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Combining biocontrol agents with different mechanisms of action in a strategy to control *Botrytis cinerea* on grapevine

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ABSTRACT

The use of several microbial biocontrol agents to combat *Botrytis cinerea,* the causal agent of grey mould, has been studied. However, only a few microorganisms have been developed as biofungicides, which are currently used in some countries, mostly in organic farming. The main reason for the limited market uptake of microbial biofungicides is their debated variable efficacy. To cope with poor survival in the canopy, due to unfavourable environmental conditions or their intrinsic lower level of disease control compared to synthetic chemical fungicides, use of a mixture of two or more microorganisms with different environmental requirements and mechanisms of action has been proposed with contrasting results. However, their use in strategies involving calculated timing of the microbial biocontrol agents, taking into consideration their mechanism of action in relation to the epidemiology and pathogenesis of the disease, has never been attempted in relation to combating grey mould on grapes. The results of four years of trials in three locations in Northern and Central Italy show that *Trichoderma atroviride*, *Aureobasidium pullulans* and *Bacillus subtilis,* applied at bunch-closure, veraison and pre-harvest, respectively, controlled *B. cinerea* on bunches very satisfactorily, and the results did not differ from those obtained with a strategy combining the three biofungicides, applied at the aforementioned stages. Colonisation of berries by each of the different microbial biocontrol agents at harvest time did not differ for individual treatments or when applied in the combined strategy, suggesting that the microorganisms did not negatively interfere with each other and that they may possibly occupy different ecological niches. The high level of efficacy of the tested biocontrol agents against grey mould can be explained with the relatively low-medium level of the disease, their integration with agronomic practices or the optimal timing of the treatment.

1. Introduction

Botrytis cinerea (= *Botryotinia fuckeliana*; Johnston et al., 2014) is an extremely polyphagous and ubiquitous pathogen and the causal agent of grey mould, one of the major diseases of the grapevine. On grapevine, it may cause significant losses in terms of quantity and quality, especially on sensitive varieties and when disease-conducive meteorological conditions prevail (Elad et al., 2007). Control of *B. cinerea* on various crops is commonly achieved with a combination of pesticide treatments and agronomic practices. On the grapevine, such practices can directly or indirectly influence the disease, by modifying both berry defence mechanisms and the microclimate of the vine. For example, avoiding excessive nitrogen fertilisation, removal of leaves around the bunches and thinning of the berries can significantly reduce the disease (Mundy, 2008; R'Houma et al., 1998). Removal of leaves in the fruiting zone increases their exposure to the sun, resulting in more epicuticular wax and a more resistant cuticle, and thanks to higher air-flow in the canopy, in a reduction of relative humidity and faster drying of the bunch following rain (Gubler et al., 1987). In addition, removal of leaves in the bunch zone at the 'pea-size berries' stage can reduce

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infestation by the second generation of *Lobesia botrana* (European grapevine moth) and consequently limit grey mould, which develops as a result of feeding damage caused by this insect (Pavan et al., 2016). Varieties with tight bunches are considered to be more susceptible to *B. cinerea,* not only because of compression among the berries, which can create wounds easily colonised by the pathogen, but also because of the intrinsically higher susceptibility of the epicuticular wax at the point of berry contact (Marois et al., 1986). The advantages of increasing bunch openness, achieved with the use of gibberellic acid, is still controversial (Ferree et al., 2003; Mundy et al., 2014) and bunch tightness should probably be considered in the wider context, being only one of the factors concurring in susceptibility to the disease in some varieties (Vail et al., 1998).

Botrytis cinerea infections may start at bloom, when the most likely site of infection is the receptacle area or the cap scar (Keller et al., 2003) and remain latent until after veraison. After veraison, the sugar concentration increases and antifungal plant compounds decrease, with a parallel increase in berry susceptibility to *B. cinerea* (Jacometti et al., 2010). *Botrytis cinerea* can easily colonise senescing floral tissues that remain trapped inside compacted bunches and result in a source of inoculum following veraison. In addition, *B. cinerea* germination is promoted by the presence of sugars, which may be exuded by ripening tissues or leached from micro and macro-wounds on the skin of ripening berries. Recent studies demonstrated that in some regions grape inflorescences are more susceptible at flowering (beginning, full, and end of flowering) than at earlier growth stages or at fruit swelling or berries groat-sized stages (Ciliberti et al., 2015).

Although very helpful, agronomic practices alone cannot prevent the disease in many grape-growing areas, so chemical treatments should normally be applied (Jacometti et al., 2010). Because of *B. cinerea* epidemiological traits (the inoculum is always present in the vineyard and the range of climatic conditions suitable for the pathogen to infect plant tissues is quite wide), disease forecasting models are commonly not used to schedule chemical treatments against grey mould on the grapevine. This is possibly owing to the fact that none of the models developed so far took into account the complexity of *B. cinerea* epidemiology. Consequently, treatments are applied at fixed phenological plant stages: full bloom, bunch closure, veraison and before harvesting. However, the full fungicide schedule is normally applied only in the event of high disease pressure, and in most locations with low-medium disease pressure, fewer sprays are carried out. For example, under the environmental conditions of most Italian vineyards, treatment at blossoming is skipped, because no or few infections commonly occur at that stage. Recently a new mechanistic, weather-driven model was developed for predicting the risk of grapevine infection by *B. cinerea* during two infection periods (from the stage 'inflorescence clearly visible' to 'berries groat-size' and from the stage 'berry touching' to 'berries ripe for harvest'). This model gave very promising results calculating the infection severity in the two periods, correctly classifying the severity of 17 out of 21 epidemics and opening new perspective for using forecasting models to schedule treatments to control grey mould (Gonzalez et al., 2015).

In recent years, the use of microbial biofungicides based on microbial biocontrol agents has increased continuously, because of public concerns regarding the risk of pesticide residues in food and their negative impact on the environment (Fillinger and Elad, 2016). An additional reason to reduce the use of synthetic chemical fungicides against *B. cinerea* is the fast, rapid and relatively easy selection of resistant strains against single-site fungicides in *B. cinerea* populations, caused by continuous use of active ingredients with the same mechanism of action (Fillinger and Elad, 2016; Schnabel, 2016). Microbial biocontrol agents may represent an alternative to these synthetic chemicals; indeed, they normally have multiple mechanisms of action (Vos et al., 2015), which are surmised to prevent or at least significantly slow down the build-up of fungicide-resistant populations. Because of the economic impact of grey mould, several microbial biocontrol agents and non-synthetic chemicals used to combat the disease have been studied (Jacometti et al., 2010). Among the microorganisms, several fungal and bacterial strains have been successfully tested against grey mould on a variety of crops, including the grapevine (Elmer and Reglinski, 2006).

The *Trichoderma* genus has been a valuable source of microbial biocontrol agents for a long time (Vos et al., 2015). *Trichoderma* spp. can be easily isolated from soil, wood and decaying plant material, but they may also be excellent root colonisers (Vinale et al., 2008). *Trichoderma* spp. strains are characterised by multiple mechanisms of action (induction of plant resistance, mycoparasitism, antibiosis and competition for space and nutrients), which may all result in the reduction of plant diseases (Rossi and Pattori, 2009; Vinale et al., 2008; Vos et al., 2015). In addition, *B. cinerea* often penetrates plant tissue through wounds and takes advantage of senescing host tissues to survive and act as an inoculum for infections of berries. Therefore, by colonising these senescing tissues and competing with *B. cinerea*, *Trichoderma* spp. can prevent or reduce grey mould infections (Card et al., 2009). A specific strain, *T. harzianum* T39, was the first biofungicide marketed to combat *B. cinerea* on the grapevine (O'neill et al., 1996).

Aureobasidium pullulans is a widespread and common fungal grapevine epiphyte. Strains of *A. pullulans* were initially developed to control post-harvest diseases, including grey mould (Bencheqroun et al., 2007; Lima et al., 1997; Zhang et al., 2010). However, *A. pullulans* was also shown to be highly effective against grey mould in greenhouse conditions, for example on cucumbers and tomatoes (Dik and Elad, 1999), and in the field on the grapevine (Elmer and Reglinski, 2006). Natural strains of *A. pullulans* present on grapes or in must/wine are good antagonists of *B. cinerea* (Raspor et al., 2010). The main mechanism of action is based on competition with the pathogen for nutrients at the infection site, although hydrolytic enzymes are also produced (Castoria et al., 2001; Di Francesco et al., 2015a). It was recently demonstrated that *A. pullulans* produces volatile organic compounds that can prevent the germination of conidia of several pathogens, including those of *B. cinerea* (Di Francesco et al., 2015b), making the mechanism of action of this biocontrol agent more complex than previously thought or determined (Spadaro and Droby, 2016).

Strains of *Bacillus subtilis*, *Ba. pumilus* and *Ba. amyloliquefaciens* can control *B. cinerea* (Elad et al., 1994; Mari et al., 1996), mainly through the production of antibiotics (Leifert et al., 1995), although induction of resistance has also been reported to occur in several crops (Choudhary and Johri, 2009). Biofungicides based on spore-forming *Bacillus* species have the advantage of a long shelf-life, a wide spectrum of activity and a generally high compatibility with most synthetic chemical fungicides (Emmert and Handelsman, 1999).

One of the main practical constraints in the use of microbial biofungicides is their variable efficacy, which is mainly due to unfavourable environmental conditions impairing their survival in the canopy or the intrinsically lower level of disease control as compared to synthetic chemicals. To partially solve these problems, a mix of two or more microorganisms has been proposed (Sylla et al., 2015). Several studies have been carried out on combinations of two or more microorganisms in one treatment (Guetsky et al., 2002; Sylla et al., 2015; Xu et al., 2011) with differing results. On the other hand, strategies in which different biocontrol agents are applied in sequence throughout the season have received little attention. In contrast to mixtures of different

microorganisms, where the mechanism of mutual antagonism or problems of compatibility may arise, application in sequence may allow an increase in efficacy. The objective of this research was to assess the effect of using three biofungicides with different mechanisms of action, applied at the phenological stages when botryticides are commonly applied in Italy. More specifically, the protocol was based on applying a good coloniser of dead plant tissues at bunch closure (*T. atroviride*), a strong competitor for space and nutrients after veraison (*A. pullulans*) and a microorganism having a fast, direct effect against pathogens, but compatible with wine fermentation, close to harvesting (*Ba. subtilis*). This strategy was compared to single applications of the same microorganisms at the specified stages and to an untreated control. In order to guarantee rapid transfer of the practices to growers, commercially formulated biofungicides were used. The trials were carried out in commercial vineyards in Northern and Central Italy from 2011 to 2014.

2. Materials and methods

2.1. Efficacy trials

The trials were carried out in commercial vineyards in three locations in Italy: San Michele all'Adige (SM, Trentino-Alto Adige region), Ziano Piacentino (ZP, Emilia-Romagna region) and Montepaldi, San Casciano Val di Pesa (MP, Tuscany region). The varieties tested were Schiava (in 2011) and Pinot gris (in 2012-2014) in SM, Barbera (in 2011-2013) in ZP, and Sangiovese (in 2011, 2012) and Trebbiano (in 2013) in MP. All these varieties are highly susceptible to *B. cinerea*. The vineyards were homogeneous in terms of soil conditions, plant vigour and age (10-year-old) and all of them well representative of each grape growing area. Leaves in the fruiting zone were removed at the 'pea-size berries' stage [corresponding to BBCH 75 (Lorenz et al., 1995)]. In all vineyards, crop protection against powdery and downy mildew was carried out following integrated pest management standards and considering local weather conditions, by using a fungicide schedule which did not include active ingredients effective against *B. cinerea*. Meteorological data were recorded throughout the seasons using automated weather stations close to the experimental sites. A randomised complete block design with three (SM) or four replicates (ZP, MP), having at least eight vines per replicate, was used.

The active ingredients applied were: *T. atroviride* SC1 (Vintec; Belchim Crop Protection; at 1000 g/ha), *A. pullulans*-DMS 14941-DMS 14940 (Botector; Manica S.p.A.; at 400 g/ha) and *Ba. subtilis* QST 713 (Serenade Max; Bayer Crop protection; at 3000 g/ha). The spray volume varied from 500 to 1000 L/ha, according to the trellis system and size of canopy. Products were applied with backpack spray equipment (Solo 450 in SM; Volpi in ZP; Fox motori in MP), carefully avoiding any drift to neighbouring plots. Untreated control plots were sprayed with water. Treatments were applied at specific stages according to the following programme: (T) *T. atroviride* SC1 at the 'berries beginning to touch' stage (corresponding to BBCH 77), (A) *A. pullulans* at the 'beginning of ripening: berries begin to develop variety-specific colour' (corresponding to BBCH 81) and (B) two treatments of *Ba. subtilis* 20 days and one week before harvesting. In the various years/locations the T, A and B treatments were carried out from 25 June to 10 July, from 27 July to 13 August and from 24 August to 20 September respectively. The combined protocol included all the treatments with each individual biofungicide at the aforementioned stages (TAB). The untreated control (U) was sprayed with water at all the stages specified above.

Symptoms on the berries were assessed one or two days before harvesting, with scoring for 20 (SM, ZP) or 25 (MP) bunches per replicate. Assessment was carried out on 22, 3, 11 and 9 September in 2011, 2012, 2013 and 2014, respectively in SM; on 5, 19 and 10 September in 2011, 2012 and 2013, respectively in ZP; on 27, 25 September and 2 October in 2001, 2012 and 2013, respectively in MP. Disease severity was assessed as the percentage of berries (on each bunch) with grey mould symptoms and disease incidence was calculated as the percentage of bunches having grey mould symptoms. In order to compare results from different locations and years displaying different levels of disease with the untreated control, disease control efficacy (%) was calculated on the disease severity or incidence with the following formula:

$$100 - (S_t, I_t / S_t, I_u \times 100)$$

where S_t, I_t is either the severity or the incidence of the disease with the treatment and S_t,I_u is either the severity or the average incidence in the untreated control. Disease control efficacy was only calculated and used in statistical analysis for locations and years when the severity and incidence on the untreated control was higher than zero.

2.2. Populations of microorganisms on berries

The populations of biocontrol microorganisms on berries were assessed before the first treatment with *Ba. subtilis* (60 and 30 days after treatment with *T. atroviride* and *A. pullulans,* respectively) and at harvest. In SM in 2013 and 2014 the microbial population (*Trichoderma* sp., *Aureobasidium* sp. and *Bacillus* sp. on the *T. atroviride*, *A. pullulans* and *Ba. subtilis* treated plots, respectively and on the untreated plots) was also assessed before and after the treatments and at harvest. For each treatment, 100 berries per replicate were randomly collected from different clusters. Samples were placed in plastic bags and immediately transferred to the lab in cool conditions. Each sample of berries was placed in 230 mL of sterile saline solution (NaCl 0.9%) with the addition of Tween 80 (100 μl/L) in 500 ml-Erlenmeyer flasks (one flask for each replicate). Flasks were shaken for 2 h at 25 °C at 100 rpm with an orbital shaker. A serial dilution was prepared (1:1 to 1:10000) and 100 μL of each dilution were plated on the following two media in 90 mm-diameter-Petri dishes. Potato dextrose agar (PDA; Oxoid; 39 g/L) with the addition of rose bengal (Sigma-Aldrich, 0.1 g/L), chloramphenicol (Sigma-Aldrich, 0.1 g/L) and streptomycin (Sigma-Aldrich, 0.05 g/L) was used to isolate and count *Trichoderma* sp. colonies, while PDA (39 g/L), with the addition of chloramphenicol (0.1 g/L) and streptomycin (0.05 g/L), was used to isolate and count total fungal flora. On the latter medium, *Trichoderma* sp. and *Aureobasidium* sp. were assessed based on colony morphology, followed by morphological identification of fungal structures under a microscope, using random sampling of these colonies, which was carried out to confirm their identity. To enumerate bacteria, Luria-Bertani broth (Sigma-Aldrich; 25 g/L), with the addition of bacteriological agar (Sigma-Aldrich; 8 g/L) and cycloheximide (Sigma-Aldrich; 0.1 g/L), was used. Three biological replicates (Petri dishes) were prepared for each dilution. The colony forming units (CFUs) were counted after 60–72 h of incubation at 25 °C. Average CFUs were calculated for each replicate in each treatment and expressed per cm^2 of berry skin. The volume of each sample of berries was calculated by immersing the berries in water and assessing the increase in final volume occupied by berries. After measuring the average diameter of the berries, the total surface of the berries was then estimated by assuming their shape to be spherical.

2.3. Statistical analysis

Average temperature and precipitation in the different locations and years were compared with the *t*-test. Pearson's test was used to correlate the number of rainy days before harvesting and the

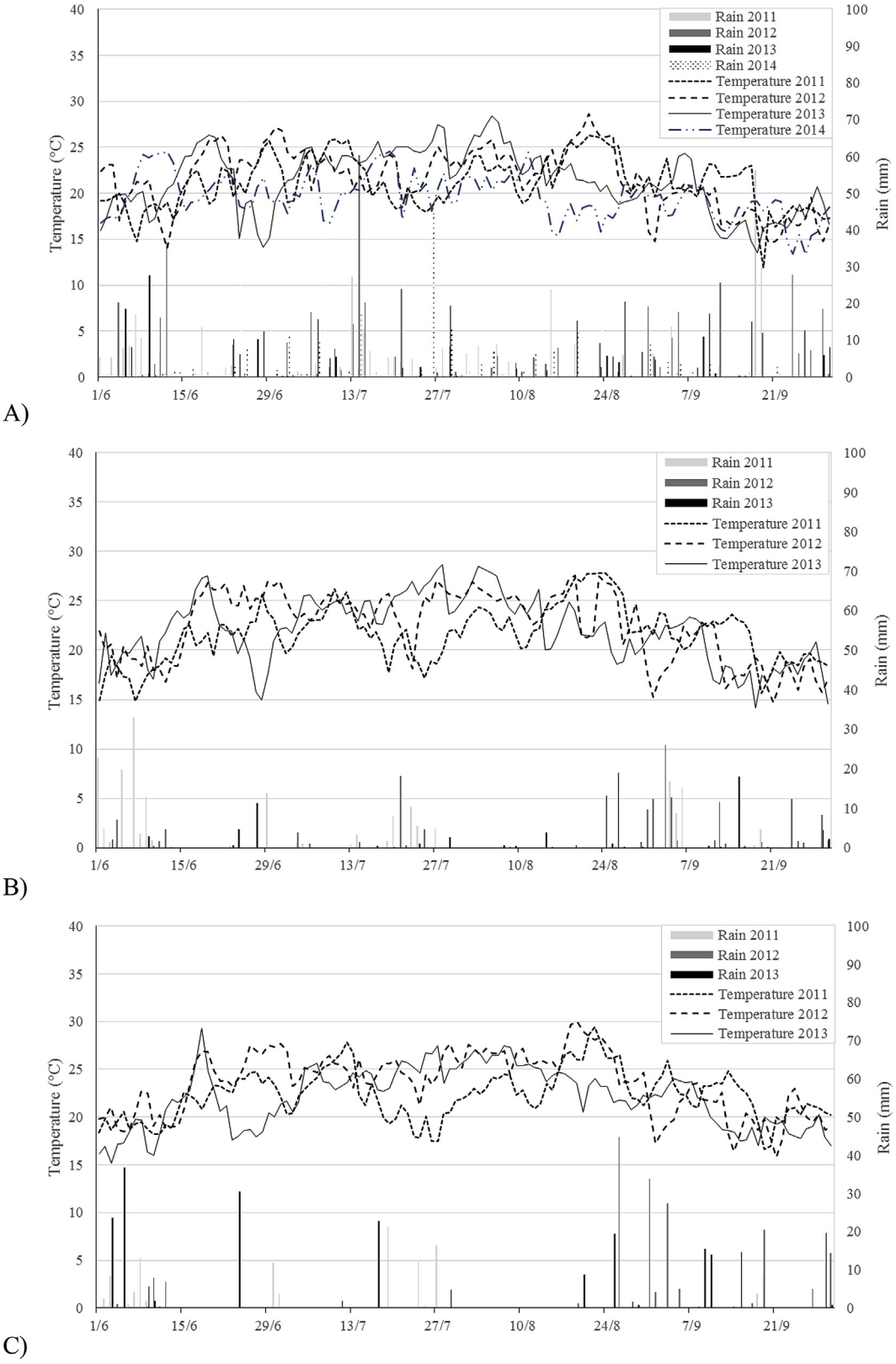

Fig. 1. Mean daily temperature and rain in the three locations, San Michele all'Adige (A), Ziano Piacentino (B) and Montepaldi (C), in the years of experiments from the beginning of June to the end of September.

severity and incidence of disease. Severity, incidence and efficacy data were 'arcsin' transformed. Multifactorial ANOVA was used for comparison of years, locations and treatments. Because significant differences between years and locations were not found, the

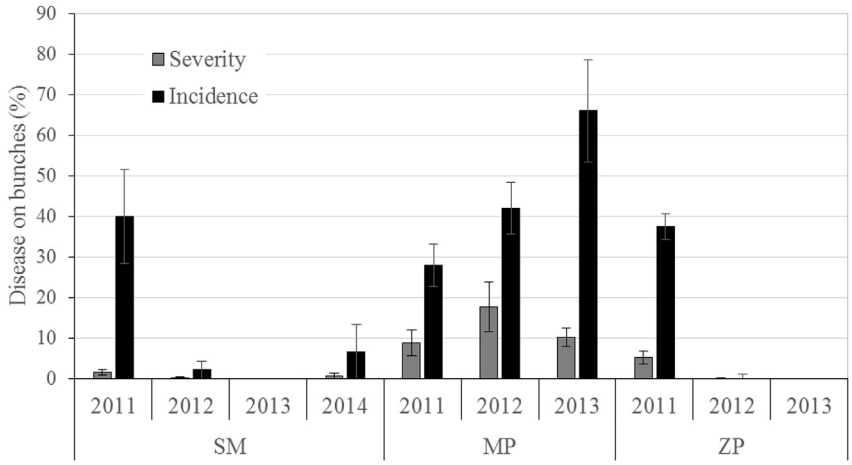

Fig. 2. Mean and standard error (error bars) of severity and incidence of grey mould (*Botrytis cinerea*) on bunches of the untreated control in the three locations, San Michele all'Adige (SM), Ziano Piacentino (ZP) and Montepaldi (MP), in the years of experiments. Assessment of severity and incidence was carried at harvest time. The level of the disease varied among years and location (ANOVA severity: $p = 0.000081$; ANOVA incidence: $p < 0.00001$).

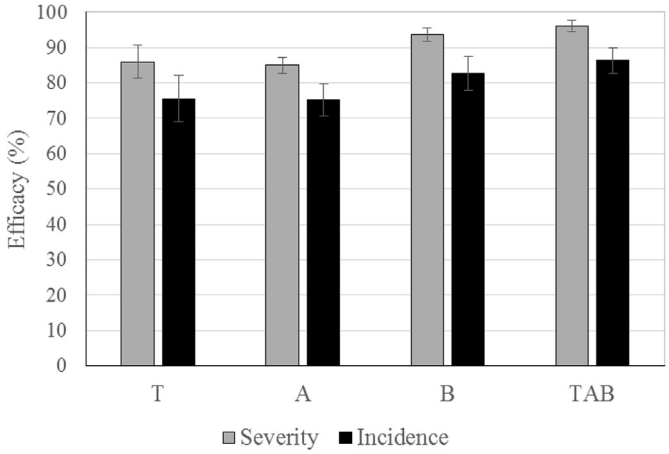

Fig. 3. Mean and standard error (error bars) of the efficacy (calculated on severity and incidence) of biocontrol agents in controlling grey mould (*Botrytis cinerea*) on bunches in the four strategies: T = *Trichoderma atroviride* at the 'berries beginning to touch' stage; A = *Aureobasidium pullulans* at the 'beginning of ripening: berries begin to develop variety-specific colour'; B = *Bacillus subtilis* approximately 20 days and one week before harvesting, TAB = *Trichoderma atroviride* at the 'berries beginning to touch' stage, *Aureobasidium pullulans* at the 'beginning of ripening: berries begin to develop variety-specific colour' and *Bacillus subtilis* approximately 20 days and one week before harvesting. Data from the three locations (San Michele all'Adige, Ziano Piacentino and Montepaldi) and different years (2011-2014) were pooled. Assessment of severity and incidence was carried out at harvest time. The efficacy values, calculated on both severity and incidence, did not differ significantly (ANOVA efficacy on severity: $p = 0.37$; ANOVA efficacy on incidence: $p = 0.27$).

efficacy data were pooled. One-way ANOVA was used for comparison of normally distributed continuous variables with homogeneity of variances. Tukey's HSD post-hoc test was used for comparison between individual treatments when ANOVA was significant. All tests were performed using Statistica 10 software (StatSoft, version 2011; USA).

3. Results

3.1. Efficacy trials

With regard to the growing season (from bunch closure to harvesting), the mean air temperature was 21.7 (2011), 22.8 (2012), 22.6 (2013), 20.1 (2014), 22.8 (2011), 23.3 (2012), 23.5 (2013), 22.7 (2011), 24.2 (2012), 22.8 °C (2013) in SM, ZP and MP, respectively (Fig. 1). Total precipitation varied from 45.2 mm (ZP, 2013) to 416.9 mm (SM, 2014) from bunch closure to harvesting in the different years and locations. The total number of days with rainfall was between 5 (MP, 2011) and 37 (SM, 2014) in the different locations between bunch closure and harvesting. For each location, the level of the disease increased with the increase of the total precipitation (mm) from bunch closure to harvesting.

The severity and incidence of grey mould on bunches of the untreated control (Fig. 2) varied significantly in different years and locations (Fig. 1; ANOVA severity: $p = 0.000081$; ANOVA incidence: $p < 0.00001$). Control efficacy, calculated on the disease severity and incidence of all years, was in general very high with little variability between years and locations (Fig. 3). The efficacy of treatments with single biofungicides at the specific phenological stage was comparable and not different from the combined strategy, with reference to both severity and incidence (ANOVA efficacy calculated on severity; $p = 0.37$; ANOVA efficacy calculated on incidence; $p = 0.27$).

3.2. Populations of microorganisms on berries

Trichoderma sp., *Aureobasidium* sp. and *Bacillus* sp. CFUs were determined based on semi-selective media and colony morphology (Fig. 4). Only a random sample of *Trichoderma* and *Aureobasidium* colonies was identified at species level based on micro morphological traits, therefore the results are reported as *Trichoderma* sp., *Aureobasidium* sp. and *Bacillus* sp. CFUs, assuming that they all belonged to the strains used in the treatments. At harvest time, the presence of *Trichoderma* sp. was detected in all the plots receiving the treatment with *T. atroviride* SC1 (T and TAB) and the concentration on the berry skin did not significantly differ for the two strategies. Only minor *Trichoderma* sp. contamination in plots treated with *A. pullulans* was noticed. *Aureobasidium* sp. was found in all plots, although at variable concentrations. The highest concentrations were found in the treated plots (A and TAB), with no statistically significant differences (Tukey's test at $\alpha = 0.05$) in concentration between the A and TAB strategy, which were, in contrast, higher than those found in T and U.

Bacillus sp. was only found in treated plots (B and TAB), which did not display significant differences (Tukey's test at $\alpha = 0.05$). *Bacillus* sp. was only found occasionally and at biologically

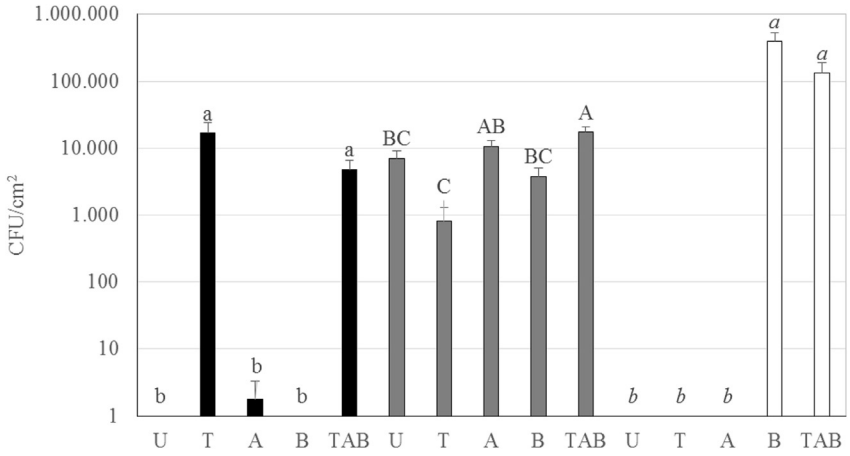

Fig. 4. Mean and standard error (error bars) of colony forming units (CFU/cm^2) of *Trichoderma* sp. (black histogram), *Aureobasidium* sp. (grey histogram) and *Bacillus* sp. (white histogram) on bunches in the four strategies: T = *Trichoderma atroviride* at the 'berries beginning to touch' stage; A = *Aureobasidium pullulans* at the 'beginning of ripening: berries begin to develop variety-specific colour'; B = *Bacillus subtilis* approximately 20 days and one week before harvesting, TAB = *T. atroviride* at the 'berries beginning to touch' stage, *A. pullulans* at the 'beginning of ripening: berries begin to develop variety-specific colour' and *Ba. subtilis* approximately 20 days and one week before harvesting; U = untreated. Data from the three locations (San Michele all'Adige, Ziano Piacentino and Montepaldi) and different years (2011-2014) were pooled. Assessment was carried out at harvest time. Significantly different values within each microorganism are shown with different small, capital and italic letters (ANOVA *Trichoderma* sp.: p = 0.000313; ANOVA *Aureobasidium* sp.: p = 0.000002; ANOVA *Bacillus* sp.: p = 0.000003; Tukey's test at $\alpha = 0.05$).

irrelevant concentrations in some plots of T and U. The presence of each of the three microorganisms in the combined TAB strategy did not differ from their presence in the related single treatments.

The population of the *Trichoderma* sp., *Aureobasidium* sp. and *Bacillus* sp. increased after the treatments and similarly in the single application (T, A or B) and the strategy (TAB) (Fig. 5). *Trichoderma* sp. and *Bacillus* sp. were absent before the treatment, while *Aureobasidium* sp. was already present before the treatments. *Aureobasidium* sp. increased similarly after the treatment in both *A. pullulans* treated (A, TAB) and untreated plots (U). The population of *Bacillus* sp. increased after the first treatment and remained at the same high levels until harvest.

4. Discussion

Grey mould is still one of the main causes of grape losses, in terms of both quantity and quality. On wine grapes, it can result in problematic vinification processes (e.g. need for bentonite fining, light press, pasteurization, etc.) and poor quality wines. Agronomic practices alone cannot always satisfactorily manage the diseases and chemical treatments have to be applied in many grape-growing areas. Eco-friendly solutions such as biofungicides are therefore highly desirable. Although some microbial active ingredients have been registered as biofungicides in Europe, the USA and elsewhere, their use is relatively limited. There are several reasons that can explain their poor uptake by the market, although inconsistency in terms of their efficacy is often claimed to be a strong limiting factor for their implementation in practice. In order to overcome the variable level of efficacy of microbial biofungicides, a combination of biocontrol agents having different modes of action has been attempted on a few crops (Guetsky et al., 2002; Magnin-Robert et al., 2013; Xu et al., 2011), however often with inconsistent or disappointing results. For example, on the strawberry, combined treatments with *Ba. amyloliquefaciens*, *A. pullulans* and *Beauveria bassiana* resulted in improved control of grey mould on fruit, however the effective combinations varied in the trials and single biofungicides were not effective (Sylla et al., 2015). In addition, simultaneous application of incompatible biocontrol agents has sometimes resulted in impaired biocontrol of this disease (Robinson-Boyer et al., 2009; Xu et al., 2011).

The use of strategies involving application of each microbial biofungicide at the stage in which it is supposed to be the most effective has never been attempted on the grapevine to combat grey mould, and thus could offer a way of exploiting the different mechanisms of action of various microorganisms without posing problems in terms of their compatibility when used in tank-mixtures. Therefore, we tested a strategy based on the assumption that *Trichoderma* spp., being a good coloniser of dead plant tissue, may be an excellent biocontrol agent at the bunch closure stage, while *A. pullulans* could be a more suitable biocontrol agent when sugar is increasing in the berries, by competing with *B. cinerea* on cracks or wounds formed as a result of bunch compression. Thanks to rapid direct activity due to antifungal metabolites, treatments with *Bacillus* spp. should be timed preferably close to harvest, when rapid, strong action against *B. cinerea* is needed.

In general, during the period of the trials, the level of disease, both in terms of severity and incidence, was higher in MP than in the other two locations (SM and ZP). On the opposite, the disease was very high in SM and ZP only in 2011. Absence of disease on bunches was noticed in 2013 in SM and ZP. In ZP, the disease was almost absent also in 2012. The different level of disease can be explained mainly by the meteorological conditions in different years and locations before harvesting (Fig. 1). In particular, rain before harvest, specifically on 18 September 2011 in SM and the rain on 4 September 2011 in ZP most probably promoted the development of disease, while in all other years the week before harvest was generally dry. Summer 2013 was, in general, characterised by higher temperatures and a lower quantity of precipitation. In each location, harvesting took place almost at the same time in different years. The high and constant level of the disease in MP can be explained either by the meteorological conditions before harvesting or by the higher susceptibility of the varieties in comparison to those at the other locations.

The most surprising result was that the disease control efficacy of the single biofungicides at the selected stages was always very high and similar to the combined strategy. To explain these results the following two hypotheses can be proposed. The single biocontrol agents applied at the specified stages fully controlled the disease because, in the tested conditions, they survived until

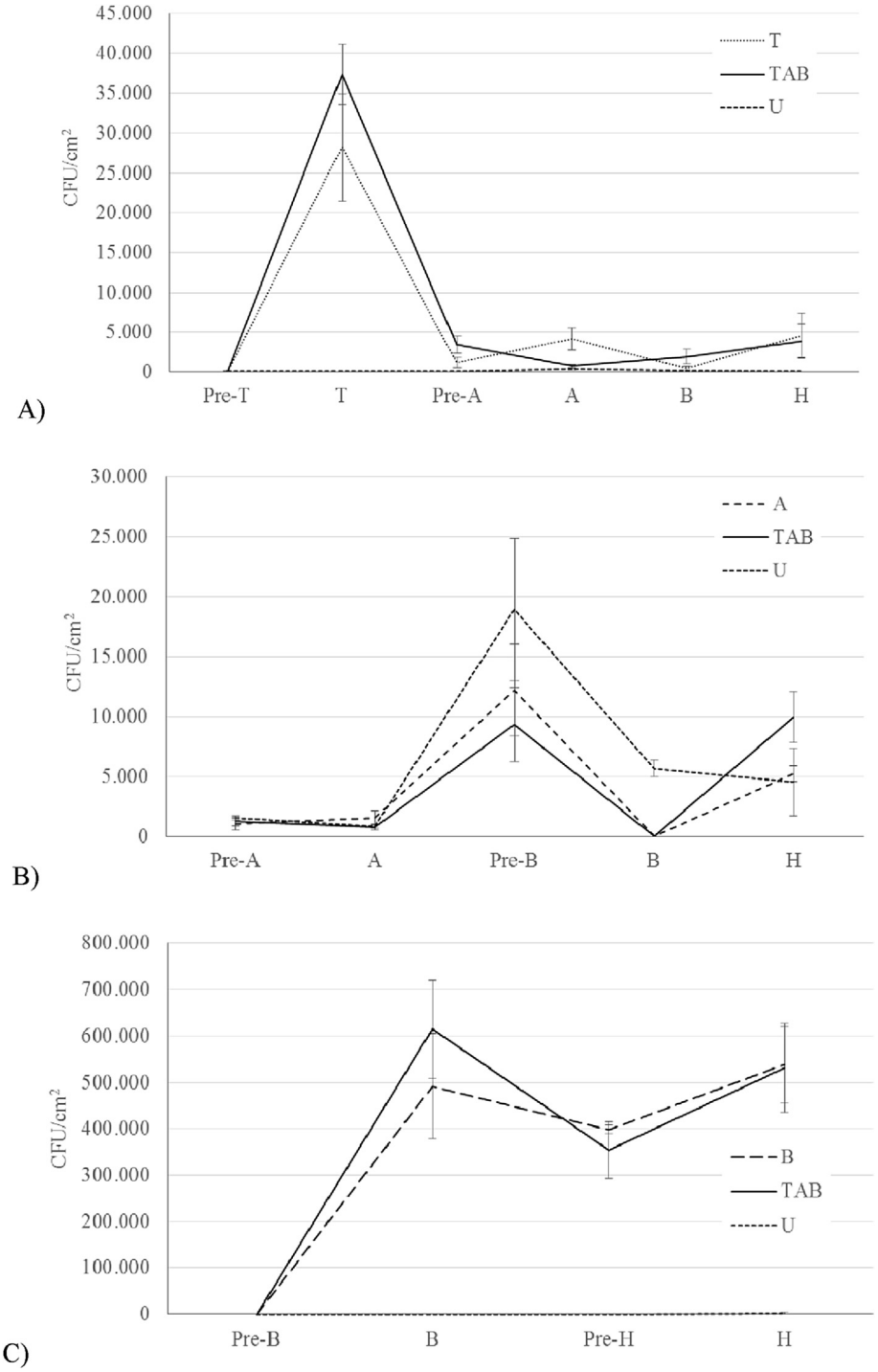

Fig. 5. Mean and standard error (error bars) of colony forming units (CFU/cm^2) of *Trichoderma* sp. (A), *Aureobasidium* sp. (B) and *Bacillus* sp. (C) on bunches in the strategies in San Michele all'Adige: T = *Trichoderma atroviride* treated at the 'berries beginning to touch' stage; A = *Aureobasidium pullulans* at the 'beginning of ripening: berries begin to develop variety-specific colour'; B = *Bacillus subtilis* 20 days and one week before harvesting, TAB = *T. atroviride* at the 'berries beginning to touch' stage, *A. pullulans* at the 'beginning of ripening: berries begin to develop variety-specific colour' and *Ba. subtilis* 20 days and one week before harvesting; U = untreated. Data from 2013 to 2014 were pooled. Assessment was carried out at before (pre-T, pre-A. pre-B), after the treatment (T, A, B) and the harvest time (H).

harvesting at concentrations sufficient to prevent *B. cinerea* infections. Any additional effect provided by the combination of different mechanisms of action in the TAB strategy cannot be highlighted, because of the high level of disease control already achieved by each single biocontrol agent.

The fact that *Trichoderma* sp. was found on all treated plots indicates that *T. atroviride* easily survives on bunches following treatment at bunch closure, probably because it colonises the dead tissues trapped in the bunch and only limited and occasional contamination may appear in untreated bunches. The presence of few colonies in plots that did not receive the treatment may be explained by occasional contamination from the treatments or by the presence of natural *Trichoderma* spp., which can naturally colonise flower residues on the bunch. Because of the methodology

used, this hypothesis cannot be rejected. *Aureobasidium* sp. CFUs were retrieved from almost all berries. This natural contamination and colonisation can be explained by the nature of the microorganism, which can easily spread naturally, or by contamination of natural strains of *Aureobasidium* spp. commonly present on mature berries. *Aureobasidium pullulans* is widespread in the phyllosphere and carposphere of plants and it has also been detected as an endophyte of grapevines (Martini et al., 2009); it is not therefore surprising that it can easily disperse and multiply after its application. *Bacillus* sp. was detected almost exclusively in treated plots, indicating that the natural spread of this microorganism following treatments is relatively limited. The similar level of colonisation of each of the microorganisms in the combined TAB strategy in comparison to the colonisation in the respective strategies receiving only the treatment with each single microorganism, suggests that different niches may be occupied by the three microorganisms when they are applied in the TAB strategy, and that they did not interfere with each other.

The level of disease control obtained with each individual biofungicide applied at the specific stages was in general very high compared to the data reported in the literature. For example, the efficacy obtained with *T. atroviride* SC1 was much higher (86.0 ± 4.5%; average ± SD) than the level obtained by O'neill et al. (1996) with *T. harzianum* T39 (36.3± 2.7%). This difference could be explained by the very good ability of *T. atroviride* SC1 to colonise dead plant tissues (Pellegrini et al., 2014) and/or by the mechanism of action of *T. harzianum* T39, which mainly relies on resistance induction (Perazzolli et al., 2008). Unfortunately, similar field studies regarding *A. pullulans* or *Ba. subtilis* on the grapevine in field conditions are not available in the public literature for a comparison. Although the experiments and strains used in previous published studies are not comparable with the methodology used here, both *A. pullulans* and *Ba. subtilis* used alone provided a very high level of disease control in field conditions compared with previous studies carried out under other conditions. For example, the degree of grape berry infection depended on the *B. cinerea* strains used and the highest control efficacy reached by *A. pullulans* reported in the literature on artificially inoculated berries was 41.5 ± 4.9%. The disease control efficacy obtained in our trials with *Ba. subtilis* was also higher than that obtained by induction of plant resistance in grapes (Magnin-Robert et al., 2013).

The good level of disease control obtained in our trials can be explained by the fact that agronomic practices known to decrease grape susceptibility to disease were applied in all the locations. For example, fertilisation was balanced, the plants were not excessively vigorous, defoliation around the bunches at the 'pea-size berries' stage was applied, there was no powdery mildew damage or *L. botrana* infestation on bunches, etc. All these factors can explain either the relatively low level of disease or the high efficacy of biofungicides. Indeed, it is already known that the combination of agronomic practices and biocontrol agents guarantees greater efficacy of the latter. The high level of efficacy of the biofungicides observed in these trials can also be explained by the relatively low level of disease. Microbial biocontrol agents are indeed known to often fail to control diseases when the latter are at very high levels. On the contrary, in these trials, the efficacy was still quite high also with 40–70% disease incidence.

The timing of the treatments with the individual biofungicides in these trials was based on the following assumptions. The *Trichoderma* species is a good coloniser of dead plant tissues and can be applied before bunch closure in order to colonise flower waste trapped in the bunch, where *B. cinerea* can survive and where the infection can start during ripening (Mundy et al., 2012). *Aureobasidium pullulans* can consume the sugar needed for *B. cinerea* to grow and can colonise wounds, therefore *A. pullulans* should be applied when sugar starts to increase at veraison. *Bacillus* spp. have a more direct effect, mainly due to the activity of the antibiotics and lipopeptides produced, so they can exert their maximal potential when applied before harvesting, when a rapid, direct effect is needed. However, further studies are needed to verify whether the high efficacy obtained in these trials is truly related to the best timing of the respective biofungicides in relation to their mechanisms of action. For instance, further studies may be performed utilising a mechanistic forecasting model in order to better time the application of BCAs, in relation to weather conditions and risk of *B. cinerea* infection.

In conclusion, although the trials were carried out over a relatively short period and in a small number of locations, meaning that generalised application of these results to all vineyards could be over ambitious, the results show that microbial biofungicides represent an alternative to synthetic chemical fungicides against *B. cinerea* on grapes in truly integrated pest management programmes.

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