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New frontiers in agriculture productivity: optimised microbial inoculants and *in situ* microbiome engineering

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Abstract

Increasing agricultural productivity is critical to feed the ever-growing human population. Being linked intimately to plant health, growth and productivity, harnessing the plant microbiome is considered a potentially viable approach for the next green revolution, in an environmentally sustainable way. In recent years, our understanding of drivers, roles, mechanisms, along with knowledge to manipulate the plant microbiome, have significantly advanced. Yet, translating this knowledge to expand farm productivity and sustainability requires the development of solutions for a number of technological and logistic challenges. In this article, we propose new and emerging strategies to improve the survival and activity of microbial inoculants, including using selected indigenous microbes and optimising microbial delivery methods, as well as modern gene editing tools to engineer microbial inoculants. In

addition, we identify multiple biochemical and molecular mechanisms and/approaches which can be exploited for microbiome engineering *in situ* to optimise plant-microbiome interactions for improved farm yields. These novel biotechnological approaches can provide effective tools to attract and maintain activities of crop beneficial microbiota that increase crop performance in terms of nutrient acquisition, and resistance to biotic and abiotic stresses, resulting in an increased agricultural productivity and sustainability.

Key words: Agricultural industry; Plant microbiome; Microbial inoculants; Microbiome engineering *in situ*; Biotechnological tools

1. Microbial communities and agricultural crops

For centuries, improving farming technologies and crop varieties have been the main drivers to increase farm productivity (Juma 2015; De Buck et al., 2016; Altieri 2018). In the past decades, the sustainability of global food production has been progressively hampered by decreasing water availability, loss of arable land due to soil degradation and urbanisation (Cerdà et al., 2017), and higher incidences of disease and pest damage (Bebber et al., 2014). Moreover, global warming is predicted to significantly impact crop yields (Lobell and Field 2007), posing significant pressure on those systems heavily relying on seasonal precipitation for profitable farming. Similarly, issues such as the indiscriminate use of chemical fertilisers and pesticides are increasingly threatening natural ecosystems, causing air, water, and soil pollution (Savci 2012). Importantly, further inorganic inputs do not increase farm productivity in many extensive farming regions due to the structural decline in farm fertility (Trivedi et al., 2017). Thus, to ensure an environmentally sustainable and socially responsible food supply to the ever-growing human population, the need for a step-change advancement in agriculture practices has been highlighted; where technological progress aimed at

improving farm productivity is paired with practices that focus on minimising soil degradation (Cerdà et al., 2017), environmental pollution (Reddy 2013), and the adverse effects of climate change (Nelson et al., 2014; Rosenzweig et al., 2014). Integrating sustainability in the management of crops is an important requirement to ensure adequate food production for current and future generations, but also to protect both environmental and human health (Van Emmerik et al., 2014; Chang et al., 2015).

Harnessing microbiome functions in the context of agricultural production holds great potential to provide solutions to key current challenges related to food security, land degradation, and crop yield (Royal Agricultural Society of NSW 2017; National Academies of Sciences and Medicine 2018). Indeed, microbes are critical drivers of soil functions and agricultural crop productivity (Nazaries et al., 2013; Singh and Trivedi, 2017). Microorganisms are immensely diverse and ubiquitous in terrestrial ecosystems (McFall-Ngai et al., 2013), and can be found both free-living in soil or in symbiotic relationships with organisms from higher trophic level (Azcón 1989; Glöckner et al., 1996; Smith and Goodman 1999). The plant microbiome comprises of microbes that colonise plants internal tissues (endophytes) and external surfaces (e.g. rhizosphere/ rhizoplane and phyllosphere) and are organised into communities that are constantly interacting with their hosts (Agler et al., 2016). Most of these microbes obtain their living source-carbon- from the plant host in exchange for supply of essential nutrients and other benefits (Bonfante and Anca 2009). The role of microorganisms in plants, particularly those inhabiting the rhizosphere (i.e., soil in direct contact with the root surface), includes core functions such as the supply of nitrogen, phosphorus, potassium, sulphur and micronutrients (Dobereiner 1961; Stewart 1973; Cole et al., 1977; Sundara et al., 2002; Schmalenberger et al., 2008) and water retention (Lehto and Zwiazek 2011). These associations have evolved together for millions of years, resulting in fine-tuned mutual recognition and communication mechanisms based on complex molecule

exchanges (Lugtenberg et al., 2002), including microbial chemotactic responses towards root-secreted organic and amino acids, and bacterial quorum sensing (Bais et al., 2004). Such host-microbiome interactions are crucial for plant health, as microbes can affect plant growth and/or development at multiple stages, including germination, morphogenesis, flowering, and hence, productivity (Mayak et al., 2004; Weyens et al., 2009). Microbial symbionts of plants also act as a functional extension in plant defence against biotic (e.g. pathogens and pests) and abiotic (e.g. drought and nutrient pressure) stresses (Droby et al., 2002; Dimkpa et al., 2009).

Given the substantial contribution of the plant microbiome to the fitness of their host (Zilber-Rosenberg and Rosenberg 2008), exploring and utilising these microorganisms could be key to unfolding agricultural constraints and achieving increased productivity sustainably. Successful manipulation of long-term and persistent plant beneficial microbial communities in farmland will greatly benefit current agricultural outcomes. Based on the current status of knowledge and available technologies, several novel approaches have the potential to improve the application of beneficial microbial communities in agriculture, and thus increase crop productivity and environmental sustainability.

In this article, we summarise and discuss issues and challenges associated with the traditional use of microbial inoculants in agriculture. We review state-of-the-art technologies related to the manipulation of culturable microbial species to sustainably increase farm productivity and food quality. Subsequently, we discuss recent emerging approaches to manipulate the whole plant microbiome *in situ*, including culture-free strategies to directly manipulate microbial communities. We conclude by highlighting knowledge gaps, and identifying priority areas in microbiome research to improve agricultural outcomes.

2. Improving manipulation, inoculation efficiency and persistence of beneficial microbes and microbial products

2.1 Use of microbial inoculants in agriculture: state-of-the-art technologies and current challenges

The inoculation of microbial species beneficial to crops has been extensively explored over the past decades. Isolation and application of beneficial plant-related microbes has been successfully exploited in some cases to improve agricultural outcomes (Bossio et al., 1998; Lupwayi et al., 1998), resulting in increased crop growth (e.g. plant growth promoting rhizobacteria (PGPR), Nelson 2004b) and control of plant pests and pathogens (e.g. bio-control microorganisms such as *Bacillus thurengenesis*; Naseby et al., 2000, and *Trichoderma* spp; Kumar and Ashraf 2017). Additionally, well-maintained plant microbial inoculants have been reported to enhance the natural plant resistance against diseases, showing the potential to, at least partially, substitute the use of antibiotics, fungicides and pesticides (Chang et al., 2015; Mueller and Sachs 2015). Similarly, microbial-based fertilisers (bio-fertilisers), consisting of living microorganisms applied to seeds, plants, or soil, are broadly promoted in organic farming as an alternative to chemical fertilisers or to increase inorganic nutrient-use efficiency (e.g. P). Bio-fertilisers increase the supply of nutrients to plants by harnessing the natural ability of microorganisms to mineralise, solubilise and mobilise nutrients (Mäder et al., 2002; Qiu et al., 2012), while reducing the costs associated with frequent fertiliser applications (Singh 2017). The application of such microbial-based crop amendments is rapidly growing globally (Timmusk et al., 2017) and could serve as a promising alternative to some traditional agricultural techniques, especially in countries where agriculture is the main driver of economic development. It is proposed that developing countries in Asia and Africa have the potential to largely benefit from the application of

multi-strain bio-fertilisers developed from rhizosphere soil, with a predicted increase in grain yields of up to 10% (Nguyen et al., 2017).

While the use of microbial inoculants in agriculture can be useful to reduce many current issues associated with extensive farming demands, their success faces some important methodological, technical, and theoretical challenges. Firstly, the introduction of microbial inoculants in agricultural systems has to overcome colonisation issues and issues revolving around the maintenance of introduced microorganisms in the new environment. While several studies have reported successful microbial colonisation in soil (e.g. Marschner and Rumberger 2004), the use of microbial inoculants in the agricultural context has often yielded inconsistent or moderate results, with rapid declines in inoculant populations and activity following introduction into soil (van Veen et al., 1997). Mechanisms responsible for decreases in inoculant numbers and activity include the physiological status of the inoculant cells, as well as biotic interactions in soil (e.g., competition with indigenous soil microorganisms), contextual edaphic properties (e.g., texture, pH, temperature, moisture content), and suitable substrate availability (van Veen et al., 1997). Agronomic practices based on the heavy use of agrochemicals can directly (e.g. simultaneous use of fungicide and fungal inoculants) and indirectly (via changes in the indigenous microbiome and soil pH) impact the efficacy of inoculants (Singh and Trivedi 2017; Trivedi et al., 2017). Additionally, plants can select which microbes they choose to associate with from the introduced microbial community in order to retain valuable colonisers, including those living within their tissue (Hardoim et al., 2012; Marasco et al., 2012; Rashid et al., 2012). This selection is mediated by the host immune system, root exudates, and/ or indigenous endophytic microbes present in the plant tissue, including bacteria (Fraune et al., 2015), fungi (Van Der Heijden et al., 2016), microalgae (Ramanan et al., 2016) and viruses (Fister et al., 2016). Introduced microbes that cannot blend or are able to overcome the local micro-/macro- interactions are at risk of being

147 eliminated. The success of the introduced microbes thus depends on the ability of these
148 microbes to cope with unfavourable or unstable soil conditions, to successfully compete with
149 indigenous microorganisms, to overcome plant selection preferences, and be able to establish,
150 proliferate and remain active.

151 Secondly, the influence of introduced microbes may be not limited to beneficial
152 effects. Indeed, ecological succession of microbial communities after inoculation with a new
153 strain is difficult to predict, as introduced microbes can be identified and displaced by better
154 host-adapted microbes (Seedorf et al., 2014). Introduced microbes can also harbour or favour
155 potential opportunistic pathogens that, in appropriate conditions, can cause dysbiosis in the
156 root environment and induce disease in plants (Cook 1993), which may cause further
157 constraints in agriculture productivity. The release of alien species has the potential risk for
158 disrupting ecological integrity, whereby indigenous communities may be vulnerable to
159 introduced species (Traveset and Richardson 2014), with unknown consequences for
160 ecosystem functionality (Delgado-Baquerizo et al., 2016; Nazaries et al., 2013). A
161 combination of classical pathogenicity tests for non-target organisms with genomic
162 approaches should be implemented before the release of microbial inoculant in agricultural
163 setting.

164 Increasing performance, persistence in the field, and inoculation efficiency of
165 introduced microbes in agriculture is thus a priority to effectively harness their potential,
166 along with reducing risks of detrimental outcomes and improving predictability of efficacy of
167 products. We summarise below the latest trends in research that offer promising avenues to
168 improve the power of microbial-based amendments on agricultural productivity, including
169 use of indigenous microbes, genetic engineering tools, and improved delivery methods.

170

171 ***2.2 Harnessing indigenous plant microbes***

Recent studies have increasingly highlighted the benefits of using indigenous microbes (a group of innate microbial communities that inhabit local soils, plant internal tissues and outer surfaces) to enhance plant resistance to biotic and abiotic stresses (Marulanda et al., 2009; Banerjee et al., 2017), suggesting that the activities of strains already adapted to the plant environment may increase the chances of inoculum survival and confer a positive effect to plant development under stress. Thus, exploiting the intrinsic ability of plants to attract beneficial microbes, combined with the positive role of indigenous microbial species for growth and resistance, could represent an appealing alternative to the introduction of alien microbes. Such an approach has been successfully applied in other situations, for example human faecal transplants, where compositional similarity of the gut microbial community between donors and recipients increases the likelihood of successful colonisation (Li et al., 2016).

A promising strategy to select and introduce beneficial indigenous inoculants is based on the breeding method developed by Mueller and Sach (2015). In this approach, individual plants showing the best performance (e.g. growth, productivity, disease resistance, etc.) under stressed conditions (e.g. disease, drought, heat, etc.) are identified, and microbes harbouring a phenotype of interest are isolated from plant compartments such as rhizosphere, leaf and stem. After removal of potential pathogens, the remaining isolates are either used alone (based on plant phenotype response) or combined and used as a composite microbial inoculum to improve overall crop performance and fitness (e.g., stress resistance, increased growth, productivity). Furthermore, for related but different crop-types, the mixed microbial consortia can be crop-optimised through successive inoculation and selection in order to maximise microbial colonisation and the plant beneficial properties (Fig. 2A). In addition, to the use of microbial consortia (vs single isolate) that include multiple plant promoting activities (e.g. disease resistance, N mobilizations, provision of plant hormones) isolated from

specific crops, can provide better efficiencies given higher chance of survival and activities in crop roots (Trivedi et al 2017: Singh and Trivedi 2017). The utility of synthetic microbial consortia has been successfully demonstrated to provide the plant with benefits including early flowering, increased nutrient acquisition and disease resistance (Gopal and Gupta, 2016 and reference within).

2.3 Contemporary genetic tools to modify microbes for beneficial activities

In the past decades, a number of genetic tools have been developed and employed to enhance productivity and reduce pest/pathogen damage (Qaim and Zilberman 2003; Godfray et al., 2010). Genetic engineering on targeted microbial species for agricultural use holds the potential of being fast and reasonably effective, due to the direct introduction of individual, heterologous traits, into well-characterised microbes. Among the most recent biotechnological developments in genetic tools, the discovery of RNA interference (RNAi) (Zamore et al., 2000) has allowed researchers to modify genes at the expression level. RNAi is initiated by double-stranded RNA (dsRNA) which activates the ribonuclease protein *Dicer*, resulting in small fragments of ~21 nucleotides called small interfering RNA (siRNA). These siRNA bind to specific proteins to form a complex, which is incorporated into the RNA-induced silencing complex (RISC). When one strand of incorporated siRNA binds to the complementary messenger RNA (mRNA) sequence, a cleavage reaction is triggered, which is the catalytic component of RISC (Filipowicz 2005), resulting in the inhibition of the gene expression or translation process. In agriculture, the utility of RNAi anti-pathogen purposes has been demonstrated. For example, Ganbaatar et al. (2017) explored an *Escherichia coli* strain containing RNA interfering sequences specifically targeting corn pathogens to eliminate *Mythimna separata*. In this case, genetically modified microorganisms did not kill the pathogen directly, but carry the dsRNA that silence targeted genes in the pathogen of

interest. RNAi technology can thus be potentially applied to engineer beneficial microbes and increase plant resistance to specific pathogens (Fig. 2B).

With the development of the CRISPR and CRISPR/Cas9 technologies (Cong et al., 2013), gene and genome editing have become easier. Cas9 functions as an RNA-guided DNA endonuclease that complexes with engineered sequence-specific single guide RNA (sgRNA) into a cell. The cell genome can then be edited with insertion/removal on targeted location. There have been several reports where this approach has been successfully demonstrated for its potential use within agricultural industries, including editing genes of crops (reviewed in Andersen et al., 2015) and enhancing resistance to pathogens (Ali et al., 2015). Using these molecular tools, we are not only able to mine molecular knowledge from both genetic and transcriptional levels, acquiring information from their functions and gene expressions, but also able to modulate genes *per se* to get desired genotypes and phenotypes such as improved nutrient mobilisation and defence against invading pathogens. With these gene editing tools, genetically modified microorganisms can be prospectively utilised in the agricultural system, which can avoid the rapid decline in introduced microbial population and thereafter benefit the crops (Fig. 2B).

While improving reliability and predictability, the incorporation of transgenic or genetically modified microbes into farming systems remains controversial. Drawbacks include the limited survival of individual genotypes (clones) of microbes in the field and gene transfer risks between strains, which pose considerable uncertainty on the efficacy, survivability, and environmental hazards associated with any newly introduced genetically modified organism (Wang et al., 2011). In addition, a key component of introducing genetically modified organisms, requires continuous monitoring of their fate and behaviours. This is a serious limitation as monitoring methods are expensive, require highly specialised personnel, and are susceptible to biosafety restrictions. This along with

247 legislative prohibitions in many countries limit the mass release of genetic modified
248 organisms into the field and scientifically informed policy decisions are needed to
249 overcome these limitations.
250

251 ***2.4 Optimising delivery methods***

252 Both natural and genetically modified microbial species are promising, with many new
253 strains harbouring plant growth promoting (PGP) and biocontrol abilities documented
254 annually. However, basic and applied strategies of inoculum delivery often represent a
255 small proportion of the research effort, despite delivery being a fundamental aspect of the
256 bio-inoculation success. Indeed, up to 90% of introduced microbes can be lost during
257 application to the field, imposing considerable costs to the farming systems in terms of
258 labour and application rates and increasing the scepticism around the use of alternative
259 farming methods in modern agriculture (Vejan et al., 2016). Therefore, finding effective
260 tools to improve dispersion in fertiliser formulations and allowing the controlled release of
261 microbial inoculants can ensure feasibility, sustainability and commercial success of
262 microbe-mediated improvements on crops.

263 Seed bio-priming (i.e., seed coating with biological agents before sowing) has been
264 proposed and used as an effective method to improve the delivery of microbial inoculants
265 (Reddy 2012). Indeed, the plant-microbial interactions from the germination stage are
266 crucial for the later stages of plant development. Seed priming can thus be expected to have
267 profound effects on plant fitness, lasting throughout the entire plant life cycle (Mendes et al.,
268 2013). Consistently, in microbial-based seed bio-priming applications, a significant
269 increase in the microbial population applied on seed surfaces has been observed (Yadav et
270 al., 2018), resulting in an early activation of the priming inoculants before interacting with
271 pathogens in the spermosphere (i.e. seed surrounding zone, Pill et al., 2009).

Seed bio-priming with PGPRs have been reported to be effective in suppressing disease infection and inducing disease resistance in many agronomic and horticultural crops (Junges et al. 2016). Recent improvements to seed treatments, such as seed coating and pelleting (Halmer 2000; Goswami et al., 2017; Mei et al., 2017), have been experimentally tested to obtain longer shelf life, as well as increase viability and resistance against soil and seed-borne pathogens. These methods consist of binding seeds with liquid polymers, adhesives as well as pellets such as gelatin, starch, methylcellulose etc. Examples showed improvements in germination, seedling vigour and growth via seed coating for multiple plant species (Gholami et al., 2009; Rubin et al., 2017) and disease resistance (Jambhulkar and Sharma 2014) was also observed using the above-mentioned methods.

In parallel to seed bio-priming, better encapsulation methods could potentially improve the utilisation rate of microbe-based fertiliser and pesticides in the farming system. Encapsulation technologies have been established since the 1990s, and are based on the use of polymeric membranes in order to achieve a controlled release of nutrients in the soil (Trenkel 1997; Jarosiewicz and Tomaszewska 2003), resulting in improved fertiliser release rate, efficiency and moisture preservation. Similarly, microbial agents can be encapsulated for their use as biocontrol/plant growth promoting agents (Fig. 2C). This approach has been tested for the field-release of bacteria and fungi (John et al., 2011), resulting in the development of a vast array of solid and liquid formulations for the effective delivery of selected microbes, including promising emulsion techniques for concentrated bio-inoculant production and encapsulation (John et al., 2010). Micro-encapsulation and micro-composites of beneficial microbes with alginate and bentonite have been demonstrated to increase the efficacy of microbial inoculants within an agricultural setting (Tu et al., 2016; He et al., 2015). Major drawbacks related to high production costs, low variety and versatility of available encapsulated inoculants still limit the use of these formulations as a large-scale

alternative to traditional fertilisers in farming practices. However, most of the current studies reported positive effects of utilising these advanced formulations (reviewed in Bashan et al., 2014), with advantages including an improved microenvironment for microbial survival, physical protection for a prolonged period to prevent a rapid decline of introduced inoculants, and increased shelf life.

2.5 Key challenges and an emerging microbial inoculant toolbox in agriculture

Using emerging technologies to optimise the plant and soil microbiome for improved tolerance to abiotic (e.g. water, nutrient) and biotic (e.g. pathogens and pests) stresses is a promising approach to increase crop productivity. We envisage that the manipulative tools listed above, in conjunction with the optimisation of delivery methods, will significantly increase our ability to design stable, controllable, and persistent functions in agricultural microbial products. However, some key challenges and technical difficulties remain. As discussed in section 2.3, genetically modified technology remains a hotly debated topic from both the ethical and environmental perspective (Azadi and Ho 2010; Ma et al., 2018), which constrains large-scale use of genetically modified microbes in agriculture (Thakur and Sharma 2005). On the other hand, using improved indigenous microbes as inoculants seems an efficient approach, with comparatively lower biosecurity risks, with an increasing number of databases of microbiomes associated with crop species being developed and curated annually (Arjun and Harikrishnan 2011; Ellouze et al., 2013; Peiffer et al., 2013). However, more data collection and analyses are necessary to validate the efficiency and applicability of indigenous microbes within an agricultural context. Indeed, the soil and plant microbiome may change seasonally or under abiotic and biotic stresses (Barnard et al., 2015; Bérard et al., 2015; Smith et al., 2015), and shifts in the microbiome and its role in mitigating those stresses need to be established to ensure effective inoculation on plants.

For microbial delivery technologies, the optimised methods highlighted here have been widely used and are subject to constant research and improvement in the biotechnological industry. If cost effective, these approaches have the advantage of being user friendly and well-accepted by farmers. Moreover, such delivery methods along with improved formulations to include the development and selection of better carrier materials (reviewed previously by Sahu and Brahmaprakash, 2016) can support the use of genetically modified microorganisms and indigenous microbes by facilitating their application and survival in soil, enough to be sustainable in the farming system. However, additional studies are warranted before bio-delivery strategies, such as the ones proposed here, can effectively represent a reliable alternative to other methods. For example, while seed-priming has the advantages of being effective on plants, and requiring low cost and work input, it is still unclear to what extent this positive effect can be maintained after long-term storage and transportation. Similarly, encapsulation methods provide long-term effects by constantly releasing microbes into the environment, but the technology itself does not address the issue of the microbial survival and persistence in the soil.

Ultimately, the microbial product efficacy depends on complex multi-trophic interactions (e.g. plant-microbes; microbes-microbes), which regulates the plant response to microbial treatment. Factors underpinning such responses include the physiological and genetic potential of the microbial inoculants, the structure and function of the pre-existing plant and soil microbiome, and environmental variables, such as contextual environmental constraints (e.g., drought, salinity, pollution). We argue that a better understanding of the functions and dynamics of these associations are needed to enable long-term survival of microbial inoculants, and more accurate predictions of their fate and activity levels in the environment.

3. Harnessing the plant microbiome *in situ*

3.1 Recent advancement on plant microbiome studies

In parallel with the development of novel technologies to introduce desired functions into single or multiple microbial species, a novel research frontier in agriculture is represented by the alteration of plant-associated microbiomes *in situ* to improve plant performance (Mueller and Sachs 2015). Indeed, plant microbes occurring in the plants phyllosphere (above-ground compartments), rhizosphere (below-ground compartments) and endosphere (inside the plant tissues) play key roles to increase plant survival in constrained environments (Brugman et al., 2018). Most environmental microbes (>95%) are unculturable (Singh et al., 2009), implying that only a small proportion of the potentially beneficial microorganisms can be cultured and 'engineered' for use in agriculture. Thus, harnessing the intrinsic capabilities of the large proportion of indigenous plant microbiome can allow for the selection of novel and improved microbial functions.

A growing body of literature suggests that plants harbour species-specific microbial communities, defined as common microbial assemblages in different plant species (Shade and Handelsman 2012; Mendes et al., 2013). Members of microbiota that are systematically and consistently associated with a particular crop species under different environmental conditions comprise the so-called plant 'core' microbiome (Lemanceau et al., 2017). Several studies characterising the composition of the core microbiota in different crops, such as maize, rice and sugarcane, have reported up to hundreds of core microbial taxa occurring on each plant species (Peiffer et al., 2013; Edwards et al., 2015; Hamonts et al., 2018), with differences in composition being linked with plant functions (Lemanceau et al., 2017). Within the core microbiota of plants, the "hub" microbiota can be defined as members of the plant microbial community that can form strong facilitative and mutualistic interactions (Toju et al., 2018), and are central to the plants microbial community assembly (Bulgarelli et al.,

2013). Recent studies have highlighted the importance of such 'hub' microbes, are expected to play key roles in orchestrating assembly of other plant-associated microbiomes within and around host plants (Shade and Handelsman 2012). It is proposed that hub microbiota can be sourced in two ways: transmission and recruitment. Similar to human microbiota, where the ability of microbial communities to be transmitted from mother to offspring before, during after (via milk) birth has been widely demonstrated (Charbonneau et al., 2016; Pendse and Hooper 2016), plant seeds carry a large number of microorganisms, with a significant proportion of them having been transferred from the parent plant (Gundel et al., 2011). From the seed germination stage, in addition to the inherited microbiota, the release of targeted signals and root exudates may become key factors for plants to control the recruitment of associated microbes (Nelson 2004a; Philippot et al., 2013; Chaparro et al., 2014). The initial hub microbiota shaped by plant selection and filtering can then facilitate the formation of the core microbiota and overall assembly (Fig. 3).

Given the strong interaction between plants and their associated hub microbiota, the intrinsic capabilities of the large proportion of the indigenous plant microbiome to recruit beneficial microbes can be harnessed for the selection of novel and improved microbial functions. Hub microbes are usually identified based on the degree of their interactions, whereby their relationship and identities can be decoded via network analysis (Shade and Handelsman 2012; van der Heijden and Hartmann 2016), which can facilitate the process of engineering these critical members of the microbiome *in situ*. Indeed, network maps can highlight hub microbiota and their associated members carrying specific functions. These microbes can be targeted for isolation and whole genome sequencing to identify their functional capability. Thus, identifying hub microbiota and their influence on a plant microbiome will reveal target microorganisms responsible for important host–microbe–

microbe relationships and enable targeted interventions to promote plant growth or/and resist pathogen infection (Fig. 3).

Plant indigenous microbiomes can be altered *in situ* by artificially implementing naturally occurring ecological processes using microbial-, biochemical-, and molecular-based tools. We summarise below some promising approaches with the potential to speed up and improve our ability to exploit these crucial interactions to improve agricultural productivity.

3.2 Microbial-based strategies

The concept of, and preliminary experimental evidence, suggest a hierarchical organisation for the core and hub microbiota, where these central members of the community have an overarching role in regulating the growth and function of other members of the microbial assemblage (Alger et l., 2016; Niu et al., 2017). Hub microbiota can thus represent an appealing target for microbial manipulation in agricultural settings, where management of the order and timing of hub microbial arrival (i.e., priority effects) can be harnessed to enhance the recruitment of other beneficial members of the plant microbiome during early stages of plant development. We suggest here an approach inspired by Wei and Jousset (2017), where a microbial-based plant breeding method is used to engineer plant hub microbiome *in situ*. In this strategy, plant microbiota can potentially be modified by inoculating vertically-transmitted microbiota to the next generation using a step-wise approach. First, plant microbiomes are profiled using next generation sequencing technologies and core and hub microbes are identified using statistical and network analysis, respectively. Subsequently, strongly linked microbes harbouring critical functions (i.e. benefit to plant growth / pathogen defence) are determined using metagenomic sequencing (Fig. 4A). These highly connected hub microbiota can then be isolated from the plant and applied as a microbial cocktail sprayed on parental flowers, resulting in seeds enriched with specific hub microbiota before a

421 seedling is established. Recent attempts using similar flower inoculation strategies suggest
422 that microbes introduced at the seed stage are likely to survive and be passed to the next
423 generation (Mitter et al., 2017). This approach may be a promising alternative to exploit
424 priority effects in order to generate plants harbouring 'improved' plant microbiota (i.e., seeds
425 carrying a microbiome with increased capability to recruit beneficial microbes) (Fig. 4B).
426
427 ***3.3 Biochemical strategies***
428 Biochemical strategies to engineer indigenous microbiomes include the exploitation
429 of chemical compounds naturally produced by both plants and microbes to attract and
430 maintain hub microbiota or other beneficial microbiota *in situ*. For example, it is well known
431 that root exudates attract beneficial microbes or reshape microbiome assembly in the plant
432 rhizosphere (Berendsen et al., 2012; Doornbos et al., 2012; Stringlis et al., 2018), with
433 exudate production being promoted under environmental stresses (Berendsen et al., 2018;
434 Kwak et al., 2018). A recent study has shown the ability of plant volatile organic carbon to
435 attract pathogen-suppressing soil bacteria from long distances, suggesting the potential
436 application of engineering the soil microbiome using certain volatile organic substances to
437 enhance plant defence (Schulz-Bohm et al., 2018). Thus, by identifying which root
438 metabolites are associated with the proliferation of particular rhizosphere microbial
439 components, targeted root compounds can be purified or synthetised, and used to enhance the
440 plants ability to attract and maintain beneficial microbes and their activities in the rhizosphere
441 (Fig. 4C).
442 An alternative approach to enrich beneficial members of the plant microbiome results
443 from the exploitation of microbial systems similar to microbial quorum sensing mechanisms.
444 Quorum sensing is a population-density-dependent regulation of gene expression in
445 microbes, and has a recognised role in modulating collective microbial behaviours through

446 the release of complex arrays of communication (signal) molecules (Papenfort and Bassler
447 2016). These signal molecules can influence important ecological microbial functions,
448 including easing nutrient or niche acquisition, modulating collective defence against
449 competitors, and facilitating community escape in the face of population destruction (Badri et
450 al., 2009). Plants are also able to detect and be positively stimulated by microbial signals,
451 such as heat shock proteins and reactive oxygen species (reviewed in Vinocur and Altman
452 2005) arising in the rhizosphere during stress response (Bauer and Mathesius 2004). In the
453 context of microbiome *in situ* manipulation, signal molecules could be used to selectively
454 promote hub or beneficial microbes, increase microbial-mediated nutrient supply (e.g.,
455 fixation, mineralisation and mobilisation), or elicit a microbial-mediated response to
456 pathogens. Signal molecules could thus represent an effective tool to control plant–microbe
457 interactions for maximising resource availability and plant protection. However, our
458 understanding of identity, functions and mechanistic interactions of signal molecules used by
459 plants or microbes for these communications remain extremely limited, hampering our ability
460 to fully harness these promising tools. An increase in the sensitivity of spectroscopy-based
461 detection technologies, in parallel with the integration of metagenomics, metatranscriptomics
462 and metabolomics approaches, will be needed to better characterise their diversity and
463 specificity.
464
465 ***3.4 Molecular strategies***
466 In plants, host genetics plays a prominent role in determining the overall microbiome
467 composition, abundance or function (Turner et al., 2013; Horton et al., 2014), with many
468 plant genes and functions being correlated with variation in the plant microbiota across
469 environmental conditions (Brachi et al., 2017). Given this tight interplay between host
470 genome and microbiome structure, harnessing the associations between the microbiome and

471 the whole host genome could provide the basis to generate plants harbouring an improved
472 microbiome. Quantitative genetic tools, such as QTL (quantitative trait loci) mapping, can be
473 particularly useful in this sense, as they allow the identification of genes or genetic loci
474 underlying important biological traits (phenotypes) of any organism of interest (Collard et al.,
475 2005; McCarthy et al., 2008). Such an approach has been widely used in mice and human
476 microbiome research to identify genetic loci that influence specific microbial taxa or
477 pathways (reviewed in Kurilshikov et al., 2017), as well as to link environmental factors to
478 shifts in microbial composition (Spor et al., 2011). This suggests a great potential for this
479 technology to generate improved plant varieties either via genetic engineering or traditional
480 plant breeding approaches. Once QTL or genetic traits of crops, which mediate the
481 interactions between crop and beneficial microbiomes, are identified, these can be used to
482 generate new and improved crop varieties which can potentially attract and harness beneficial
483 indigenous microbiota. With advent of CRISPR/Cas9 technology, such approaches hold great
484 potential to improve or induce the ability of plants to preferentially recruit beneficial
485 microbiota (Schaeffer and Nakata 2015) (Fig. 4D). A similar approach can be used to
486 manipulate the initial colonisation by hub microbiota which may ultimately shape the core
487 and whole plant microbiomes in predictable fashions. In addition, if genetic pathways for
488 microbe-microbe signalling (see section 3.3) or biochemical pathways (section 3.4) can be
489 identified, these genes can be transferred into crops to elicit beneficial response from plant
490 microbiomes.
491
492 ***3.5 Future perspectives for microbiome engineering in situ***
493 Plant microbiomes play critical roles in crop development and health. Thus, maintenance of a
494 healthy microbiome would benefit the crop growth and yields in the farming system. Hub
495 microbes in particular show great potential in overcoming many of the issues associated with

the selection, survival and maintenance of plant-associated beneficial microbes. However, our current ability to harness the plant microbiome in agriculture, and to manipulating microbiomes *in situ* remain limited, and more studies and trials are needed to increase our understanding of the nature and mechanisms underpinning the hub microbiota-plant relationship before such an approach can be applied and commercialised at large scales. One major challenge in this regard is addressing the effect of abiotic or environmental factors, which can influence the activity of hub microbes (Santoyo et al., 2017; Vacher et al., 2016). Better designed and more informed frameworks integrating ecology, physiology, genetics and genomics of both host and microbiome (holobiome), in conjunction with better characterisation of the environmental context in short- or long-terms, will be critical to harness beneficial microbes in agriculture. Additionally, concentrated efforts to identify the core and hub microbiota (both structural and functional), biochemical signal molecules, and molecular markers involved in the plant-microbial and microbe-microbe interactions, can provide effective tools for microbiome manipulation. Future research should also include methodical isolation of these hub microbiota, and *in situ* testing for their use as microbial inoculants. From a practical perspective, we envisage that the identification of stable, stress tolerant microbiomes able to improve crop productivity in different soil types and climates (Mueller et al., 2016) would be extremely helpful to advance sustainability in agriculture.

4. Concluding remarks

Based on the above discussion, three potential approaches have been highlighted for the purpose of enhancing the use and impacts of plant beneficial microbial inoculants: (i) selecting indigenous microbes as inoculants, ii) improving microbial inoculants using genetically modifying technologies, and iii) optimising microbial delivery methods, which

can improve survival, activities and efficacy of microbial inoculants. We propose that microbial engineering *in situ*, using microbial-, biochemical-, and molecular- based approaches targeting the hub microbiota can provide the most effective outcomes in the long-term. These approaches can provide tools for predictable changes in microbiome structure and functions. For example, if crop growth is P-limited, biochemical molecules (i.e. signal molecules derived from the plant or microbiome) to activate P-solubiliser activity could be sprayed directly onto the crop root zone to release fixed or organic P for plant uptake. There are other approaches which are being tested globally but not covered in this article, however, we propose that our article provides a good starting point for an initial debate, and for concerted global effort to harness biotechnological-based solutions for current challenges associated with agriculture productivity. To improve these strategies, establishing a global database of plant microbiomes and their response to biotic and abiotic stresses will be an important milestone towards successful translational research. Such databases can help (1) assess and predict local conditions to identify and apply effective microbial consortium used as inoculants, and (2) develop future tools for *in situ* microbiome engineering for sustainable increase in farm productivity, food security and environmental sustainability. However, to achieve these goals, significant resource inputs from both public and private sectors, and globally coordinated approaches are needed to fill critical knowledge gaps and develop an efficient translational research pipeline. In addition to these emerging approaches, if issues linked to regulatory and policy development, and social acceptability of microbial/ microbiome products can be simultaneously addressed, these bio-based tools can potentially contribute significantly to the sustainable increase in agricultural productivity.

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Figure 1. Beneficial Plant-Microbe Interactions. An immense number of microbes are living in the rhizosphere soil, on leaf surfaces and in the plant endosphere. These microbes have intimate interactions with the entire life of plants and serve important host functions that are mainly involved in plant nutrient provision and enhancement of plant defence. The presence of microbes in rhizosphere soils could increase the availability of soil nutrients. Additionally, certain plant microbes could induce the plant primed conditions that allow plants to quickly respond to pathogen and pest invasions. Dynamic changes in the soil and plant microbiome and structure will influence the functions that are delivered to the plant host. Under biotic and abiotic stresses, plants could actively modify their physiological conditions, that change the root exudate profile and manipulate their associated microbiome. There are also complex interactions between above and belowground plant microbes, which directly or indirectly influence plant health.

Figure 2. Strategies to improve microbial inoculants and inoculation. A. Isolation, selection and application of beneficial indigenous microbes. Plants with the best phenotype under environmental stresses are selected. Microbes isolated from the plants rhizosphere are screened, and species with negative effects on crops are removed. The remaining beneficial microbes are applied onto the plant roots. Plant phenotyping and following steps are repeated for several generations. A similar approach can be employed to isolate hub microbiota and their host functions can be identified by inoculating with different

568 combinations of hub microbiota and the combination which provide best outcomes for plant
569 health can be developed as microbial inoculants. **B. Utilisation of genetically modified**
570 **inoculants.** Microbial genes are modified with gene editing tools (RNAi, CRISPR/Cas9, etc.)
571 to achieve a specific purpose (e.g. gene silencing, adding microbial functions, adaptation to
572 local environment, etc.). Genetically modified microbes (GMMs) harbouring the desired
573 functions are inoculated to promote plant growth and/or pathogen resistance. While wild type
574 microbes can be significantly reduced after application to the soil due to poor adaptation to
575 the local soil and plant and microbe selection, GMMs are expected to be better adapted to the
576 local environment. **C. Improved delivery method of microbial inoculants.** Microbial
577 inoculants can be delivered by several approaches including encapsulation. Encapsulation
578 methods provide a slow release approach of the microbial product, which could ensure the
579 survival and supply of microbial inoculants for an extended period of time if adequate
580 resources (pre-biotics) are provided within formulations.
581
582 **Figure 3. Core and hub microbiota: mode of transmission.** During the germination stage,
583 seeds release exudates that attract specific microbes in the soil environment. Simultaneously,
584 seed microbiota inherited from parental plants, co-effect with exudates to regulate initial
585 microbial assemblages. In the early seedling stage, the initial hub microbiota are developed
586 and recruit the linked microbes to the plant rhizosphere. Plants continue releasing root
587 exudates to select and filter microbes in the soil environment. Initial hub microbes along with
588 plant root exudates shape the microbial assemblages including plant hub microbiota, core
589 microbiota and other microbes associated with plants.
590
591 **Figure 4. Microbiome engineering *in situ*.** As hub microbiota can influence other linked
592 microbes in the environment, manipulating hub microbiota can largely and efficiently

593 optimise microbial networks. Meta-omics tools and network analysis can be used to identify
594 the hubs that link with more beneficial microbes. To enrich these hub microbes in the plant
595 microbiome we suggest three different approaches could be developed: microbial tools,
596 biochemical tools and molecular tools.
597 **A. Microbial tools optimising hub microbiota.** First, key hub microbes are isolated and
598 sub-cultured under laboratory conditions. Second, cultured hub microbes are transferred to
599 seeds by, for example, spraying to the parental flowers according to Mitter et al. (2017).
600 Parental plants can transfer the sprayed microbes to offspring seeds, thereby passing them to
601 the next generation. **B. Biochemical tools optimising hub microbiota.** Under certain
602 environmental stresses, plants release specific chemical compounds (e.g. signal molecules in
603 root exudates) into the rhizosphere to actively attract certain microbes. Multi-omics
604 approaches (e.g. metagenomics, metabolomics, etc.) are then used to reveal microbial taxa
605 that are increased in abundance and over-secreted chemical compounds during disease
606 infection or abiotic stress. Thereafter, the chemical compounds can be extracted and their
607 properties and interactions with plants and microbes investigated. Synthesised compounds
608 can be added to crops to attract/favour the growth of beneficial microbes. **C. Molecular tools**
609 **optimising hub microbiota.** Plant genomes can affect their associated microbes, including
610 the assemblages of hub microbiota. Thus, modifying plant genomes can potentially optimise
611 plant hub microbes. In this molecular approach, targeted functional genes linked to hub or
612 beneficial microbes could be identified with QTL mapping, followed by use of traditional
613 breeding or genetic modification using modern genetic editing tools (e.g. CRISPR/Cas9) can
614 be used to develop improved crop varieties. Improved crop varieties are then expected to
615 recruit hub microbiota which facilitates the assembly of more plant beneficial microbes.
616
617

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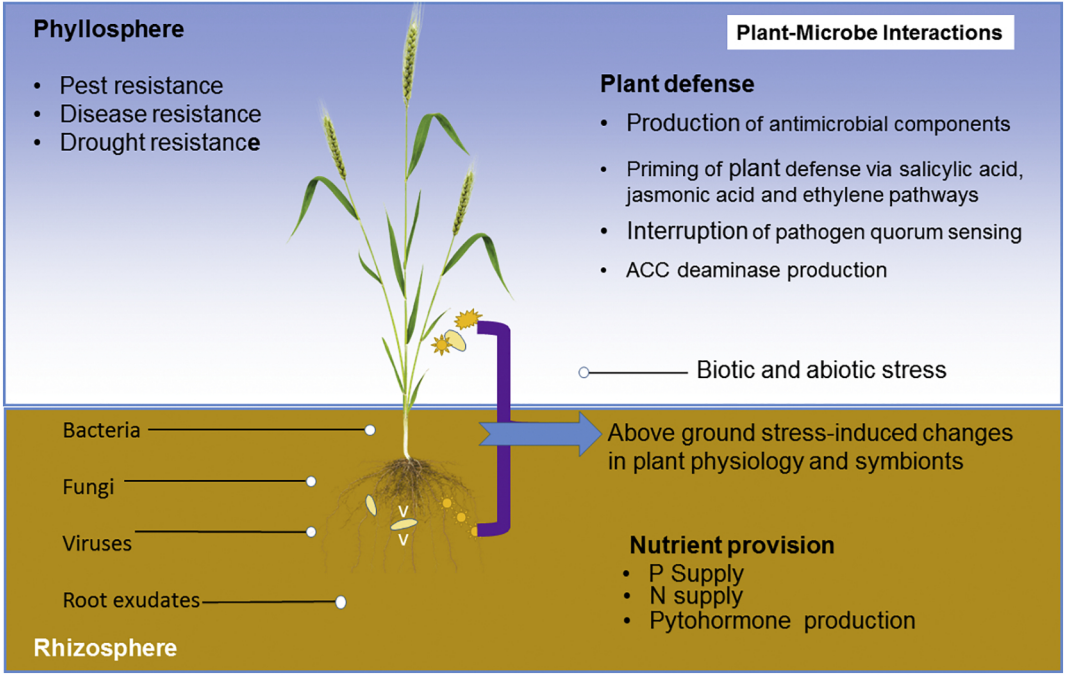
Phyllosphere
• Pest resistance
• Disease resistance
• Drought resistance
Plant-Microbe Interactions
Plant defense
• Production of antimicrobial components
• Priming of plant defense via salicylic acid, jasmonic acid and ethylene pathways
• Interruption of pathogen quorum sensing
• ACC deaminase production
Biotic and abiotic stress
Bacteria
Fungi
Viruses
Root exudates
Above ground stress-induced changes in plant physiology and symbionts
Nutrient provision
• P Supply
• N supply
• Pytohormone production
Rhizosphere

Figure 1

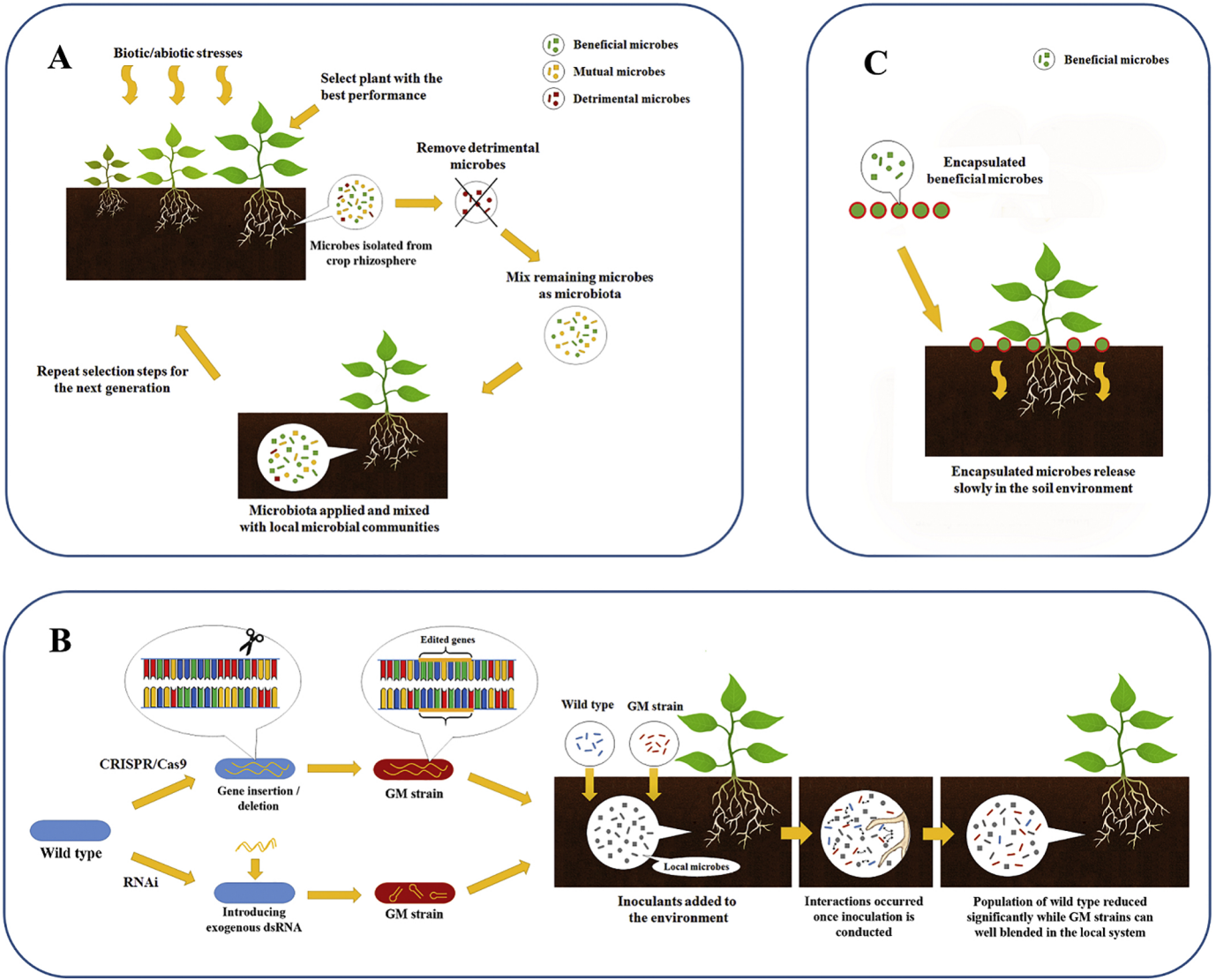
A
Biotic/abiotic stresses
Select plant with the best performance
Beneficial microbes
Mutual microbes
Detrimental microbes
Remove detrimental microbes
Microbes isolated from crop rhizosphere
Mix remaining microbes as microbiota
Repeat selection steps for the next generation
Microbiota applied and mixed with local microbial communities
C
Beneficial microbes
Encapsulated beneficial microbes
Encapsulated microbes release slowly in the soil environment
B
Edited genes
CRISPR/Cas9
Gene insertion / deletion
GM strain
Wild type
RNAi
Introducing exogenous dsRNA
GM strain
Wild type
GM strain
Local microbes
Inoculants added to the environment
Interactions occurred once inoculation is conducted
Population of wild type reduced significantly while GM strains can well blended in the local system

Figure 2

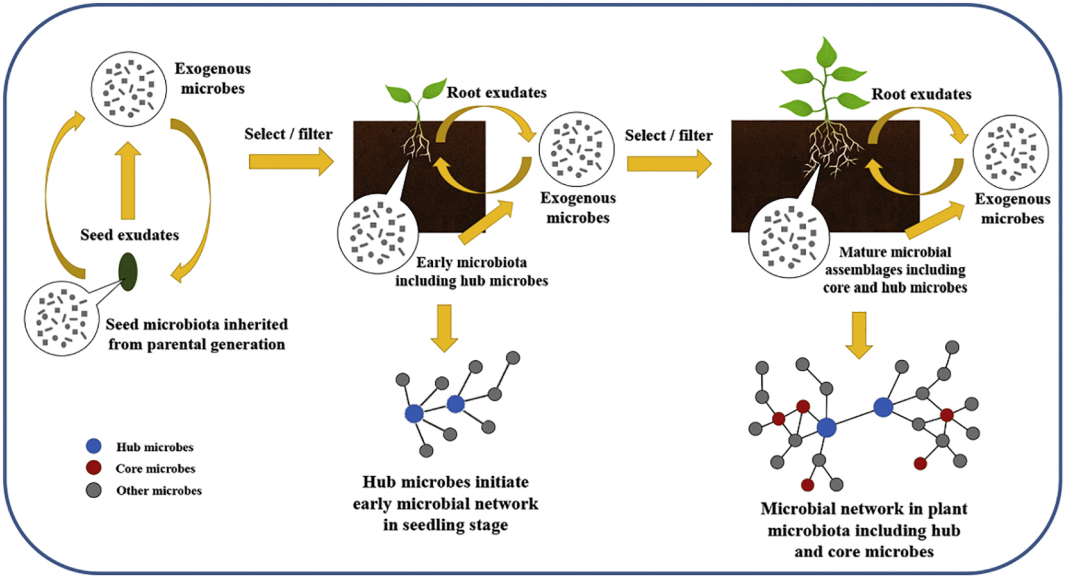
Exogenous microbes
Seed exudates
Seed microbiota inherited from parental generation
Select / filter
Root exudates
Exogenous microbes
Early microbiota including hub microbes
Hub microbes initiate early microbial network in seedling stage
Select / filter
Root exudates
Exogenous microbes
Mature microbial assemblages including core and hub microbes
Microbial network in plant microbiota including hub and core microbes
Hub microbes
Core microbes
Other microbes

Figure 3

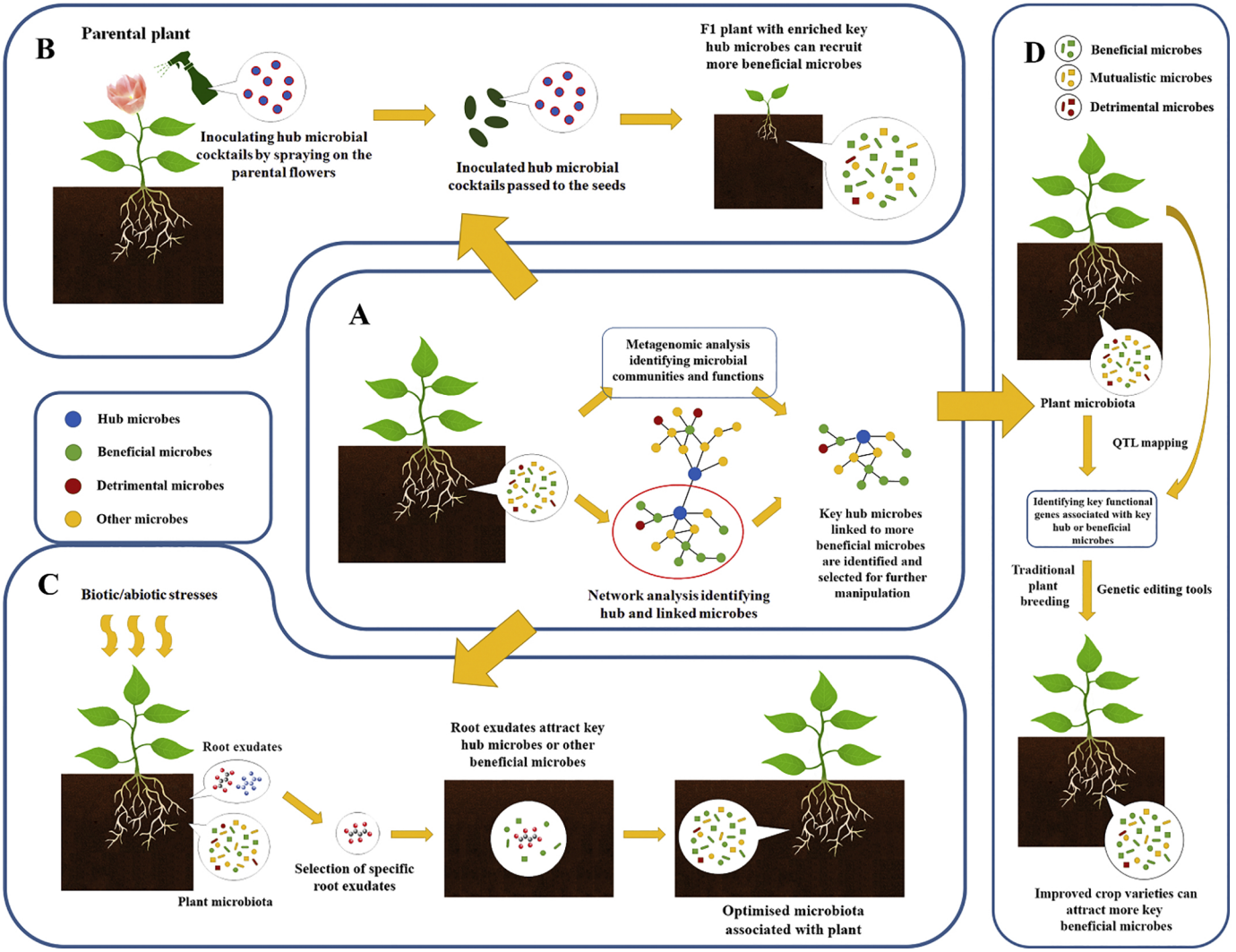
B
Parental plant
Inoculating hub microbial cocktails by spraying on the parental flowers
Inoculated hub microbial cocktails passed to the seeds
F1 plant with enriched key hub microbes can recruit more beneficial microbes
A
Metagenomic analysis identifying microbial communities and functions
Network analysis identifying hub and linked microbes
Key hub microbes linked to more beneficial microbes are identified and selected for further manipulation
Hub microbes
Beneficial microbes
Detrimental microbes
Other microbes
C
Biotic/abiotic stresses
Root exudates
Plant microbiota
Selection of specific root exudates
Root exudates attract key hub microbes or other beneficial microbes
Optimised microbiota associated with plant
D
Beneficial microbes
Mutualistic microbes
Detrimental microbes
Plant microbiota
QTL mapping
Identifying key functional genes associated with key hub or beneficial microbes
Traditional plant breeding
Genetic editing tools
Improved crop varieties can attract more key beneficial microbes

Figure 4