Chapter 6
Challenges, Regulations and Future Actions in Biofertilizers in the European Agriculture: From the Lab to the Field

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Abstract Microorganisms have been used in agriculture for more than a century, beginning with the rhizobia inoculants and, more recently, the so-called plant growth-promoting rhizobacteria (PGPR). Generally, bacteria have proven to be a valid and useful biotechnology for crop production. In spite of the existing knowledge about functional aspects of the interaction between microorganisms and plants and their effects on plants growth, adoption of such products by farmers is still incipient in some regions of the world, especially in industrialised areas While in Asia and Latin America they are widespread, in Europe they are still emerging. This chapter analyses the challenges of the European sector, including: (i) avoiding inconsistences in field performance, and (ii) informing and training farmers about this technology. Emerging regulation in Europe are also examined. Last, it discusses the prospective actions to help overcome challenges while also staying within the current regulation guidelines, including: (i) searching for autochthonous strains, (ii) optimisation of the industrial production and formulation, (iii) development of techniques for precise strain identification in products, especially for non-sterile carriers, (iv) field experiments at the "farmers scale," and (v) screening action mechanisms from a genetic viewpoint. This chapter reviews the scientific information about field trials from a critical standpoint.

Keywords Biofertiliser · PGPR · PGPB · Microbial biostimulant · Field trial

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D. Zúñiga-Dávila et al. (eds.), *Microbial Probiotics for Agricultural Systems*, Sustainability in Plant and Crop Protection,
https://doi.org/10.1007/978-3-030-17597-9_6

6.1 The Challenges for Microorganism-Based Products in Agriculture

Products based on plant growth-promoting rhizobacteria (PGPR), or plant growth-promoting bacteria (PGPB) for use in agriculture have received widespread attention in recent years (Pastor-Bueis et al. 2017). It has been demonstrated that these kind of products lead to an increase in crop yields when used properly, which results in a reduced need for chemicals (Bhardwaj et al. 2014). This technology is compatible with, and may be complementary to, conventional technologies based on mineral, synthetic or organic products. Eventually, microorganism-based products could partially, or even totally replace conventional agricultural products. However, microbial products face several challenges, which pose a threat towards their more generalised use in agriculture (Fig. 6.1).

Avoiding the well-known inconsistences in the performance of microorganisms on the field scale is one of the most important challenges (Morrissey et al. 2004; Vejan et al. 2016). The success of microorganisms in the field depends on the

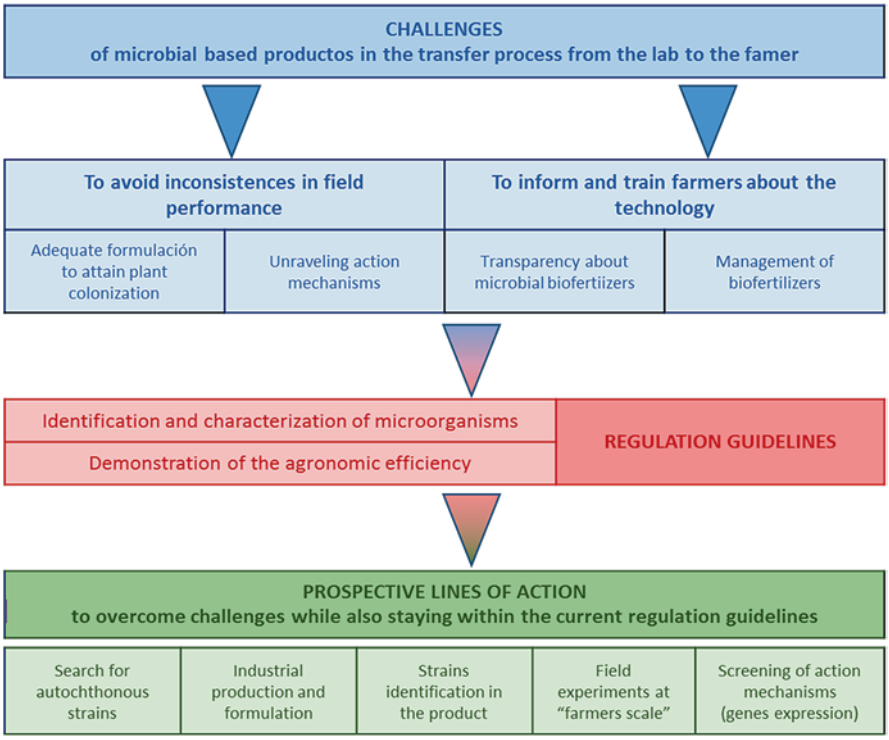

Fig. 6.1 Addressing the challenges of using microorganisms in agriculture, the emerging regulations in Europe, and prospective actions to overcome challenges while also staying within the current regulation guidelines

effective plant colonisation of bacteria, which is influenced by the intrinsic bacterial properties, as well as the physical, chemical and biological nature of the environment. Among the factors affecting bacterial colonisation, some of the most significant are soil particle aggregation, quantity and quality of available carbon, temperature and pH (Timmusk et al. 2017), as well as the ability to interact with indigenous soil microflora (Martínez-Viveros et al. 2010). Usually, populations of inoculated bacteria decline rapidly after initial inoculation. Consequently, they do not attain a sufficient number of viable cells to successfully colonise the root and thus cannot trigger a plant response. Therefore, many efforts are being concentrated in overcoming this issue (Herrmann and Lesueur 2013). Keeping a sufficient number of viable cells is imperative, and preparing the correct bio-formulation is a key challenge. The microorganisms must be prepared methodically in order to provide an appropriate micro-environment, including physical protection for a sustained period of time to avoid decline (Bashan et al. 2016; Timmusk et al. 2017). Unfortunately, there is a lack of available scientific knowledge about the formulation of biofertilisers. In fact, most of this information is either patented or declared an industrial secret. However, even taking into account the significant efforts of private companies in formulation development, the existing information is far from being optimised.

Another contribution to the apparently inconsistent effects of these kind of products from the farmer's viewpoint is given by the diversity of the modes of action (Choudhary et al. 2011; Vejan et al. 2016). For example, a single microorganism can have multiple simultaneous actions in a crop, and can also exhibit multiple mechanisms for a given action (Etesami and Maheshwari 2018). This effect can make it sometimes difficult to identify the action of a given product in the crop, and ultimately confuses the farmer.

A third challenge involves the information that farmers receive and their training in this technology. The rhizobia for legumes, and the PGPR or PGPB, either directly or indirectly facilitate or promote plant growth under nutritional, abiotic or biotic stress conditions. In the last case they are called biocontrol-PGPB (Cassán et al. 2014). When the primary action mode is nutritional or relates to abiotic stress, the microorganism-based products are generally called biofertilisers or microbial-biofertilisers (Pastor-Bueis et al. 2017). However, there is general confusion about what a biofertiliser is. Frequently, anaerobic digestates and their derivatives (Mekki et al. 2017; Du et al. 2018), along with several kinds of composts, are wrongly considered biofertilisers, solely due to the fact that they have a high microbial load (Mulas et al. 2013). Instead, microbial biofertilisers are products that contain specific bacteria strains, which have been carefully selected after the isolation and the biochemical and/or genomic processes of identification and characterization. Afterwards, the products are tested in plants, including crop testing in field situations. Such confusion has been detrimental to the image of microbial biofertilisers and may preclude the use of appropriate products. Therefore, another challenge is to provide a clear explaination to farmers about the differences between organic fertilisers and microbial biofertilisers, along with what they can expect from these products and how they must manage them. Such products consist in fact of living

organisms, and their management requires specific care. Moreover, it is unlikely that farming practices will significantly change to accommodate biofertiliser requirements. Therefore, more effort must be put towards developing farmer-friendly products (Bashan et al. 2014). Private companies play an important role in transferring knowledge to farmers. Thus they need to train technicians and sales agents in such products. Regardless, a lack of responsibility in the commercial distribution of microorganism-based products in the past also contributed to a general distrust among farmers.

Finally, the "Nagoya Protocol on Access to Genetic Resources" pursues a fair and equitable sharing of the benefits arising from the utilization of genetic resources, including access to these resources, technology transfer and funding (IEEP, Ecologic and GHK 2012). However, it poses a threat regarding the bureaucratic procedures, which can become a hindrance for the development of new and more effective products, if based on new isolates.

6.2 Regulations on Microorganism-Based Products in European Agriculture

Products based on PGPR burst into the market in the decade 1980–1990, but their presence was dramatically reduced shortly thereafter. Among other reasons, this was due to the lack of formal and standard regulation of the sector, which resulted in situations of poor quality and low efficiency in the field. Currently, the quality is still far from being adequate, and in some cases it is considered poor. In order to increase the agricultural use of microorganism-based products, the desired quality and stability should be maintained (Bashan et al. 2014, Stamenković et al. 2018).

Nevertheless, during the last decade, microorganism-based products have made a strong comeback in certain regions, such as Latin America and southern Asia (Bashan et al. 2014). Conversely, Europe has always been reluctant to use microbial products in agriculture because of the strength of the chemical industry, which until a few years ago did not show interest in microorganism-based products because they were outside of their scope. Still, the most important companies in agricultural enterprise are currently creating production lines for microbial products, and several small or medium sized companies are entering into the business as well.

A key aspect of the safe commercialisation of products based on microorganisms, as well as for the safeguard of the farmers and the consumers' rights, is the development of standard regulations. Hence, Europe has been conscious of the interest of small and large companies in this business and has started creating a regulation to define rules for the availability of microorganism-based products on the market of what is called "CE-marked fertilising products". This new regulation will replace (EC) No 2003/2003 (the existing "Fertilisers Regulation"), and it will amend two other regulations, including (EC) No 1107/2009 concerning the placement of plant protection products on the market. The proposal of the European

Parliament and the Council of the European Union is still in draft form (No 2016/0084, COD), but it already allows for an understanding of the forthcoming regulation. On the one hand, it considers the biocontrol-PGPB agents, which will be out of the range of the new regulation on marked fertilising products. Moreover, it recognises that substances, mixtures and microorganisms commonly referred to as plant biostimulants are not as such nutrients, but nevertheless stimulate the plants nutrition processes. The draft indicates that since such products are aimed solely at improving the plants nutrient use efficiency, tolerance to abiotic stress, or crop quality traits, they are by nature more similar to fertilising products than to other categories of plant protection products. These products should therefore be eligible for CE-marking under the regulation on CE-marked fertilising products, and excluded from the scope of Regulation (EC) No 1107/2009 of the European Parliament and of the Council on plant protection products.

Some European Union countries already regulate the sector, such as Spain for example, with the RD 999/2017 about fertilising products. This regulation includes a special section for "special products with micro-organisms," which includes (Spain 2017): (i) mycorrhizal fungi, (ii) fertiliser with mycorrhizal fungi, (iii) non-mycorrhizal microorganisms, (iv) fertiliser with non-mycorrhizal microorganisms, (v) a mix of mycorrhizae and non-mycorrhizal microorganisms, and (vi) a mix of fertilisers with mycorrhizae and non-mycorrhizal microorganisms. According to the European regulations, the key aspects required to register a microorganism-based product by manufactures are: (i) the identification and characterisation of the microorganisms, and (ii) the demonstration of its agronomic efficiency.

6.2.1 Identification and Characterisation of Microorganisms

At the moment, no specific list of accepted microorganisms taxa exists. The European regulation draft tentatively includes nitrogen fixing bacterial (*Azospirillum, Azotobacter* and *Rhizobium*) and mycorrhizal fungi. In any case, any addition to the component material category (CMC) will include the following data on the new microorganism: (i) name, (ii) taxonomic classification, (iii) historical data of safe production and use, (iv) taxonomic relation to micro-organism species, which fulfills the requirements for the Qualified Presumption of Safety as established by the European Food Safety Agency, (v) information on the residue levels of toxins, (vi) information on the production process, and (vii) information on the identity of residual intermediates or microbial metabolites in the component material. The identification of the microorganisms included in a registered product must be based on molecular sequences, such as the 16S rRNA ribosomal gene in bacteria and the ITS-18S rRNA in the case of mycorrhizal fungi.

The minimum microorganism concentration has been tackled by the Spanish regulation, and for bacteria it has been set at 10^7 CFU/ml or 10^7 CFU/g, depending on the product formulation. The regulation does not take into account the estimated final number of microorganisms per plant in the field, as other regulations do

(Herrmann and Lesueur 2013), but only the concentration in the product. However, the Spanish regulation accepts products with a lower concentration of bacteria, provided that their effectiveness is proven with statistical significance in two different microcosm experiments (one experiment with one crop, and another with a second crop, or two different experiments with the same crop).

6.2.2 Demonstration of the Agronomic Efficiency

A vital aspect of the successful registration of a microbial biostimulant is proving its agronomic proficiency in field experiments, using the necessary controls and an adequate experimental design, with a statistical evaluation of results. According to the Spanish regulation, a different experiment is necessary for each group of crops (i.e. horticultural crops, open field crops, trees, products for plants nurseries, etc.), and the registration of products in different groups will follow parallel processes.

6.3 Field Evidences in Scientific and Academic Literature of Effective Microorganisms for Agricultural Use

Multiple advanced "-omics" technologies have enabled us to gain insights into the structure and function of plant-associated microbes (Quin et al. 2016), as the number of scientific and academic studies in such disciplines does not stop growing. The evaluation of biofertilisers and strain selection still chiefly remains in controlled environments rather than under field conditions, whereas scientifically sound field experiments are a necessary step in the development of innovative products based on microorganisms (Herrmann and Lesueur 2013). Table 6.1 gathers recent existing worldwide information about field experiments on microorganism-based products on a medium or large scale. As can be observed in the Table 6.1, there is a broad range of microorganisms used in inoculants, including PGPR, PGP, rhizobia as N-fixing with legumes and mycorrhizal fungi. Likewise, the field assays include inoculations with only one microorganism and those with cocktails containing two or more microorganisms. Although a few of the experiments tested commercial inoculants, most of them reflected the results of the initial stage of strains testing, which are applied directly to the seed (not formulated). In other cases, the microorganisms have been mixed (formulated) with a carrier, such as peat or compost, but a microbial protectant has not been added, and the survival of the inoculum has not been evaluated. Even though the product is tested in the field, rarely its expiry date has been appraised, nor the shelf-life of the product in which its efficiency and quality can be assured. What this means is that such products cannot be released from the commercial viewpoint.

Table 6.1 Medium or large scale field experiments performed with microorganism-based products, published in scientific and academic literature during the last 4 years. The intended action of the microorganisms refers to the main action reported

Type of microorganism	Intended action of the microorganism in the crop	Formulation	Product development phase	Crop	Geographical area of testing	Number of different environments covered by the experiment	Size of an individual field experiment (where available)	Brief summary of the results obtained	References
PGPR: Several species from *Pseudomonas*, *Azotobacter* and *Bacillus*	Not specified	Formulated	Commercial: Phylazonit MC®	Tomato (*Solanum lycopersicum* L.)	Gödöllő, (Hungary)	2	Not available	Positive effect on yield with irrigation.	Le et al. (2018)
PGPR: *Azospirillum brasilense, Pseudomonas fluorescens*	Not specified	Formulated (commercial) and not formulated (experimental)	Commercial: Rhizoflo premium Maíz ™ (mix of *A. brasilense* and *P. fluorescens* and experimental (strains of *A. brasilense*)	Maize (*Zea mays* L.)	Buenos Aires province (Argentina)	1	3.150 m^2	PGPR + nitrogen fertilisation increased grain yield and modified rhizosphere microbial communities.	Di Salvo et al. (2018)
PGPR: *Paenibacillus mucilaginosus*	N fixation, P and K solubilization	Not formulated	Experimental	Soybean (*Glycine max* L.)	Shandong Province (China)	1	90 m^2	Positive effects on soybean growth, nodulation and yields. Improved soil bacterial community.	Ma et al. (2018)

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Table 6.1 (continued)

Type of microorganism	Intended action of the microorganism in the crop	Formulation	Product development phase	Crop	Geographical area of testing	Number of different environments covered by the experiment	Size of an individual field experiment (where available)	Brief summary of the results obtained	References
PGP: *P. fluorescens*, *Pseudomonas* sp., *Serratia* sp., *Enterobacter* sp.	P solubilization, ACC-deaminase activity, siderophore production	Not formulated	Experimental	Oilseed rape (*Brassica napus* L.)	Carlow (Ireland)	1	3.312 m^2	Increased crop height and aerial/pods biomass. Best results with consortium. Not a statistically significant increase of seeds or oil yields.	Lally et al. (2017)
PGPR: *Bacillus siamensis*	Not specified	Formulated	Experimental	Sweet pepper (*Capsicum annuum* L.)	León (Spain)	2	45 m^2	PGPR + decreased mineral N fertilisation (80%) produced significantly better yields than the N–80% and full N (100%) controls. Improved N use efficiency.	Pastor-Bueis et al. (2017)
PGPR: *Pseudomonas oryzihabitans, Bradyrhizobium japonicum*	ACC-deaminase activity, auxin production	Formulated (commercial) and not formulated (experimental)	Commercial: Rhizotorfin (*B. japonicum*) and experimental (*P. oryzihabitans*)	Soybean	Village Lavrovo, Orel, (Russia)	1	160 m^2	Increased plant growth driven by soybean genotype, explained by the interaction of PGPR and root exudates.	Kuzmicheva et al. (2017)

PGPR: *Arthrobacter scleromae*	Not specified	Formulated	Experimental	*Lactuca sativa* L., *Raphanus raphanistrum*, *Brassica pekinensis* L.	Paltan-myeon, Hwaseong-si, Gyeonggi-do, (South Korea)	1	250 m^2	Increased shoot lengths in three crops and increased leaf number in lettuce. Especially effective in salinised environments.	Hong and Lee (2017)
PGPR: *Azorhizobium* spp., *Azoarcus* spp., *Azospirillum* spp.	Not specified	Formulated	Commercial: TripleN®	Wheat (*Triticum aestivum* L.)	Padua province (Italy)	2	540 m^2	PGPR + N-fixing bacteria improved root growth and increased plant resilience to environmental stressors.	Dal Cortivo et al. (2017)
PGPR: *Serratia. marcescens*, *Microbacterium arborescens, Enterobacter* sp.	N fixation	Not formulated	Experimental	Wheat (*T. aestivum* L.)	Uttar Pradesh (India)	2	280 m^2	Increased growth and yield; best results with the treble consortium.	Kumar et al. (2017)
PGPR: *Azospirillum lipoferum*	Not specified	Formulated	Commercial: *Azospirillum* strain	Maize	Sérézin-de-la-tour, Chatonnay, saint Savin and Corg, (France)	8	5.750 m^2, 3.080 m^2, 4.600 $m^2$and 2.300 m^2	Improved yield through effects on sugar metabolism and securing mature plant density; no suggested impact on N and P assimilation.	Rozier et al. (2017)
PGPR: Several species of *Bacillus* and one of *Virgibacillus*	P solubilization	Formulated	Experimental	Wheat (*T. aestivum* L.)	Not specified	1	445 m^2	Increased several plant growth parameters.	Mukhtar et al. (2017)

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Table 6.1 (continued)

Type of microorganism	Intended action of the microorganism in the crop	Formulation	Product development phase	Crop	Geographical area of testing	Number of different environments covered by the experiment	Size of an individual field experiment (where available)	Brief summary of the results obtained	References
PGPR: *Pseudomonas aeruginosa*	Not specified	Formulated	Experimental	Sunflower (*Helianthus annuus* L.)	Faisalabad (Pakistan)	2	2.250 m^2	PGPR + N-enriched compost optimised N uptake efficiency, reduces N fertiliser losses.	Arif et al. (2017)
PGPR: *P. aeruginosa*	Not specified	Formulated	Experimental	Sunflower (*H. annuus*)	Faisalabad (Pakistan)	2	2.700 m^2	PGPR + N-enriched compost improved yield and soil fertility in nutrient-poor agrosystems in drylands.	Arif et al. (2016)
PGPR: *Pseudomonas rhodesiae*, *Paenibacillus polymyxa*, *Rahnella* sp., *Serratia* sp.	N fixation, P solubilization	Formulated	Experimental	Switchgrass (*Panicum virgatum*)	Quebec (Canada)	9	Not available	Inoculation with biochar + consortium improved crop height.	Shanta et al. 2016
PGPR: *Rhizobium* sp., *Burkholderia* sp. AMF: *Claroideoglomus etunicatum*, *Acaulospora* sp.	Not specified	PGPR: Not formulated AMF: Formulated	Experimental	*Schizolobium parahyba* var. *Amazonicum*	Pará state, (Brazil)	1	5.670 m^2	AMF + PGPR + fertiliser increased wood yield by 20% compared to fertiliser alone.	Cely et al. (2016)

PGPR: *Bacillus amyloliquefaciens*	Indole-3-acetic acid (IAA) production	Formulated	Experimental	Banana (*Musa* AAA cv. Dwarf Cavendish)	Hainan Province (China)	2	1.410 m^2	Application of a bio-organic fertiliser significantly promoted banana growth/fruit yield while suppressing *Fusarium* wilt disease.	Wang et al. (2016)
PGPR: *Pseudomonas plecoglossicida*, *Pseudomonas mosselii, Pseudomonas taiwanensis*	IAA production, siderophore production,	Not formulated	Experimental	Banana *(Musa* AAA Cavendish cv. Brazil)	Azua, (Dominican Republic)	1	432 m2	Improved banana fruit yield and controlled the incidence of black Sigatoka disease.	Marcano et al. (2016)
PGPR: *Enterobacter cloacae, Bacillus drentensis*	IAA production, P solubilization, ACC-deaminase activity, siderophore production.	Formulated	Experimental	Mung bean (*Vigna radiata* L.)	Jeddah, (Saudi Arabia)	2	648 m^2	PGPR + silicon enhanced salinity tolerance.	Mahmood et al. (2016)
PGPR: *Exiguobacterium oxidotolerans, AMF: Glomus fasciculatum*	PGPR halotolerant	PGPR: Not formulated, AMF: Formulated	Experimental	Mentha (*Mentha arvensis* L.)	Uttar Pradesh (India)	1	150 m^2	PGPR + AMF improved plant growth and AMF colonisation under salt stress conditions; better if combined with vermicompost.	Bharti et al. (2016a)

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Table 6.1 (continued)

Type of microorganism	Intended action of the microorganism in the crop	Formulation	Product development phase	Crop	Geographical area of testing	Number of different environments covered by the experiment	Size of an individual field experiment (where available)	Brief summary of the results obtained	References
PGPR: *Dietzia natronolimnaea,* AMF*: Glomus intraradices*	Not specified	PGPR: Not formulated, AMF: Formulated	Experimental	Indian basil (*Ocimum basilicum* L.)	Uttar Pradesh (India)	1	72 m^2	PGPR + AMF + vermicompos improved plant growth under salt stressed, improved indigenous microbial community structure.	Bharti et al. (2016b)
PGPR: *Azospirillum* sp., *Azotobacter* sp.	Not specified	Not formulated	Experimental	Safflower (*Carthamus tinctorius* L.)	Islamabad (Pakistan)	2	36 m^2	PGPR + significantly reduced use of NP fertilisers (up to 75%) improved quality/ quantity of seed protein.	Nosheen et al. (2016)
PGPR: *Bacillus* sp., *Pseudomonas* sp.	Not specified	Formulated	Experimental	Sunflower (*H. annuus*)	Faisalabad (Pakistan)	2	Not available	PGPR + different P rates increased yield and P use efficiency.	Sarwar et al. (2016)
PGPR*: P. polymyxa, Pantoea agglomerans* AMF: *Glomus mosseae*	Not specified	Formulated	Experimental	French beans (*Phaseolus vulgaris* L.)	Bangalore (India)	1	216 m2	Microbial consortium saved 25% of the recommended NPK fertiliser.	Chauhan and Bagyaraj (2015)

PGPR cocktail: 13 different *Bacillus* species. AMF cocktail.	Not specified	Formulated	Commercial PGPR cocktail: Symbio (*Bacillus* sp. on bran) Commercial AMF cocktail: (micronised Endo mycorrhizae; Symbio)	Durum wheat (*Triticum durum* Desf.)	Sicily (Italy)	1	270 m^2	Soil inoculation with AMF consortium and PGPR consortium (alone or in combination) improved nutrient uptake.	Saia et al. (2015a)
PGPR cocktail: 13 different *Bacillus* species. AMF cocktail.	Not specified	Formulated	Commercial PGPR cocktail: Symbio (*Bacillus* sp. on bran) Commercial AMF cocktail: (micronised Endo mycorrhizae; Symbio)	Durum wheat (*T. durum* Desf)	A typical semiarid Mediterranean area	2	445 m^2	AMF negatively affected amination activity in the root. Combination of AMF + PGPR increased concentrations of amino acids in roots. Exotic AMF reprogrammed primary metabolism in plants.	Saia et al. (2015b)
PGPR: *Bacillus megaterium, Bacillus subtilis*	N fixation, IAA production, ACC-deaminase activity	Formulated	Experimental	Lettuce (*L. sativa* L)	Erzurum (Turkey)	2	110 m^2	Alleviated the deleterious effects of irrigation shortage in plant growth and yield.	Sahin et al. (2015)

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Table 6.1 (continued)

Type of microorganism	Intended action of the microorganism in the crop	Formulation	Product development phase	Crop	Geographical area of testing	Number of different environments covered by the experiment	Size of an individual field experiment (where available)	Brief summary of the results obtained	References
PGPR: *Achromobacter xylosoxidans, P. oryzihabitans, Variovorax paradoxus*	ACC-deaminase activity	Not formulated	Experimental	Potato (*Solanum tuberosum* L.)	St. Petersburg (Russia)	2	160 m^2	Increased tuber number and crop yield.	Belimov et al. (2015)
PGPR: *P. fluorescens, Bacillus lecheniformis, Bacillus* sp., *Mesorhizobium ciceri*. FUNGUS*: Trichoderma harzianum*	Not specified	Formulated	Mix of commercial and experimental	Chickpea (*Cicer arietinum* L.)	New Delhi (India)	2	128 m^2	Combination of fungal and bacterial bio-agents had a synergistic effect, increasing grain yield.	Dubey et al. (2015)
PGPR: *B. subtilis, Bacillus mucilaginosus*	N fixation, P and K solubilization	Formulated	Experimental	Tomato, spinach (*Spinacia oleracea* L.)	Suzhou (China)	1	121 m^2	Vermicompost + PGPR enhanced soil's nutrient availability, microbial biomass, and crop yield and quality.	Song et al. (2015)

PGPR: *Enterobacter* sp., *Bacillus* sp., *Klebsiella pneumoniae*, *Serratia* sp., *Staphylococcus saprophyticus, Klebsiella* sp.	N fixation, P, K and Zn solubilization, IAA production, ACC-deaminase activity, siderophore production	Formulated	Experimental	Rice (*Oryza sativa* L.)	Coimbatore (India)	1	Not specified	Improved growth parameters and crop yield.	Sarathambal et al. (2015)
PGPR: *Azotobacter chroococcum, A. brasilense*	N fixation	Not formulated	Experimental	Onion (*Allium cepa* L.)	Kafr El-Sheikh (Egypt)	2	94,5 m^2	PGPR + compost extract + reduced chemical fertilisation improved yield and soil characteristics.	Mahmoud et al. (2015)
PGPR: *Burkholderia* sp.	N fixation, IAA production, P solubilization, ACC-deaminase activity, biocontrol activity	Not formulated	Experimental	Tomato (*S. lycopersicum*)	Beijing (China)	1	48 m^2	Improved crop growth/yield and enhanced soil enzymatic activity.	Gao et al. (2015)
PGPR: *Pseudomonas moraviensis, Bacillus cereus*	Not specified	Not formulated	Experimental	Wheat (*T. aestivum* L.)	Islamabad (Pakistan)	2	100 m^2	PGPR improved growth and physiological activity. In combination with tryptophan there was a synergic effect.	Hassan and Bano (2015)

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Table 6.1 (continued)

Type of microorganism	Intended action of the microorganism in the crop	Formulation	Product development phase	Crop	Geographical area of testing	Number of different environments covered by the experiment	Size of an individual field experiment (where available)	Brief summary of the results obtained	References
PGPR: *B. amyloliquefaciens, S. marcescens*	Not specified	Not formulated	Experimental	Ginger (*Zingiber officinale* Rosc.)	Calicut (India)	2	Not available	Growth promotion with two bacteria; only B. Amyloliquefaciens was recommended for safety reasons.	Dinesh et al. (2015)
PGPR: *Azospirillum* sp. *Bacillus pumilus*	High transfer factor of cesium (Cs)	Not formulated	Experimental	*Brassica rapa* L. var. *Perviridis*, *B. juncea* (L.) Czern., *Fagopyrum esculentum*	Nihonmatsu, (Japan)	1	160 m^2	Increased biomass production and 137Cs final content (not statistically significant). Despite a positive effect of inoculation, the removal of 137Cs from soil was very low.	Djedidi et al. (2015)
PGPR*: Bacillus aquimaris, B. subtilis*	Not specified	Formulated	Experimental	Wheat (*T. aestivum* L.)	Not specified	1	480 m^2	Induction of proline and sugar accumulation in plants. Increased N, P, and K content in leaves, reduction of Na.	Upadhyay and Singh (2015)

PGPR: *Bradyrhizobium. diazoefficiens, B. japonicum, B. subtilis, Staphylococcus* sp.	N fixation, IAA production, ACC-deaminase activity, siderophore production	Not formulated	Experimental	Soybean (*G. max* L.)	Ratchasima province, Buriram province (Thailand)	2	7296,75 m^2, 2923 m^2	Coinoculation increased number of active nodules, plant yield and nitrogen fixation.	Prakamhang et al. (2015)

Of the 37 studies, published between 2015 and the present moment, in the first or second quartile of the Journal Citation Index, and which performed PGPR or mycorrhizal fungi field tests, only seven were tested in the EU. Thus, it is necessary for European scientists to increase the efforts in formulating products based on native microbes, in order to design successful products that are ready-to-market under the EU regulations.

6.4 Prospective Actions for a Microorganism-Based Agriculture in Europe

The EU is emerging regarding the use of microorganisms for agriculture. The European regulations on "plant biostimulants", which, independently of the products nutrient content, aim to improve the efficiency of nutrients uptake, the tolerance to abiotic stress, and/or the crop quality traits, is the starting point for a promising future of this kind of products. In light of the challenges posed in Sect. 6.1 and the new regulation, the prospective lines of action are discussed below and shown in Fig. 6.1.

One important line of action is the use of autochthonous strains. The advantages of using autochthonous microorganisms is still a controversial aspect, even though there is enough scientific evidence to verify the better adaptation and field performance of autochthonous microorganisms (Mulas et al. 2013). For instance, in *Phaseolus vulgaris* L. the use of allochthonous rhizobia usually does not produce a response to inoculation (Rodríguez-Navarro et al. 2000; Daza et al. 2000), while the use of autochthonous strains have produced a good response in field studies in South America (Motasso et al. 2002; Hungría et al. 2003; Díaz-Alcántara et al. 2014), Africa (Mrabet et al. 2005), and Europe (Mulas et al. 2011, 2015).

The genes encoding an adaptation to a given environment are generally located in the bacterial chromosome (García-Fraile et al. 2010; Mulas et al. 2011; Cao et al. 2017). It has been demonstrated that native rhizobia strains, which are well adapted to environmental conditions, incorporated the plasmid containing the nodC gene typical of biovar phaseoli into their genome (García-Fraile et al. 2010; Mulas et al. 2011, 2015; Díaz Alcántara et al. 2014). This plasmid comes from America, the centre of origin of the common bean, and was persistently transferred to the native rhizobia species, up to date. Such a plasmid confers to the native rhizobia the ability to successfully fix nitrogen. According to manufacturing companies, the main drawback of using autochthonous strains is the increase of the portfolio, as it is necessary to design several products for the same crop, depending on the geographic region. An alternative solution is to use multi-strain inoculants; however, even though there are many laboratory-based studies describing the advantages of strain combinations, there is still a lack of information about the performance of these kind of formulations, on the field scale (Bashan et al. 2014).

Another aspect is to define the range of autochthony, that is the adaptation ranges of each strain. There is a lack of information about this issue. Unraveling this question would involve testing each strain in a set of field experiments in the same and different agroclimatic regions. A study by Marcano et al. (2016) showed that within the same agroclimatic region in a transect of 150 km, the biodiversity of cultivable soil bacteria was mainly located within populations, indicating homogeneity between populations in the agroclimatic region. However, Marasco et al. (2013) observed a broader transect of 1000 km across the agroclimatic region known as the "Mediterranean basin" and found greater diversity between distantly located populations in the transect. Hence, the bacterial diversity across different regions depends not only on the agroclimatic region, but also on the transect length and plant genotype. This must be taken into account when designing biofertilisers based on autochthonous bacteria because locations that are very far apart, but which belong to the same agroclimatic region, could need different strains in order for them to be considered autochthonous.

Another action line is the optimisation of the fermentative process for microorganism production and the formulation at an industrial scale (Bashan et al. 2014). The cost of the growth media for microorganisms needs to be feasible, and for this reason the use of residues has been proposed as a cheap option (Pastor-Bueis et al. 2017). More basic research is needed to develop formulations that maximise the shelf life of the microorganisms, while also optimising plant colonisation.

One important bottleneck in research is identifying the inoculated strain or strains in a product in order to count the CFU per g or ml, as requested by the regulation. This is especially important when the carrier is not sterile, for instance in the mix of a fertiliser with microorganisms. In addition, another major point for microorganism tracking in the field is the development of specific strain markers. Even if the regulations do not require this assessment at this moment, it is of high interest to control the populations change in soils and survival across the different growth stages of the crop. In such cases, it is necessary to have a strain-specific marker to precisely identify the inoculated strain and to distinguish it from other resident microorganisms, even from the same species. Moreover, the identification system must be cheap and effective. For this reason, it has been proposed to design a Sequence Characterized Amplified Region (SCAR) marker for each strain (Reddy Priya et al. 2016), although due to the decrease of the price in genome sequencing, in the future it will be more feasible to find distinctive sequences, based on the analysis of the full genome.

Finally, future research has to be carried out under field situations in order to concentrate the efforts only on those strains which are consistently effective in field conditions. Theoretically, it is possible to achieve a very large range of responses in plants using adequate microorganisms (Etesami and Maheshwari 2018). However, at this moment it is sometimes difficult to see the effects of some actions at the field scale, even if it has been observed in crop tests, at the lab scale or in controlled conditions. Moreover, it is necessary to gain a better understanding of the mechanisms of action of the microorganisms in the plant. Frequently, several mechanisms are working simultaneously. Presently, several studies have sequenced

and characterised the plant genes whose expression is affected by interactions with PGPRs, resulting in an improved plant performance in stress situations. For example, studies by Kaushal and Wani (2016), Jatan et al. (2018) and Tiwari et al. (2017) identified several drought stress-related genes that were up-regulated in plants inoculated with PGPR, which resulted in a growth promotion under drought and salinity stress. Similarly, nitrate, ammonium and phosphorus transporter genes in wheat were up-regulated after inoculation with PGPR and mycorrhizal fungi (Saia et al. 2015a). The list of known genes is continuously increasing, and therefore, in the near future it will be relatively easy to screen for the molecular mechanisms of action for a given strain.

6.5 Conclusion

This chapter reviews the challenges facing microorganism-based products in the market of agricultural inputs, such as products for improving the plants efficiency of nutrient uptake, the tolerance to abiotic stress, and the quality traits of crops. Main challenges of using microbial stimulants reside in overcoming the inconsistences in field response and in adequately informing and training farmers. The response of the EU regulation to tackle such challenges was discussed. Such regulations aim to guarantee the quality of the product and their effectiveness in the field, as well as defining what has to be demonstrated in field experiments. In this scenario, the following prospective lines of action are discussed: (i) searching for autochthonous strains, (ii) optimising industrial production and formulation, (iii) developing techniques for precise strain identification in the product, (iv) performing field experiments at the "farmer's scale", and (v) screening action mechanisms from a genetic viewpoint.

Acknowledgements The research line "Biofertilisers for Sustainable Agriculture" belonging to the research group IQUIMAB (University of León), has been financially supported since 2014 to date by the Spanish Ministry of Science, Innovation and Universities (project RTC2014-1793-2), by the Spanish Ministry of Foreign Affairs and Cooperation (project 2015/ACDE/1339), and by the European Comission call H2020 (project NEWFERT).

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