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EFFECTIVENESS OF FUNGICIDES AND AN ESSENTIAL-OIL-BASED PRODUCT IN THE CONTROL OF GREY MOULD DISEASE IN RASPBERRY

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Abstract

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Field experiments were conducted in two commercial raspberry fields to evaluate effectiveness of some fungicides and an essential oil based product in the control of Botrytis fruit rot. The experiments consisted of four fungicides - fenhexamid, vinclozolin, benomyl, and pyrimethanil, two essential oil product treatments (0.5% and 1% emulsion) and untreated control, in randomized block design with four replicates per treatment. Afterwards, the pathogen was isolated from infected fruits and identified based on colonial and conidial morphology. Sensitivity of 10 randomly chosen isolates to all mentioned fungicides and tea tree oil was determined in radial growth assay on PDA supplemented with a range of concentrations of the relevant product. At both localities, the highest efficacy was achieved by pyrimethanil (97.4% and 98.2%) and fenhexamid (93.6% and 97.6%), while the efficacy of tea tree oil, applied at both concentrations, was less than satisfactory and ranged from 13.3% to 55.9% compared to the control. *In vitro* sensitivity of *B. cinerea* was determined based on EC-50 values which ranged between: 0.14 mg/l and 0.20 mg/l for vinclozolin, 0.16 mg/l and 0.46 mg/l for benomyl, 0.22 mg/l and 3.81 mg/l for pyrimethanil, 0.06 mg/l and 0.19 mg/l for fenhexamid, and 383.3 mg/l and 1500.6 mg/l for tea tree oil product.

Key words: Botrytis cinerea; raspberry; disease control; fungicide efficacy; fungicide resistance

Introduction

Botrytis cinerea Pers.: Fr., the anamorph of *Botryotinia fuckeliana*, is phytopathogenic fungus that affects a wide range of plant species. It is a necrotroph which can cause soft rotting or grey mould of almost all aerial plant parts at all growth stages, accompanied by collapse of parenchima tissues covered by grey masses of conidia. Small fruits and vegetables are the most severely affected (Williamson et al., 2007). It is well documented that Botrytis fruit rot is one of the major limiting factors in raspberry (*Rubus idaeus* L.) production worldwide. The fungus affects vegetative and flowering structures throughout the growing season, often causing reductions in yield and fruit quality deterioration. Stamens and stigmas of raspberry flowers are major pathways by which the pathogen invades the fruit. However, flower infections usually remain dormant until the fruit ripens (Dashwood and Fox, 1988). After ripening, fruit rot develops rapidly, followed by profuse sporulation of the pathogen and secondary infections of ripe fruits occur. Mature fruits are very

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susceptible to the pathogen, especially during periods of rainy, wet weather before harvest. Additionally, *B. cinerea* causes significant losses during shipping and marketing.

Control of *B. cinerea* in raspberry is mainly based on the use of chemicals during flowering period. The most commonly used fungicides in raspberry farms in Serbia are: iprodione, vinclozolin, pyrimethanil and fenhexamid. However, growers are frequently complaining about decreased efficacy of some products and seek some environmentally friendly alternatives to synthetic pesticides.

Application of substances of natural origin as crop protectants could be a convenient solution, safe for both humans and the environment. Antimicrobial properties of certain essential oils have already been known for a long time (Chamberlain, 1887; Daferera et al., 2003), but their efficacy as crop protectants is not well documented. Some of them, like tea tree oil from *Melaleuca alternifolia* (Maiden & Betche) Cheel, are effectively used in human medicine (Hammer et al., 2003). Our previous *in vitro* experiments (Tanovic et al., 2005) showed that volatile phase of tea tree oil was toxic to some plant pathogens including *B. cinerea* and could be considered for future investigation. Similarly, tea tree oil expressed toxic effect to some mycopathogenic fungi *in vitro* (Tanovic et al., 2009). It was also shown that Timorex 66 EC, a formulated tea tree essential oil, provided some level of protection of white button mushroom from *Cladobotryum dendroides* (Potocnik et al., 2010).

Taking into account growers needs and the fact that both the pathogen and the fungicides with specific mode of action are well-known for their high risk for resistance development, it was reasonable to evaluate the effectiveness of several fungicides and a tea tree essential oil based product in *B. cinerea* control in commercial raspberry fields, and to test sensitivity of the pathogen *in vitro.*

Material and Methods

Field trials. Field experiments were carried out in commercial raspberry fields at two localities in raspberry growing area in Serbia. In the past few years fungicide treatments in fruit rot control were applied regularly at both fields. Cultivar 'Willamette', the most commonly grown and highly susceptible to Botrytis fruit rot, was used in both trials. Inter-row space was 2.5 m, with plants set 0.25 m apart within the row. Similar cultivation measures were performed at both localities. The experiments were set as randomized blocks with four replicates per treatment. The plot size was 25 m^2 and consisted of 90-100 plants.

Products used in the experiments, rates and timing of applications are summarized in Table 1. Conventional fungicides (fenhexamid, vinclozolin, benomyl, and pyrimethanil) were applied three times- until the end of flowering, while essential-oil based product was applied five times – until the beginning of harvest. The control plot did not receive any applications. No other fungicides or insecticides were applied during the growing season.

The effect of the tested products on fruit infection was assessed 20 days after the last fungicide treatment. Hundred mature fruits were randomly picked from the central plants of each plot, placed in plastic boxes (15x10x9 cm) and incubated for two days at 7°C. The fruits were grouped into classes according to the intensity of fruit infection as follows: 0 = no infection, 1 = only one drupelet of a berry is affected, 2 = less than 30 % of a berry is affected and 3 = more than 30% affected. Total number of healthy fruits per plot was also recorded.

The disease severity index (DSI) was calculated using the following formula:

$$DSI = \Sigma(nv)/V$$

where: n = degree of fruit infection according to the scale, v = number of fruits in the class and V = total number of fruits screened.

The data were processed by standard statistical methods (analysis of variance and Duncan's multiple range test). The efficacy of treatments was calculated based on the DSI using Abbott's formula (Abbott, 1925).

$$\text{Efficacy (\%)} = (DSI_{control} - DSI_{treatment}) / DSI_{control} \times 100$$

Isolation. All the isolates were derived from decaying fruits, originating from control plots from both localities, after incubation of separate fruits in moist chambers for seven days at 20°C. Mycelial fragments, cut from thick and woolly mycelia developed on the incubated diseased

Table 1
Fungicide and biofungicide treatments, rates and timing of application at both localities

No	Active ingredient / Product	Supplier of the product	Product rate	Timing of application
1	Tea tree essential oil (Timorex 66EC)	Stockton Chemical Corp. Israel	0.5% 1%	I - beginning of flowering (BBCH 61)[1] II - full flowering (BBCH 65) III – end of flowering (BBCH 71) IV - beginning of ripening (BBCH 81) V - beginning of harvest(BBCH 85)
2	Vinclozolin (Ronilan-FL)	BASF, Germany	1.5 l/ha	I - beginning of flowering (BBCH 61) II - full flowering (BBCH 65) III – end of flowering (BBCH 71)
3	Pyrimetanil (Mythos)	Bayer.CropScience, Germany	2.5 l/ha	I - beginning of flowering (BBCH 61) II - full flowering (BBCH 65) III – end of flowering (BBCH 71)
4	Benomyl (Benfungin)	Galenika-Fitofarmacija, SCG	0.06%	I - beginning of flowering (BBCH 61) II - full flowering (BBCH 65) III – end of flowering (BBCH 71)
5	Fenhexamid (Teldor 500 SC)	Bayer.CropScience, Germany	0.1%	I - beginning of flowering (BBCH 61) II - full flowering (BBCH 65) III – end of flowering (BBCH 71)

[1] according to Meier (1997)

fruits, were transferred on potato-dextrose-agar medium (PDA), purified by monospore isolation and assigned numbers. The obtained isolates were cultured at 20°C on PDA and stored on slants at 4°C.

Identification. The isolates were identified according to colony morphology and microscopic observations of conidiophores and conidia after 10-day cultivation of the isolates on PDA medium at 20°C.

Fungicide sensitivity testing *in vitro.* Sensitivity of 10 randomly chosen *B. cinerea* isolates was determined in radial growth experiments according to the method described by Leroux and Gredt (1972). To obtain repeatable results, sensitivity to pyrimethanil was tested on asp-agar medium (Hilber and Schuepp, 1996), while for the other fungicides and essential-oil based product PDA medium was used. Fungicides were suspended in sterile distilled water and aseptically added to autoclaved media that had cooled to 50°C. The ranges of fungicide concentrations for the experiment were obtained by conducting preliminary experiments and the final concentrations of active ingredients (a.i.) in the media were the following: for pyrimethanil 0.01, 0.1, 0.25, 0.5, and 1 mg/L, for benomyl 0.15, 0.175, 0.20, 0.25, and 0.30 mg/l, for vinclozolin and fenhexamid 0.03, 0.06, 0.125, 0.25, and 0.5 mg/L, respectively. Essential oil was added at concentrations of 1000, 500, 250, 125, and 62.5 mg a.i./l. Petry dishes were inoculated centrally with an inverted mycelial plugs (10 mm), cut from the edge of actively growing 4-day-old colonies on PDA, and incubated for three days at 20°C. All experiments were conducted twice in four replicates. Mycelial growth on fungicide-amended media was presented as percentage of the control. All the data were pulled together and the fungicide concentration that inhibited mycelial growth by 50% (EC-50) was obtained using probit analysis (Finny, 1964).

Results

Field trials. Compared to the control, all applied treatments at both localities significantly reduced the disease incidence in raspberry fruits. The disease severity index values in the control plots were 1.76 and 0.42 (Table 2), showing that infection pressure in the investigated fields was not the same. Treatments with commercial fungicides were significantly more effective than the treatments with tea tree oil based product, regardless the infection pressure. At both localities, the highest efficacy was achieved by pyrimethanil (97.4% and 98.2%) and fenhexamid (93.6% and 97.6%), while the efficacy of tea tree oil, applied at both concentrations, was less than satisfactory (13.3% – 55.9%). In the field with lower disease incidence in the control plots, efficacy of the tea tree oil was significantly higher than in the field with higher disease pressure (Table 2).

Isolates. After 7-day incubation of diseased fruits in moist chamber at 20^{o}C, thick and woolly micelium was developed. In total, more than 100 isolates forming white, uniform, areal mycelium with entire margin were derived. According to colony morphology, microscopic observations of conidiophores and conidia, and comparison to the literature data, the isolates were identified as *Botrytis cinerea*, the anamorph of *Botryotinia fuckeliana*. Ten randomly selected isolates were chosen for sensitivity tests (Table 3).

***In vitro* sensitivity of the isolates.** Sensitivity of *B. cinerea* to tested fungicides was determined based on EC-50 values which ranged between: 0.14 mg/l and 0.20 mg/l for vinclozolin, 0.16 mg/l and 0.46 mg/l for benomyl, 0.22 mg/l and 3.81 mg/l for pyrimethanil, 0.06 mg/l and 0.19 mg/l for fenhexamid, while for tea tree oil product the same value was between 383.3 mg/l and 1500.6 mg/l (Table 3). In most cases, results obtained in the *in vitro* experiment were in agreement with the conclusion from field trials that the pathogen is sensitive to all investigated conventional fungicides. However, two isolates (Bc18 and Bc26) were moderately resistant to pyrimethanil. It was also found that sensitivity of all the

Table 2
The disease incidence and efficacy of the treatments in the fields

Treatment	Locality Pozega			Locality Valjevo		
	No. of healthy fruits/plot	DSI[1,2]	Efficacy, %[3]	No. of healthy fruits/plot	DSI	Efficacy, %
Tea tree oil 0.5%	32.0±7.0	1.53±0.18 bc	13.3	87.0±9.6	0.22±0.07 b	47.6
Tea tree oil 1%	32.8±15.0	1.38±0.44 b	21.6	88.3±1.5	0.19±0.04 b	55.9
Vinclozolin	90.3±4.5	0.18±0.09 a	89.6	96.5±2.6	0.01±0.01 a	96.4
Pyrimetanil	87.8±5.8	0.04±0.03 a	97.4	95.5±4.0	0.01±0.01 a	98.2
Benomyl	97.3±2.6	0.18±0.09 a	89.6	99.5±1.0	0.03±0.02 a	93.0
Fenhexamid	94.8±3.3	0.11±0.03 a	93.6	99.3±0.5	0.01±0.01 a	97.6
Control	20.5±1.3	1.76 ±0.11c		78.0±10.7	0.42±0.02 c	

[1]Based on 400 berries per treatment;
[2]Disease severity index; values within column followed by different letters are significantly different ($p< 0.05$) based on Duncan's multiple range test
[3]Efficacy of the product based on the DSI

Table 3
***In vitro* sensitivity of selected *B. cinerea* isolates to fungicides and tea tree oil**

Isolate	EC-50, mg/l*									
	vinclozolin		benomyl		fenhexamid		pyrimethanil		tea tree oil	
	mean	range	mean	range	mean	range	mean	range	mean	range
Bc2	0.15	0.13-0.18	0.25	0.22-0.33	0.09	0.08-0.10	0.22	0.16-0.29	476.6	378.3-627.4
Bc8	0.24	0.21-0.26	0.06	0.05-0.07	0.06	0.05-0.07	0.36	0.27-0.53	474.7	295.5-1265.6
Bc9	0.16	0.14-0.18	0.16	0.16-0.17	0.12	0.10-0.15	0.40	0.36-0.44	1134.5	629.0-4568.0
Bc10	0.14	0.11-0.16	0.46	0.36-1.16	0.19	0.16-0.23	0.41	0.18-3.19	751.7	633.0-925.0
Bc12	0.14	0.11-0.18	0.20	0.19-0.22	0.10	0.07-0.13	0.54	0.44-0.70	989.1	554.0-9617.4
Bc17	0.18	0.16-0.22	0.24	0.22-0.26	0.08	0.06-0.10	0.32	0.24-0.46	1500.6	806.0-6289.7
Bc18	0.20	0.17-0.22	0.16	0.14-0.17	0.10	0.06-0.14	3.56	0.91-185.02	1142.5	831.0-1846.5
Bc26	0.14	0.12-0.24	0.17	0.16-0.18	0.10	0.09-0.12	3.81	0.77-2409.32	774.7	620.7-1041.0
Bc27	0.16	0.12-0.24	0.29	0.26-0.36	0.07	0.06-0.09	0.30	0.25-0.37	383.3	308.7-489.6
Bc30	0.17	0.15-0.19	0.20	0.19-0.20	0.10	0.08-0.18	1.00	0.38-12.34	657.0	516.6-908.5

* Fungicide concentration that inhibited mycelial growth by 50%

isolates to conventional fungicides is much higher than to the formulated essential oil product.

Discussion

All conventional fungicides in our experiments provided effective control of *B. cinerea*. Our findings in raspberry have confirmed high efficacy of fenhexamid in reduction of *B. cinerea* infections in *Rubus* species (Duben et al., 2002; Walter et al., 2005). However, in some cases, fungicide effectiveness was seriously affected by the development of resistance in target fungi (Brent and Hollomon, 1998). Without effective product management, resistance could arise very quickly, as happened with the methyl benzimidazole carbamates (mbc), dicarboximides and phenylamides in the early 1980's (Russel, 2004). Yourman and Jeffers (1999) reported benzimidazole and dicarboximide resistance in *B. cinerea* isolates from greenhouses, while Raposo et al. (1996) and Sun et al. (2010) found isolates of *B. cinerea* with multiple fungicide resistance. The fact that resistant *B. cinerea* isolates have been found in commercially treated fields indicates the need for active resistance management (Anonymous, 2010). Our *in vitro* testing of the sensitivity of the isolates from natural populations showed that antiresistance strategy that had been applied in investigated commercial fields prior to our experiments was adequate and did not cause shift in pathogen's sensitivity. According to the criteria proposed by Faretra and Polastro (1993), all the isolates are sensitive to vinclozolin because EC-50 values were bellow 1 mg/l. Choi et al. (1997), however, gave EC-50 value of 0.5 mg/l for vinclozolin as an upper limit for sensitive isolates. Even if this stronger criterion, proposed by Choi et al. (1997) would be used, all the tested isolates would still be considered sensitive to vinclozolin. This finding is very important having in mind that practical resistance in *B. cinerea* to dicarboximides is usually a consequence of the presence of moderately (EC-50 values 1 – 3 mg/l) and not highly resistant strains (Pollastro et al., 1996).

All the tested isolates were sensitive to benomyl despite the fact that benzimidazoles have been used in raspberry crops in Serbia for many years. It is well known that combination *B. cinerea* - benzimidazoles poses a high risk for resistance development. Shortly after introduction of benzimidazoles into the market several cases of resistance across Europe were recorded (Bollen and Scholten, 1971; Leroux and Clerjeau, 1985). In Greece, selection of resistant strains to benzimidazoles appeared in early 1970s (Malathrakis, 1979). Most of benzimidazole-resistant strains exhibit high resistance levels with resistance ratio greater than 1000 (Ben R1 phenotype) without fitness cost, as a consequence of a mutation at position 198 on ß-tubuline gen (Leroux et al., 1999). It was also found that another mutation at position 200 in the Ben R2 phenotype leads to resistance level of 30-100 (Leroux, 2004). None of these resistant phenotypes were found among our isolates.

The history of pyrimethanil and fenhexamid use in raspberry fields does not imply possibility of resistance development. The results of *in vitro* tests mostly confirmed this hypothesis. However, two isolates were moderately resistant to pyrimethanil with EC-50 values of 3.81 mg/l and 3.65 mg/l. Further investigation will clarify both the importance of this finding and resistance mechanism in the isolates. According to the Fungicide Resistance Action Group (FRAC) reports, pathogen strains with high resistance to anilinopyrimidine fungicides have been found in Europe, but their frequency remained stable, while the performance of the fungicides in the field was not affected after more than 10 years of commercial use (Anonymous, 2009). On the contrary, resistance development to anilinopyrimidines was detected in vegetable crops on the island of Crete (Myresiotis et al., 2007) and in Switzerland (Baroffio et al., 2003). Anilinopyrimidine resistant *B. cinerea* strains were also frequently found in table grapes in California (Smilanick, 2010). It is well documented that resistance to anilinopyrimidine fungicides is monogenically controlled and has a qualitative disruptive pattern (Bardas et al., 2008). Therefore, continuous monitoring program of the pathogen sensitivity is required to detect any change in the frequency of resistant strains on time and to insure a long-term effective use of these fungicides.

Even though a volatile phase of a pure tea tree oil had shown strong lethal effect on *B. cinerea* isolate *in vitro* (Tanovic et al., 2005), the formulated product, based on the same essential oil, did not provide effective disease control in the field. The efficacy of the product, applied at both concentrations, was less than satisfactory. Higher efficacy was achieved in the field with lower disease pressure, when the product was applied at higher concentration. *In vitro* testing of sensitivity of the isolates also showed that this formulation is not sufficiently toxic to *B. cinerea*. The EC-50 values ranged from 383.3 mg/l to 1500.6 mg/l, but this effect was fungistatic not fungicidal. Since the formulated product has never been tested or applied against *B. cinerea* in raspberry before, the results of our experiments are initial findings and should be continued.

Conclusion

Our results showed that all investigated fungicides are very effective in *B. cinerea* control confirming appropriate resistent management strategy that is used in the investigated fields. Even though *B. cinerea* control by application of essentional oils may not work as efficient as chemical pesticides and may only provide partial levels of disease control, further investigation considering better formulation of the oil product should be done to obtain complete evaluation of the potential of tea tree oil use in the disease management. Taking into consideration the market demand, effective alternatives to conventional fungicides must be identified or, at least, methods of control acceptable from the standpoint of organic agricultural production found. Additionally, formulations containing mixtures of essential oils and conventional fungicides could also be tempting for future investigations.

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