PLANT-BENEFICIAL MICROBIAL INOCULANTS AND THEIR FORMULATION – A REVIEW

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Abstract: Agriculture plays a crucial role in the society and global economy and has a huge impact on the environment. Human overpopulation require higher amounts of food, and due to the overwhelming increase of health disorder it is a consumer demand for high quality food products. However, intensive agricultural practices involve the use of synthetic substances, with negative effects on human health and environmental safety. These triggered the concern of global regulatory agencies for new strategies and harsh regulations regarding agricultural inputs. Sustainable agriculture practices, including the use of renewable resources are now promoted. Biofertilizers, biopesticides and biostimulants contribute to agricultural yield and quality improvement, having a low detrimental impact on the environment. Microbial inoculants based on selected microorganisms are promising products that can improve plant growth and productivity and prevent crops from pest and diseases attack, being an environmental friendly approach. Plant beneficial microorganisms trigger various mechanisms for soil improvement, nitrogen fixation, nutrients solubilization and uptake in plants. Some beneficial microorganisms can release active biomolecule involved in plant protection, or suppress biotic and abiotic stress factors, revealing plant or environmental benefits. This study aims to review plant beneficial microbial agro-inoculants, successful formulations and application methods.

Keywords: *microbial inoculants, microbial formulation, organic agriculture*

INTRODUCTION

Agriculture is indispensable in providing the nutritional requirements of humanity (Berners-Lee et al., 2018). Moreover, apart from the human nutritional needs, agriculture should assure the required feed beside the animal needs for grazing. Agriculture delivers also many beneficial traits to human wealth by ornamental, aromatic and medicinal plants, as well as raw materials for some non-food industries, such as cosmetics and pharmaceuticals. Considering technical crops, agriculture is providing row materials, derivatives and sub-products for biofuels and various industries such as constructions, textiles, detergents, paints etc. In order to deliver all of the above, agriculture needs several inputs to be efficient. Fertilizers, pesticides and soil amendments are needed to sustain the agricultural production. Intensive agricultural systems make use of higher quantities of such inputs (Alori & Fawole, 2017a). Although the productivity is increased and crops benefit of a good biotic and abiotic protection, the quality of such products is not always satisfactory. This is due to the lack of flavor, lower active principles, and sometime increased risk of pesticides residues and other chemical or heavy metal contamination (Alori & Fawole, 2017b). To counteract these inconveniences and to improve environmental protection and biodiversity, some biologic alternatives to synthetic pesticides and fertilizers were conceived.

Nowadays, for a sustainable agriculture production, various biologic agro-inoculants, biofertilizers, biopesticides, biostimulants and biocontrol agents are available. Such products can be used both for organic farming but also as complementary products along with synthetic chemicals inputs for agriculture. The active ingredients of such bio-based products could include viable microorganisms or microbial metabolites, plant or algae extracts, vegetal or animal derivatives along with aminoacids, humic and huminic acids. This review is focused only on microbial based agro-inoculants, in order to describe their benefits and active principles, as well as some of their formulation types and application methods.

Agricultural used microbial bioproducts Microbial bioproducts used in agriculture aim to increase plant cultivation performance by improving their productivity. In order to induce such beneficial traits microbial inoculants should be based on selected microorganisms capable to promote plant growth, to activate plant defense mechanisms against detrimental stress factors or to control plant diseases or pest attack.

Biofertilizers and their benefits Biofertilizer are products derived from vegetal wastes and animal manures, minerals or microbial inoculants. Regarding microbial inoculants, they can be based on active or latent viable microorganisms. The biofertilizers are capable of improving soil fertility, increasing the supply of available nutrients, and promoting plant growth and development when applied in the substrate, seed or aerial parts of the plants (El-Ghamry et al., 2018). Beside the fact that they are not produced by chemical synthesis, biofertilisers are distinguished from the homolog chemical fertilizers, as they do not generate deleterious side effects on the environment (Mishra et al., 2013).

Regarding the microbial inoculants, they could be based on single strains or consortia with synergic effects. These beneficial microorganisms are good colonizers of the rhizosphere, phylosphere or plant endosphere increasing nutrient up-take in their hosts, improving photosynthesis and promoting plant growth and productivity (Vessey, 2003).

Biopesticides and their benefits Pesticides are plant protection products (PPPs) that are commonly used in agricultural production in order to control or prevent pests and diseases attack or suppress weeds, and to maintain good yield and decrease biologic contamination of the harvest. The active ingredient of these PPPs could be synthetic or originated from biologic resources. These second category is more environmental friendly than chemical pesticides (Cornea et al., 2019).

Microbial inoculants used for plant protection purposes are a class of biopesticides. As any other type of PPP, the active ingredients of biopesticides, including microbial based products, should demonstrate their efficacy and safety for the crops, consumers and environment. In the evaluation protocols, no differences are made between biologic and synthetic active ingredients, regarding their approval as PPP. There are no indulgences for the biologic compounds compared to the ones chemically synthetized. If meeting all required traits according to the regulations, a microbial strain can be accepted as potential active ingredient for plant protection (http://ec.europa.eu). Other authorization is than needed for the microbial inoculant to be commercialized as biopesticide (http://ec.europa.eu). To fasten-up a microbial inoculant on the market, it could be classified as soil improver (Fătu et al., 2019). However, this is only available for those bioproducts that are intended to be applied in the soil.

Biostimulants and their benefits Agricultural biostimulants are another type of biological products designed to be used in small or large scale farming. They contain organic substances or microorganisms aimed to be applied to plants, or in their culture substrate. Their role is to stimulate the natural processes of nutrient absorption, increase plant tolerance to stress factors, boost plant productivity and improve production quality.

Microbial inoculants Microbial inoculants for agricultural use can be classified based on different criteria, such as active ingredient, purpose of their use, or application type. Regarding the active ingredient(s), they are more commonly based on viable microorganisms than microbial metabolites. However, microbial derivatives are not excluded, especially if they are used as biopesticides or biostimulants. A well-known metabolite of microbial origin, used in agriculture and environmental protection, is the bacterial *Bt* endotoxin form *Bacillus thuringiensis*, that has insecticidal effects (Azizoglu, 2019). The agro-active compounds of microbial origin include amino acids, phytohormones, enzymes, lipopeptides, and antimicrobial compounds.

The microorganisms commonly used as agro-inoculants include mostly bacteria and fungi, in single strains or in consortia. The most representative gender and species used as active biofertilizers are listed below (Table 1).

Table 1. Plant beneficial microorganisms commercially used as biofertilizers

Microbial category	Microorganisms	Applications
Bacteria	Various species and strains of *Azotobacter*, *Azospirillum*, *Beijerinkia*, *Ensifer*, *Herbaspirillum*, *Paenibacillus*, *Rhizobium* and *Bradyrhizobium*	Nitrogen fixation
Cyanobacteria	*Anabaena* sp. and *Nostoc* sp.	
Actinomycetes	*Frankia* sp.	
Bacteria	*Bacillus megaterium, Pseudomonas* sp.	Phosphorus solubilization
Arbuscular mycorrhizae	*Glomus* sp., *Gigaspora* sp., *Acaulospora* sp., *Scutellospora* sp., and *Sclerocystis* sp.	
Ectomycorrhizae	*Laccaria* sp., *Pisolithus* sp., and *Boletus* sp.	
Ericoid mycorrhiza	*Pezizella ericae*	
Bacteria	*Bacillus* sp.	Silicate and zinc solubilizers
Bacteria	Various species and strains of *Azospirillum, Azotobacter, Bacillus, Bradyrhizobium, Cellulomonas, Ensifer, Herbaspirillum, Paenibacillus, Pseudomonas, Rhizobium, Serratia*	Plant-growth-promoting microorganisms
Actinomycetes	*Frankia*	
Fungi	*Beauveria, Trichoderma*	
Microalgae	Various species of eukaryotic (*Chlorella, Desmodesmus, Dunaliella, Spirulina, Scenedesmus*) and prokaryotic blue-green algae (*Anabaena, Nostoc*)	

Nitrogen fixing microorganisms Nitrogen is an important component of many essential structural, genetic and metabolic compounds in plant cells. It is an elementary constituent of important organic compounds including amino acids, proteins, enzymes and nucleic acids. Therefore, nitrogen is a key macronutrient in all enzymatic reactions in plants' cells and is a vital constituent of the chlorophyll molecules (Wagner, 2011). Nitrogen sustains plant growth and development and plays a crucial role in crop production (Thilakarathna et al., 2016).

Best known nitrogen fixing bacteria belong to the *Rhizobiaceae* family of Alphaproteobacteria. The most studied nitrogen fixers are *Rhizobium*, *Bradyrhizobium*, and *Ensifer* (formerly known as *Sinorhizobium*), although there are also *Azorhizobium, Allorhizobium*, and *Mesorhizobium* commonly called rhizobia (Patel & Sinha, 2011). The beneficial effects of symbiotic nitrogen fixing bacteria application has been widely documented, revealing an increased dry matter and root nodulation of the compatible leguminous plants, improved protein content and yield. A special interest is currently given to the identification of symbiotic and nonsymbiotic association in order to establish best plant-microbial association (Fagorzi et al., 2020).

Beside leguminous-rhizobia symbioses, the actinobacteria of genus *Frankia* are also known as nitrogen-fixers able to induce nodule formation on their hosts. *Frankia* are filamentous, sporulating, gram positive bacteria with diazotrophic attributes. Nodulated plants are mostly woody species, from three orders: Fagales, Rosales, and Cucurbitales (Rascio & La Rocca, 2008).

Among nitrogen fixing bacteria, *Azotobacter* and *Azospirillum* are free-living rhizospheric colonizers, widely used as biofertilizers in cereal and legume crops (Gupta et al., 2016; Vurukonda et al., 2016). These diazotrophyc bacteria are commonly found in the soils of tropical, sub-tropical and temperate ecosystems. Although they are not able of nodulating their host roots, they colonize plant radicular system creating a special relationship called rhizospheric association (Gangwar et al., 2018). Some strains of such diazotrophyc bacteria are also mentioned as endophytic colonizers of certain plant species (Fujita et al., 2017).

Another category of plant growth promoting bacteria (PGPB) capable of nitrogen fixation are members of the *Paenibacillus* genera (Backer et al., 2018). Although they are not capable of plant roots nodulation, they can enhance the nodulation process in leguminous plants if co-inoculated with rhizobia (Oancea et al., 2013). Such microorganisms are also considered as microbial plant biostimulants (Ruzzi & Aroca, 2015) as they are proven to enhance nutrient uptake, increase plant tolerance to abiotic stress and improve crop quality (Calvo et al., 2014).

Another important class of nitrogen-fixing microorganisms includes cyanobacteria or blue-green algae (BGA). These are free-living oxygenic Gram-negative organisms, commonly occurring in ponds, lakes, water streams and rivers (Singh et al., 2016), which transform N_2 into nitrogenous and ammonium compounds. Most of BGA, such as *Nostoc, Anabaena, Aulosira, Cylindrospermum, Calothrix, Tolypothrix* and *Stigonema* are endowed with heterocyst, specialized thick-walled modified cells, which are known to be the place of nitrogen fixation by nitrogenase (Kumar et al., 2010).

Phosphorus solubilizing microorganisms Similar to nitrogen, phosphorus is another indispensable macronutrient for plant growth and development. It is involved in vital enzymatic pathways regulating plant metabolism, having an active role in energy transfer mechanisms, photosynthesis, and several other important processes. To be available for plants, both organic and inorganic phosphorus are required in assailable forms (Macik et al., 2020). The common way to manage phosphorus deficiency is to apply P-mineral fertilizers such as monocalcium phosphate or monopotassium phosphate (Sharon et al., 2016).

Microbial inoculants based on P-solubilizing microorganism have showed a higher uptake of this nutrient in treated crops. Bacteria that have been found to increase the availability of soil phosphates and phytates are pseudomonads. Other bacteria capable of P-solubilization include various strains of *Azotobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Kushneria, Paenibacillus, Ralstonia, Rhizobium, Rhodococcus, Serratia, Bradyrhizobium, Salmonella, Sinomonas*, and *Thiobacillus* (Alori et al., 2017; Elias et al., 2016). Fungi have also been reported as phosphorus solubilizers, best results being obtained with mycorrhizae. However, studies performed with insoluble forms of phosphorus inoculated with selected microbial fungi, revealed various strains with P-mobilization ability, such as: *Achrothcium, Alternaria, Arthrobotrys, Aspergillus, Cephalosporium, Cladosporium, Curvularia, Cunninghamella, Chaetomium, Fusarium, Glomus, Helminthosporium, Micromonospora, Mortierella, Myrothecium, Oidiodendron, Paecilomyces, Penicillium, Phoma, Pichia fermentans, Populospora, Pythium, Rhizoctonia, Rhizopus, Saccharomyces, Schizosaccharomyces, Schwanniomyces, Sclerotium, Torula, Trichoderma* and *Yarrowia* (Alori & Babalola, 2018).

Microbes increasing micronutrient availability for plants Zinc is the essential trace element, required in low concentrations (5-100mg/kg), for optimum plant growth and development. In plant' cells it takes part in carbohydrate and auxin metabolism, in energy transfer reactions and behaves as anti-oxidant. Zinc deficiency causes plant chlorosis, leaf size reduction, and increased vulnerability to heat and light stress, as well as fungal phytopathogenic attack (Alloway, 2008; Goteti et al., 2013). The occurrence of plants deficiency for zinc is mostly due to the low solubility of zinc compounds, rather than a low total amount of Zn in the soil. The application of zinc mineral fertilizers is the common way to increase its content but, overloading soil with chemical inputs may pose a threat to the natural environment and trigger higher financial expenses (Gontia-Mishra et al., 2017). To overcome these detrimental, selected microbial strains can be used as agro-inoculants. Various microorganisms demonstrate the ability to improve zinc bioavailability in soil, including *Azospirillum, Bacillus* and *Thiobacillus, Gluconacetobacter diazotrophicus, Pseudomonas*, *Rhizobium* and *Serratia* species (Kamran et al., 2017; Vyas & Meena, 2018). Zinc solubilization depends on the soil pH, and involves two mechanisms. The first takes place in acidic soils and it is based on cation exchange. The second mechanism occurs by Zn chemisorption on $CaCO_3$. Microbial Zn solubilization usually involves siderophores synthesis (Saravanan et al., 2011) or organic acids production such as gluconate or its derivative, 2-ketogluconate (Macik et al., 2020).

Despite the fact that silicon is the second most abundant element in Earth's crust, its availability for plant nutrition is limited. This is mainly due to its presence within indissoluble compounds which cannot be readily assimilated by roots. In plants growth, silicon is considered as a non-essential element. However, it has been demonstrated that available silicon positively influences plants' growth, mechanical strength, and resistance to several unfavorable growth conditions. Silicon boosts plant resistance to biotic (fungi, nematodes, viruses) and abiotic stress (salinity, heating, UV-B radiation), decreases the content of cadmium and arsenic in edible plant parts. It also influences the macronutrients uptake and distribution into plants (Frew et al., 2018; Greger et al., 2018).

Silicon is absorbed by plants only as silicic acid. Studies regarding this aspect evidenced that silicic acid primes the defense response in both Si-accumulators and non-accumulator plants (Luyckx et al., 2017). Due to the proven effects of Si in plant growth and stress mitigation, the microorganisms that can convert an insoluble silicon source into silicic acid are of great interest. Silicate solubilizing bacteria are thought to play an important role in transforming silicates unavailable forms into compounds that plants are capable to absorb (Naureen et al., 2015).

Plant-growth-promoting microbial inoculants Microorganisms enhancing plant growth and development produce a variety of agro-active substances such as phytohormones, organic acids, nutrient solubilizing enzymes, 1-aminocyclopropane-1-carboxylate deaminase (ACC-deaminase), extracellular polysaccharides, siderophores, volatile organic compounds (VOCs), antimicrobial compounds, cyanides and fungal cell-wall-degrading enzymes, and various classes of elicitors (Gouda et al., 2018).

Plant growth promoting microorganisms (Table 1) include a wide spectrum of bacterial (Vejan et al., 2016), fungal (Yadav et al., 2020) and microalgae species (Chiaiese et al., 2018). PGPR (plant growth promoting rhizobacteria) are the most studied and used agro-inoculants. Among these, a high interest for actinomycetes has risen. Due to their abilities to sporulate and produce a wide variety of biologically active compounds, actinomycetes are highly appreciated as plant growth promoters (Anwar et al., 2016).

Currently a great attention is accorded also to microbial endophytes, both fungi (Murphy et al., 2018) and bacteria (Mburu et al., 2021). The preferences for this type of microbial inoculants are mainly due to their intimate relationship with their host plants.

Plant beneficial microorganisms and their mechanism of action Despite the wide variability among plant beneficial microorganisms, their working principles are similar (Mahanty et al., 2016). They can promote plant growth and mediate plant protection by direct and indirect mechanisms. According to Umesha et al. (2018) the role played by PGPM is correlated to: phytohormones and other plant-growth regulators production, improved plant nutrition by nitrogen fixation, phosphorus solubilization and micronutrient mobilization, natural resources preservation and environmental protection, suppressed pests and diseases attack, priming defense mechanisms into inoculated plants, induced resistance to biotic and abiotic stress by elicitation.

Microbial formulation Microbial formulation is one of the key factors for agro-inoculants success. Although the active ingredient(s) are selected microorganisms, the bioproduct formulation can influence microbial performance and viability, as well as product stability and preservation.

The formulation is described as a process during which the selected microbial strains are unified with carrier (Bargaz et al., 2018). Microbial inoculants are often improved with additives designed to increase product stability and to protect the microorganisms during formulation, storage and manipulation (Namasivayam et al., 2014). Various formulation types were developed with time, all being improved to meet market requirements. Main formulation types for microbial inoculants are listed below (Figure 1).

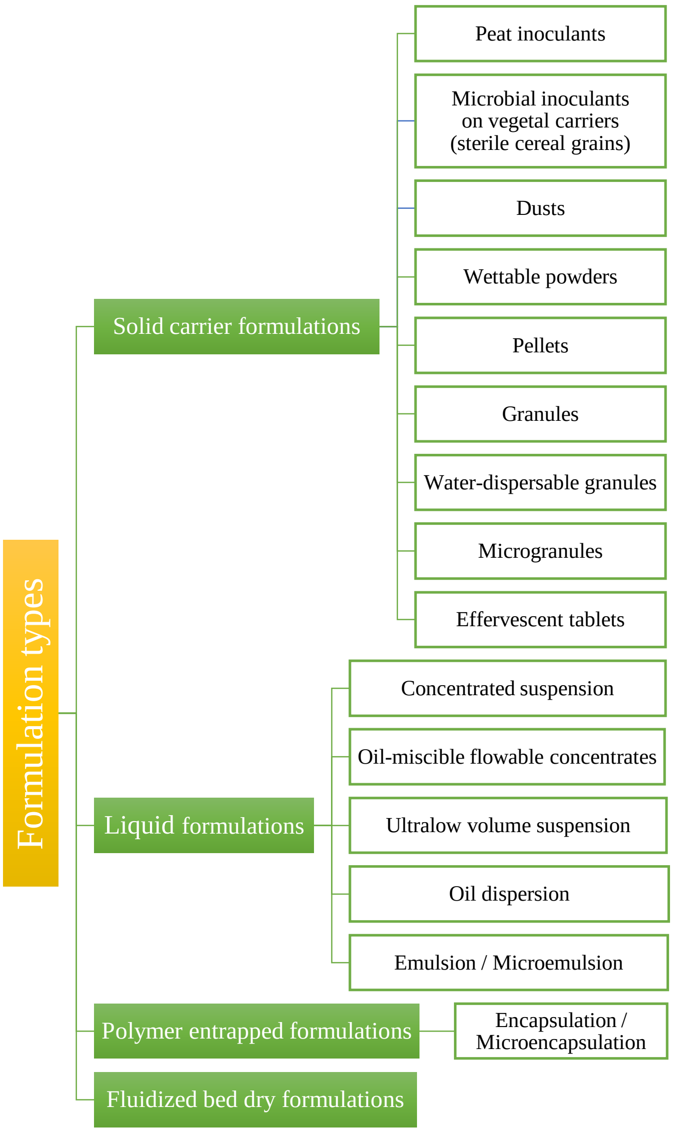

Figure 1. Microbial inoculants formulations types (after Macik et al., 2020)

The first formulation types were those prepared as concentrated suspensions, were submersed cultures were used as they were, or supplemented with some additives. Such formulations were used for rhizobia inoculants. Microbial cultures were also packed in suitable carrier, such as peat, lignite powder, vermiculite, clay, talc, charcoal, soil, rock phosphate pellet, paddy straw compost, cereal bran, or a mixture of such materials, which provides better shelf life to the microbial based bioproduct (Umesha et al., 2018). An efficient and low cost method is the colonization of natural carriers such as sterile cereal grains. This method is commonly used for mycoinsecticides (Kaiser et al., 2019). Various formulation types were developed with time. Microbial based bioproducts being prepared in similar forms as for synthetic products. The obtained agro-inoculant were formulated as: dusts, wettable powders, pellets, granules, microgranules, oil dispersion, microemulsion end various others (Macik et al., 2020).

Encapsulation of microbial cells is another important technique to develop microbial formulations. This method has several advantages than free cell formulations. It provides (i) biotic and abiotic stress tolerance, (ii) increased viability and better physiological activity, (iii) controlled release, (iv) enhanced cell densities, and (v) superior cell growth in various internal aerobic and anaerobic zones of the encapsulating gel. Such method can provide enhanced efficacy for endophytes formulations (Aswani et al., 2020).

Effervescent tablets are another approach in the field of microbial-inoculants. Such tablets are designed to produce a rapid solution, easily diluted and correctly dosed. The tablets are prepared by pelleting the active ingredient with a mixture of organic acids such as citric acid or tartaric acid and sodium bicarbonate (Nair & Brahmaprakash, 2017).

Microbial inoculants production To obtain competitive microbial inoculants, the strains used must have certain characteristics, such as: plant beneficial traits, high efficacy, easy and fast multiplication capacity, high competence in the rhizosphere and compatibility with indigenous soil microorganisms, and lack of side effects on the non-target organisms and natural environment.

Large scale growth of the selected microbes takes place on appropriate medium which should be inexpensive, easily available and provide all fundamental nutrients indispensable for obtaining microbial strains in adequate amount. This step is achieved by liquid, semisolid and solid state fermentation techniques (Stamenkovic et al., 2018). The impregnation of the carrier(s) with fully grown microbial broth or immobilized cells is the next step. The carrier selection should be done based on microbial types and desired form of the final product. Before approval, the bioproduct must undergo a number of greenhouse and field trials and fulfill certain requirements that will confirm: (i) lack of eco-toxicological effects and (ii) expected plant beneficial effects and (iii) increasing crop yields. Registration and regulatory approval of the agro-inoculants must be done prior commercialization (Backer et al., 2018).

The packaging should be marked with the following information: name of the product, microbial strain(s) contained, microbial load, cultures for which the product is intended, name and address of the manufacturer, production and expiry date, instructions and recommendations for application (Bhattacharjee & Dey, 2014; García-Fraile et al., 2015).

Environmental impact of microbial inoculants The application of microbial agro-inoculants has an inevitable impact on physicochemical soil properties, structure and functions of soil microorganisms (Javoreková et al., 2015). Effects are significantly variable between indigenous microbial populations. The techniques used to analyze shifts in microbial communities under the influence of microbial inoculants include denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (t-RFLP), amplified ribosomal DNA restriction analysis (ARDRA), single strand conformation polymorphism (SSCP) and community level physiological profiling (CLPP) which uses a BIOLOG technique (Rastogi & Sani, 2011).

Agro-inoculants application Microbial agro-inoculants can be applied to seeds or plantlets, as well as incorporated into the soil. The recommended application is taking into account the microbial properties, formulation type, environmental conditions and needed equipment. Seed treatment remains the most common practice of applying microbial inoculants due to its simplicity and small amount of required product (Asif et al., 2018). Inoculants can be applied to the seeds by dusting, slurry or seed coating (Malusá & Ciesielska, 2012). Dusting dry seeds may result in weak adherence of microorganisms to seeds, therefore is thought to be least effective. With slurry, inoculant is mixed with wetted seeds or directly with water and then with seeds. Alternatively, the seeds may be left in the slurry overnight (Muraleedharan et al., 2010). Due to the fact that each seed must be coated with the appropriate number of microorganisms, adhesives such as arabic gum, carboxymethyl cellulose, sucrose solutions, vegetable oils and non-toxic commercial products are used (Bashan et al., 2014).

Soil inoculation is recommended in case of introducing a large population of microbial strains directly to the soil. In this technique, carrier material in form of granules (0.5-1.5mm) is preferred and granular forms of peat, perlite, talcum powder or soil aggregates are commonly used. Generally, soil inoculation consists in placing the inoculant next to the seed or under seedbed. In case of liquid formulations, seeds can be sprayed in furrows with the inoculants, also compatible with hydroponic systems (Macik et al., 2020).

Marketed microbial inoculants The commercial use of microbial inoculants has begun more than 120 years ago, when rhizobia based “Nitragin” was registered as plant biofertilizer (O’Callaghan, 2016). Approximately 5% of total fertilizer market is represented by the biofertilizers and more than 150 products based on microbial strains are registered for agricultural purposes. According to Owen et al. (2014) *Rhizobium* inoculants are the most popular microbial inoculants in last few years and constitute approximately 79% of the worldwide demand. Phosphate solubilizing biofertilizers share ~15% of global market and mycorrhizal fungi represent approximately 7% (Verma et al., 2019).

Regarding plant protection products, among the microbial strains approved in the European Union most have fungicidal / bactericidal activity (52.7%) and insecticidal effects (29.1%), the rest (18.2%) being elicitors, nematicides and virus inoculants (Cornea et al., 2019).

CONCLUSION

Environmental pollution and climate change are major problems affecting the quality of life on Earth. It is thus mandatory to find sustainable solutions as an alternative to chemicals inputs used in agriculture. Microbial inoculants formulated as biofertilizers, biopesticides and biostimulants are an effective tool for controlling pest and disease attacks, weed management, plant abiotic stress and nutrient demand of plants. Such agro-inoculants are renewable and eco-friendly resources that increase plant productivity, yield and quality of agricultural products.

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