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Advantages and limitations involved in the use of microbial biofungicides for the control of root and foliar phytopathogens of fruit crops

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Benefici, limiti e risvolti pratici nell'utilizzo di prodotti fitosanitari a base microbica per il controllo di patogeni radicali e fogliari

Riassunto. Nonostante i prodotti fitosanitari di sintesi chimica abbiano un ruolo determinate nella protezione delle colture, negli ultimi decenni il loro uso eccessivo o scorretto ha portato a preoccupazioni crescenti nell'opinione pubblica. La normativa europea è molto restrittiva per quanto concerne la registrazione dei prodotti fitosanitari e negli ultimi anni sono state messe in campo numerose azioni volte ad favorire un uso sostenibile degli stessi, tra cui la sostituzione con mezzi non chimici. I prodotti fitosanitari basati su microrganismi costituiscono una valida alternativa e, nonostante richiedano maggiori attenzioni nella loro applicazione, presentano spesso efficacia comparabile. I microrganismi attualmente approvati come biofungicidi appartengono principalmente a due generi: *Trichoderma* e *Bacillus*. Il successo di questi ceppi è principalmente legato al loro ampio spettro d'azione, alla facilità ed economicità di produzione e al mantenimento di una buona vitalità nel tempo. I meccanismi di azione degli agenti di biocontrollo a base microbica possono essere classificati in quattro gruppi: antibiosi diretta, competizione per spazio e sostanze nutritive, induzione della resistenza e iperparassismo, anche se in genere più di un meccanismo coesiste nello stesso microrganismo. La comprensione del meccanismo di azione è fondamentale per applicare correttamente i biofungicidi ed ottenere i risultati migliori in termini di efficacia. L'antibiosi diretta è il meccanismo che più si avvicina a quello dei prodotti fitosanitari di sintesi chimica. Infatti i metaboliti/enzimi prodotti dal microrganismo esercitano un'azione tossica/inibitoria diretta contro il patogeno. Per colonizzare specifiche nicchie ecologiche, oltre a produrre tossine e enzimi, i microrganismi spesso competono con altri per lo spazio e le sostanze nutritive. Su questo principio si basa l'azione di alcuni biofungicidi microbici, usati soprattutto contro le malattie di post-raccolta. Numerose molecole o microrganismi, essendo percepiti dalla pianta come segnali di pericolo, attivano una complessa rete di risposte alla difesa classificabili come resistenza sistemica acquisita o resistenza sistemica indotta. Questo meccanismo, molto efficace in condizioni sperimentali, è però piuttosto limitato in campo. L'iperparassismo (micoparassismo), invece, è il meccanismo mediante il quale l'agente microbico cresce a spese del patogeno, limitandone la crescita ed i danni. Ci sono diversi vantaggi nel sostituire un fungicida di sintesi chimica con un biofungicida. Il principale vantaggio è l'assenza di residui tossici nel prodotto finale che è particolarmente utile nel caso di colture per le quali la raccolta è prolungata nel tempo e quando le piante devono essere trattate in prossimità della raccolta. L'assenza di tossicità non è solo un vantaggio per i consumatori, ma è estremamente vantaggiosa anche per gli operatori. Inoltre, i biofungicidi microbici non sono tossici per gli insetti utili e gli impollinatori, tanto che in alcuni casi le api sono state utilizzate per distribuirli sui fiori. A causa del loro complesso meccanismo di azione è improbabile che sviluppino resistenza nelle popolazioni patogene e quindi, sono anche strumenti validi nelle strategie anti-resistenza. In alcuni casi rappresentano l'unico prodotto efficace contro alcune malattie, come nel caso delle malattie del legno della vite (mal dell'esca ed eutipiosi). Oltre a poche molecole naturali autorizzate, i biofungicidi microbici sono gli unici strumenti utilizzabili nella produzione biologica. D'altra parte, ci sono però anche diversi fattori limitanti che ne rallentano la diffusione. In particolare essendo organismi viventi, le condizioni ambientali, al momento dell'applicazione o successivamente, sono fondamentali per la loro sopravvivenza e la conseguente l'attività. In particolare, le condizioni ambientali estreme e la competizione con la microflo-

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ra naturale concorrono ad una rapida riduzione delle popolazioni dopo l'applicazione. Hanno inoltre un'azione più lenta rispetto ai fungicidi di sintesi chimica, aspetto particolarmente evidente nel caso degli iperparassiti. La maggior parte dei biofungicidi fungini non può essere combinata con i fungicidi chimici ed il rame è generalmente tossico per tutti i microorganismi. Di conseguenza è necessario prestare attenzione alla pulizia del serbatoio dell'atomizzatore dopo l'utilizzo di fungicidi chimici ed mettere in atto altre azioni volte a preservane la vitalità, incluso il rispetto della data di scadenza. La vite è la coltura che ha ricevuto la maggior attenzione in termini di identificazione ed autorizzazione di biofungicidi microbici. Numerose sono state anche le ricerche condotte nei confronti di altri agenti patogeni in frutticoltura, in particolare per l'utilizzo in post-raccolta, purtroppo però con scarse ricadute sul mercato. Ad esempio, nonostante i significativi sforzi per ridurre l'inoculo invernale o la sporulazione di *Venturia inaequalis,* non sono disponibili ancora prodotti commerciali contro questo patogeno. Similmente non esistono biofungicidi commerciali contro *Stemphylium vesicarium* o *Monilinia laxa*. Le ragioni vanno dalla competizione sul mercato con i fungicidi chimici che spesso sono più economici, e la conseguente bassa attrattività per gli investitori dell'agrofarmaco, al comportamento distruttivo di numerose malattie dei fruttiferi se non controllate totalmente. L'incremento delle malattie dell'apparato radicale sono in costante aumento a seguito al divieto dell'impiego di noti fumiganti. Sebbene il trattamento del suolo prima del reimpianto con biofungicidi microbici possa ridurre significativamente l'inoculo di vari agenti patogeni, il raggiungimento della concentrazione efficace dell'antagonista a costi ragionevoli e la sua sopravvivenza nel terreno per un periodo sufficiente, sono ancora due punti irrisolti. In conclusione, sono già disponibili vari biofungicidi microbici e la loro efficacia può essere nettamente migliorata con un uso corretto, formulazioni migliori o diminuendo i costi di produzione, consentendo un aumento della concentrazione. Per sviluppare una seconda generazione di prodotti fitosanitari di microbica, con caratteristiche intrinsecamente migliori, sarà però necessario esplorare nuovi substrati o nuove tecniche d'isolamento.

Parole chiave: difesa integrate, biopesticidi, pesticidi, produzione biologica.

Reasons for the increasing interest in active ingredients offering an alternative to synthetic chemical fungicides

Although synthetic chemical fungicides provide undoubted advantages in terms of plant protection, their overuse and/or misuse may raise several safety concerns for the environment and human health (Fantke *et al.,* 2012). For this reason, their placement on the market is strictly regulated in almost every country. In the European Union, active substances and the related plant protection products can only be approved if they do not pose toxicological and ecotoxicological risks, and the authorisation only lasts for a limited number of years, after which it must be renewed [Regulation (EC) No. 1107/2009]. In addition, action is currently taken to achieve the sustainable use of pesticides (Directive 2009/128/EC) by increasing training and information, improving application equipment and handling and storage, regulating specific uses and promoting low pesticide-input pest management, by giving priority to non-chemical methods. Of these, microbial biofungicides are the most promising tools to replace synthetic chemical active substance used to control fungal diseases. In microbial fungicides, the active component is commonly a bacterial or fungal microorganism, produced on industrial scale in large fermenters (submerged or solid-state fermentation). Being of natural origin, they are commonly approved for use in organic production, where they represent an alternative to copper or sulphur. They are valid tools in integrated pest management (IPM), because they have negligible or no toxicity/eco-toxicity and they are supposed not do not develop pathogen resistance, because no reports of resistance to microbial biofungicides have been documented so far. In addition, they are safe for operators and are commonly exempt from the 'maximum residue level' [Regulation (EC) No. 396/2005), which makes them attractive in integrated pest management due to the fact that their use in strategies can lower the final chemical residues in the food.

Although the need for the registration of biopesticides has often been seen as an economic burden, which slows down their entry to the market and increases the final cost for the end-user, nowadays there is an increasing consensus about the need for a rigorous evaluation process before these products are placed on the market. Indeed, the registration not only guarantees safety for the consumers and the environment, but also the efficacy, therefore protecting growers from 'fake' plant protection products or frauds. Before Regulation No. 1107/2009 came into force, several microbiological products were offered on the market, claiming efficacy as biopesticides, but without any guarantee of quality (viability of the microorganism) and proven efficacy.

The microorganisms currently approved as biofungicides belong to a few genera (tab. 1). Specifically, species from the genera *Trichoderma*

(12 strains) and *Bacillus* (four strains) account for half of the approved active strains (figs. 1 and 2). The success of these species as biofungicides is mainly related to their biological characteristic, in relation to their wide spectrum of activity, relatively cheap and easy production methods and satisfactory shelf-life. Indeed, *Bacillus* spp. and *Trichoderma* spp. can form respectively spores or conidia that stay viable for a long time, even in adverse environmental conditions (Harman et al., 1991; Checinska *et al.,* 2015).

Mechanism of action of microbial biofungicides

The mechanisms of actions of microbial biocontrol agents in biofungicides have been classified into four groups (fig. 3): direct antibiosis, competition for space and nutrients, induction of resistance and hyperparasitism (Howell 2003, Narayanasamy, 2013). In general, more than one mechanism of action coexists in the same microorganism (Vos *et al.,* 2015). Some strains are highly specific against a single or a few pathogen species (e.g. *Coniothyrium minitans* against *Sclerotinia sclerotiorum* and *S. minor*; *Ampelomyces quisqualis* against Erysiphaceae), while others (e.g. *Bacillus* spp., *Pseudomonas* spp. and *Trichoderma* spp.) have a wide spectrum of activity (Whipps *et al.,* 1992; Kiss, 2003; Weller, 2007; Vinale *et al.,* 2008; Chowdhury *et al.,* 2015).

Understanding the mechanism of action is crucial in order to apply biofungicides in optimal conditions for their activity and to obtain the best results in term of efficacy.

Tab. 1 - Microbial strains approved as active substances in the European Union and member States where they have been authorized (from http://ec.europa.eu/food/plant/pesticides_en; 10th April 2017 update).
Tab. 1 - Ceppi microbici approvati come sostanze attive nell'Unione europea e negli Stati membri in cui sono stati autorizzati. (da http://ec.europa.eu/food/plant/pesticides_en; aggiornamento del 10 aprile 2017).

Microorganism	Authorised or authorization in progress (Member States)
Ampelomyces quisqualis strain AQ10	BE, CY, DE, DK, EL, ES, FR, IT, LU, NL, SI, SK, UK
Aureobasidium pullulans (strains DSM 14940 and DSM 14941)	AT, BE, DE, EL, ES, FR, HU, IT, NL, PL, PT, SI, SK
Bacillus amyloliquefaciens strain MBI 600	CZ, EL, FI, HU, NL, SE
Bacillus amyloliquefaciens subsp. plantarum strain D747	FR, IT, ES
Bacillus pumilus strain QST 2808	FR
Bacillus subtilis strain QST 713	AT, BE, CY, CZ, DE, DK, EE, EL, ES, FI, FR, IE, IT, LU, NL, PL, PT, SE, SI, UK, LT, LV
Candida oleophila strain O	AT, FR, NL, UK, EL, HU, IT
Coniothyrium minitans strain CON/M/91-08 (DSM 9660)	AT, BE, CZ, DE, DK, EL, ES, FR, HU, IE, IT, LU, NL, PL, PT, SE, SK, UK
Gliocladium catenulatum strain J1446	AT, BE, CY, DE, DK, EE, ES, FI, FR, IE, NL, SE, UK, SI
Phlebiopsis gigantea (several strains)	DK, EE, FI, FR, LT, LV, PL, SE, UK
Pseudomonas chlororaphis strain MA342	AT, BE, DE, DK, ES, FI, FR, IT, LT, LU, NL, PT, SE, UK
Pseudomonas sp. strain DSMZ 13134	AT, CZ, EL, IE, IT, NL, SE, ES, SI
Pythium oligandrum strain M1	CZ, FR, HU, PL, SK, UK, AT, IT
Saccharomyces cerevisiae strain LAS02	In progress
Streptomyces K61 (formerly *S. griseoviridis)*	BE, CY, DK, EE, ES, FI, FR, HU, IT, LT, LV, NL, SE, UK, AT, IE
Streptomyces lydicus WYEC 108	In progress
Trichoderma asperellum (formerly *T. harzianum)* strains ICC012, T25 and TV1	DE, EL, ES, FR, IT, NL, PT
Trichoderma asperellum (strain T34)	BE, IE, NL, UK
Trichoderma atroviride (formerly *T. harzianum*) strains IMI 206040 and T11	EL, IT, SE
Trichoderma atroviride strain I-1237	FR
Trichoderma atroviride strain SC1	AT, CZ, ES, HU
Trichoderma gamsii (formerly *T. viride*) strain ICC080	DE, EL, ES, FR, IT, NL, PT
Trichoderma harzianum strains T-22 and ITEM 908	BE, DK, EL, FR, IE, IT, NL, SE, SK, UK
Trichoderma polysporum strain IMI 206039	DK, SE
Verticillium albo-atrum (formerly *Verticillium dahliae*) strain WCS850	NL, SE, UK

Fig. 1 - Colony of *Bacillus amyloliquefaciens* growing on artificial medium in Petri dish.
Fig. 1 - Colonia di Bacillus amyloliquefaciens *che cresce su un mezzo artificiale in una capsula di Petri.*

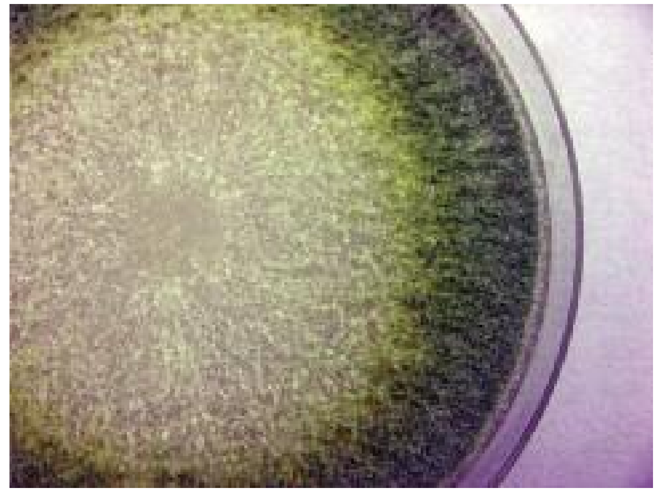

Fig. 2 - Colony of *Trichoderma atroviride* growing on artificial medium in Petri dish.
Fig. 2 - Colonia di Trichoderma atroviride *che cresce su un mezzo artificiale in una capsula di Petri.*

Direct antibiosis

This mechanism resembles the activity of the synthetic chemical pesticides more closely. It is based on the fact that the metabolites/enzymes (antibiosis?) produced by the microorganism and released in the target environment (leaf, fruit and soil) have a toxic effect that kills the pathogen (Leifert *et al.,* 1995, Markovich *et al.,* 2003). Therefore, the efficacy is directly related to the presence of the active compound at the time of pathogen inoculation. Being living organisms, the survival of the microbial biocontrol agents may be jeopardised when they are exposed to a harsh environment and competition with resident microflora, therefore their persistency on plants is often very limited. In addition, rain can easily wash-off hydrosoluble metabolites. For this reason, treatments with microorganisms acting through direct antibiosis should be sufficiently frequent and they should preferably be

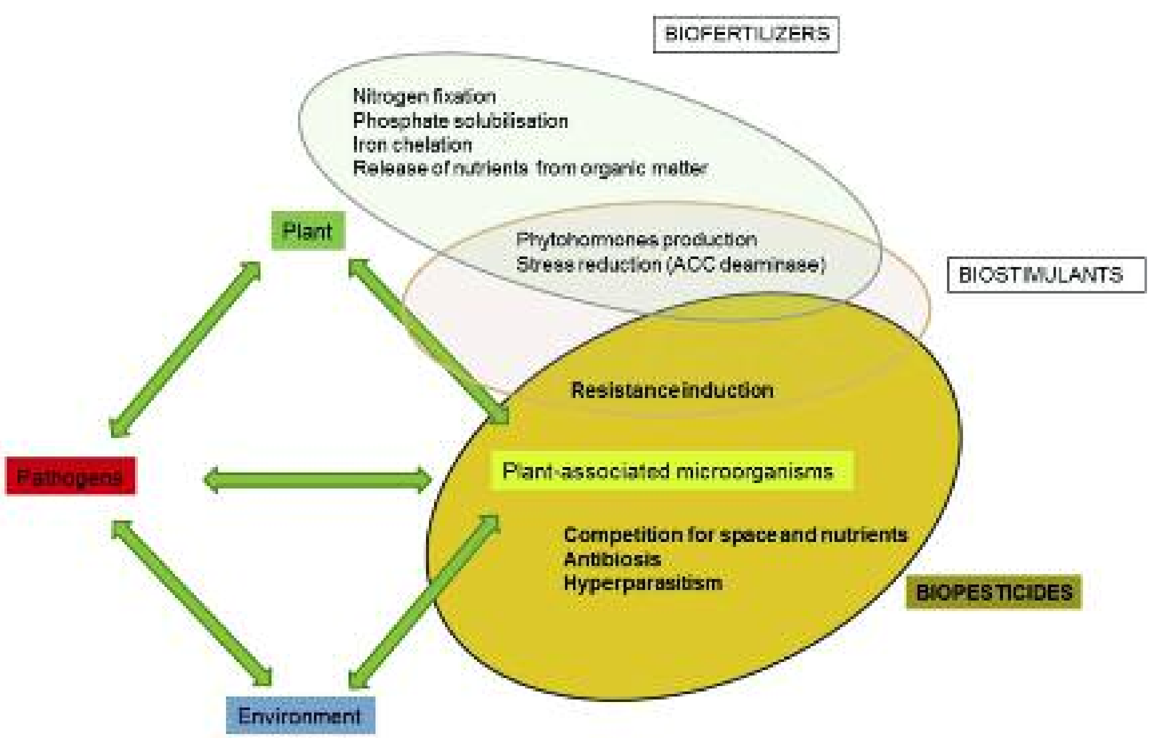

Fig. 3 - Microorganisms associated with plants, their mechanism of actions and use in agriculture.
Fig 3 - I microrganismi associati alle piante, il loro meccanismo di azione e loro uso in agricoltura.

applied immediately before the pathogen infection in order to exert the maximum effect.

There is a wide variety of different metabolites that can be produced by microbial biocontrol agents (Stein, 2005, Vinale *et al.,* 2008) and recent sequencing of the genomes of several microorganisms suggests that their number is likely underestimated (Puopolo *et al.,* 2016). The microorganism commonly releases enzymes and secondary metabolites only at the site of competition, in some cases following the perception of other microorganisms (Vinale*, et al.* 2008). Recently also the importance of volatile organic compound in the antibiosis was highlighted (Di Francesco *et al.,* 2015). The most accredited hypothesis is that in nature microorganisms produce small volatile compounds to reach a wider space and bigger hydrosoluble/liposoluble molecules to control the immediate vicinity, while more complex molecules, such as enzymes, are only produced in an inducible way when the prey is present (Vinale *et al.* 2008). The production of such toxic compounds is one of the critical aspects in the registration process, because one of the criteria is that the production of metabolite of concern is absent. Genome sequencing can also be helpful in this case. For example, the absence of certain specific genes makes it possible to exclude the existence of pathways that can lead to unwanted metabolites (Puopolo*, et al.* 2016).

Most of the microbial biocontrol agents exerting direct antibiosis were identified using dual culture tests in Petri dishes, in which the putative antagonist faces the pathogen on a jellified nutritional medium. Although this approach is the easiest and cheapest for identifying microbial biocontrol agents, it is biased by the fact that the type of medium can strongly affect the production of metabolites (Guerra *et al.,* 2001). As a consequence, the positive results obtained on rich media under optimal conditions in the lab, relatively often cannot be confirmed by application to plants in the natural environment.

Induction of resistance in the plant

In addition to pathogen-associated molecular patterns (PAMPs), specific molecules from microorganisms or plants can trigger the innate immune response of the plant (Delaunois *et al.,* 2014). They are referred to microbe-associated molecular patterns (MAMPs), when related to microorganisms or damage-associated molecular patterns (DAMPs), when related to plant endogenous molecules produced in response to pathogen attack. Both are perceived by the plant as danger signals, activating a complex network of defence responses: systemic acquired resistance and induced systemic resistance (Pieterse *et al.,* 2009). While systemic acquired resistance often results in metabolic costs for the plants because the defence pathways are continuously activated, induced systemic resistance, also known as the 'priming effect', involves a pre-activated physiological status for the plant, which reacts faster and stronger in case of subsequent pathogen attacks (Perazzolli *et al.,* 2011; Delaunois*, et al.* 2014; Nesler *et al.,* 2015).

Several beneficial microorganisms and compounds such as laminarin, chitin, plants extracts (e.g. *Salix* spp., *Reynoutria sachalinensis* and *Rheum* spp.), hydrolysed proteins and carbohydrates and etc. can induce resistance in plants (Perazzolli *et al.,* 2008), however the level of plant protection under field conditions is commonly very limited. Several hypotheses can explain the limited effect of the resistance inducers (environmental conditions, physiological status of the plant, etc.). However, the main explanation is that induced resistance does not give immunity to pathogens, making the plant only partially resistant. Therefore, under high disease pressure (i.e. in the epidemic stage of polycyclic diseases), partial control is not sufficient to stop the evolution of the disease. In addition, in nature plant resistance is commonly already induced by several factors, therefore the treatment with resistance inducers does not provide additional protection compared to the untreated controls (Delaunois *et al.,* 2014).

Almost all microbial biofungicides (e.g. *Bacillus* spp., *Pseudomonas* spp. and *Trichoderma* spp.) induce resistance, although with various levels of efficacy. Biofungicides acting only by inducing resistance are generally registered for use against powdery mildews, while they are generally less effective against downy mildews or fruit and root rots (van Aubel *et al.,* 2014; Nesler *et al.,* 2015)

Hyperparasitism

Hyperparasitism (mycoparasitism) is the mechanism with which the microbial biocontrol agent grows on/in the host (pathogen). The mycoparasite forms austoria or invades the mycelium of the pathogen to absorb nutrients for its survival and growth. In general, mycoparasites weaken, but never completely kill the host, resulting in a slow reduction of the disease (Kiss, 2003; Xu *et al.* 2010). Mycoparasites are often found in nature, however at levels that are insufficient to control diseases on crops (Angeli *et al.,* 2009). Two well-studied specific mycoparasites are *A. quisqualis* and *C. minitans* (Whipps *et al.,* 1992; Kiss, 2003), but several strains of *Trichoder*ma spp. can also parasitise other fungi (Howell 2003).

Ampelomyces quisqualis and *C. minitans* can be used to control disease, but their most effective use is in reducing overwintering) and the soil pathogen inoculum, respectively (Whipps *et al.*,1992; Caffi *et al.,* 2013). Mycoparasites need a host to survive; therefore, it is crucial to apply them when pathogen starts to be present.

Competition for space and nutrients

In order to colonise specific niches in nature, besides producing toxins and enzymes, microorganisms often compete for space and nutrients (Hibbing *et al.,* 2010). This mechanism of action is based on efficient consumption of key nutritional factors needed by other microorganisms and physical occupation of the space. Some microbial biofungicides especially those used against post-harvest disease (Spadaro e Gullino*,* 2004) are specifically based on this mechanism (Castoria *et al.,* 2001; Bencheqroun *et al.,* 2007). They indeed grow efficiently on wounds and cracks, consuming the sugars that are leaching, so they are not available for the growth of other microorganisms. This mechanism is common in yeast and several bacteria, but it is also observed in filamentous fungi such as *Trichoderma* spp., especially when used to prevent the colonisation of pruning (fig. 4) or grafting wounds by microorganisms associated with grapevine trunk diseases (Di Marco *et al.,* 2004, Pertot *et al.,* 2016) or flowers residues by *Botrytis cinerea* (Pertot *et al.,* 2017). In general, competition for space and nutrients is always associated with the release of some antibiotic substances or lytic enzymes.

Advantages and limiting factors in the use of microbial biofungicides

There are several advantages in substituting synthetic chemical fungicide with biofungicides in IPM. The main one is the fact that they do not leave residues in the final product, therefore they can be extremely useful in reducing the number of chemical treatments especially in the latter part of the season, as they have a limited or no requirements in terms of time to harvest. This specific advantage is particularly useful in the case of crops for which harvesting is prolonged over time and when plants need to be treated close to harvest. They are also very useful in pre-harvest treatment targeting post-harvest pathogens (e.g. *Aureobasidium* spp., *Bacillus* spp.).

The minimal or absent toxicity for humans is not only an advantage for the consumers, but can also benefit the workers in the field. The risks associated with operator exposure to these biofungicides are minimal, as possible accidental contamination is a matter of major concern. A short or absent re-entry interval after spraying is an additional advantage. None of the existing microbial biofungicides have displayed any phytotoxicity on crops, thus they are also suitable tools at plant stages when the tissues are soft and tender or to treat flowers.

Fig. 4 - Pruning wound treated with a biofungicide based on *Trichoderma atroviride* in late winter. After the application the microorganism colonises the dead wood below the wound.
Fig. 4 - Potatura trattata con un biofungicida basato su Trichoderma atroviride *nel tardo inverno. Dopo l'applicazione il microrganismo colonizza il legno morto sotto la ferita.*

They can be classified as 'low risk substances', which not only have the advantage of a longer authorization time (fifteen instead of ten years), but they can also be used in sensitive areas. Although not yet widely promoted for domestic use, they can be useful tools for home gardening and in areas with sensitive population groups.

None of the known genera registered as biofungicides are known to have any negative side effects on *Saccharomyces cerevisiae*, therefore they can be used on grapes to prevent grey mould or sour rot, with applications possible close to harvesting without influencing the fermentation during the vinification process, as several botryticides often do.

Microbial biofungicides are not toxic for pollinators and in some case bees have been used to deliver them to flowers (Hokkanen *et al.,* 2007). Furthermore, they are not dangerous for beneficial insects, such as hyperparasites and predators.

Because of their complex mechanisms of action (production of metabolites and enzymes, induction of resistance, competition for space and nutrients and mycoparasitism), microbial biofungicides are unlikely to develop resistance in pathogens populations. Thus, they are also valid tools in plant protection strategies to avoid building up resistance.

Although they are often accused of being less effective than synthetic chemical fungicides, in some cases they represent the only effective products against certain diseases. This is the case of grapevine trunk diseases, where only *Trichoderma* spp. strains have been proven to prevent infection by the causal agent of Eutypa dieback, or the microorganisms associated with Esca, Petri disease, and Botryosphaeria dieback. In this case, chemicals fail to control the infections mainly because their persistence in the pruning wound is limited and the length of the pathogens infection period is quite long (from March to September in most areas). Conversely, *Trichoderma* spp. strains efficiently colonize the wood for several months, providing a biological barrier to the entry of the pathogens (Kotze *et al.,* 2011).

They are the only tools usable in organic production, because they are of natural origin and do not have a negative impact on the environment. In addition, from an ecological point of view, they are fully biodegradable and renewable resources. Bioaccumulation of their metabolites has never been reported.

On the other hand, there are also several limiting factors that slow down market up-take of microbial biofungicides. As mentioned above, they are living organisms therefore the environmental conditions at the time of application or afterwards are crucial for their survival and activity. In particular, temperatures outside their range of survival, UV and desiccation are the most important factors conditioning their persistence in the phyllosphere. Survival on the phyllosphere is commonly enhanced by combining them with suitable additives (e.g. UV protectants, hydrating agents and nutritional factors) in the formulation (fig. 5) (Segarra *et al.,* 2015).

Fig 5 - Formulation (wettable granules) of *Trichoderma atroviride* SC1 conidia.
Fig. 5 - Formulazione (granuli bagnabili) di conidi di Trichoderma atroviride *SC1.*

When applied to soil, the most significant factors responsible for a decline in the biocontrol microorganisms is competition with resident microflora. After application, the concentration of microorganism's viable propagules remain high for a while, but then slowly decreases to reach the natural levels for similar microorganisms in that specific environment (Savazzini *et al.,* 2009). In several cases, this time is insufficient to reduce the pathogens inoculum to a no-risk level, as the case of several root rot diseases. The effective concentration threshold is also an important factor to be considered and often makes the treatment too expensive for growers and not economically sustainable. In several cases, the costs of the amount of of the microbial biofungicide required in order for it to be effective, amply exceeds the potential losses that could be caused by the disease. In other cases, when the biofungicides are targeted at reducing the pathogen inoculum in the soil, sufficient time should be given to the antagonist to kill the pathogen. This may be incompatible with common agronomic practices. For example, to prevent Rosellinia root rot in apple plants, the microbial biofungicide must stay in the soil for several weeks before transplanting new plants, in order to lower the *Rosellinia necatrix* inoculum. However, it is common practice to explant the previous orchard to be carried out in spring immediately before transplanting and, if this is done in autumn, the soil temperature may be too low to allow the microbial biofungicide to be active (Pasini *et al.,* 2016).

Compared to synthetic chemical fungicides several microbial biofungicides have slower effect. This is particularly true in the case of the hyperparasites, which need sufficient time to colonize and kill the pathogen. For this reason when a fast action is needed, biofungicides acting through direct antibiosis should be preferred (Pertot*, et al.* 2017). On the other hand, when the scope is the reduction of the inoculum, competition for space and nutrient and mycoparasitism are commonly more suited (e.g. a reduction of chasmothecia of *Erysiphe necator*, or reduction of sclerotia of *Sclerotinia* spp.) (Whipps*, et al.* 1992).

Most fungal biofungicides cannot be combined with fungicides and particular attention should be paid on the negative effects caused by chemical additives in chemical pesticides, which can be toxic for the microorganism. Copper is in general toxic for all microbial biofungicides, except those where the active microorganism is capable of extruding heavy metals (Puopolo *et al.,* 2014, Puopolo*, et al.* 2016) or has other detoxification mechanisms. Care should be taken in cleaning the sprayer tank after treatments with active ingredients that may kill the microbial active agent. Other precautionary measures to preserve the viability of the microorganism include a prompt use of the water suspension, which cannot be stored for too many hours without affecting the viability of the microorganism, correct storage in the conditions stated on the label and respect for the expiry date.

Practical issues involved in the application of microbial biofungicides to perennial fruit crops

Grapevine is the crop that has received the most attention in terms of identification of pathogen antagonists and authorisation of microbial biofungicides (Pertot *et al.,* 2017). However significant research have been carried out on antagonists of other fruit crops pathogens, particularly in post-harvest (Sharma *et al.,* 2009). In spite of the commercial importance of foliar diseases of pome and stone fruits, little knowledge is available on their biocontrol. Significant efforts have been made to reduce the overwintering inoculum (Carisse *et al.,* 2000) or the sporulation of *Venturia inaequalis* (apple scab) with microbial biocontrol agents (Köhl *et al.,* 2009), but in spite of numerous reports in the literature, no commercial products have been developed yet. Similarly, none of the known antagonists of *Stemphylium vesicarium* (brown spot of pear) or *Monilinia laxa* (peach twig blight), have been further developed as microbial biofungicides (Melgarejo *et al.,* 1986, Montesinos *et al.,* 1996, Rossi e Fattori*,* 2009). There are various reasons for this limited market uptake, ranging from the availability of cheap chemical competitors to the limited attractiveness for investors, however the destructive behaviour of the above-mentioned diseases when they are not fully controlled may also explain the limited success of microbial biofungicides.

The importance of soil-borne disease in perennial crops is increasing after the banning of chemical fumigants and microbial fungicides are often seen as the only practicable alternative. Soil treatment before replanting the new orchard/vineyard can efficiently reduce the inoculum of several soil-borne pathogens (Pasini*, et al.* 2016). However, obtaining a sufficient concentration of the antagonist and its survival in the soil after the treatment are still issues that need to be solved. No reports in the literature have shown that treatment of plants roots before transplanting or injections of microbial biofungicide into the soil of the infected orchard are sufficiently effective against root disease in perennial fruit crops.

Several studies have been carried out on consortia of two or more microorganisms in one treatment. Although consortia can theoretically improve the performance of the single microorganisms, results were contrasting (Guetsky *et al.*, 2002; Xu *et al.*, 2011; Sylla *et al.*, 2015). More studies are necessary to understand how consortia of microorganisms can be efficiently used in practice.

Future perspectives in microbial biofungicide research

To respond to the main weakness of microbial biofungicides when released into the environment, namely insufficient survival, new and more persistent strains must be identified or the focus must move to the industrial production of active compounds. In this respect, there is a need for more robust screening protocols and/or new selection criteria in the mining of new strains (marker genes, functional selection, etc.). New sources or substrates must be explored to screen candidates. For example, the endophytic microbial populations of plants, which are often highlighted as providing benefits to the hosting plant, may represent a largely unexplored source of useful isolates isolation (Gimenez *et al.,* 2007). The formulation can also help to prolong survival and more efforts should be put into identifying suitable additives for microbial biocontrol agents. Another challenge would be to use specific nutritional substances to shape the composition of resident microbial populations on the plant in such a way that the proportion of natural biocontrol active microorganisms is enhanced (Cappelletti *et al.*, 2017). In conclusion, several microbial biofungicides are already available and their efficacy may be improved by a correct use, better formulations or by decreasing production costs, allowing an increase in the concentration. However, more resources should be invested to develop a second generation of microbial plant protection products, with intrinsically better characteristics.

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Abstract

Although synthetic chemical fungicides provide undoubted advantages in terms of plant protection, their overuse and/or misuse may raise several safety concerns for the environment and human health. Microbial biofungicides, where the active substance is commonly a bacterial or fungal microorganism, are the most promising tools to substitute synthetic chemical active substance used to control fungal diseases. The mechanisms of action of microbial biocontrol agents contained in biofungicides have been classified into four groups (direct antibiosis, competition for space and nutrients, induction of resistance and hyperparasitism). The main advantages of using biopesticides are: they do not leave residues and can be applied close to harvest, they are biodegradable, renewable, safe for workers and can be used in strategies to prevent pathogen resistance. On the other hand, they have a few limitations: low persistency, often a slower effect, and an expiry date. In addition, biopesticides require care in the application and storage and their cost is generally higher than synthetic chemicals. Although they are promising tools, more efforts and resources should be put in identifying and developing a second generation of more performant microbial biofungicides overcoming their major limiting factors.

Key words: integrated pest management, biopesticides, pesticides, organic agriculture

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