Biocontrol Agents

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Biocontrol agents (BCAs) are living organisms and their derivatives that act against plant diseases and pests via direct antagonistic effects but also indirectly via the induction of plant resistance. They have been proposed as an alternative to standard fungicides but their disease management capacity is usually incomplete and heavily relies on uncontrollable environmental conditions. An integrated approach of combining BCAs with fungicides can reduce the fungicide doses to manage plant diseases and thereby their residual effects that affect the environment and human health.

Keywords: Biocontrol ; fungicide combinations ; integrated pest management ; induced resistance ; antagonism

1. Introduction

Plant diseases and pests are a major threat to global food availability. For example, the potential food losses due to diseases, caused by pathogenic micro-organisms and animal pests, are estimated to be up to 38.2% of total yield losses in rice and 36.5% in potatoes [1]. According to the United Nations, the world population is expected to increase to 9.7 billion by 2050, which means that a dramatic increase in global crop harvest is required in order to satisfy the population's food needs [2]. This can be realized by increasing the area of cropland, disrupting natural ecosystems or by intensifying crop yields [3]. However, crops with increased yield are often associated with even higher vulnerability to diseases and pests. In particular, fungal plant pathogens are attracted to nutrient-dense plant tissues. As such, the potential loss of wheat production due to fungal diseases increases from less than 10%, with an attainable yield of 2 tons/ha, to more than 20% when the intensity of production increases to 12 tons/ha [1]. The use of synthetic pesticides has therefore become an integral part of agriculture. As such, since the discovery of the first synthetic fungicide, phenylmercury acetate in 1913, over 110 new fungicides have been developed during the last century, allowing food production to increase with a value of USD 12.8 billion in the US annually [4][5][6] However, their extensive use has encountered two main challenges. First, concerns have been raised over the residual effects and toxicity that affect the environment and human health. For example, fungicides, and other types of pesticides, have recently been linked to cancer and respiratory and hormone imbalance diseases, thereby depending on the level of exposure [7][8][9]. Driven by the opinion of consumers, who perceive pesticides as a threat, and the vast amount of research supporting this view, regulators have approved laws that result in either banning or restricting their use by imposing lower maximum residue limits (MRLs) [10][11]. In the European Union (EU), the MRL review program was implemented under Regulation 396/2005 to restrict the use of synthetic pesticides. Second, the efficacy of fungicides has decreased due to the emergence of resistant pathogens [12]. However, the discovery of new types of fungicides has become more difficult and more costly [13]. As such, the cost of the discovery and development of one new active ingredient increased from USD 195 million in 1995 to USD 286 million in 2016 [13].

In response to the increasing knowledge about the negative side effects of pesticide overuse, integrated pest management (IPM) was implemented. IPM is defined as the best mix of plant disease control strategies, taking into account the crop yield, profit and safety profile, as presented by the Food and Agriculture Organization of the United Nations (FAO) [14]. Worldwide, IPM is an accepted strategy to reduce pesticide usage in pest management [15]. In the EU, the sustainable application of pesticides is required by directive 2009/128 [16]. Particular emphasis is placed on the prevention of infection and the consideration of all available plant disease management tools while taking into account their economic benefits and toxicity. In this regard, biocontrol has been proposed as an alternative to conventional pesticides.

The term biological control or biocontrol often causes confusion as different meanings circulate in the scientific literature. In the most narrow definition, biological control can be defined as the use of a living organism to act against a specific plant pathogen or pest via parasitism, antibiosis or competition for nutrients or space [17]. However, plant diseases and pests are induced and regulated by complex processes on different levels: the invader—being the plant pathogen or pest—the environment and the plant itself. They only thrive if conditions are optimal on all three levels [18]. Therefore, a broader definition of biological control, covering all levels, is needed to in order to achieve its true potential in disease and pest management. This broader term includes the application of living organisms and their derivatives to control plant diseases and pests, not only via direct antagonistic effects against plant pathogens and pests but also indirectly via the induction of resistance [19]. The differences with the narrow definition of biocontrol are therefore that derivatives of living organisms and inducers of resistance, which activate the defense mechanisms of plants, are also defined as biocontrol agents (BCAs) [20]. Examples include parasitoid wasps, predatory mites against several pests such as potato tuber moth and pathogenic bacteria and fungi, like *Bacillus* spp. and *Trichoderma* spp., which act against different types of plant pathogens [21][22][23][24]. Along with biocontrol organisms, there are BCAs such as chitosan and derivatives such as chitooligosaccharides (CHOS) originating from the fungal cell wall [25]. The potential of these has challenged researchers to develop chemical analogs with similar characteristics or distinct from known natural inducers of the plant's immunity [26][27]. However, despite intensive research, success in field trials using BCAs is very limited due to variations in ecological parameters like plant physiological and genetical status, climatological conditions, etc., which increase the variability of the desired BCA effect [28][29][30]. Therefore, their use is more restricted to the cultivation of greenhouse crops, where environmental conditions are more controllable [31]. In the field, a more reliable disease control could rely on combinations of BCAs and fungicides. As these types of combinations could reduce the fungicide dose (under the MRLs) or the frequency of application and improve disease control, they translate the principles of IPM into practice. In addition, such a strategy of combining antifungal treatments with different modes of action would fit within the advice of the fungicide resistance action committee to reduce the selection pressure on pathogens and thereby the chances of resistance development [32].

2. Biological BCAs

To inhibit fungal pathogens, fungicides have been developed that target different components or mechanisms of the fungal cell, including respiration, nucleic acid metabolism, cell membrane integrity, protein synthesis, signal transduction and cell mitosis [32]. However, some fungicides perform these activities without distinguishing between harmful pathogens and nontarget organisms such as beneficial micro-organisms in soil and living BCAs [33]. As such, fungicides could impact the growth of BCAs or reduce their population size, making the biocontrol treatment ineffective. Therefore, knowledge of compatibility of fungicides and BCAs is crucial to allow combined applications. For example, by using bacterial BCAs, the biocontrol effect could be less impacted by fungicides acting on more fungi-specific targets [34]. Alternatively, fungal BCAs can be used that are selected or developed for enhanced resistance to specific fungicides [35]. Each combination of a BCA and a fungicide should therefore always be examined. Usually, the inherent resistance of a BCA against a fungicide is first examined in vitro [36]. Combinations of such resistant BCAs and the corresponding fungicide can subsequently be confirmed in vivo and further fine-tuned for optimized disease management capacity.

2.1. Combinations of Fungicides with Biological Antagonists

BCAs can manage plant diseases through direct antagonistic effects on plant pathogens via parasitism, antibiosis or competition for nutrients or space. Parasitism is a relationship between two organisms in which one directly gains nutrients from the other. A specific type of parasitism well-known in the biocontrol field is mycoparasitism, in which fungal plant pathogens are parasitized by biocontrol fungi, reportedly often *Trichoderma* spp. [37][38]. The second mechanism, antibiosis, takes place between two organisms when one produces antimicrobial metabolites that directly impact the growth or metabolism of the other organism. These antimicrobial products are produced at very low concentrations, and they are only locally distributed and have a short life-span; therefore, their toxicological risks to humans are low [19]. Finally, competition occurs when two organisms require the same limited nutrients or space. These protective mechanisms of direct BCAs are often complex and rely on different and multifaceted modes of action, which is expected to lower the chances of resistance development. Another advantage of direct BCAs includes the possibility to investigate inhibitory effects via simple bioassays between only the antagonist and the pathogen, which is more straightforward than investigating indirect effects between the pathogen, inducer of resistance and the plant [20]. Despite this, complete disease control can only be obtained when BCAs are combined with fungicides [39]. In the following paragraphs, such combinations will be described but no distinction will be made between competition, antagonism and parasitism since the main mechanism of control is not always clear; instead, a distinction will be made based on their time (either pre- or post-harvest) or method of application or origin [19].

Microbial antagonists with a direct action have reportedly been combined with fungicides to control post-harvest diseases. An advantage of the post-harvest application of antagonists and fungicides includes the simple treatment via dipping of harvested fruits in one solution. However, as mentioned before, the application in a mixture implies that the antagonist is inherently resistant against certain fungicides. As such, improved control of ber fruit rot (caused by *Alternaria alternata*) was obtained when harvested fruits were dipped in a mixture of fungicide-resistant *Trichoderma* spp. and various systemic and non-systemic fungicides at low doses of 50 or 100 ppm [40], as compared to the 10-times higher doses typically applied for fungicides on ber fruit [41]. Some *Trichoderma* isolates caused a latent infection which was completely suppressed with the combination. In a different study, on stored apples, a mixture of the biocontrol yeast *Cryptococcus laurentii* and thiabendazole, at 10% of the standard dose, resulted in the highest and longest control of another important post-harvest pathogen, *B. cinerea* [42]. The combination was even more effective against a thiabendazole-resistant isolate of *B. cinerea*, also providing longer disease control compared to treatment with the biocontrol yeast alone. Therefore, BCA–fungicide combinations could have potential against populations of fungicide-sensitive and fungicide-resistant populations, which are becoming more and more prevalent [43]. Similarly, on harvested apples but using newer fungicides, a solution of the biocontrol yeasts (*Rhodosporidium kratochvilovae* or *C. laurentii*) with a low dose of either boscalid or cyprodinil was more effective against blue mold caused by *Penicillium expansum* than the treatment by itself [44]. Interestingly, lower fungicide residues were observed with the combination treatment even when compared to single treatment with the same fungicide at the same low dose. Most successful post-harvest treatments involve the combined application of biocontrol yeasts and fungicides, which is likely due to the ability of yeasts to tolerate extreme environmental conditions, making them appealing for food application. As such, yeasts can survive in routinely used storage conditions, including low oxygen levels, low temperatures and UV radiation, but also in conditions specific to foods, such as low pH and high sugar concentrations [45]. Sometimes, the pre-harvest application of fungicides is more efficient against post-harvest pathogens, but also this approach could be improved via combinations with BCAs. As such, the combined pre-harvest application of *Epicoccum nigrum* and various fungicides could reduce the fungicide dose three-fold without affecting the management of brown rot (caused by *Monilinia* spp.) on harvested peaches during four different field trials [46]. Disease reduction was most effective in years with lower disease severity.

Fungal antagonists also improve disease control when combined with traditional fungicides against pre-harvest pathogens. In particular, the application of *Trichoderma* spp. against soilborne pathogens is known for these reasons. *Trichoderma* spp. are inherently resistant against some fungicides, allowing the combined application in a mixture. Such a combination of *T. virens* and thiophanate-methyl was found to be compatible and more effective than either treatment alone against *Fusarium solani* and *Fusarium oxysporum* in field trials of dry bean production [47]. The vegetative growth of the plants and yield was also significantly increased for the combination compared to single treatment. Similarly, the combined application of *Trichoderma* spp. with a low dose of fluazinam was found to be more effective to control avocado white rot (caused by *Rosellinia necatrix*) than either treatment alone [48]. Finally, though *Trichoderma* spp. were found not to be effective against *F. oxysporum* and *Acremonium strictum* in an in vitro setting, combining them with a low dose of the broad-spectrum fungicide tolclofos-methyl was superior to the fungicide only [49].

The rhizosphere of plants forms a source of bacteria not only important for plant resistance but also for direct biocontrol in pre-harvest applications. Similar to fungal antagonists, these bacterial antagonists mainly improve disease control against soilborne pathogens. For example, the incomplete disease control of *Bacillus megaterium* against *F. oxysporum* on tomato could be improved when combined with a low dose of the fungicide carbendazim in plant-packs [50]. The combination provided full disease control, even outperforming application with the fungicide at a 10-fold higher dose. Similarly, in the same set-up, combined application of rhizobacteria *P. fluorescens* and a 10-fold reduced dose of benomyl was more effective than treatment with either alone and reduced the disease as much as a full dose of the fungicide alone [51]. Interestingly, some biological antagonists can survive on the leaves of plants, which allows spray application against foliar pathogens. As such, *Bacillus subtilis* is a rhizobacterium that has been widely tested for its production of antibiotics that affect the cell wall of plant pathogens [52]. In multiple greenhouse trials, the foliar application of *B. subtilis* with azoxystrobin provided the highest yield and the best disease control against powdery mildew (caused by *Podosphaera xanthii*) on zucchini, compared to both treatments alone [53].

Fungicides have also been combined with multiple fungal and bacterial BCAs to enhance their disease management capacity pre-harvest. A combination of *P. fluorescens*, *Mesorhizobium cicero* and *T. harzianum* with the fungicide Vitavax® (active ingredients: carboxin and thiram; Haryana, India) provided the highest seed germination, grain yield and the lowest wilt incidence (caused by *F. oxysporum*) in pot and field experiments of chickpea [54]. Moreover, in field experiments of rice, the combination of *T. harzianum*, *P. fluorescens* and carbendazim was more effective against *Magnaporthe oryzae* in comparison to their individual application [55].

Derivatives of living organisms like plant extracts are also known as direct BCAs that can be combined with fungicides. Synergy was observed between either CHOS or chitosan and various synthetic fungicides on strawberry flowers [56]. The combination of the fungicide at a 100-fold reduced dose and chitosan or CHOS yielded a protection level against *B. cinerea* similar to the fungicide at full dose. A similar combination with a 10-fold reduction of the fungicide dithianon was more effective in controlling scab (caused by *Venturia inaequalis*) than the fungicide alone at the recommended dose in field trials of apple [56]. Moreover, plant extracts of *Inula viscosa* combined with the fungicide iprodione at a reduced rate were as effective against *B. cinerea* on bean plants as the full dose of fungicide [57].

2.2. Combinations of Fungicides with Biological Inducers of Resistance

Various biotic and abiotic stresses are well known to regulate the natural plant defense mechanisms by triggering induced resistance, which can be defined as an enhanced physiological state of defense that prepares plants against future pathogenic attacks. There are two main reported types of induced resistance: systemic acquired resistance (SAR) and induced systemic resistance (ISR). Both provide long-lasting resistance against plant pathogens but differ in the signaling molecules and pathways that result in such an increased state of alertness [58]. As such, the induction of SAR is usually activated by pathogen infection and requires the signaling molecule salicylic acid (SA) to accumulate pathogenesis-related proteins [59]. In contrast, ISR is triggered by beneficial micro-organisms and usually does not rely on SA but is dependent on pathways regulated by jasmonate and ethylene [60]. Moreover, there are other types of interactions between biological BCAs and plants (such as symbiosis) that can induce the defense mechanisms of plants. For example, endophytic fungi have been shown to colonize banana plants and thereby induce systemic resistance against *Radopholus similis* [61]. However, these types of symbiotic interactions fall outside the scope of this review.

The amount and variety of mechanisms involved, and the absence of a direct interaction with the pathogen, implies that there is limited selection pressure on pathogens [62]. It is therefore unlikely that resistance develops against inducers of plant resistance. In addition, as these inducers activate the plant defense response that produces molecules which are generally present in natural environments for the communication between plants and micro-organisms, it is assumed that the induction of resistance poses very low toxicological and ecological risks to nontarget, beneficial organisms and humans [19]. Despite this, induced resistance often provides only 20–85% disease control and is thus rarely complete [63]. Moreover, the unpredictability of disease control due to environmental variations in crop nutrition, genotype and disease severity has raised further concerns [64]. To maximize efficiency and allow commercial application, they can be combined with fungicides. In the first instance, it is expected that, in such combinations, the systemic effect of inducers of resistance improves the disease control of non-systemic fungicides that only provide local disease control at the site of application. However, such an improved effect on disease control is also true for systemic fungicides, which do translocate through the plant but rarely move to all plant parts [51][65]. Another advantage of systemic inducers of resistance includes the possibility to apply the BCA as a seed treatment or on the roots of plants against foliar pathogens. Therefore, these types of combinations are more regularly effective against leaf spots, blights and mildews. Moreover, the chances of resistance development decrease by using these types of combinations [66].

2.2.1. Combining Fungal Inducers of Resistance and Fungicides

Trichoderma spp. are beneficial fungi in the rhizosphere of plants of which some species are reported to act as BCAs either by directly antagonizing other pathogens or indirectly by inducing ISR [67]. When applied in alteration with a fungicide, the latter does not impact the growth of the BCA, and disease control performance is enhanced. In corn, for example, the spray application of difenoconazole-propiconazole followed by *Trichoderma harzianum* SH2303 was as effective in reducing southern corn leaf blight caused by *Cochliobolus heterostrophus* as a sequential spray with the fungicides, while the BCA alone was not effective [68]. Thereby, the combination allows a two-fold reduction of the fungicide dose to control southern corn leaf blight. Similarly, alteration of *T. harzianum* with dicarboximide fungicides was found to be as effective against grey mold (caused by *Botrytis cinerea*) on tomato plants as single dicarboximide treatment, while treatment with *Trichoderma* alone resulted in variable disease control [69]. Nevertheless, the combination allows a two-fold reduction in the number of fungicide sprays.

Even in combination with fungicides, the use of biological inducers of resistance can result in variable disease control, as their mode of action might be independent and just additive, or more variable results might be related to the lowered dose rate of the fungicide. A combination of carbendazim and *Trichoderma* sp. Tri-1 could reduce the fungicide dose by 25–50% to control *Sclerotinia sclerotiorum* on oilseed rape [70]. The highest disease control was obtained in fields where a rice–oilseed rotation was used, which generally is associated with lower disease occurrence. In the same way, alteration of chitosan with the fungicide chlorothalonil against *Didymella bryoniae* was found to be as effective as a continuous spray with the fungicide during a field trial on watermelon when disease severity was low [71]. However, during another field trial with high disease severity, alteration with chitosan was found to be ineffective against *D. bryoniae*. Therefore, sufficient research must be performed in different environmental conditions to reveal the true potential of combining a biological inducer of resistance with a fungicide.

When applied as a mixture, the compatibility of *Trichoderma* spp. with fungicides needs to be examined [72]. If the fungicide impacts the survival of the BCA and the compounds cannot be administered separately, alternatives need to be explored. The combination of *Trichoderma* spp. and the fungicide iprodione against *S. sclerotiorum* required the selection of iprodione-resistant isolates of *Trichoderma* spp. [35]. Soil application of iprodione-resistant *Trichoderma virens* combined with iprodione resulted in a synergistic interaction and managed disease most effectively on cucumber. Alternatively, the administration of fungicide-sensitive *Trichoderma* spp. can be physically separated from the fungicide to allow such a combination. The main diseases threatening cotton seedlings are pre-emergence damping-off by *Pythium* spp. and *Rhizopus oryzae* and post-emergence damping-off by *Rhizoctonia solani* [73]. *Trichoderma* spp. can effectively manage pre-emergence damping-off via the local induction of phytoalexins in the cotton root. However, it cannot access the hypocotyl and therefore there is no phytoalexin production in this part of the plant, leaving it unprotected from post-emergence damping-off [74]. On the contrary, fungicide seed treatments can control post-emergence pathogens but they are not effective against pre-emergence pathogens. Hence, a combinatorial seed treatment, in which the fungicide chloroneb is first applied, followed by a coating of the seeds with a latex sticker after which *Trichoderma* spp. are applied to the seeds, manages both phases of damping-off effectively.

2.2.2. Combining Bacterial Inducers of Resistance and Fungicides

In addition to fungal inducers of resistance, such as some *Trichoderma* spp., also bacterial ones have been successfully used in combination with fungicides. Maneb and mancozeb are non-systemic fungicides that need to be repeatedly applied to manage fungal plant diseases as they are only effective on contact. When roots of corn were drenched in a solution containing *Bacillus cereus* C1L, a rhizobacterium known to induce plant resistance, the number of leaf sprays with maneb which was necessary to control southern corn leaf blight (caused by *C. heterostrophus) could be reduced two-fold [75]. In addition, while treatment with the fungicides alone negatively affected plant growth, the combinatorial treatment significantly increased plant growth, as compared to untreated plants under natural conditions. Similarly, seed treatment of B. cereus, which induces systemic resistance,* could reduce the number of sprays of another non-systemic fungicide, chlorothalonil, to manage early blight (caused by *Alternaria solani*) in tomato [76]. The frequency of fungicide sprays therefore could be scaled down from 20 to 10 applications while the yield was unaffected over a 90-day field study, confirming the long-lasting effect of inducers of resistance on plant defense mechanisms. Combinations of bacterial inducers of resistance and systemic fungicides are also relevant against various diseases on crops with economic importance. For example, leaf spots caused by *Cercospora beticola* reduce the harvest of sugar beets. In the past, the disease was controlled by fungicides such as triphenyltin hydroxide, benomyl and thiophanate-methyl; however, the pathogen has become resistant. The biocontrol agent *Bacillus mycoides* was able to induce plant resistance and thereby reduce Cercospora leaf spot by 38–91% in six different field trials [77]. The addition of an alternative fungicide, propiconazole, in a mixture with *B. mycoides* reduced the variability and always allowed effective disease management while lowering the chances of resistance development. Similarly, in field trials with different wheat varieties, ranging from susceptible to moderately resistant, the combination of *Lysobacter enzymogenes* strain C3, known to induce disease resistance in the plant, and the fungicide tebuconazole was consistently effective against Fusarium head blight (caused by *Fusarium graminearum*) while disease control with the fungicide or BCA alone was variable [77]. Indeed, while in half of the field trials, application of the BCA alone was effective on susceptible varieties but not on moderately resistant varieties, in the other field trials, it was either not effective on any variety or effective on all. As mentioned before, such variable differences in the disease control activity of a BCA are assumed to be dependent on environmental conditions. Interestingly, the combined treatment of BCA and tebuconazole in these field trials did not show this high variability and was always effective [78]. Finally, these types of combinations are also reported to be relevant against powdery mildews and fruit rots. When applying the biocontrol bacterium *Pseudomonas fluorescens* to treat powdery mildew and fruit rot (caused by *Leveillula taurica* and *Colletotrichum capsici,* respectively) in chili cultivation in the field, disease control was found to be incomplete [79]. However, combined application of the BCA with a two-fold reduced dose of the standard fungicide, azoxystrobin, was as effective as the fungicide at standard dose.

2.3. Conclusions

To conclude, fungal disease control can be improved when fungicide-compatible BCAs are combined with fungicides. These treatments may have the potential to develop new antifungal strategies for integrated pest management since the chances of resistance development are lower and the fungicide dose might be reduced compared to traditional treatment with single fungicides. If the fungicide impacts the growth and development of the BCA, they should be separated in time or space from fungicides, which is evidently not possible for most direct BCAs that are applied together with fungicides. Advantages of indirect inducers of resistance include the long-lasting, systemic disease control. As a result, the application will protect the entire plant, even parts that are hard to reach by spray applications. It seems that the potential of these types of combinations has not been completely explored, since, to the best of our knowledge, there are no reports on the combined use of biological inducers of resistance and fungicides in post-harvest applications. However, it is reported that biological inducers of resistance do provide incomplete disease control in this setting [80][81]. In contrast to BCAs directly antagonizing pathogens, which have been used since the 1980s, these ISR-inducing BCAs have been more recently developed for treatment in post-harvest disease control, which could explain the research gap [39]. However, since these combinations of fungicides and ISR-inducing BCAs might also be valuable against post-harvest diseases, they should be included in future research. Nevertheless, disease control through the use of BCAs might remain variable, even in combination with fungicides, and therefore such combinations need to be fully investigated under natural conditions [70][71].

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