Phytopathologia Mediterranea (2012) 51, 2, 276–282

Research Papers

A natural product for the control of olive leaf spot caused by *Fusicladium oleagineum* (Cast.) Ritschel & Braun

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Summary. This study examined the effects of a liquid formulation (Formulation A) on olive leaf spot (OLS), caused by *Fusicladium oleagineum*. In the laboratory test the formulation, consisting in a dispersion of Brassicaceae meal in vegetable oil, was tested in comparison to vegetable oil alone and to a chemical fungicide (dodine). Vegetable oil, Formulation A, and dodine reduced conidium germination (47, 20 and 23% respectively), compared with untreated experimental controls (56%). Field trial results confirmed the efficacy of the formulation and dodine with respect to the untreated controls. Two applications of formulation in spring plus three additional sprays in autumn maintained OLS incidence below the harmful threshold, especially if applied on cultivars showing medium to low susceptibility to the disease.

Key words: *Brassica carinata*, environmentally-friendly products, glucosinolates, vegetable oil.

Introduction

Olive leaf spot (OLS, also "peacock spot") is considered to be one of the most important olive diseases. It is caused by *Fusicladium oleagineum* (=*Spilocaea oleagina*), a specific biotrophic pathogen with subcuticular development manifesting as circular lesions on the leaves of olive tree (Gonzales-Lamonthe *et al*., 2002). *Fusicladium oleagineum* infections lead to leaf abscission and weakening of whole plants. Moreover, on olive fruit, the infections can cause unacceptable blemishes, especially on table olives. Peacock spot is common worldwide and causes yield losses in olives estimated to be as high as 20% (Wilson and Miller, 1949). In Italy the fungus is endemic, but unusual climatic conditions of the last few years, characterized by moist weather and temperate winters, have caused widespread disease in some olive growing regions. Chemical fungicides (dodine) applied before the onset of the main infection periods (spring and autumn) represent the principal method used to control OLS (Prota, 1995).

The continuous use of chemical fungicides to control plant diseases has had considerable environmental impact, and has resulted in the onset of resistance phenomena within some populations of fungal pathogens. This situation has led to an increased demand for environmentally-friendly products in order to reduce the effects of widespread fungicide utilization in crop protection (Coats *et al*., 2003). The use of natural products together with chemical fungicides at low dosage in the framework of integrated pest management programs could achieve the aims of reducing costs and limiting the environmental impact of crops. Several studies using natural products have demonstrated the possibility of their use to control pests and diseases.

Rue extract (from *Ruta graveolens* L.) and sodium bicarbonate significantly reduced tobacco powdery mildew (*Erysiphe orontii*). In greenhouse trials, rue extract gave good disease control (up to 90%) at a concentration of 100 g hL^{-1} (Lahoz *et al*., 2001). Emulsi-

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ISSN (print): 0031-9465
ISSN (online): 1593-2095
www.fupress.com/pm

fied oils were effective against powdery mildew and other epiphytic fungi (Hagiladi and Ziv, 1986), while Martin *et al*. (2005) showed that mineral, vegetable and fish oils reduced severity of grapevine powdery mildew. Northover and Schneider (1993) suggested that oil treatments were more effective if applied as curative rather than preventive products against grapevine powdery mildew. Natural oil-based formulations could represent good alternatives to chemical fungicides (Hagiladi and Ziv 1986; Horst *et al*., 1992; Northover and Schneider, 1993; 1996; Henriques *et al*., 1998; Osnaya and Schloser, 1998; Wicks *et al*., 1999). These formulations are effective in controlling some plant pathogens at low doses and induce little or no resistance in target fungi (Martin *et al*., 2005). Furthermore, they show excellent spreading and leaf surface adhesion characteristics, have low toxicity for human beings due to rapid biodegradation, and have limited environmental impact. The effects of a natural fungicide for the control of *Erysiphe betae* and *Erysiphe cichoracearum* were investigated by Rongai *et al*., (2009); laboratory and field trials demonstrated good control of sugar beet and cucumber powdery mildews.

In the present study, the effects of a natural formulation on OLS were investigated. The formulation (Rongai *et al*., 2006), used at the dose of 2% emulsion in water, was obtained from vegetable oils of *Brassica carinata* added to meal obtained from the same species and Arabic gum. The meal contains glycosidic compounds whose enzymatic hydrolysis degradation products (isothiocyanates and nitriles) are well-known for their high cytotoxic activity (Lazzeri *et al*., 2004; Marciano *et al*., 2004). The experiments reported here were carried out in 2009 and 2010 under laboratory and field conditions. The results were compared to those obtained using a vegetable oil alone and a chemical fungicide (dodine).

Materials and methods

Formulation and fungicide used

Formulation A (vegetable oil of *Brassica carinata* + arabic gum at 10% of oil + 2 g L^{-1} of *B. carinata* meal), a chemical fungicide (dodine, Venturex 35L, Agriphar s.a., Italy), and *B. carinata* vegetable oil alone were tested in the laboratory trials. Formulation A was applied at a dose of 2% emulsions in water while dodine was used at the recommended label dose. Vegetable oil alone was excluded in field experiments since it showed low activity in laboratory trials compared with the formulation.

Laboratory trials on conidium germination, appressorium formation and infection hyphae

To obtain a large number of *F. oleagineum* conidia, leaves with characteristic OLS lesions, from naturally infected olive trees, were submerged in water and shaken for 2 h at 80 rpm at room temperature (Benitez *et al*., 2005). The resulting conidium suspension was filtered through a double layer of cheesecloth to remove leaf debris and then centrifuged at 2500 g for 3 min. Conidia were than counted and 10 μL of suspension at a concentration of 5×10^4 conidia mL^{-1} were used to inoculate the upper leaf surface of 12 detached olive leaves for treatments (three or four replicates) (Obanor *et al*., 2008). After 2 h, 10–15 mL of product (formulation, vegetable oil, dodine and water) were sprayed onto 12 leaves for each treatment, using a hand pump dispenser. The leaves were then incubated at 20°C, RH 100% in the dark for 48–96h. The leaves were cut into 0.75 × 0.75 cm pieces such that each contained the site of the conidium droplet inoculations, and cleared in a 1:1 solution of acetic acid and ethanol (95%) for 48h (Saad and Masri, 1978). A fuchsine solution (lactic acid 25%, fuchsin 0.01%, glycerol 50%) was applied to the upper surface of the leaf pieces, which were then each enclosed with a cover slide and examined using an optical microscope at 250× magnification to determine conidia germination, appressorium formation and infection hyphae (Benitez *et al*., 2005). About 270 conidia for four replicates were observed. A conidium was considered germinated if the length of the germ tube exceeded half the length of the conidium. The infection hyphae were counted on 1 mm of leaf surface.

Field experiments

The natural fungicide was tested in an olive grove naturally infested by the OLS located in the hills near Pescara (Italy) (Lat. 42° 31′ 15″; Long. 14° 07′ 00″). The trials were carried out with the *Olea europea* cultivars "Gentile di Chieti" (resistant to *F. oleagineum*) and "Nocellara del Belice" (susceptible) (Iannotta and Monardo, 2004). The resistant cultivar was used both to increase the number of replications and to obtain additional information from the trials. The

trees were 20 years old with diameters of approx. 20 cm. A randomized block experimental design was used with four blocks and three treatments. A total of eight plants for each treatment (two plants for four replicates) were sprayed (using a motor pump at 20 atmospheres) with formulation, dodine or water (untreated control).

The plants were sprayed (80 L per treatment) five times (7 April, 6 May, 24 July, 20 August and 20 October 2009) using formulation and twice (29 April and 20 October 2009) with dodine. Chemical fungicide was applied following integrated pest management protocols (spring and autumn) suggested for the Abruzzo region. On the other hand, the formulation, whose efficacy is linked to a permanent overall cover of leaf surfaces, was also sprayed in the summer period, because it is easily washed off by rainfall. Two hundred olive leaves per treatment were examined (50 leaves for four replicates). Leaf samples were detached at random from the mid section or lower half of the plants on 18 March (before treatment applications) and on 18 May, 18 June, 22 September, 29 December 2009; leaves from the shoots of the year were collected. Leaf samples were also detached on 22 February 2010.

The OLS infection was estimated visually, determining the percentage of infected leaves by an early diagnosis method (Loprieno and Cenerini, 1959). The percentage of infected leaf surface was also evaluated using a scale from 1 to 5. The values were assigned on the basis of the following proportion of leaf surface area affected: 1 = 1–20%; 2 = 21–40%; 3 = 41–60%; 4 = 61–80% and 5>80%. Disease level was calculated by the sum of these incidence and severity assessments.

Two separate trials were carried out on two cultivars: "Gentile di Chieti" (resistant to *F. oleagineum*) and "Nocellara del Belice" (susceptible).

Statistical analyses

Statistical analyses of the data collected were performed by ANOVA, testing for statistical significance of trial and trial x treatment interactions. The data were arcsine-transformed before the analysis. Mean values were compared by Fisher's protected Least Significant Difference (LSD) test at $P \leq 0.05$. SigmaPlot version SPW10 was used to create graphics.

Results

Fusicladium oleagineum conidia germination, appressorium formation and infection hyphae

The mean percentages of germinated conidia, appressorium formation and infection hyphae are reported in Table 1. The mean percentage of germinated conidia was significantly different for untreated and treated leaves. In Formulation A and dodine treatments, 20 and 23% of conidia were germinated, values significantly lower than with vegetable oil (47%) and untreated control (56%) (Table 1). Appressorium formation is an essential prerequisite for the infection process. Only 16 and 13% appressorium formations were observed for formulation and dodine against 26 and 37% for vegetable oil and untreated control. Ninety-six h after treatment, 14 in-

Table 1. Mean proportions of germinated conidia and appressorium formation for *F. oleagineum* 48 h after different treatments had been applied, and mean number of infection hyphae on 1 mm^2 of leaf 96 h after treatment. The data were arcsine-transformed before the analysis. Values with different letters are statistically different (P<0.05) as determined by Fisher's protected least significant difference (LSD).

Treatment	48 h after treatment		96 h after treatment
	Germinated conidia (%)	Appressorium formation (%)	Infection hyphae (No. of infection hyaphae mm^{-2})
Untreated	56 a	37 a	14 a
Vegetable oil	47 b	26 b	9 ab
Formulation A	20 c	16 c	5 b
Dodine	23 c	13 c	3 b

fection hyphae per mm^2 of leaf surface were counted in the untreated control, versus five and three in the formulation and dodine treatments respectively. There were nine infection hyphae in vegetable oil, a figure not significantly different from the others (Table 1). The ANOVA of trial and trial × treatment interactions showed no significant effects.

Field efficacy of Formulation A against *Fusicladium oleagineum*

Formulation A and dodine significantly decreased the mean percentage of infected olive leaf tissue in comparison with the untreated control (Figures 1 and 2). The percentage of infected leaf tissues in the resistant cultivar (Gentile di Chieti) was always very low, except for untreated control, where on 29 December 2009 and on 22 February 2010 the percentage of leaf tissues with *F. oleagineum* infection was about 10% (Figure 1).

Different results were obtained for the more susceptible cultivar Nocellara del Belice. The mean percentage of tissues with *F. oleagineum* increased from 18 June to 22 September 2009. In the untreated control, a mean of 45.4% of tissue was infected, while for the dodine and formulation treatments the mean leaf area with *F. oleagineum* spots was 29.6 and 11.6% respectively (Figure 2). Three months later, the level of infection decreased slightly in the untreated control and dodine treatment, while it remained constant in formulation. From 18 June onward, the plants treated with formulation showed lower disease severity than untreated control and dodine treatments.

In the cultivar Gentile di Chieti (Table 2), the proportion of infected leaves in the treated plants was always less than 10%. In the untreated control, the fungus activity, while lower in the summer, increased in autumn when proportions of infected leaves reached 41% at the end of December, confirmed in the sampling on February 22.

The cultivar Nocellara del Belice (susceptible) (Table 3) showed high infestation on old leaves (18 May) in all theses while on subsequent observation (18 June), carried out by sampling leaves taken from the new shoots of the year, the infestation decreased below 20%. At the end of summer (22 September) the mean infestation was 90% for the untreated control and 75% for the dodine treatment, while in the plants treated with formulation the proportion was 40%. At 29 December the disease level slightly decreased in the untreated control and dodine treatments, to 78 and 65% respectively, while for the Formulation A treatment 51% of the leaves were infected, which was a statistically significant reduction.

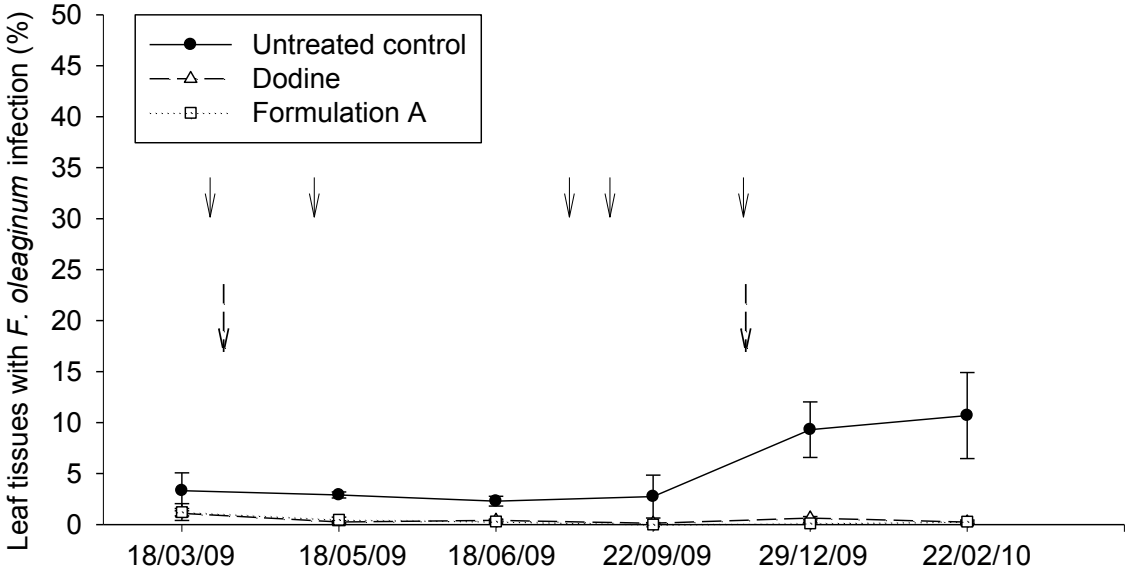

Figure 1. Mean proportions of olive leaf tissues with *F. oleagineum* infection in the cultivar Gentile di Chieti. Treatments with Formulation A ↓ and dodine ↓ are indicated.

No phytotoxic effects were observed on olive trees treated with the Formulation A.

Discussion

In laboratory tests, the values measured showed the efficacy of Formulation A and dodine in reducing *F. oleagineum* activity. Both treatments reduced percentage of germinated conidia, and reduced formation of appressoria and infection hyphae compared with the untreated control and vegetable oil (Table 1). In the untreated control, the values of germinated conidia and appressorium formation were similar to those reported by Saad and Masri, (1978) and Obanor *et al*. (2008). In these studies, after 48h and at 20°C, the authors measured about 60% of germinated conidia and 35% appressorium formation, values comparable to those obtained in the present study. The relationship between germinated conidia and infection hyphae in the Formulation A and dodine treatments id noteworthy. For the Formulation A treatment, about 25% of germinated conidia produced infection hyphae, while in dodine treatment this proportion was 13%. One explanation might be that the chemical fungicide, being more persistent, was also more effective than Formulation A in inhibiting the infection hyphae after 96 hours (Table 1).

Regarding the field experiments, the results obtained confirmed the different behaviour of the cultivars tested with regard to relative susceptibility to the fungus, as reported previously by Iannotta and Monardo (2004). The untreated control for the resistant cultivar showed a low infection level until 22 September; at the same sampling the susceptible cultivar had almost all foliage infected. The spread of disease was also quite different in the two cultivars, since in Gentile di Chieti leaf spots were fewer and smaller than in the susceptible cultivar. The climatic conditions of the autumn season led to an increase of the proportion of infected leaves in Gentile di Chieti but, on the contrary, Nocellara del Belice showed a slight decrease. One possible explanation could be that, in the susceptible cultivar, leaf spots easily expand and coalesce to cover large proportions of leaf area, causing premature leaf-fall, and leaf-fall probably led to a decrease of infected leaves detached during the sampling on 29 December 2009. For the same reason, despite the still large number of infected leaves (Table 2), the severity of disease was less because of the small leaf surface that was damaged (Figure 1). In the plants treated with Formulation A, on the other hand, both the percentage of infected leaves and leaf tissues increased probably due to the fact that leaf-fall did not take place.

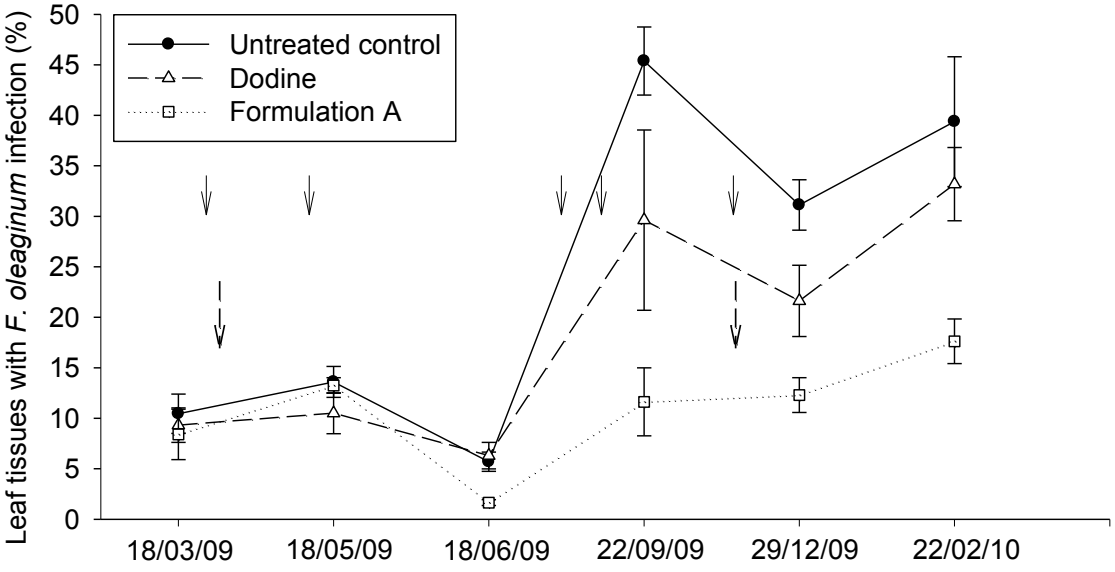

Figure 2. Mean proportions of olive leaf tissues with *F. oleagineum* infection in the cultivar Nocellara del Belice. Treatments with Formulation A ↓ and dodine ↓ are indicated.

Table 2. Mean proportions of *F. oleagineum* infected leaves of the resistant olive cultivar Gentile di Chieti (G.C.). The data were arcsine-transformed before the analysis. Values with different letters are statistically different ($P<0.05$) as determined by Fisher's protected least significant difference (LSD).

Treatment	Percentage of infected leaves					
	Pre-treatment	Post-treatment				
	18/03/2009	18/05/2009	18/06/2009*	22/09/2009*	29/12/2009*	22/02/2010
Untreated	13 a	16 a	15 a	10 a	41 a	47 a
Dodine	10 a	5 b	5 b	3 a	10 b	5 b
Formulation A	8 a	7 b	4 b	0 a	3 b	10 b

* Leaves of the year

Table 3. Mean proportions of *F. oleagineum* infected leaves of the susceptible olive cultivar Nocellara del Belice (N.B.). The data were arcsine-transformed before the statistical analysis. Values with different letters are different ($P<0.05$), as determined by Fisher's protected least significant difference (LSD).

Treatment	Percentage of infected leaves					
	Pre-treatment	Post-treatment				
	18/03/2009	18/05/2009	18/06/2009*	22/09/2009*	29/12/2009*	22/02/2010
Untreated	36 a	64 a	16 a	90 a	78 a	88 a
Dodine	33 a	54 a	19 a	75 ab	65 b	77 ab
Formulation A	28 a	52 a	8 b	40 b	51 c	64 b

* Leaves of the year

Data observed on September 22 suggested that treatment is also needed in the summer season to protect the new foliage, which is more susceptible to disease than old leaves (Graniti, 1993). As reported by Saad and Masri (1978), during the dry season the viability of conidia did not greatly vary but only the inoculum density varied, expressed as number of conidia per cm^2. The germination of conidia depends upon humidity, temperature and leaf wetness duration, and the optimum of those conditions for *F. oleaginum* activity in this region of Italy can also occur in summer.

The effects of treatments in the resistant cultivar were not appreciable compared with the untreated control until the December sampling, while in the susceptible cultivar (Nocellara del Belice) olive leaves treated with Formulation A showed lower proportions of infected leaves than the untreated control already in September (Table 3). Moreover, the reduction of the number of infected leaves relative to the untreated control was always less than the same percentage of infected tissues, showing that the effect of Formulation A was probably related to some effect on the subcuticular growth of the pathogen rather than on conidia germination.

The greater efficacy of Formulation A than dodine could be due to the number and time of the treatment applications; while the Formulation A was sprayed five times, dodine was sprayed only twice. The percentage of olive leaf tissues with OLS on leaves treated with dodine was not different compared with the untreated control, as was the case in New Zealand (Obanor *et al.*, 2008). These authors noted that when dodine was applied 7 days before or after inoculation, the severity of disease was not significantly different than untreated experimental controls.

The different behaviour of the two cultivars with respect to OLS disease, confirmed in this study, is demonstrated both by the lower proportion of infected leaves on the resistant cultivar and, to a greater degree, by the lesser disease severity expressed as infected leaf tissues. This result seems to disagree with the observation by Benitez *et al.* (2005), who reported that the difference between resistant and susceptible plants, respectively Lechin de Sevilla and Picual, depended on differences in germtube penetration by *F. oleaginum*.

In conclusion, this study has shown that the natural formulation tested against OLS, regularly applied throughout the vegetative period when climatic conditions are favourable to fungus activity, could control infections better than the two dodine applications of the commonly used chemical fungicide dodine, allowed in integrated pest management programs.

Acknowledgements

This research was supported by the projects "EXTRAVALORE – Valorizzazione dei sottoprodotti della filiera del biodiesel" and "RGV/FAO" – Risorse genetiche vegetali, financed by the Italian Ministry of Agriculture, Food and Forestry.

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Accepted for publication: September 24, 2011