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# A Holistic Approach for Enhancing the Efficacy of Soil Microbial Inoculants in Agriculture: From Lab to Field Scale

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## ABSTRACT

Microbial inoculants can be an efficient tool to manage the soil and plant microbiomes providing direct beneficial effects, and for modulating native soil and plant-associated microbiota. However, the application of soil microbial inoculants as biofertilizers and biopesticides in agriculture is still limited by factors related to their formulation, application method, and the knowledge about the impact and interactions between microbial inoculants and native soil and plant host microbiomes. The review is thus describing and discussing three major aspects related to microbial-based product exploitation, namely: i) the discovery and screening of beneficial microbial strains; ii) the opportunities and challenges associated with strain multifunctional features; iii) the fermentation and formulation strategies also based on the use of wastes as growth substrates and the technical and regulatory challenges faced in their path to field application. All these issues are addressed in activities performed by the EXCALIBUR project ([www.excaliburproject.eu](http://www.excaliburproject.eu)), which aims to expand the current concept about microbiomes interactions, acknowledging their interactive network that can impact agricultural practices as well as on all living organisms within an ecosystem.

# 1. Introduction

Microbes and communities thereof (microbiomes) have been shown to play critical roles in the diversification and functioning of all other living organisms, driving evolution, ecological adaptation, and organizing biodiversity from the origin of life [1]. Host-microbiome association is known to influence the capacity of a host to cope with abiotic and biotic stresses and have biological (e.g., on physiology or metabolism) and ecological (e.g., in plant-pest interactions) implications for economic and social humans activities [2-4], plants [1, 5-8] and animals [9-10]. Soil is an important reservoir for environmental and host-associated microbiomes as well [9-12]. The outstanding and unique role of soil as a microbiome reservoir opens new perspectives for applications of soil microbial inoculants to address several agronomical and environmental challenges of our time [13].

Soil microbial inoculants can be one efficient tool to manage the microbiome, which includes i) microbiome transplants, ii) microbial inoculants, iii) microbial extracts as well as iv) methods to change environmental conditions [14]. For example, they may be used for their direct beneficial properties as well as for modulating native soil and plant-associated microbiota, thus providing intriguing options for sustainable agriculture and circular bio-economy [15]. Currently, microbial-based products as potential alternatives or complements to synthetic fertilizers and pesticides in agriculture are one of the fastest-growing sectors in agriculture. The market value of plant growth-promoting rhizobacteria/bacteria (PGPR/PGPB), biological control agents, and biostimulants was valued at USD 6.00 Billion in 2016, growing at an annual rate of 13.8% up to more than 14.5 Billion by 2023 [16]. However, the application of soil microbial inoculants as biofertilizers and biopesticides in agriculture is still limited and hindered by several factors [17-18]. Despite the enormous research efforts made in the last years, there is still much to be learned about the underlying processes affecting their efficacy in crop systems, especially under open field conditions. In fact, we still have little understanding on the impact and interactions between microbial inoculants and native soil microbiome, and our knowledge of microbiome assembly, host-microbiome interactions, and communication remains largely incomplete. Moreover, these complex interactions are also greatly influenced by the selection [19] and formulation [16] of beneficial microbial strains. Currently, it is assumed that a host-microbiome is generally structured by host genetics and nutrients [20]. However, host-microbiome interactions are likely driven by evolutionary and ecological relationships, and the microbiome of single host species is not only influenced by host genetics, nutrients, and abiotic factors but also by biotic inter-kingdom interactions (e.g., soil, plant, and animals) via microbial loops within the ecosystem, in the so-called eco-holobiont concept [21]. The interlinked microbiota is a concept already embraced by the One Health concept [22] as well as by the European exposome concept for health issues.

In this review, we describe and discuss the main aspects related to microbial-based product exploitation that are investigated by the EXCALIBUR project ("Exploiting the multifunctional potential of belowground biodiversity in the horticultural farming" - [www.excaliburproject.eu](http://www.excaliburproject.eu)). In EXCALIBUR, the holistic approach proposed aims to deepen our knowledge on the interactions between plant, soil, micro-, meso-, and macroorganisms as influenced by formulated bio-inocula, in an effort to understand the links and dynamics with native soil biodiversity and agricultural practices. More specifically, the review addresses issues related to i) the discovery and selection of the microbial strains; ii) their multifunctional features; iii) the production and formulation of bio-inoculants; iv) the challenges to assure their efficacy under field conditions.

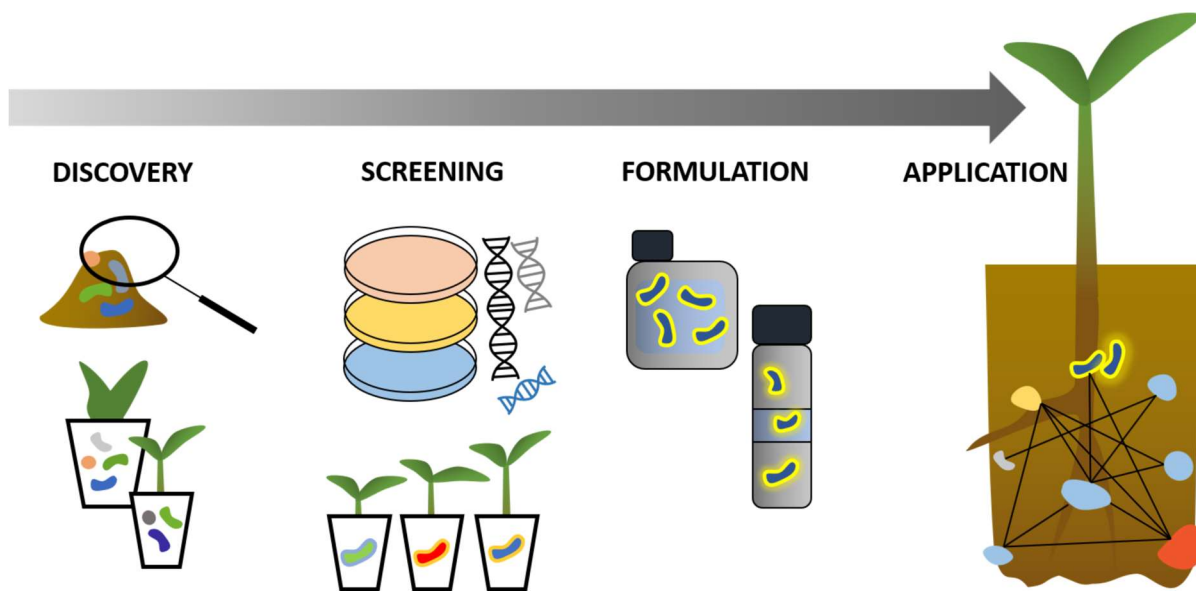
## 2. Discovery and Selection of Microbial Strains

### 2.1. Environmental Microbiomes as Sources for Beneficial Microbial Strains

Potentially beneficial microbes can be isolated from a variety of sources, and in particular from soil and plant microhabitats (rhizosphere, endosphere, phyllosphere, spermosphere). Traditionally, one approach to exploit environmental microbiomes is through isolation and enrichment using culture-dependent methods. High throughput sequencing approaches developed over the last decades showed their great potential in exploring the complex microbial networks within plant microbiomes. Recently, this sequencing approach demonstrated its potential by discovering new highly efficient microbial strains [23-26]. However, the proportion of beneficial

microbial strains within microbiomes differs greatly and depends on many other factors, e.g., plant species, cultivar, microhabitat, soil properties, climate, and anthropogenic activities [27].

Interestingly, extraordinarily high proportions of plant growth promoting and antagonistic strains were found in mosses, representing the first land plants on earth [28-29]. This can be explained by different plant-microbe interaction strategies developed by vascular and non-vascular plants [30]. Indeed, vascular plants can filter or select specific microorganisms from the environment through a chemical signaling mechanism influenced particularly by plant secondary metabolites [31]. Nevertheless, endophytic microhabitats are an important source of beneficial microbial strains as endophytes are known for their intimate interaction with host plants [32].



**Figure 1:** Schematic representation from the discovery of potential microbial strains to screening, formulation, and application.

Ecological knowledge is always a good basis to identify sources of beneficial microbial strains. Isolation from the target plant is a common strategy to enrich candidate strains for later applications [33], even though allochthonous strains from non-host plants have sometimes shown stronger effects [34]. During the last centuries, domestication and breeding have changed the microbiota of crop plants [35]. Recent approaches using wild relatives of modern plants showed the potential of discovering new strains from microbiomes of long-forgotten ancestors [36]. Another ecological strategy is to explore the “pathobiome”, the microbiome of pathogen affected plants, to isolate beneficial strains. Kusstatscher *et al.* [37], for instance, showed a higher share of antagonistic strains among those isolated from diseased sugar beet fields than from healthy ones.

Moreover, suppressive soils harbor an especially great variety of potential candidates for microbial applications [38]. Compost, especially earthworm compost, was shown to contain a high proportion of beneficial microbial strains. Biocontrol agents, including *Trichoderma* and antagonistic *Fusarium* strains as well as antagonistic *Pseudomonas* and *Bacillus* strains, are present in suppressive compost and able to control specific soil-borne pathogens [39]. Recently it was shown that plants are able to select genotype-specific microorganisms from these rich sources [40].

Altogether, there are thus manifold possibilities to find appropriate sources for novel beneficial microbial strains; however, following isolation, potential microbial strains need to undergo various screenings to fully evaluate their characteristics, efficacy, and mechanisms of action.

## 2.2. Screening Strategies for Beneficial Microbial Strains

For decades, screening microbial strains for plant disease management and plant growth promotion has been performed [41]. Nevertheless, there is still not a single perfect screening method available: most of the screening

methods are based on the well-known tests of the mode of interaction of microbial strains with plants or pathogens [14, 42]. Biocontrol strains exhibiting direct antagonistic activity (by parasitism, the release of antimicrobials and/or enzymes, or competition for nutrients) can be screened using solid or liquid media assays as well as *in planta* assays. Moreover, selected strains can be screened for desired enzymatic activity such as chitinase, cellulase, protease, and glucanase secretion produced metabolites or volatile organic compounds (VOCs), as well as the release of plant growth hormones such as auxins. Results obtained from plate assays need to be confirmed with *in planta* assays [43]. Screening strains for inducing systemic resistance (ISR) in plants is rare since extensive *in planta* assays are needed [44].

Additionally, implementing molecular methods into the screening process is valuable to assess the full potential of prospective strains [24]. Recently two independent studies based on microbiome analysis discovered specific microbes that confer holistic disease resistance against plant pathogens in agricultural plants [23-25]. Zachow *et al.* [45] proposed and showed the effectiveness of a multi-faceted screening approach to obtain the best microorganisms with beneficial traits from several environmentally conditioned and host-adapted bioresources. Additionally, microbiome modulation was recently identified as an efficient mechanism for screening microbial strains [15].

Combining different strains into consortia is a good approach to increase the diversity of the inoculum, which may provide a higher efficacy to the final formulated product and opens the way toward broad-spectrum or multifunctional microbial products. However, the screening of consortia implies more difficulties than single strains. Compared to single strains, complex inocula provided better plant protection [46, 47] and growth promotion [48, 49]. Microbial consortia can use so-called “helper strains” to improve the efficacy of individual beneficial microbes as well as the overall traits beneficial to plants. For example, Loján and colleagues [50] highlighted the mechanisms used by PGPR (stimulation of hyphal branching and germ tube elongation) to support arbuscular mycorrhiza fungi (AMF) in the development of the symbiotic relationships with plants that were previously described [51]. Co-inoculation of a *Pseudomonas* strain with a *Stenotrophomonas* strain, both known to produce VOCs, resulted in greater plant growth promotion due to VOC production [52]. Even though single strain applications are by far the easiest and widely used strategy, current research trends indicate the potential of multi-strain applications in the future.

Based on strain activity assays, genetic profile, and interaction of strains with other microbes or plants, promising candidates/consortia can then be chosen for formulation to achieve stable products for testing in large-scale trials before registration. However, besides the efficacy, other important criteria shall be considered when selecting beneficial microorganisms: safety, environmental risks, and ecological behavior, aspects of intellectual property rights and registration, production costs, and potential market [53]. Multifunctional characteristics can also be suitable for strain selection, as they can allow targeting different market segments.

### 3. The Multifunctional Role of the Root and Rhizosphere Microbiome

The rhizosphere microbiome can play crucial roles in sustainable agriculture, nature conservation, phytoremediation, the development of bio-energy crops, and the mitigation of climate change [54, 55]. The multifunctionality of the rhizosphere microbiome arises from the ability of rhizosphere microbes to impact a wide variety of soil biogeochemical processes; they include mineral cycling, carbon sequestration, and the emission of greenhouse gases, as well as affecting interactions with other organisms by producing and detoxifying a wide range of metabolites, hormones, and enzymes. A group of rhizosphere microbes can also form associations with a host plant to modulate a broad set of plant functional traits during the interaction with their host.

The multifunctional potential of beneficial rhizosphere microbes to enhance crop production and quality can be broadly subdivided into effects on (1) plant growth and quality, (2) abiotic stress mitigation, and (3) biotic stress mitigation [e.g.,56-59]. Plants are dependent on microbial activity for nutrient cycling and availability through decomposition of organic matter and mineralization. Although plants produce their own nutrient solubilizing enzymes such as phosphatases, acquisition of, e.g., P, Zn, and Fe are considerably enhanced by microbial

acidification, solubilization, and siderophore production [60]. Furthermore, free-living biological nitrogen fixers (BNF) increase N nutrition, while symbioses with AMF and symbiotic BNF that trade P and N for plant carbon, respectively, generally enhance plant growth under P and N limited conditions [61, 62]. Many PGPR also produce phytohormones such as auxins, gibberellins, and indole acetic acid that stimulate plant growth, whereas they can decrease stress-induced levels of ethylene, maintaining plant growth under stress [59]. Importantly, PGPR and AMF can not only enhance crop production but also crop quality. Examples are microbial induction of primary and secondary metabolites, some of which are phytochemicals for which the crop is intentionally grown, or phytochemicals associated with increased product health [57]. Other examples are microbially enhanced attractiveness of flowers for pollinators, leading to enhanced fruit set and quality [63].

Beneficial root and rhizosphere microbes also play an important role in mitigating plant biotic stresses, making their utilization as bio-inoculants an attractive and promising way to reduce the input of pest and pathogen control chemicals in sustainable agriculture and horticulture. Several mechanisms underlie the disease suppression incurred by rhizosphere microbes [58-59]. Some of these are based on direct interference of beneficial microbes with pathogen proliferation in the rhizosphere, such as competition for nutrients or space [56], the production of antibiotics [64], or the production of hydrolytic enzymes, e.g., the chitinolytic enzymes produced by mycoparasitic bacteria and fungi [65]. Others are based on the induction of changes in the host plant by beneficial microbes that alter plant's susceptibility or suitability as a host for the pathogen. For instance, many PGPR and AMF have the ability to induce systemic resistance in plants (ISR) [66].

Whereas disease suppressive effects of beneficial microbes have been recognized for a long time, the interest in their use for controlling arthropod pests has emerged more recently. Like for pathogen control, pest control by beneficial microbes can be based on a variety of mechanisms, e.g., on direct interactions with pest arthropods, such as pest control by entomopathogenic bacteria and fungi, or on plant-mediated effects, including the production of metabolites by plant endophytes that are toxic to the plant's insect pests [67], as well as microbial induction or priming (ISR) of plant defenses [68]. Interestingly, many entomopathogenic fungi have endophytic stages during which they confer additional functions, including enhanced growth [69] pathogen resistance [70] and priming plants for enhanced pest resistance [71], illustrating the multifunctionality of these microbes. Furthermore, many beneficial rhizospheres and root microbes can modulate the profile of volatile organic compounds that plants produce in response to herbivory (herbivore-induced plant volatiles, HIPV) that play an important role in the attraction of the natural enemies (biocontrol agents) of the herbivores such as predators and parasitoids and hence affect the indirect defense of their host plant [72].

Genome sequencing allows obtaining a detailed insight into the genomic properties and functional potential of microorganisms, which helps to gain better taxonomic resolution and understanding of metabolic pathway potential as well as multifunctionality, for instance, shown for *Bacillus aryabhatai* [73]. Furthermore, the combination of *in vitro* experiments with RNA-based transcriptomic profiling allows identifying the genes responsible for regulating specific pathways as demonstrated for accessing nutrients such as potassium and phosphate by ectomycorrhizal fungi under nutrient limitations [74, 75].

All the approaches and methods useful to shed light on the multifunctionality of beneficial microbial inocula described above are utilized within EXCALIBUR. However, one of the challenges for the optimization of the multifunctional potential of rhizosphere microbes in sustainable agriculture is to design consortia in which the constituent strains have complementary beneficial functions and that are at least compatible with each other but preferably have synergistic effects. Compatibility may be challenging for combinations that include, e.g., AMF and bacteria or fungi with potential mycoparasitic activity. On the other hand, promising synergisms have been observed, e.g., for several PGPR and AMF species [76, 77]. Synergistic effects could also play an important role in the successful applications of beneficial microbes as biostimulants [78]. For instance, Meena *et al.* [79] found that co-inoculation of the endophytic fungus *Piriformospora indica* with a phosphate-solubilizing bacterium *Pseudomonas striata* strain led to a higher uptake of phosphate and plant dry weight than in treatments with only one of these strains. Fermentation and formulation technologies are thus necessary to be adapted to accommodate and foster the exploitation of microbes' multifunctionality.

## 4. Production and Formulation of Bio-Inocula: A Challenge for their Successful Marketing and Field Application

### 4.1. Fermentation and Formulation of Bioinoculants

Several techniques can be utilized to produce microbial-based formulations: from isolation and selection of microbial strains, through testing their best fermentation performances, up to formulating them into commercial products [80, 81]. Even though there is abundant scientific literature on the effects of many bio-inoculants on plant growth or crop protection properties, studies on fermentation processes and formulation techniques are still limited [82]. A recent analysis has emphasized the need for a better, integrated view of soil inoculants, including their production and formulation processes, particularly for the possible use of additives that can increase either the products' shelf life or efficacy [18]. Such an approach has been introduced in the development of bio-inocula as well as in the assessment of their efficacy and effect on soil biodiversity in the EXCALIBUR project.

Bioinoculants can be composed of different microbiome members: bacteria, archaea, fungi, algae, protists, or even combinations thereof. Each group requires its own fermentation and formulation technique. Currently, the majority of bio-inoculants are based on spore-forming bacteria and fungi, which are relatively easy to produce. However, the majority of plant-beneficial microbes belong to Gram-negative bacteria [83, 84]. Recently a technology was developed, which opens the exploitation of this promising group as well as of consortia together with other microbiome members [85].

When fermentation processes are concerned, submerged liquid and solid-state fermentation are the main biotechnological techniques utilized to produce microbial biomass or spores [86]. Bioinocula can be formulated as a solid commercial product when solid carriers or solid-state fermentation is utilized or as liquid formulations after submerged fermentation processes. In the latter case, adding substances directly to the fermentation broth or during the formulation process can ensure a high-quality product (high cell number/ml and metabolic activity) with a sufficiently long shelf life [81]. Selection of the fermentation mode and its parameters, as well as the optimization of the medium components, are the critical points that must be addressed to achieve a high biomass production of bio-inoculants with high metabolic activity at the industrial scale [16, 87, 88]. Having a circular bioeconomy approach in mind, exploiting agricultural wastes for bio-inocula production should become a major effort in developing fermentation processes. Several waste products have been proven to be suitable for industrial inoculant production [99, 90]; using waste products can lower production costs, which are a main limiting factor in the commercialization of bio-inocula, thus making their use attractive to manufacturers. Moreover, the production of cell-free fermentation liquids with strong phyto-stimulating and biocontrol properties [6, 91] should increase manufacturers' interest due to the independence from interactions with the soil micro- and macro-biota. A specific production method is required for AMF-based products: the obligate biotrophic nature of AMF has, indeed, complicated the development of cost-efficient large-scale production methods to obtain high-quality AMF inoculum [92]. AMF can be produced following two methods: *in vivo* [93, 94] and *in vitro* [95, 96], with their advantages and disadvantages (Table 1).

Co-culturing microorganisms and hence exploiting possible synergistic interactions between strains can also ease the formulation of microbial consortia that may better overcome environmental stresses than single strains and provide a better plant protection or growth promotion effect [97, 98]. Even though the reasons for consortium benefit are not always well known, complementary functional mechanisms and/or a greater chance of environmental colonization are likely to support a more consistent efficacy [99].

AMF-based products may be considered as an example demonstrating the benefits deriving from microbial consortia. Commonly, commercial AMF products contain only one fungal strain: frequently *Rhizophagus irregularis* (*Rhizophagus intraradices*) or *Glomus iranicum*. Only a few products on the market contain two or more strains, and in this case, different species of *Glomus* are generally used. However, the interest in a "multi-strains" product, like those tested in EXCALIBUR, derives from the possibility of applying it to a large diversity of soils and

**Table 1: Advantages and disadvantages of AMF production methods.**

Production Methods of AMF	Advantages	Disadvantages
<i>In vivo</i>		
Nursery plots with soil (Sieverding 1991)	<ul style="list-style-type: none"> <li>• simple</li> <li>• adapted for local use</li> <li>• low costs</li> </ul>	<ul style="list-style-type: none"> <li>• limited application</li> <li>• easily contaminated</li> <li>• not well adapted for the development of an industrial activity</li> </ul>
Containers (pots) with different substrates (Feldmann and Idczak 1994; Feldmann and Grotkass 2002)	<ul style="list-style-type: none"> <li>• low technology input</li> <li>• fairly easily elimination of undesirable contaminations</li> <li>• reasonable costs</li> </ul>	<ul style="list-style-type: none"> <li>• not pure cultures</li> <li>• limited in its industrial development</li> </ul>
Hydroponic and Aeroponic systems (Jarstfer and Sylvia 1994)	<ul style="list-style-type: none"> <li>• easy control of contaminants</li> <li>• carrier-free inoculum</li> <li>• adapted for plants micropropagation method</li> </ul>	<ul style="list-style-type: none"> <li>• relatively complicated technological setup</li> </ul>
<i>In vitro</i>		
Dual-compartment culture system (Bécard and Fortin 1988; Declerck <i>et al.</i> 1996; Rosikiewicz <i>et al.</i> 2017)	<ul style="list-style-type: none"> <li>• pure cultures</li> <li>• reduced contamination</li> <li>• allows industrial development</li> </ul>	<ul style="list-style-type: none"> <li>• high technological investment</li> <li>• high costs</li> <li>• not all AM fungi can be successfully cultured in this system</li> <li>• fungi obtained <i>in vitro</i> could "lose" their mycorrhizal potential when used in soil</li> </ul>

crops, possibly also benefiting from the synergistic interactions between PGPR and AMF regarding alleviation of abiotic stress have been observed [76]. Furthermore, the application method will define which type of formulation is the most suitable: (i) for mixing with plant substrates (e.g., in nursery production), "granule" products should be favored, (ii) soluble or wettable powder is best suited for application through ferti-irrigation systems or by means of sprayers, (iii) for seed treatment, a "sticky" powder can be used [100].

The multifunctional use of bio-inocula [101-104] could be exploited to support the development, marketing, and application of microbial-based products. However, the current legal framework in the European Union, as well as in other countries, on the production and marketing of microbial-based products poses serious challenges to exploit the multifunctionality of beneficial microbes. Legislation and registration entities have embraced the distinction between biofertilizer (enhancing the acquisition and efficient use of resources) and biopesticide (mitigating the losses due to pests and pathogens) effects. The distinction has also been useful to rank the potential risks for human health associated with using these microbial products, which are arguably higher for biopesticides than for biofertilizers. However, a microbial species inherently cannot be classified as either an organism with biofertilizer properties or an organism with biopesticide properties. The current requirement to register microbial products either as biostimulants or as biopesticides is, therefore, a denial of biological reality and forms an important stumble block for the development and marketing of microbially based multifunctional products.

Moreover, for example, in the European Union, the registration process and data requirements for microbial-based pesticides are similar to those needed for chemical pesticides (Regulation EC 1107/2009) [103], thus not taking into consideration the characteristics of the biopesticide mechanisms of action [105]. Similar limitations were pointed out also in the Indian legislation, resulting in unfair competition from sub-standard or misbranded biopesticides [106]. A situation limiting the use of microbial-based products could result from the newly enacted EU Regulation on biofertilizers (named microbial-based biostimulants, Reg. EU 2019/1009) [107], which foresees at present only four groups of genera allowed to be marketed as biofertilizers (*Azotobacter* spp., mycorrhizal fungi, *Rhizobium* spp., *Azospirillum* spp.), in contrast with the plethora of genera and species that are recognized to have positive effects on plants.

The potential risk for humans and animals due to the application of microbial inoculants has been addressed in the scientific literature [108]. To reduce such risk and assure a microbiological quality of microbial-based biostimulants, the new EU Regulation 2019/1009 foresees limits for the contamination from human pathogens. However, an additional issue is represented by possible contaminations with not declared species or strains as well as by the not compliance of the product with the composition declared on the label [109]. Besides the possible commercial damage, the main concern in both these cases derives from the difficulty of distinguishing between plant beneficial and pathogenic microorganisms, as they have similar characteristics [110].

Related to the registration requirements, but also useful to design a correct application method, are the needs for methods to detect and monitor bioformulants' strains under natural conditions [111]. Protocols suitable for regulatory or commercial purposes need to be developed to assure a level of discrimination suitable for tracking and monitoring bioformulations in the soil and plants. A polyphasic approach, combining classical (culture-dependent and microscopic methods) and molecular techniques, should be used to monitor bioformulation strains. The evaluation of the impact of bioformulations on soil biodiversity should also concern the time factor since their effects might change over time [112-114]. In this respect, a broad range of "omic" approaches are being used in the EXCALIBUR activities, in parallel to the design of custom-specific probes with high specificity to increase the success of detection and monitoring.

#### **4.2. Challenges of Field Application of Microbial-Based Products**

In order to reduce the input of synthetic plant protection products and fertilizers, microbial-based products are needed to play a key role in agricultural systems. Recently, increasing knowledge of the role of soil health and biodiversity for the production of healthy and high-quality crops has oriented researchers and companies to focus on application methods and field efficacy testing of microbial-based products. This technology industry sector is growing at a faster pace than the knowledge and regulations governing the production and marketing of microbial products. Although microbial-based products (both biofertilizers and biopesticides) are not expected to replace synthetic chemical pesticides and fertilizers fully, they will play an important role in improving resource use efficiency and protection from pests and diseases [114, 115].

Microbial strains have demonstrated the ability to enhance crop nutrition [116] and to improve plant health or innate immunity by priming plants' defense mechanisms [117] or directly boosting their photosynthesis [118]. Some other mechanisms are also speculated for microbial biopesticides based on entomopathogenic fungi, such as insect repellence [119, 120]. However, even though a number of microbial-based products are commercially available worldwide, a challenge to foster broad field application of microbial-based products relates to the fact that their beneficial traits are not always consistently expressed under the applied cultivation conditions. The interactions between plants and beneficial microorganisms are complex, and the mechanisms that regulate the plant-soil-microorganisms system remain largely to be discovered. This is, in particular, relevant when comparing results from experiments that are carried out *in vitro* in the laboratory and *in situ* under greenhouse and complex agricultural conditions [55]. Therefore, there is a need to understand better the context-dependency of the expression of these traits [121, 122]. Obviously, part of this context-dependency is a simple consequence of the fact that inoculated microbes or microbial consortia may fail to establish sufficiently high densities in the rhizosphere to exert their effects due to interactions and competition with resident microbes in the soil, despite efforts to design formulations that ensure the best possible conditions for establishment. However, some desired functions have more fundamental sources of context-dependency because the expression of these microbial traits is contingent upon the biotic and abiotic environment in which the microbes function and the host plant with which they interact. For instance, bacterial production of antibiotics or volatile signals that can provide bioprotective effects for the plant are often only triggered in the presence of particular microbial antagonists that are not necessarily present under the cultivation conditions [123]. Even stronger context-dependency is expected for beneficial effects of microbes that can induce plant responses, such as ISR. This is because plants tend to tailor their responses based on the information that they receive from multiple signaling pathways, and their final response is the result of cross-talk between these different signaling pathways [124, 125]. Therefore, for instance, a microbe with the potential to trigger ISR may fail to trigger it because, under the prevailing conditions, the plant prioritizes a different challenge. Understanding the processes governing such cross-talk and prioritization are



therefore needed to identify the abiotic and biotic cultivation conditions that maximize the chances that the desired trait is expressed.

The field efficacy of plant protection products and fertilizers is influenced by many factors, including soil characteristics, climate and weather conditions, application methods, and crop management practices. This is particularly true for microbial-based products, which can also interact with soil native biodiversity and with the plant microbiome as well as be influenced by abiotic factors [126]. Often what seems to be working under laboratory conditions fails in subsequent field trials [127]. For these reasons, within the EXCALIBUR Project, experimental field trials are carried out in three different pedoclimatic regions (Atlantic, Continental, and North Mediterranean) under integrated and organic management methods, both under open field or protected conditions. Thirty-one field trials are being carried out using experimental and commercial formulations in eight European countries on three economically important model crops (apple, tomato, and strawberry) (Table 2) to address specific cropping issues. For example, apple replant disease is a debilitating soil problem affecting trees when they are replanted on the same site. Due to the inconspicuous nature of the reduced tree growth, although various approaches have been employed in an effort to characterize the etiology of replant disease, differences continue to exist in terms of quantifying the relative importance of individual pathogens. Convergence has evolved around a group of fungal, oomycete, and nematode agents that appear to contribute to the disease worldwide [128]. Due to the recent withdrawal of broad-spectrum chemical fumigants, apple replant disease has re-emerged as an important issue facing the apple industry. Since the relative importance of replanting causal agents can vary greatly between orchards, a single disease control measure is unlikely to manage apple replant disease consistently and effectively across regions. Thus, in EXCALIBUR, we study the single and combined use of selected microbial products also in combinations with rotating rootstock genotypes [129] and verifying the effect of application timing (in the nursery, at planting time, too young orchards).

To evaluate the activity of the formulations, several indicators (such as phenological and growth indexes, quality, and yield parameters) are measured, together with a number of microbial and physico-chemical soil properties. All these analyses, having a special focus on soil biodiversity, its dynamics, and the plant-soil-microorganisms interactions, are expected to highlight the effects of bio-inocula on plant responses to stresses providing bioindicators and supporting the development of molecular diagnostic tools for monitoring the persistence of bio-inocula and their impact on soil and plant-associated biodiversity [111].

**Table 2: Overview of the field trials carried out in the Excalibur project.**

CROP	Bioinoculum Type	Management	INHORT (PL)	CRPV (IT)	UNITO (IT)	TU-GR (AT)	FOEKO (D)	KOB (D)	NIAB (UK)	KIS (SI)	UCPH (DK)	IN+ (FR)	TOTAL	TOTAL CROP
APPLE	Biofertilizers	Organic		1		1	2	1					5	14
		IPM	1	1		1							3	
	Biopesticides	Organic	1				2	1					4	
		IPM							2				2	
STRAWBERRY	Biofertilizers	Organic											-	10
		IPM		1		1							2	
	Biopesticides	Organic	1	1						1	1		4	
		IPM	1		1					1	1		4	
TOMATO	Biofertilizers	Organic										2	2	7
		IPM	1	1									2	
	Biopesticides	Organic		1									1	
		IPM	1		1								2	
TOTAL			6	6		3	4	2	2	2	2	31		

## 5. Conclusions

Microbial inoculants are expected to partially replace synthetic pesticides and fertilizers in agriculture in the near future and represent one of the most intriguing and technical demanding approaches toward sustainable agriculture. However, even though microbial-based products have been successfully applied in agriculture so far, failures still occur under field conditions. This is due to limited knowledge about the impact of soil characteristics, climate and weather conditions, application methods, and crop management practices. Moreover, knowledge for field application is limited in terms of dosage requirements, specific interactions of the bioinoculant with the native soil biota, as well as the ecological behavior of the inoculants. The knowledge gap must be addressed with a vision that considers the complexity of soil and plant microbiome.

Fermentation and formulation approaches that exploit co-cultivation methods, use of wastes as substrates, and synergic interactions between strains represent a perspective strategy to boost the commercial production of microbial consortia that can overcome environmental stresses compared to single strains thus assuring a lower risk of field failures. Moreover, the potential multifunctionality of many beneficial strains might represent an additional opportunity to develop innovative microbial-based products, though regulatory issues may contrast their commercial application.

Besides direct beneficial effects on plants, microbial inoculants might provide indirect benefits through the modulation of native soil and plant-associated microbiomes, also affecting animal and human health. This potential may represent a promising option towards sustainable agriculture, especially when integrating microbiome research into breeding and plant protection strategies. The multifunctional potential of beneficial microbes needs to be fully integrated into a broader perspective towards the “One Health-One Environment” approach that is at the heart of both UN’s Sustainable Development Goals and EU Green Deal strategy. Such a perspective includes the recent advances in microbiome research related to human health [2-3], plant health, and ecosystem functioning [5, 6]. Due to the importance of the microbiome, the underlying communication and interaction mechanisms between the microbiomes and the environment require better knowledge, which has to expand our current holistic concept, acknowledging that biotic (plants and animals) and environmental (soil, water, and air) microbiomes form an interactive network that can impact the assembly and the functions of holobionts of all living organisms within an ecosystem, as recently proposed [21]. Such an approach is at the core of the EXCALIBUR project concept.

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