UNLOCKING NATURE'S SECRETS:

beneficial microorganisms can transform crop production

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Edited by Eligio Malusà and Krzysztof Ambroziak

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INTRODUCTION

Microorganism and their communities have been shown to play critical roles in the diversification and functioning of all other living organisms. The interaction between plants and microorganisms is known to influence the plant's capacity to cope with abiotic and biotic stresses with important implications for plant production. Because of this understanding, during the last 20–30 years, a large number of microorganisms have been isolated, characterized and tested as biofertilizers and biocontrol agents. The results confirmed the beneficial effect of the selected microorganisms on plant growth and health, also positively impacting soil properties.

The use of beneficial microorganisms in crop production is thus becoming a common practice, since the microbial-based products are recognized as potential alternatives or complements to synthetic fertilizers and pesticides particularly in a modern, sustainable agriculture and in the context of environmental and climate concerns. In this regard, it is noteworthy that at EU level, the microbial-based products are regulated by EU Regulation 2019/1009 on fertilising products or EU Regulation 1107/2009 on the marketing of plant protection products.

Microorganism-based products used for plant nutrition, named by the EU legislation "microbial biostimulants", are an effective means of promoting plant growth and production in a context where the use of mineral or synthetic fertilisers is reduced. The microbial biostimulants are products that stimulate plant nutrition processes independently of the nutrient content of the product, with the sole aim of improving one or more of the following plant or rhizosphere characteristics: nutrient use efficiency, tolerance to abiotic stress, quality characteristics, availability of nutrients confined in the soil or rhizosphere. Among the microbial biostimulants, four groups of microorganisms are currently listed among the fertiliser products: three atmospheric nitrogen-fixing bacteria (*Azotobacter* spp., *Rhizobium* spp., *Azospirillum* spp.) and mycorrhizal fungi, which mainly promote phosphate nutrition, but can also contribute to improving nitrogen and trace element nutrition.

On the other hand, plant protection products based on microorganisms are instead considered similar to the synthetic ones and to be registered must efficiently control the targeted pest. When considering the control of insects, entomopathogenic fungi are the group of microorganisms that has provided the most useful solutions. The main species used for this purpose include *Beauveria bassiana*, *Beauveria brongniartti*, *Metarhizium anisopliae* and *Isaria fumosorosea*. In the case of biofungicides, microbial formulations are available for both pre-harvest and post-harvest uses. However, also in this case, the multitude of species and strains known for biocontrol activity is not matched by the limited number of microorganisms registered as active substances.

Even though microbial-based products have been successfully applied in agriculture so far, farmers are still reluctant to apply them broadly. This is due to limited knowledge about the impact that application methods and crop management practices can have on the microbial products efficacy. Knowledge for field application is limited in terms of dosage requirements or the specific interactions of the bioinoculant with the native soil biota. Moreover, a recent analysis of the products present on the market at global level has emphasized the need for an integrated development of microbial-based products, including their production and formulation processes, particularly for the possible use of additives that can increase either the products' shelf life or efficacy. From this point of view, the formulation of the microbial biostimulant is a key factor in ensuring the efficacy of the crop treatment. Indeed, while the formulation of products based on a single strain or species simplifies the production and registration process for marketing, various studies indicate the usefulness of applying consortia of micro-organisms composed by species able to 'collaborate' among them and possibly also with the soil microbiome. In this respect, consortia of mycorrhizal fungi and bacteria proved particularly effective in various crops, making it possible to reduce the application of mineral fertilisers by up to 50%.

Along with the industry's search for the best formulation, a crucial contribution to ensuring successful treatment with a microbial biostimulant or pesticide must be made by farmers themselves, through the adoption of agronomic practices that do not adversely affect the action of the product itself. In fact, the persistence or efficacy of microbial biostimulants and pesticides is generally influenced by agricultural management practices: practices such as tillage, pest management with other means, mineral and organic fertilisation, or water regime can heavily modify the efficacy of a microbial product. In case of fertilization management, considering that in general 60–90% of applied mineral fertilisers are washed away and that only 30–50% of nitrogen fertilisers and 10–45% of applied phosphate fertilisers are absorbed by crops, the application of microbial biostimulants can favor an integrated fertilisation management system, supporting agricultural productivity with a low environmental impact. In case of microbial pesticides, the dose and environmental conditions at the time of product application are also important factors affecting their efficacy.

All the aspects mentioned above have been explained in detail, but with a practical and easily understandable approach in the chapters of this book. The book has been conceived as an effort to favor the transfer of knowledge acquired within the EXCALIBUR project, which has been funded by the EU under the Horizon 2020 research programme, and other projects on this topic. It is the hope of the authors that such effort will foster the reduction of chemical inputs use through a reasoned application of microbial-based products among farmers and other professionals, to avoid any negative impact on the crops' productivity. Such thoughtful use would instead positively affect the environment, animal and human health, which have been shown to be all connected.

Prof. dr hab. Eligio Malusà[1] and Dr Krzysztof Ambroziak[2]

[1]The National Institute of Horticultural Research, ul. Konstytucji 3 Maja 1/3, 96-100 Skierniewice, POLAND

[2]INTERMAG sp. z o.o., Al. 1000-lecia 15G, 32-300 Olkusz, POLAND

Chapter 1
Mycorrhizal fungi for the improved uptake efficiency and growth of horticultural crops

Lidia Sas-Paszt[1], Sławomir Głuszek[1], Beata Sumorok[1], Edyta Derkowska[1], Anna Lisek[1], Louisa Robinson Boyer[2], Maria Grazia Tommasini[3]

[1]The National Institute of Horticultural Research, ul. Konstytucji 3 Maja 1/3, 96-100 Skierniewice, POLAND
[2]NIAB, East Malling, West Malling, Kent, ME19 6BJ, UNITED KINGDOM
[3]RI.NOVA, via dell'Arrigoni 120, 47522 Cesena – FC, ITALY

The aim of this chapter is to introduce mycorrhizal fungi and to promote a better understanding of these fungi and their potential to improve horticultural production systems. It also describes how or when it is more appropriate to use these beneficial microorganisms, as well as the conditions or practices that can limit their beneficial effects.

1. Types of mycorrhizal fungi and their ability to colonize plants

There are four major types of Mycorrhizal fungi. These consist of the Ectomycorrhizae, the arbuscular mycorrhizae, the ericoid mycorrhizae, and the orchid Mycorrhizae. Ecto-mycorrhizae are associated with many tree species, they form coiled structures within roots and are often host specific, each favoring particular species. Ericoid Mycorrhiza are associated only with the ericaceous plants such as blueberry and cranberry and Orchid Mycorrhizae with their orchid hosts. In this chapter we will focus on the Arbuscular mycorrhizal fungi (AMF). The AMF are ubiquitous and found in all corners of the globe and in all terrestrial environments.

Arbuscular mycorrhizal fungi are represented by about two hundred and fifty species belonging to the division Glomeromycota divided into four orders (Archaeosporales, Diversisporales, Glomerales, and Paraglomerales), which include a total of 11 families. The AMF are considered to be one of the factors responsible for the success of land colonization by primary plants, and have a fossil record dating back 450 million years. They enter into symbiosis with nearly 80 percent of the terrestrial plant species found on Earth, which includes many of the global food crops, however it is important to note that AMF do not form associations with the *Brassicacea*.

Mycorrrhizal fungi are considered to be asexual organisms, they reproduce by forming large spores, on the ends of hyphae that spread out into the soil. In the presence of a living plant spores are able to germinate. The mycelium colonizes the roots of plants and transfer water and nutrients to the plant in return for carbon. As there are benefits to both the fungus and the plant this relationship is a mutual symbiosis (Smith and Read 2008).

When hyphae of AMF come into contact with a plant root, they form appressoria on its surface, where the fungi release chemical signals to the plant to allow them enter the cortex. Once inside the root the mycelium usually forms intracellular coils, and then spreads by growing within the primary cortex cells. Within the plant cortical cells, branches of the mycelium form into structures called arbuscules (Fig. 1.1). It is through these arbuscules that transfer of water and nutrients occurs between the plant and the fungus and from which the name of this type of mycorrhiza originates. In the root, some species can also form vesicles which perform storage functions, and the organic substances contained in them are used by the fungi during periods of low metabolic activity of host plants.

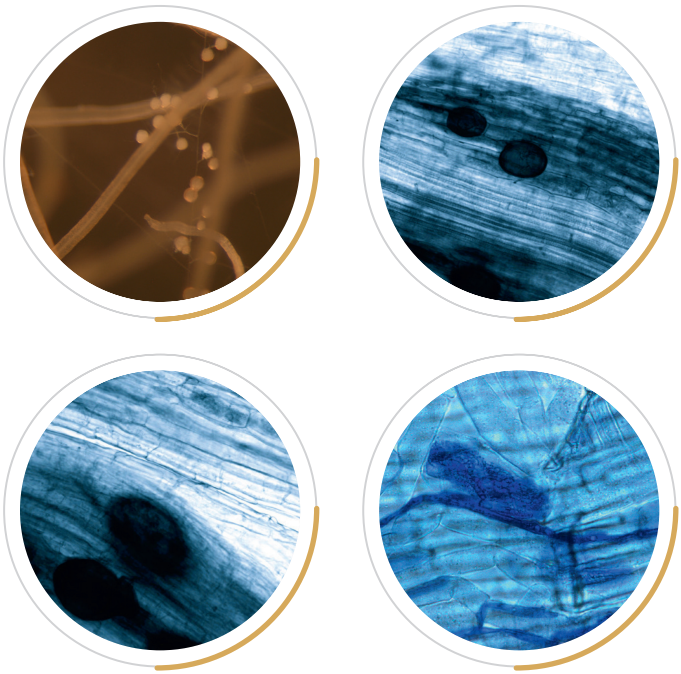

Fig. 1.1. Photo of AMF. Root organ culture showing roots hyphae and spores, strawberry roots stained with trypan blue showing root cells, hyphae, vesicles and arbuscules.
Author: Louisa Robinson Boyer

Due the necessity to reduce chemical input in agriculture for restrictions on their use as well as for their increased costs, farmers are calling for alternative sustainable solutions to maintaining cropping yields. AMF have a great potential to be used in many species of crop plants that play an important role in agriculture, including numerous species of fruit trees, grapevine, shrubs, strawberry, vegetable crops, salads and ornamentals. Recently there has been a great interest in the use of AMF in commercial system, thanks also to the availability of such commercially inoculums and to the known good practices intervention in cropping systems to increase native populations of soil microbes.

Some species of fruit plants from the Ericaceae family, e.g., highbush blueberry, medium blueberry, bilberry, lingonberry, cranberry, form ericoid mycorrhiza, which are quite different from AMF and in which the hyphae of the mycorrhizal fungus penetrate both the intercellular spaces and the interior of the root cells, creating structures in the form of coils. Commercial inoculum of Ericoid Mycorrhiza is also available.

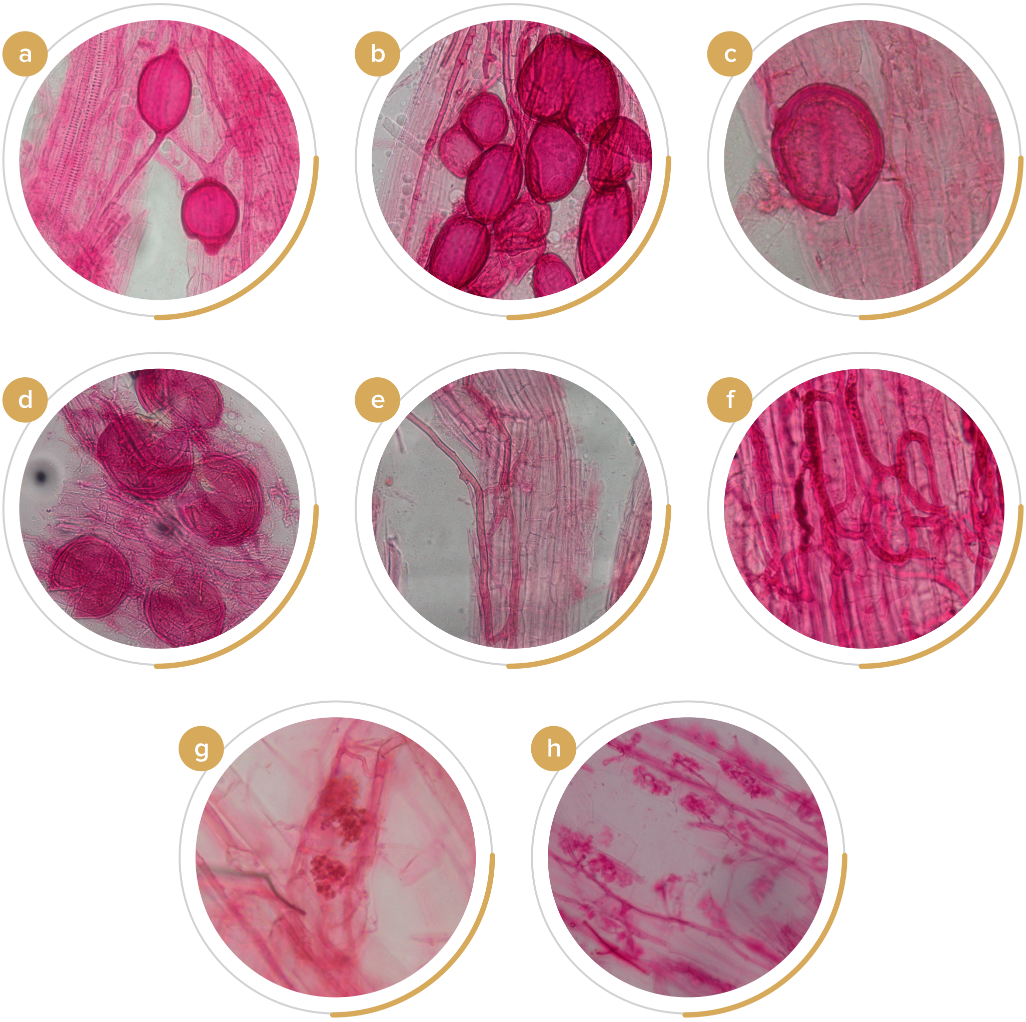

Fig. 1.2. Structures of mycorrhizal fungi in roots. (a) Vesicles in the root of a control strawberry plant, (b) Vesicles in strawberry root after inoculation with beneficial microorganisms, (c) Spore in the root of a control strawberry plant, (d) Spores in strawberry root after inoculation with beneficial microorganisms, (e) Mycelium of an arbuscular mycorrhizal fungus in the root of a control strawberry plant, (f) Mycelium of an arbuscular mycorrhizal fungus in the root of strawberry plants after inoculation with beneficial microorganisms, (g) Arbuscule in the root of a control strawberry plant, (h) Arbuscules in the root of strawberry plants after inoculation with beneficial microorganisms. *Source: Department of Microbiology and Rhizosphere, The National Institute of Horticultural Research.*

Fig. 1.3. Ectomycorrhizal fungi in the roots of forest plants. (a) Ectomycorrhiza on the roots of European Beech growing in the Podanin forest nursery, (b) Ectomycorrhiza on the roots of silver birch growing in a forest nursery in Radom, (c) Ectomycorrhiza on the roots of the European hornbeam growing in the Białogard forest nursery, (d) Ectomycorrhiza on the roots of an English oak growing in the Białogard forest nursery, (e) Ectomycorrhiza on the roots of Scots pine growing in the Gościniec forest nursery, (f) Ectomycorrhiza on the roots of Scots pine growing in the Gościniec forest nursery, (g) Ectomycorrhiza on the roots of Norway spruce growing in the Białogard forest nursery, (h) Ectomycorrhiza on the roots of Norway spruce growing in the Wałcz forest nursery. *Source: Department of Microbiology and Rhizosphere, The National Institute of Horticultural Research.*

2. Effect of AMF on plant growth, yield and tolerance to stresses

Research into the practical application of mycorrhizal products has been carried out for many years in several research centers around the world. A number of studies have shown positive effects of AMF inoculation on the growth of a range of horticultural crops and model plants. AMF fungi has the effect to modify the growth, morphology and number of roots, making the root system of plant more efficient in taking up water and mineral compounds from the soil. In many cases, plant roots colonized by arbuscular mycorrhizal fungi are better formed and have more lateral roots.

The mutualistic symbiosis with AMF brings to plants many benefits, the most important of which, for farmers and growers, is the increased water and nutrient uptake and a rather wide increased resistance to various environmental stresses, both biotic, such as pest and pathogen attack, and abiotic such as drought, waterlogging and heat stress. In particular, by improving water and nutrition assumption AMF are well known to benefit plant growth and often yield. Mycorrhization increases also leaf area and plant photosynthetic activity facilitating for growth.

The AMF are especially important for phosphorus, nitrogen, potassium, magnesium and microelements uptake increasing plant health especially in poor conditions. A deficiency of phosphate significantly limits the growth of plants, especially as phosphate ions are not very mobile in the soil. They form a number of insoluble complexes slowing down their diffusion. The external mycelium of the AMF penetrate the soil to a much greater extent than the roots alone forming a hyphal network that facilitates the uptake of water and mineral compounds from the soil by plants and the release of organic compounds from the roots into the soil .The hyphal network not only reaches far beyond the nutrient uptake zone of the roots providing increased surface area for uptake, the fine hyphal strands can penetrate into smaller particles extracting otherwise inaccessible nutrients, other minimal compounds and water from pockets in the soil. (Helgason and Fitter 2005). Marschner and Dell (1994) have shown that up to 80% of the phosphorus contained in plants can be provided by fungi of the division Glomeromycota. It has been demonstrated that an efficient use of Phosphorus, even under soil deficiency conditions is aided by AMF secreting enzymes into the soil that dissolve forms of phosphorus that are not available to plants. Phosphorus in the filaments is translocated with the movement of the cytoplasm and occurs in a water-insoluble form (polyphosphates), so that low concentrations of metabolically active phosphorus allow its continuous uptake from the soil solution. It has been shown that on phosphorus-poor soils, mycorrhiza in apple plants increases the efficiency of phosphorus uptake, while on phosphorus-rich soils mycorrhiza facilitates the uptake of zinc and copper ions. Nevertheless, there are several studies that show a reduction in the level AMF association and dependence in situations where phosphorus is in abundance. Very high levels of phosphorus often have a negative effect on the degree to which plants put their resources into AMF partnership.

Other key minor elements and minerals are also transported to the plant by AMF such as potassium, zinc, calcium, magnesium and copper. It has been shown that plum and cherry trees inoculated with an AMF species, *Glomus intraradices*, had a beneficial effect on the increased micronutrient content of plant tissues and the growth of plants growing on low-phosphorus soils. Also, the improved growth of grapevines of different varieties resulting from mycorrhization is closely linked to the uptake of the following minerals from the soil: phosphorus, nitrogen, iron, copper and zinc.

It is now well understood that AMF play an important role also in the transfer of nitrogen to plants, facilitating uptake from the soil and assimilation of nitrogen. Researchers have observed transporters in the arbuscules for nitrogen and other elements along with aquaporins for water. Certain AMF species shown also to transport amino acids i.e. glycine and glutamine taken up from the soil to the roots. Please see image below for nutrient exchange.

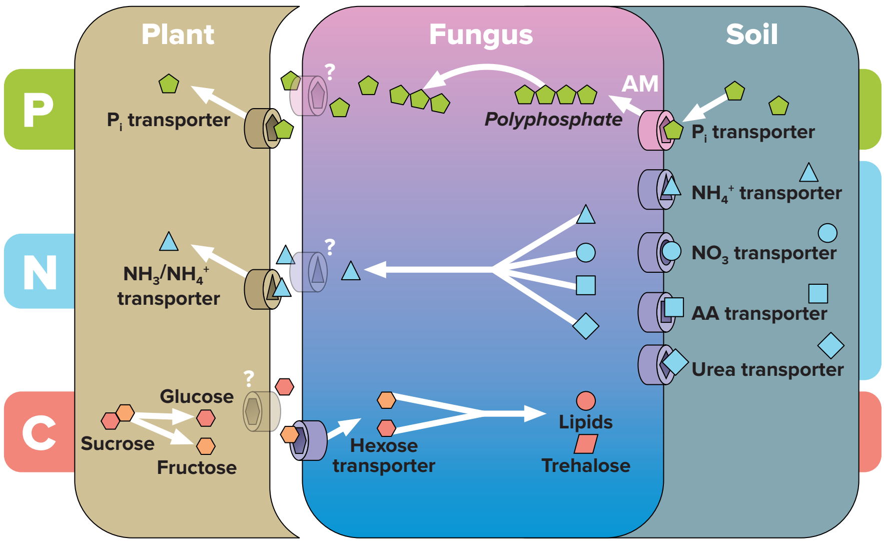

Fig. 1.4. The main nutrients exchange in AMF (modified from Bonfante and Genre 2010)

In general, beneficial microorganisms directly influence plant growth by synthesizing growth regulators, organic acids, siderophores, biofilm formation and antagonistic action against soil and plant pathogens. The results obtained indicate a high potential in this respect for both beneficial rhizosphere bacteria and fungi, resulting in increased yield and improved yield quality, compared to that of plants fertilized with standard mineral fertilizers.

With global climate change and the increase of extreme weather events it is crucial to find mechanisms to increase the resilience of crop plants to these stresses. There is much evidence that AMF play a large role in water regulation in plants and to increase, for example, plants tolerance to drought. It has been found that the roots of plants growing in arid environments are intensively colonized by mycorrhizae and colonization by AMF of crop plants growing in these areas can effectively increase the size and quality of plant yields (Al-Karaki et al. 2004) and resilience to drought episodes. Likewise, there is increasing evidence to show that AMF may help plants to tolerate waterlogging (from increased rainfall events and heat stress).

It has been shown that AMF dynamics change within roots according to stress and season effects. For example, the degree of root colonization of grapevines (Pinot Noir) growing in a 20-year-old vineyard varied over two years from 20% during bud burst to 50% in late June/early July. In contrast, in late October/early November, during a period of very heavy rainfall, the degree of AMF colonization decreased to almost zero. Another effect saw is the inoculation of vine roots with *Glomus iranicum* mycorrhizal fungi reduces the effects of water stress, improves photosynthesis rate and grapes yield, accelerating table grape ripening too.

Experiments by Martins et al. (1997) showed positive effects of chestnut (*Castanea sativa*) mycorrhization on vegetative growth and photosynthetic activity of plants. Arbuscular mycorrhizal fungi can also increase the yield of non-halophilic plants growing on saline soils. Another effect of AMF is the production of glomalin, aggregate soil particles increasing soil aggregation, this has a stabilizing effect on the soil and helping to prevent soil erosion and leaching. AMF support soil-forming processes and contribute to plant cover diversity, especially on eroded and highly contaminated soils.

Mycorrhizal fungi play a significant role in resilience to biotic stress such as pest and pathogen tolerance in plants via a number of mechanisms. The colonization of roots by AMF can stimulate the plant's natural defense mechanisms eliciting factors of induced systemic resistance, which can reduce the effect of pests and pathogens. Additionally, the improved plant health and vigor from increased nutrition and water makes them less susceptible to attacks. Furthermore, AMF can compete with pathogens for resources, such as space and nutrients, thereby reducing pathogen colonization and proliferation in the plant's root zone. For example AM fungi in some grapevines varieties activate defense mechanisms against parasitic nematodes together with increase their drought tolerance.

However, this symbiotic interaction means a cost for the plant hosting its AMF partners. Carbon is transferred from the plant to the fungus, this can lead to reduced growth of very young plants, in early establishment and when there are scenarios where plants are not provided with all their nutritional needs. Generally, the benefit to cost balance increases as the plant grows or indeed faces stress or challenges.

In some cases, mycorrhizae also increase the efficiency of uptake and accumulation of heavy metal ions (i.e., copper, zinc, cadmium, lead or cobalt), as well as chloride and sulphate ions. Mycorrhizal fungi also increase the resistance of plants to the harmful effects of excessive heavy metals and soil contaminants, e.g., aromatic compounds**.** Nevertheless, due to that mycorrhization of plants contributes to a significant clean-up of organic pollutants present in the soil and for this reason such AMF are used in the phytoremediation of contaminated areas.

3. Formulations and application guidelines to different horticultural cropping systems

Recent years have seen an increase in the demand for and production of mycorrhizal inoculum products to serve the agriculture and horticulture industries with many growers seeing the potential for use in sustainable cropping. Formulations come in a variety of formats, including dry granules and powders, liquids, seed coatings and dressings. Most formulations will contain either a mixture of species of AMF or selected individual species depending on the use. The production of mycorrhizal inoculum products can be challenging as they can only be grown in the presence of host plants (i.e., obligate symbionts).

Mycorrhizal fungal products are regulated and in the EU under the regulation of fertilizers and plant biostimulants Annex II Regulation (EU) 2019/1009. They are classed as fertilizing products because that are intended to improve plant nutrition and soil fertility. While mycorrhizal fungal products do not fall under the same regulation as biocontrol agents, as they are not classified as having plant protection properties. National laws vary amongst the EU countries, consequently local guidance and policy would need to be followed for registration of mycorrhizal fungal products. Consequently, manufacturers of these products need to register the product in each EU country where they intend to sell the product.

Many products contain species of AMF that are commonly found in many agriculture and horticulture environments, in particular species of *Rhizophagus* and *Funneliformis* (formerly known as *Glomus*). As the technology for production increases so do the number of AMF species included into products and of tailored products for different environment. Even though AMF are considered to be non-specific in their colonization potential of different species, it is important to classifying the diverse pool of AMF species according to their host and environmental preferences. This allows identifying the best strategies for inoculum formulation and production and application methods suitable for different conditions (e.g., open field, greenhouse, transplant stage, in vitro propagation stage, urban greening, etc.). Therefore, appropriate technology for the production of inocula as well as formulation media is key to their effective application.

Although the inoculation of plants with mycorrhizal fungi is a well-known practice, their wider use is still hampered in some cases by the formulation of inocula able to assure a reliable and consistent effect under field conditions. However, it is key to raise awareness for the growing community about the use of these products and how their application differs from standard chemical approaches. In particular individual products vary in their application rates and instructions on application. Consequently, the manufacturers' guidelines should be followed for use.

In general, when applying AMF in cropping environments there are a number of key factors to consider:

- Application - Any AMF inoculum needs to be applied directly to the roots of plants: either as a root dip, applied during transplanting in a hole near young plant or below/ adjacent to seed at germination. It is preferable to inoculate plants during their early stages of development.
- Environment - The environment can affect AMF and certain formulation may be better suited in some specific environmental conditions. In particular, crop type, soil conditions and planting methods can play an important role in the effect of AMF application and its formulation. For example, it has to be known that high chemicals application can compromise AMF efficacy (i.e., very high levels of nutrients especially P will determine a risk in the association of AMF with roots). Extreme environmental conditions like excessive heat, low pH, high water may suggest to choose specific species of AMF that better adapt to such conditions. It is worth taking care of the correct soil pH, as a pH below 5.5 limits root colonization by arbuscular mycorrhizal fungi.
- Post-planting treatment - The effect of post planting could have an effect on the successful association of AMF. Very high application of fungicides soon after planting could highly reduce chances of colonization. Many fungicides and herbicides are compatible with AMF, however careful selection and timing of applications may be appropriate. AMF need a living host plant in order to survive, processes that leave soil devoid of living plants like tillage, grubbing of orchards, weed control or fallow periods, would rapidly deplete AMF viability within the soil.
- Cost-benefit considerations - The cost to the farmer of applying inoculum needs to be considered reasonable and economically sustainable. Assessment of benefit to short-cycle crops (e.g., herbs and salads) vs perennial crops (fruit trees and forest) would need to be analyzed along with the benefit to crops, especially for those high demanding of nutrient environments. Some cropping systems may be better suited to AMF application than others.
- Interactions with other organisms - The interaction between AMF and other beneficial microbes appears to increase their potentialities. For example, in many studies, tailored communities comprising difference AMF species, and other beneficial fungi such as Trichoderma and beneficial bacteria's show increased benefits and higher levels of colonization of AMF.

4. Management of crops to better exploit AMF activity by proposing management practices increasing AMF efficiency in crop growing and yielding

Many aspects of conventional agricultural management practices affect the diversity and abundance of AMF. The increased use of monocropping reducing above ground biodiversity reduces the below ground soil microbiology, including AMF. High levels of chemistry used in farming, rates of fertilizer (with excess phosphate levels) necessary to support modern cropping yields plus pesticide and fungicide applications all reducing populations of these important microbes. Crops have been highly domesticated over many decades and most breeding programs do not consider how new genotypes interact with soil microbes and many newer varieties have a reduced requirement for uptake of AMF. Soils in high input farming are likely to give less favorable conditions to harbor microbes with low levels of organic matter and reduced soil structure. Many crops are also now grown in soil free substrates, such as coir (coco fiber). These substrates are devoid of microbes and as such commercially available preparations may be key. All of these modern practices are decreasing the soil biology within agriculture and horticulture soils.

If the use of beneficial microorganisms and AMF is to be better utilized in commercial horticulture we need to consider the practices that encourage the development of AMF. If commercial preparations are applied to commercial systems we need to consider how best to ensure their survival and longevity in commercial cropping. Many farmers are also considering ways in which native populations of AMF can be revived or encouraged by adopting sustainable methods and interventions.

There are many studies that have been carried out examining the effects of crop management techniques carried out under different soil and climatic conditions. Although environment, soil type and location all add variability to the data collected in these studies, universal patterns emerge:

- Soil disturbance - The use of tillage and soil disturbance (which would include grubbing in orchard) has a big effect on populations of AMF in the soil. Excessive disturbance of soil breaks established hyphal networks in soils reducing the beneficial effects from the AMF. Mechanical soil tillage, affects AMF communities and leads to lower spore counts, lower root colonization, lower MF taxonomic diversity and decrease extra-radical mycelium when compared to undisturbed soil. Oehl et al [2003] found that intensive soil cultivation reduced the diversity of mycorrhizal fungi. This work was conducted on arable land and grassland, but the results can also be applied to intensive orchard crops. In order to maintain and encourage AMF populations in soils growers should reduce as far as possible disturbance to their soils.
- Chemical application - Although many fungicides are compatible with AMF, care should be taken to reduce their application and seek advice from inoculum producers to ensure the correct chemistry is applied. Timing for fungicides is also important, as well as excessive drenching in early establishment of AMF would not be recommended. The compatibility of glyphosate with AMF has been a matter of debate over the past years and it is still not entirely clear how AMF populations are affected by this chemical.
- Fertilizers application - Very high inputs of fertilizers clearly negate the need for plants to put their resources into forming association with AMF. To ensure sufficient crop production growers need to apply fertilizer to crops, but this should be reduced if AMF are to be encouraged in the system, saving the grower money on expensive

chemicals and working towards sustainability. Very high levels of readily available Phosphorus are going to have the most detrimental effects. Maintaining adequate levels of soil organic matter is also important to provide resources for the AMF to explore for nutrition and to provide better soil structure and drainage.

- Crop diversity - Above ground biodiversity is important to encourage species diversity of AMF. In commercial farms this can be achieved by increasing the diversity of non-crop plants, such as wildflower strips, cover crops, interplanting and rotations. Due to the obligate symbiotic nature of AMF having bare soil for long periods with no living roots would vastly reduce populations.

Implementing these management strategies into commercial farms can ensure environments conducive for AMF can be achieved. By understanding that different strategies need to be considered when biology (rather than traditional chemistry) is applied to commercial systems the use of AMF could be a key driver in achieving sustainable cropping. More research is needed to design simple and reliable field trials in commercial horticulture cropping to quantify the impact of AMF communities on crop growth, yields and agroecosystem sustainability.

Studies do show a difference between AMF community composition at the genus level between organic and conventional farming systems with organic farming practices shown to increase richness of AMF families in soils, however often the most abundant genera are seen in both systems. Agricultural practices, such as organic farming, can have a positive or neutral effect on AMF communities on the soil (Manoharan et al. 2017), organic farming practices can increase richness of AMF Gigasporaceae family in soils. The main factor, however, in conventional farming system which cause decline in AMF richness was related to the high concentrations of soil C and N (Wadhan et al. 2021). Moreover, soil factors like content of N, P, K, and also C/P and N/P ratios shaped total AMF community.

Some studies show that AMF communities in agricultural soils respond to long-term agricultural systems and are resistant to short-term summer droughts. It is not known whether the ability of AMF communities to tolerate drought depends on soil properties, AMF community diversity and composition, or on associated plant communities. It remains clear that given increased global temperatures and the frequency of extreme weather events including heat and precipitation, we need to understand how this affects the AMF populations and how we can better utilize these fungi to increase the resilience of major horticulture crop plants to face with these challenges.

The lack of sound inoculation practices and large-scale studies with cost-benefit analysis of AMF application remains a major obstacle to the stable introduction of AMF into cultivation protocols.

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Chapter 2
Microbial products for biocontrol of horticultural crops

Magdalena Ptaszek[1], Małgorzata Tartanus[1], Massimo Pugliese[2]

[1]The National Institute of Horticultural Research, ul. Konstytucji 3 Maja 1/3, 96-100 Skierniewice, POLAND
[2]University of Turin - Agroinnova, Largo Paolo Braccini 2, 10095 Grugliasco (TO), ITALY

Plant protection is one of the key challenges in modern horticulture. Therefore, new approaches are being developed, including the use of microorganisms as sustainable and effective biopesticides. The aim of this chapter is to demonstrate the possibilities of using beneficial microorganisms in plant protection against both foliar and soil-borne pathogens and pests. In addition, the multifunctionality of microorganisms in plant production is presented as well as the advantages and disadvantages of their use. Much attention was paid to the conditions that should be fulfilled in the application of microbial products and the factors affecting the achievement of their high effectiveness, as well as to the practical aspects of using biopesticides containing various microorganisms. Finally, the issue of legal regulations for the marketing of bioproducts was also discussed.

1. Uses of microorganisms for biocontrol

Microorganisms and their communities (microbiome) play a fundamental role in the biodiversity and functioning of all other living organisms, driving evolution and ecological adaptation since the beginning of life on Earth. The host-microbiome relationship, irrespective of the organism - plant, animal or human - affects the host's ability to cope with abiotic and biotic stresses and has biological (e.g. in physiology or metabolism) and ecological (e.g. in plant-parasite interactions) implications relevant to human economic and social activities. It is therefore increasingly clear that proper management of the microbiome in agricultural systems can promote sustainable production with respect for the environment. Products based on or derived from microorganisms can therefore be an effective tool for managing the soil and/or plant microbiome.

The history of the use of beneficial microorganisms in agriculture began in the 19th century. Since then, many important studies have been carried out on the isolation of individual bacterial and fungal strains, their characterisation and potential use as biopesticides or biofertilizers. Biopesticides are defined as plant protection products that contain groups of microorganisms such as viruses, bacteria and fungi, as well as nematodes. The list of „microbiological active substances" currently authorized by European Union is available on: https://food.ec.europa.eu/plants/pesticides/eu-pesticides-database_en. Considering the regulations in the European Union Biodiversity Strategy to reduce the use of chemical plant protection products by 50% until 2023, biopesticides will play a key role in agricultural practices worldwide. For this reason, the use of microbial products is becoming a viable alternative to chemical plant protection products, as confirmed by the increasing number of microbial based products appearing on the market in the context of integrated disease and pest management. It should also be mentioned that many products containing microbial inoculants and showing pathogen and pest control activity are not registered as plant protection products, but as microbial preparations that stimulate microbial activity in soils or have a beneficial effect on plant growth and yield. This is due to the fact that in the European Union, the registration process, requirements and costs for microbial based pesticides are similar to those needed for chemical pesticides (Regulation EC 1107/2009).

However, the chemical paradigm, in which a product is used to effectively, easily and quickly eliminate harmful pests, still persists, and the use of biopesticides is too often seen as a simple substitute for chemical pesticides. Such an approach is certainly not appropriate, as it does not take into account the various factors that influence the efficacy of control with so-called 'living material' biopesticides. An alternative, and perhaps better, complementary approach to the use of biopesticides is to manipulate the crop environment to increase the population of the native microbiome to provide some pest or disease protection (conservative biocontrol). When considering pest (insect and other arthropod) control, entomopathogenic fungi are the group of biopesticides that has provided the most useful solutions. A recent worldwide survey showed that 129 mycoinsecticidal products are available (de Faria and Wraight 2007). Species mainly used for this purpose belong to the ascomycetes and include *Beauveria bassiana*, *Beauveria brongniartti*, *Metarhizium anisopliae* and *Isaria fumosorosea*. These species are often generalists, i.e. they parasitise a variety of species of harmful insects, but some are specialised (e.g. *B. brongniartii* parasitizes only species of the genus Melolontha) (Zimmermann 2007). However, viruses that are pathogenic to insects are of increasing interest, although the development of resistance of a harmful insect to an insecticide strain appears to be an important factor in determining the correct use of such products, as highlighted in the case of carpocapsa apple granulovirus.

The approach of spraying crops with a microbial agent, i.e. the distribution of biopesticide formulations, is analogous to that used for synthetic pesticides: insect control is solely by means of the organism used (Jaroński 2010). The technical reasons for using inundation are related to the biology of entomopathogenic species, but also to the fact that most cropping systems and their associated pests are transient, occurring for only one growing season, sometimes only for a few weeks. Temporary interruption of the cultural habitat not only removes the pests, but in many cases also eliminates the microbial agent that can control them. Repeated applications are therefore necessary, thus adapting to biopesticides a chemical paradigm familiar to the farmer, who simply applies beneficial fungi as he does chemical pesticides. In the case of biofungicides, which are used to control plant diseases, formulations are available that can be applied both pre-harvest and post-harvest. However, even in this case, the variety of species and strains known for their biocontrol activity is not matched by the number of microorganisms registered as active substances. Moreover, most of the authorised strains (e.g. *Trichoderma* sp.) are concerned with the control of soil-borne pathogens (Ptaszek et al. 2023). Recently, however, there has been growing interest in biofungicides that offer the possibility of controlling infection occurring in the field, but also during storage (e.g. caused by *Botrytis cinerea*, *Penicillium* sp., *Alternaria* sp. etc.) (Sellitto et al. 2021). The use of biofungicides prior to harvest avoids the risks arising from the presence of pesticide residues that do not comply with the limits set by EU legislation. In this regard, it should be noted that in the latest update of Regulation (EC) 1107/2009 on pesticide active substances that do not require an establishment of maximum residue levels, 21 of the 39 listed active substances are microbial-based. Nevertheless, in addition to the factors influencing the effectiveness of the bioinoculants used during cultivation, it is important to consider that the post-harvest phase is characterised by physico-chemical interventions (e.g. washing or modification of the storage atmosphere) used to preserve or extend the shelf-life of the products, which therefore represent another factor of variability.

Furthermore, the use of microbial based product reduces the risk of pathogen resistance, which often occurs with the application of pesticides. The action of microorganisms in plant protection can be based on different mechanisms, both direct and indirect. The direct effects on the pathogen are based on microbial antagonism through antibiosis, competition for nutrients or colonization nisches and parasitism. Microorganisms can also disrupt the growth conditions necessary for pathogens development. The indirect mechanism involves the induction of resistance in the plant which may lead to reduced proliferation, aggressiveness or pathogen damage.

Microorganisms used in biological plant protection must fulfil certain requirements in order to effectively help to protect plants against pests or pathogens:

- they must be safe for human health,
- they must be genetically stable and have a high viability and resistance to environmental fluctuations,
- they should have moderate nutritional requirements and fast growth rates,
- they should be specific to the pest or pathogen concerned, in order to avoid adverse effects on beneficial organisms and to avoid undesirable side-effects,
- they should be compatible with different cultivation systems, including the use of fertilisers and other plant protection products,
- they should be effective in controlling pests or pathogens. Their ability to colonise and compete with pathogens is a key factor.

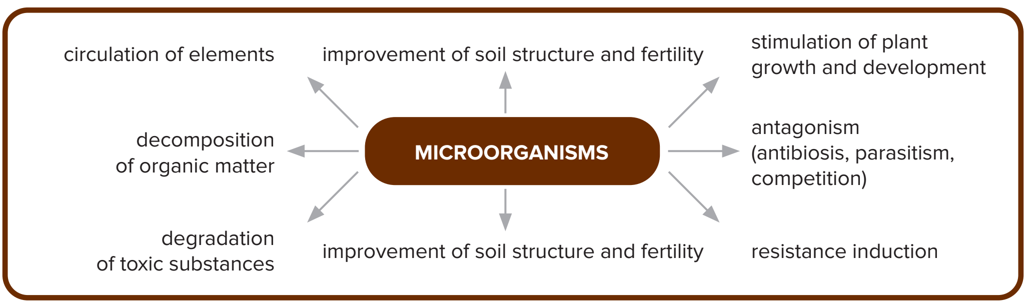

Fig. 2.1. The most important function of microorganisms

2. Biocontrol of soil-borne pests and pathogens: factors to consider for achieving high efficacy

It should be noted that currently most of the biological plant protection products based on microorganisms contain single strains, and only few are registered as consortium (*Trichoderma spp.*). While the efficacy of selected bacterial or fungal isolates in laboratory or greenhouse tests is satisfactory, under field conditions the effectiveness of bioproducts is still one of the major challenges in modern agriculture (Kowalska et al. 2020; Michev et al. 2021). The factors limiting the achievement of high efficacy of such microorganism-based formulations may be due to biotic and abiotic factors i.e. like competition from native microorganisms, susceptibility to variable environmental conditions, but also climate and weather conditions, application methods and crop management practices (Trivedi et al. 2020; Malusà et al. 2021). For practical purposes, the above-mentioned factors can be divided into four categories: crop-dependent (Plant), soil properties (Soil), formulation process (Product) and farming practices (Farmer) (Figure 2.2.).

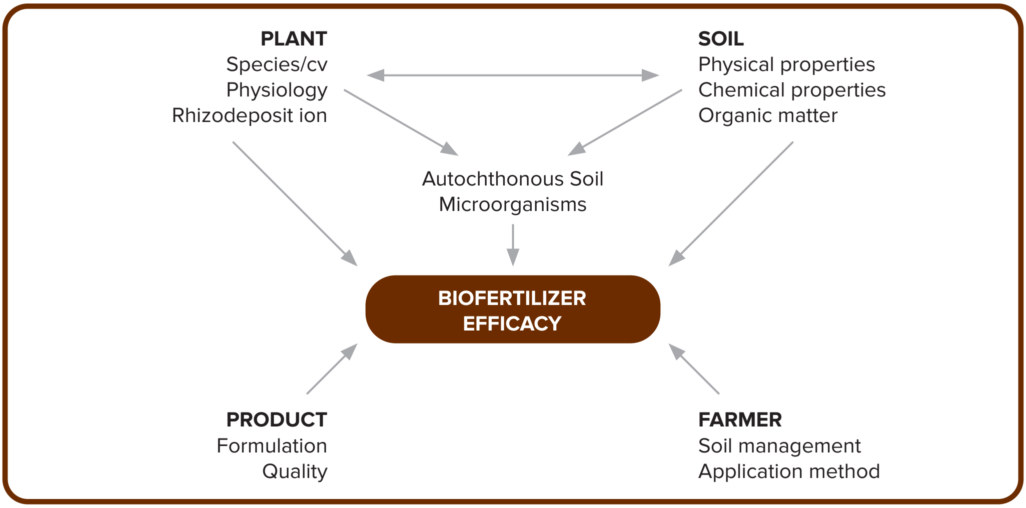

Fig. 2.2. Factors influencing the effectiveness of bioproducts

The physiological state of the plant is important in interactions with biopesticides. Leaf components can affect spore persistence and the susceptibility of insects to infection by bioinsecticides. The nutritional level of the plant also influences the overall health of insect pests and thus may mediate the effect of fungal entomopathogens. In addition, it has been observed that the presence of phytopathogens can affect the susceptibility of the pest to insecticidal fungi: for example, *M. anisopliae* has been found to cause 100% mortality of the pest when the pest is on leaves infected with the fungus, but only 50% mortality when the pest feeds on uninfected leaves (Rostas and Hilker 2003).

Difficulty in ensuring consistent efficacy against plant pathogens and pests increases complications and difficulties in the use of biopesticides despite their safety. In practice, the achievable level of efficacy is definitely a ‘numbers game’: enough spores must be applied to reduce the population of harmful insects or prevent infection by pathogens. In the case of bioinsecticides, the mode of action by which the strain parasitizes (via cutaneous infection or via the digestive system), the behaviour of the insect (on the plant or in the soil) and the morphology of the plant determine the best method of application to ensure effective control. Similarly, for biofungicides, the biological cycle of the pathogen and the site of infection determine the time and method of application to allow preventive colonisation with a biocontrol agent against the pathogen.

The chemical (pH, organic matter and nutrient content) and physical (texture) properties of the soil influence the bacterial and fungal communities and thus the colonisation potential and effectiveness of the biopesticides applied to the soil. Competition from the native microbiome, which is also influenced by soil properties, increases the variability of bioinoculants’ adaptability and persistence. However, optimal conditions for microbial growth (moisture, temperature, organic matter content, etc.) generally favour persistence and root colonisation by bioinocula.

The inoculum production process is crucial to achieve a high-quality formulation, as there is a direct correlation between the abundance of microorganisms and the quality of the final product. Since the active agent in microbial formulations is viable bacteria or fungi, a key problem is to develop a formulation with a long shelf life. Additives are used to improve the physical and chemical parameters of the inocula (adhesives, emulsifiers, buffering compounds) and to extend the life of the microorganisms. The optimum formulation protects the microbial cells during storage and transport, potentially increasing the shelf life of the inoculum in the soil or on the plant while being inert to the environment. There are two basic forms of microbial preparations: dry or granular and liquid. In general, granular formulations show better efficacy in soil conditions, while liquid formulations, although easier to apply, have a shorter shelf life. Another important factor affecting the effectiveness of microbial products is the conditions in which they are stored. Storing products at extreme temperatures or allowing a dry formulation to become damp leads to a faster decline in microbial numbers. Farmers using preparations containing bacteria or fungi should be aware of factors affecting the shelf life of the formulation.

The establishment of minimum legal standards for the registration and marketing of microbial products is important to ensure a minimum quality standard, which is another factor influencing the effectiveness of bioproducts in the field. In the case of biopesticides, their quality is ensured by a lengthy and costly registration process and monitoring of product quality at market level, which must be complied with by each EU Member State.

Understanding the factors influencing beneficial microbial activity is essential for farmers to successfully incorporate such products into practice. The structure of the soil microbiome, and consequently the sustainability or efficacy of inoculated bioproducts, is fundamentally influenced by agricultural management practices, with contrasting effects when comparing intensive and more environmentally friendly systems: practices such as tillage, crop protection, mineral and organic fertilisation or water regime can alter the efficacy of soil-applied biopesticides.

For biopesticides, the practice that can have the greatest impact on their efficacy is certainly resistance to synthetic products (Karpouzas et al. 2022). The site of chemical interaction between bioinsecticides and synthetic product residues is the leaf surface, where the beneficial fungal spore is dormant under most conditions until it comes into contact with the insect's epidermis. Some agrochemicals are rapidly absorbed by the leaf after application: for example, strobilurin fungicides are toxic *in vitro* to fungal entomopathogens and a wide range of fungi, but are absorbed by the leaf within 15 minutes of application, making contact between fungal spores already present or applied at the same time very short and therefore not particularly problematic for the bioinsecticide. Obviously, biopesticides applied after synthetic fungicides must not come into contact with their residues, so the withdrawal period of previously applied products must be taken into account before applying a biopesticide.

Another limitation to the use of biopesticides on a commercial scale is their correct application and dose rate. The effect of dose is particularly important for biopesticides. In general, when applying insecticidal fungi to field or greenhouse crops, at least 10^{-10} propagul ha^{-1} should be applied to ensure a good level of efficacy (Jaroński 2010). This converts to 10^{5} propagul cm^{-2} on a flat surface or, theoretically, 10^{4} propagul cm^{-2} on a crop with a normal leaf area index. Similar doses are also necessary for biofungicides. The situation becomes more complicated for soil-based pest control treatments, where, in addition to the appropriate dose, it is necessary to facilitate contact between the biopesticide and the pest or pathogen. The application technique therefore plays a key role in ensuring defensive action. By concentrating biopesticide propagules on a small, targeted area, a 6-30-fold increase in their concentration can be achieved. However, it should be remembered that after application to the leaves, the concentration of propagules decreases depending on various factors, i.e. sunlight, rain, temperature, humidity, chemical composition of the leaf surface and plant-associated microbiota. A similar situation exists in the soil: in many cases, especially for bacterial preparations, the persistence of bioinoculants in the soil or root rhizosphere is generally limited to 30-40 days after inoculation (Bashan et al. 1995). It is therefore necessary to ensure multiple applications (2-4) per growing season, with frequency depending on weather conditions and pest/pathogen development in the case of biopesticides. This is not considered a disadvantage for microbial preparations, as several treatments per growing season are also made for synthetic preparations.

The method of application can also affect the performance of biopesticides. For their distribution, especially granular formulations, machines are usually already available. The application of bioinoculants by seed dressing or to the soil in liquid form provides comparable efficacy in the production of different plant species (Deaker et al., 2004). The application of liquid formulations by hydraulic sprayer only slightly affects the viability of the bacteria used for crop protection, but the extended working time reduces the number of viable cells reaching the leaf surface by up to 50% (Świechowski et al. 2012). The volume of water used for application and the presence of adjuvants can also affect the effectiveness of fungal biopesticides. Particular attention should be paid to the timing of distribution of the bio-product: overexposure to light (especially UV light) or low humidity conditions can reduce the viability and longevity of the microorganisms and consequently their effectiveness.

It is also important to ensure that the microorganisms introduced into the environment have the appropriate conditions conducive to their proliferation. Also important will be the aspect of proper storage of biopesticides, which depends on their composition.

For these reasons within Excalibur project (Horizon 2020), experimental field trials aimed at investigating the new multifunctional soil microbial inoculants are tested on three model crops in different pedoclimatic regions.

Biocontrol of soil-borne pathogens: practical examples

Biocontrol of soil-borne pathogens involves the use of beneficial microorganisms to suppress the growth of pathogenic organisms in the soil. Several mechanisms are employed by these microorganisms to achieve biocontrol, including antibiosis, hyperparasitism, induction of resistance, and competition for space and nutrients. For instance, certain bacterial strains, such as fluorescent pseudomonads, produce extracellular secondary metabolites that inhibit the growth of soil-borne pathogens. Additionally, some biocontrol agents elicit induced systemic resistance in host plants, while others interfere specifically with fungal pathogenicity factors. The efficacy of microbial-based products in controlling soil-borne pathogens is influenced by various factors, such as the diversity and abundance of pathogenic taxa, soil microbiome, and environmental conditions. Understanding the interactions between the soil, plants, pathogens, and biocontrol agents is crucial for the practical application of biocontrol strategies. Therefore, ongoing research aims to improve the efficacy of microbial-based products and assess their impacts on the soil microbiome (Haas and Défago 2005; Ptaszek et al. 2023; Handelsman and Stabb 1996).

Some examples of microorganisms used for the biocontrol of soil-borne pathogens include:

- *Pseudomonas* spp. – Members of the genus *Pseudomonas*, such as fluorescent pseudomonads, are known for their ability to produce antifungal antibiotics, elicit induced systemic resistance in host plants, and interfere with fungal pathogenicity factors (Haas and Défago 2005).
- *Bacillus* spp. – Certain strains of *Bacillus* bacteria, such as *Bacillus amyloliquefaciens*, have been used as biocontrol agents to suppress soil-borne pathogens. They can produce a variety of antifungal compounds and induce systemic resistance in plants (Bonaterra et al. 2002).
- *Streptomyces* spp. – Some species of *Streptomyces* bacteria have been studied for their potential in biological control. They are known for producing a wide range of

bioactive compounds, including antibiotics and antifungal agents (Bonaterra et al. 2002).

- *Trichoderma* spp. – *Trichoderma* is a well-known biocontrol fungus used to suppress soil-borne pathogens. It acts through various mechanisms such as mycoparasitism, antibiosis, and competition, and has been widely studied for its potential in controlling a range of soil-borne pathogenic fungi, including *Fusarium*, *Pythium*, and *Rhizoctonia* (Graham and Strauss 2021).
- *Fusarium* sp. – The use of antagonistic *Fusarium* strains to control soil-borne pathogens has been studied in the context of biological control. For example, the *Fusarium* strain K5 has shown a disease control of 69% and an increase in biomass production of basil compared to the inoculated control (Pugliese et al. 2008), and Fusarium strain MSA35 is known to produce volatile compounds to control Fusarium wilts (Gilardi et al. 2005).

These microorganisms employ various mechanisms, such as antibiosis, induced systemic resistance, and interference with pathogenicity factors, to control the growth of soil-borne pathogens.

Various methods are used to apply antagonistic microorganisms to control soil-borne pathogens, including:

- Seed treatment - antagonistic microorganisms can be applied to seeds before planting. This method ensures that the microorganisms are present in the rhizosphere, where they can establish and provide protection to the emerging seedlings.
- Soil application - microorganisms can be applied directly to the soil, either as a drench or through irrigation systems. This allows the microorganisms to colonize the rhizosphere and provide long-term protection against soil-borne pathogens.
- Incorporation into growing media - for greenhouse or containerized crop production, antagonistic microorganisms can be incorporated into the growing media to provide protection against soil-borne pathogens (Panth et al. 2020; Pandit et al. 2022).

These methods aim to ensure the establishment and activity of antagonistic microorganisms in the rhizosphere, where they can effectively control soil-borne pathogens and contribute to plant health.

3. Biocontrol of pests and pathogens affecting above ground plant organs: practical aspects

Currently, biopreparations based on microorganisms such as viruses, bacteria and fungi are used in plant protection against diseases and pests.

Viruses

They constitute a huge group of insect pathogens, the most numerous of which is the family *Baculoviridae*. Representatives of this group are viruses with high selectivity for their hosts - insects. Butterflies (caterpillars of the apple fruit fly, leafrollers, etc.) are most often attacked, hymenoptera and beetles much less frequently. Their virulence is often limited to a family, genus or even a particular insect species. Moreover, they do not multiply in the cells of vertebrates and plants, representing an environmentally safe agent of biological pest control. The effects of their presence in insects were observed as early as the 19th century, but their practical use only became possible with a closer understanding of the biology of the hosts, as well as the mechanism of infection and the development cycle of the viruses themselves. Baculoviruses are large viruses containing deoxyribonucleic acid (DNA) packed into proteinaceous rod-shaped capsids, forming mainly in the nuclei of host cells. The capsids are fused into a matrix, proteinaceous molecules called inclusion bodies. This protein matrix, called polyhedrin in nuclear polyhedrosis viruses (NPVs) and granulin in granulosis viruses (GVs), protects them from adverse environmental factors and enables them to survive outside the host organism. Their exclusivity to infect arthropods and their occurrence in protective inclusion bodies make them valuable biological agents of great practical importance. Intrusion bodies carried by wind and rain and by some vertebrates and parasitic invertebrates acting as transporters (vectors) can persist in nature for many years and provide a source of infection for susceptible insects. Ingested by the insect with its food, they dissolve in its intestine and, infecting most host tissues, lead to its death. *Baculoviruses* are present in almost all natural insect populations in Poland. However, additional introduction of them in the form of biopreparations increases their infection frequency or, with the introduction of highly concentrated viral preparations, allows rapid reduction of the pest population. However, it should be remembered that temperature plays an important role in the effective action of virus preparations. It has an important influence on the course of the viral infection. Temperatures below 20°C slow down the rate of virus multiplication, while higher temperatures (25-30°C) accelerate the viral disease process. There is also varying sensitivity of the different stages of the pest to viral infection - younger stages are more susceptible.

Bacteria

Among the large group of insecticidal bacteria in nature, the most important for plant protection against pests is the species *Bacillus thuringiensis*, which produces a protein crystal containing the so-called delta-endotoxin responsible for the death of the insect. The spores (endospores) are the survival form of this bacterium, allowing it to survive even in adverse environmental conditions. The endotoxin crystals and the spores of the various subspecies and isolates of *B. thuringiensis* are usually the basic ingredients of bacteria-based biopreparations. They are used against a number of important pest species: butterfly caterpillars use an isolate of *B. thuringiensis* var. *kurstaki*, fly larvae *B. thuringiensis* var. *israelensis* and beetle larvae *B. thuringiensis* var. *tenebrionis*. The crystal in question is a protein compound that needs to be activated before it can take effect. Under normal conditions, this protein shows very low solubility, making it completely safe for humans, higher animals and most insects. However, it dissolves easily in an alkaline environment (pH>9.5), such as that found in the midgut of, for example, butterfly caterpillars. Therefore, *B. thuringiensis* is a very specific insect control agent. Delta-endotoxin damages the intestinal epithelium of the insect and prevents it from absorbing food. The insect stops feeding and successive generations of bacteria develop in its body, leading to its death within the next few days. The inhibition of feeding and the delayed death of the insect is a characteristic feature of infection by this bacterium.

The use of bacteria to control foliar diseases has been studied, and some beneficial bacteria have shown potential for this purpose. For example, in a study on the biocontrol of root and foliar diseases in tomato, it was found that microbial consortia, including *Pseudomonas chlororaphis* and *Pseudomonas azotoformans*, demonstrated antagonistic effects against the foliar pathogen *Botrytis cinerea* when applied as a foliar spray. These treatments reduced the area of necrotic lesions caused by *B. cinerea*, with the microbial consortia achieving up to a 70% reduction in lesion area (Minchev et al. 2021).

Additionally, bacteria of the genus *Bacillus* (e.g. *B. subtilis*, *B. amyloliquefaciens*) are also commonly used in plant protection against diseases. These bacteria exhibit antifungal activity by producing various compounds (antibiotics, enzymes, chemicals) that inhibit pathogen growth, compete with pathogens for food and living space, and induce resistance in plants. The use of *Bacillus* bacteria in plant protection is often part of sustainable cultivation strategies, as these bacteria are safe for the environment and do not have negative effects on other organisms.

Furthermore, research has demonstrated the potential of bacterial biocontrol agents, such as *Bacillus* and *Pseudomonas* strains, to reduce the incidence and severity of common bacterial diseases when applied as foliar sprays (Minchev et al. 2021).

Yeasts

The most commonly used yeasts for biocontrol of plant diseases in the EU are *Candida oleophila, Aureobasidium pullulans, Metschnikowia fructicola, Cryptococcus albidus*, and *Saccharomyces cerevisiae* (Freimoser et al. 2019). These yeast species have been recognized for their potential as biocontrol agents against various plant pathogens, highlighting their importance in sustainable agriculture practices. *Candida oleophila*, for instance, is known for its strong antagonistic activity against mold and postharvest diseases of pome fruits. *Aureobasidium pullulans* has been used for the biocontrol of various plant pathogens, including fungi and bacteria, and it has been registered for use in the EU as a biocontrol product targeting *B. cinerea* on grape, tomato and strawberry. *Metschnikowia fructicola* has been found to be effective in controlling postharvest diseases of fruits, such as apple and pear. *Cryptococcus albidus* has been shown to have antagonistic activity against various plant pathogens. *Saccharomyces cerevisiae*, commonly known as baker's yeast, has been used for the biocontrol of various plant pathogens, including fungi and bacteria, and different formulated products containing dried yeast cells are also applied as elicitors. These yeast species have been registered for use in the EU as biocontrol products due to their antagonistic activity against various plant pathogens, their undemanding cultivation requirements, and their limited biosafety concerns. The use of these yeast species as biocontrol agents has the potential to reduce the use of chemical pesticides, which can have negative effects on human health and the environment (Kowalska et al. 2022). Therefore, the use of biocontrol yeasts represents a promising approach for the development of sustainable agriculture practices (Kowalska et al. 2022).

Fungi

In addition to fungi, which can be the causal agents of dangerous plant diseases, there is also a large group of these organisms showing specific abilities to act as antagonists and parasitise on or in harmful insects and nematodes. Fungal infections are easily recognised by the characteristic appearance of the diseased or dead insect, as many species produce mycelium and spores on the outside of the host's body after death, covering it with a thick coating often with a characteristic colour e.g. *Metarhizum anisopliae* - greenish, *Beauveria bassiana* – white. The development cycle of most insecticidal fungi is similar: the infective stage is usually the spore, which, after reaching the insect's cuticle, germinates and enters the insect via the germ tube. After entering the body of the host, the fungus multiplies in the haemolymph leading to paralysis of the insect, which dies after some time. After the insect dies, an abundant mycelium develops on the insect's surface, giving rise to new spores that can be carried by wind, water and by insects and other animals.

Of the almost 100,000 species of fungi currently known, approx. 800 species of insecticidal fungi, of which about 12-15 species are used in biological protection against insects harmful to plants, mainly in greenhouse crops, but also in the field. Insecticidal fungi are very common in the environment and play a crucial role by reducing insect populations. The most common species are *Beauveria bassiana, Beauveria brongniartii*, *Metarhizium anisopliae*, *Paecilomyces farinosus*, *Paecilomyces fumosorosea*, *Verticillium lecanii*, which are used to a greater or lesser extent in biological preparations. Insecticidal fungi are very sensitive to temperature and humidity. They act by contact and must be on the surface of the insect's body to germinate and penetrate the insect to cause its death. Due to the requirements of a suitable microclimate, in practice fungal biopreparations are mainly used in greenhouses, although there are also many reports of their good efficacy under field conditions, e.g. *B. brongniartii* for reducing populations of May beetle larvae. In cover crops, they are used to reduce populations of e.g. whiteflies, and in field crops also potato beetle, cabbage leafhopper and brown rot. It has been found that annually about 30% of cereal aphid populations are infected with insecticidal fungi. Epizootics caused by this group of fungi have also been observed in populations of cocksfoot, apple fruit borer, crossbill tansy, apple honey beetle, rape whitewash and others.

In the biological plant protection against pathogens, the most widely used are *Trichoderma* strains. For example, research carried out by various scientific centres all over the world has shown that *Trichoderma* strains or their secondary metabolites exhibit strong antagonistic activity against various plant pathogenic fungi, they compete with and suppress the growth of pathogenic organisms through mechanisms such as the production of antibiotics and enzymes that degrade the cell walls of other fungi, enhance plant growth, and promote the development of the root system. *Trichoderma* can also induce systemic resistance in plants by triggering the plant's defense mechanisms. This includes the activation of signaling pathways that lead to the production of defense-related compounds, making plants more resistant to infections by pathogenic microorganisms. This genus is also known for their ability to produce a variety of enzymes such as chitinases, glucanases, and proteases, which play a crucial role in breaking down the cell walls of pathogenic fungi, contributing to the control of diseases (Waghunde et al. 2016; Zin and Badaluddin 2020).

Ampelomyces quisqualis is another example of biocontrol fungus that has proven effective against foliar pathogens like powdery mildews and rusts. It acts as a hyperparasite, infecting and parasitizing other fungi, thereby suppressing a wide range of plant diseases, including those affecting the foliage. *Clonostachys rosea* has also been investigated for its biocontrol potential against various foliar pathogens. It employs multiple mechanisms, including mycoparasitism, antibiosis, and induced resistance, to suppress foliar diseases in different crops (Elad 2003).

The challenges associated with using fungi for the biocontrol of foliar diseases include:

- Variability and inconsistency - Large-scale use of biocontrol fungi is limited due to the variability and inconsistency of their biocontrol activity, which can be influenced by environmental factors. This variability hinders their widespread application.
- Sensitivity to environmental influences - Some biocontrol fungi are sensitive to environmental influences, which can affect their efficacy. This sensitivity may pose challenges in maintaining consistent disease suppression under varying environmental conditions.
- Integration with chemical fungicides - Integrating biocontrol with chemical fungicides on a calendar basis or according to ecological requirements can be challenging. While this integration can enhance disease suppression, it requires careful management to ensure the compatibility and effectiveness of both control methods.

Fig. 2.3. Photo of adult *Otiorhynchus sulcatus* infected by *Beauveria bassiana*
Author: Małgorzata Tartanus

Fig. 2.3. Foto of Larvae *Melolontha melolontha* infected by *Beauveria bassiana*
Author: Małgorzata Tartanus

4. Multifunctional capacity of microorganisms: an opportunity for integrated pest management

In the past, biological control based on microorganisms has generally consisted of using a single strain of bacteria or fungi, also as a result of regulatory approaches that have been considered microbial-based products similar to chemical compounds. The effectiveness of microbial inoculants, especially those applied as single strains to the environment, varies widely. So, currently the main trend in plant protection is the use of inoculants consisting several microorganisms with different mode of action to increase the effectiveness of microbial products and to extend the spectrum of their activity toward various pathogen species. The consortia could be designed as a mixture of different strains belonging to the same species or composed of species of different genera (Sarma et al. 2015; Minchev et al. 2021; Ptaszek et al. 2023). Consortia based on the complex of beneficial microorganisms potentially could enhance biocontrol effects because different bacteria and fungi have distinct mechanisms of action. Moreover, different microorganisms occupy distinct nishes in the root zone and thus reduce competition among them (Sarma et al. 2015; Minchev et al. 2021). The consortia are superior to the single strain formulation at multiple task level. They can induce different mechanisms of action of the various microorganisms present, which can sometimes also include plant protection mechanisms (e.g. antagonism towards pathogens or induction of metabolic processes that increase tolerance to pathogens or make the plant less 'palatable' to pests), and can therefore be considered as 'multifunctional' products (Kowalska et al. 2020; Minchev et al. 2021). Several biopesticides, including some insecticidal fungi (e.g. *Beauveria* spp.) and bacteria (e.g. *Bacillus thuringiensis*), currently used in crop protection, have shown stimulating effects on plant growth (Kowalska et al. 2020). The dual effect of protecting plants and promoting nutrient uptake has been observed in some studies with *Trichoderma*-based biopesticide strains. Among the bacteria used for protection against pathogens, bacteria of the genus *Bacillus* have, in general, a commercial application as biopesticides, but are also active in promoting plant growth.

The multifunctional capacity of microorganisms provides a promising avenue for developing harmless and sustainable alternatives within the framework of IPM. The Food and Agriculture Organisation (FAO) considers IPM to be the best combination of pathogen and pest control strategies, taking into account yield, profit and safety profile. Numerous field trials and practical applications provide evidence of the successful integration of microorganisms into IPM programs. These studies demonstrate the feasibility and effectiveness of using microorganisms in diverse agricultural settings. Microorganisms are a key element in maintaining the biological balance in the agroecosystem. By exploiting the diverse mechanisms of action of microorganisms, sustainable strategies for pest and disease control can be developed, promoting environmental health and ensuring the long-term sustainability of agriculture. The multifunctionality of microorganisms extends to nutrient cycling and soil health. However, the production and marketing of microbial consortia presents some technical problems for producers and, above all, regulatory issues.

Beneficial microorganisms in the rhizosphere play an important role in alleviating biotic stresses in plants, making their use as bioinoculants to reduce the use of synthetic pest control agents in agriculture attractive. The mechanisms of pathogen reduction by microorganisms in the rhizosphere are diverse (Ptaszek et al. 2023). Some are based on direct interference by beneficial microorganisms with pathogen proliferation in the rhizosphere through various mechanisms of competition, e.g. for nutrients or space, or antibiotic production. Indirect mechanisms are based on the induction of changes in the host plant that alter the susceptibility of the plant to the pathogen, e.g. by inducing systemic resistance in the plant.

On the other hand, many plant growth-promoting microorganisms exhibit biocontrol functions. The observation that root colonisation by mycorrhizal fungi is not always associated with improved plant nutrition and growth has led to the conclusion that increased tolerance to biotic stresses is another important benefit of symbiosis. Increased resistance of mycorrhized plants to infection by soilborne pathogens has been linked to the accumulation of phytoalexins, flavonoids and isoflavonoids in root tissues colonised by these fungi. Biological protection using mycorrhizal fungi has also been demonstrated on plant-parasitic nematodes, e.g. by reducing populations of *Meloidogyne incognita* or *Pratilenchus penetrans* (45% and 87%) in mycorrhizal roots compared to non-mycorrhizal roots. The complexity or multifunctionality of the relationship between host plant and mycorrhizal fungi was demonstrated, for example, in a study in which mycorrhized tomato plants in the presence of the pathogen *Alternaria solani* showed significantly less disease symptoms than non-mycorrhized plants, but increased doses of phosphate fertiliser, in parallel with reduced mycorrhiza formation, led to increased disease severity even in mycorrhized plants. The induction of defence activity by mycorrhizal fungi was also demonstrated in the epigeic organs of the plant: for example, tomato moth larvae (*Helicoverpa arimigera*) reared on the leaves of mycorrhized plants were significantly smaller (62.3% weight reduction) than those reared on non-mycorrhized plants.

In this framework, which encompasses scientific, commercial and regulatory aspects, the development of tools to monitor introduced microbial species becomes extremely important, especially to ensure the correct assessment of risks to the environment and human health, but also to assess their sustainability, which is a useful aspect to determine the optimal method of application (time and doses) that allows for maximum effectiveness. Research for this purpose, which also uses innovative methods, is ongoing, and it can therefore be expected that decision support systems (DSS) will be made available to technicians and farmers to improve the application and effectiveness of bioproducts.

In summary, problems associated with insufficient efficacy of microbiological products may be due to production technology (choice of strains, formulation, application and survival) that has not been fully developed. The pressure to reduce the use of chemical plant protection products and fertilisers from non-renewable sources will result in the further development and improvement of microbial products, despite their current limitations, and they will become a viable alternative to pesticides. It is also expected that with technological progress and increasing knowledge of the viability of microorganisms introduced into the environment, the current barriers to the use of bioinoculants will be overcome.

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Chapter 3

Microorganisms for plant growth promotion

Samuel Bickel[1], Paweł Trzciński[2], Stefano Mocali[3]

[1]Graz University of Technology, Petersgasse 12, 8010 Graz, AUSTRIA

[2]The National Institute of Horticultural Research, ul. Konstytucji 3 Maja 1/3, 96-100 Skierniewice, POLAND

[3]Council for Agricultural Research and Economics, Research Centre for Agriculture and Environment, Via di Lanciola 12/A, 50125 Cascine del Riccio, ITALY

Specific soil, rhizosphere and plant microorganisms play crucial roles in promoting plant growth and agriculture. In this chapter we explain how commonly used microorganisms and commercial strains can promote plant growth. We address the exploitation and selection of pre-, pro-, and postbiotics for plant growth promotion and improvement of soil fertility and soil health. Finally, the practical aspects for the correct application of plant growth-promoting microorganisms are discussed.

1. Microorganisms able to promote plant growth

Plant growth-promoting microorganisms (PGPM) are a key group of predominantly bacteria and fungi that contribute significantly to enhancing plant growth (Glick 2012) and

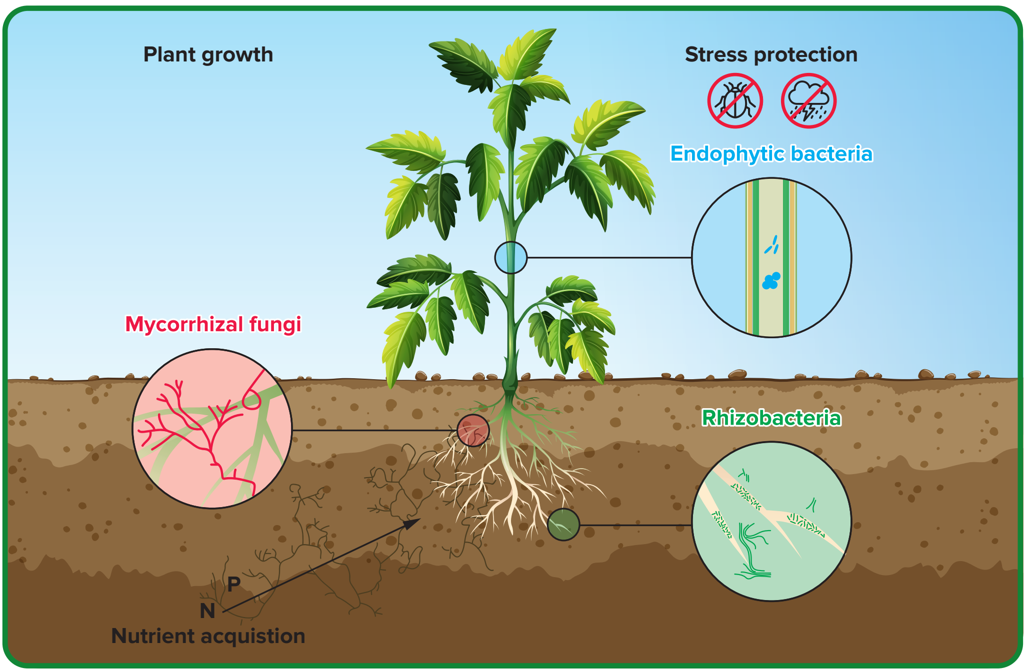

Fig. 3.1. Three main groups of plant growth-promoting microorganisms.

agricultural practice is moving toward this more sustainable and environmentally friendly approach. This includes both the increasing use of transgenic plants and plant growth-promoting bacteria as a part of mainstream agricultural practice. Here, several mechanisms of the mechanisms utilized by plant growth-promoting bacteria are discussed and considered. It is envisioned that in the not too distant future, plant growth-promoting bacteria (PGPB).The utilization of commercial PGPM strains and ongoing research on novel microorganisms can enhance our understanding of how these beneficial microbes can be harnessed to improve agricultural sustainability and crop productivity. The three main groups of PGPM are: (1) plant growth-promoting rhizobacteria that colonize the surface of plant roots, (2) endophytic bacteria that live inside of the host plant, and (3) mycorrhizal fungi that form symbiotic relationships with plant roots. The main functions provided by PGPM and their association with the plant host are illustrated in figure 3.1.

Additionally, the soil and plant microbial diversity play a crucial role in promoting plant growth through various mechanisms that benefit plant health and development. A diverse microbial community in the rhizosphere can increase the availability of nutrients to plants. This diverse community includes microorganisms capable of promoting plant growth by facilitating nutrient uptake and cycling. High microbial taxonomic and functional diversity in the rhizosphere is associated with the suppression of diseases caused by soilborne pathogens and can promote plant growth. The diversity and activity of microbial communities can contribute to improved plant performance and reduced disease incidence, ultimately supporting plant growth and health. The abundance of beneficial microbes in the soil is positively correlated with soil quality, leading to better plant growth, lower disease incidence, and improved nutrient content. The presence of diverse microbial communities' influences soil biological and chemical properties and contributes to enhanced plant health and overall ecosystem functioning. Also, the below-ground microbial community can impact plant community dynamics and ecosystem processes. Furthermore, the interactions between plants and a diverse microbial community can shape plant traits and influence plant adaptation over time.

The intricate interactions between plants and diverse microbial communities underscore the importance of microbial diversity in supporting plant health and ecosystem functioning.

1.1. Mode of action – how do microorganisms promote plant growth?

As mentioned above, microorganisms play a crucial role in promoting plant growth through various mechanisms. These mechanisms include the production of phytohormones, aiding in nutrient acquisition, providing systemic resistance against pathogens, and offering stress protection to plants.

Phytohormones

One of the primary ways microorganisms promote plant growth is through the production of phytohormones. Phytohormones are signaling molecules that regulate various aspects of plant growth and development. Many PGPM can synthesize phytohormones such as auxins, cytokinins, and gibberellins, which influence plant growth processes. For example, *Azotobacter spp.*, *Rhizobium spp.*, *Pantoea agglomerans*, *Rhodospirillum rubrum*, *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Paenibacillus polymyxa* have been reported to synthesize cytokinins, which are essential phytohormones influencing plant growth processes like cell division, elongation, and differentiation (Glick 2012). Several bacterial species can produce indolic compounds such as the auxin phytohormone indole-3-acetic acid (IAA), which plays an important role in bacteria-plant interactions and can stimulate plant growth (Souza et al. 2015). The production of IAA by *Pseudomonas putida* has been associated with significant increases in plant growth parameters and yield in tomato plants. Other taxa, such as Bacilli species associated with the wheat rhizosphere (including *Bacillus endophyticus*, *Paenibacillus xylanexedens*, *Planococcus citreus*, *Planomicrobium okeanokoites*, *Sporosarcina sp.*, and *Staphylococcus succinus*) exhibit multifunctional plant growth-promoting attributes, potentially including the production of phytohormones. PGPM that can synthesize phytohormones can contribute to enhanced plant growth and development through multiple mechanisms.

Nutrient acquisition

Another essential mode of action by which microorganisms enhance plant growth is through the promotion of nutrient acquisition by plants. For example, PGPM can solubilize minerals containing phosphorus, fix atmospheric nitrogen, and produce siderophores that chelate iron to make it more accessible to plant roots. By improving nutrient availability, these microorganisms support the overall growth and development of plants. Microbial inoculants with root growth-promoting and nutrient-mobilizing properties have been proposed as a strategy to improve plant nutrient acquisition. By interacting with organic and inorganic fertilizers, these inoculants can facilitate nutrient uptake by plants and contribute to improved growth and development. Certain beneficial rhizobacteria have been reported to increase plant nutrient uptake by enhancing the efficiency of nitrogen uptake from fertilizers, as demonstrated by studies utilizing ^{15}N isotope techniques to track the movement of nitrogen in plant tissues. Interestingly, microbial inoculants containing *Bacillus spp.* and arbuscular mycorrhizal fungi (AMF) can enhance plant growth and yield, leading to improved nutrient uptake efficiency. These PGPM can help plants extract more nutrients, including nitrogen, phosphorus, and potassium, from the soil, thereby enhancing nutrient acquisition. On the other hand, *Sphingomonas spp.* that produce auxins and siderophores can enhance plant growth and nutrient uptake by stimulating the development of root hairs and lateral roots. Diverse interactions of PGPM play a significant role in enhancing nutrient acquisition by plants, ultimately promoting better growth, nutrient uptake efficiency, and overall plant health.

Biotic stress protection and systemic resistance

Microorganisms can also contribute to plant growth by inducing systemic resistance in plants. In fact, PGPM can activate the plant's defense mechanisms, making them more resistant to pathogens and diseases. This systemic resistance response helps plants combat various stresses and maintain their health and vigor (Vacheron et al. 2013). PGPM have been identified as inducers of systemic resistance in plants through various mechanisms, including siderophore production, antibiotics secretion, phytohormone generation, and the activation of systemic resistance pathways. The ability of PGPM to produce siderophores contributes to their antagonistic effects against pathogens and further supports the induction of systemic resistance in plants. The induced systemic resistance by rhizosphere bacteria confers broad-spectrum resistance, enhancing the plants' ability to defend against different types of pathogens and has been observed in a range of plant species including Arabidopsis, bean, carnation, cucumber, radish, tobacco, and tomato. Here, also the microbial diversity in contact with the plant plays a key role because it offers variability for "training" the plant immune system. By triggering induced systemic resistance, PGPM can enhance the plant's defense mechanisms and improve its resilience against pathogen attacks.

Abiotic stress protection

Moreover, microorganisms provide stress protection to plants, especially under adverse environmental conditions. By colonizing the rhizosphere and interacting with plant roots, PGPM can help plants tolerate abiotic stresses like drought, salinity, and heavy metal toxicity. This stress protection capability enhances the resilience of plants and ensures their survival in challenging environments. Rhizosphere bacteria isolated from harsh environments have been shown to improve drought tolerance in wheat. These bacteria enhance biomass production and reduce emissions of stress volatiles, contributing to increased drought resilience in plants. The biofilm of rhizosphere bacteria, along with soil mulch, plays a protective role against drought stress (Timmusk et al. 2014). For example, *Paenibacillus polymyxa* isolated from the rhizosphere microbiome of wild barley in northern Israel demonstrated the ability to improve plant resilience to drought conditions, highlighting its potential for promoting drought resistance in crops. Likewise, *Stenotrophomonas rhizophila* and other beneficial microbes, demonstrate a strong capacity to colonize root tissues allowing the bacteria to interact closely with the host plant, potentially facilitating the exchange of beneficial compounds and enhancing plant stress tolerance. *S. rhizophila* has been shown to exhibit pronounced saline tolerance by producing highly effective osmo-protectants such as glucosyl glycerol, trehalose, and spermidine that aid plant survival under harsh environmental conditions. These stress-protecting agents promote plant growth and aid in root protection, contributing to enhanced plant resilience in the face of various stressors (Alavi et al. 2013). In summary, PGPM employ a combination of mechanisms, including the production of osmo-protectants, enhanced environmental adaptability, root protection against stresses, and colonization of root tissues, to protect plants from stress and promote their growth and survival in challenging environments.

1.2. Commercial PGPM strains – translating research to application

In research settings, scientists are continuously exploring novel strains of microorganisms with plant growth-promoting abilities. These "research" strains undergo rigorous evaluation to understand their mechanisms of action, effectiveness in enhancing plant growth, and potential applications in agriculture. By studying these strains, researchers aim to expand the knowledge base on plant-microbe interactions and develop innovative strategies for sustainable crop production. PGPM-based inoculants have shown promising results for reducing chemical fertilizer application rates and are being further evaluated as components of integrated nutrient management strategies (Adesemoye et al. 2009).

In commercial agriculture, the use of specific PGPM strains has gained prominence. These commercial strains are selected for their efficacy in promoting plant growth and improving crop yields. By harnessing the beneficial effects of these microorganisms, farmers can enhance the productivity and sustainability of their agricultural practices. Among the most popular PGPM used commercially are *Rhizobia*, *Pseudomonas spp.*, *Azotobacter spp.*, *Bacillus spp.*, *Trichoderma spp.*, *Aspergillus spp.*, and *Glomus spp*. These commercially available microbial inoculants are commonly used as biofertilizers or bioenhancer products that contain single species or multiple strains of beneficial microorganisms that have been extensively studied for their ability to promote plant growth, improve nutrient uptake, enhance plant tolerance against stresses, and contribute to sustainable crop production in smallholder agroecosystems. Table 1 provides an overview of common PGPM, their mechanisms of action, and the benefits they offer to plants.

Tab. 1. Common plant growth-promoting microorganisms (PGPMs).

Microorganism	Mechanisms of action	Benefits for plants	Example
Rhizobacteria	• Production of phytohormones • Nutrient solubilization (e.g., phosphorus) • Induction of systemic resistance	• Enhanced plant growth and development • Improved nutrient uptake • Increased resistance to pathogens	*Bacillus subtilis* produces phytohormones like auxins, solubilizes nutrients such as phosphorus, and induces systemic resistance in plants
Endophytic bacteria	• Establishment of close associations with plants • Production of growth-promoting substances	• Enhanced plant growth • Protection from diseases and stresses	*Pseudomonas fluorescens* establishes symbiotic relationships with plants, secretes growth-promoting substances like indole acetic acid (IAA), and aids in nutrient uptake
(Arbuscular) mycorrhizal fungi	• Facilitation of nutrient uptake, especially phosphorus • Enhancement of plant tolerance to stresses	• Improved nutrient acquisition • Increased plant resilience to environmental challenges	*Rhizophagus irregularis* (formerly *Glomus intraradices*) forms arbuscular mycorrhizal associations with plant roots, facilitating phosphorus uptake and enhancing plant stress tolerance

2. Exploiting pre-, pro- and postbiotics for plant growth promotion and improvement of soil fertility and health

During the last 20–30 years, a large number of PGPM have been isolated, characterized and tested as biofertilizers and biocontrol agents in controlled and natural conditions. The results confirmed the beneficial effect of the selected microorganisms on plant growth and health, enhancing nutrient content and improving soil properties, as reported in the previous section. Now, the emphasis of the scientific activity in the field of microbial inoculants is on developing environmentally friendly and efficient microbial formulations and analyze how the introduced microorganisms affect microbial community, diversity, and the specific plant–microorganism interactions.

In this framework, the EXCALIBUR project[1] activities tackled biodiversity from various angles focusing on soil health in horticulture, thus strengthening cros-sector interactions humans, soils, plants, and ecosystems towards a „One Health" approach. Considering the complexity of the soil system, we moved beyond the simplified view of individual plant–microbe or soil-plant interactions and considered the key factors that influence this complex ecosystem, including the plant, the soil, and the soil organisms as a unique "meta-organism" able to mediate and influence the various exchanges (flows) that contribute to plant health and productivity. A combination of pre-, pro- and postbiotics has been thus applied to manage and stimulate the native soil beneficial microbiome. Briefly, we 'artificially' promoted soil biological functions and diversity integrating management practices with newly developed formulations containing beneficial microbial bio-inocula ('probiotic approach') and bio-effectors ('prebiotic approach'), to understand how they are affecting crop productivity, soil biodiversity and fertility. The 'prebiotic approach' refers to the improvement of plant capacities to exploit the surrounding biodiversity by stimulating soil microbiota and endophytes, while the 'probiotic approach' introduces beneficial living microorganisms into the soil/crop to improve plant nutrition or protection. Excalibur addressed this challenge by a systems approach, evaluating: (i) how cropping systems affect soil biodiversity and their dynamics; (ii) how the native biodiversity influences the efficacy of new bio-products, especially in terms of plant nutrition and protection; (iii) how crop management strategies involving bio-inocula can be improved to assure consistent benefits to growers at the farm level and relevant biodiversity protection at regional/land level.

Implementing a strategy that integrates prebiotics, probiotics, and postbiotics can lead to several benefits for plant growth and soil fertility. Prebiotics and postbiotics improve plant nutrient availability, suppress disease, enhance soil structure, and stress tolerance. Overall, they contribute to sustainable agriculture by promoting a healthy soil microbiome, reducing reliance on chemical fertilizers and pesticides, and enhancing plant resilience to environmental stresses. However, it is essential to consider factors such as microbial compatibility, application methods, and environmental conditions when implementing prebiotic, probiotic,

[1] https://cordis.europa.eu/project/id/817946

and postbiotic strategies in agriculture. Ongoing research is needed to optimize these approaches and understand their long-term effects on soil health and plant productivity.

2.1. How to select a plant beneficial Prebiotic, Probiotic or Postbiotics?

Based on the above considerations, three strategies for microbiome-driven management of soil–plant systems could be selected based on prebiotics, probiotics, and postbiotics (Fig. 3.2.).

Prebiotics

Prebiotics are products which improve microbial diversity and soil health by promoting the growth of soil microorganisms already present within the soil–plant system. Prebiotics are natural products, normally agro-industrial wastes, including biochar, sewage sludge, compost, humus, animal manure, and chitin-bearing wastes, among others, which ameliorate (particularly in degraded soils) the soil structure, biochemical activity, and increase microbial population and diversity. Compost and animal manure, however, can be considered as so-called "*symbiotic*" products as they contain microorganisms (some of them with beneficial properties). Solid-state fermentation based inoculants can also be defined as symbionts, as they are multifunctional mixtures of mineralized organic matter (with both prebiotic and carrier functions) and plant beneficial microorganism (with probiotic plant growth promoting or biocontrol functions) (Vassileva et al. 2020). For example, when the probiotic microorganism is a P-solubilizing agent, the symbiotic mixture could additionally be enriched with plant available P. Similar symbiotic characteristics were observed in microbial inoculants encapsulated in natural gels in the presence of additives with beneficial microbial stimulating action.

Probiotics

Probiotics are commonly accepted as beneficial microorganisms which exert health promoting and nutrient-mobilizing properties. Once introduced into soil, probiotics should develop a critical biomass level to exert their plant beneficial traits. Particularly attractive are bacteria with high enzyme (ACC-deaminase) activity, production of phytohormones (auxins, cytokinins, gibberellins), osmotic metabolites (e.g. trehalose, glycine betaine). As microbial growth depends on the soil–plant characteristics and environmental conditions, it seems difficult for a single microorganism or a microbial consortium to reach the critical cell numbers needed. Therefore, after a long period of studies on isolation, selection, and characterization of PGPM, research scientists are focused on development of economic biotechnological processes for biomass/spores production and formulation that will ensure survival and growth of the inoculum. One of the most promising formulation techniques is the encapsulation in macro- and micro-beads of polysaccharides which guarantees a continuous delivery of the inoculant into soil preventing the effect of soil and environmental stress factors including indigenous microbial community. Double/multiple inoculants combined with biostimulants and other additives including seeds (all-in-one smart bio-formulates) should be developed to complete with the traditional chemical fertilizers.

Another option, to avoid problems during each phase of production, formulation, storage, and establishment/action of the PGPM in soil, is to use their plant beneficial metabolites (postbiotics).

Postbiotics

Postbiotics are metabolic byproducts or substances produced by PGPM, which exert specific growth promoting and/or biocontrol effects on plants thus avoiding the risks associated with applying microbial cells. Specific examples of such metabolite include phytohormones, volatiles, and quorum-sensing compounds. Which are the risks of using microorganisms in soil–plant systems? Wrong formulation procedures without osmo-protectants, UV-protectors, fillers with nutrient value, and other plant benefiting additives can provoke inconsistent results under field conditions. Further risks include various abiotic and biotic factors, which affect the rate of microbial colonization, the presence of other, more competent, components of the microbial population, the level of plant needs and capacity to attract and feed beneficial microorganism. It is important to note that the protocols for field applications of PBM are not assuring that they will find their niche of establishing and function. Moreover, it is yet not clear what kind of metabolites the introduced microorganisms will release in the soil–plant system. This complex set of conditions determines the rate of survival of the inoculants and the performance of their target functions (Kaminsky et al. 2019). Analyzing all these aspects, it appears that endophytic microorganisms are better protected from adverse environmental conditions and, in addition, more efficient functionally.

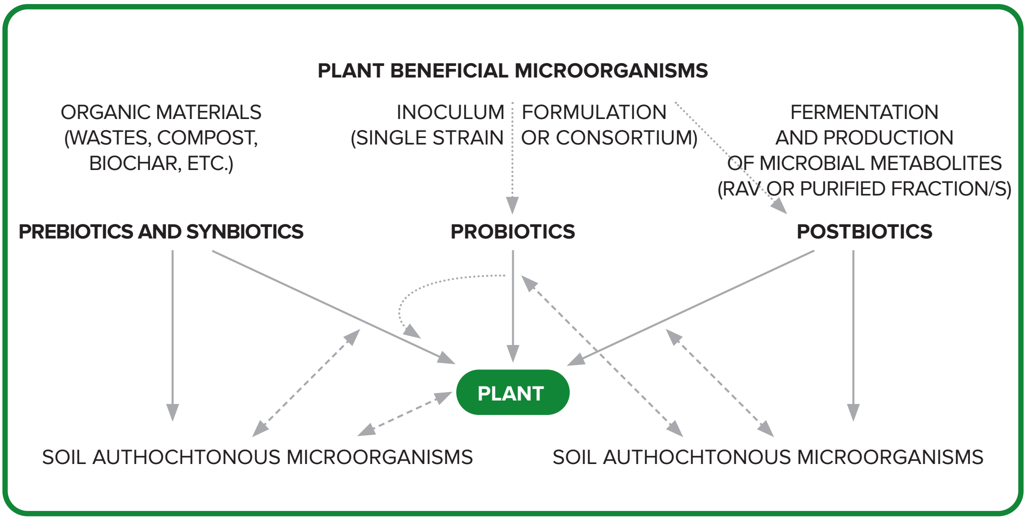

Fig. 3.2. Diagram showing the three strategies for microbial management of soil and plants based on prebiotic, probiotic, and postbiotic approaches. Full lines show the direct effect, dashed lines show the interactions, dotted lines – the formulation/production processes (modified from Vassileva et al. 2020).

3. Practical aspects for the correct application of plant growth-promoting microorganisms

Since microorganisms are living things, the product based on them has to be applied properly to keep the organisms alive. There are many crucial aspects affecting the survival and effectiveness of bacterial and fungal inocula but there are some general rules:

1. The proper storage condition - exposition of the preparations to high or low temperatures, on strong light or moisture could quickly decrease the viable cell count in the product.
2. The usage depends on the type of the microorganism - some beneficial microorganisms like arbuscular mycorrhizal fungi (AMF) have to be applied directly into the soil, preferably, near the root zone, while the others, acting as direct contact biological control agents or endophytes, could be sprayed on the leaves or other parts of the plants.
3. The weather conditions should be suitable for the use of the microorganisms – applying the microbial-containing products should be similar to the usage of plant protection products (avoiding high or low temperatures, rain etc.).
4. Some agrotechnical practices could decrease the effectiveness of microbial inocula – for example fertilization, especially with phosphorus, will inhibit the growth of AMF; application in the same time plant protection products and beneficial microorganisms could destroy part or all of the microbes.
5. Unlike chemical protection products or fertilizers, microorganism products are not fast-acting ones and should be used for prevention. Additionally, the biological control agents do not act as systemic fungicides and due to this they cannot be used to 'remove' pathogens from the plants.

Treatment methods can be divided depending on the purpose, i.e.: spray application, soil treatment or seed treatment. Additionally, in some cases, the treatment will differ between field and greenhouse cultivation.

<u>Spray application</u> is mainly used to treat the above-ground parts of the plants. Due to harsh conditions via the exposition of plants to sunlight, lack of nutrient sources and drying, the treatments have to be repeated several times during the vegetative season. The exception to this rule are endophytic bacteria, for example, from *Methylobacterium* genus. There is also a possibility to apply an inocula on the soil surface to enrich it with beneficial microorganisms. However, this method is prone to be ineffective since the microbes will have problems with penetrating the soil and/or reaching the root zone. The advantage of the spraying method is that it is easy to perform with the use of common agricultural machinery. However, there are restrictions regarding the use of this method. Prolonged and intensive liquid circulation in the sprayer liquid system will induce the death of the bacterial cells due to mechanical damage (Doruchowski et al. 2015). Additionally, the size of the aerosolization rate (lower survival was connected with the smaller droplets), temperature (the highest survival was noticed at 12°C) and relative humidity (the optimal moisture level was 70-80%) could impact the survival of the bacterial cells (Marthi et al. 1990).

Seed treatment is performed to inoculate plants with symbiotic microorganisms, protect them from pathogens or hasten seed germination. There are several methods of seed treatment, but in general, this method relies on coating the seeds with inoculum mixed with binding agents and optionally with cells protectants. It is commonly used to inoculate plants with arbuscular mycorrhizal fungi or legumes with symbiotic nitrogen-fixing bacteria like *Rhizobium* and *Bradyrhizobium*. Also, the seeds could be treated with some biological control agents like *Trichoderma* spp or *Bacillus* spp to decrease the chance of infestation by plant pathogens. Apart from seed inoculation, the same technique could be used to treat seedlings during repotting or before planting. The advantage of seed or seedlings treatment with symbiotic bacteria or fungi is that the inoculation is at majority performed only once and, in most cases, the seed treatment could be done with the use of simple machinery. In contrast, the treatment of seedlings is more complicated as the young plants are fragile and require human labor or specialized equipment.

Soil application is used to apply the beneficial microbes directly into the root zone of the plants or the bulk soil. Another method is mixing the microorganism with the growing substrate to enrich it with the beneficial bacteria or fungi (more commonly performed in greenhouse conditions) before planting the plants or seeds. The application of microorganisms into the bulk soil (with special fertilizers or as a microbial preparation) could be done easily with the use of common agricultural machinery. However, due to economic limitations, the dosage of commercial preparations, depending on the formulation, usually amounts to several kilograms or liters per hectare. Therefore, the ratio of applied microorganisms per cm^3 of soil is low as the inoculum is spread over the entire acreage of the field (so, in places where there are plants and in places where there are no plants). In contrast, the direct incorporation of inoculum into the root zone enables a

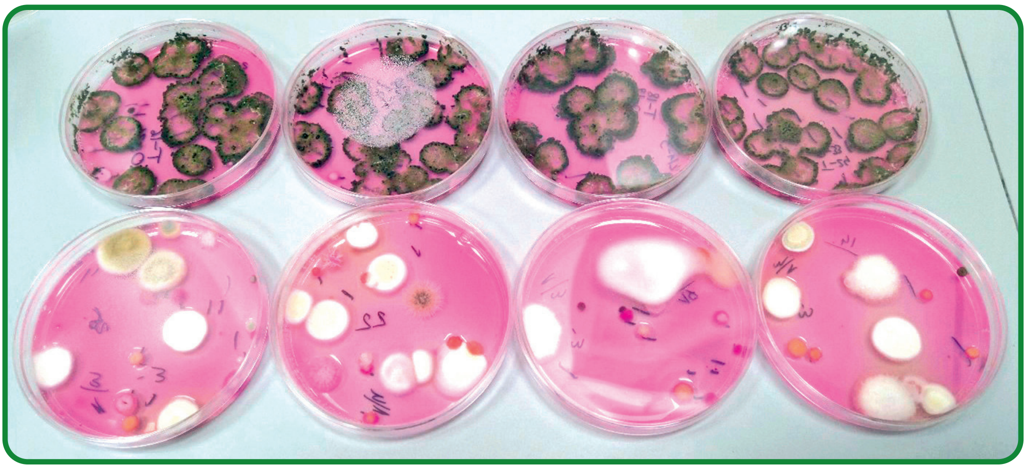

Fig. 3.3. The comparison of fungal populations in soil treated with *Trichoderma* (the upper row of plates) and in untreated control soil (lower row of plates). The green colonies belong to the *Trichoderma* genus.
Author: Paweł Trzciński.

higher concentration of microbes in the place where they can influence of roots or affect the microbiome of the rhizosphere. Science, higher concentration, the introduced microorganisms have a greater chance of success in the soil. For example, in the Excalibur project, the *Trichoderma*-based biological control agent was applied to planting holes just before planting the seedlings. After the vegetative season, the fungi population in the treated soil was dominated by the introduced fungus (Fig. 3.3.). However, this method is hard to perform on already growing plants and is labor-intensive and time-consuming when applying microorganisms to transplanted seedlings. The most commonly used microorganisms in soil application are AMF, nitrogen-fixing bacteria, phosphorus solubilizers and biological control agents.

A separate type of soil application is the introduction of microorganisms through fertigation systems. This method is easy to perform as the microorganisms are introduced to the plants with the irrigation water. However, the disadvantage of this method is that only water-soluble formulation can be used, and after each application, all elements of the irrigation system should be rinsed with water to clean them from the residues of the inoculum. Science, the microbial products, aside from the bacteria or fungi, contain the carriers or bulking agents that could often be used by the microorganisms as a food and energy source. As a result, some parts of the irrigation system, especially drip emitters, could be clogged by the biomass. The most common microorganisms found in the clogged drip emitters are fungi from the *Trichoderma* genus (Boari et al. 2008; Trzcinski et al. 2013).

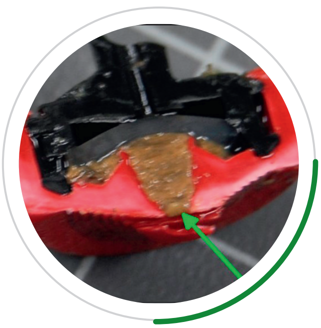

Fig 3.4. Drip emitter clogged by fungal based biomass. *Author: Paweł Trzciński.*

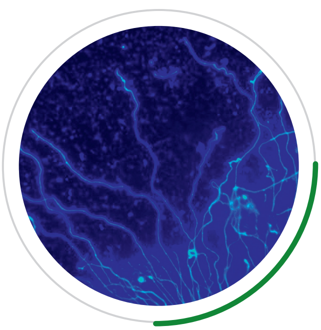

Fig 3.5a. Fungi growing on the inner surface of the drip emitter. Preparation was stained with the use of KOH and calcofluor white and observed on a microscope equipped with UV light. *Author: Paweł Trzciński.*

Fig 3.5b. Fungi growing on the membrane of the drip emitter. Preparation was stained with the use of KOH and calcofluor white and observed on a microscope equipped with UV light. *Author: Paweł Trzciński.*

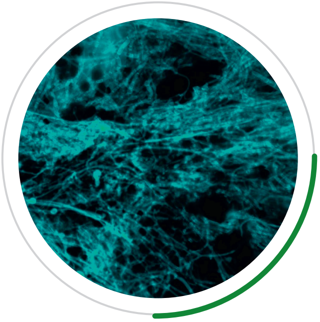

Fig 3.6. Structure of the fungal biomass collected from clogged drip emitter. Preparation was stained with the use of KOH and calcofluor white and observed on a microscope equipped with UV light. *Author: Paweł Trzciński.*

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Chapter 4
Production technology of microbiological products

Katarzyna Góralska[1], Magdalena Jopek[1], Anna Gierut-Kot[1], Jakub Drewniak[1], Roksana Rakoczy-Lelek[1], Maria Bylica[1], Radosław Wilk[1]

[1]INTERMAG sp. z o.o., Al. 1000-lecia 15G, 32-300 Olkusz, POLAND

The purpose of this chapter is to present the fascinating field of microbial technology, which not only transforms the way we look at microorganisms but also has the potential to have a revolutionary impact on our daily reality. By understanding and advancing this field, we can open the door to discoveries, innovative products, and sustainability on a global scale.

1. Types of Formulation (in a practical context – for what, which ones we use and why).

In the design and development of microbiological products with plant beneficial properties, the key points to obtain a good final product are (Vassileva et al. 2021):

1. isolation and characterization of an efficient microbial strain;
2. development of an efficient fermentation process for the production of biomass and/or spores;
3. an efficient formulation procedure

The less studied but with decisive impact is the selection of the adequate formulation technique to ensure the product's highest efficiency and stability in soil-plant systems. Among the formulations available on the market, liquid and solid products dominate (Ibañez et al., 2023). The choice of the formulation procedure is interrelated with the mode of fermentation – it is the technological process, which finally produces the biomass/spores, that should be formulated. For example, some companies produce biocontrol spore-bearing products based on solid state fermentation, while other companies prefer liquid cell-containing products based on liquid submerged fermentation.

Liquid formulations are based on aqueous solutions, oil solutions, or oil-in-water emulsions, with less frequent use of polymers. Microorganisms in liquid solutions often require additives such as nutrient solutions containing basic nutrients or substances that stabilize the entire formulation (Chaudhary et al. 2020). Conversely, carrier materials must be used to support the persistence of microorganisms in the case of free-flowing products. These materials should be environmentally neutral and characterized by a specific, cell/spore-retaining, porous structure. Materials such as peat, talc, lignite, kaolinite and zeolite are commonly used carriers. In INTERMAG, we use bacteria to form bulk formulations, which are obtained by drying biomass at low temperatures and pressure (freeze drying).

It should be mentioned that carriers used in bulk formulations increase the survival rate of the bacteria, protecting them from changes in moisture content and increasing the product's volume, resulting in an even dispersion of microorganisms during application (Singleton et al. 2022), thus increasing their development and efficacy in soil-plant systems.

Liquid and free-flowing formulations have their own unique characteristics that should be considered when producing a microbial product. What is not discussed in scientific works is that the liquid form of the products allows for the more accessible and faster preparation of the working liquid for application. In addition, it does not require prior preparation of a slurry, as in the case of free-flowing formulas. Another important advantage is that liquid formulations show better miscibility with other agrochemicals, which reduces the risk of nozzle clogging during spraying. From practical point of view, the lower risk of nozzle clogging and the ability to mix with other formulations increases flexibility and application efficiency. It is worth noting that liquid formulations can be more susceptible to storage conditions that can affect the degradation or oxidation of formulation components.

Packaging tends to be larger, and the concentration of microorganisms in solution is lower than that of bulk formulations. The high concentration of microorganisms in free-flowing products allows the use of smaller packaging, making them lighter and easier to transport and store. However, it is potentially more challenging to apply these products. The need to evenly disperse the powder in the working liquid and the risk of precipitation of insoluble fractions during application entails greater involvement when preparing the solution. It is worth mentioning that free-flowing formulations show higher stability over a longer storage period, providing a longer shelf life.

All types of formulations containing microorganisms intended for marketing must be tested for their stability over time, temperature and compatibility with other agrochemicals when recommending combined use. From industrial point of view, the introduction of several different types of agrochemicals into a single tank mix that has not been previously tested for combined use can lead to adverse interactions between microorganisms and the active ingredients contained in the different types of formulations, which can result in a loss of biological effectiveness of such a tank mix. The reservoir mixture prepared for testing with the microbial formulation under test should also be stable for at least 4 hours. In mixability tests, the abundance and survival of microorganisms are determined at intervals.

In our work, when developing a new product, we tried to establish a rigorous quality control regarding formulation and testing for efficacy in a given crop species, as well as environmental impact and possible phytotoxicity of above-normal doses. Accordingly, our intention is to develop new formulated products, which should be stable and suitable after storage for at least 12 months.

2. Microbial product manufacturing technology

In today's dynamic world, microbial product manufacturing technology is a fascinating industry revolutionizing many fields, from agriculture and medicine to environmental protection. Microorganisms, such as bacteria and fungi, are becoming objects of intensive research and a source of innovative solutions and modern products.

The microbial technology is based on the use of microorganisms to produce a variety of substances, from enzymes and antibiotics to specialized agricultural preparations (Kuila and Sharma 2018). There is a close-knit interaction between biological science, chemistry, process engineering and the practical aspects of industrial production. Microorganisms play a key role in this technology, acting as biological factories capable of synthesizing various chemical compounds. Microorganisms are adapted to produce desired substances through fermentation, or genetic modification, introducing new industrial and agricultural production possibilities. The technology for producing microbial products is applicable in a wide range of fields. In agriculture, microorganisms produce metabolites that improve crop yields, plant resistance, and soil structure. In medicine, they are a source of antibiotics and biotechnology drugs. In the food industry, they produce fermented products. While in environmental protection, they are used to treat wastewater and soil.

Despite many advantages, the technology also presents challenges. Introducing innovative solutions, managing complex processes and maintaining sustainable practices are some of the issues that require our attention. The development of modern microbial genetic engineering techniques simultaneously opens new perspectives and raises ethical and regulatory questions.

2.1. Selection and isolation of microorganisms

Microbes have colonized environments where there is a chance for their survival, which creates a wide range of places, from those where there are optimal conditions for most bacteria to extreme ones where highly specialized bacteria live. Microorganisms play a huge role in the environment. They are responsible for circling biogenic elements in the biosphere (carbon, nitrogen and phosphorus) and distributing a large portion of biogenic elements in living organisms. Molecular nitrogen is a major air component but inaccessible to higher organisms. Thanks to the activity of microorganisms, it is converted from compounds available to plants. The number of bacterial species in a gram of soil varies from several hundred to more than eleven thousand (Elander and Chang 1979). The quantitative and qualitative composition of the bacterial community depends on several physicochemical factors, as well as the type of soil and plant species inhabiting the soil, agricultural practice, geographic zone, nutrient content, salinity and environmental contamination. They affect the weathering of minerals, thus contributing to changes in soil-forming processes (Bednarski and Fiedurek 2012). In addition, they decompose and mineralize organic matter. By renewing humic compounds, they shape and improve soil structure. They prevent erosion and protect the soil from drying out. They facilitate soil cleanup by taking part in the decomposition of pesticides, hydrocarbons and antibiotics.

Selection of microorganisms for agricultural products such as fertilizers, biostimulants and crop protection products is a process that aims to select strains of microorganisms with beneficial properties. The first step is to define the objectives to be met by the microorganism. These may include, for example, the ability to fix nitrogen, produce substances that promote plant growth, or the ability to suppress pathogens.

The stages used to select the beneficial microorganisms are as follows:

- primary selection and isolation of a pure culture,
- secondary selection.

Primary isolation of microorganisms from specific environments involves the selection of microorganisms exhibiting the desired properties. Their thorough characterization follows the isolation of microorganisms. Their physiological, biochemical and genetic properties are studied. This makes it possible to determine whether the microorganisms have the desired characteristics. Secondary selection involves isolates with the highest expression of the chosen trait (Steele and Stowers 1991).

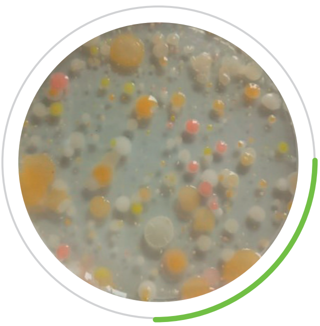

Fig. 4.1. Colonies of bacterial isolates grown on trypticase soy agar (TSA).medium confirming the microbial diversity during isolation and selection of plant beneficial microorganisms.
Source: Intermag sp. z o.o.

Screening tests of specific activity play a major role in selecting specific microorganisms. Laboratory tests to confirm the properties of microorganisms include a variety of methods and techniques that allow a thorough analysis of their morphology, physiology, genetics and other characteristic features.

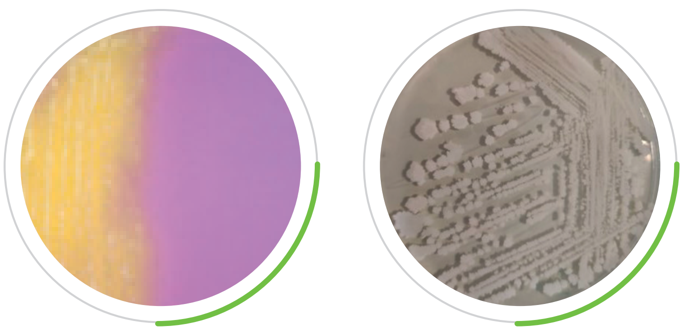

Fig. 4.2. An example of selective isolation of microorganisms of the genus *Bacillus* on solid media. Left image - chromogenic medium for differentetion of acidifying micoorganisms. Right image - mannitol salt agar medium for diversifcation the source of carbohydrates for gram-positive bacteria. *Source: Intermag sp. z o.o.*

The basic selection technique is the medium enrichment method. This is the process of providing a suitable environment which is suitable for the growth of specific microorganisms simultaneously inhibitory or lethal for non-target ones. For example, anaerobic microorganisms do not survive in the presence of oxygen. Resistance to heavy metals, antibiotics, oxidants and organic solvents can also be applied to selective environments for microorganisms with specific properties.

Significant advances in the development of analytical tools significantly affect the ability to screen for specific compounds. Instrumental analysis is useful in both secondary and primary screening when simpler techniques are unavailable. Instrumentation such as high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), nuclear magnetic resonance (NMR) spectrometry and others allow for faster, selective and highly sensitive detection of metabolic products. Of increasing importance is the use of molecular biology and bioinformatics tools to rapidly verify the genetic potential of microorganisms. The specific set of tests depends on the type of microorganism and the purpose of the study (Olicón-Hernández et al. 2022).

Microorganisms that show promise under laboratory conditions are tested under field conditions. This makes it possible to assess their effectiveness under realistic agricultural conditions. The best microorganisms are selected for further breeding based on the test results. This process involves the selection of genotypes with desirable characteristics and their mass breeding under controlled conditions.

2.2. Banking

Once the microorganism has been identified and characterized, it should be stored in a condition that ensures physiological, biotechnological and genetic stability. This process is called banking and includes maintaining the isolated selected strains for further experiments and their characterization.

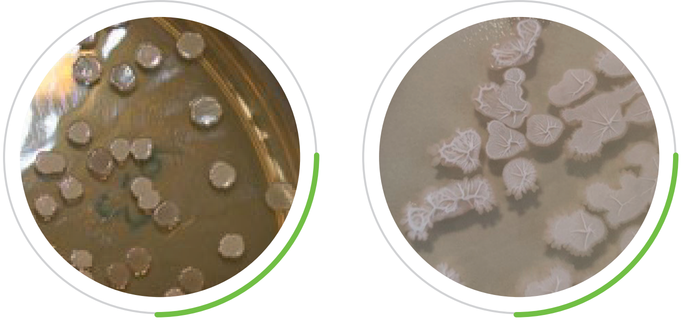

Fig. 4.3. Pure bacterial cultures isolated from environments.. Left image *Bacillus pumilus*, right image *Bacillus subtilis*.

Source: Intermag sp. z o.o.

Selected isolates are stored in our own resources and/or external institutions - collections (banks) of microorganisms. Banks of microorganisms are an important tool in science, medicine, industry and agriculture. New species or strains with previously unknown characteristics described in scientific publications should be available to all interested parties. For security reasons, isolates should be stored by several alternative means. Microorganisms are stored in the collection in a freeze-dried state, in a deep-frozen state at -80°C in liquid nitrogen at temperatures down to -195°C, or in special containers with a preservative (Stackebrandt et al. 2014).

Fig. 4.4. Container systems for storing microorganisms at low temperatures

Each culture is carefully documented, containing information on the species, strain, culture conditions, physiological characteristics and other important data. Microbial banking plays a crucial role in preserving biological resources, contributing to scientific and technological progress and ensuring the continuity and reproducibility of production of preparations based on active microorganisms.

2.3. Inoculum

A microbial inoculum is a term describing a microbial culture grown at specific conditions used to start a large-scale cultivation process. In the context of microbial formulation technology for agriculture, the inoculum plays a key role as a source of microorganisms that that after a fermentation process will be introduced into formulations that affect soil and plants. It serves as a starting point, providing the necessary number of active and healthy microorganisms to positively impact the agrotechnical environment. The process of microbial inoculum production is a carefully controlled step. It begins with collecting an active strain of microorganisms, then cultured on a smaller scale to form an inoculum. In this process, care is taken to ensure the right culture conditions, such as medium composition, temperature and humidity, to ensure the microorganisms' ability to multiply effectively. The quality of the inoculum is a key factor in the effectiveness of microbial preparations. The microorganisms in the inoculum must be active, able to compete with soil pathogens, improve nutrient availability and promote healthy plant growth. Producers take care to control culture parameters by monitoring microbiological and genetic quality. The effectiveness of microbial inoculum in agriculture depends mainly on its ability to adapt to soil conditions.

Producers must adapt the process of growing microorganisms to become accustomed to the specific environmental conditions in which they will be applied. This adaptation allows the microorganisms to colonize the soil better and positively affect plants.

Once the optimum inoculum has been achieved on a smaller scale, the process is scaled up to produce larger quantities. Bioreactors and fermenters grow microorganisms under controlled conditions to produce large quantities of active microorganisms ready for use in agricultural formulations.

The ultimate effectiveness of microbial preparations in agriculture is directly related to the quality of the inoculum. Properly prepared inoculum ensures the delivery of an adequate number of active microorganisms to the field, which translates into increased plant biostimulation, reduction of soil pathogens and improved overall soil health. Monitoring the inoculum production process and its microbial composition is key to maintaining high standards of quality and efficiency in the production technology of microbial preparations for agriculture.

2.4. Media

Nutritional media are a basic element in the technology of microbial preparations for agriculture. They are specially composed nutrients designed to provide microorganisms with the right ingredients for growth and activation. A growth medium or culture medium is a solid, liquid or semi-solid combination of substances designed to support the growth of microorganisms. In producing microbial formulations, an adequately selected growth medium is crucial for the quality and productivity of the microorganisms introduced into the agricultural environment. The composition of microbial media is tailored to the requirements of the individual microorganisms being cultured. They usually contain sources of carbon, nitrogen, phosphorus, macro- and micronutrients and other nutrients. Depending on the type of microorganisms, nutrient solutions can be adjusted to maximize their growth and activity. The optimal composition of the nutrient solution is crucial for obtaining high quantities of active microbial cultures. In some cases, it could be economically attractive to use agroindustrial wastes as a production medium (Vassilev et al. 1998).

Fig. 4.5. Microbiological media commonly used in the laboratory.
Source: Intermag sp. z o.o.

Microbiological media play a key role in the process of growing microorganisms on a larger scale. Manufacturers of microbiological preparations carefully tailor the media to the specifics of the cultured strains, which enables rapid and healthy growth of microorganisms. They provide nutrients, stability, and uniformity of culture conditions, which is important for obtaining effective preparations. The composition of culture media influences the characteristics of microorganisms cultured in the production of microbiological preparations. Properly adjusted culture media can affect metabolite production, enzymatic activity, or the ability to compete with pathogens. Careful control of the composition of nutrient solutions allows production of to tailor microorganisms for specific agricultural applications, increasing formulations' effectiveness.

Producing microbial media involves careful mixing and controlled delivery of nutrients. During this stage, manufacturers must take care to maintain the proper proportions of ingredients to meet the requirements of the microorganisms being cultured. Controlling parameters such as pH, temperature and nutrient concentration is crucial to achieving optimal culture conditions.

The final quality of microbial preparations in agriculture is closely related to the quality of the microbial media used. Effective composition of the medium positively affects the reproduction, activity and viability of microorganisms, which translates into the formulation's effectiveness. Manufacturers precisely control this element of the process, ensuring that the media supports the expected characteristics of the microorganisms and ensures the consistency of the final product. Taking care to precisely tailor the media to the needs of the cultured microorganisms is integral to achieving optimal results in this area.

2.5. Key components of the production line

The key element of any biotechnology plant is the bioreactor, around which the entire plant is built to produce the finished product. Depending on the needs, this may include mixers, centrifuges, filtration systems, dryers, lyophilizers, clarifiers and decanters.

Most biotechnological processes using microorganisms seek to move them from their natural habitat through a petri dish into bioreactors, otherwise known as fermenters. Bioreactors and their processes are usually the centerpiece of any biotechnology. It is in them that the growth of the microorganism and the production or processing of the desired product occurs. In strictly technical terms, a bioreactor is considered: "An enclosed space of any size and shape in which there is the possibility of exchanging momentum, heat and mass with the environment and the possibility of a chemical reaction. The bioreactor should contain any biocatalyst to which such operation conditions are created to obtain the desired effect in the form of a predetermined course of a biochemical reaction or growth of cells of a living organism." (Olicón-Hernández et al. 2022).

As quoted above, the definition of a bioreactor is very general, so bioreactors are subjected to many classifications based on different structure elements, modes of operation, or biocatalysts. The most general division relates to their differentiation in terms of how the process is carried out:

- bioreactors for submerged culture,
- bioreactors for culture on solid media,
- bioreactors for culture with an immobilized biocatalyst.

The type of bioreactor used for the process depends mainly on the growth requirements of the microorganism. The most commonly used fermenters are those for aerobic submerged processes. In this type of fermenter, the medium is in a liquid state and the biocatalyst is suspended in it.

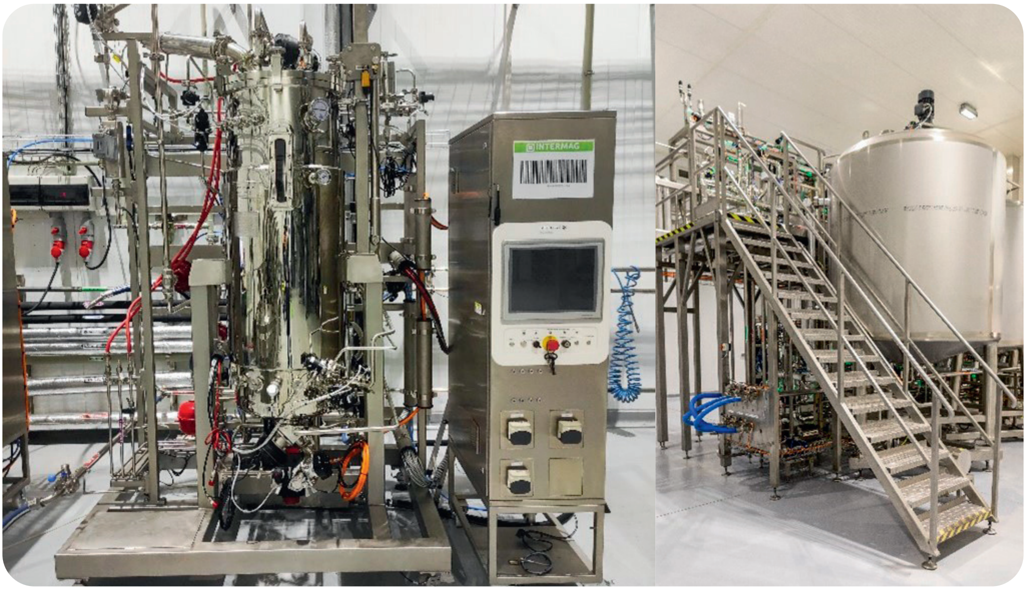

Fig. 4.6. Bioreactors for the production of microbial products at INTERMAG.

Source: Intermag sp. z o.o.

As indicated above, the bioreactor is an essential point of most biotechnology production processes, and each subsequent component is selected accordingly under the operation of a particular bioreactor or its assemblies. Such a plant may include mixers for combining biomass with formulation components, filtration membranes or centrifuges capable of separating biomass from culture fluid, dryers or freeze-dryers for obtaining a bulk product, homogenizers for releasing the bioproduct from within the cells, and many other pieces of equipment often developed and built specifically for producing a particular product.

Fig. 4.7. Microbial product production line at INTERMAG.
Source: Intermag sp. z o.o.

2.6. Biopreparation production technology

A biopreparation can be: a microorganism in various forms metabolite, post-digestion medium, or a mixture of the three. Theoretically, the easiest to obtain is a post-digestion mixture containing both the microorganism and the post-digestion medium with all the metabolites. Such production is based on the multiplication of the microorganism in batch cultivation, i.e., preparation of the medium, inoculation, culture and collection of the produced biomass. In practice, several problems arise here related to the viability of the microorganism in the finished product, stability of secondary metabolites constituting the properties of the product, storage of the biomass itself as well as the product, and above all, appropriate packaging to eliminate the risk of contamination with undesirable microflora, for which the biomass itself may serve as a medium.

In order to preserve the properties of the bioproduct for as long as possible, a reduced storage temperature is used, which works well for products based on bacteria such as *Rhizobium* spp. or *Azotobacter* spp. Products containing spore-forming bacteria, e.g., *Bacillus* spp., are characterized by greater shelf life precisely because of the stability of the spore forms.

Solid formulations require further processing of post-culture biomass, i.e., appropriate compaction and addition of protective and carrier substances. The most common methods for obtaining dry formulations are spray and freeze drying. Both processes aim to remove water from the biomass, but they do so differently.

Lyophilization, otherwise known as sublimation drying, is a process that takes place under low temperatures and vacuum conditions. Preparation of the microorganism for the freeze-drying process involves concentrating the biomass as much as possible, i.e., removing water initially by filtration or centrifugation. The concentrated biomass is then mixed with a cryoprotectant, i.e., a substance that is supposed to protect the microorganism's cells from being ruptured by ice crystals during the first freeze-drying stage, i.e., freezing to as low as -80°C. Popular protective agents include maltodextrin, sucrose, skimmed milk powder, trehalose and glycerol. The biggest advantage of freeze-drying is the high concentration of the product, which almost wholly removes water and extends the product's shelf life. However, it is a costly process, which ultimately exemplifies the higher price of the commercial product.

Fig. 4.8. The production freeze-dryer at INTERMAG.
Source: Intermag sp. z o.o.

Spray drying is a high-temperature process in which biomass is pulverized in hot air. Preparation of the biomass, as with freeze-drying, involves compacting it to obtain the greatest amount of dry matter possible, which will produce the appropriate grains of the finished product in the dryer. Spray drying is used with microorganisms exposed to high temperatures (mainly spore-forming bacteria, e.g., *Bacillus* spp.) or appropriate stabilizing substances will be used, e.g. whey protein, maltodextrin, starch, polysaccharides.

For products based on a specific metabolite, such as NOD factors or specific enzymes, the culture of microorganisms must be carried out under conditions that direct the metabolism of the microbe for production. This can be achieved by altering the physical conditions of the culture or the appropriate composition of the culture medium.

2.7. Monitoring and control of variables affecting microbial growth and quality control

Monitoring the culture of microorganisms is a key process in laboratories, the biotechnology industry, pharmaceutical production and scientific research. This includes controlling culture conditions, evaluating microbial growth, tracking metabolite production, and maintaining culture purity and quality. Monitoring microbial culture is essential in research and industry to ensure process control, optimize performance and produce consistent results. In the field of industrial biotechnology, microbial culture is a fundamental step in the production of a variety of substances, from drugs to agricultural products. To ensure optimal growth conditions, monitoring the variables affecting microorganisms closely is necessary. This process involves controlling environmental parameters, culture density, metabolite production and culture purity.

A well-known chart of microorganism growth during stationary culture is known as batch culture. The growth curve of a microorganism, as well as the production of a biotechnology product, has four characteristic stages:

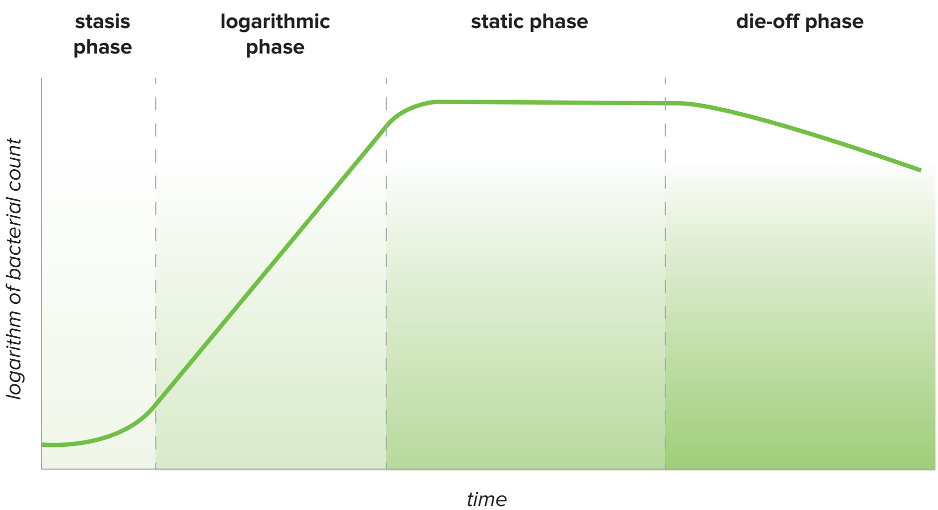

Fig. 4.9. Batch culture growth curve.

1. Stasis or pre-stasis or the period in which the microorganism adapts to the new culture environment and begins its growth;
2. The logarithmic phase or rapid growth phase depends on the availability of nutrients and the accumulation of metabolites that limit the growth of the culture, as is the case with alcoholic fermentation;
3. The stationary phase, otherwise known as the lag phase or the number of cell divisions, is equal to the number of dying cells;
4. The die-off phase begins when cells rapidly begin to die.

This graph is important for monitoring the production process, as it is necessary to correctly place the relevant moments of the production process on the above curve. Skillful control of the process based on knowledge of the culture's condition and the microorganism's requirements in the various growth phases allows efficient control of the process and high production efficiency. The process control and the cultures are influenced by physical and chemical factors such as temperature, pH, oxygen and carbon dioxide concentrations.

Temperature has a decisive effect on enzyme activity and microbial metabolism. Temperature control in bioreactor cultures is carried out by heating and cooling the water jacket of the bioreactor (used most often in large industrial fermentors) or heat exchangers located in the culture vessel (popular in small laboratory reactors. Temperature control in bioreactors is multistage and includes control of the temperature of the culture vessel, the temperature of the heating jacket or heat exchanger, and the heating and cooling medium.

Proper pH is essential for proper protein structure and enzyme function. Each microorganism has a specific pH tolerance range of the solution in which it can survive and a specific narrow optimal range that allows it to grow most efficiently. The pH is controlled based on the readings of pH electrodes placed in the bioreactor.

Adequate oxygen levels are crucial for aerobic microorganisms, while anaerobes require an oxygen-poor environment. Gas distribution is the most critical issue when culturing aerobic microorganisms such as *Bacillus* spp. The shape of the reactor, the agitator used and its power, the shape of the aeration device and its location in the culture vessel are important factors in gas distribution. Control of the relative saturation of the medium with oxygen is carried out using oxygen electrodes. In the case of anaerobic organisms, oxygen should be removed from the medium by saturating it with an inert gas, such as nitrogen, because for many organisms, it is harmful, such as methanogenic bacteria. In bioreactors with a mechanical stirrer, maintaining the desired oxygenation conditions of the culture involves intensifying stirring during the increased oxygen demand phase, which can result in high shear forces within the stirrer.

In addition to the physicochemical variables of the bioreactor, it is very important to control the microorganism's growth and the medium's quality during culture. Depending on the target product, measurements are made of the microorganism's utilization of substrate components, e.g., glucose, using strip tests, or in more advanced systems using near-infrared spectroscopy, the growth of a metabolite over time, e.g., antibiotic production by *Streptomyces*. On the other hand, one can control the growth of microaggregates per se by counting bacteria in situ using OD 600 optical density probes or more sophisticated solutions performing cytometric and electrical capacity measurements of the culture, which correlates with the number of viable cells in solution.

2.8. Hygienic considerations for the entire line

In an ideal set-up, all biotechnology production should occur under sterile conditions, from the inoculation of the first flask to the confection of the finished product. Ensuring culture sterility is the key and most resource-intensive process in industrial microbiology, as it begins at the design stage of the premises and production lines and the target products. The basic quality standard defining production lines in biotechnology are the requirements established by the US FDA and 3A specifying several requirements, such as perfectly smooth, electropolished weld surfaces that come into contact with the product. This procedure translates into further use of the plant, as fine roughness can trap biofilm, leading to contamination porosity of the material, which can prevent degassing of the vessel during sterilization, leading to non-sterility of the bioreactor or other vessel. The next step is to select appropriate materials for the anticipated production, i.e., resistant to high temperatures during steam sterilization and aggressive cleaning chemistry during cleaning and disinfection processes.

3. Biological efficacy and commercialization

3.1. Verification of biological efficacy under laboratory conditions

Verification of the biological efficacy of microbial formulations is a key step in the process of their development and evaluation. It involves a series of laboratory tests to confirm whether the microorganisms in the formulations exhibit the expected biological and physicochemical activity. In this context, efficacy can include the ability to solubilize phosphorus, assimilate nitrogen, control pathogens, improve plant health, or affect soil microbial structure.

Various laboratory methods are used to evaluate the biological effectiveness of microbial preparations. These can be in vitro tests, microscopic analysis, molecular biology techniques, or studies on the production of metabolites by microorganisms. Specific procedures are used depending on the purpose and type of preparation to obtain a complete picture of biological effectiveness. One commonly used test is antagonistic analysis, which tests the ability of microorganisms in a formulation to compete with pathogens. For example, antimicrobial tests can show whether a microbial formulation inhibits the growth and development of pathogenic microorganisms, which is an important indicator of effectiveness.

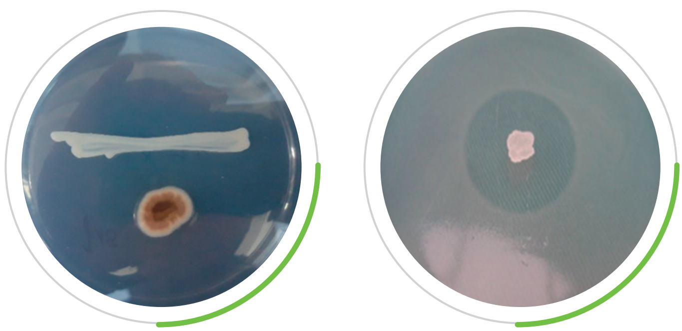

Fig. 4.10. An example of verifying the properties of microorganisms in terms of biocontrol activity – *Bacillus amyloliquefaciens* versus *Botrytis cinerea* (left) and silicon solubilization of *Paenibacillus polymyxa* (right). *Source: Intermag sp. z o.o.*

Another test of the properties of microorganisms is their ability to solubilize nutrient elements such as phosphorus or assimilate nitrogen.

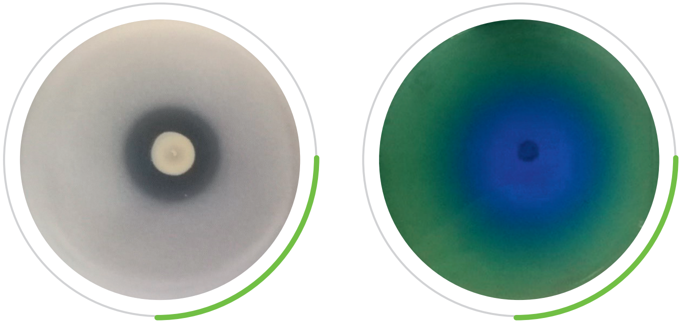

Fig. 4.11. Property test - (left) phosphorus solubilization by *Bacillus pumilus*- discoloration around the microorganisms indicates a correctly occurring process; (right) nitrogen fixation by *Paenibacillus polymyxa* - the appearance of blue around the bacterial colony indicates active nitrogen fixation.
Source: Intermag sp. z o.o.

Genetic and molecular analyses make it possible to identify microorganisms in preparations and monitor their biological activity; accurate analysis of gene expression amounts of DNA or RNA can provide information on the response of microorganisms to laboratory conditions, which helps assess their performance. Monitoring the growth and activity of microorganisms in microbiological preparations is crucial in assessing their ability to colonize and reproduce under specific conditions. Measuring parameters such as the number of cells, the amount of chemicals secreted or the rate of cell division provides important information on biological efficacy (Sana et al. 2023).

The final verification of the biological efficacy of microbial formulations involves tests conducted under conditions that simulate natural environments, such as soil or water. This verifies that the microorganisms also retain their desired properties under more complex conditions, which is crucial for assessing their suitability under real agricultural conditions.

3.2. Verification of biological effectiveness under controlled conditions

Verification of biological efficacy under controlled conditions is the process of evaluating the effectiveness of a strain or formulation under controlled conditions, i.e., phytotron, greenhouse in crop cultivation. This process aims to understand how well a substance or product under test performs in a biological context (setting), i.e., the effect on plant growth and development.

Stages of verification of biological effectiveness (in plant cultivation) under controlled conditions:

1. Design of the experiment - defining the purpose of the experiment and its methodology, defining the expected results and selecting the appropriate parameters.

2. Preparation of a controlled space - creating an experimental environment where factors such as temperature, humidity and lighting can be controlled to ensure reproducibility and comparability of results.
3. Application of the substance or product - application of the substance or product as directed.
4. monitor biological parameters during growth - study biological parameters such as plant height, chlorophyll content, macro and micronutrient content, and more.
5. Determination of biological parameters after completion of cultivation - the study of biometric parameters of plants, i.e., vegetative weight.
6. Statistical analysis of data - processing and analysis of the collected data, comparison of the experiment results with the control group and interpretation of the obtained results.

Verifying biological efficacy is a key step in scientific research, especially in fields such as agriculture, biology, biotechnology and medicine. It allows scientists, researchers and agricultural formulation companies to assess whether a new product or substance has the intended effect under controlled conditions, which is essential before it can be used in agricultural practice.

3.3. Verification of biological efficacy under field conditions

Verification of biological efficacy under field conditions refers to evaluating the effectiveness of substances or products in the environment under field conditions. Unlike the controlled conditions found in a phytotron, field conditions include a variety of environmental factors, such as varying weather conditions, varying soil composition, and the effects of different organisms in an ecosystem. Steps for verifying biological efficacy under field conditions:

1. Design of the experiment under field conditions - developing a research plan that considers the specific field conditions under which the experiment will be conducted;
2. Site preparation - selecting a suitable area for the experiment, preparing the site in terms of research objectives, and applying necessary safeguards, such as ethical rules or experimental permits;
3. Application of the substance or product - application of the substance or product under natural conditions, considering the specifics of the substance or product, the crop type and the environment;
4. Monitoring of biological parameters of the crop in the field - conducting measurements of selected plants in plots, determining the emergence, vigor and healthiness of plants, measuring chlorophyll content, monitoring vegetation indices, i.e. NDVI.
5. Measurement of yield quantity and quality;
6. Data collection and statistical analysis - analyzing the collected data in the context of the effect of the substance or product on plant growth, development and yield, and the influence of external factors.
7. Verifying biological efficacy under field conditions is important because it provides information on how products or substances affect the plant in the actual environment.

This approach is fundamental in agriculture, conservation and ecology, where interactions between plants and their environment are complex and can be challenging to reflect under laboratory conditions.

In short, the process of verifying the biological efficacy of microbial formulations is a multi-step process that combines a variety of testing methods. Attention to precise, reliable and representative testing allows manufacturers to provide microbial formulations that are effective under controlled conditions and meet expectations in a diverse and dynamic agricultural environment.

3.4. Solubility, miscibility and stability of biopreparations

Checking the stability of microbial formulations is an important step in their manufacture and use. Stability refers to the ability of a preparation to maintain its biological, physicochemical and microbiological properties for a specified period and under various storage conditions. Laboratory testing assesses whether the microorganisms in the formulation retain their activity ability to grow and do not degrade when exposed to various factors such as temperature, light or humidity. Stability tests also include monitoring for possible changes in the chemical composition of the formulation and assessing whether degradation of active substances is taking place. This is important because the effectiveness of a microbial formulation in agriculture depends largely on maintaining the integrity of the microorganisms during storage. Careful stability testing allows manufacturers to accurately determine the shelf life of a formulation and assure farmers that they will benefit from a product with consistent quality and effectiveness. Tests are used to assess how long a biopreparation retains its effectiveness under storage conditions, which can include storing the biopreparation at different temperatures, conducting microbial composition analyses such as assessing the number of viable microorganisms and analysing genetic diversity after a certain period, and evaluating physical and chemical changes. Stability is checked on various scales, from laboratory tests to pilot-scale tests to large-scale production tests, to assess whether the product maintains its properties during various stages of production and storage.

Checking the solubility of microbiological preparations is an important aspect of their effectiveness and use. In this context, the process aims to assess how much the formulations can dissolve and distribute uniformly in various carrier substances, such as water or other auxiliary agents. Solubility tests are essential because they affect the uniformity of a formulation's application in the target area, determining the effectiveness and efficiency of the microorganisms in the field. Manufacturers usually conduct these tests under laboratory conditions, simulating various application conditions. In practice, solubility tests include assessing the time required for the product to dissolve fully, the degree of dissolution at different temperatures or pH, as well as evaluating any physicochemical changes. Careful monitoring of these parameters allows manufacturers to adjust microbial formulation formulations to ensure optimal application methods. Effectively preparing microbial formulations for application is important for successful use in agriculture, horticulture, and environmental protection. Solubility tests evaluate how a bioproduct dissolves in water of different hardness levels or other substances. This can be accomplished by preparing biopreparation solutions in various solubilizing agents and evaluating the degree of solubility. Solubility tests are not required for solid products containing active microorganisms applied directly to solid ford.

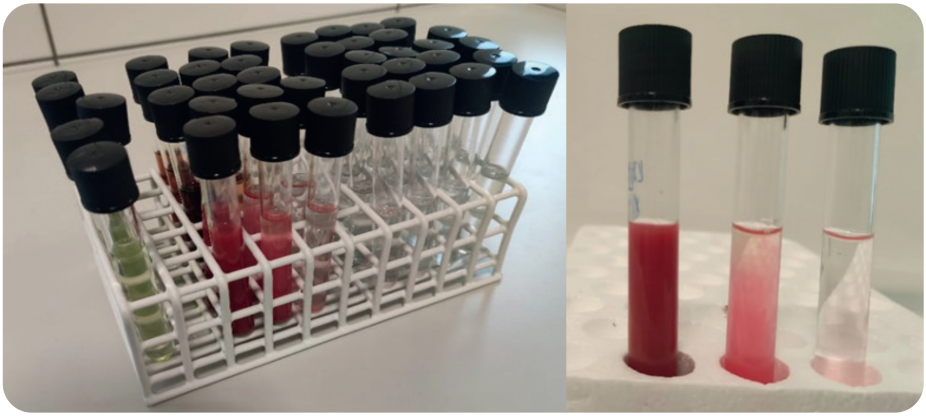

Fig. 4.12. Mixability tests of agrochemicals with microbial products in a multicomponent spray.
Source: Intermag sp. z o.o.

Checking the miscibility and compatibility of microbial formulations with other substances is a key step in their application in agricultural practice. Miscibility refers to the ability of a formulation to mix evenly with other substances, such as fertilizers or pesticides, without forming undesirable deposits or changes in physicochemical structure. Compatibility, on the other hand, refers to the ability of a formulation to interact with different chemicals without losing its effectiveness, i.e., in the case of microbiological formulations, maintaining the viability of active microorganisms. Mixability and compatibility tests are carried out under laboratory conditions, where the stability of the formulation in different mixtures and possible interactions with other substances are evaluated. As a result, manufacturers can provide farmers with microbial formulations that are readily miscible with other agents used in agrotechnology while not losing their effectiveness. Meticulous research in this area helps minimize the risk of undesirable chemical interactions, ensuring the effective and safe use of microbiological preparations in agricultural practice (Ledakowicz 2012).

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 817946.