

Microbial inoculum development for ameliorating crop drought stress: a case study of *Variovorax paradoxus* 5C-2

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Abbreviations

PGPR: plant growth promoting rhizobacteria

ABA: abscisic acid

ACC: aminocyclopropane-1-carboxylate

acdS: aminocyclopropane-1-carboxylate deaminase

QS: quorum sensing

AHL: acyl homoserine lactone

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ACC deaminase; ethylene, pan-genome, rhizosphere colonisation, *Variovorax paradoxus*, sustainable agriculture, climate change

Abstract

Drought affects plant hormonal homeostasis, including root to shoot signalling. The plant is intimately connected below-ground with soil-dwelling microbes, including plant growth promoting rhizobacteria (PGPR) that can modulate plant hormonal homeostasis. Incorporating PGPR into the rhizosphere often delivers favourable results in greenhouse experiments, while field applications are much less predictable. We review the natural processes that affect the formation and dynamics of the rhizosphere, establishing a model for successful field application of PGPR utilizing an example microbial inoculum, *Variovorax paradoxus* 5C-2.

Introduction

The world faces a major water crisis as a consequence of unsustainable human water withdrawal (primarily for irrigation) and alterations in natural hydrological cycles through agriculture, industrial and urban activities [1, 2]. Climate change will stimulate additional water demands in many agricultural regions [3]. The need to increase food production by at least 50% to feed an increasing population with greater wealth and food demands, could create feedbacks increasing the loss of freshwater quantity and quality [4]. Consequently, changes in agricultural practices are needed, based on multi-disciplinary scientific research and working in cooperation with farmers and policy-makers.

Knowledge from basic and applied sciences can be employed to fulfil the needs of modern producers. Although classical plant breeding methods have seen extensive application culminating in the development of more drought-tolerant crops, both ethical and practical limitations have constrained the development of genetically modified (GM) drought-resistant crops [5]. Specifically, drought tolerance is usually regulated by finely synchronized multi-gene systems that are difficult to manipulate [6]. Moreover, logistical difficulties occur in producing GM crops when considering variations of drought tolerant genes networks between different species, or even among different cultivars [6, 7, 8].

Another biotechnological option has been to use soil-dwelling microbes that interact with plant root systems, namely plant growth promoting rhizobacteria (PGPR) [9], which dwell on, and sometimes in, plant roots and confer stress tolerance via multiple mechanisms [10, 11]. This review is focused on utilising PGPR to enhance crop fitness under drought conditions, especially since they are more effective in promoting growth and crop yields under drought conditions. By presenting a case study of the development of an exemplar PGPR, namely *Variovorax paradoxus* 5C-2, as a microbial inoculum, key concepts are introduced to help match an agricultural scenario with successful PGPR activity.

Plants adapt to changing soil water availability by “measuring” soil water status [12, 13]. When the soil water status is low enough (below -0.05 MPa), root metabolism changes. Transcriptomics data [14] and headspace analysis [15] indicate that plants respond to abiotic stresses by producing the volatile plant hormone ethylene, which is aerially transported from one

plant to another plant within a community. Elevated ethylene biosynthesis decreases yield by inhibiting growth, photosynthesis and seed set [16]. Although ethylene promotes fruit ripening, it accelerates leaf senescence and inhibits leaf growth. Ethylene also induces lateral cell expansion in stems and roots. In addition, ethylene increases root hair density and length [17], but inhibits primary root growth [18]. Ethylene-mediated growth inhibition has made the genetic, chemical or microbial manipulation of ethylene levels *in planta* an attractive biotechnological target.

ACC as a key inter-kingdom signal molecule

In terrestrial plants, ethylene biosynthesis involves the methionine metabolic pathway (**Figure 1**) [14]. In this process, the conversion of 1-aminocyclopropane-1-carboxylate (ACC) to ethylene is regarded as a rate-limiting step. Moreover, ACC can also be bound with organic compounds to produce malonyl-ACC (M-ACC), γ -glutamyl-ACC (G-ACC) and jasmonoyl-ACC (JA-ACC). Additionally, ACC can be transformed by plant [19] and microbial enzymes known as 1-aminocyclopropane-1-carboxylate deaminases (ACC deaminase, gene notation *acdS*) into 2-oxobutanoate and ammonia. Hence, ACC is a central biochemical player in integrating plant and microbial (inter-kingdom) metabolic networks and its root to shoot signalling is an important component of plant adaptation to drought [20].

Although the root system is regarded as the primary sensor of soil conditions, microorganisms inhabiting the surface of, or inside, the root can influence root-to-shoot signalling processes [9, 10, 11]. Indeed, some PGPR producing ACC deaminase modulate root ACC levels and hence root [11] and shoot [21] ethylene evolution. When plants grown in drying soil were exposed to bacterial ACC deaminase, they developed a longer root system and had increased biomass and yield [22] suggesting this plant-microbe interaction is of adaptive significance in increasing plant drought tolerance. Interestingly, wild barley (*Hordeum spontaneum*) plants exposed to more stressful environments in natural settings have a higher proportion of ACCd-containing rhizobacteria on their root systems [23]. Moreover, withholding irrigation from wheat (*Triticum aestivum*) plants increased the relative abundance of *acds*-containing rhizobacteria on the root system [24]. Understanding how plants regulate the recruitment of such PGPR represents a potential biotechnological opportunity to enhance plant drought tolerance.

Agricultural practices and climate warming decrease soil biodiversity, community composition and activity, affecting the delivery of ecosystem services [25, 26]. The important meaning of richness and diversity in a given soil community lies in the number of species that undertake specific ecosystem services such as inducing plant drought fitness. The rhizosphere can be defined as the soil adjacent to the root systems that is biologically, chemically and physically influenced by the root system, distinct from the remaining bulk soil that is not [27, 28]. The rhizosphere is enriched, both in numbers and in the proportion of certain taxa, with certain soil microbes compared to the bulk soil “starting” community composition. Evidence based on qPCR of all *acdS* alleles and Illumina sequencing of *acdS* from DNA of rhizosphere of different *Poaceae* species (including maize) shows that contrasting plant genotypes preferentially recruit *acdS* alleles from the bulk soil [29, 24]. When microbial biodiversity is impaired, plant selection of different *acdS* alleles may be compromised. Efforts to understand the regulation of rhizosphere microbial dynamics is fundamental for the compatible application of PGPR to drought-stressed crops.

Characteristics and dynamics of the plant holobiont

Each plant species and its habitat have a unique consortium of microbes, the microbiota [30, 31]. Altogether the microbiota’s genetic information is referred as the microbiome, and the concurrence of the host organism with the microbiota is the “holobiont”. Although each organism presents its own independent mechanism of survival, their co-evolution coordinates their metabolic processes resulting in increased fitness of the holobiont. Moreover, the holobiont possesses its “hologenome”: the aggregate of microbiome and host genomes. Such integration of genetic material from diverse organisms results in the formation of superorganism genetic systems. Therefore, altering the composition of the holobiont may potentially affect inter-kingdom signalling thereby changing fitness/growth of the host [30].

High throughput sequencing surveys of many rhizospheric microbiota show consistent repetition (at phylum level) between different plant species. Bacteria encompass 90% of the holobiome and the dominant phyla are the Proteobacteria, followed by Actinobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, and Acidobacteria [11]. Nevertheless, different bacterial species occur even between different plant cultivars growing in the same soil [32].

Bacterial population dynamics (species composition and number) are strongly influenced by their environment, such as soil water availability. Soils affected by drought events tend to be enriched with bacteria that tolerate desiccation. The spore-forming phyla Actinobacteria and Firmicutes are described as drought-resistant [33], even though several members of the Actinobacteria are drought-sensitive [34]. While the Verrucomicrobia are regarded as drought-tolerant, they have not yet been described as having ACC deaminase activity. Thus while there is no absolute association of ACC deaminase and bacterial drought-tolerance, drought-stressed plants tend to select for the occurrence of *acdS* alleles in their rhizosphere [29, 24]. Consequently, there has been much interest in isolating and culturing free-living PGPR containing the ACC deaminase gene [11]. When developing microbial inocula for agriculture, it is crucial to understand the factors influencing PGPR activity in agricultural ecosystems to mitigate plant drought stress responses.

Challenges of using PGPR to alter hormonal dynamics *in planta*

Soil inoculation with PGPR to promote crop yields has delivered some promising results. Furthermore, these techniques are environmentally friendly, since inoculant impacts on rhizospheric community dynamics are either positive, i.e. cooperative or synergistic, or null [35]. Applying PGPR has generally been accepted by farmers, since there has been a long history of using rhizobacterial inoculants, with the first patent granted in the USA in 1896 for inoculating leguminous field crops with nitrogen-fixing *Rhizobium* spp [36, 11]. The first non-symbiotic microorganism (*Azospirillum*) to promote plant growth was commercially released in 1981 [36]. However, most PGPR products are marketed as bio-control (70%) and bio-fertilizers (25%), with only 5% formulated to enhance plant stress tolerance [36]. Nevertheless, PGPR inoculation sometimes fails under field conditions, often due to diminished fitness of the inoculum in competing with native microbial communities [37, 38]. Successful inoculation of the soil requires that it supplies the minimum bacterial physiological requirements to grow and colonize the appropriate niche: the rhizosphere, rhizoplane or plant root cells.

Three major environmental components drive the holobiont assemblage in agricultural plant hosts. Firstly, soils determine both physical and chemical characteristics of environmental

niches for bacteria, via variation in structure, porosity, texture, nutrients, and pH [39, 40]. Secondly, plant genotype governs the quantity and quality of nutrients and chemicals signals accessible to the microorganism. Finally, agricultural practices/field history (e.g. application of herbicides and soil amendments such as lime, manure and microbial inoculants) will affect microbial populations and community diversity [40].

To demonstrate how these environmental components affect the success of microbial inoculation, an example PGPR bacterium, *Variovorax paradoxus*, is used. This species shows valuable traits such as osmotic stress resistance, resistance to some biocidal compounds applied in agriculture and high competitiveness [41, 42, 43], which will be advantageous when it is applied to a field in which there is limited information concerning its management history.

The genetic diversity of *V. paradoxus* was probed by studying the total collection of genes within the genomes of seven *V. paradoxus* strains, the pan-genome [44], containing an average of 6000 genes (Table 1). The pan-genome of *V. paradoxus* strains shares 3400 genes in common, the core genome, but also has an average of 2000 genes shared by some strains, the variable or accessory genome, and an average of 65 unique genes for each strain [45]. The percentage of genes covering functions in metabolism of amino acids, carbohydrates, lipids, xenobiotic biodegradation and motility are shared by both core, variable and unique pan-genome (Figure 2). Thus pan-genomes and core genomes of *V. paradoxus* strains are functionally linked to the metabolic versatility and plant growth promotion traits. In particular, multi-drug resistance features present in *V. paradoxus* confer pesticide-antibiotic cross-resistance, implying the organism can adapt to different environmental constraints [42, 46].

V. paradoxus 5C-2 was first isolated from the roots of *Brassica juncea* L. Czern plants growing in soils contaminated with cadmium, based on its growth of ACC as a sole nitrogen source [43]. It is a facultative oligotrophic, Gram negative bacterium that can grow in both nutrient-poor and nutrient-rich media. To compete, endure and settle in the rhizosphere, *V. paradoxus* 5C-2 displays quorum sensing (QS). In addition, *V. paradoxus* 5C-2 may shape the bacterial population around the root as it can also degrade QS signals and utilize AHLs as a carbon source (A.A. Belimov, unpublished data). The N-Acyl homoserine lactone lactonase (AIIA) activity of *V. paradoxus* provides “quorum quenching”, allowing the bacteria to degrade

and grow on acyl-HSL signals [43]. *V. paradoxus* 5C-2 has multiple PGPR features including the synthesis of indole-3-acetic acid (IAA), the expression of ACC deaminase activity [43] and enhancing nutrient uptake and partitioning *in planta* [47].

Successfully applying *V. paradoxus* 5C-2 as a soil inoculant will depend on its adaptive capacity to new, potentially hostile soil conditions, e.g. temperature, pH and water content, and competition with native organisms [48]. Ultimately, the physiological needs of the inoculum must match those it will encounter in the new environment.

Soil temperature

Soil temperature is one of the most important factors affecting the dynamics of the rhizosphere [49, 50]. Microbial activity rate doubles for every 10°C rise in temperature until a point, depending on the temperature sensitivity of the microorganism, when high temperatures disrupt enzyme structure and cellular material. Moreover, temperature changes the physical properties of the environment such as surface adhesivity, thus affecting microbial motility [49]. Soil temperature depends on the irradiation energy, soil reflectivity (albedo), rainfall and topology, and increases during drought events when surface evaporation is decreased by low soil water content [51]. Soil temperature decreases with depth, which may influence the inoculation technique used in arid environments.

V. paradoxus 5C-2 is a mesophile microorganism, with optimal growth at 28°C (Figure 3A). However, maximum motility was measured at 30°C, with no measurable motility at temperatures less than or equal to 15°C, or more than or equal to 37°C (Figure 3A). This information helps determine the correct ecosystem climate zone and time of the year to apply this bacterium to encourage its proliferation [52]. It will proliferate when spring-summer minimum soil temperatures average 18°C, but local agricultural practices that trigger temperatures above 28°C should be avoided. In crops grown in black plastic mulch to minimise weed growth, surface soil temperature can exceed 28°C [53], thus *V. paradoxus* 5C-2 inoculation may be ineffective when plants are mulched.

In agro-ecosystems from lower latitudes, drought events combined with high air temperatures trigger surface soil temperatures higher than 40°C in more than half the soils in

Europe [49]. In this scenario, thermophile PGPRs may be better able to establish following inoculation, depending on the method of inoculation. Since most of the PGPRs currently isolated are mesophile, there is increasing interest in isolating thermophilic PGPRs from desert environments [54]. *V. paradoxus* isolation from such environments suggests that it manages to find a temperature niche that allows its proliferation.

Soil pH

In agricultural soils, pH determines the availability of nutrients and metals, affecting microbial composition and biomass [55]. Bacterial species exhibit various tolerances to pH, with most growing in a pH range between 6 and 9, but generally growing best at neutral conditions [48]. pH effects on bacterial motility and fitness will mediate the rhizosphere bacterial community. Alkaline pH (>8.5) suppresses bacterial motility [56], while catabolic reactions that consume acids are favoured at acidic pH (<6.5). Alkaline pH represses chemotaxis systems, perhaps due to their energetic cost [57].

Of those pHs tested, minimal growth of *V. paradoxus* 5C-2 occurred at pH 4 and pH 9 (Figure 3.B). *V. paradoxus* 5C-2 g with growth and motility maximal between 5 and 7 (Figure 3.B). It resisted acidic pHs, because the growth and motility curve was high between pH 5 and 8 and skewed towards an acidic range (Figure 3.B). This optimal pH embraces the range recommended by agronomists (pH 6-7) for good crop nutrition. Nevertheless, highly leached tropical soils are often acidic, which mobilises aluminium in the soil solution which can be toxic for plant growth and inhibits root elongation [58]. Since ethylene is involved in root growth inhibition under aluminum toxicity, the possibility that *acdS*-containing rhizobacteria such as *V. paradoxus* 5C-2 can overcome Al-mediated root growth inhibition [59] merits further research.

Soil water availability

Bacteria maintain a certain cellular water content for optimal metabolism and soil water availability is perhaps one of the most influential factors that shape a microbial community [50]. Drought destabilises the bacterial community network, changing the trophic network dynamics [34]. The community tends to reorganize their populations towards drought tolerant bacteria (phylum Verrucomicrobia and the class Alphaproteobacteria). The bacterial semipermeable outer

membrane allows rapid equilibration of cellular contents with soil water, consequently soil drying, which decreases water potential, decreases bacterial internal water potential. Bacterial accumulation of solutes, or osmolytes, is one survival strategy to maintain a favourable water potential gradient, but leaves the bacteria riskily dependent on nutrient availability [60]. Drought-induced morbidity can result from damage to the outer cell membrane, which may explain the higher resistance of Gram positive bacteria to desiccation. This effect varies between species and should be determined for each bacterium [61].

Since *V. paradoxus* 5C-2 lives naturally in the rhizosphere, it can be exposed to a wide range of water potentials, which may be as low as -10 MPa [62]. To simulate the effects of soil drying on bacteria, glycerol was added to TSB medium to impose a range of osmotic potentials (-0.14 to -12 MPa). There was no significant effect on *V. paradoxus* 5C-2 survival but the bacteria were not motile (Figure 3.C). Indeed, motility steadily decreased from -0.17 MPa to -0.24 MPa and bacteria were immobile below -0.54 MPa. After cells were exposed to different osmotic potentials in liquid media for 12 hours, motility tests were conducted to study whether *V. paradoxus* 5C-2 survives at low osmotic potential but loses its ability to resume motility (Figure 3D). Exposure to even the lowest osmotic potential tested (-12 MPa) did not diminish subsequent *V. paradoxus* 5C-2 motility.

Compared to some other bacteria, *V. paradoxus* 5C-2 seems more resistant to osmotic shocks. In *Rhizobium* sp., the growth is minimal at -5 MPa [63] while this osmotic stress had no effect in *V. paradoxus* 5C-2. Lower osmotic tolerance of *R. leguminosarum* is correlated with its production of low molecular weight external polysaccharides (EPS). High-molecular-weight EPS increase water retention around the bacteria cell wall thus protecting against low external osmotic potential [64], [65]. While decreased rhizobial motility and growth at low soil water potentials may decrease nodulation frequency [66], co-inoculation with *V. paradoxus* 5C-2 partially alleviated negative effects of drought on nodulation [22]. Taken together, these data suggest that *V. paradoxus* 5C-2 survives drought conditions, and recovers its motility when a favourable water regime is re-established, which may assist rhizosphere colonisation following inoculation of plants grown in drying soil.

Plant influences on the microbial community

Rhizosphere microbial activity can influence plant nutrient status while, *vice versa*, plants can also influence the physiology of microbial communities with their root exudates [67]. Root exudates vary from one species to another and are affected by plant age and nutrient status [68]. Plants may invest more than the 20% of the total carbon fixed in photosynthesis in rhizosphere carbon efflux [40]. This can benefit the plant if root exudate composition 'selects' beneficial microorganisms that colonize the roots. Soil porosity will affect the amount and distance which root exudates can travel [48], in turn affecting the chemo-attraction of the rhizobacteria. Since bacterial response time, or detection limit for root exudates, may alter bacterial fitness, bacteria utilize chemotaxis to assess a cocktail of exudates.

Root exudation varies along the root surface, determined by different root cell characteristics, thus affecting rhizospheric chemical composition from tip to base [69]. Moving away from the root tip defines different zones such as the root tip, with the root cap and the meristem, the elongation zone without any cell division, the maturation zone with differentiated xylem vessels and root hairs and finally the mature zone with dead root hairs (Figure 4C). For maize, root surface pH changed along the root axis. Root tip pH was 7.6 and decreased linearly within the first 4 mm distance by 0.75 units and then increased linearly by 0.50 pH units [70]. This is consistent with the release of organic anions, which will attract different microorganisms. *Pseudomonas* species preferentially colonise the root tip and the elongation zone of wheat [71]. *Rhizobium leguminosarum* and the fungal pathogen *Nectria haematococca* are attracted to the maturation zone of pea roots [72] while *Bacillus sp.* preferentially colonize the *Arabidopsis* root elongation zone [73]. Confocal microscopy is often used to determine the location of fluorescently labelled bacteria on the roots of plants grown in artificial media *in vitro* [71]. Alternatively, the numbers and distribution pattern of *V. paradoxus* 5C-2 along the roots of both pea (*Pisum sativum* cv. Progress No. 9) and corn (*Zea mays* cv. ZB677) was measured, since inoculation increased growth of both species [22, 74]. Colonization of pea roots was minimal near the tip and maximal near the base of the root where root hairs were present (Figure 4A, C). In contrast, colonization of corn roots showed no specific spatial distribution and was 10-fold lower than in peas (Figure 4B). Differences in the relative concentrations of organic components

such as amino acids and sugars exuded from corn and pea roots [75] were correlated with bacterial attraction to the roots of each species.

***V. paradoxus* 5C-2 dispersal in a soil mesocosm**

Like other bacteria, *V. paradoxus* can form biofilms on the plant root [76]. Bacterial dispersal occurs when they detach from the biofilm, switching to a planktonic lifestyle allowing new plant roots to be colonised. The dispersal process is complex and can be regulated by nutrient status and QS signals. It involves several steps such as the up-regulation of flagellar and chemotactic proteins and down-regulation of EPS [77]. Thus bacterial dispersal was tested in a mesocosm with field soil to determine bacterial movement in the rhizosphere and bulk soil.

When peas were grown in the mesocosm (Figure 5), *V. paradoxus* 5C-2 moved gradually away from the initial zone of inoculation, such that after 30 days, it was recovered from pea roots over 50 cm distant from the initial site of inoculation (Figure 5A). However, in the absence of plants, it moved only 30% of this distance and no change in movement was detected from 12 days after inoculation (Figure 5B). Since *V. paradoxus* 5C-2 has a high dispersal capacity by moving between pea roots, field experiments were designed with substantial buffer strips between inoculated and uninoculated plots to prevent contamination. In other systems, runoff from sloping soil may risk cross-contamination of uninoculated plots [36] and should be considered in field trial design.

Field application of a PGPR inoculum

Having understood the physiological requirements of *V. paradoxus* 5C-2, a field experiment was designed to determine the effects of *V. paradoxus* 5C-2 on pea (*Pisum sativum*) growth and development under soil drying conditions. It was performed in a field situated at 53°50'44.42"N and 2°46'23.55"W (Lee Farm, Bilsborrow, Lancashire, UK). The mineral content of this soil at this site was predominantly sandy (93%), while silt and clay were 2.8% and 4.2% respectively. The organic matter content of the soil was 7.7 % with a pH of 5.8 (water extraction). The field site (13 m x 7 m) was covered by a semi-transparent plastic roof ("poly-tunnel") to exclude rainfall and the irrigation system installed. Soil moisture was monitored by an irrigation controller and data logger (Model GP1, Delta-T Devices, Burwell, UK) connected to soil

moisture sensors (Delta-T Devices, Burwell, UK, SM-200 sensors). Half of the 24 plots were randomly assigned for *V. paradoxus* 5C-2 inoculation. During this field experiment, soil temperature at 5 cm depth varied between 15°C (week 1, 01/09/2009) and 19°C (week 5, 06/10/2009), while soil pH and water availability matched those optimal for *V. paradoxus* 5C-2 (Figure 3) [48].

Pea (*Pisum sativum* ‘Progress No. 9’) plants were germinated in seedling trays filled with an organic substrate (Levington’s M3, Levington, UK) in a greenhouse at the Lancaster Environment Centre. The inoculation was done on the day of transplanting by submerging the peat blocks containing one seedling per block) in a bacterial solution (1 litre, 10^{10} CFU.ml⁻¹). At the field site, seedlings were transplanted on 01/09/2009 and well watered initially, then water was withheld (reaching soil water potential of -0.06 MPa at 20 cm depth) from half the plants 3 days after transplanting. Shoot fresh weight was determined 21 and 47 days after transplanting. Roots were also sampled at these times to determine *V. paradoxus* 5C-2 colonizing the rhizosphere, using selective agar growth medium containing the antibiotics rifampicin, kanamycin and streptomycin to which this strain is resistant [22]. Colonies that resembled authentic *V. paradoxus* 5C-2 colonies were counted, and samples were taken to spread again on selective medium containing ACC as a sole nitrogen source for confirmation that bacteria contained *acdS*.

While uninoculated plants were not colonized (since the plot arrangement reflected the results of Figure 5), *V. paradoxus* 5C-2 root colonization decreased 20-fold by the end of the experiment (Figure 6C). Nevertheless, well-watered plants inoculated with *V. paradoxus* 5C-2 had 50% more nodules than uninoculated controls (Figure 6B). Drying soil decreased nodule number by 30%, but the addition of *V. paradoxus* 5C-2 restored nodule number to a level that exceeded control plants grown in well-watered conditions. *V. paradoxus* 5C-2 inoculation increased shoot biomass by 23% in well-watered soil (Figure 6A). Drier soil significantly decreased shoot biomass of uninoculated controls by 22%, but applying *V. paradoxus* 5C-2 increased biomass above that of the well-watered controls. Thus in the field experiment *V. paradoxus* 5C-2 colonization in the roots was not adversely affected by drought, while drought decreased the number of nodules by 40% (Figure 6B). These results were qualitatively similar to

those obtained in greenhouse experiments [22], even though soil type (and thus likely indigenous bacterial community) was very different.

Considering the expense of developing microbial inoculants, an important question is whether they can successfully colonize, and promote growth of, multiple plant species. Using the technique described above, multiple studies have measured *V. paradoxus* 5C-2 colonisation of the root system in a range of substrates, with levels of root colonisation similar in pea and other species, and between plants that were grown in well-watered *versus* drying soil. Thus *V. paradoxus* 5C-2 is competitive in the rhizosphere of a range of species, and promoted growth of plants grown *in vitro*, pot trials and field trials [78]. In further field experiments, *V. paradoxus* 5C-2 promoted potato, *Solanum tuberosum*, shoot growth and tuber yield [79]. Nevertheless, it can show specificity in its interaction and growth promotion according to the host genotype [80]. Of 4 tomato (*Solanum lycopersicum*) recombinant inbred lines tested for their growth and physiological responses to drought, only one was more drought resistant following *V. paradoxus* 5C-2 inoculation. While there is abundant evidence that this specific bacterial strain is a promising inoculant to boost growth of plants grown in drying soils, it is of interest to know if other *V. paradoxus* strains could have the same effect. *V. paradoxus* is a widespread bacterium across different soils and environments [43] and generally promotes plant growth under stress conditions such as drought [22], salinity [81], heavy metals [43] and phytopathogens [82]. Isolation of local strains, their enrichment and inoculation is theoretically possible. However, each strain has different properties and thus effectiveness. For example, 10 different strains of *V. paradoxus* were isolated, in which ACC deaminase activity *in vitro* varied by an order of magnitude [43]. Interestingly, *in vitro* root growth promotion also differed by *circa* 25%. Thus not all the strains share the same effectiveness, and in the case of *V. paradoxus*, there will be differences in plant host specificity, that affect the capacity to integrate into the holobiome.

Bacterial mechanisms to cope with hyperosmotic stress comprise complex genetic regulation involving a large set of genes arranged in operons [83]. The *V. paradoxus* core genome possesses genes that configure the bacteria for coping with hyper-osmotic stress (Table 2), with two main mechanisms identified. First, osmotic adjustment allows the accumulation of physiologically compatible organic osmolytes utilising betaine-aldehyde dehydrogenase, and

choline/betaine ABC transporters. Secondly, selective influx of inorganic ions such as potassium and chlorine occurs via trans-membrane channels. These genetic traits allow *V. paradoxus* to survive in the rhizosphere of extreme environments such as desert grasses [84], even if the soil matric potential in this niche has not been directly measured. While conserved genetic material across multiple strains suggests that *V. paradoxus* is well-adapted to proliferate in drying soil, it is less clear whether all strains can promote growth of plants in drying soil.

Final remarks and future outlook

One concern of introducing a new organism to the field is that it may alter microbial community structure: displacing some organisms and/or favouring the activity of plant pathogens [85]. However, this risk is likely minimal since bacterial colonization of the root systems decreased with time following inoculation (Figure 6C) [85]. While the rhizosphere is a very complex chemically interconnected niche, with plant-microbe and microbe-microbe communication forming trophic networks, the plant holobiome is dynamic and reacts following any disturbance [86]. Soil inoculation with PGPR sometimes facilitates their integration within soil trophic networks [22]. On the other hand, microbial stress tolerance traits are of pivotal importance for PGPR success under abiotic stress conditions and such information can help design field applications to mitigate drought-induced crop losses. This model of bacterial ecophysiology (Figure 7) and targeted experimentation in gradually more complex systems (Figures 3-5) leading to controlled field trials (Figure 6) provides a roadmap to lessen the probability of inoculum failure in the field.

PGPRs often possess more than one beneficial characteristic and complete genomic information can identify multiple potential growth-promoting mechanisms of each isolate [38]. Moreover, putative PGPRs can be identified by their specific genes rather than conventional bioassays. Information on the species dwelling in the plant holobiont (metagenomics) can help understand rhizosphere microbial community dynamics of a specific crop. Studying these effects in agroecosystems could help to deliver inocula that change bacterial dynamics towards the restoration of a microbial community promoting plant drought tolerance. In addition, with metatranscriptomics, it is possible to foresee the active bacteria or target genes and determine synergistic behaviours of the inoculum with native microbial population. Integrating the working

model (Figure 7) with improved understanding of the microbial ecology of droughted agroecosystems may increase the reliability of microbial inocula, thereby enhancing grower acceptance.

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Figure and Table legends

Figure 1. Ethylene biosynthesis. Methionine is converted by (1) S-adenosylmethionine synthetase (SAM synthetase) to S-adenosyl methionine (SAM). Next, SAM is converted to 1-Aminocyclopropane-1-carboxylate (ACC) and 5'-methylthioadenosine (MTA) by (2) 1-aminocyclopropane-1-carboxylate synthase (ACS). MTA can enter in the Yang cycle that refills the pool of l-methionine. (3) 1-aminocyclopropane-1-carboxylate oxidase (ACO) turns ACC into ethylene, HCN and CO₂. Alternatively, (4) ACC deaminase deaminates ACC resulting in 2-oxobutanoate and ammonia. (5) γ -glutamyl-transpeptidase (GGT) forms γ -glutamyl-ACC (GACC) by addition of a glutamyl group (6) ACC-N-malonyl transferase (AMT) forms 1-malonyl-ACC (MACC) from malonyl-CoA and ACC while (7) an enzyme encoded by Jasmonic acid resistance 1 (JAR1) yields jasmonyl-ACC (JA-ACC).

Figure 2. Core and Strain-Specific Gene Extrapolation. A pan-genome study was implemented to determine the *Variovorax paradoxus* core genome, variable and unique genome. Seven strains of *V. paradoxus* (Table 1) were downloaded from NCBI Genome and their pan-genome analysis was accomplished with bacterial pan-genome analysis (BPGA) program [60]. Specific functions of the *V. paradoxus* pan-genome were delivered based on KEGG (Kyoto Encyclopedia of Genes and Genomes) biological pathways database.

Figure 3. Effects of temperature (A), pH (B) and osmotic potential (C) on *V. paradoxus* 5C-2 growth after 18 hours of incubation (◆) and motility after 72 hours (X). For (A) and (B) growth was assessed in TSB (liquid medium) while motility was tested in M8/M9 minimal medium solidified with 0.5% agarose. For (C) and (D), TSB (survival test) and semi-solid M8/M9

(motility test) plates were amended with glycerol. (D) Resumption of *V. paradoxus* 5C-2 motility (on MS, 0.25% agar) after exposure (12 hours) to different osmotic potentials. Symbols are means \pm SE of 9 replicates.

Figure 4. *V. paradoxus* 5C-2 distribution along pea (A) and corn (B) roots. Root anatomy (C). Data are means \pm SE of 3 replicates.

Figure 5. *V. paradoxus* 5C-2 dispersal between the rhizosphere of pea plants (A) and in bulk soil without plants (B). Root colonisation by bacteria was assessed at fixed distances from the initial inoculation zone (yellow line), four times after inoculation. Symbols are means \pm SE of 3 replicates.

Figure 6. Shoot biomass (A), nodule number (B) and *V. paradoxus* 5C-2 root colonisation (C) of inoculated and uninoculated plants grown in well-watered and drying soil in a field experiment. Bars are means \pm SE of 6 plots. Different letters above the bars indicate significant treatment differences determined by LSD test ($p < 0.05$). (D) Field experiment design. Seedlings were transplanted to coloured plots while uncoloured plots were fallow. Plots were inoculated with *V. paradoxus* 5C-2 (red cells) or not (control – blue cells). The outer two beds (each comprising 6 pea plots) were watered throughout the experiment while water was withheld from the middle two (red arrows).

Figure 7. Roadmap for soil and PGPR monitoring, leading to inoculum application in field experiments, as outlined in this manuscript.

Table 1. Pan-genome characteristics of seven strains of *Variovorax paradoxus*

Table 2. Operons for hyperosmotic stress resistance found in the core genome of *Variovorax paradoxus*. The genes are represented in the genomic context of the reference strain EPS NC_014931.1 (directions coordinates). Information on putative operons were obtained through UniProtKB (<https://www.uniprot.org>).