
Efficacy of Biofertilizers: Challenges to Improve Crop Production

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Abstract

Different kinds of soil microorganisms belonging to several taxa of the bacteria, fungi, and possibly, protozoa kingdoms, colonizing the rhizosphere or the plant tissues and promoting plant growth (PGPM), can be utilized for the production of microbial-based fertilizers (biofertilizers). However, their application in agricultural practice is still hindered by several factors. The main reasons derive from the unpredictability of results, problems to identify and track inoculated strains in the field, the poor understanding of the interrelationships between microorganisms and plants, and the technology of production. After describing in brief which microorganisms have been utilized up until now to improve plant productivity through enhanced nutrition, we mention for possible exploitation of new groups of microorganisms (e.g. non-mycorrhizal fungi). Furthermore, we review the factors affecting the efficacy of biofertilizers on crop productivity, from the point of view of the farmers, who appraise their application on the base of their efficacy. In particular, we consider the factors related to the production process (including quality and marketing standards), the persistence and traceability of inoculants, the relations between plant, soil conditions and microorganisms, as well as the effect of farmers' practices (fertilization, soil management practices, application method). In conclusion, it emerges that biofertilizers could allow obtaining a crop productivity similar to that obtained with mineral fertilizers, but with a significant reduction of their use. Therefore, biofertilizers can play a key role to develop an integrated nutrient management system, sustaining agricultural productivity with low environmental impact.

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2.1 Introduction

Exploiting microbial-based fertilizers can be traced back to ancient times—already Classical Greek and Roman writings (namely Virgil's *Georgics* or Pliny the Elder's *Naturalis Historiae*) described agricultural practices for improving yield, that we can today link to microbiological activity (e.g. rotation with legumes or use of animal manure); the ancient Maya were managing water in the Mexico wetlands to support a complex mixture of algae, cyanobacteria, and other microorganisms also with the purpose of increasing the content of nutrients in the soil (Morrison and Cozatl-Manzano 2003). However, a conscious use of microorganisms for soil fertilization started in the late nineteenth century, when patenting and marketing of microorganisms for fertilization purposes began (Kilian et al. 2000; Nobbe and Hiltner 1896, cited in Bashan 1998). Since then, particularly in the last couple of decades, the development and use of microbial-based fertilizers has gained significance in the effort of reducing the negative environmental effects generated by the excessive and/or improper application of chemical fertilizers. However, despite the huge amount of studies and findings of beneficial strains, the application of microbial-based fertilizers in agricultural practice is still hindered by several factors. The main reasons derive from the unpredictability of results, problems to identify and track inoculated strains in the field, the poor understanding of the interrelationships between microorganisms and plants, and the technology of production (Bashan et al. 2014; Lucy et al. 2004; Owen et al. 2015). In this chapter, we aim to describe which factors we consider as mostly affecting a widespread exploitation of microbial fertilizers. Moreover, we want to foster actions by the different stakeholders interested in this sector that could promote a wider practical application of these

products. We also briefly describe which microorganisms have been utilized up until now to improve plant productivity through enhanced nutrition, also providing information about new groups of microorganisms not widely exploited yet.

2.2 Microorganisms for Biofertilizers

Different kinds of soil microorganisms belonging to several taxa of the bacteria, fungi, and possibly, protozoa kingdoms, colonizing the rhizosphere or the plant tissues and promoting plant growth (PGPM), can be utilized for the production of biofertilizers (Lucy et al. 2004; Smith and Read 2008; Vessey 2003). Their contribution to plant nutrition can be limited to a single nutrient element, as in the case of N-fixing bacteria, or to a variety of elements, such as for arbuscular mycorrhizal fungi (AMF) (Bardi and Malusà 2012, and references therein). However, they can have a remarkable impact on the yield and quality of plants, increasing the nutrient uptake capacity and the use efficiency of applied chemical or organic fertilizers. Rhizobia, the best known N₂-fixing bacteria symbionts of legume plants, are able to provide up to 90 % of the N requirements of the host through atmospheric N₂ fixation (Franché et al. 2009), but they can also behave as plant growth promoting rhizobacteria (PGPR) with non-legumes such as maize, wheat, rice, and canola (Hayat et al. 2010; Yanni et al. 2001). Non-symbiotic free-living N-fixing bacteria species have been proved to enhance N uptake of plants (Bardi and Malusà 2012; Lucy et al. 2004; Okon and Labandera-Gonzalez 1994), which can derive nitrogen from biological nitrogen fixation in 7–58 % range in cereals (Baldani et al. 2000; Malik et al. 2002) and up to 60–80 % in sugarcane (Boddey et al. 1991). Cyanobacteria

(*Anabaena*, *Aulosira* and *Nostoc*), as free-living or in symbiosis with *Azolla*, a small free floating fresh water fern, were found to fix N and to release it for rice uptake in the range of 30–40 up to 70–110 kg N ha⁻¹ (Wagner 1997). Arbuscular mycorrhizal fungi (AMF) may supply more than 50 % of plant N requirements (Govindarajulu et al. 2005; Leigh et al. 2009), which is particularly important under arid and semi-arid conditions, where water availability limits uptake of mobile nutrients such as inorganic N (Subramanian and Charest 1999). AM fungi can take up nitrogen both as inorganic (either ammonium or nitrate) and organic (Hawkins et al. 2000).

Arbuscular mycorrhizal fungi form the major group of microorganisms contributing to plant phosphorus (P) uptake (Smith and Read 2008) by increased exploitation of the soil (Cavagnaro et al. 2005), the solubilization of inorganic P forms (Tawaraya et al. 2006), and the hydrolization of organic P (Richardson et al. 2009). Several PGPR are very effective in solubilizing P from the highly insoluble tricalcium phosphate, hydroxyl apatite and rock phosphate (Rodríguez and Fraga 1999; Owen et al. 2015).

A wide array of bacterial genera (e.g. *Pseudomonas*, *Burkholderia*, *Acidithiobacillus*, *Bacillus* and *Paenibacillus*) are able to release potassium from minerals such as mica, illite, muscovite, biotite and orthoclases (Bennett et al. 1998, 2001; Liu et al. 2012), increasing K availability up to 15 % (Supanjani et al. 2006).

The search of new strains of microorganisms showing beneficial effects for plant nutrition has fostered studies on species that were less considered in the past. Following, we present an overview of results and potentialities which could derive from the introduction of non-mycorrhizal fungi into biofertilizers.

2.2.1 Potentialities of Non-Mycorrhizal Fungi as Inoculants for Biofertilizers

Fungi are ubiquitous in soil, and can be dominant components of the microbiota in many soil types

(Gadd 2004; Burford and Gadd 2003). For example, fertile soil may contain a fungal network up to 10,000 km/m² (Burford et al. 2003). By adapting their metabolism to the availability of varying nutritive compounds in the soil environment, fungi produce a wide range of oxidative and hydrolytic enzymes that allow them to efficiently break down organic matter like ligno-cellulosic materials but also other natural or human-derived compounds, like in the field of xenobiotic and organic pollutant degradation (Harms et al. 2011).

The plasticity of fungi biology and the plethora of functions that can be attributed to fungal metabolism suggest that there are several potential uses and forms of exploitation of non-mycorrhizal fungi for the production of biofertilizers. The ability of some fungal groups or species in the dissolution or leaching of minerals and elements' chelation and translocation has been very little evaluated and even less exploited as a potential for the production of innovative soil amendments.

The biological activity of fungi can cause the enrichment of C, N, and S in the soil, making these as well as other nutrients available to plants. Moreover, fungi are capable of transporting substances in their hyphae that act as pipes connecting microenvironments with different concentration of nutrients and can actually transport ions against a chemical osmotic gradient (Banitz et al. 2011, 2013). Translocation across distant parts of the mycelium enables fungi to colonize places with low initial resource availability and to actively change the microenvironment and the availability of nutrients in the substrates, turning the colonizing mycelium from a resource sink into a source (Banitz et al. 2011, 2013). Jongmans et al. (1997) proposed that tunnels formed inside weatherable mineral grains were likely to have been formed by fungal hyphae and coined the term “rock-eating fungi” to describe such microscopic tunnels within feldspar and hornblende grains in the eluviated horizon of podzol soils. Within soils, a vertical distribution can be distinguished regarding fungal type in terms of their ecology (Pinzari et al. 2001). Organic layers are mostly colonized by

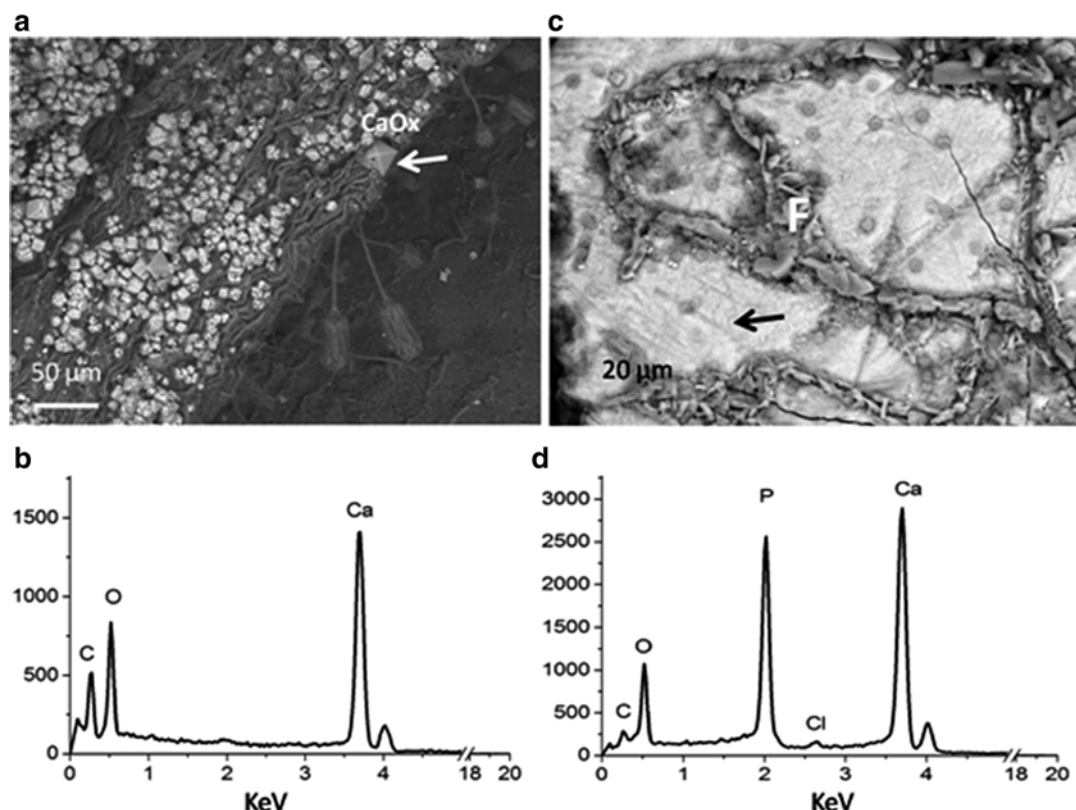


Fig. 2.1 Precipitation of calcium oxalates by filamentous fungi and solubilization of P-containing minerals observed with a Zeiss EVO 50 variable pressure scanning electron microscope (VP-SEM) operating at an accelerating voltage of 20 kV equipped with a detector for backscattered electrons (BSE) (Pinzari et al. 2012): (a) bipyramidal structures of calcium oxalate produced in vitro by an *Aspergillus (A.terreus)*; (b) energy dispersive spectroscopy (EDS) spectra with chemical characterization of the crystals mainly containing Ca; (c) solubilization of apatite (P-containing minerals) by fungal hyphae (*Aspergillus niger*); tracks of dissolution of the mineral material are caused by fungal growth. In the tracks, around fungal threads other biogenic crystals are deposited. (d) X-ray area scan of the apatite that contains P and Ca. The y-axis on the spectra represents the EDS counts in arbitrary units

saprophytic fungi, whereas mineral layers are colonized by mycorrhizal fungi (Van Schöll et al. 2008).

Fungi can dissolve rocks and leach minerals by different mechanisms that involve the excretion of H^+ , or the production of primary and secondary metabolites with mineral solubilization or metal-chelating properties like siderophores, phenolic compounds, carboxylic acids, and amino acids. The potential of some fungal species in the breakdown of mineral phosphates could be very high, as shown in some recent

papers (Pinzari et al. 2012) (Fig. 2.1). Fungi are more efficient than bacteria in P solubilisation, on both solid agar and in liquid cultures (Saxena et al. 2013). According to some authors, sub-culturing of most of the P-solubilizing bacteria results in the loss of the phosphate solubilizing activity (Halder et al. 1990) while fungi maintain their ability to leach P-containing rocks even after prolonged culturing (Kucey 1983). Such features could be important in the industrial manufacturing of biofertilizers for P nutrition.

P mobilization, particularly from Fe and Al phosphates, has been shown to be performed also by non-symbiotic fungi from different species of genera such as *Penicillium*, *Aspergillus*, *Trichoderma*, *Mucor*, *Candida*, *Discosia*, *Eupenicillium*, and *Gliocladium* (Ahmed and Shahab 2009; Jain et al. 2012; Saxena et al. 2013; Wakelin et al. 2007; Whitelaw 2000). The solubilizing ability of P minerals by the different organic acids produced by fungi also allows the mobilization of minerals other than phosphates (Achal et al. 2007; Ahmed and Shahab 2009; Asea et al. 1988). Esterase type enzymes released by fungi are known to be involved in liberating phosphorus from organic P compounds (Ahmed and Shahab 2009).

Fungal dissolution mechanisms can release also other cations like Si^{4+} , Fe^{3+} , Al^{3+} , and Ca^{2+} (Boberg et al. 2009). In general, fungi are strong solubilizing agents of K containing minerals such as feldspar, biotite, and phyllosilicates by organic acid release (Ahmed and Shahab 2009; Gadd 1999; Sayer et al. 1995; Singhal et al. 1994). *Piloderma* was able to extract potassium and/or magnesium from biotite, microcline, and chlorite to satisfy plant nutritional requirements (Glowa et al. 2003).

Fungal cells can also represent elective sites for biogenic mineral precipitation. This is the case of calcite or metal oxalates precipitation, which would also influence the availability of phosphates for plants that have been widely documented as coupled to fungal growth in near-surface limestones (calcretes), calcic and petrocalcic horizons in soils (Gadd 2007). Reduced forms of metals (such as Ca, Cd, Co, Cu, Va, Mn, Zn, Ag, Ni, and Pb) can be precipitated by many fungi within and around fungal cells (Gadd 2007). Mechanisms of fungal mineral weathering, translocation or bio-precipitation are still little known, but could represent useful tools especially in the perspective of using fungi in the formulation of biofertilizers aimed at improving soils' chemical and structural properties as well as plant nutrition (Table 2.1).

Table 2.1 Fungal properties that can be further explored for new biofertilizers

Fungal property	Potential applications for improving crop production	Examples or reference studies
Hyphae highly suited to growth across soil physical structure (surfaces, pores, and air gaps)	Fungi as highways or pipelines for nutrients translocation	Wick et al. (2010), Furuno et al. (2012), Banitz et al. (2011, 2013)
Ability to develop in patchy environments	Improvement of soil fertility and treatment of extreme heterogeneous soils (i.e. saline soils)	Green et al. (2008), Bashan and de-Bashan (2010)
Translocation and redistribution of biogenic elements	Improvement of soil fertility	Boberg et al. (2009)
Growth in low nutrient habitats	Widening the possibility of crop production in sites with low resource availability	Green et al. (2008)
Dissolve rocks, leach minerals, precipitation of calcium oxalate	P solubilization and availability	Sudhakara et al. (2002), Kucey (1983), Chuang et al. (2007)
	Si solubilization	Meena et al. (2014), Pradhan and Sukla (2005)
Chitin as elective sites for biogenic mineral precipitation	Ca insolubilization	Gadd (2000), Burford et al. (2003)
	Toxic metals precipitation	
Precipitation of reduced forms of metals (like Ca, Cd, Co, Cu, Va, Mn, Zn, Ag, Ni, and Pb) within and around fungal cells	The precipitation of metal oxalates may provide mechanisms that allow fungi tolerating high concentrations of toxic elements	Gadd (2007), Gadd (2008), Harms et al. (2011), Fomina et al. (2003, 2004, 2005), Daghighi et al. (2010)
Degradation of organic compounds	Compost stabilization	Harms et al. (2011), Gadd (2008), Wick et al. (2010)
	Organic pollutants decomposition	

2.2.2 Fungal Inocula for Micronutrients Mobilization: The Case of Silicon

Lack of trace elements in soil is not uncommon (Bell and Dell 2008). However, the limitation of vital micronutrients can be attributed to some factors that reduce their availability for crops such as low organic matter content, high amounts of sand (soils with coarse textures), use of chemical fertilizers that change the equilibrium between soil fungi and bacteria as well as between the mineral substrates and microorganisms, or to other menaces that alter soil functions and fertility (compaction, desertification, etc.) (Brevik and Burgess 2012). Although these elements could be abundant in rocks, they are not always available to plants, as in the case of silicon (Si). Silicon is present in plants, and several studies have shown beneficial effects of silicon fertilization for agricultural crops (Belanger et al. 1995; Savant et al. 1997, 1999; Meena et al. 2014). The beneficial effects seem mainly associated with Si deposition in plant tissues, which enhances their rigidity and resistance to mechanical stress. This increased strength improves the light-receiving posture of the plant, benefiting photosynthesis, and enhances the resistance to biotic and abiotic stresses (Gascho 2001). Plants absorb silicon from the soil solution in the form of monosilicic acid, also called orthosilicic acid (H_4SiO_4) (Meena et al. 2014). Typical silicon absorbers and accumulator crops are rice, wheat, millet, and sugarcane, which require a relative large amount of silicon. However, inorganic materials such as quartz, clays, micas, and feldspars, although rich in Si, are poor sources because of their low solubility (Meena et al. 2014).

Fungi and bacteria can solubilize insoluble silicates (Wainwright et al. 1997; De Mico et al. 2004). Fungi, while degrading silica-based rocks, can release other mineral nutrients (e.g. potassium, iron and magnesium) (Daghino et al. 2010). The solubilization process occurs mainly via the production of organic and inorganic acids and complexing agents (Gadd 2008) and it is faster than that of bacteria (Castro et al. 2000; Gadd

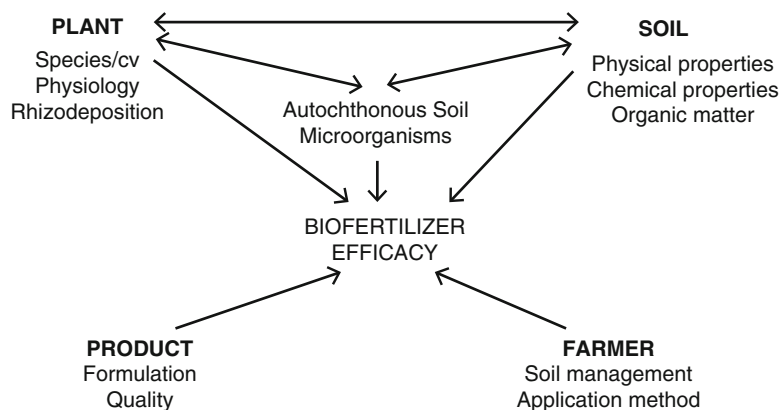
2008; Daghino et al. 2010). The ability of *Aspergillus niger* in weathering olivine, serpentine, feldspar and other minerals, of *Penicillium simplicissimum* disgregating basalt, of *Penicillium expansum* and *Scopulariopsis brevicaulis* solubilizing aluminosilicates has been demonstrated (Daghino et al. 2010). The solubilization of silica by fungi (and bacteria) is considered as a source of supply for several crops such as cotton, maize, wheat, potato and tomatoes (Meena et al. 2014). Therefore, to fully exploit this capacity and benefit of Si nutrition by plants, further studies on application of bioinoculants for improving Si availability are needed.

From this general brief overview it appears that the availability of several beneficial strains from the different groups of microorganisms is not hindering the possibility of formulating an efficient biofertilizer.

2.3 Factors Affecting the Efficacy of Biofertilizers

The various mechanisms involved in plant promotion may be host plant-specific and strain-specific. Furthermore, once introduced into the soil, plant growth promoting microorganisms (PGPM) face competitive conditions that may severely reduce their beneficial effects (Bashan 1998). Therefore, the beneficial effects deriving from the application of a specific biofertilizer may differ greatly under different agro-environmental conditions and this has resulted in contesting the efficacy of microbial-based products (Cummings 2009; Owen et al. 2015). However, to overcome such perception and improve the propensity of farmers in using biofertilizers, it is useful to consider which factors affect the efficacy of biofertilizers on crop productivity trying to meet the point of view of the farmers, who appraise the application of a biofertilizer as for any other technical mean, on the base of its efficacy. For practical purposes, we have grouped the factors that could mainly be considered as those mostly affecting biofertilizers efficacy with relation to the plant, the soil, the farmers and the products themselves (Fig. 2.2).

Fig. 2.2 Major factors affecting the efficacy of biofertilizers in improving crop nutrition, growth and yield



2.3.1 Factors Related to the Product

2.3.1.1 Production Process

The production process of the inoculum is key to a final high-quality product (Bashan et al. 2014), since there is a direct relationship between the population density of mother culture and the quality of the final products (Stephens and Rask 2000). Commonly, the inoculum is formed of one strain. However, the understanding of the complex relationships among the microorganisms interacting in the rhizosphere has fostered the study on inocula composed of more than one microorganism which have showed promising results both in legumes and non-legume plants. Successful examples, in case of legumes, comprised the co-inoculation of rhizobia with arbuscular mycorrhizal fungi (AMF) (Wang et al. 2011), dual inoculation of *Rhizobium* and phosphate solubilizing bacteria (Alagawadi and Gaur 1988), an inoculum formed of *Rhizobium* together with a plant growth promoting rhizobacteria (PGPR) and a phosphorous solubilizing bacteria (PSB) (Prasad and Chandra 2003). In non-legumes, nutrients uptake comparable to chemically fertilized plants have been reported with dual inoculations involving AMF and free-living N-fixing bacteria (Adesemoye et al. 2008; Barea et al. 2002; Lisette et al. 2003; Wang et al. 2011), also under dry conditions (Aseri et al. 2008). Consortia of AMF and different PGPR were beneficial for different annual and horticultural crops (Malusà et al. 2007; Wu et al. 2005), leading to a reduction of fertilizer application by

up to 50 % (Singh and Adholeya 2003). Better nutrient efficacy was reported also in the case of PSB and KSB mixture inocula (Han and Lee 2005; Vassilev et al. 2006a).

When designing a consortium for a biofertilizer, it should be considered that certain bacterial groups appear to associate more frequently with AM fungi or to be inhibited by them by several mechanisms (Filion et al. 1999; Mansfeld-Giese et al. 2002; Sood 2003; Toljander et al. 2006; Vestergård et al. 2008; Wamberg et al. 2003), including the fungal release of stimulatory or inhibitory compounds (Johansson et al. 2004), which could result in a higher or limited colonization of the roots, respectively. Also the species specificity of the strains, even in case of AMF, or the differences in adaptation to environmental condition should be considered when selecting strains to formulate a biofertilizer (Antunes et al. 2011; Zoppellari et al. 2014; Malusà et al. 2012).

A PGPM consortium could be more efficient due to the different mechanisms of action of the various microorganisms present, sometimes overlapping also plant protection mechanisms (e.g. Vassilev et al. 2001, 2006b), which tend to match the requirements of both farmers, in using “multifunctional” products, and manufacturers, preferring to market a product for several purposes. A potential example of such kind of product could be that patented in the USA by Reddy and Janarthanam (2014) or already marketed in Europe under the brand name Micosat (CCS Aosta). However, not always the efficacy of consortia has proven to be consistent, and also their

production and commercialization raises some technical problems (Herridge 2008; Stephens and Rask 2000). This could derive from the observation that a key aspect determining the relationship of microorganisms with plants is not their taxonomic diversity, but rather their functional diversity (Nannipieri et al. 2003; Maherali and Klironomos 2007).

A sufficiently long shelf life of the inoculant (up to at least one season), maintaining its biological traits at an adequate level, is key for assuring the efficacy of the biofertilizer, though being a major challenge for any kind of formulated product (Bashan et al. 2014). Therefore, the formulation of the inocula, i.e. a multistep process which results in mixing one or more strains of microorganisms (inoculum) with a particular carrier, with or without additives (e.g. sticking agents or other additives), plays an important role in assuring the efficacy of the biofertilizer. It allows the protection of the cells during storage and transport, possibly enhancing the persistence of the inocula in soil, in order to obtain the maximal benefits after inoculation of the host plants (Manikandan et al. 2010; Schoebitz et al. 2012).

Different carriers can be used in the formulation process, and each of them presents specific positive qualities and drawbacks, affecting thus the overall quality and efficacy of the biofertilizers (Bashan et al. 2014; Herrmann and Lesueur 2013; Herridge 2008; Malusà et al. 2012). Nevertheless, granular inoculants are showing better results under harsh soil conditions (Clayton et al. 2004; Lupwayi et al. 2006; Rice et al. 2000). Liquid inoculants, though easier to distribute, have shorter shelf life (Bashan et al. 2014; Date 2001; Stephens and Rask 2000). Encapsulation into polymers, though theoretically allowing very diverse compositions and structures (Vassilev et al. 2005), has been mainly limited in practice to formulations based on alginate, which still presents some limitations for industrial production (Bashan et al. 2014; John et al. 2011).

Besides the various additives used to improve the shelf life of the product (Bashan et al. 2014; Malusà et al. 2012; Herrmann and Lesueur 2013), specific compounds can be introduced into the formulation to enhance the efficacy of biofertil-

izers. Legume biofertilizers containing elicitors of nodulation are already marketed (Mabood et al. 2006; Skorupska et al. 2010; Smith and Smith 2012), but other rhizobial metabolites related to the nodulation process (Nod factors) were successful in enhancing the performance of N-fixing bacteria inoculants on soybean and maize (Marks et al. 2013). Strigolactones, also in the form of synthetic analogous, could be used to foster the establishment of the mycorrhizal symbiosis (Ruyter-Spira et al. 2011; Xie et al. 2010).

2.3.1.2 Marketing of Biofertilizers and Quality Standards

The development of a new kind of products based on microorganisms is requiring a general agreement on the definition of the terminology or name used. Frequently, in the scientific literature the term 'biofertilizer' has been used to describe a simple microorganism showing plant growth promotion effects (Bardi and Malusà 2012, and references therein). However, as mentioned above, to be used within agronomical practices, any beneficial microorganism (inoculum) requires to be formulated to allow the effective delivery to the soil or plant. Along with the increased understanding of the mechanisms of action of the different kinds of beneficial microorganisms, the term biofertilizer has been defined in different ways (Okon and Labandera-Gonzalez 1994; Vessey 2003; Fuentes-Ramirez and Caballero-Mellado 2005), sometimes associating also a confusing terminology that does not take into consideration the legal definitions in place for other kinds of fertilizers or amendments (Owen et al. 2015). Recently, in an effort to propose a definition that could be useful also for regulatory purposes, Malusà and Vassilev (2014) have proposed to define biofertilizers, in analogy to the mineral or organic fertilizers, as "the formulated product containing one or more microorganisms that enhance the nutrient status (the growth and yield) of the plants by either replacing soil nutrients, and/or by making nutrients more available to plants and/or by increasing plant access to nutrients". An agreed, legally binding, definition of these products, as well as the establishment of minimum legal standards for registration and marketing of biofertilizers are

also important to assure a minimum quality standard, which is another factor affecting the efficacy of biofertilizers' field performance. Indeed, it seems that the quality of biofertilizers has not been improving in the last few decades. Surveys carried out in the 1990s on products containing rhizobia showed a high level of contamination, with alien bacteria outnumbering the rhizobia in the great majority of products (Olsen et al. 1994, 1996). A similar situation emerged from a recent survey where 40 % of 65 tested commercial bacterial products (formulating also PSB and free-living N-fixing strains) did not contain the claimed strain, but only contaminants (Herrmann and Lesueur 2013). The situation does not appear more promising in case of AMF-based biofertilizers: surveys of products showed a very low quantity of viable propagules and reduced colonization potentials (Corkidi et al. 2004; Faye et al. 2013; Rowe et al. 2007; Tarbell and Koske 2007). Such frauds, together with insufficient label information, are probably the major reason for inconsistency of outcomes in field use of biofertilizers and are thus causing a lack of confidence in this kind of products which is affecting their market potential (Bhattacharyya and Jha 2012; Gemell et al. 2005; Husen et al. 2007). Marketing of biofertilizers should thus be regulated assuring a minimum quality standard of the final product (Herrmann and Lesueur 2013; Malusà and Vassilev 2014).

The distribution chain can also further affect the overall quality of biofertilizers. Indeed, several studies have demonstrated the decline of microbial populations in inoculants over time, particularly under non-optimal storage conditions, resulting in lower inoculation efficiency (Biederbeck and Geissler 1993; Maurice et al. 2001) and reduced quality (Hartley et al. 2005).

2.3.1.3 Persistence and Inoculant Traceability in Soil: Need for a Standard Method

The assessment of the persistence and traceability in soil of the strains applied with biofertilizers can be very difficult to investigate due to the complex web of microorganisms present in the soil and the rhizosphere, which can exceed hun-

dred million units (Torsvik and Ovreas 2002), and the high variability of the microbial communities which reflects ecological, environmental and structural soil characteristics, as well as the large variety of agricultural management systems (see headings below). Therefore, no single qualitative and quantitative approach of traceability can capture the persistence of a bioinoculant in soil because of the variety of organisms marketed as biofertilizers. This raises questions about which methods should be considered suitable to monitor the persistence of the different inoculated strains. Such information is fundamental to evaluate the success of inoculation, thus helping to fine tune its application strategy. There is a perceived need for accurate and standard methods that can identify and trace the inoculants in soils.

During the past two decades, phenotypic and PCR-based methods have been developed to better characterize the structure, dynamics and diversity of soil microbial communities. The different methods address different questions, and therefore can all be suitable for the monitoring of the effects in soil due to the introduction of bioinoculants, and to give a picture of different aspects of the microbial community. For the detection of microorganisms released in the environment, molecular methods based on PCR techniques that use natural genome polymorphism have largely facilitated and allowed the discrimination at the strain level, of natural and introduced organisms, minimizing the costs and the time efforts (Öpik et al. 2010; Stockinger et al. 2010; Sýkorová et al. 2012). There are several molecular DNA fingerprinting methods that can be adopted to probe the inoculated strains, but they are mainly qualitative and not quantitative. Among the culture-independent methods available, commonly used to investigate soil microbial communities, traditional molecular fingerprinting, sequencing, or combination of different approaches can be used (Trabelsi and Mhamdi 2013; Schwieger and Tebbe 2000; Hirsch et al. 2010; Han et al. 2012).

The fingerprinting method, based on universal bacterial primers, was found not sufficient to discriminate between non-native and native micro-

organism when used singly (Pellegrino et al. 2012). However, combining a community level fingerprinting approach such as T-RFLP, with phylogenetic strain identification after a culture-dependent approach, proved to be a sound approach to highlight differences in community structure and at the same time to track inoculants (Pellegrino et al. 2012). To widen the understanding of the effect of the inoculant on the autochthonous microbial community, the real-time PCR with probes targeting the genes of interest, together with quantifying their copy number, can provide information on the relative abundance of the introduced strains within the microbial community; this approach could be used to follow the dynamics of the microbial community after the application of the biofertilizer (Babić et al. 2008).

The molecular marker-assisted approach, such as T-RFLP, DGGE, TGGE, can also be particularly useful for monitoring purposes. The combination of two culture-independent methods can allow assessing the persistence of microbial inoculants introduced in the soil, also evaluating at the same time, the possible changes occurring at species level for native strains. In this case, the community-level fingerprinting profile can be the preliminary method that allows to define the size of the clone library and the sequencing analysis. Nevertheless, in order to avoid inconsistent results due to the spatial heterogeneity of soil microbial populations, either horizontally or vertically, the soil sampling protocol shall follow a methodology that considers such variability. Successful examples of the application of such methodology can be found in some recent papers. Combining a community-level T-RFLP analysis, with phylogenetic strain identification by culture-dependent approach, made tracking the inoculants possible (Pellegrino et al. 2012). The tracing of an inoculated AMF isolate in the roots of target plants was carried out on the base of a nested PCR protocol (Sýkorová et al. 2012). Habteselassie et al. (2013) used, for the purpose of AMF tracing, the PCR amplification of a target gene followed by clone-assisted or direct sequences analysis. A PCR coupled with a novel combination of NS31 and Glomeromycota-

specific LSUClom1 primers targeting the nuclear rDNA cistron, and classified amplicons by T-RFLP were designed to trace two inoculants of arbuscular mycorrhizal fungi discriminating them from native strains in roots up to two year post-inoculation (Pellegrino et al. 2012). Ceccarelli et al. (2010) used sequencing to better trace AMF applied strains showing that the marker-assisted fingerprinting analysis and the associated cloning and sequencing approach represents a multi-approach effective method for traceability of inoculants in soil.

2.3.2 Factors related to the plant

The plants can exert a significant effect on the strain(s) forming the biofertilizer and on their efficacy in promoting growth and yield, which are intimately related to the plant physiological status and phenological phase of growth. Indeed, depending on their nutritional status, plants can modify the release of compounds from the roots resulting in both quantitative and qualitative differences in rhizodeposits (Hartmann et al. 2009; Uren 2007), varying in time and space with respect to the position on the root (Dennis et al. 2010) and growth stage (van Overbeek and van Elsas 2008), which can lead to the selection of specific rhizosphere bacterial communities (Paterson 2003; Marschner et al. 2004; Marschner and Timonen 2005). Furthermore, root exudates contain compounds with stimulatory and inhibitory effect on rhizosphere microorganisms that affect their capacity of establishing beneficial relations with the plant (Hartmann et al. 2009; Bais et al. 2006). Under P-deficiency, plants release more chemical signals stimulating hyphal branching (Akiyama et al. 2005) and colonization (Akiyama et al. 2002) of AM fungi than under P-sufficient conditions. Plants can also influence the functions of soil microorganisms, such as nitrification (Smits et al. 2010). However, root exudates from a long-term monoculture of soybean had little effect on the nitrifier community, but reduced nitrification in the rhizosphere; in contrast, total AMF hyphal length was significantly stimulated by the increased release of

genistein (Wang et al. 2012), a phenylpropanoid compound probably involved in the chemical signaling leading to AMF root colonization (Cesco et al. 2010). Phenolic acids, also exuded by roots, are responsible for the shift in soil microbial communities (Qu and Wang 2008).

However, it has been suggested that rhizosphere microbial communities respond to other rhizosphere carbon pools (e.g. microbial exudates) for the majority of their coexistence with their plant host, thus limiting in reality the role of rhizodeposits in shaping the rhizosphere microbial community (Dennis et al. 2010), therefore also of the strains inoculated with the biofertilizer. Nonetheless, root exudates are likely to be of great importance in initiating the rhizosphere effect in very young seedlings and on emerging lateral roots. In this respect, the application of biofertilizers on seeds and seedlings would increase the efficacy of the treatment.

2.3.3 Factors Related to Soil Conditions

2.3.3.1 Abiotic Interactions

Soil chemical (pH, nutrient content) and physical (texture) characteristics have been found to shape both bacterial and fungal communities (Girvan et al. 2003; Fierer and Jackson 2006; Lauber et al. 2008; Marschner et al. 2004). Soil pH has been found to be the most important predictor of bacterial community structure at the ecosystem level, with higher diversity associated with neutral soils and lower diversity in acidic soils, likely due to the relatively narrow pH growth tolerance of bacterial taxa (Fierer and Jackson 2006; Rousk et al. 2010). The field surveys of AMF communities in a wide range of soil pH suggest that it is also the major driving force for structuring these communities (Dumbrell et al. 2010; Wang et al. 1993), thus affecting the colonization potential, and efficacy, of all kinds of PGPM included in biofertilizers. Adaptations of AMF to abiotic factors such as soil temperature and nutrient availability can strongly influence the effect of the AMF symbiosis on plant growth (Treseder and Allen 2002; Antunes et al. 2011).

2.3.3.2 Interaction with Autochthonous Soil Microorganisms

When introduced into the soil, the biofertilizer strain(s) face the competition from indigenous microorganisms. However, the knowledge of the ecological interactions among soil microorganisms and about the impact of those included into biofertilizers with the soil microbial populations are still limited and do not allow to effectively predict the effect of inoculants introduced with the biofertilizers. Nevertheless, there is a great effort in evaluating these interrelationships and their impact on biofertilizers efficacy, both on the short- and long-term, using methods such as the analysis of soil microbial biomass, soil microbial activity, soil microbial community structure and diversity (Trabelsi and Mhamdi 2013). It has been demonstrated that inoculation with products containing different PGPM (e.g. fluorescent *Pseudomonad*, symbiotic and free-living N-fixing bacteria, AM fungi, etc.) affects in different ways various taxonomical or functional groups of autochthonous soil microorganisms. The application of inocula based on N-fixing bacteria was either increasing (Trabelsi et al. 2011) or strongly reducing the local bacterial community structure and diversity (Trabelsi et al. 2012), also when the inoculation was carried out with a consortia of strains (Naiman et al. 2009; de Salamone et al. 2010). A symbiotic N-fixing strain was shown to particularly affect a specific group of Proteobacteria (Robledo et al. 1998). Many studies have confirmed a high degree of specificity of the bacteria species associated with the AMF that was reflected on the increased presence of these species after inoculation with AMF (Albertsen et al. 2006; Olsson et al. 1996; Marschner and Timonen 2006). However, inoculation with AMF also significantly affected the general development of rhizospheric bacterial and fungal biomass (Linderman 1988). Once established successfully, introduced AMF showed to decrease the species richness of indigenous AM fungal communities in host roots (Koch et al. 2011).

The selection of strains expressing features that support the colonization process, and the “fight” for the roots’ environment, is key to

assure the efficacy of any biofertilizer. In this respect, quorum sensing confers an enormous competitive advantage on bacteria, improving their chances to survive (e.g. through biofilm formation) and the ability to explore more complex niches (Gera and Srivastava 2006) even by ‘swarming’ (i.e. moving in the soil owing to motility – Fray 2002). Such characteristics are strongly related to the need of assuring a minimum population level of the initial PGPR inoculum to promote plant growth (Persello-Cartieaux et al. 2003).

The efficacy of the biofertilizers seems to be also mediated by protozoan grazing, particularly by naked amoeba, which is the most important bacterial grazer in soil (Bonkowski 2004). An increase of the bacterial and fungal feeding nematodes population was observed after application of a biofertilizer composed on both AMF and PGPR (Malusà et al. 2012). Wheat rhizosphere colonization by two *Pseudomonas* species and *Bacillus subtilis* was substantially reduced by three species of nematodes (*Caenorhabditis elegans*, *Acrobeloides thornei* and *Cruzinema* sp.) (Knox et al. 2003).

However, it must be underlined that the observed relationships between indigenous and introduced microorganisms would depend largely on the techniques used to address the dynamics of soil microbial communities (Trabelsi and Mhamdi 2013). Indeed, although the number of microbial taxa could be clearly identified through novel metagenomic approaches combined with culture-dependent method, it is still very difficult to identify which functions are attributable to a specific microorganism or group, what are the metabolic potential of soil microbial communities in response to inoculation, and what is the link between the effects on soil microbial communities structure and the functional capabilities of soil microbial population. The study of genes coding for important enzymatic activities or key genes in the interaction process between the inoculant and native microbial population may contribute to gain such knowledge, which could unveil possible functions for the application of biofertilizers specifically designed for particular soil/crops.

2.3.4 Factors Related to Farmers’ Practices

2.3.4.1 Fertilization

Fertilization is surely the agronomical practice that affects the efficacy of biofertilizers the most. The application of large quantities of mineral fertilizers has profound effects on soil microorganisms (Gosling et al. 2006; Johansson et al. 2004) and is thus expected to strongly affect the inoculated ones. Long-term application of mineral nitrogen has been shown to reduce soil microbial activity, with both quantitative and qualitative effects on soil bacterial and AMF communities which negatively impacted natural mycorrhizal colonization of roots (Mäder et al. 2002; Johnson et al. 2005; Hartmann and Widmer 2006; Oehl et al. 2004; Toljander et al. 2008). P-accumulation in the soils due to 10-year (Jensen and Jakobsen 1980) or 90-year application of P fertilizers (Cheng et al. 2013) or irrigation with wastewater (Ortega-Larrocea et al. 2001) decreased the spore density, colonization and communities of AM fungi. However, a lower level of differences was observed in sporulating AMF diversity despite 70 years of different soil fertilization regimes (Antunes et al. 2012). Duan et al. (2010) found low AMF colonization levels in maize, soybean, and wheat grown on fertilized soils. The kind of nitrogen fertilizer used can also impact on the AMF community: the occurrence of *Glomus intraradices*, a nitrophilic taxon (Jumpponen et al. 2005), among the most frequent taxa in arable soils (Hijri et al. 2006), was drastically reduced by ammonium nitrate while it was favored by calcium nitrate inputs (Toljander et al. 2008). However, in case of AMF, it has been suggested that the fertilizer rate might have a larger impact than fertilizer nature, mineral or organic, under some conditions (Beauregard et al. 2013).

Nevertheless, in terms of expected efficacy of AMF-based biofertilizers, it is important to consider that the overall fertility of the soil is supposed to regulate the kind of relation between the AMF and the plant. According to the trade balance model (Johnson 2010), parasitic, commensalism or mutualistic outcomes in the AMF symbiosis might be determined according to the

relative abundance or availability of N and P and their interaction with carbon supply and demand among plants and fungi. When N and P are available in sufficient amounts, then AM fungi are more likely to cause growth depression; on the other hand with sufficient N availability, but limited P, the plant benefits from the mutualistic symbiosis (Johnson 2010).

Organic fertilizers generally affect rhizosphere microorganisms positively, though this is not necessarily a favorable condition for inocula introduced with biofertilizers. Root colonization by AMF and development of AM fungal mycelia in soil can be stimulated by amendment of different organic substrates (Gryndler et al. 2005, 2006). Manure application can induce a general increase of bacteria and AMF richness (Esperschütz et al. 2007; Toljander et al. 2008), but can differently impact on specific groups of rhizosphere microorganisms such as denitrifying, aerobic N-fixing or sulfate reducing bacteria (Enwall et al. 2005). Compost treatments increased the frequency of Gram-positive bacteria to more than 80 % of total isolates and to a major frequency of rhizobacteria populations exhibiting PGPR characters (Viti et al. 2010). Application of two liquid organic fertilizers, derived from alternative sources of organic matter (a stillage and a vermicompost extract), with strikingly different composition and nature, differentially affected the size and biodiversity of rhizospheric Archaea and Eubacteria populations even after a short period of the plant growth, in contrast with common mineral fertilizers (Canfora et al. 2015). However, not all organic fertilizers can exert positive effects on AMF bioinoculants. Sewage sludge applications, for example, proved to reduce AMF richness and strongly altered the local bacterial community (Esperschütz et al. 2007; Toljander et al. 2008).

Considering that higher efficacy of colonization and activity of PGPM is expressed under low nutritional conditions, it is thus advised to reduce, but not to eliminate, the quantity of chemical fertilizers applied to favor the establishment of inoculated strain(s). A reduction by 20–50 % of chemical fertilizers has been proved feasible with several crops (Adesemoye et al. 2009; Jeffries

et al. 2003). A medium level of N fertilization resulted in a higher N uptake from mycorrhizal plants with respect to high or low N fertilization rates (Azcón et al. 2008). In case of PGPR, when two strains of *Pseudomonas fluorescens* were tested on wheat in combination with varying levels of N, P, and K (at 0, 25, 50, 75, and 100 % of recommended doses), the efficacy was reduced with the increasing rates of NPK added to the soil and the maximum nutrient use efficiency was recorded with the 25 % of recommended NPK fertilizers dose (Shaharoon et al. 2008).

The use of biofertilizers can also allow utilizing different inorganic fertilizers, with lower nutrient availability, thus cheaper for farmers in comparison to synthetic fertilizers. For example, co-inoculation of PSB and KSB together with direct application of phosphate and potassium rocks, characterized by low solubility, increased yield and N, P and K uptake with different vegetable plants grown on soils deficient in P and K (Han and Lee 2005; Supanjani et al. 2006; Vassilev et al. 2006a).

2.3.4.2 Other Soil Management Practices

The structure of the soil microbiome is generally influenced by agricultural management practices (Bernard et al. 2012; Lumini et al. 2011; Reeve et al. 2010; Watt et al. 2006), with contrasting effects when comparing intensive and more environmental-friendly systems: the higher the management intensity (i.e. high inorganic fertilization, no rotation, deep tillage) the lower the microbial diversity (Franke-Snyder et al. 2001; Jansa et al. 2002, 2003; Oehl et al. 2003, 2004). Twenty years of organic management altered soil bacterial and fungal community structure compared to continuous conventional management with the bacterial differences caused primarily by a large increase in diversity (Berthrong et al. 2013). Practices, such as tillage, pest management, combined mineral and organic fertilization, and water regime can modify the efficacy of AMF inoculation (Lumini et al. 2011; Van Der Gast et al. 2011; Malusà et al. 2013; Alguacil et al. 2014). Regular disturbance by plowing in the arable soils strongly reduce AMF survival

(Helgason et al. 1998). Furthermore, some AMF taxa like *Acaulospora*, *Gigaspora*, *Paraglomus*, and *Scutellospora* appear to be more sensitive to some soil management practices (e.g. tillage) (Hijri et al. 2006; Maherali and Klironomos 2007) probably due to fewer intramycelial anastomoses (hyphal fusions) with respect to *Glomus* species (De La Providencia et al. 2005), a feature that could lead to using different species for biofertilizers adapted to specific crops. Several investigations on the diversity of AMF communities in conventional versus low input agricultural sites concluded that the status of nutrients and soil disturbance play a more influential role in homogenizing fungi diversity than any differences due to the employed farming systems (Franke-Snyder et al. 2001; Viti et al. 2010).

Finally, when considering the practice of substrate preparation for potted crops (e.g. in nursery), it was found that the characteristics of the peat used could differently affect root colonization by AMF (Linderman and Davis 2003; Ma et al. 2007).

2.3.4.3 Application Methods

Farmers need to better understand how microorganisms are acting in soil in order to learn the appropriate methods to perform a successful crop inoculation (Date 2001). The method of application can indeed affect the performance of the biofertilizer (Deaker et al. 2004). However, very little work has been done to assess and optimize the application of biofertilizers, in order to meet the farmers' requirement of using technologies already available in the farm or to verify how much the application method utilized can affect the viability of the distributed inocula (Bashan et al. 2014; Malusà et al. 2012). Among the few efforts in this regard can be mentioned by the development of machines to apply biofertilizers having different physical form (Malusà and Sas Pász 2009).

The already available machines can be normally used to distribute biofertilizers, particularly granulated formulations. However, some machines have been developed for their distribution, by small adaptation of existing machines to be used in horticultural crops (Wawrzyńczak

et al. 2011), or for specific purposes, e.g. to inject a slurry containing AMF to inoculate big trees (Symbiom© Inject System), which have shown to support a better performance of the biofertilizer (Hołownicki 2014). Application of inoculants by seed treatment or in furrows by mixing the inocula with soil or vermicompost provided comparable efficacy with regard to grain and straw yields in *Cicer* (Bhattacharjya and Chandra 2013). The application of liquid formulations with a normal sprayer based on hydraulic atomization system only slightly affected bacteria viability, but a prolonged working time reduced the amount of living cells up to 50 % (Świechowski et al. 2012). Effect of water volume and adjuvants were also affecting the amount of spores delivered and their efficacy in case of a fungus (Bailey et al. 2007). Foliar application can also be considered for PGPM application, particularly for endophytic species. Examples of growth and yield promotion using of such application method were demonstrated with several fruit species (Esitken et al. 2003; Pirlak et al. 2007; Sudhakar et al. 2000).

Since the recovery of the inoculated strains in the soil or on root rhizosphere was found to be limited to 30–40 days after inoculation in case of PGPR (Bashan et al. 1995), it would be more efficient to foresee repeated applications (2–4) during the growing season, with an interval of 3–4 weeks. This is not considered an additional drawback for biofertilizers in comparison to the inorganic ones, since normally, even for cereal crops, at least two fertilization treatments are performed, also to fulfill legal requirements or quality standards (e.g. Directive 676/91/CE concerning the protection of waters against pollution by nitrates).

2.4 Conclusion

The global market for biofertilizers was estimated to be worth about five billion USD in 2011 and is forecasted to double by 2017 (Marketsandmarkets 2013), actively in Latin America, India and China (Fuentes-Ramirez and Caballero-Mellado 2005; Bashan and de-Bashan

2010; Bashan et al. 2014). Improvement of quality standards of production and a clear legal framework that guarantees both manufacturers and farmers are needed to sustain such potential economic development.

Considering that, in general, 60–90 % of the total applied fertilizer is lost and only 30–50 % of applied N fertilizers and 10–45 % of P fertilizers are taken up by crops (Adesemoye et al. 2008, 2009), the application of biofertilizers can play a key role to develop an integrated nutrient management system, sustaining agricultural productivity with low environmental impact (Adesemoye et al. 2009; Adesemoye and Kloepper 2009; Malusà and Sas Paszt 2009). However, even though in some cases the application of biofertilizers resulted in an increased yield over respective un-inoculated controls in the presence of 100 % of recommended fertilizer doses, we believe that a valid target for this practice would be reaching the same crop productivity obtained without biofertilizers, but with a significant reduction of mineral fertilizers use. Biofertilizers have the potential to help reducing the buildup, leaching, or runoff of nutrients from fields when used in the framework of an integrated nutrient management system, participating in nutrient cycling and benefiting crop productivity (Singh et al. 2011). It has been demonstrated that such approach, combining in different ways, depending on the crops, the use of organic fertilizers and no or limited tillage, is promising and can support an economically and environmentally sustainable management of the crops (Adesemoye et al. 2009; Grzyb et al. 2012; 2013).

More impetus for a wider and effective use of biofertilizers can derive from recent knowledge on microorganisms and technological development. Use of strains cooperating with autochthonous microorganisms, such as endophytic bacteria (Reinhold-Hurek and Hurek 2011; Ryan et al. 2008), or exploiting the synergies with microbial communities (Bernard et al. 2012), as well as the inclusion of protozoa in the formulation of biofertilizers (Bonkowski 2004; Ronn et al. 2002) could also be key for the development of new kinds of biofertilizers. The observation that dimethyl sulfide, a volatile organic

compound, is released by legumes to attract nematodes that transport rhizobia toward the roots is also supporting a wider use of the different microorganisms forming the soil web (Horiuchi et al. 2005).

New kinds of biofertilizers can benefit from the inclusion in the inoculum of yeasts, since isolates from genera such as *Williopsis*, *Saccharomyces*, *Candida*, *Meyerozyma* and *Pichia* have been shown to promote plant growth and nutrition with different crops (Amprayn et al. 2012; Agamy et al. 2013; El-Tarabily and Sivasithamparam 2006; Xiao et al. 2013) also with an integrated nutrient management (Nakayan et al. 2013).

The use of non-obligate endosymbiont mycorrhizal fungi, of the order Sebaciniales, could ease the production process of this kind of biofertilizers, which have shown beneficial effect also in association with PGPR and with non-mycorrhizal species (Kumar et al. 2012).

New kind of additives could derive from biological substances that are involved in the colonization of roots such as the strigolactones synthetic analogs for the AMF–plant symbiosis (Ruyter-Spira et al. 2011), or that can support the root colonization by inoculated microorganisms such as vitamins (Palacios et al. 2014).

Biofertilizers could also be developed for in-vitro grown plant material leading to enhanced growth of seedlings, being more resistant to biotic and abiotic stresses (Sekar and Kandavel 2010), as well as for their quantitative and/or qualitative enhancement of plant secondary metabolites content in medicinal plants (Zubek et al. 2012).

From the data presented, it emerges that the several biotic, abiotic and anthropogenic factors pose challenges in successful application of commercial biofertilizer and are responsible for the efficacy of the biofertilizers as a field practice. Mathematical simulations showed that the most significant factors affecting PGPR survival, and thus the ability of providing beneficial effect to plants, were the competition with autochthonous bacteria, the compatibility with the exuded compounds by the plant host (rhizodeposition) and their ability to utilize them (Strigul and

Kravchenko 2006). On the other hand, there are several tools and actions which can be already utilized and implemented to improve the field efficacy of biofertilizers. The assurance of efficacy for a biofertilizer in a particular soil with a specific variety of crop is thus a complex task, which shall be considered by researchers, manufacturers, agricultural advisors and farmers when designing and applying a specific biofertilizer: a challenge to transform the fertilization with these products into a common practice for twenty-first century agriculture.

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