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Borderline Products between Bio-fertilizers/ Bio-effectors and Plant Protectants: The Role of Microbial Consortia

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Abstract: In the delicate normative balance, at European Union (EU) level of the borderline products (i.e., between plant protectants and bio-fertilizers/bio-effectors) containing microbial consortia (MC) instead of single microbial strains, the most relevant factors influencing the categorization of the products are the intention of use, the cell density and the mode of action. For the latter, the basic difference between the two types of products is that a plant protectant has a targeted activity on plant pathogens, while a bio-fertilizer acts indirectly by nourishing and fortifying the host plant (healthier plant), thus inducing a generalized resistance to the onset of pathological status, irrespective of its origin and nature. Case-studies are presented on the effectiveness of MC as bio-fertilizers/bio-effectors on different crops. Bio-fertilizers exhibit a double effect—biotic and abiotic, leading to the fortification of the crop plant linked to its more effective water and nutrient uptakes as well as to a generalized healthier status. This in turn leads to a higher resistance to diseases. In addition, bio-fertilizers play a relevant role on the reduction of environmental impacts due to chemical fertilizers, e.g., by facilitating the uptake of phosphorus (P), thus reducing the need of P fertilization. Although finding a scientifically-based balance between regulatory need and marketing constraint is not always an easy task, the availability of scientific advancements combined to common sense should help in describing positive effects and risk profiles of MC in agriculture.

Key words: Bio-fertilizers/bio-effectors, plant protection products, MC.

1. Introduction

The microorganisms are internationally recognized to play a pivotal role as ecosystem service suppliers [1-4]. Indeed, to accomplish their roles, microorganisms provide the turnover of organic matter in soil, mobilize plant nutrients and establish tight or loose relationships with plant roots (such as symbiotic nitrogen (N) fixation of legume crops with rhizobia, mycorrhizal symbioses, biocoenoses between cereal crops and *Azospirillum* spp.), thus contributing to plant growth by providing essential nutrients (e.g., N, C, P), water and phytostimulatory substances. In addition, microorganisms contribute to plant quality by altering (e.g., often enhancing) their nutritional and nutraceutical traits [5-7], and finally they can contribute to plant health by antagonizing harmful organisms [8]. Furthermore, microorganisms significantly reduce soil non-synthetic toxicants via their bioremediation potential [9, 10], contribute significantly to soil functional biodiversity, act as primary agents in the biogeochemical cycles, facilitate the carbon sinks build-up and contribute to animal welfare and nutrition [11]. There are several ways in which microbial activities can help plants to grow better and healthier, as it has been demonstrated in the last two decades by using conventional and molecular approaches. These include: (1) the production of secondary metabolites which are toxic to pathogens; (2) the induction of host plants to produce secondary metabolites which are toxic to pathogens; (3) direct hyper-parasitism of a single microbial strain towards one harmful organism; (4) the competition with plant

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pathogens for trophic/spatial niches; (5) the induction of resistance in the crops; (6) the alteration of the fertilization status and chemical traits of the host plants. This paper aimed at identifying the scientifically-based pros and cons of the European legislative framework about the borderline products based on microorganisms between bio-fertilizers/ bio-effectors and plant protectants. The paper focused in particular on the role of the microbial consortia (MC), which on the basis of a wider scientific evidence has gained in the last few years more popularity than the "microbials" based on single strains, and aimed at defining the boundaries of the two groups of microbial products, actually undefined, on scientifically-based criteria.

2. Microbes as Single Strains or MC

Very seldom microbes occur, survive and persist as single cells, strains or even single species in bulk soil, in the proximity of plant root canopy, as phyllospheric bacillus (i.e., on the plant leaf) or even as endophytes (i.e., present within the plant shoot or other parts) [12]. Most commonly, if not generally, they occur as members of a more complex microbiota [13], just as it happens in the human intestine [14], the stomach of ruminants [15], a biogas digestor [16], grape must and beer fermentation [17-19], cheese ripening [20], a legume root nodule [21], a termite nest [22-24] or in bio-mineralization mediated by anaerobic methane-consuming cell consortia [25]. Despite their intrinsic diversity, MC tends to respond to the environmental stressors as a unique organism, because they can have more chances than any microbial strain living as a single population to adapt one or more of their components to the stressor and can take advantage of internal beneficial interactions among members. Since each of a given ecosystem's physiological functions can be carried out by more than one microbial species, the functional biodiversity and the possibility of replacement among different microbial components play a fundamental role in maintaining an active life of the ecosystem. Furthermore, the balance among the different components of a MC, in quantitative terms, will ultimately consist of a continuous shift between actively growing (i.e., viable and culturable) cells and non-dividing (i.e., viable but non-culturable) cells of the various components of the total population. It is known that different microbial populations in a given environment "talk" to each other (e.g., through the "quorum sensing" mechanism) by exchanging precise chemical signals [26]. Natural MC holds many appealing properties also in a bioprocessing context, such as stability, functional robustness and the ability to perform complex tasks. The powerful features of natural consortia have inspired an interest in engineering synthetic consortia for industrial biotechnology applications [27].

3. Microbes Used as Bio-fertilizers/ Bio-effectors and Plant Protectants

For more than the last two decades (except rhizobia, a bio-fertilizer in use since 1896), dozens of plant protection products and a few bio-fertilizers have been used based on single microbial strains. More recently, the MC is receiving increasing attention for use in agriculture and agro-industry as either bio-fertilizers/bio-effectors or plant protectants. Single strains used as plant protectants are produced at high cell density in the formulated products and are subjected to extensive risk assessment [28]. The new regulatory framework for plant protection products is laid out in Commission Regulation (EC) No. 1107/2009 [29] and Commission Regulation (EU) No. 283/2013 [30], and explicitly requires consideration of impacts on non-target species, their ongoing behavior and the biodiversity and ecosystem, including potential indirect effects via alteration of the food web. Single microbial strains and thereof products used as bio-fertilizers or bio-effectors are present on the EU market as high cell density commodities and are subjected to risk assessment in the framework of national legislations. These products generally do not require the same extensive risk assessment as for plant protectants and are therefore marketed with more limited registration requirements.

The density (expressed as CFU/g of product) of each of the components of a MC used as plant protectant remains considerably lower than the density which characterizes the products based on single strains, and several endpoints fall below the threshold of toxicological concern in the authorization process. However, at the moment, plant protectants consisting of MC should undergo the same procedures as the products based on single strains, despite the fact that it is scientifically very hard to imagine that the overall risk can be assessed basically as a summation of the risk of each of the components, i.e., without taking into consideration the interactions.

Bio-fertilizers/bio-effectors consisting of MC have the same registration requirements as the ones based on single microbial strains. The overall matter appears even more complex when considering that: (1) some microorganisms, either as single strains or as members of a MC, can have both effects, i.e., as bio-fertilizer/bio-effectors and plant protectants; (2) some microorganisms, having a potential for acting as plant protectants, play instead a role as members of a consortium in the organic matter turnover in soil environment or in a composting process. A few soil and rhizosphere microorganisms, such as *Bacillus subtilis*, are quoted in the annexes of the EU Regulation 1107/2009 [29], which deals with plant protection products. This has generated considerable uncertainty in the interpretation of the provision: if any *B. subtilis* strain must be considered functionally equivalent to a phyto-pharmaceutical, then any plant (with its rhizosphere where this bacterial species is quite common) or organic manures (where this species is also commonly present) should also be considered a plant protectant, thus requiring strict risk assessment and registration to be delivered or marketed. In addition, if a microbial species is quoted as such in the abovementioned regulation, all the strains of that species actually should not be marketed for purposes other than plant protectants, such as a bio-fertilizer or a component of a yogurt. This is in a sharp contrast with the requirements for registration of active microbes as plant protectant, which is always at strain level and not at species level.

At the moment, it appears that the intention of use and the mode of action should play a major role in the process of making decision about the categorization of the microorganism and the related registration requirements in the EU. In the authors' view, the third parameter affecting the assignment to the category of plant protection products or to the one of bio-fertilizers/bio-effectors is the cell density in the product to be marketed. The example is provided by composts, including mature manure compost, which are characterized by the presence of MC formed by strains with clear activity as bio-effectors (for both plants and functional diversity in soil) and strains with suppressive potential towards common plant diseases. As a logic consequence, the mature manure compost, which has the longest history of use as a bio-fertilizer in agriculture since thousands years, should be nowadays submitted to the registration procedures after undergoing the extensive risk assessment of the plant protection products, owing to its potential to help crops to grow better and healthier. This situation clearly represents an open question for industry and regulators, and offers a good opportunity for further improvement of scientifically-based registration procedures.

4. MC Is Different from Plant Extracts

As a consequence of the above considerations, it is advisable, for normative purposes, to clearly distinguish the bio-effectors in two sub-categories. One is represented by plant/algae extracts and one is represented by microbial single strains/consortia. The latter sub-category is internationally designated with the generic name of "bio-fertilizers", which very well defines their nature and functions. Based on the different nature and origin, the two sub-categories clearly require different analytical and methodological approaches. Plant/algae extracts methods are widely available [31, 32], while for bio-fertilizers, some methodology is already included in national legislations, e.g., the Italian Fertilizers Act [33], in particular for products based on mycorrhizal consortia and other rhizospheric microorganisms. For the MC, high-throughput DNA sequencing has been proven invaluable for investigating diverse environmental and host-associated microbial communities. Recently, Franzosa et al. [34] have comparatively discussed the emerging strategies for microbial community analysis that complement and expand traditional metagenomic profiling. These include novel DNA sequencing strategies for identifying strain-level microbial variation and community spatial and temporal dynamics, for measuring multiple “omic” data types that better capture community functional activity, such as transcriptomics, proteomics and metabolomics, and for combining multiple forms of “omic” data in an integrated framework. The “multi-omics” approach has led to improved mechanistic models of microbial community structure and functions.

5. Case Studies of MC

5.1 Mycorrhizal Inoculants as Bio-fertilizers

Since mycorrhizal plants are more efficient in the uptake of specific nutrients in exchange of plant-assimilated carbon [35], arbuscular mycorrhizal fungi (AMF) inoculation of plants offers the possibility of reducing fertilizer applications. Therefore, AMF has gained popularity as “bio-fertilizers” both in the field [36, 37] and containers [38, 39]. A recent meta-analysis [40] of 38 published field trials with 333 observations to determine the effects of inoculation and root colonization by inoculated and non-inoculated (resident) AMF on P, N and Zn uptake. The growth and grain yield of wheat has shown that field AMF inoculation increases aboveground biomass, grain yield, harvest index, aboveground biomass P concentration and content, straw P content, aboveground biomass N concentration and content, grain N content and grain Zn concentration. Indeed, grain yield has been shown to be positively correlated with root AMF colonization rate, whereas straw biomass was negatively correlated. The most important drivers of wheat growth response to AMF have been shown to be soil inorganic matter, pH, total N and available P concentration, texture of soil, as well as climate and the AMF species inoculated. The meta-analysis shows that AMF inoculation of wheat in field conditions can be an effective agronomic practice, although its economic profitability should still be addressed for large-scale applications in sustainable cropping systems. The industry of mycorrhizal inoculants production is expanding around the world (e.g., http:// mycorrhiza.ag.utk.edu/). Although some studies indicate that inoculation with more than one AMF isolate may not bring more benefit to the host plant [41], a mixture of AMF with complementary functions appears to be more beneficial to the plant than a single isolate [5, 42]. In the field [43], performances of *Trifolium alexandrinum* inoculated with the exotic AMF, both single and mixed, were compared to those obtained with a native inoculum, showing that field AMF inoculation increased crop productivity and quality and that the native inoculum was as effective as, or more effective than the exotic AMF isolates. The persistency of the beneficial effects of AMF was also shown until the second year after inoculation with yield increases of the following crop (maize). In a second field study, Pellegrino and Bedini [6] tested the agronomic relevance of field-inoculated locally sourced and foreign inocula on chickpea (*Cicer arietinum* L.), one of the most important worldwide grain legumes, evaluated not only the yield but also the improvement of the nutritional value of chickpea grain by protein, Fe and Zn bio-fortification and showed in the field the role of AMF as bio-fertilizers of crops and bio-fortification tools. Recent advances have shown that MC containing mycorrhizal inocula is more effective [44, 45]. The patented commercial product "Micosat F" (MF) contains a mixture of AMF (*Glomus coronatum*, *G. caledonium*, *G. intraradices*, *G. mosseae*, *G. viscosum*) and helper bacteria (*Pseudomonas* spp., *Bacillus* spp., Actinobacteria *Streptomyces* spp. and the saprophytic fungi *Trichoderma* spp.). Delivery of the inoculant is done via roots or seed coating, and for trees through localized soil treatment with granular formulations. The use of the AMF alone and the MC of MF was comparatively studied on some major crops to measure the quantitative response and final quality of the epigeal parts. The quantitative response on average was higher for MF: for maize 19% in cut up, 12% in spikes with bracts and 6.4% of grain yield; 13% for wheat grain; 11% for total yield of tomato, due to an increase of 6% of the fruit mass; 11% for cucumber; 8%-20% in the development of the olive trees; null in melon a normal mycotrophic species [45]. The rapid scan by UV-Vis-NIR rays from 350 nm to 2,500 nm of the leaves, flower and fruit parts, which was associated with a rapid examination by an electronic nose (EN) for a total of more than 1,400 analyses, revealed that the cultures submitted to the microbial treatments appeared different from the control samples, with linear regression *R* values of 0.40-0.70, but with oscillations between the different species and run-test. Grain- and forage-maize, aromatic plants, camellia, apple (flowers and leaves), melon and water melon, ryegrass *Lolium* spp., oat and clover are strongly responsive to the treatment with the MC. Tomato was mediumly respondent, while alfalfa and vetch were lowly respondent in an EN test. In some cases, the results of the rapid methods were fairly corroborated by fine chemical analyses. The modern wheat cultivar "Blasco" treated with MF gave consistent results, predicted by the EN test, in a bread-making panel test: the panel appreciated the bread from the treated "Blasco" flour as very similar and as good as the bread obtained from the ancient wheat cultivar "Sieve", "Inallettabile" and "Gentil Rosso" [45]. Another study on tri-trophic consortium *Azospirillum*-*Pseudomonas*-*Glomus* [46] showed that the three-component inoculants may be useful in promoting maize growth. Application of a consortium of AMF and the plant growth-promoting rhizobacteria (PGPR) was studied by Mäder et al. [47] and found to positively affect crop yield, grain, soil quality and nutrient uptake of the staple food crop wheat (*Triticum aestivum* (L.)) in a rotation with either rice (*Oriza sativa* (L.)) or black gram (*Vigna mungo* (L.) Hepper). Recently, Berta et al. [48] have shown that the inoculation with MC containing bacteria and AMF promote the growth of maize cultivated in field conditions and differentially affect the grain nutritional content.

The induction of a healthier status of crop plants (e.g., increased content protein, starch and microelements) due to the usage of MC containing AMF may encompass a natural, decreased susceptibility of the plants to pathogens. It has been proposed to call this trait "mycorrhiza-induced resistance (MIR)" [49-51], providing systemic protection against a wide range of attackers and sharing characteristics with systemic acquired resistance after pathogen infection and systemic induced resistance following root colonization by non-pathogenic rhizobacteria. It is commonly assumed that fungal stimulation of the plant immune system is solely responsible for MIR. However, the latter could be the result of a cumulative effect of direct plant responses to mycorrhizal infection and indirect immune responses (ISR) to ISR-eliciting rhizobacteria in the mycorrhizosphere. The mycorrhizal MF-induced resistance has been verified in the case of flavescence of vines in the Pedimont area in Italy [52]. Continuous cropping of vines in the same soils during the last 70 years and over-usage of chemical fertilizers has produced the well-known soil degradation effects on one side [53] and a generalized impairment of vines towards phytoplasms responsible for the so-called “flavescence”. In two different farms (Santa Caterina in Grazzano Badoglio and Torelli in San Grato di Bubbio, both in the Province of Asti), which had 30% of the vines affected by flavescence, the new vines in the first farm were soil-inoculated with the MC, and the old vines were equally treated in order to re-establish the microbial functional biodiversity in soil [52]. In both cases, the entire area has become flavescence-free and still is after 10 years from treatment, despite the presence of the latter in all the surrounding vine-farms.

5.2 Composts as Bio-fertilizers

During composting, microbial decomposition aerobically transforms organic substrates into a stable, humus-like material [54]. The composted organic matter is an excellent tool to contrast soil erosion and desertification, and there is a generalized need to provide these soils with an adequate return of the organic carbon subtracted by the continuous cropping. When deeply humified, compost can substantially help in contrasting the increased carbon dioxide emissions. In EU Southern zone, where the majority of soils contain less than 2% organic matter, and more in general in the entire Mediterranean Basin, the wet olive husks provide annually 20-30 million tons of biomass, which could help contrasting the continuous deprivation of organic matter due to intensive agricultural management. This end use of wet olive husks can be achieved through composting, which is a less environmentally impacting process compared to the production of electric power or heat as end uses [55]. Recently, Echeverria et al. [56] have described a method to industrially transform the wet olive husks as a sole raw material in a high quality “green” compost by using MC as starters. The compost produced in 60 d is a mature, deeply humified organic matter useful to restore soil fertility and soil texture in both agriculture-intensive and less intensive areas. In addition, it has been found to effectively substitute for turf as a cultivation substrate in horticulture at greenhouse level, with beneficial effects on nutraceutical traits of tomato fruits. The composting process can be run by using the same MC as starters, also at farm scale [57], and the final product is equally characterized by a high microbial biodiversity. The use of “green” composted amendments should be encouraged, also considering the continuous decrease of organic matter content in agricultural soils. Incidentally, apple orchards treated with this soil amendment, in which the MC contains bacteria, such as *Bacillus amyloliquefaciens* subsp. *plantarum* and microfungi like *Trichoderma atroviride*, proved to be healthier, e.g., not affected by the attacks of *Alternaria alternata* than all surrounding orchards which were severely affected by the fruit- and leaf-spots. Similar observations were made by Alfano et al. [58] for another compost obtained from olive mill waste. This type of indirect disease control, which includes the activation of induced systemic resistance in the plants by the microbial compost population and the improved plant nutrition and vigor leading to enhanced disease resistance, is clearly different from a direct plant disease control, and looks like a secondary non-tailored effect compared with the primary effect of the amendment as a bio-fertilizer.

5.3 Suppressive Composts as Plant Protectants

Direct biological control of soil-borne plant pathogens by suppressive composts now is an established horticultural approach [59]. The mechanisms of direct disease control suggested for disease suppression by composts, include direct parasitism of pathogens and competition for nutrients, such as carbon or iron and antibiosis. The delivery mode of the biological control agent is its addition and subsequent sorption of the plant protectant to a compost. Plant growth media enriched with the biological control agent *Trichoderma asperellum* strain T-34 reduced the incidence of *Rhizoctonia solani* disease. In composts aged 0.5-1 year, this strain was only efficient when added to spent mushroom and cork compost, although it remained well established in all of them. The fact that strain T-34 transformed all composts aged 1.5-3 years into highly suppressive composts was attributed to the low levels of easily biodegradable substances. *Rhizoctonia* damping-off in cucumber plants can be reduced by using composts and/or the biological control agent *T. asperellum* strain T-34. In addition, the extent, to which the composts suppress this disease, depends on the chemical-physical nature of the composted materials and increases with the compost maturity [60]. More in general, composts can be transformed into suppressive compost by the addition of one or more biological control agent, specifically active against a plant disease. Fortifying composts with beneficial microorganisms is one possible factor that can help increasing the efficacy and reliability of disease control [61]. The distinction between bio-fertilizers/ bio-effectors and plant protectants is clearly reported by SANCO [62]. However, a few soil and rhizospheric microbial species are mentioned in the EU legislation [29] as bio-pesticides, along with the many chemicals for use as phyto-pharmaceuticals, collectively called “plant protection products”. The uncertainties deriving from this quotation of given microbial species (then all strains of this species should be considered plant protection products even if they have nothing to do with protection of plants) should be solved as soon as possible. Indeed, an incorrect interpretation of this provision can negatively affect common agricultural practices at EU level (e.g., usage of manure and composts as bio-fertilizers, use of MC as bio-fertilizers) and the human welfare via the nutritional value of food, considering that human microbiota depends also on the quality of the MC of different foods [63]. A depression of microbial diversity of our intestine may cause important human pathologies, such as diabetes type II or inflammatory processes. A well balanced nutrition and the correct presence of microbiota in the intestinal tract of humans and animals increase their natural defense potential [64] without being considered *per se* a medicine. The same logics should be used for plant health, allowing the use of beneficial MC not having a specific activity against plant pathogens, such as bio-fertilizers, simply because they promote plant growth and plant nutrition. Therefore, in agreement with Malusà and Vassilev [65], it seems relevant at normative level: (a) avoid the interpretation of EU Regulation 1107/2009 [29] that any strain belonging to a quoted species must be considered a plant protection product, therefore not allowing the usage different strains belonging to the same species for other purposes, such as bio-fertilizers; (b) promote a sensible legislation at EU level of bio-fertilizers (i.e. fertilizers based on living microorganisms), which in turn would stimulate their industrial production, thus setting appropriate qualitative standards and defining a science-based risk assessment; (c) consider that bio-fertilizers can promote the intrinsic ability of plants to counteract abiotic stresses, e.g., drought [66] as well as biotic stresses, e.g., plant pests [67, 68] without being *per se* phyto-pharmaceuticals, but rather beneficial rhizospheric microorganisms; the microbial strains or consortia would not necessarily need to be registered as plant protectants, but could be registered as bio-fertilizers or bio-effectors, with an appropriate risk assessment.

6. MC as Bio-effectors: An Approach to Risk Assessment

In order to provide a frame for an approach to risk assessment of MC to be marketed in EU as bio-effectors, the following elements should be taken into consideration: (1) the MC should be evaluated and properly assessed before entering the market; (2) the risk assessment should take into consideration on the features and traits of MC, which are different from the ones characterizing microbial products based on single strains at high density. To achieve this goal, the product based on MC should be assessed by: (1) describing in full the taxonomically identified components of the MC and their biological traits; (2) describing the physical-chemical traits of the product and the methods used, including statistical evaluations; (3) assessing the toxicological traits as a whole consortium with respect to the production of (secondary) metabolites of toxicological concern *ex planta* as well as *in planta* after short/extended exposure of the plant to the MC at mesocosm (greenhouse) level, i.e., looking at whether a (rhizospheric) soil extract contains relevant metabolites in concentration of toxicological concern; (4) describing the efficacy of the consortium; (5) describing the invasiveness and persistence of the MC in the environmental compartments for an appropriate number of crop cycles; (6) identifying the ecotoxicological consequences of the use of the MC on ecologically relevant non-target species or non-target species providing ecosystem services.

7. Conclusive Remarks

In the delicate normative balance of the borderline products (plant protectants and bio-fertilizers/ bio-effectors), the intention of use was mentioned in the first instance and the mode of action was in a second place. For the latter, the basic difference between the two type of products is that a plant protectant mostly has a targeted activity on plant pathogens, while a bio-fertilizer acts indirectly by fortifying the host plant to make it become a healthier plant, thus inducing a generalized resistance to the onset of pathological status, irrespective of its origin and nature. Therefore, bio-fertilizers exhibit a double effect—biotic and abiotic, leading to the fortification of the crop plant linked to its more effective water and nutrients uptake, as well as to a generalized healthier status. This in turn means a higher resistance to diseases. In addition, bio-fertilizers play a relevant role for the reduction of the environmental impacts of chemical fertilizers by facilitating the uptake of P, thus reducing the need of P fertilization. Actually, the reservoirs on the earth are expected to extinguish by the year 2050, at the actual rate of extraction/ consumption. Although finding a scientifically-based balance between regulatory needs and marketing constraints is not always an easy task, the availability of scientific advancements combined to common sense should help in describing the risk profile of MC satisfactorily for all interested parties.

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