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Effects of fungicide treatments against Anthracnose in Calabrian (southern Italy) Olive Orchards during 2014-2015

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In recent years, severe natural olive anthracnose infections have been observed in orchards of Southern and Central Italy, favoured by mild and rainy seasonal trends, making disease control with the sole use of copper fungicides difficult. Besides, European restrictions in the use of copper compounds for eco-toxicological aspects have forced researchers towards the use of available plant protection products. Thus, 2 year investigations (2014-2015) was carried out in a Calabrian olive orchard (Southern Italy) to evaluate the efficacy of different commercial fungicides (copper oxychloride, tebuconazole/ trifloxystrobin mixture and tebuconazole/trifloxystrobin mixture plus copper oxychloride) in controlling olive anthracnose on 2 CV. Ottobratica (highly susceptible) and Carolea (susceptible). In 2014, anthracnose incidence on Ottobratica ranged from 2% (April) to 21% (November), while in 2015 it was 15 and 29%, respectively. In both years, CV. Carolea showed a higher disease incidence ranging from 22% (April 2014 and 2015) to 56 and 46% in November, 2014 and 2015, respectively. In both years, 3 applications, in April, June and October, with the mixture of tebuconazole/trifloxystrobin and copper oxychloride were the most effective, significantly reducing anthracnose incidence on Ottobratica (11 and 6%) and Carolea (13 and 11%), respectively in November 2014 and 2015. Moreover, the mixture showed a satisfactory activity on primary and secondary infections. The results also showed that respecting fungicide decay curves, the residue levels in extra-virgin olive oil for all tested pesticides were well below European MRLs.

Key words: *Olea europaea*, *Colletotrichum* spp., leprosy, epidemiology, fungicides, Integrated Pest Management (IPM).

INTRODUCTION

Olive anthracnose (OA) is caused by different species of *Colletotrichum* spp.; fruit rot and mummification are the typical and severe symptoms that are responsible for a heavy decrease in olive yields of up to 80-100% and affecting oil quality (high acidity and peroxide values, reduction of polyphenols and α-tocopherol) (Carvalho et

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al., 2008). Leaves, flowers and immature drupes are susceptible to *Colletotrichum* infections, but the pathogen remains latent on plant symptomless tissues for several months, becoming active when environmental conditions are favourable or when fruit begin to ripen. Infected olive trees also show chlorosis, necrosis of the leaves, defoliation and dieback of twigs and branches. OA occurs in many olive-growing areas around the world including Mediterranean countries (Italy, Spain, Portugal, Greece, Tunisia, Morocco), South Africa, South America (Uruguay, Argentina, Brazil), California, Australia and New Zealand (Cacciola et al., 2012; Talhinhas et al., 2018).

OA infections are variable both in incidence and severity depending on environmental conditions (mild temperatures and high rainfall), cultivar susceptibility and pathogen virulence. Since the first appearance of OA in some Mediterranean Basin countries (Portugal, Greece, Spain, Italy) (Petri, 1930), the pathogen pressure is very different, with epidemic outbreaks occurring in low and sporadic attacks. During the last decade, severe infections of OA occurred in Italy, especially in 2010, 2011, 2014, not only in Calabria and Apulia, regions where the disease became endemic after epidemic outbreaks, but also in Tuscany, Umbria, Latium and Sicily. Climate change and approximate cropping practices appear to have created relevant conditions which favoured the olive fruit fly (*Bactrocera oleae* Gmelin) (Gutierrez et al., 2009; Pautasso et al., 2012); larval throphic activity encourages *Colletotrichum* colonisation contributing to the spread of pathogen conidia. A similar effect is also caused by the olive moth (*Prays oleae* Bern.), which is often underestimated by olive-growers. Copper fungicides, commonly applied during pre-flowering and post-fruit set to control olive leaf spot [*Spilocaea oleagina* (Castagne) Hughes] and also to prevent OA infections, has failed to provide an effective OA control especially in regions where a high pathogen pressure and a mild and rainy seasonal trend favour the disease. Moreover, European restrictions in the use of copper compounds such as negative effects on soil and water and the regulations (128/2009/EC, 1107/2009/EC) on plant protection require a new and systematic approach in eco-sustainable integrated control strategies.

Since 2011, the Italian Ministry of Health has temporarily authorised a single application of pyraclostrobin, between June and August, to control OA epidemics, however, in calabrian olive orchards this treatment resulted as being ineffective in controlling OA (Cacciola et al., 2012; Talhinhas et al., 2018). In the following year, the Italian Ministry of Health, in accordance with the emergency situations in plant protection (article 53 of EC Regulation No 1107/2009), authorized a single application of Flint Max (Tebuconazole + Trifloxystrobin) for a period not exceeding 120 days up to pre-flowering for OA control, and therefore the aim of the present study is to evaluate the effect of different commercial fungicides recently registered for OA control under field conditions in Calabria (Southern Italy).

MATERIALS AND METHODS

Field trial location

The study was carried out during the 2014 and 2015 olive growing seasons. The trials were executed in a olive orchard, located at San Giorgio Morgeto (38° 23' N, 16° 5' E, 550 m a.s.l.), representative of the main commercial olive groves in the province of Reggio Calabria.

Field trial design

The experimental field has a soil texture mainly constituted by sand and siltose clays. The field has olive trees (CV. Ottobratica and Carolea) aged 25 years old which were selected because they have been seriously affected by OA and are spaced on 8 x 5 m grid. The olive orchard is not irrigated, and other integrated management practices are those recommended by the Region of Calabria (Disciplinari produzione integrata-Regione Calabria, 2014 and 2015) for commercial olive growers. The trial was laid out in a randomised-block design with 3 replicates and plot size of about 300 m^2. Each plot was separated from the next one, in the row, and from the adjacent rows by a buffer row of 3-5 olive trees.

The treatments and doses of applied fungicides are listed in Table 1. Treatments were applied with a water volume of 1200-1500 L ha^{-1}, depending on the size of olive trees. Each fungicide treatment was applied three times, during both years, specifically in April, June and October, using the growers' nozzle atomiser equipped with an extended lance to spray into the upper branches of olive plants. Control plots consisted of untreated olive trees. All plants were also treated with imidacloprid/Nuprid® Supreme SC, (Nufarm, Italia s.r.l.) (18.08.2014 and 16.08.2015) as recommended by regional olive management practices against olive fruit fly. Monthly average temperatures and rainfall, recorded during the two experimental years as well as the data series of the period 1916 (1924)-2016 were provided by Arpacal Centro Funzionale Multirischi (http://www.cfd.calabria.it).

Disease monitoring

The efficacy of the treatments on OA was assessed by determining the disease incidence before each fungicide treatment for the three application times in both years and at the end of the trials, 30 days after the last fungicide application to respect the safety interval before the olive harvest. OA incidence was evaluated by humid chamber to promote pathogen sporulation (acervuli). Particularly, at the beginning of each trial, on 5 plants per each replicate, ten twigs (approximately 15-20 cm) with leaves and flowers were randomly collected and then placed in Petri plates (∅150 mm), one twig per plate, containing filter paper moistened with deionised water. All plates were maintained at 20 ± 2°C for 5 days. Each twig was then examined with the aid of a stereomicroscope to observe the presence of acervuli formed on the tissues surface and disease incidence was expressed as a percentage of infected twigs per plant. Before the other 2 application times (June and October) and before the olive harvest (November), 100 drupes were randomly collected from each plant and then placed in Petri plates (20 drupes /plate) as described above. Drupes were then examined to determine pathogen sporulation. Disease incidence was expressed as % of infected fruits. Since fruit susceptibility increases with ripening and rot symptoms become visible, drupe samples collected in October and November, were also used to determine disease

Table 1. Fungicide treatments applied in the field trials during 2014 and 2015.

Treatment	Commercial product and formulation[a]	Active ingredient (%)	Commercial product rate (g hl^{-1})	Application dates[b] 2014	2015
Untreated control	↓-	-↓-	-↓-	-↓-	
Copper oxychloride	Cupravit® Blu 35, WG	35	350	3 April	13 April
Tebuconazole + Trifloxystrobin	Flint Max, WG	50 + 25	20	25 June	15 June
Copper oxychloride and Tebuconazole + Trifloxystrobin mixture	Cupravit® Blu 35 and Flint Max mixture	35 and 50 + 25	350 + 20	1 October	1 October

a = Commercial products are supplied by Bayer
b = Each fungicide treatment was applied at each application dates; for more details see Materials and methods.

severity by scoring OA infections according to a scale from 0 to 3 (0= no fruit rot; 1= 10-25% fruit rot; 2=26-50% fruit rot; 3 over 50% fruit rot).

After 1 month safety interval, drupe samples (about 2.3 kg) from plants of each replicate were randomly collected and transported to the laboratory to obtain extra virgin olive oil. Drupes were ground by an “Olio Mio 50” continous cycle mini-mill (Toscana Enologica Mori, Tavernella Val di Pesa, Florence, Italy). Drupes were crushed in cold and the malaxing was lasted for 20 min. The oil was then centrifuged at 7000 rpm for 3 min by a Sorvall centrifuge and stored in dark glass bottles at 9±2°C. Pesticide residue analyses and estimation on all oil samples were conducted by Centro Analisi Biochimiche s.a.s. (Rizziconi, Reggio Calabria), certified Accredia no.0859.

Statistical analysis

Data regarding disease incidence, expressed as a percentage of infected twigs or infected fruits, were subjected to the analysis of variance (ANOVA) followed by LSD post hoc test to separate the means and to evaluate the effect of treatments. Disease severity was calculated using the McKinney index (McKinney, 1923) by scoring OA infections according to a percentage scale expressing the fruit rot. Data were subjected to the analysis of variance and to the Tuckey test.

RESULTS

Examining the monthly average temperatures and rainfalls recorded during the two experimental years (2014-2015), it is possible to note that temperatures were generally similar to those recorded in the period 1924-2016 (Figure 1). On the contrary, the amount and the distribution of rainfalls were above the average of the years included in the period 1916-2016 (Figure 1). In particular, a variable distribution among months was observed in the years 2014 and 2015 and OA infections were favoured by mild and rainy autumns (October-November).

Indeed, olive anthracnose incidence on untreated control plants of both susceptible cultivars showed a progressive increase throughout the growing season during 2014 and 2015. In particular, in 2014 OA incidence on CV. Ottobratica (high susceptible) ranged from 2% (April) to 21% (November) while in 2015 OA incidence was 15 and 29%, respectively (Figures 2 and 3).

In both years, CV. Carolea (susceptible) showed a higher disease incidence ranging from 22% (April 2014 and 2015) to 56 and 46% in November 2014 and 2015, respectively (Figures 2 and 3). This apparent discrepancy between the two cvs could be explained by the larger size of Carolea drupes compared to Ottobratica fruits, and therefore they are more affected by punctures or exit holes of the olive fruit fly. Larval throphic activity encourages *Colletotrichum* colonisation. Moreover, under favourable environmental conditions the longer harvest period of Carolea, compared to Ottobratica (which takes its name as it is harvested in October), contributes to severe OA infections.

All the treatments applied during April-October generally reduced OA latent and secondary infections. In both years, two applications for each fungicide reduced disease incidence. The effects of these fungicides, after two applications, are shown in Figures 2 and 3. In particular, in October 2014 and 2015, Flint Max (Tebuconazole + Trifloxystrobin) and the mixture Flint Max + Cupravit® Blu 35 (Copper oxychloride) significantly reduced disease incidence on both cvs. In October 2014, for example, disease incidence on Carolea was 15 and 8% on olive plants that received two applications of Flint Max and the Flint Max + Cupravit® Blu 35 mixture, respectively compared with 47% of untreated plants. Flint Max and the mixture Flint Max + Cupravit® Blu 35 resulted particularly effective against OA latent infections while Cupravit® Blu 35 generally showed a modest effect in disease control and resulted in a significant OA incidence reduction only on CV. Carolea in both years.

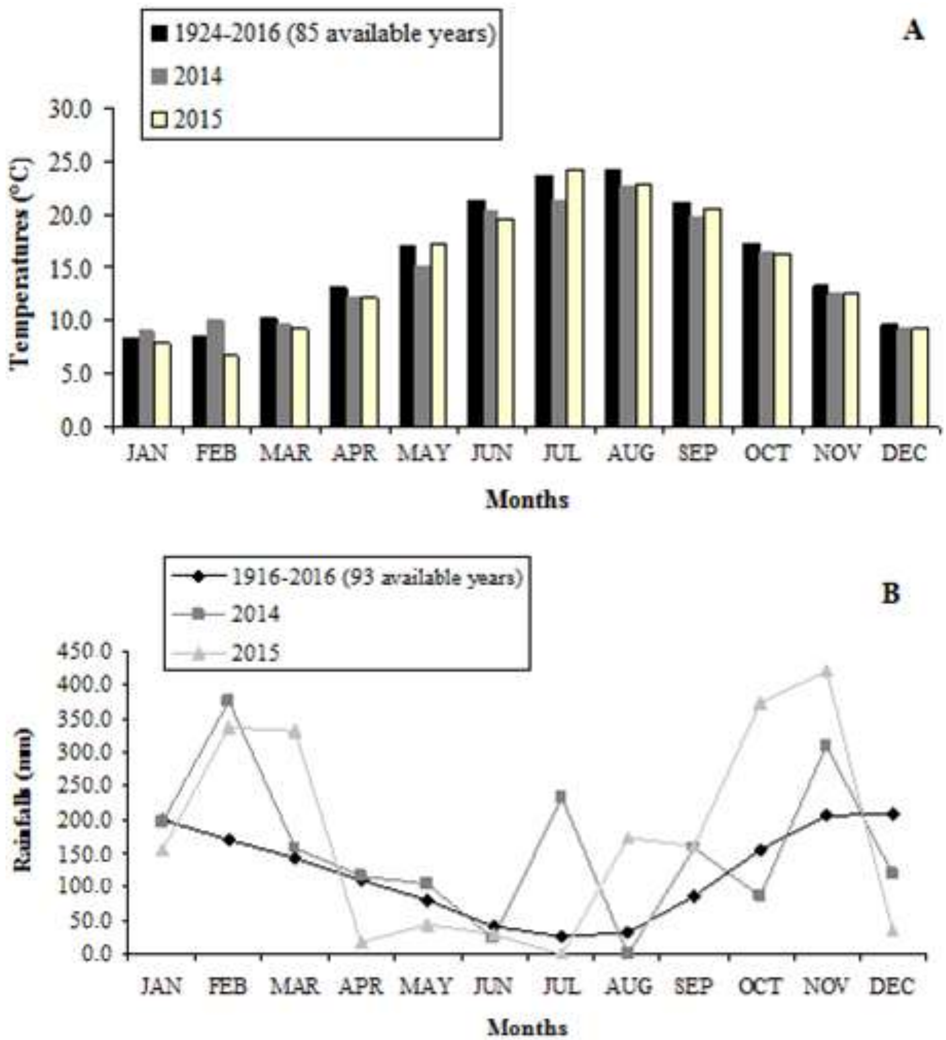


Figure 1. Monthly average temperatures (A) and rainfalls (B) recorded during 2014 and 2015.

In November, after the third application, Flint Max and the mixture Flint Max + Cupravit® Blu 35 resulted to be effective in reducing OA secondary infections compared to the untreated control plants (Figures 2 and 3). Considering all fungicide treatments, the best disease control was observed with the Flint Max + Cupravit® Blu 35 mixture which was effective on both cultivars and in both years.

The performance of each fungicide is also confirmed by the average OA severity observed during October-November in 2014 and 2015 (Figure 4). In particular, the application of the Flint Max + Cupravit® Blu 35 mixture showed a statistically significant higher efficacy compared to the untreated control for both cultivars. By the end of the field experimental trials (November 2014 and 2015), this fungicide treatment showed the lowest OA severity of 22 and 6%, respectively for CV. Ottobratica and of 17% for CV. Carolea compared with Ottobratica (42 and 23%) and Carolea (70 and 50%) untreated controls, respectively (Figure 4). A satisfactory disease control was observed with Flint Max. Modest results were achieved with Cupravit®Blu 35 which, however, resulted in a significant reduction in OA severity on CV. Carolea in both years, but disease control on CV. Ottobratica was unsatisfactory (Figure 4).

The results of fungicide residue analyses on all oil samples are shown in Table 2. Residue levels of the applied systemic fungicides (tebuconazole and trifloxystrobin) and the insecticide (imidacloprid) were determined by a multi-residue pesticide analysis. The average recovery of fungicides was determined at value of uncertainty k = 2 and confidence level 95%. The residue levels for all tested pesticides were well below European MRLs (Table 2). On the basis of the three recommended copper treatments to prevent olive leaf spot (*Spilocaea oleagina*), useful for their collateral action

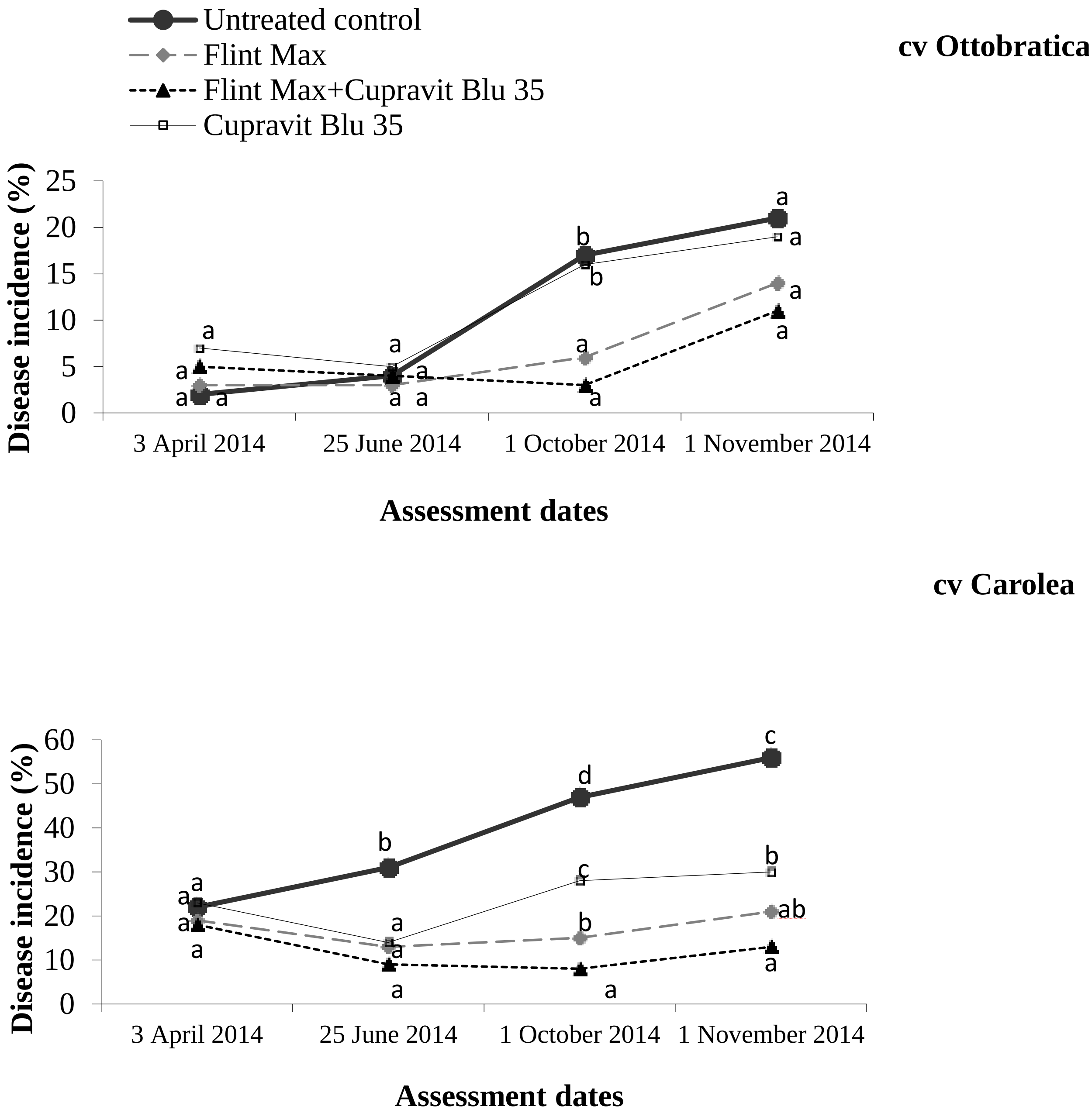


Figure 2. Effect of fungicide treatments on cvs Ottobratica and Carolea applied during April-October 2014 on olive anthracnose incidence. Within each assessment date, the values of incidence average with the same letter do not differ statistically from each other (P < 0.05).

against OA infections, in this study the residue level of copper was not determined as it was assumed that the copper MRL on olives was below European MRL (30 mg kg^{-1}, according to the European Regulation No 396/2005). Results of pesticide residue monitoring for olive and olive oil, show that the metal concentration was always found at a value lower than the MRL. Copper is concentrated in the olive water fraction and it is easily eliminated with the vegetation waters by centrifugation (Simeone et al., 2009).

DISCUSSION

The results obtained during the two-year investigations performed in Calabria on olive anthracnose and its chemical control confirmed the seriousness of this olive disease. Under natural conditions, OA incidence on untreated trees of both susceptible cultivars (Ottobratica and Carolea) increased during the growing season reaching highest incidence (56%) and severity (70%) on Carolea in November 2014. Weather conditions favoured

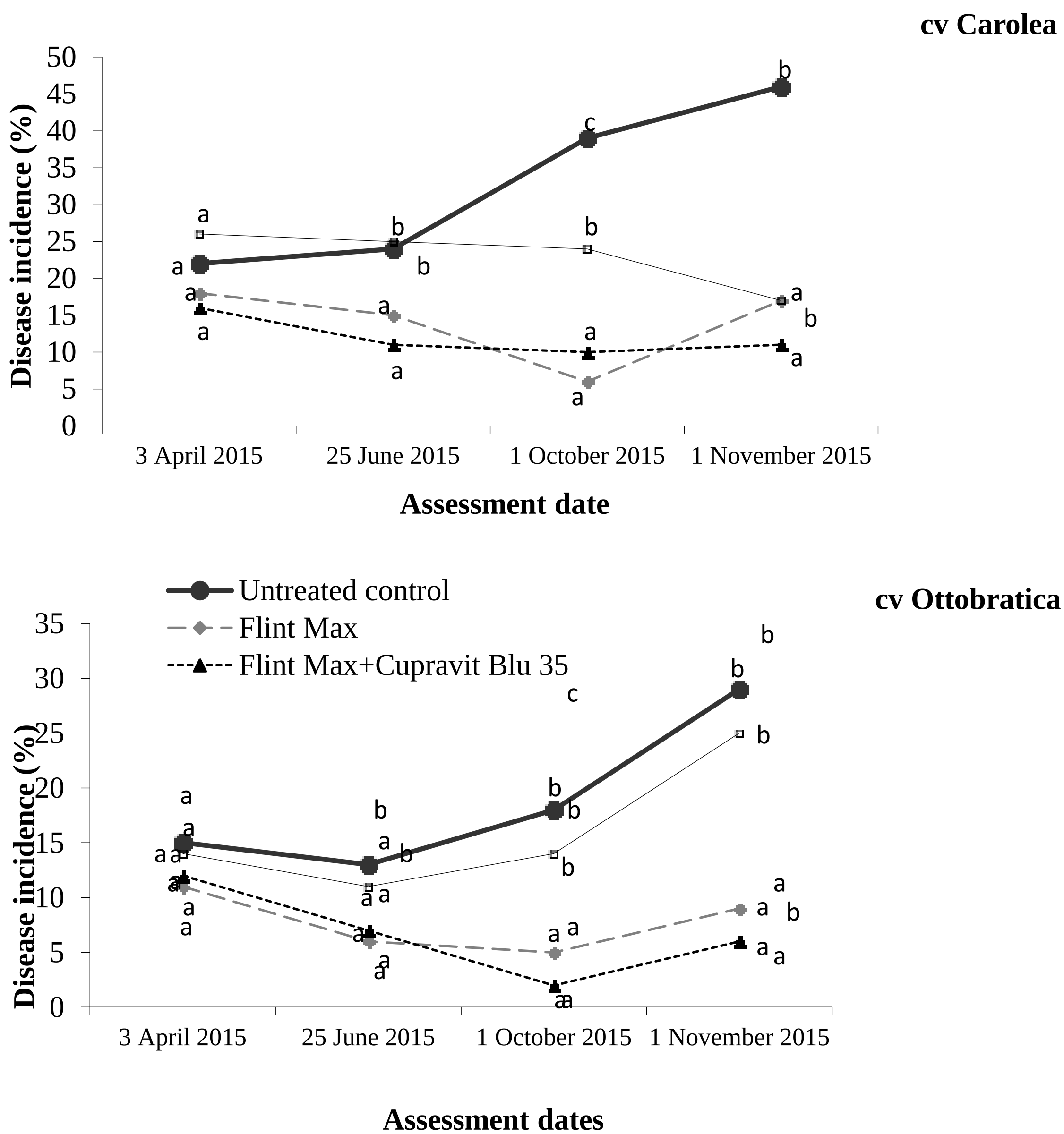
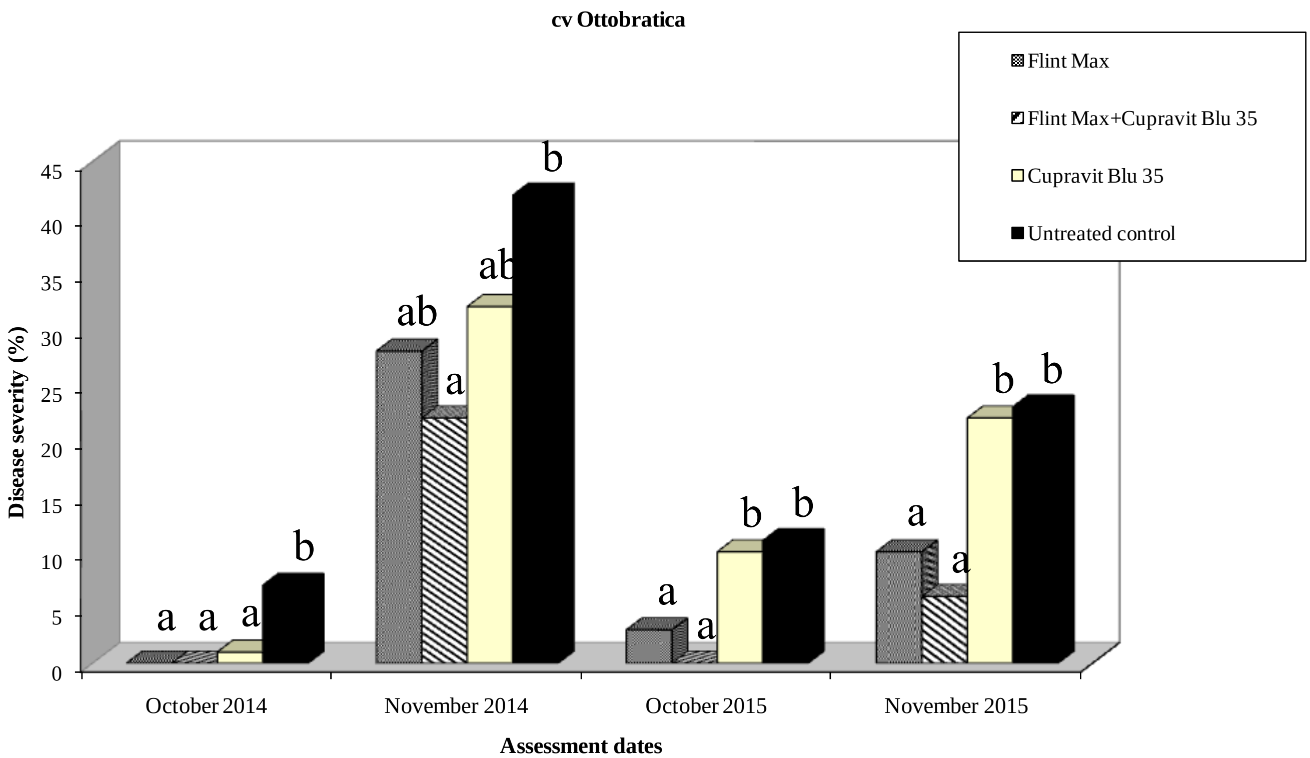


Figure 3. Effect of fungicide treatments on cvs Ottobratica and Carolea applied during April-October 2015 on olive anthracnose incidence. Within each assessment date, the values of incidence average with the same letter do not differ statistically from each other (P < 0.05).

the rapid increase of OA incidence, shaped disease severity among cultivars and also the virulence variability existing among *Colletotrichum* species populations (Talhinhas et al., 2009, 2015). Therefore, as a consequence of future changes in climate and also in current annual variability of weather conditions, chemical control is necessary to prevent serious OA epidemics.

Our results show that Flint Max (Tebuconazole + Trifloxystrobin) and the mixture Flint Max + Cupravit® Blu 35 (Copper oxychloride) were the most effective fungicides in reducing latent and secondary infections when sprayed in three applications (April, June and October). Moreover, the fungicide residue levels of the commercial products applied in accordance with the label recommendations were well below European MRLs. Furthermore, application of the Flint Max + Cupravit® Blu 35 mixture resulted as being very active in controlling the pathogen and reducing OA severity in both cultivars.

In consideration of eco-sustainable integrated protection strategies, the Italian Ministry of Health should authorise up to three application of the Flint Max only when weather conditions are very conducive for OA infections, depending on the cultivar susceptibility and the history of the disease in the previous years, to reduce epidemics in



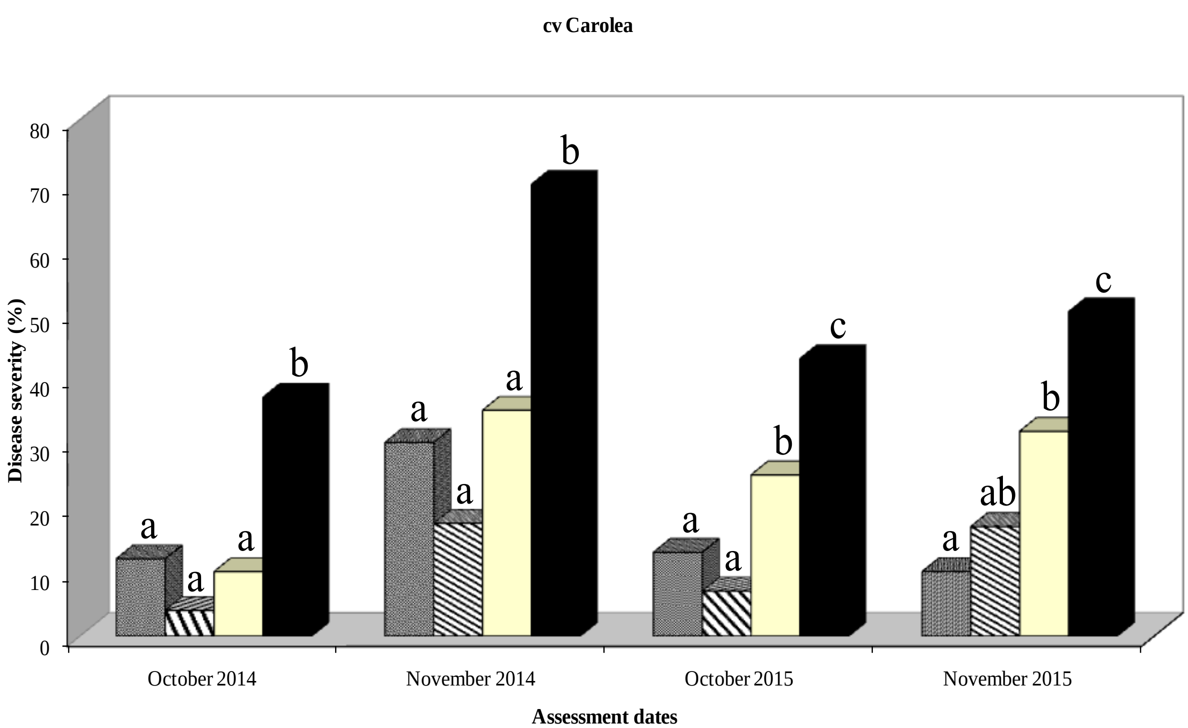


Figure 4. Olive anthracnose severity observed on cvs Ottobratica and Carolea sprayed with different fungicides during October-November in 2014 and 2015. Within each assessment date, columns with different letter are significantly different at (P <0.05).

accordance with the principles of good agricultural practice. Nevertheless, OA chemical control should necessarily be combined with cultural management such as balanced fertilization and pruning to remove infected

Table 2. Average residue levels of applied systemic pesticides recovered in olive oil samples collected in the field trials in November 2014 and 2015.

Commercial product	Active ingredient[a]	November 2014		November 2015		MLR[b] (mg kg^{-1})
		Ottobratica	Carolea	Ottobratica	Carolea	
Flint Max	Tebuconazole	0.061 ± 0.065*	0.058 ± 0.013	0.107 ± 0.024	0.119 ± 0.026	0.25[c]
	Trifloxystrobin	0.064 ± 0.014	0.014 ± 0.003	0.023 ± 0.006	0.020 ± 0.006	0.3
Nuprid	Imidacloprid	0.014 ± 0.003	0.014 ± 0.003	0.022 ± 0.006	0.012 ± 0.004	1.0
Flint Max + Cupravit® Blu 35	Tebuconazole	0.029 ± 0.006	0.060 ± 0.013	0.081 ± 0.019	0.157 ± 0.033	0.25[c]
	Trifloxystrobin	0.058 ± 0.002	0.029 ± 0.006	0.031 ± 0.008	0.052 ± 0.013	0.3
Nuprid	Imidacloprid	0.008 ± 0.002	0.022 ± 0.005	0.012 ± 0.004	0.014 ± 0.004	1.0

[a]LOQ max (mg kg^{-1}) for 0.01;
*concentration expressed as mg kg^{-1} ± standard deviation;
[b]MLRs according to the European Union legislation framework Regulation (EC) No 491/2014 for imidacloprid and trifloxystrobin;
[c]for tebuconazole, the MLR value of 0.05 [Regulation (EC) No 61/2014] must be rectify applying a correction factor for the pro-cessed products, as reported in Regulation (EC) No 400/2014 [*Virgin olive* oil (unless a specific oil processing factor is available, oil pro-cessing factor =5, taking into account an olive oil production standard yield of 20% of the olive harvest. Member States are requested to report the pro-cessing factors used in the 'National Summary report].

twigs and mummified fruits, to reduce potential source of infection, and to improve air movement in the canopy.

In organic olive orchards, where copper is the only preventive fungicide to control olive leaf spot and anthracnose, the adoption of efficient and reliable control measures becomes more strict and the cupric ion, due to its antibacterial activity, is also effective against olive knot (*Pseudomonas savastanoi* pv. *savastanoi*) and olive fruit fly because its repellent and anti-oviposition effects interrupt the bacterium-fly symbiosis and so jeopardize nutrition of the larval stages (Sacchetti et al., 2008; Caleca et al., 2010).

The uncertain future of copper-based fungicides, due to reduction of soil microbial biomass and potential influence on physical and chemical processes, and the resulting European regulatory restrictions make it necessary to develop effective alternatives such as selected biological agents, antifungal compounds, plant and compost extracts inducing plant resistance. Recently, the biocontrol potency of olive epiphytic and endophytic fungal communities was evaluated *in vitro* tests against *Colletotrichum acutatum* J.H. Simmonds (Preto et al., 2017) and two treatments with a pomegranate (*Punica granatum* L.) peel extract were very effective in reducing OA infections and bacterial populations, when applied under natural conductive conditions of Southern Italy (Pangallo et al., 2017). Moreover, further researches developping more eco-friendly anthracnose control alternatives are needed to replace synthetic and copper fungicides, contribute to a successful integrated and biological olive disease management systems and improve olive productions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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