (100), 161 (12), 133 (22), 92 (7)


g) Nine-week-old shoot differentiated from the organogenic calli induced in the BM supplemented with BA10⁻⁵ M + IBA10⁻⁷ M

The retention times (Rₜ) for nicotine (10.81 min), hyoscyamine (19.64 min) and scopolamine (20.86 min) were within ± 0.01 min, ± 0.02 min and ± 0.02 min of those in the mixed standard respectively analysed on the same day. The m/z values and their relative abundances were comparable to those of the literature and NIST 62 library (Section 6.3.4.2.1a). A typical GC-MS total ion chromatogram and mass spectra for nicotine, hyoscyamine and scopolamine are presented in Fig. 6.8 a-d respectively (page 281-282). Typical results obtained are: MS m/z (rel. int)
Nicotine: 162 [M⁺]^+ (15), 84 (100), 161 (13), 133 (25), 92 (6)
Hyoscyamine: 289 [M⁺]^+ (7), 124 (100), 140 (13), 83 (23), 82 (25), 67 (12)
Scopolamine: 303 [M⁺]^+ (21), 94 (100), 154 (32), 138 (67), 108 (51)

h) Fourteen-week-old calli collected from the base of the shoot differentiated from the organogenic calli induced in the BM supplemented with BA10⁻⁵ M + IBA10⁻⁷ M

The retention time (Rₜ) for hyoscyamine (15.55 min) present in the sample was within ± 0.01 min of that in the mixed standard analysed on the same day. The m/z values and their relative abundances were comparable to those of the literature and NIST 62 library (Section 6.3.4.2.1 a and b). A typical GC-MS total ion
**Text Figure 6.7a** Typical GC-MS total ion chromatogram obtained from analysis of the 6-week-old shoot (without any root initiation) differentiated from the non-organogenic callus induced in the MS basal medium supplemented with Kin10⁻⁴M + 2,4-D10⁻⁵M. Column: SGE BPX5 (30m x 22mm, 0.25 μm film thickness) (for analysis details see Section 6.2.5.2). Nicotine ($R_t = 8.97$ min).

**Text-Figure 6.7b** Mass spectrum of nicotine
**Text-Figure 6.8a** Typical GC-MS total ion chromatogram obtained from analysis of the 9-week-old shoot differentiated from the organogenic callus induced in the BM supplemented with BA10^{-5}M + IBA10^{-7}M. Column: SGE BPX5 (30m x 22mm, 0.25 μm film thickness) (for analysis details see Section 6.2.5.2). Nicotine (R_t = 10.81 min), hyoscyamine (R_t = 19.64 min) and scopolamine (R_t = 20.86 min).

**Text-Figure 6.8b** Mass spectrum of nicotine
Text-Figure 6.8c  Mass spectrum of hyoscyamine

Text-Figure 6.8d  Mass spectrum of scopolamine
Text-Figure 6.9a Typical GC-MS total ion chromatogram obtained from analysis of the 14-week-old calli (collected from the base of the 9-week-old shoot) induced in the semi-solid MS basal medium supplemented with BA10⁻³M+IBA10⁻¹M. Column: SGE BPX 5 (30m x 22mm, 0.25μm film thickness) (for analysis details see Section 6.2.5.2). Hyoscyamine (Rᵣ = 15.55 min).

Text-Figure 6.9b Mass spectrum of hyoscyamine
chromatogram and mass spectrum for nicotine are shown in Fig. 6.9 a and b (page 283). Typical results obtained are MS m/z (rel. int):
Hyoscyamine: 289 [M⁺] (6), 124 (100), 140 (7), 83 (26), 82 (27), 67 (10)

i) Roots regenerated from the non-organogenic calli induced in the semi-solid MS basal medium supplemented with BA10⁻⁵M + NAA10⁻⁵M

The retention times (Rₜ) for nicotine (10.82 min), hyoscyamine (19.63 min) and scopolamine (20.37 min) in the sample were within ± 0.02 min of those in the mixed standard analysed on the same day. The m/z values and their relative abundances were comparable to those of the literature and NIST 62 library (Section 6.3.4.2.1 a and b). Typical results obtained are MS m/z (rel. int):
Nicotine: 162 [M⁺] (14), 84 (100), 161 (13), 133 (24), 92 (6)
Hyoscyamine: 289 [M⁺] (8), 124 (100), 82 (25), 67 (9)
Scopolamine: 303 [M⁺] (24), 94 (100), 154 (45), 138 (81), 108 (60)

j) Roots regenerated from the organogenic calli induced in the BM supplemented with BA10⁻⁵M + IBA10⁻⁵M

The retention times (Rₜ) for nicotine (10.80 min), hyoscyamine (19.65 min) and scopolamine (20.85 min) in the samples were within ± 0.02 min, ± 0.01 min and ± 0.01 min with those in the mixed standard analysed on the same day. The m/z values and their relative abundances were comparable to those of the literature and NIST 62 library (Section 6.3.4.2.1a and b). A typical GC-MS total ion chromatogram and mass spectra for nicotine, hyoscyamine and scopolamine are presented in Fig. 6.10 a-d. Typical results obtained are MS m/z (rel. int):
Nicotine: 162 [M⁺] (14), 84 (100), 161 (13), 133 (24), 92 (6)
Hyoscyamine: 289 [M⁺] (11), 124 (100), 140 (5), 83 (23), 82 (21), 67 (8)
Scopolamine: 303 [M⁺] (26), 94 (100), 207 (21), 154 (42), 138 (91), 108 (63)
Text-Figure 6.10a Typical GC-MS total ion chromatogram obtained from analysis of the 4-week-old root regenerated from the organogenic callus induced in the semi-solid MS basal medium supplemented with BA10⁻⁵M+IBA10⁻⁵M. Column: SGE BPX5 (30m x 22mm, 0.25µm film thickness) (for analysis details see Section 6.2.5.2). Nicotine (Rt = 10.80 min), hyoscyamine (Rt = 19.65 min) and scopalamine (Rt = 20.85 min).

Text-Figure 6.10b Mass spectrum of nicotine
Text-Figure 6.10c  Mass spectrum of hyoscyamine

Text-Figure 6.10d  Mass spectrum of scopolamine
k) Cell aggregates with shoot cultured in the MS basal suspension medium supplemented with BA$10^{-5}$M + IBA$10^{-7}$M

The retention time ($R_t$) for nicotine (10.80 min) in the sample was within ± 0.01 min of that present in the mixed standard analysed on the same day (Fig. 6.11a). The m/z values and their relative abundances were comparable to those of the literature and NIST 62 library (Section 6.3.4.2.1 a and b). A typical GC-MS total ion chromatogram and mass spectrum for nicotine are presented in Fig. 6.11 a and b. Typical results obtained are MS m/z (rel. int):
Nicotine: $162 [M^+](15)$, $84 (100)$, $161 (14)$, $133 (24)$, $92 (7)$

l) Roots regenerated in the B5 basal suspension medium supplemented with IBA$25x10^{-4}$M

The retention times ($R_t$) for nicotine (10.79 min), hyoscyamine (19.63 min) and scopolamine (20.35 min) were within ± 0.01 min, ± 0.01 min and ± 0.02 min of those in the mixed standard respectively analysed on the same day. The m/z values and their relative abundances were comparable to those of the literature and NIST 62 library (Section 6.3.4.2.1 a and b). Typical results obtained are MS m/z (rel. int):
Nicotine: $162 [M^+](16)$, $84 (100)$, $161 (17)$, $133 (28)$, $92 (7)$
Hyoscyamine: $289 [M^+](8)$, $124 (100)$, $82 (24)$, $67 (9)$
Scopolamine: $303 [M^+](24)$, $94 (100)$, $154 (43)$, $138 (81)$, $108 (57)$
Text-Figure 6.11a Typical GC-MS total ion chromatogram obtained from analysis of the 3-week-old cell aggregates with shoot cultured in the MS basal suspension medium supplemented with BA10^-5 M + IBA10^-7 M. Column: SGE BPX5 (30m x 22mm, 0.25μm film thickness) (for analysis details see Section 6.2.5.2). Nicotine (Rt = 10.80 min).

Text-Figure 6.11b Mass spectrum of nicotine
6.4 DISCUSSION

The presence of nicotine, hyoscyamine and scopolamine in the 6-week-old shoots differentiated from the non-organogenic callus induced in the BM supplemented with BA10⁻⁶M + NAA54x10⁻⁵M observed in the standardization of plant regeneration of this study indicates that tropane alkaloids can be produced in the differentiated shoots without root formation. My literature search failed to find any report about the presence of tropane alkaloids in the shoot differentiated by organogenesis without root initiation.

The presence of nicotine, hyoscyamine and scopolamine in the 11-week-old non-organogenic calli induced on the leaf explant grown on the BM supplemented with BA10⁻⁵M + NAA10⁻⁶M was observed in this study. Kitamura et al.,(1985a) reported the presence of nicotine and anabasine in the stem callus of *D. myoporoides* induced on the MS basal medium supplemented with Kin4.65x10⁻⁸M+2,4-D4.5x10⁻⁶ M. However, these authors did not detect any tropane alkaloids in their studies. It appears that some cytokinin and auxin combinations are required for the biosynthesis of nicotine in the non-organogenic callus.

The presence of tropane alkaloids i.e., hyoscyamine and scopolamine in the 11-week-old non-organogenic callus induced in the BM supplemented with BA10⁻⁵ M + NAA10⁻⁶M was also found in this study. Sipple and Friedrich (1975) found atropine and scopolamine in the 15-week-old leaf callus of *D. myoporoides* induced in the MS basal medium supplemented with 2,4-D1.35x10⁻⁵M +15% coconut milk. Yamada and Endo (1984) reported hyoscyamine and scopolamine in the 12-week-old non-organogenic callus induced on the leaf explant of *D. leichhardtii* incubated in the B5 basal medium supplemented with BA5x10⁻⁶M + NAA5x10⁻⁵M. These authors did not give any values for nicotine.

No alkaloids were detected in the 11-week-old non-organogenic and 2-week-old organogenic calli induced in the BM supplemented with the other selected cytokinin and auxin combinations used in this study. Endo and Yamada (1985) found no alkaloid in the non-organogenic calli of the 3 *Duboisia* species induced on
the leaf explant incubated in the Gamborg B5 basal medium supplemented with BA5x10^-6M + NAA5x10^-5M. Lin and Griffin (1992 a) also reported no alkaloid in the non-organogenic callus of Duboisia hybrid induced on the shoot tip and seed calli incubated in the MS basal medium supplemented with BA10^-6M + NAA54x10^-6M. It appears that the presence of alkaloids in the non-organogenic calli depends on factors other than cytokinin and auxin combinations.

Similar results on the presence of alkaloid in the non-organogenic calli induced in the medium supplemented with various cytokinin and auxin combinations were observed in other tropane alkaloid-producing plant species as well. Tropane alkaloids were detected in the callus cultures of Atropa belladonna (Eapen et al.,1978; Sharma and Khanna,1982), Hyoscyamus muticus (Basu and Chand, 1998) and a small amount of tropane alkaloid was detected in the callus cultures of Scoporia japonica and several species of Datura (Tabata et al.,1972). On the other hand, the absence of tropane alkaloid has been reported in the callus cultures of Atropa belladonna (Hamilton et al.,1986). It seems that the presence of tropane alkaloids in the callus culture is not solely dependent on the plant species but may also depend on the physiological and biochemical status of the callus cells which express an unreproducible alkaloid biosynthesis in the callus cultures (Lindsey and Yeoman,1983).

Although reported results showed the presence of pyridine and tropane alkaloids in the non-organogenic calli of various Duboisia species, a consistent relationship between the presence of alkaloids in the calli and cytokinin/auxin combination, explant or basal medium formulation was not found. Further investigation of cytokinin and auxin combinations and medium requirements is necessary to find out the relationship.

No alkaloids were detected in the 2-week-old green calli obtained from the non-organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations and incubated in the BM supplemented with BA0.1x10^-6M+NAA27x10^-6M. Again, tropane alkaloids present in the 11-week-old non-organogenic calli induced in the BM supplemented with BA10^-5M + NAA10^-6M were no longer detected in the green calli. These observations are partially
consistent with previously published results. Yamada and Endo (1984) found tropane alkaloids in the 12-week-old callus of *D. leichhardtii*, induced on the leaf, stem and flower-bud explants, grown on the B5 basal medium, supplemented with BA5x10^{-6}M + NAA5x10^{-5}M. The alkaloids present in the 12-week-old non-organogenic calli were no longer detected in the 16-week-old calli. Dhoot and Henshaw (1977) reported hyoscyamine and scopalamine in the cultured cells of *Hyoscyamus niger* which disappeared after a series of subcultures. It appears that the decline in alkaloid biosynthesis in the non-organogenic calli may be related to organ formation as well as the age of the calli.

No alkaloids were detected in the shoot-buds induced on the non-organogenic (Table 6.1) and organogenic (Table 6.2) calli incubated in the BM supplemented with the selected cytokinin/auxin combinations. These observations are consistent with previously reported results obtained from different *Duboisia* species. Yamada and Endo (1984) reported the absence of alkaloids in the *D. leichhardtii* shoot-buds induced on the leaf, stem and flower-bud calli incubated in the B5 basal medium supplemented with BA10^{-5}M. Lin and Griffin (1992 b) reported the absence of alkaloid in the *D. hybrid* shoot-buds induced on the shoot tip and seed calli incubated in the MS basal medium supplemented with BA22x10^{-6}M. These results indicate that tropane alkaloid biosynthesis in *D. myoporoides* needs more differentiation than that present in the shoot-buds.

Nicotine was detected in the 6-week-old shoots differentiated from the non-organogenic calli induced in the BM supplemented with 2,4-D10^{-7}M, BA10^{-5}M + 2,4-D10^{-7}M or Kin10^{-5}M + 2,4-D10^{-7}M (Table 6.1). These results are consistent with the previously reported results. Kitamura *et al.*, (1985 a) reported the presence of small amounts of nicotine in the shoots differentiated from the non-organogenic callus induced on the stem callus of *D. myoporoides*, incubated in the MS basal medium supplemented with Kin4.65x10^{-8}M+2,4-D4.5x10^{-6}M, without root formation. My results indicate that some cytokinin and auxin combinations and 2,4-D10^{-7}M alone or in combination with 10^{-5}M BA or Kin were sufficient either to express nicotine biosynthesis or cell differentiation (necessary for alkaloid biosynthesis) to occur in the regenerated shoots (without root formation).
My studies detected tropane alkaloids in the 6-week and 9-week-old shoots, differentiated from the non-organogenic (Table 6.1) and organogenic (Table 6.2) calli induced in the BM supplemented with BA10^{-5}M + NAA10^{-6}M and BA10^{-5}M + IBA10^{-7}M respectively without any root initiation. These results are in contrast to those reported for differentiated shoots of Atropa belladonna and Duboisia species regenerated by organogenesis (Saito et al., 1991; Lin and Griffin, 1992b). These authors failed to detect any tropane alkaloid in the shoot differentiated from the non-organogenic calli by organogenesis. My results indicate that, under the conditions used in this study, tropane alkaloid formation is possible in the differentiated shoots without any root initiation. A longer incubation period may have allowed cellular differentiation in the shoots which was adequate for the tropane alkaloid formation without any root initiation.

Higher amounts of hyoscyamine than scopolamine produced in the shoots differentiated in the BM supplemented with BA10^{-5}M + NAA10^{-6}M (Table 6.1) and BA10^{-5}M + IBA10^{-7}M (Table 6.2) indicate two possibilities: Firstly, the activity of the enzyme catalysing hyoscyamine formation is higher than that of the enzyme catalysing the hydroxylation and epoxidation of hyoscyamine to scopolamine. Secondly, the hydroxylation and epoxidation of hyoscyamine to scopolamine, both catalysed by H6H (Section 1.2.7) is affected in the differentiated shoots. A further investigation on manipulation of culture medium composition is needed to find out the answer as well as to increase the conversion rate of hyoscyamine to scopolamine.

The presence of tropane alkaloids in the shoots (without any root formation) differentiated by organogenesis is a novel finding of this study. Basu and Chand (1998) reported the presence of tropane alkaloids in the shoots of Hyoscyamus muticus L. differentiated from the embryogenic calli. It appears that differentiation is necessary for the tropane alkaloid formation in the cultured shoots.

Nicotine was present in the shoots differentiated from the non-organogenic calli induced in the MS basal media supplemented with 2,4-D10^{-7}M, BA10^{-5}M + 2,4-D10^{-7}M and Kin10^{-5}M + 2,4-D10^{-7}M (Table 6.1). On the other hand, tropane alkaloids were present in the shoots differentiated from the non-organogenic (Table 6.1) and organogenic (Table 6.2) calli induced in the BM supplemented with BA10^{-5}.
M + NAA10^{-6}M and BA10^{-5}M + IBA10^{-7}M respectively. It may be that tropane alkaloid formation in the regenerated shoot needs differentiation similar to that in the whole plant whereas nicotine formation does not.

The presence of tropane alkaloids in the differentiated shoots without root initiation depends on the cytokinin and auxin combinations used at the induction stage of the non-organogenic and organogenic calli from which the shoots were differentiated. No such relationship between tropane alkaloid formation and cytokinin/auxin combinations has previously been reported for *D. myoporoides* R. Br. However, Badaoui *et al.*(1996) reported a variation of solasodine contents in the callus culture of *Solanum paludosum* with different cytokinin and auxin combinations. Since tropane alkaloid biosynthesis in *D. myoporoides* is related to differentiation, it appears that some cytokinin and auxin combinations affect cell differentiation in the regenerated shoots which express tropane alkaloid biosynthesis in the regenerated shoots without any root initiation.

An effect of incubation period on the presence of alkaloids in the differentiated shoots was found in this study. No alkaloid was detected in a 4-week-old shoot whereas the 3 alkaloids were found in a 9-week-old shoot differentiated from the organogenic callus induced in the BM supplemented with BA10^{-5}M + IBA10^{-7}M (Table 6.2). It appears that after a longer incubation period, differentiation in the shoots regenerated in the BM supplemented with BA10^{-5}M + IBA10^{-7}M was sufficient for tropane alkaloid biosynthesis without any root initiation. However, further studies should investigate longer incubation periods and other cytokinin and auxin combinations as well as concentrations.

The presence of hyoscyamine and absence of nicotine and scopolamine was observed in the 14-week-old calli collected from the base of the shoots differentiated from the organogenic callus induced in the BM supplemented with BA10^{-5}M + IBA10^{-7}M (Table 6.2). The tropane alkaloid biosynthetic pathway (Section 1.2.7) shows that nicotine is produced early, whereas hyoscyamine and scopolamine form at the end of the biosynthetic pathway. Absence of nicotine and scopolamine in the 14-week-old calli collected from the base of the shoot indicates that biosynthesis of alkaloids did not take place at the base of the shoot embedded in the callus tissues.
from which it originated. The presence of hyoscyamine indicates its translocation from the shoot to the base of the shoot for storage. These results are consistent with those of Kitamura et al., (1996) who reported the recycling of atropine in *D. myoporoides*. Nicotine and scopolamine may have also recycled in the analysed samples but in levels too low to be detected.

Nicotine was present in the 3-week-old cell aggregates with shoots cultured in suspension (Table 6.5) but was absent in the 3-week-old shoot-buds induced on the non-organogenic (Table 6.1) and organogenic (Table 6.2) calli cultured in the semi-solid media. These results indicate that an early alkaloid biosynthesis can be expressed with a change in the physical form of the culture medium. According to Kitamura et al., (1985 a), nicotine biosynthesis is possible in the differentiated shoots. My results show that, under the conditions used in this study, nicotine biosynthesis can be expressed without complete differentiation of the organs. A manipulation of culture environment and medium composition is needed to express the complete biosynthetic pathway in the shoot-buds cultured in suspension.

No alkaloid was detected in the turbid suspension medium (Table 6.5). This result indicates that alkaloid biosynthesised in the shoots was not released into the culture medium. According to Payne et al., (1991), the presence of secondary metabolites in the extracellular medium is due to either true secretion or lysis of the glandular hairs. It appears that nicotine formed in the shoots regenerated in suspension culture of this study is intracellular and not released into the liquid culture medium.

Roots regenerated on the calli cultured in the semi-solid (Table 6.4) as well as in the suspension (Table 6.5) media contained nicotine, hyoscyamine and scopolamine. This is consistent with results of Endo and Yamada (1985) and Kitamura et al., (1985 a). Since the regenerated roots in my study were without any aerial parts, tropane alkaloid biosynthesis took place in the root cells, a result consistent with the earlier studies of Waller and Nowacki (1978) observed on complete plant root and Endo and Yamada (1985) observed on independently regenerated roots.
No alkaloid was detected in the root culture suspension media. This result is in contrast with the results of Endo and Yamada (1985) who reported the release of tropane alkaloids in the suspension media of *D. myoporoides* and *D. leichhardtii* roots cultured in the B5 basal liquid medium supplemented with IBA10^{-5}M. It may be that culture conditions or incubation periods used in the present experiment were not suitable for release of tropane alkaloids in the culture medium.

A lower amount of nicotine was found in the leaves and a higher amount in the root samples collected from Mount Annan Botanic Garden and greenhouse-grown plants. These results indicate that after biosynthesis of nicotine and tropane alkaloids in the root, nicotine remains in the root cells whereas tropane alkaloids are translocated to the aerial organs. Endo and Yamada, (1985) reported a similar selective translocation of alkaloids in *D. leichhardtii* and *D.myoporoides*.

The amount of hyoscymamine and scopolamine analysed in the differentiated shoots was comparable to that in the differentiated roots. These findings demonstrate the value of shoot culture for production of tropane alkaloids. By manipulating the culture environment and medium constituents, the amount of tropane alkaloids can be increased in the differentiated shoots without any root formation.

The GC-MS results of this study show a relationship between organogenesis, differentiation and alkaloid localization in *D. myoporoides* R. Br. cultured tissues. Alkaloid biosynthesis is related to root differentiation but under certain conditions, alkaloid biosynthesis may take place in the differentiated shoots without root initiation. However, adequate differentiation for tropane alkaloid biosynthesis in the cultured shoot is related to the cytokinin and auxin combinations used at the callus induction stage.
Mycorrhization of the
*Duboisia myoporoides* R. Br.
Roots and Alkaloid Contents in
the Leaves of the
Field-Grown Trees
7.1 INTRODUCTION

Roots of most higher plant species live in symbiosis with soil fungi and are mycorrhizal. The most common type of mycorrhiza is the arbuscular mycorrhiza (AM) which usually increase the growth of plants by enhancing nutrient uptake (Smith and Read, 1997). However, there are conflicting reports so far as the occurrence of AM fungal infections in the roots of medicinal plants are concerned. Medicinal plants are regarded as non-mycorrhizal, probably due to the presence of various secondary metabolites (Mohankumar and Mahadevan, 1984). However, roots of field grown garlic, a secondary metabolite producing plant were found to be colonized by AM fungi (Shuja and Khan, 1977). This observation was supported by many recent workers who examined roots of various secondary metabolite producing plants and found them mycorrhizal (Abu-Zeyad et al., 1999; Srivastaba and Basu, 1995; Maheshwaran and Khan, 1994; Ueda et al.1992; Sharma and Roy, 1991; Lakshman and Raghavendra, 1990; Selvaraj and Subramanian, 1990; Rao et al.1989).

Various authors have reported increased alkaloid, flavonoid, essential oils and other secondary metabolites in AM plants compared to the non-mycorrhizal controls (Kape et al.,1992; Volpin et al.,1994). Recently, Abu-Zeyad et al.,(1999) conducted the first correlational study to show the positive effects of AM fungal infection in the roots on the biosynthesis of the secondary metabolite, castanospermine, in the leaves of a native Australian medicinal plant, Castanospermum australe.

The presence of AM fungal infection has also been found in Duboisia myoporoides by Ratti and Janardhanan (1995) in India but no root colonization values as well the mycorrhizosphere physico-chemical characteristics were given by the authors. No study, as far as I am aware of, has been conducted regarding the occurrence, biodiversity and taxonomy of AM fungi in this native Australian plant in Australia. The present study is the first detailed report of AM in this native species.
This study was aimed at investigating the occurrence of AM propagules in the mycorrhizospheres of the field-grown trees of *D. myoporoides* R. Br. and their roots colonization by the AM fungi. It was also aimed at analysing their relationship with AM fungal colonization.

The specific objectives were:

1. to collect roots and mycorrhizosphere soil samples from the field-grown trees of *D. myoporoides*
2. to study the physical, chemical and biological characteristics of the mycorrhizosphere soil samples collected from the field
3. to examine the field collected roots for the presence of AM colonization
4. to recover and identify the AM fungal propagules present in the rhizosphere soil samples
5. to analyze field collected leaves for tropane alkaloid (hyoscyamine and scopolamine) contents
7.2 MATERIALS AND METHODS

7.2.1 Chemicals and Reagents

All chemicals used in this work were obtained from Sigma Chemical Company (USA) and reagents were freshly prepared.

7.2.2 Source of Plant Materials

_Duboisia myoporoides_ R. Br. plant materials were collected from Blue Mountains National Park, 20km from Richmond, NSW (Fig 7.1). The trees in the National park were well established and grown wild in its natural habitat (Fig 7.2). The plant materials were also collected from the small trees cultivated at the Mount Annan Botanical Gardens near the University of Western Sydney Macarthur campus (Fig. 2.1 – 2.3; page 77, 78).

7.2.3 Collection and Preservation of Materials

Mycorrhizosphere samples and plant material including roots were collected and put in polythene bags and within 2-3 hours of collection, stored in the cold room at 4°C till further use.

7.2.3.1 Rhizosphere Soil

Using an auger, 5 rhizosphere samples around 6 different trees were collected at a depth of 20-30cm and about 1 meter away from a 2-3 m tall tree trunks. The six
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**Text-Figure 7.1** Location of *Duboista myoporoides* R.Br. sample collection from Blue Mountains National Park (collection site; near Wheeny creek, arrow), 20 km from Richmond (33°35' S 150°42' E) NSW Australia.

**Fig. 7.2**

**Text-Figure 7.2** *Duboista myoporoides* R.Br. tree (d) grown wild in the natural habitat of Blue Mountains National Park near Wheeny creek Richmond, NSW, Australia.
10 cm deep core samples collected from 1 tree were mixed to form one composite sample for further study.

**7.2.3.2 Root**

The roots were separated from the rhizospheres, washed with tap water to remove the attached soil, cut into 1 cm pieces and fixed in FAA fixative (Appendix 10) till further study.

**7.2.3.3 Leaves**

The alkaloid contents in the air-dried leaves were analyzed for alkaloid contents within 3 days of collection using GC-MS on a SGE BPX5 column (Sections 6.2.3 and 6.2.5.2).

**7.2.4 Physical and Chemical Soil Characteristic**

Moisture content, pH and total P contents of the rhizosphere soil were measured by using the following procedures:

**7.2.4.1 Moisture Content of the Soil**

Fifty gram of the soil sample was dried at 100°C for 30min taken in a pre-weighed evaporating dish. After taking out from the oven, the dish was put in a desiccator and allowed to stand for 30min. The dish with the soil sample was then weighed and again put in the oven for another 30min. This procedure was repeated until a constant weighed was achieved.
7.2.4.2 pH of the Soil

The pH of the soil was determined as described by Reid and Haeslaylo, (1982). Five gram of the soil sample was mixed with 5ml of distilled water and stirred with a glass rod for 5min. The pH of the solution was measured by using a calibrated pH meter (T.P.S. Digital pH meter, TPS PTY LTD. Model No 1852mV, Serial No. FC 17-2.).

7.2.4.3 Total P content of the Soil

The sodium carbonate fusion procedure (Blackmore et al., 1987) was used to determine the total P in the soil samples. The values were determined at 882nm using spectrophotometer (Shimadzu UV-Visible recording spectrophotometer UV-160). The P concentrations were read as P mg (100mL)^1 from a calibration curve (Appendix 19) of the absorbance prepared against the concentrations of the standard solutions.

7.2.5 Biological Soil Characteristics

7.2.5.1 Extraction of Spores from the Soil Sample

The fungal spores present in the soil sample were extracted by a modified (Daniels and Skipper, 1982; Tommerup, 1992) wet sieving and decanting method (Fig. 7.3). The soil sample stored in polyethylene bag was mixed properly and 50 gram of the sample was soaked with 500ml tap water before sieving. The sample was stirred with a glass rod for about 10min and allowed to stand for a few seconds to settle the heavy soil particles. The suspension was decanted through a column of sieves varying from 750μm to 40μm mesh pore diameter. This washing and decanting process was repeated until the water was clear. The materials over
Text-Figure 7.3 The Wet-sieving and decanting technique
(Brundrett et al., 1996)
different sieves were transferred to labelled Petri dishes by using a jet of water and used for microscopic observation. The spores were transferred to microscopic slides. A drop of glycerine was used as mounting medium and covered with a cover slip and sealed with nail polish.

### 7.2.5.2 AM Fungal Infection Assessment

The root pieces were stained as per method of Brundrett et al., (1996). The fixed root pieces were taken out from the fixative and washed several times with tap water to make it free from the fixative. From the clean root pieces, thin roots were selected. They were cut into small pieces and put into a beaker containing enough 10% KOH solution. The beaker was then covered and heated at 90°C in a water bath for 1 hour. The KOH solution was poured off and the root pieces were washed properly in tap water. The pigments present in the root pieces were removed by bleaching in alkaline H₂O₂ solution (Appendix 20) for about 20min at room temperature. The bleached root pieces were put in 1% HCl for 3min. The acidified root pieces were stained with Trypan blue solution (Appendix 20) for a few hours. The stained root pieces were put in lactoglycerol (Appendix 20) for de-staining and storage. The percentage of roots colonized by AM fungi was determined by the root-slide of Giovannetti and Mosse (1980).

### 7.2.6 Alkaloid Extraction

The alkaloids present in the leaves of the field-grown trees collected from 2 different sites were analyzed as described in Chapter 6.
7.3 RESULTS

7.3.1 Field Soil Characteristics

Results are summarised in Table 7.1. The moisture contents of the soil collected from Blue Mountains National Park was lower (13.02%) compared to that collected from the Botanic Gardens (23.8%). The analysis of the mycorrhizosphere soil samples revealed that the pH values ranged 5.3 (Blue Mountains National Park sample) to 4.6 (Mount Annap Botanic Gardens sample). Total P content of the soil from around the roots of the trees grown at the Blue Mountains National Park was higher (25.61 ppm) as compared to that collected from the Mt. Annan Botanic Gardens (9.79 ppm) (Table 7.1)

TABLE 7.1 Moisture contents, pH and total P contents of the mycorrhizospheres of Duboisia myoporoides R. Br. collected from the 2 sites studied (values are means of duplicate analyses).

<table>
<thead>
<tr>
<th>Study site</th>
<th>Moisture content (%) *</th>
<th>pH</th>
<th>Total P (ppm) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue Mountains National Park</td>
<td>13.02 ± 0.01</td>
<td>5.3</td>
<td>25.61 ± 0.15</td>
</tr>
<tr>
<td>Mt. Annap Botanic Garden</td>
<td>23.8 ± 0.14</td>
<td>4.6</td>
<td>9.79 ± 0.01</td>
</tr>
</tbody>
</table>

*: Mean ± standard deviation.
7.3.2 AM Fungal Spores in the Mycorrhizospheres

7.3.2.1 AM Fungal Spore Number

No AM fungal spores were extracted from the mycorrhizosphere samples collected from around the small trees cultivated at the Mt Annan Botanic Garden which contained lower amounts of total P. The mycorrhizosphere soil samples collected from the field-grown mature trees at the Blue Mountains National Park, however, harboured a small population of AM fungal spores [42 (100 g soil)$^{-1}$] (Table 7.2)

7.3.2.2 AM Fungal Spore Morphology and Taxonomy

AM fungal spores ranging in size from small (150 – 200 μm) to large (200 – 380 μm), colour from brown to yellow, with two-layered to composite cell walls and with or without subtending hyphal attachments, were recovered from the mycorrhizospheres of the Blue Mountains National Park collected samples (Fig. 7.4).

Large, singly produced and globose spores of *Acaulospora* spp. Were found in the mycorrhizospheres of field grown trees of *D. myoporoides*. The spores contained oily contents, were sessile and born laterally on the subtending hypha (Fig. 7.4a). The spore wall consisted of two distinct layers, the outermost being continuous with wall of the subtending stalk. A collapsed hyphal terminus or saccule was evident. No sporocurps were recovered.
Text-Figure 7.4  Arbuscular mycorrhizal (AM) fungal spores extracted from the mycorrizospheres of *Diobosia myoporoides* R. Br. trees grown at the Blue Mountains National Park, NSW Australia. (a) *Acaulospora* spp. x 400; (b) *Glomus microcarpum* x 400; (c) *Glomus* spp. x 400 (For description please see text).
The mycorrhizosphere of the field grown trees of *D. myoporoides* also contained a small number of *Glomus* spp., most probably *G. microcarpum*, spores (Fig. 7.4 b). Chlamydospores were yellow to brown colour, 40 – 45 \( \mu \text{m} \) size with one 4 - 5\( \mu \text{m} \) thick composite spore wall and born terminally on the subtending hyphae. No sporocarps were recovered.

A few small, cream to yellow coloured spores without any attachments and containing lipids were also recovered from the mycorrhizosphere samples from the trees of *D. myoporoides* grown at the Blue Mountains National Park (Fig. 7.4c). Based on the spore colour and wall structure, it is probably a *Glomus* spp.

### 7.3.3 Mycorrhizal Status of the Roots

Microscopic examination of the stained root pieces collected from the Mt Annan Botanic Gardens showed that they were non-mycorrhizal whereas those collected from the Blue Mountains National Park were colonized (39%) with both intra and inter-cellular AM fungal hyphae with angular projections and vesicles (Figs. 7.5 & 7.6) indicating that AM fungal infection in secondary metabolite producing *D. myoporoides* is possible under field conditions (Table 7.2). An extensive network of AM fungal hyphae was observed around and on the surface of the roots (Fig.7.6 a-c). No arbuscules were observed in the field collected stained root segments.
Text-Figure 7.5 a, b, c  Intercellular and intracellular fungal hyphae (fh) with angular projections (ap) and terminal vesicle (v) in the root cortices (rc) of the *Dubausta myoporoides* R. Br. tree grown at the Blue Mountains National Park, NSW Australia. (For explanation/description please refer to the text). x 400.
Text-Figure 7.6 a, b, c  Arbuscular mycorrhizal fungal hyphae (fh) around and on the surface of the roots (r) of *Daboecia myoporoides* R. Br. trees grown at the Blue Mountains National Park, NSW Australia. (For explanation please refer to the text). x 400
7.3.4 Alkaloid Contents in the Leaves

The scopolamine content was lower in the leaves collected from the naturally grown older trees of *D. myoporoides* at the Blue Mountains National Park than in those collected from the younger cultivated trees at the Mt. Annan Botanic Gardens (Table 7.2). The leaf samples from the Blue Mountains National Park as well as those from the Mt. Annan Botanic Gardens contained both atropine as well as scopolamine but the amounts were variable. The mature leaves from the 2 – 4 years old shrubs grown at the Mt Annan Botanic Gardens contained higher amounts of both the alkaloids as compared to those in the mature leaves of the field-grown older tree (Table 7.2). Incidentally, the roots from the well fertilized small trees grown at the Mt. Annan Botanic Gardens were devoid of any AM fungal infection and no AM fungal propagules were recovered from their rhizospheres.

**TABLE 7.2** AM Mycorrhizal colonization (%) of the roots, the AM mycorrhizal propagules (100g)\(^{-1}\) mycorrhizosphere soil samples and alkaloid contents in the leaves of the field collected *Duboisia myoporoides* R. Br. samples (values are means of 5)

<table>
<thead>
<tr>
<th>Study site</th>
<th>Mycorrhizal colonization %</th>
<th>AM Fungal spores (100g soil)(^{-1})</th>
<th>Atropine mg (g dw leaf)(^{-1})</th>
<th>Scopolamine mg (g dw leaf)(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue Mountains National Park</td>
<td>39.4 ± 1.14(^*)</td>
<td>42.6 ± 0.89</td>
<td>0.80 ± 0.03</td>
<td>1.12 ± 0.04</td>
</tr>
<tr>
<td>Mt. Annan Botanic Garden</td>
<td>0</td>
<td>0</td>
<td>3.05 ± 0.04</td>
<td>2.25 ± 0.06</td>
</tr>
</tbody>
</table>

\(^*\) : Mean ± standard deviation  
dw : Dry weight
7.4 DISCUSSION

My observations of AM fungal association are the first detailed account of *Duboista myoporoides* R. Br. and confirm the passing reference of Ratti and Janardhanan (1995) in India. The roots of the field-grown trees were found to harbor AM fungal hyphae, vesicles and arbuscules indicating that the secondary metabolite, atropine and scopolamine did not prevent AM fungal colonization. These findings are also consistent with those of the earlier investigators (Srivastava and Basu, 1995; Ueda *et al.*, 1992; Abu-Zeyad *et al.* 1999) who reported the occurrence of AM fungal infections in the roots of many medicinal plants and AM fungal propagules in their rhizospheres.

Our results revealed higher hyphal and vesicular AM fungal infection. These findings are in agreement with those of others (Selvaraj and Subramanian, 1990; Ratti and Janardhanan, 1995; Abu-Zeyad *et al.*, 1999) who also reported mainly vesicular infections in the medicinal plants. Ueda *et al.*, (1992), however, reported both vesicles and arbuscules in the roots of Japanese medicinal plants. Although no AM fungal infection was found in the roots of the cultivated trees of *D myoporoides* in the present study, Maheswaran and Khan (1994) reported lipid containing vesicles and arbuscules in the native insecticidal plants cultivated at the Mt. Annan Botanic Gardens, Sydney. Roots of the trees grown at the Blue Mountains National Park were heavily pigmented with phenolic and other secondary metabolites and required bleaching. This may have reduced the subsequent staining of the delicate hyphal branches of the arbuscles, explaining the absence of arbuscular infection observed.

A significant feature arising from my study reporting the absence of AM fungal infection in the roots and AM fungal propagules in the rhizospheres of *D. myoporoides* plants cultivated at the Mt. Annan Botanic Gardens, indicates that, probably, the moist habitat inhibited mycorrhization and sporulation of AM fungi. Khan (1993) noted a positive correlation between redox potential values and AM fungal infection levels in the aquatic trees of Australia. Many researchers (Khan, 1972; Allsopp and Stock, 1994; Chandrashekara *et al.*, 1995) have reported a
decrease or absence of AM fungal infection in the roots of plants with increasing P in their rhizospheres.

My results of the mycorrhizosphere soil analysis of *D. myoporoides* showed that the soil pH was mildly acidic. This observation is consistent with those of Ueda *et al.*, (1992) and Abu-Zeyad *et al.*, (1999) who also found the pH values in the mycorrhizospheres of the medicinal plants they studied ranged from 4.2 to 5.9.

Most of the AM fungal spores, recovered from the mycorrhizospheres of the field grown trees of *D. myoporoides* studied, belonged to the *Glomus* spp. On the contrary, Ratti and Janardhanan (1995), the only report of AM fungal infection in *D. myoporoides*, did not recover any *G. mosseae* spores associated with the mycorrhizospheres of this native Australian tree cultivated in India. Abu-Zeyad (1995) recovered *Glomus fasciculatum, G. claroideum* and *G. mosseae* from the mycorrhizospheres of the secondary metabolite producing and Australian endemic trees of *Castanospermum australe* grown around Sydney. Whereas, researchers elsewhere have recovered AM propagules belonging to many different genera including *Glomus* spp., in the rizospheres of other medicinal plants (Ueda *et al.*, 1992; Sullia and Sampath, 1990; Ratti and Janardhanan, 1995).

In the present study, leaves collected from the mycorrhizal trees grown at the Blue Mountains National Park contained lower amounts of tropane alkaloids as compared to those collected from the non-mycorrhizal small trees cultivated at the Mt. Annan Botanic Gardens. These observations are in contrast with those of many workers who found that AM infection significantly changes the chemical constituents of the plant tissue. Baltruschat and Schonbeck (1975) reported the effect of AM infection on increasing arginine accumulation in tobacco. A report by Morandi and co-workers (Morandi *et al.*, 1984; Morandi and Gianinazzi-Pearson, 1986) found an increase in the concentration of isoflavonoids in AM vs non-AM soybeans. Flavonoid contents in alfalfa roots were increased by AM fungal infection (Volpin *et al.*, 1994). Peipp *et al.*, (1996) suggested that there is a correlation between the continuous accumulation of secondary metabolites in the roots of some Poaceae members and the establishment of the AMF in these plants.
Role of AM fungal infections in the medicinal plants has yet to be elucidated. There is a need for more basic and practical information if mycorrhizal fungi are to be fully utilized into enhancing secondary metabolite production by these plants.

My studies also revealed that tropane alkaloid contents in the leaves from the naturally grown older trees of *D. myoporoides* at the Blue Mountains National Park were lower than those from the younger cultivated trees at the Mt. Annan Botanic Gardens. These observations are in contrast to those of the other researchers (Nash *et al.*, 1988; Donaldson *et al.*, 1990) who reported higher alkaloid contents in the mature leaves than those in the young ones. Future work could involve correlation studies to investigate the effects of mycorrhization on alkaloid biosynthesis.
General Discussion, Conclusions and Future Investigations
8.1 GENERAL DISCUSSION

This study reports the relationship between organogenesis, differentiation and alkaloid localization in Duboisia myoporoides R. Br. The effect of cytokinin/auxin combinations used at the callus induction stage on differentiation in the cultured organs and the presence of tropane alkaloids in the differentiated shoots, regenerated by organogenesis, without any root formation is a novel finding of this study.

Two different types of calli i.e., non-organogenic and organogenic were induced on the leaf explant incubated in the semi-solid MS basal medium supplemented with 65 different (including control) cytokinin and auxin combinations. These results are consistent with the previous results reported by other researchers observed in different alkaloid-producing as well as plant species which do not produce alkaloid (Chapter 2).

Histochemical analysis of the 11-week-old non-organogenic calli induced in the MS basal medium supplemented with 7 different cytokinin/auxin (selected from 65 different cytokinin/auxin combinations) combinations showed the presence of meristemoids i.e., regions of active cell division. These observations are consistent with those of Thorpe (1980).

Alkaloid colour reagents did not localize alkaloid in the meristemoid regions or outside the meristemoid regions of the 11-week-old selected non-organogenic calli induced with 7 different cytokinin/auxin combinations. It may be that either 1) the cell arrangement in the non-organogenic calli was not suitable for the expression of alkaloid biosynthesis or 2) alkaloid was produced in the calli but the amount was not identifiable by the alkaloid colour reagents used.

According to GC-MS spectra, no alkaloid was detected in the 11-week-old non-organogenic calli induced with 7 different selected cytokinin/auxin combinations except BA10^-5M+NAA10^-6M, which contained small amounts of nicotine, hyoscyamine and scopolamine. However, when shoot-buds were induced
in these calli the alkaloid was no longer detected. Yamada and Endo (1984) also
detected tropane alkaloids in the 11-week-old non-organogenic calli of *D.
leichhardtii* incubated in the B5 basal medium supplemented with BA10^{-6}M+
NAA10^{-5}M which disappeared after 16 weeks. It may be that some cytokinin and
auxin combinations cause unstable alkaloid formation in the non-organogenic calli
cells.

Histochemical analysis of the 2-week-old shoot-bud producing organogenic
calli induced in the MS basal medium supplemented with 2 different cytokinin and
auxin combinations (selected from 4 different combinations and concentrations of
cytokinins and auxins) detected an area where cells were organised. Similar areas
with organised cells were also found in the 2-week-old root-producing organogenic
calli. It appears that some cytokinin and auxin combinations as well as
concentrations can organise cells at an early stage of culture.

Alkaloid colour reagents did not localise alkaloids in or outside the region of
the organised cells in the 2-week-old shoot-bud and root-producing organogenic
calli. These results indicate that cell organisation in the shoot-bud and root
producing organogenic calli was not suitable for alkaloid formation.

Cytokinin and auxin combinations BA10^{-5}M+NAA10^{-6}M and BA10^{-5}M+
IBA10^{-7}M caused both shoot and root organogenesis from the non-organogenic and
organogenic calli. Endo and Yamada (1985) reported shoot-bud and root induction
from the *D. myoporoides* calli induced with BA5×10^{-6}M+NAA5×10^{-5}M. These
results indicate that some cytokinin and auxin combinations are optimal to cause
organogenesis in *D. myoporoides*.

No alkaloid was detected by GC-MS in the shoot-buds regenerated from the
non-organogenic and organogenic calli. These results are consistent with those of
other researchers for different *Duboisia* species (Yamada and Endo,1984; Endo and
Yamada,1985; Lin and Griffin,1992b). It appears that the less differentiated state is
not suitable for alkaloid formation in *D. myoporoides*. 
Shoots with more and longer leaves and elongated basal stems were obtained when the non-organogenic callus induction medium was supplemented with BA10^{-5} M+NAA10^{-6}M. Moreover, shoot regeneration was optimal when the callus induction medium contained low concentrations of NAA. A similar effect of cytokinin/auxin (used in the induction medium) on shoot differentiation of Aeschynomene americana, A. falcata, A. fluminensis, A. sensitiva, A. villosa was reported by Rey and Mroginski (1996). They proposed that shoot differentiation depended on the callus induction medium and regeneration was highest when the callus induction medium contained a relatively low concentration of NAA. Although cytokinin and auxin interaction plays a role in the shoot regeneration from the non-organogenic calli, my results indicate an effect of auxin used at the callus induction stage on shoot differentiation. NAA appears the most effective of all auxins used in this study for induction of non-organogenic calli.

Basal stem elongation of the 6-week-old shoots differentiated from the non-organogenic calli and 9-week-old shoots differentiated from the organogenic calli was variable in extent and related to the cytokinin/auxin combinations used at the calli induction stage. Whereas, an elongated basal stem (hypocotyl) was observed in the seedlings produced from the seeds in the greenhouse-grown plants produced from cuttings. Flygh et al. (1998) also reported variation in the basal stem elongation in the differentiated shoots depending on the cytokinin and auxin combinations and an elongated basal stem (hypocotyl) in the seedlings of Pinus contorta. It appears that the synergistic effect of cytokinins and auxins caused various basal stem elongation in the differentiated cultured shoots.

An elongated basal stem equivalent to that in the seedlings was observed in the differentiated shoots when the calli induction media were supplemented with either BA10^{-5}M+NAA10^{-6}M or BA10^{-5}M+IBA10^{-7}M. However, less elongated basal stems were observed in the shoots differentiated from the non-organogenic and organogenic calli induced with the other selected cytokinin and auxin combinations. Morholt and Brandwein (1986) reported that during seed germination, auxin causes elongation and cytokinin stimulates cell division in the embryo. My results showed that, during shoot differentiation in culture, 2 of 9 cytokinin and auxin combinations
caused elongation of the basal stem of the differentiated shoots similar to that of seedlings.

Histological study of the basal stem sections of the shoots differentiated from the selected non-organogenic and organogenic calli indicates a relationship between the cytokinin and auxin combinations and the pattern of xylem differentiation during 6 and 9 weeks incubation periods respectively. Cytokinin and auxin combinations BA10⁻⁵M+NAA10⁻⁶M and BA10⁻⁵M+IBA10⁻⁷M used for induction of the non-organogenic and organogenic calli respectively caused formation of the same pattern (with large and small vessels in the secondary xylem) of xylem in the differentiated shoots (without root formation). The pattern of xylem formed in the 6 and 9-week-old shoots differentiated from the non-organogenic and organogenic calli was like that of the mature plant. The other cytokinin/auxin combinations used were either not suitable for cell differentiation in the shoots similar to those of the complete plant without root induction (complete plant regeneration). Alternatively, the shoots need further incubation for cell differentiation (without root formation) to be comparable to the shoots of the complete plant.

Histological study of the basal stem sections of the shoots differentiated from the selected non-organogenic and organogenic calli indicates a relationship between basal stem elongation and various patterns of xylem formation in the vascular regions. Vascular regions in the basal stems of a complete plant shoot have large and small xylem vessels in the secondary xylem. My results showed that “normal” (i.e., equivalent to shoots from seedlings) elongation of the basal stem took place where well differentiated xylem cells with large vessels in the secondary xylem were present.

Alkaloid colour reagents localized alkaloids in the basal stem sections of the shoots (without any root formation) differentiated from the non-organogenic and organogenic calli where large vessels in the secondary xylem were present. However, no alkaloid was localized in the basal stem sections of the shoots differentiated from the non-organogenic and organogenic calli without any large vessel in the secondary xylem. It appears that formation of large vessels in the
secondary xylem may be related to alkaloid accumulation/storage in the differentiated shoots without any root formation.

In some alkaloid-producing plant species, alkaloid transport is mediated by carrier proteins (Warner and Matile, 1985; Matern et al., 1986). A significant alkaloid production occurred when gene expression for alkaloid biosynthesis and transport (which is related to accumulation) took place at the same time (Wink, 1987). Gene expression for both alkaloid biosynthesis and transport takes place in the presence of a suitable storage site where accumulation without degradation occurs (Guern et al., 1987). Since large xylem vessels are dead cells, biosynthesis of enzyme is not possible in those cells. It appears that, biosynthesis of all enzymes of the alkaloid biosynthetic pathway and carrier proteins of alkaloid transport take place in cells other than large xylem vessels.

Nicotine was detected in the 6-week-old shoots differentiated from the non-organogenic calli induced in the MS basal medium supplemented with 2,4-D10^{-7} M, BA10^{-5} M+2,4-D10^{-7} or Kin10^{-5} M+2,4-D10^{-7} M by GC-MS analysis. Kitamura et al., (1985a) reported nicotine in the shoots differentiated from the non-organogenic calli induced on the stem calli of D. myoporoides, incubated in the MS basal medium supplemented with Kin4.5x10^{-6} M+2,4-D4.65x10^{-8} M without any root formation. My results showed that BA or Kin in combination with either 2,4-D or 2,4-D10^{-7} M was required either to express nicotine biosynthesis or stimulate cell differentiation (necessary for alkaloid biosynthesis) to occur in the regenerated shoots.

The presence of nicotine, hyoscyamine and scopolamine in the shoots (without any root formation) differentiated from the non-organogenic and organogenic calli induced induced in the BM supplemented with BA10^{-5} M+NAA10^{-6} M and BA10^{-5} M+IBA10^{-7} M respectively was detected by GC-MS analysis. These results indicate that under the conditions applied in this study, tropane alkaloid can form in the differentiated shoots without any root initiation. It also appears that, formation of nicotine in the regenerated shoots (without any root formation) does not require differentiation in the basal stem like the complete plant. However, tropane alkaloid formation in the regenerated shoots (without root formation) requires differentiation in the basal stem like that in the complete plant. Similarly, analyses
of leaves of differentiated shoots may also detect alkaloid. This work is yet to be done.

Cytokinin and auxin combinations BA10^{-5}M+NAA10^{-6}M and BA10^{-5}M+IBA10^{-7}M caused elongated basal stem, differentiation and alkaloid localization in the cultured shoots whereas other combinations did not. In the complete plant, cytokinin and auxin biosynthesis takes place in the root and shoot cells respectively (Matthysse and Scott, 1984). Again, auxin levels produced in the shoot are related to other PGRs produced in the root (Tal et al., 1979). However, the shoot culture media of this study were supplemented with cytokinin and auxin only. Shoot cultures in this study lacked roots and therefore, the effect of roots in a normal system was absent. After long incubation (6-week and 9-week for shoot differentiation from the non-organogenic and organogenic calli respectively) 2 cytokinin and auxin combinations produce differentiation like the parent plant. Perhaps, the older regenerated shoots were able to supplement the PGRs provided to produce the required differentiation and thereby alkaloid formation like that in the parent plant.

The above view is consistent with comparison of the biosynthetic ability of the seedlings and the regenerated plant. Kitamura et al. (1985b) reported that leaves of the D. myoporoides seedlings contained the largest amounts of alkaloids and the main alkaloids were present throughout the development. On the other hand, leaves of the plantlets regenerated from the callus induced in the BM supplemented with Kin4.5x10^{-5}M+2.4-D4.65x10^{-8}M contained no or few alkaloids and the amounts of alkaloid were extremely low at early stages of development. Later, the contents resembled amounts found in the intact plant.

Alkaloid colour reagents localised alkaloids in the 14-week-old calli collected from the base of the 9-week-old shoots differentiated from the organogenic calli induced in the BM supplemented with BA10^{-5}M+IBA10^{-7}M. However, cell arrangement in the calli was unlike that in the differentiated organs. Since alkaloid formation in the cultured shoot is related to differentiation, it appears that calli cells are used for alkaloid accumulation.

The presence of hyoscymamine and absence of nicotine and scopolamine was detected by GC-MS analysis in the 14-week-old calli collected from the base of the
9-week-old shoots differentiated from the organogenic calli induced in the BM supplemented with BA10^{-5}M+IBA10^{-7}M. Tropane alkaloid biosynthetic pathway shows that nicotine is produced early, whereas hyoscyamine and scopolamine form at the end of the biosynthetic pathway. The presence of hyoscyamine and absence of nicotine in the calli tissues indicates that formation of alkaloids did not take place at the base of the shoot embedded in the callus tissues from which it is originated. The presence of hyoscyamine indicates its translocation from the shoot to the base of the shoot for storage. These results are consistent with those of Kitamura et al. (1996) who reported the recycling of hyoscyamine in D. myoporoides. It appears that calli cells may be used for accumulation and storage of the alkaloids biosynthesised in the differentiated shoots without any root formation. Nicotine and scopolamine may have also recycled in the analysed samples but in levels too low to be detected.

Roots were regenerated from the non-organogenic calli induced in the semi-solid MS basal medium supplemented with BA10^{-5}M+NAA10^{-6}M. These results are consistent with those of Endo and Yamada (1985) who also reported regeneration of roots from the non-organogenic calli induced in the B5 basal medium supplemented with BA10^{-6}M+NAA10^{-5}M. These results indicate that cytokinin and auxin combination BA+NAA is suitable for root induction in the non-organogenic calli.

Roots were regenerated from the organogenic calli induced in the semi-solid MS basal medium supplemented with BA10^{-6}M+IBA10^{-5}M. These results indicate that roots and shoots can be regenerated from the organogenic calli using different concentrations of BA and IBA. However, regeneration of roots on the organogenic calli induced in the semi-solid MS basal medium supplemented with BA10^{-6}M+IBA10^{-5}M has not yet been reported in D. myoporoides.

Histological analysis of the 4-week-old roots regenerated from the organogenic calli indicated a xylem cell arrangement similar to that of the mature plant root, except with fewer xylem cells in the cultured roots compared to the mature plant root. Comparing the incubation period for differentiation in the root (4 weeks) and shoot (9 weeks), it appears that the organization of xylem vessels in the differentiated roots takes place earlier than that in the basal stems of the shoots.
Alkaloid colour reagents localized alkaloids in the xylem cells of the 4-week-old roots regenerated from the organogenic calli. James (1950) also localized alkaloids in the xylem vessels of the mature Atropa belladonna plant roots using alkaloid colour reagents. These results indicate that xylem vessels in the D. myoporoides cultured and mature plant roots may be used for accumulation or storage of alkaloids.

The presence of nicotine, hyoscyamine and scopolamine in the 4-week-old roots regenerated from the non-organogenic and organogenic calli was indicated by GC-MS analysis. Yukimune et al. (1994 a) reported the presence of nicotine, hyoscyamine and scopolamine in the cultured roots of D. myoporoides. Endo and Yamada (1985) reported that tropane alkaloids are present in the cultured roots and absent in the cultured shoot. These results indicate that tropane alkaloid formation in the roots, independently regenerated from non-organogenic and organogenic calli takes place earlier than that in the regenerated shoots. Comparing the incubation period for differentiation and alkaloid biosynthesis in root (4 weeks) and shoot (9 weeks), it appears that the organization of xylem vessels (necessary for alkaloid biosynthesis) and alkaloid biosynthesis in the differentiated roots takes place earlier than in the stems of the differentiated shoots. Earlier xylem vessel organization in the regenerated roots than in the regenerated shoots may account for the early biosynthesis of alkaloids in the former tissue.

Shoots regenerated in the MS basal suspension medium supplemented with BA10^{-5}M+IBA10^{-7}M grew for 4 weeks in shake flasks. Shoot cultures of some plant species are also able to thrive in submerged conditions (Levin et al., 1988). These results indicate that D. myoporoides shoots can be cultured in submerged conditions and the cytokinin and auxin combination BA10^{-5}M+IBA10^{-7}M is optimal for shoot regeneration both in semi-solid and suspension media.

Nicotine was detected by GC-MS analysis in the 4-week-old shoots differentiated from the calli induced in MS basal suspension medium supplemented with BA10^{-5}M+IBA10^{-7}M. However, no nicotine was detected in the 4-week-old shoots differentiated from the organogenic calli induced in the semi-solid MS basal
medium supplemented with BA10^{-5}M+IBA10^{-7}M. These results indicate that nicotine formation takes place earlier in the shoots differentiated in suspension than in the shoots differentiated in the semi-solid medium.

Nicotine, hyoscyamine and scopolamine were detected by GC-MS in the 4-week-old roots regenerated in B5 basal suspension medium supplemented with IBA10^{-5}M. Similar results were also obtained in different tropane alkaloid-producing plant species (Tabata et al., 1972; Endo and Yamada, 1985; Yukimune et al., 1994d).

The leaves of the intact plant contained hyoscyamine and scopolamine but the nicotine content was very low. However, roots of the intact plant contained more nicotine. Again, hyoscyamine and scopolamine contents of the cultured shoot were lower than that of the cultured roots. Endo and Yamada (1985) reported the presence of higher amounts of tropane alkaloids and lower amounts of nicotine in the aerial parts and higher amounts of nicotine in the roots of the intact plant. It appears that the root is the main biosynthetic site for alkaloids and tropane alkaloids are translocated to the aerial parts whereas nicotine remains in the root cells. However, in certain conditions, tropane alkaloid formation may take place in the differentiated shoots without any root formation.

Since an explanation for tropane alkaloid formation in the cultured shoot and root on the basis of cell differentiation is lacking, the following generalizations can be made from the present study:

1) some cytokinin and auxin combinations are optimal for differentiation of xylem in the regenerated shoots without root formation
2) for differentiation of xylem for tropane alkaloid formation in the roots and shoots, cytokinin BA and auxins NAA and IBA are optimal
3) different PGRs or other factors related to xylem differentiation may be mainly present in the root cells
4) xylem differentiation in the root takes place earlier than in the shoot
5) after induction of root in the cultured shoot, various factors related to xylem differentiation are translocated to the shoot and these influence "normal" differentiation of xylem

6) when xylem cells are well differentiated, the basal stem of the cultured shoot elongates as for a seedling

7) tropane alkaloid biosynthesis takes place in the independently regenerated shoots and roots only after proper xylem differentiation in those cultured organs

8) some cytokinin and auxin combinations, optimal to the plant species enhance xylem differentiation in the cultured shoot

9) tropane alkaloid biosynthesis may independently take place in the root and aerial organs of the seedling as well as in the whole plant

My results show that different environmental factors such as light and the chemical factors such as basal media, extra-factors and alkaloid precursors can affect in vitro shoot growth. My literature survey did not reveal relevant studies on D. myoporoides R. Br. Most studies on the effect of environmental factors on secondary metabolite producing shoot cultures have been conducted to increase the amount of secondary metabolites (Hagimori et al., 1983; Mantel and Smith, 1983; Benjamin et al., 1987; Colllin, 1987; Chung and Staba, 1988). However, further investigation on chemical analysis of the shoots grown under various physical and chemical environments will show their effectiveness for the production of tropane alkaloids by shoot culture.

Since this study showed that tropane alkaloid biosynthesis is related to xylem differentiation, tropane alkaloid biosynthesis in the calli with differentiated xylem, cultured in suspension, may allow commercial tropane alkaloid production without the need to differentiate complete plant.
8.2 CONCLUSIONS

This experimental results showed that:

1. A stable tropane alkaloid formation is possible in the cultured shoots of *Duboisia myoporoides* R. Br. without root formation
2. Biosynthesis of tropane alkaloids in the cultured shoot depends on xylem differentiation
3. Differentiation of xylem in the cultured shoot is related to the cytokinin/auxin combinations used at the callus induction stage
4. The amount of hyoscyamine biosynthesised in the cultured shoots without root formation is lower than the amount biosynthesised in the cultured roots
5. Nicotine biosynthesis takes place earlier in shoots cultured in suspension than in semi-solid medium

8.3 FUTURE INVESTIGATIONS

1. Investigate xylem differentiation in the callus using different cytokinin and auxin combinations
2. Shoot culture should be tested for biosynthesis of tropane alkaloids without root formation using different cytokinin/auxin combinations
3. Investigate the enzymes responsible for biosynthesis of tropane alkaloids in the differentiated shoots without root formation
4. Investigate cellular chemistry/biochemistry in relation to alkaloid biosynthesis in the cultured shoots without root formation
5. Optimize culture conditions using various environmental factors for increasing the amount of tropane alkaloids in the differentiated shoots without root formation
6. Study the effect of various environmental factors on the expression of tropane alkaloid biosynthesis in the shoots cultured in suspension.


APPENDICES
APPENDIX 1  Source of *Duboisia myoporoides* R. Br. plant

The plants in Mount Annan Botanic Garden were grown from cuttings collected from a wild plant at Raymond Terrace NSW (Registration No. 273674, special geographical unit: central coast, Hunter Region Botanic Garden) by Peter Cuneo of Mount Annan Botanic Garden (collection no. 94, date of collection 03 Jun 1993, Acc. No. 931439). The cuttings were grown in the green-hose-conditions and then planted on organic grey/black sand on deep sand in the botanic garden natural habitat (bed/area-23). The plants are now growing there as undisturbed understorey shrubs in eucalypt forest. These plants are vigorous and about 3 to 4m high.
### APPENDIX 2  Murashige and Skoog (MS) (1962) plant tissue culture medium (ICN catalogue, 1996)

<table>
<thead>
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<th>Component</th>
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<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>3. Vitamins</td>
<td></td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>100.00</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.50</td>
</tr>
<tr>
<td>Pyridoxine.HCl</td>
<td>0.50</td>
</tr>
<tr>
<td>Thiamine.HCl</td>
<td>0.10</td>
</tr>
<tr>
<td>4. Glycine</td>
<td>2.00</td>
</tr>
</tbody>
</table>
Appendix 3  Measurement of light intensity

LI – COR: Terrestrial Radiation Sensors, Type – SA, Model Number: LI – 200SA
Pyranometer Sensor; Serial Number: PY 13321; LI – COR, inc. / LI COR, Ltd.

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Units Label</th>
<th>Readout Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantum</td>
<td>μmol</td>
<td>μmol s⁻¹m⁻²</td>
</tr>
</tbody>
</table>

1 μmol s⁻¹m⁻² = 1 μE s⁻¹m⁻² = 6.02 x 10¹⁷ photons s⁻¹m⁻²

(LI – COR Radiation Sensors Instruction Manual 1986;
Publication No. 8609 – 56
LI – COR, Lincoln, Nebraska USA)
## APPENDIX 4  UNE #B medium
(de Fossard, 1976)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca (NO₃)₂</td>
<td>2 mmol</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1 mmol</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.5 mmol</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.9 mmol</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>15 μmol</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>1.5 μmol</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>8 μmol</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0.1 μmol</td>
</tr>
<tr>
<td>Na₂MoO₄</td>
<td>0.1 μmol</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>50 μmol</td>
</tr>
<tr>
<td>Na₂ EDTA</td>
<td>50 μmol</td>
</tr>
<tr>
<td>Sucrose</td>
<td>6 mmol</td>
</tr>
<tr>
<td>Agar</td>
<td>8 g</td>
</tr>
</tbody>
</table>
**APPENDIX 5**  Standard error (from %)  
(Freund, 1984)

The quantity \( \sqrt{\frac{p(1-p)}{n}} \) is the standard error of a proportion

Where, \( p = \) proportion; \( n = \) number of replicates

**APPENDIX 6**  Least Significant Difference (LSD)  
(Montgomery, 1991)

\[
\text{LSD} = t_{a/2,N-a} \sqrt{\frac{2 \text{MS}_E}{n}}
\]

Where,
\( n = \) Number of replicates
\( \text{MS}_E = \) Mean square error
\( N = \) Total number of observations
\( a = \) Number of treatments
\( \alpha = 0.05 \) (chosen to show significance at 5%)
APPENDIX 7 Gamborg et al.,(1968) (B5) Plant Tissue Culture Medium (Sigma catalogue, 1995)

<table>
<thead>
<tr>
<th>Component</th>
<th>mg L(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO(_3)</td>
<td>2500.00</td>
</tr>
<tr>
<td>(NH(_4))(_2)SO(_4)</td>
<td>134.00</td>
</tr>
<tr>
<td>NaH(_2)PO(_4)</td>
<td>130.00</td>
</tr>
<tr>
<td>MgSO(_4)</td>
<td>122.09</td>
</tr>
<tr>
<td>CaCl(_2) (anhydrous)</td>
<td>113.24</td>
</tr>
<tr>
<td>FeSO(_4).7H(_2)O</td>
<td>27.85</td>
</tr>
<tr>
<td>Na(_2)EDTA</td>
<td>37.25</td>
</tr>
<tr>
<td>MnSO(_4).H(_2)O</td>
<td>10.00</td>
</tr>
<tr>
<td>ZnSO(_4).7H(_2)O</td>
<td>2.00</td>
</tr>
<tr>
<td>H(_3)BO(_3)</td>
<td>3.00</td>
</tr>
<tr>
<td>KI</td>
<td>0.75</td>
</tr>
<tr>
<td>NaMoO(_4).2H(_2)O</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO(_4).5H(_2)O</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl(_2).6H(_2)O</td>
<td>0.025</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>100.00</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>1.00</td>
</tr>
<tr>
<td>Pyridoxine.HCl</td>
<td>1.00</td>
</tr>
<tr>
<td>Thimine.HCl</td>
<td>1</td>
</tr>
</tbody>
</table>
**APPENDIX 8**  
Equation for Dunnett's test  
(Montgomery, 1991)

The equation for Dunnett’s test is presented as follows:

\[ |\bar{y}_i - \bar{y}_a| > d_\alpha (a - 1, f) \sqrt{MS_E \left( \frac{1}{n_i} + \frac{1}{n_a} \right)} \]

Where,

- \( \bar{y}_i \) = mean of the control
- \( \bar{y}_a \) = mean of each treatment
- \( d_\alpha \) = constant obtained from statistical table
- \( a \) = number of treatments
- \( f \) = number of degrees of freedom for error
- \( MS_E \) = mean square error
- \( n_i \) = number of replicates in control
- \( n_a \) = number of replicates in treatments

**APPENDIX 9**  
Test of correlation coefficient significance  
(Freund, 1984)

\[ Z = \frac{1}{2} \ln \frac{1+r}{1-r} \]

\[ z = Z \cdot \sqrt{n - 3} \]

if \( z < -1.96 \) or \( z > 1.96 \) (significant regions when \( \alpha = 0.05 \))

where,

- \( r \) = correlation coefficient
- \( n \) = number of treatments x number of replicates
APPENDIX 10  Formalin – acetic acid – alcohol (FAA)
(Jensen, 1962)

Ethyle alcohol, 50%  90ml
Glacial acetic acid  5ml
Commercial formalin (40% formaldehyde)  5ml

APPENDIX 11  TBA series for dehydration of tissues
(Jensen, 1962)

The series consists of the following mixtures:
50  50 ml H₂O, 40 ml 95% alcohol, 10 ml TBA
70  30 ml H₂O, 50 ml 95% alcohol, 20 ml TBA
85  15 ml H₂O, 50 ml 95% alcohol, 35 ml TBA
95  45 ml 95% alcohol, 55 ml TBA
100  25 ml absolute alcohol, 75 ml TBA
TBA  100% TBA

The number of the series (50, 70, .......TBA) indicates the total alcohol concentration of each member.
APPENDIX 12  Construction of paper embedding boat
(Jensen, 1962)

APPENDIX 13  Mayer's fixative
(Chamberlain, 1932)

White of egg  50 cc
Glycerin  50 cc
Salicylate of soda  1 g

Shake well and filter through cheesecloth
APPENDIX 14  Warming plate (arrowed)
(Chamberlain, 1932)
**APPENDIX 15**  Hydration and staining of microtome sections  
(Sass, 1958)

Xylene  
2 - 5 min  
(de-waxing)  

↓  
absolute  
(anhydrous)  
alcohol  
2 - 5 min  

↓  
95%  
alcohol  
2 - 5 min  

↓  
70%  
alcohol  
2 - 5 min  

↓  
50%  
alcohol  
2 - 5 min  

↓  
30%  
alcohol  
2 - 5 min  

↓  
distilled water  
2 - 5 min  

↓  
Staining reagent  

↓  
Distilled water  
1 min  

↓  
Tap water  

---  

resin and  
cover glass  

↑  
Xylene III  
5 min  

↑  
Xylene II  
5 min  

↑  
Xylene I  
5 min  

↑  
Carbol xylene  
5 - 10 min  

↑  
absolute alcohol II  
5 - 10 min  

↑  
absolute alcohol I  
5 - 10 min  

↑  
95 % alcohol  
5 - 10 min  

↑  
70 % alcohol  
5 - 10 min  

↑  
50 % alcohol  
5 - 10 min  

↑  
30 % alcohol  
2 - 5 min
APPENDIX 16  Staining reagents for macromolecular constituents
(Chamberlain, 1932; Sidman et al., 1961; O’Brien & McCully, 1981)

1) Methyl green
   1% solution in water

2) Safranin
   1% aqueous solution

3) Toluidine blue O
   0.05% in benzoate buffer (benzoic acid, 0.25 g sodium benzoate, 0.29 g; water, 200 cc) at pH 4.4

APPENDIX 17  Alcoholic tartaric acid solution
(Johansen, 1940)

5% tartaric acid solution in 95% ethanol

APPENDIX 18  Staining reagents for alkaloid localization
(Henry, 1924; James, 1950; Cromwell, 1955; Stevens, 1986)

a) 5% platinic chloride solution (aqueous)

   Platinic chloride  1 g
   Water            20 ml
b) Iodoplatinate

Add 2 ml of a 5% solution of platinic chloride and 5 g of potassium iodide to 98 ml of water and shake until dissolve.

c) Ferric chloride

5% aqueous solution

d) Gold chloride

Dissolve 6.57 g gold metal in 50 ml aqua regia, evaporate to dryness and dissolve the residue in 100ml water

e) Mayer’s reagent

Mercuric chloride 6.8 g L⁻¹
Potassium iodide 25 g L⁻¹

f) Dragendorff’s Reagent

Dissolve 1 g of bismuth subnitrate in 3 ml of 10M hydrochloric acid with the aid of heat, dilute to 20 ml with water, and dissolve in the mixture 1 g of potassium iodide. If black bismuth tri-iodide separates, add 2M hydrochloric acid and more potassium iodide to dissolve it.
g) Wagner's Reagent

0.1 g KI in 10 ml water
0.1 g iodine flake

h) Cyanogen bromide

(i) Decolorise bromine solution by the addition of solid potassium cyanide
    and then add more bromine solution until the solution is pale yellow.
(ii) Prepare a saturated solution of aniline in water.

Mix equal volumes of the 2 solutions immediately prior to the test.
APPENDIX 19  Calibration curve for total phosphorus

\[ \text{WORKING CURVE} \quad \text{CONC.} \quad \text{MAX. CHANGE Y/N} \]

\[ +0.09 \ \text{A} \]

\[ 0.010 \quad \text{(A/DIV.)} \]

\[ +0.00 \ \text{A} \]

\[ 0.0 \quad \text{CONC.} \quad 10.000/\text{DIV.} \quad 50.000 \]

1:20 1/29 '96

882.0 NM  0.078 A
APPENDIX 20  Reagents for AM fungal infection assessment

a) Alkaline H₂O₂

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄OH (concentrated)</td>
<td>3ml</td>
</tr>
<tr>
<td>H₂O₂ (10%)</td>
<td>30ml</td>
</tr>
<tr>
<td>Tap water</td>
<td>567ml</td>
</tr>
</tbody>
</table>

b) Trypan blue stain solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypan blue</td>
<td>0.05g</td>
</tr>
<tr>
<td>Lactoglycerol</td>
<td>100ml</td>
</tr>
</tbody>
</table>

c) Lactoglycerol

Lactic acid : Glycerol : Water 1 : 1 : 1
APPENDIX 21  ABSTRACT


EFFECT OF AUXIN AND / OR CYTOKININ ON THE BIOSYNTHESIS OF ALKALOID IN THE ROOT-LESS SHOOT OF DUBOISIA MYOPOROIDES R. Br. IN TISSUE CULTURE

N. Khanam, Khan, A. G., Khoo, C. Department of Biological Sciences, Faculty of Informatics Science and Technology, University of Western Sydney Macarthur, P. O. Box 555, Campbelltown, NSW, Australia, 2560.

The organogenic response of leaf explant of Duboisia myoporoides R. Br. to single and comibined auxin and cytokinin were studied and correlated to alkaloid formation in regenerated complete shoot in solid and suspension media before root formation. The cultured tissues were analysed by validated analytical method and identification and quantification of nicotine, hyoscyamine and scopolamine were carried out by GC-MS technique. The root-less shoot regenerated from single auxin induced callus were found to contain only nicotine whereas the root-less regenerated from combined auxin and cytokinin via callus formation as well as via bud formation contain 3 mentioned alkaloids. The results indicate that the presence of both auxin and cytokinin is necessary at the induction stage for the expression of the complete biosynthetic pathway in root-less shoot. On analysis of the callus with which the shoot was attached, only hyoscyamine was detected.

The 5 weeks old regenerated shoot-bud from established suspension was found to contain only nicotine. These results indicate that the biosynthesis takes place in the aerial shoot rather than the callus part and the pathway was expressed earlier than in the suspension medium. The presence of 3 mentioned alkaloids in the root-less shoot indicate that under certain condition secondary metabolite biosynthesis can start in the cultured healthy shoot before the formation of the root.

The abstract was selected as a “Hot Topic” silent presentation and was published (# SP – 1029) in In Vitro Cellular and Developmental Biology- Plant vol. 34 (4) (October-December) p – 345.
RELATIONSHIP BETWEEN ORGANOGENESIS DIFFERENTIATION AND HISTOLOCALIZATION OF SELECTED ALKALOIDS IN DUBOISIA MYOPOROIDES R. Br.

by

NURUSSABA KHANAM

B. Sc. (Hons.), M. Sc. (Chemistry), M. Sc. (Biochemistry)
University of Rajshahi, Bangladesh

A thesis presented to the University of Western Sydney Macarthur in partial fulfilment of the requirements for the degree of Doctor of Philosophy

June, 1999
In memory of my
father-in-law and my parents
ACKNOWLEDGMENTS

I would like to express my gratitude and sincere appreciation to my supervisor Dr. A. G. Khan for his guidance and advice during the course of my study and preparation of this thesis. I would like to thank my co-supervisor Dr. Cheang Khoo for his guidance in my analytical work and preparation of this thesis.

I am especially grateful to my husband for his financial support for undertaking my post-graduate study in Australia. I am grateful to the University of Western Sydney, Macarthur for producing facilities and exempting my tuition fees for the last year of my study. I am also grateful to the University of Rajshahi, Bangladesh for granting me study leave.

Special thanks are due to Associate Professor Robert Close and Dr. Mary Campbell for valuable advice and unstinted help in preparation of this thesis.

I should also like to thank the following: Mt. Annan Botanic Gardens staff for the supply of *Duboia myoporoides* samples, Peter Abel of Cobbity Plant Breeding Institute, University of Sydney for help in preparation of *D. myoporoides* cuttings, Dr. Philip Tong of AGAL for performing GC-MS analyses, Michael Balding, Chris Simkin, Pedro Sampaio for scanning and photography and Dr. Heather Winskel of the Learning Center at UWS Macarthur for assisting me with my English expression in the first draft of my thesis.

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I am grateful to my 6-year-old son for his patience during my study. Together with the help above, it would have been very difficult to submit my thesis without the support of my husband. I am very grateful for his love and patience during my study.

Finally, my great thanks to my relatives for their best wishes and encouragement throughout my study.
Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in whole or in part, for a degree at this or any other institution.

NURUSSABA KHANAM
CHAPTER 1 Introduction and Literature Review

1.1 Introduction .................................................................................. 2
1.2 Literature review ........................................................................... 5
  1.2.1 Plants as a source of chemicals ........................................... 5
  1.2.2 Secondary metabolism ......................................................... 8
  1.2.3 Alkaloids .............................................................................. 9
  1.2.4 Duboisia myoporoides R. Br. ................................................ 10
     1.2.4.1 Early history ................................................................. 12
     1.2.4.2 Alkaloids of Duboisia myoporoides .............................. 14
  1.2.5 Tropane alkaloid .................................................................. 15
  1.2.6 Importance and poisoning syndrome of tropane alkaloids ....... 16
  1.2.7 Biosynthesis of tropane alkaloids ....................................... 17
  1.2.8 Biosynthetic and accumulation sites for tropane alkaloids ...... 19
  1.2.9 Degradation of alkaloids in the plant tissues ......................... 21
  1.2.10 Plant tissue culture: Organogenesis, differentiation and secondary
         metabolites with special reference to tropane alkaloid production
         ......................................................................................... 22
     1.2.10.1 Plant tissue culture in production of secondary metabolites
             ...................................................................................... 22
     1.2.10.2 Plant tissue culture ....................................................... 24
     1.2.10.2.1 Medium constituents ............................................. 24
     1.2.10.2.2 Plant growth regulators ......................................... 25
1.2.10.2.3 Initiation and maintenance of plant tissues in culture........................................ 33
1.2.10.2.4 Vitrification or hyperhydricity ...................... 35
1.2.10.3 Organogenesis and differentiation ..................... 35
   1.2.10.3.1 Explant ........................................... 36
   1.2.10.3.2 Light .............................................. 37
   1.2.10.3.3 Physical form of the medium ....................... 37
   1.2.10.3.4 Plant growth regulators .......................... 38
1.2.10.4 Effects of environmental factors on secondary metabolite biosynthesis............................... 39
   1.2.10.4.1 Plant growth regulators ......................... 40
   1.2.10.4.2 Precursors ....................................... 41
1.2.10.5 Secondary metabolite biosynthesis using different plant tissue culture techniques .............. 41
   1.2.10.5.1 Callus culture .................................. 42
   1.2.10.5.2 Suspension culture .............................. 42
   1.2.10.5.3 Organ culture ................................... 43
   1.2.10.5.4 Transformed organ culture ....................... 46
   1.2.10.5.5 Plant regeneration .............................. 48
1.2.11 Some considerations regarding this research strategies ...... 49
1.2.12 Histochemical analysis: Organogenesis, Differentiation and Alkaloid Localization ...................... 51
   1.2.12.1 Histochemical technique ............................ 51
   1.2.12.2 Stains (dyes) and staining of plant tissue sections ...... 52
   1.2.12.3 Organogenesis ...................................... 53
   1.2.12.4 Differentiation .................................... 54
   1.2.12.5 Alkaloid Localization .............................. 56
1.2.13 Analysis of plant materials for nicotine, hyoscyamine and scopolamine........................................... 59
   1.2.13.1 Analysis of the field-grown plant materials .......... 60
   1.2.13.2 Analysis of cultured plant materials ............... 62
      1.2.13.2.1 Different Duboisia species .......................... 63
      1.2.13.2.2 Plant species other than Duboisia ................ 65
CHAPTER 2 Production of cultured tissues and organs of *Duboisia myoporoides* R. Br. in semi-solid medium

2.1 Introduction

2.2 Materials and Methods

2.2.1 Chemicals and tissue culture vials

2.2.2 Source of plant materials

2.2.3 Preparation of cuttings

2.2.4 Surface sterilization of explants

2.2.5 Preparation of tissue culture medium

2.2.6 Incubation conditions for cultured tissues and regenerants

2.2.7 Standardization of complete plant regeneration

2.2.7.1 Callus induction

2.2.7.2 Production of green callus

2.2.7.3 Shoot-bud induction

2.2.7.4 Shoot elongation

2.2.7.5 Root induction

2.2.8 Some considerations regarding experimental design

2.2.9 Induction and subculture of the calli and the regenerants

2.2.10 Shoot culture from the non-organogenic calli

2.2.10.1 Production of green callus

2.2.10.2 Shoot-bud induction

2.2.10.3 Shoot elongation

2.2.11 Shoot culture from the organogenic calli

2.2.12 Root culture from the non-organogenic calli

2.2.13 Root culture from the organogenic callus

2.2.14 Selected cytokinin and auxin combination studied

2.2.15 Effects of TDZ on shoot-bud induction

2.2.16 Seed germination
CHAPTER 3 Production of organs (shoot, root) in suspension cell cultures of *Duboisia myoporoides* R. Br. .......................... 153

3.1 Introduction ................................................................................. 154
3.2 Materials and Methods ................................................................. 156

3.2.1 Chemicals and equipments ................................................... 156
3.2.2 Preparation and storage of media .......................................... 156
3.2.3 Establishment and subculture of suspension culture for shoot culture .............................................................................................................................................. 157
3.2.4 Shoot culture in suspension .................................................... 157
3.2.5 Production of friable callus for root culture in suspension ........ 158
3.2.6 Establishment and subculture of suspension culture for root culture ......................................................................................................................................................... 159

3.3 Results ......................................................................................... 160

3.3.1. Shoot culture in suspension ................................................. 160
3.3.2. Root culture in suspension .................................................... 161

3.4 Discussion .................................................................................. 164

CHAPTER 4 Effects of environmental factors on shoot growth in *Duboisia myoporoides* R. Br. ................................................................. 167

4.1 Introduction ................................................................................ 168
4.2 Materials and Methods ................................................................. 171

4.2.1 Chemicals and culture vials ................................................... 171

4.2.2 Shoot culture in the B5 basal medium .................................... 171

4.2.3 Shoot-bud induction for investigating the effects of extra-factors, alkaloid precursors and different light regimes on shoot growth................................. 172

4.2.3.1 Shoot culture in the BM supplemented with various extra-factors .......................................................................................................................... 172
4.2.3.2 Shoot culture in the BM supplemented with various alkaloid precursors ........................................................................................................... 172
4.2.3.3 Shoot culture in different light regimes ................................ 173
4.2.4. Data collection and statistical analyses .................................... 175
4.3 Results ........................................................................................................... 176
  4.3.1 Effects of basal media on shoot-bud induction and shoot growth. 176
  4.3.2. Effects of extra-factors on shoot growth ........................................... 180
  4.3.3. Effects of alkaloid precursors on shoot growth ............................. 180
  4.3.4. Effects of different light regimes on shoot growth ...................... 185
4.4. Discussion ..................................................................................................... 191

CHAPTER 5 Organogenesis, differentiation and localization alkaloids in
  Duboisia myoporoides R. Br. ................. 194

5.1. Introduction ................................................................................................. 195
5.2. Materials and Methods ........................................................................... 199
  5.2.1 Chemicals and reagents ...................................................................... 199
  5.2.2 Source of plant materials .................................................................... 199
  5.2.3 Preparation of sections ....................................................................... 199
    5.2.3.1 Microtome sections ..................................................................... 200
      5.2.3.1.1 Microtome ........................................................................... 200
      5.2.3.1.2 Fixation of plant material .................................................... 200
      5.2.3.1.3 Dehydration ........................................................................ 200
      5.2.3.1.4 Paraffin infiltration ............................................................. 200
      5.2.3.1.5 Embedding .......................................................................... 201
      5.2.3.1.6 Section cutting ...................................................................... 201
      5.2.3.1.7 Mounting the ribbon on the glass slide ................................ 201
      5.2.3.1.8 Hydration and staining ......................................................... 202
    5.2.3.2 Free-hand sections ......................................................................... 202
  5.2.4. Histochemical investigation for organogenesis ............................... 202
    5.2.4.1. Staining .................................................................................... 203
  5.2.5. Histological investigation for differentiation .................................... 203
  5.2.6. Histolocalization of alkaloids in the mature and cultured
        plant materials ....................................................................................... 203
    5.2.6.1. Preparation of alkaloid-free sections ...................................... 204
    5.2.6.2. Alkaloid colour reagents used ................................................. 204
    5.2.6.3. Staining of the fresh and alkaloid-free sections ......................... 204
5.2.7. Light microscopy and photography.................................................. 205
5.2.8. Sectioning protocols for histochemical investigation......................... 205
5.3. Results............................................................................................................. 207
5.3.1. Selection of a general purpose histochemical staining reagent... 207
5.3.2. Cell arrangement and macromolecular constituents of the non-organogenic calli............................................................. 208
  5.3.2.1 Callus induced in the BM supplemented with
          2,4-D10^{-7}M................................................................. 208
  5.3.2.2 Callus induced in the BM supplemented with
          BA10^{-5} M+ NAA10^{-6}M.............................................. 208
  5.3.2.3 Callus induced in the BM supplemented with
          BA10^{-5}M+2,4-D10^{-7}M............................................. 208
  5.3.2.4 Callus induced in the BM supplemented with
          Kin10^{-5}M+IAA10^{-6}M.............................................. 211
  5.3.2.5 Callus induced in the BM supplemented with
          Kin10^{-5}M+IBA10^{-5}M.............................................. 211
  5.3.2.6 Callus induced in the BM supplemented with
          Kin10^{-5}M+NAA10^{-5}M.............................................. 211
  5.3.2.7 Callus induced in the BM supplemented with
          Kin10^{-5}M+2,4-D10^{-7}M............................................. 213
5.3.3. Cell arrangement and macromolecular constituents of the organogenic calli............................................................. 213
  5.3.3.1 Shoot-bud producing callus induced in the BM
          supplemented with BA10^{-5}M+IAA10^{-6}M..................... 213
  5.3.3.2 Shoot-bud producing callus induced in the BM
          supplemented with BA10^{-5}M+IBA10^{-7}M..................... 213
  5.3.3.3 Root producing callus induced in the BM supplemented
          with BA10^{-6}M+IBA10^{-5}M........................................... 216
5.3.4 Differentiation in the tissues and organs cultured from the non-
  organogenic calli...................................................................................... 216
  5.3.4.1 Calli induced in the media supplemented with the
          selected cytokinin/auxin combinations............................... 216
  5.3.4.2 Shoot differentiated from the callus induced in the BM
          supplemented with 2,4-D10^{-7}M...................................... 218
5.3.4.3 Shoot differentiated from the callus induced in the BM supplemented with BA10^{-5}M+NAA10^{-6}M............ 218

5.3.4.4 Shoot differentiated from the callus induced in the BM supplemented with the other selected cytokinin and auxin combinations.................................................. 218

5.3.5 Differentiation in the tissues and organs cultured from the organogenic calli.......................................................... 219

5.3.5.1 Calli induced in the BM supplemented with the selected cytokinin and auxin combinations............................. 219

5.3.5.2 Shoot-bearing organogenic callus induced in the BM supplemented with BA10^{-5}M+IBA10^{-7}M................. 219

5.3.5.3 Shoot differentiated from the callus induced in the BM supplemented with BA10^{-5}M+IAA10^{-6}M .............. 219

5.3.5.4 Shoot differentiated from the callus induced in the BM supplemented with BA10^{-5}M+IBA10^{-7}M ............ 222

5.3.5.5 Root differentiated from the callus induced in the BM supplemented with BA10^{-6}M+IBA10^{-5}M ............ 222

5.3.6 Histolocalization of alkaloid in the mature and cultured plant materials......................................................... 222

5.3.6.1 Histolocalization of alkaloids in the mature plant materials. 222

5.3.6.2 Histolocalization of alkaloids in the tissues and organs cultured from the non-organogenic calli....................... 229

5.3.6.2.1 Non-organogenic calli induced in the BM supplemented with the selected cytokinin/auxin combinations.................................................. 229

5.3.6.2.2 Shoot differentiated from the callus induced in the BM supplemented with 2,4-D10^{-7}M........... 229

5.3.6.2.3 Shoot differentiated from the callus induced in the BM supplemented with BA10^{-5}M+NAA10^{-6}M... 231

5.3.6.2.4 Shoot differentiated from the calli induced in the BM supplemented with the other selected cytokinin and auxin combinations.................................................. 231

5.3.6.3 Histolocalization of alkaloid in the tissues and organs cultured from the organogenic calli............................... 231
5.3.6.3.1 Organogenic calli induced in the BM supplemented with the selected cytokinin and auxin combinations ........................................... 232

5.3.6.3.2 Shoot-bearing organogenic callus induced in the BM supplemented with BA10^{-5}M+IBA10^{-7}M........... 232

5.3.6.3.3 Shoot differentiated from the callus induced in the BM supplemented with BA10^{-5}M+IAA10^{-6}M... 232

5.3.6.3.4 Shoot differentiated from the callus induced in the BM supplemented with BA10^{-6}M+IBA10^{-7}M... 232

5.3.6.3.5 Root differentiated from the callus induced in the BM supplemented with BA10^{-6}M+IBA10^{-5}M... 233

5.4. Discussion................................................................................................................. 236

  5.4.1 Cell arrangement and macromolecular constituents of the non-organogenic and organogenic calli.................. 236

  5.4.2 Differentiation in the cultured tissues and organs ...................... 238

  5.4.3 Histolocalization of alkaloids in the mature plant organs........... 241

  5.4.4 Cell differentiation and histolocalization of alkaloids in the cultured tissues and organs .......................................................... 243

CHAPTER 6 Identification and quantification of the selected alkaloids of *Duboisia myoporoides* R. Br. plant materials ................. 246

  6.1 Introduction............................................................................................................. 247

  6.2 Materials and Methods......................................................................................... 249

    6.2.1 Chemicals, stock solutions and analytical equipments.................. 249

    6.2.2 Systematic validation of the analytical method used...................... 249

    6.2.3 Plant materials analyzed.............................................................................. 250

    6.2.4 Extraction of alkaloids from plant materials................................. 250

    6.2.5 Gas chromatography..................................................................................... 251

      6.2.5.1 GC-FID................................................................................................. 251

      6.2.5.2 GC-MS.............................................................................................. 251

      6.2.5.3 Quantification....................................................................................... 252
6.2.6 Statistical analyses ................................................................. 252

6.3 Results ......................................................................................... 254

6.3.1 Alkaloid production in the semi-solid medium ...................... 254
  6.3.1.1 Standardization of plant regeneration ............................ 254
  6.3.1.2 Shoot culture from the non-organogenic calli ............. 254
  6.3.1.3 Shoot culture from the organogenic calli .................. 257
  6.3.1.4 Root culture from the non-organogenic and organogenic calli ........................................... 259

6.3.2 Alkaloid production in the suspension culture ................. 262
  6.3.2.1 Shoot culture in suspension ................................. 262
  6.3.2.2 Root culture in suspension .................................. 263

6.3.3 Alkaloid contents in the mature plant materials ............... 264

6.3.4 Gas chromatography ............................................................... 266
  6.3.4.1 Screening of alkaloids ........................................... 266
  6.3.4.2 Confirmation of alkaloids ..................................... 268
    6.3.4.2.1 The ions and relative abundance values from literature and NIST 62 library ........... 268
    a) Literature ......................................................... 268
    b) NIST 62 library ............................................. 269
  6.3.4.2.2 GC-MS total ion chromatogram and the mass spectra of the mixed standard ............... 269
  6.3.4.2.3 GC-MS total ion chromatogram and mass spectra of the cultured samples ............... 269
    a) Standardization of plant regeneration.. 272
    b) Eleven-week-old non-organogenic calli induced in the BM supplemented with BA10^{-5}M+NAA10^{-6}M ................. 272
    c) Six-week-old shoots differentiated from the non-organogenic calli induced in the BM supplemented with 2,4-D10^{-7}M ........ 272
    d) Six-week-old shoots differentiated from the non-organogenic calli induced in the BM supplemented with BA10^{-5}M+NAA10^{-6}M ...................................................... 275
e) Six-week-old shoots differentiated from the non-organogenic calli induced in the BM supplemented with BA$10^{-5}$M+2,4-D$10^{-7}$M

f) Six-week-old shoots differentiated from the non-organogenic calli induced in the BM supplemented with Kin$10^{-5}$M+2,4-D$10^{-7}$M

g) Nine-week-old shoot differentiated from the organogenic calli induced in the BM supplemented with BA$10^{-5}$M+IBA$10^{-7}$M

h) Fourteen-week-old calli collected from the base of the shoot differentiated from the organogenic calli induced in the BM supplemented with BA$10^{-5}$M+IBA$10^{-7}$M

i) Roots regenerated from the non-organogenic calli induced in the semi-solid MS basal medium supplemented with BA$10^{-5}$M+NAA$10^{-6}$M

j) Roots regenerated from the organogenic calli induced in the BM supplemented with BA$10^{-6}$M+IBA$10^{-5}$M

k) Cell aggregates with shoot cultured in the MS basal suspension medium supplemented with BA$10^{-5}$+IBA$10^{-7}$M

l) Roots regenerated in the B5 basal suspension medium supplemented with IBA25$x10^{-6}$M

6.4 Discussion

275

279

279

279

284

284

287

287

289
CHAPTER 7 Mycorrhization of the *Duboisia myoporoides* R. Br. roots and alkaloid contents in the leaves of the field-grown trees

7.1 Introduction......................................................................................................................... 297
7.2 Materials and Methods........................................................................................................ 299
  7.2.1 Chemicals and reagents. ................................................................................................. 299
  7.2.2 Source of plant materials. .............................................................................................. 299
  7.2.3 Collection and preservation of materials. ...................................................................... 299
    7.2.3.1 Rhizosphere soil ..................................................................................................... 299
    7.2.3.2 Root ...................................................................................................................... 301
    7.2.3.3 Leaves .................................................................................................................. 301
  7.2.4 Physical and chemical soil characteristic. ...................................................................... 301
    7.2.4.1 Moisture content of the soil .................................................................................... 301
    7.2.4.2 pH of the soil ......................................................................................................... 302
    7.2.4.3 Total P content of the soil ...................................................................................... 302
  7.2.5 Biological soil characteristics ....................................................................................... 302
    7.2.5.1 Extraction of spores from the soil sample ............................................................... 302
    7.2.5.2 AM Fungal infection assessment ........................................................................... 304
  7.2.6 Alkaloid extraction ......................................................................................................... 304
7.3 Results................................................................................................................................ 305
  7.3.1 Field soil characteristics ............................................................................................... 305
  7.3.2 AM Fungal spores in the mycorrhizospheres ................................................................. 306
    7.3.2.1 AM Fungal spore number ...................................................................................... 306
    7.3.2.2 AM Fungal spore morphology and taxonomy ......................................................... 306
  7.3.3 Mycorrhizal status of the roots ..................................................................................... 308
  7.3.4 Alkaloid contents in the leaves ...................................................................................... 311
7.4 Discussion........................................................................................................................... 312

CHAPTER 8 General discussion, conclusions and future investigations.................................. 315

8.1 General discussion ............................................................................................................. 316
8.2 Conclusions ....................................................................................................................... 326
8.3 Future investigations................................................................. 326

LITERATURE CITED.................................................................................. 327

APPENDICES............................................................................................... 357

Appendix 1 Source of Duboisia myoporoides R. Br. plant....................... 358
Appendix 2 Murashige and Skoog (MS) (1962) plant tissue culture medium. 359
Appendix 3 Measurement of light intensity.............................................. 360
Appendix 4 UNE #B medium................................................................. 361
Appendix 5 Standard error (from %)....................................................... 362
Appendix 6 Least significant difference (LSD)........................................ 362
Appendix 7 Gamborg et al., (1968) (B5) plant tissue culture medium....... 363
Appendix 8 Equation for Dunnett’s test.................................................. 364
Appendix 9 Test for correlation coefficient significance....................... 364
Appendix 10 Formalin-acetic acid-alcohol (FAA)..................................... 365
Appendix 11 TBA series for dehydration of tissues................................. 365
Appendix 12 Construction of paper embedding boat................................ 366
Appendix 13 Mayer’s fixative................................................................. 366
Appendix 14 Warming plate................................................................. 367
Appendix 15 Hydration and staining of microtome sections.................... 368
Appendix 16 Staining reagents for macromolecular constituents............. 369
Appendix 17 Alcoholic tartaric acid solution......................................... 369
Appendix 18 Staining reagents for alkaloid localization......................... 369
Appendix 19 Calibration curve for total phosphorus............................... 372
Appendix 20 Reagents for AM fungal infection assessment.................... 373
Appendix 21 Abstract.............................................................................. 374
LIST OF TABLES

1.1 Secondary metabolite from plants .................................................. 6
1.2 Plant alkaloids and their pharmaceutical activities ......................... 11
1.3 Organ-specific biosynthesis and accumulation of alkaloids and other secondary metabolites ......................................................... 20
1.4 Secondary metabolite produced by plant tissue culture ..................... 23
1.5 Commonly used plant growth regulators ........................................... 26
1.6 Effects of TDZ on organogenic response on various plant species........ 34
1.7 Shoot differentiation is necessary for enhanced secondary metabolism ................................................................. 44
2.1 Responses obtained at different stages of standardization of complete plant regeneration.............................................................. 96
2.2a Effects of various cytokinin /auxin combinations and concentrations on the frequency (%) of callus induction........................................ 100
2.2b Summary analysis of variance of effects of various cytokinin / auxin combinations and concentrations on the frequency (%) of callus induction.......................................................... 102
2.3 Morphogenetic responses on the leaf explant of Duboisia myoporoides R.Br............................................................. 104
2.4a Effects of cytokinin and auxin combinations on the fresh weight and appearance of the 11-week-old non-organogenic calli................. 106
2.4b Summary analysis of variance of effects of various cytokinin/auxin combinations on the fresh weight of the 11-week-old non-organogenic calli ........................................................................ 107
2.5a Effects of various cytokinin and auxin concentrations on the fresh weight of the 11-week-old non-organogenic calli............................................... 109
2.5b Summary analysis of variance of effects of various cytokinin and auxin concentrations on the fresh weight of the 11-week-old non-organogenic calli .......................................................... 110
2.6a Effects of cytokinin and auxin combinations on the number of shoot-bud and root induction on the 2-week-old organogenic calli ............... 116
2.6b Summary analysis of variance of effects of various BA+IAA concentrations
on shoot-bud induction on the 2-week-old organogenic calli.......................... 116

2.7a Effects of cytokinin / auxin combinations on green callus formation, shoot-bud
induction and morphological characteristics of the shoots differentiated on the
11-week-old non-organogenic calli............................................................... 119

2.7b Summary analysis of variance of effects of the selected cytokinin / auxin
combinations on shoot-bud induction and morphological characteristics of the
shoot........................................................................................................ 120

2.8a Effects of cytokinin and auxin combinations on the shoot-bud induction and
morphological characteristics of the shoots differentiated from the organogenic
calli........................................................................................................ 128

2.8b Summary t test of effects of cytokinin and auxin combinations on the shoot-
bud induction and morphological characteristics of the shoots differentiated
from the organogenic calli........................................................................ 128

2.9 Effects of different medium additives on root regeneration from the non-
organogenic calli ...................................................................................... 132

2.10 Effects of various TDZ+IBA combinations on frequency of callus induction
on the leaf explant..................................................................................... 136

2.11 Effects of various concentrations of TDZ in combination with IBA on the
morphogenetic response on the leaf explant.......................................... 137

2.12a Effects of various TDZ+IBA concentrations on the shoot-bud induction
.................................................................................................................. 139

2.12b Summary t test of effects of various TDZ+IBA concentrations on shoot-bud
induction...................................................................................................... 140

3.1 Effects of cytokinin/auxin combinations on shoot-bud induction on the calli
incubated in the light in an agitated suspension culture medium for 3-weeks. 160

4.1a Effects of basal media on shoot-bud induction and morphological
characteristics of the 9-week-old shoot............................................... 176

4.1b Summary of t test of effects of B5 and MS basal media on shoot-bud induction
and morphological characteristics of the 9-week-old shoot................. 177

4.2a Effects of various alkaloid precursors on morphological characteristics of the
9-week-old shoot...................................................................................... 181
4.2b Summary analysis of variance (treatment-standard) of effects of various alkaloid precursors on shoot growth ................................................. 181
4.2c Summary analysis of variance (treatments) of effects of various alkaloid precursors on shoot growth ......................................................... 182
4.3a Effects of different light regimes on the morphological characteristics of the 9-week-old shoot ................................................................. 186
4.3b Summary analysis of variance (treatment-standard) of effects of different light regimes on shoot growth ......................................................... 186
4.3c Summary analysis of variance (treatment) of effects of different light regimes on shoot growth ................................................................. 187
4.4 The correlation coefficient (r) between light intensity and the morphological characteristics of the 9-week-old shoot ................................................. 187
5.1 Staining reactions for the presence or absence of different macromolecules in the root sections ................................................................. 207
5.2 Cell arrangement and macromolecular constituents of the 11-week-old non-organogenic calli ................................................................. 209
5.3 Cell arrangement and macromolecular constituents of the 2-week-old organogenic calli ................................................................. 214
5.4 Cell organization and histolocalization of alkaloid in the basal stem sections of the 6-week-old shoot differentiated from the non-organogenic calli .... 217
5.5 Cell organization and histolocalization of alkaloid in the tissue, root and basal stem sections of the shoot differentiated from the organogenic calli .... 220
5.6 Staining reactions for localization of alkaloids in the free-hand fresh sections ......................................................................................... 223
6.1 Alkaloid contents at different stages of shoot differentiation from the non-organogenic calli ................................................................. 256
6.2 Alkaloid contents at different stages of shoot differentiation from the organogenic calli ................................................................. 258
6.3 Summary analysis of variance of effects of the selected cytokinin/auxin combinations on alkaloid contents of the differentiated shoot ........ 260
6.4 Alkaloid contents of the 4-week-old roots ........................................ 261
6.5 Summary analysis of t test of alkaloid contents in the 4-week-old root and 9-week-old shoot ................................................................. 262
6.6 Alkaloid contents in the cell aggregates, suspension medium and shoots... 263
LIST OF FIGURES

1.1 Key intermediates from which primary and secondary metabolite forms…... 8
1.2 Distribution of Daboisia in Australia ........................................... 13
1.3 Major alkaloids of Daboisia myoporoides R. Br. ............................... 13
1.4 Structure of nicotine, hyoscyamine and scopolamine .................... 15
1.5 The proposed biosynthetic pathways of nicotine and tropane alkaloids... 18
1.6 Sites of plant growth regulator synthesis and transport in a typical
   whole plant ............................................................................. 27
1.7 Role of cytokinin in a typical whole plant ...................................... 29
1.8 Role of auxin in a typical whole plant .......................................... 29
1.9 Some cytokinins and auxins used in plant tissue culture ................. 30
2.1 Location of Mount Annan Botanic Gardens at Mount Annan............. 77
2.2 The bed / area in Mount Annan Botanic Gardens where Daboisia
   myoporoides R. Br. were planted.............................................. 77
2.3 Daboisia myoporoides R.Br. plants in the natural habitat of Mount Annan
   Botanic Gardens ....................................................................... 78
2.4 Daboisia myoporoides R. Br. cuttings in a chamber of controlled conditions
   of temperatue and humidity....................................................... 80
2.5 Two-year-old Daboisia myoporoides R. Br. cuttings in the greenhouse-
   conditions of UWS Macarthur.................................................. 80
2.6 Flow chart for analysis of tissues and organs at different stages of organ culture
   .................................................................................................. 85
2.7 Incubation chamber for culturing tissues and regenerants.................. 97
2.8 Eleven-week-old non-organogenic callus ...................................... 97
2.9 Two-week-old green callus ....................................................... 97
2.10 Shoot-bud induction on the green callus....................................... 98
2.11 Elongation of the shoot-buds .......................................................... 98
2.12 Six-week-old differentiated shoot .................................................. 98
2.13 Root induction in the differentiated shoot ........................................ 98
2.14 Non-organogenic callus induced in the BM supplemented with
    BA10^{-5}M+2,4-D10^{-7}M ............................................................... 103
2.15 Shoot-bud induction on the organogenic callus induced in the BM
    supplemented with BA10^{-5}M+IBA10^{-7}M .................................. 103
2.16 Root induction on the organogenic callus induced in the BM
    supplemented with BA10^{-6}M+IBA10^{-5}M .................................. 103
2.17 Effects of cytokinin and auxin concentrations on the fresh weight of the
    11-week-old non-organogenic callus ............................................. 111
2.18 Effects of cytokinin and auxin combinations on the frequency (\%) of
    Non-organogenic callus induction ............................................... 114
2.19 Effects of cytokinin and auxin concentrations on shoot-bud induction on
    the organogenic calli .............................................................. 117
2.20 Effects of cytokinin and auxin combinations on the frequency (\%) of green
    callus formation ........................................................................ 121
2.21 Effects of cytokinin/auxin combinations on shoot-bud induction and the
    morphological characteristics of the 6-week-old shoot differentiated from the
    non-organogenic calli ................................................................. 123
2.22 Six-week-old shoot differentiated from the non-organogenic callus induced
    in the BM supplemented with 2,4-D10^{-7}M .................................. 125
2.23 Six-week-old shoot differentiated from the non-organogenic calli induced
    in the BM supplemented with BA10^{-5}M+NAA10^{-6}M .................... 125
2.24 Six-week-old shoot differentiated from the non-organogenic calli induced
    in the BM supplemented with Kin10^{-5}M+NAA10^{-5}M .................... 125
2.25 Effects of cytokinin and auxin combinations on shoot-bud induction and
    morphological characteristics of the 9-week-old shoot differentiated from the
    organogenic calli ...................................................................... 129
2.26 Nine-week-old shoot differentiated from the organogenic calli induced in the
    BM supplemented with BA10^{-5}M+IAA10^{-6}M ......................... 130
2.27 Nine-week-old shoot differentiated from the organogenic calli induced in the
    BM supplemented with BA10^{-5}M+IBA10^{-7}M ......................... 130
2.28 Four-week-old root regenerated from the non-organogenic calli ...... 130
2.29a Effects of BA+IBA concentrations on morphogenetic response on the greenhouse-grown leaf explant................................. 134
2.29b Effects of BA+IBA concentrations on morphogenetic response on the
in vitro grown leaf explant.......................................................... 134
2.30 Nine-week-old shoot differentiated from the organogenic calli induced in the
BM supplemented with BA10^{-5}M+IBA10^{-7}M.............................. 135
2.31 Formation of shoot-buds on the organogenic calli induced on the leaf explant
from in vitro grown shoot............................................................ 135
2.32 Root induction on the organogenic calli induced on the leaf explant from
in vitro grown leaf explant............................................................ 135
2.33 Root initiation on the 9-week-old shoot...................................... 135
2.34 Two-week-old callus induced in the BM supplemented with
TDZ5x10^{-6}M+IBA10^{-6}M.............................................................. 138
2.35 Shoot-bud induction on the 6-week-old organogenic calli induced in the BM
supplemented with TDZ5x10^{-6}M+IBA10^{-7}M.............................. 138
2.36 Four-week-old shoot-bud formed on the calli induced in the BM supplemented
with TDZ5x10^{-6}M+IBA10^{-6}M.......................................................... 138
2.37 Four-week-old shoot-bud formed on the calli induced in the BM supplemented
with TDZ5x10^{-6}M+IBA10^{-7}M.......................................................... 138
2.38 Duboisia myoporoides R. Br. seeds........................................... 141
2.39 Duboisia myoporoides R. Br. seed germination.............................. 141
3.1 Shoot regenerated on the suspension cultured friable callus .......... 162
3.2 Production of friable calli (for root regeneration) from the non-organogenic calli
................................................................................................. 162
3.3 Root regenerated on the friable callus cultured in the agitated liquid medium
................................................................................................. 162
4.1 Light arrangement for incubation of cultured shoots under different light
regimes....................................................................................... 174
4.2 Effects of basal media on shoot-bud induction and morphological characteristics
of the 9-week-old shoot................................................................. 178
4.3 Nine-week-old shoot grown on the B5 basal medium.................... 179
4.4 Effects of alkaloid precursors on shoot growth............................. 183
4.5 Axillary-bud formation on the shoot-buds.................................. 179
4.6 Effects of different light regimes on shoot growth

5.1 Cell arrangement and macromolecular constituents of the 11-week-old callus induced in the BM supplemented with 2,4-D10⁻⁷M

5.2 Cell arrangement and macromolecular constituent of the 11-week-old callus induced in the BM supplemented with BA10⁻⁵M+NAA10⁻⁶M

5.3 Cell arrangement and macromolecular constituent of the 11-week-old callus induced in the BM supplemented with BA10⁻⁵M+2,4-D10⁻⁷M

5.4 Cell arrangement and macromolecular constituents of the 11-week-old callus induced in the BM supplemented with Kin10⁻⁵M+IAA10⁻⁶M

5.5 Cell arrangement and macromolecular constituents of the 11-week-old callus induced in the BM supplemented with Kin10⁻⁵M+IBA10⁻⁵M

5.6 Cell arrangement and macromolecular constituents of the 11-week-old callus induced in the BM supplemented with Kin10⁻⁵M+NAA10⁻⁵M

5.7 Cell arrangement and macromolecular constituents of the 11-week-old callus induced in the BM supplemented with Kin10⁻⁵M+2,4-D10⁻⁷M

5.8 Cell arrangement and macromolecular constituents of the 2-week-old shoot-bud producing organogenic callus induced in the BM supplemented with BA10⁻⁵M+IBA10⁻⁷M

5.9 Cell arrangement and macromolecular constituents of the 14-week-old organogenic callus induced in the BM supplemented with BA10⁻⁵M+IBA10⁻⁷M

5.10 Cell arrangement and macromolecular constituents of the 2-week-old root producing organogenic callus induced in the BM supplemented with BA10⁻⁶M+IBA10⁻⁵M

5.11 Cell differentiation in the basal stem of the 6-week-old shoot differentiated from the non-organogenic callus induced in the BM supplement with 2,4-D10⁻⁷M

5.12 Cell differentiation in the basal stem of the 6-week-old shoot differentiated from the non-organogenic callus induced in the BM supplemented with Kin10⁻⁵M+NAA10⁻⁵M

5.13 Cell differentiation in the basal stem of the 9-week-old shoot differentiated from the organogenic calli induced in the BM supplemented with BA10⁻⁵M+IAA10⁻⁶M
5.14 Cell differentiation in the basal stem section of the 9-week-old shoot differentiated from the organogenic callus induced in the BM supplemented with BA10⁻⁵M+ IBA10⁻⁷M.............................................. 221
5.15 Free-hand fresh root section was stained with cyanogen bromide........ 224
5.16 Free-hand fresh root section was stained with cyanogen bromide and de-stained with water................................................................. 224
5.17 Free-hand fresh root section was stained with Dragendorff’s reagent.... 224
5.18 Free-hand fresh root section was stained with Dragendorff’s reagent and then de-stained with water......................................................... 224
5.19 Free-hand fresh root section was stained with iodoplatinate alkaloid colour reagent and then de-stained with water................................. 226
5.20 Free-hand alkaloid-free root section was stained with iodoplatinate alkaloid colour reagent................................................................. 226
5.21 Free-hand fresh root section was stained with platinic chloride (5%) alkaloid colour reagent and de-stained with water................................. 226
5.22 Free-hand alkaloid-free root section was stained with platinic chloride (5%) alkaloid colour reagent............................................................. 226
5.23 Free-hand fresh basal stem section of the shoot stained with iodoplatinate alkaloid colour reagent............................................................. 227
5.24 Free-hand fresh basal stem section was stained with platinic chloride (5%) alkaloid colour reagent................................................................. 227
5.25 Free-hand fresh basal stem section was stained with iodoplatinate alkaloid colour reagent................................................................. 227
5.26 Free-hand alkaloid-free basal stem section was stained with iodoplatinate alkaloid colour reagent............................................................. 227
5.27 Free-hand fresh section of leaf mid-rib was stained with iodoplatinate alkaloid colour reagent and de-stained with water................................. 228
5.28 Free-hand fresh section of leaf mid-rib was stained with platinic chloride (5%) alkaloid colour reagent............................................................. 228
5.29 Free-hand alkaloid-free leaf mid-rib section was stained with iodoplatinate alkaloid colour reagent............................................................. 228
5.30 Commercial atropine sample was stained with iodoplatinate alkaloid colour reagent................................................................. 228
5.31 Free-hand fresh basal stem section of the 6-week-old shoot differentiated from non-organogenic calli induced in the BM supplemented with 2.4-D $10^{-7}$M

5.32 Free-hand fresh basal stem section of the 6-week-old shoot differentiated from non-organogenic calli induced in the BM supplemented with Kin $10^{-5}$M+IAA $10^{-6}$M

5.33 Free-hand fresh basal stem section of the 9-week-old shoot differentiated from the organogenic calli induced in the BM supplemented with BA $10^{-5}$M+IAA $10^{-6}$M was stained with platinic chloride (5%) 

5.34 Free-hand fresh basal stem section of the 9-week-old shoot differentiated from the organogenic calli induced in the BM supplemented with BA $10^{-5}$M+IBA $10^{-7}$M was stained with iodoplatinate

5.35 Free-hand alkaloid-free basal stem section of the 9-week-old shoot differentiated from the organogenic calli induced in the BM supplemented with BA $10^{-5}$M+IBA $10^{-7}$M was stained with iodoplatinate

5.36 Free-hand alkaloid-free basal stem section of the 9-week-old shoot differentiated from the organogenic callus induced in the BM supplemented with BA $10^{-5}$M+IBA $10^{-7}$M was stained with iodoplatinate

5.37 Free-hand fresh section of the 14-week-old callus was stained with iodoplatinate alkaloid colour reagent

5.38 Free-hand alkaloid-free section of the 14-week-old callus was stained with iodoplatinate alkaloid colour reagent

5.39 Free-hand fresh section of the 4-week-old root was stained with iodoplatinate alkaloid colour reagent

5.40 Free-hand alkaloid-free 4-week-old root section was stained with iodoplatinate alkaloid colour reagent

6.1 Typical chromatogram of alkaloid mixed standard and leaf extract analyzed by GC-FID

6.2a-e Typical GC-MS total ion chromatogram and mass spectrum of mixed alkaloids

6.3a-c Typical GC-MS total ion chromatogram and mass spectrum from analysis of 6-week-old shoot differentiated for the standardization of plant regeneration
6.4a-b Typical GC-MS total ion chromatogram and mass spectrum from analysis of
the 6-week-old shoot differentiated from non-organogenic calli induced in the
BM supplemented with 2,4-D10^{-7}M .............................................. 274

6.5a-b & 6.5a-c Typical GC-MS total ion chromatogram and mass spectra from
analysis of 6-week-old shoot differentiated from the non-organogenic calli
induced in the BM supplemented with BA10^{-5}M+NAA10^{-6}M ............ 276
................................................................................................. 277

6.6a-b Typical GC-MS total ion chromatogram and mass spectrum from analysis of
6-week-old shoot differentiated from the non-organogenic calli induced in the
BM supplemented with BA10^{-5}M+2,4-D10^{-7}M .............................. 278

6.7a-b Typical GC-MS total ion chromatogram and mass spectrum from analysis of
6-week-old shoot differentiated from the non-organogenic calli induced in
the BM supplemented with Kin10^{-5}M+2,4-D10^{-7}M ....................... 280

6.8a-d Typical GC-MS total ion chromatogram and mass spectra from analysis of
9-week-old shoot differentiated from the organogenic calli induced in the
BM supplemented with BA10^{-5}M+IBA10^{-7}M .................................. 281
................................................................................................. 282

6.9a-b Typical GC-MS total ion chromatogram and mass spectrum from analysis of
14-week-old calli ........................................................................... 283

6.10a-d Typical GC-MS total ion chromatogram and mass spectra from analysis of
4-week-old root regenerated from the organogenic calli induced in the BM
supplemented with BA10^{-6}M+IBA10^{-5}M ..................................... 285
................................................................................................. 286

6.11a-b Typical GC-MS total ion chromatogram and mass spectrum from analysis
of 3-week-old cell aggregates with shoot cultured in suspension ....... 288

7.1 Location of Blue Mountains National Park ................................ 300

7.2 Daboecia myoporoides R. Br. tree grown in Blue Mountains National Park.. 300

7.3 Wet-sieving and decanting technique ...................................... 303

7.4 Arbuscular mycorrhizal (AM) fungal spores ............................. 307

7.5 Intercellular and intracellular fungal hyphae in the root cortex .......... 309

7.6 Arbuscular mycorrhizal fungal hyphae around and surface of the root... 310

xxiii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>B5</td>
<td>Gamborg’s B5 medium</td>
</tr>
<tr>
<td>BAP (BA)</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>BM</td>
<td>basal medium</td>
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<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>FID</td>
<td>flame ionisation detector</td>
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<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>H6H</td>
<td>hyoscyamine 6β-hydroxylase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IAA</td>
<td>indole acetic acid</td>
</tr>
<tr>
<td>IBA</td>
<td>indole-3-butyric acid</td>
</tr>
<tr>
<td>IR</td>
<td>infrared spectroscopy</td>
</tr>
<tr>
<td>Kin</td>
<td>kinetin (6-furfurylaminopurine)</td>
</tr>
<tr>
<td>Me</td>
<td>CH₃</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MS medium</td>
<td>Murashige and Skoog plant tissue culture basal medium</td>
</tr>
<tr>
<td>NAA</td>
<td>1-naphthaleneacetic acid</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NN</td>
<td>Nitsch and Nitsch plant tissue culture basal medium</td>
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<tr>
<td>OECD</td>
<td>organization for economic cooperation and development</td>
</tr>
<tr>
<td>PCA</td>
<td>plate count agar</td>
</tr>
<tr>
<td>PGR</td>
<td>plant growth regulator</td>
</tr>
<tr>
<td>Phy A</td>
<td>phytochrome A</td>
</tr>
<tr>
<td>PTC</td>
<td>plant tissue culture</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmuno assay</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>TBA</td>
<td>3° butyle alcohol</td>
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<tr>
<td>TDZ</td>
<td>thidiazuron</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<td>UV</td>
<td>ultra violet</td>
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ABSTRACT

The cultured tissues and organs of Duboisia myoporoides R. Br., an endemic medicinal plant of Australia, were investigated with the aim of establishing a relationship between organogenesis, differentiation and alkaloid localization. Histological analyses explained the relationship between cell arrangement in the cultured tissues and organs and the cytokinin/auxin combinations used at the callus induction stage. The cultured tissues and organs were analysed histochemically to localize alkaloids in different types of cells by using selected alkaloid colour reagents i.e., platinic chloride (5%) and iodoplatinate. The presence or absence of nicotine, hyoscyamine and scopolamine in the cultured tissues and organs was then confirmed by GC-MS analysis.

Two different types i.e., non-organogenic and organogenic calli were induced on the leaf explant incubated in the MS basal medium supplemented with 65 different cytokinin/auxin combinations (2 cytokinins and 4 auxins). Of the 9 different selected cytokinin and auxin combinations, BA10^{-5}M+NAA10^{-6}M and BA10^{-5}M+IBA10^{-7}M caused an elongated basal stem in the differentiated shoots similar to the hypocotyl of the seedling.

Large xylem vessels in the secondary xylem were observed in the vascular region of the elongated basal stems of the shoots differentiated from the calli induced in the BM supplemented with BA10^{-5}M+NAA10^{-6}M or BA10^{-5}M+IBA10^{-7}M respectively. Alkaloid colour reagents localized alkaloids in the vascular region and GC-MS analysis detected nicotine, hyoscyamine and scopolamine in those shoots. However, no large xylem vessels in the secondary xylem were observed in the vascular region of the less elongated basal stems of the shoots differentiated from the calli induced in the BM supplemented with the other selected cytokinin/auxin combinations. Only nicotine was detected by GC-MS analysis in the shoots differentiated from the non-organogenic calli induced in the BM supplemented with 2,4-D10^{-7}M, BA10^{-5}M+2,4-D10^{-7}M or Kin10^{-5}M+2,4-D10^{-7}M. Only hyoscyamine was detected in the 14-week-old calli induced in the BM supplemented with BA10^{-5}M+IBA10^{-7}M collected from the base of the shoot.

Xylem cell arrangement in the 4-week-old roots regenerated from the organogenic calli induced in the BM supplemented with BA10^{-6}M+IBA10^{-5}M was
similar to that of the mature plant root, except that the amount of xylem cells in the vascular region was smaller in the cultured roots compared to the roots from the mature plant. Alkaloid colour reagents localized alkaloids in the vascular region of those cultured roots and the presence of nicotine, hyoscyamine and scopolamine was confirmed by GC-MS.

The presence of nicotine was detected by GC-MS in the 4-week-old shoots regenerated in the suspension culture medium containing MS basal salts, sucrose (3%) and BA10^{-5}M+IBA10^{-7}M without any root initiation. However, nicotine, hyoscyamine and scopolamine were detected in the 4-week-old roots cultured in the suspension medium containing B5 basal salts, sucrose (3%) and IBA10^{-5} M.

This work is the first to show that tropane alkaloid formation in the separated cultured organs is related to xylem differentiation and tropane alkaloid formation in the calli cultured in suspension may allow commercial tropane alkaloid production without regenerating the organs.

The MS basal medium enhanced basal stem elongation of the shoot more than did the B5 basal medium. Shoot growth was inhibited in the MS basal medium supplemented with 2g L^{-1} yeast extract, peptone and casamino acids separately. No significant difference was observed between basal stem elongation of the shoots grown in the MS basal medium alone or those supplemented with phenylalanine, tropic acid or L-ornithine separately. The optimal light intensity was 15.2 μmol s^{-1} m^{-2}, under which the shoot cultures produced a more elongated basal stem as compared to the other light intensities used.

The number of shoot-buds induced on the leaf explant incubated in the BM supplemented with TDZ5x10^{-5}M+IBA10^{-6}M or TDZ5x10^{-6}M+IBA10^{-7}M was lower than those induced in the BM supplemented with BA10^{-5}M+IBA10^{-7}M. However, the cytokinin like activity of TDZ observed in this study showed that TDZ in combination with IBA could be used for shoot-bud induction on the leaf explant of Duboisia myoporoides R.Br.

The roots of the D. myoporoides field-grown trees were colonized by the AM fungi and the mycorrhizal infection was ranged from 0-30% which indicates that the secondary metabolite atropine and scopolamine did not prevent AM fungal colonization.