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In vivo evaluation of essential oils and biocontrol agents combined with heat treatments on basil cv Genovese Gigante seeds against *Fusarium oxysporum* f. sp. *basilici*

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Abstract Seed treatments with essential oils (from savory and thyme) and biocontrol agents (*Pseudomonas* spp. and *Fusarium oxysporum*) have been evaluated *in vivo* after dry hot air treatments against *Fusarium oxysporum* f. sp. *basilici* on basil seeds. The savory and thyme essential oils showed a significant pathogen control activity because of their innate antifungal activity and because of the seed application method, but the dry hot pre-treatment did not show any obvious effect on the performance of the essential oil treatments. The dry heat treatment improved the *Pseudomonas* seed dressing effect against *F.oxysporum* f. sp. *basilici*, and showed important reductions in plant infection and the disease index on the treated seed plants, without any negative effect on seed germination. However, the pathogen control provided by the heat treatments combined with the application of the biocontrol agents never reached the same performance as the chemical treatments considered as the reference. Thus, short dry heat treatments on basil seeds have been shown to be a valid but complementary seed disinfection method against Fusarium wilt.

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Introduction

Basil (*Ocimum basilicum* L.) is an economically important herb crop in Mediterranean area countries. However, its intensive nature and the limited size of specialized farms make the crop highly prone to the development of Fusarium wilt (Gullino *et al.* 2012). *Fusarium oxysporum* f. sp. *basilici* has been identified extensively as the causal agent of this wilt in basil cultivations in Europe and the United States (Davis *et al.* 1993; Dutky and Wolkow 1994; Elmer *et al.* 1994; Biris *et al.* 2004; Moya *et al.* 2004; Summerell *et al.* 2006; Felgueiras *et al.* 2010), and recently also in South America (Lori *et al.* 2014). It has been found that *F. oxysporum* f. sp. *basilici* is seedborne, and seeds are therefore considered an important factor in pathogen dissemination (Martini and Gullino 1991; Elmer *et al.* 1994; Vannacci *et al.* 1999; Elmer 2001), and its presence in the seeds could be internalor external (Vannacci *et al.,* 1999; Chiocchetti *et al.,* 2001).

Seed dressing is a direct control measure adopted against *F. oxysporum* f. sp. *basilici* (Garibaldi *et al.* 1997), and it represents an easy, low-cost method for basil disease management (Gullino *et al.* 2012). Commercial chemical-based seed treatments usually consist of mixtures of different active compounds with a complementary spectrum of activities to cover a wide range of pathogenic agents. Mixtures of active

compounds, such as mefenoxam, fludioxonil, azoxystrobin, and thiabendazole, have been designed for the control of *Fusarium* spp., with successful results on maize (Munkvold *et al.* 2014). Besides the use of synthetic pesticides, alternative non-chemical methods for the treatment of seeds against seedborne pathogens have also been studied. These methods mainly consist of biocontrol agents, physical treatments, and the use of natural products.

As far as treatments with biocontrol agents on vegetable seeds are concerned, promising results have already been reported against *F.oxysporum* f. sp. *lactucae* (Gilardi *et al.* 2005; Lopez-Reyes *et al.* 2014) and other seedborne, vegetable crop pathogens, such as *Phoma valerianellae* on lamb's lettuce, *Colletotrichum lindemuthianum* on beans, *Ascochyta* spp. on peas, and *Alternaria* spp. on carrots (Schmitt *et al.* 2009; Tinivella *et al.* 2009; Koch *et al.* 2010). Some bacterial strains can even induce resistance, increase stress tolerance, and promote growth in the host when applied to seeds (Fürnkranz *et al.* 2012; Joe *et al.* 2012). However, few biological products are currently available for the control of seedborne pathogens, often due to commercial constraints (Koch and Roberts 2014). Among the natural products, essential oils have been identified as possible alternatives against some seedborne pathogens belonging to the *Fusarium* genus, including *Fusarium oxysporum* (Koul *et al.* 2008; Dal Bello and Sisterna 2010). Savory and thyme essential oils have been shown to be active against *F. oxysporum* f. sp. *basilici* on basil seeds and their effect on seed germination has been found to be safe when they are applied by means of fumigation (Lopez-Reyes *et al.* 2015). Despite the proven antimicrobial activity of essential oils, their single principal compounds, or other plant extracts, commercialization of these plant-derived pest management products have had to face similar constraints to those of biological products (Koch and Roberts 2014).

The physical seed treatments in the seed industry mainly involve immersion in hot water, exposure to dry hot air, or exposure to aerated steam. Hot water treatments are well known as being effective against many seedborne pathogens (Baker 1962; Gabrielson 1983; Nega *et al.* 2003; McGrath *et al.* 2013), including *F. oxysporum*f. sp. *vasinfectum* (Bennett and Colyer 2010), but since basil seeds produce a thick layer of mucilage around the testa within minutes after hydration (Western 2012), immersion in hot water is not practical and could directly affect the seed germination process. Dry heat treatments do not require sophisticated equipment, and are consequently easy to apply (Agarwal and Sinclair 1997); they can also reduce or eliminate the presence of *Fusarium graminearum* on wheat seeds without causing important detrimental effects on their agronomic performance (Clear *et al.* 2002; Gilbert *et al.* 2005). Exposure of seeds to aerated steam leads to a more accurate temperature control, less impairment of seed germination, and the seeds are left much dryer than after moist treatments (Koch and Roberts 2014). Treatment time and seed moisture are also important factors in seed thermotherapy, and those parameters can be controlled precisely in aerated steam applications in fluidized beds, as shown by Forsberg *et al.* (2005) on wheat and barley seed treatments against *Fusarium* spp. and other seed pathogens.

Combinations of different treatments have been suggested for seed disinfection (Schmitt *et al.* 2008; Lopez-Reyes *et al.* 2014). Considering an external infection of the seeds, this study has evaluated the efficacy of chemicals, biocontrol agents, and essential oils as seed treatments, combined with dry heat, on basil seeds against artificially inoculated *F. oxysporum* f. sp. *basilici*. The impact of the treatments on seeds performance has been evaluated using seed germination and fresh biomass as indicators.

Materials and methods

Preparation of the artificially-inoculated seed lot

The experiments considered an external infection of the seeds. We decide then to reproduce in our experiments an external infection that guaranteed clear and reliable Fusarium wilt symptoms *in vivo* if the seed was still infected after the treatment. The FOB 009 RB isolate (*F. oxysporum* f. sp. *basilici*) was isolated from basil plants which showed evident Fusarium wilt symptoms and maintained at 8°C on PDA + streptomycin sulphate plates and slants before use. The pathogen was grown in a shake culture for ten days in PDB (Sigma-Aldrich / Fluka) at 25°C with 12 hours of fluorescent light per day for the inoculation preparation. The culture suspension was then centrifuged (20 minutes at 17000 RCF), and the resulting pellet was mixed with talc (1:2 w/w), spread on clean paper, and stored at 25°C for ten days in order to dry and induce the production of chlamydospores. The chlamydospore concentration in the talc was

determined by suspending a talc sample in sterilized-deionized water and plating a ten-fold serial dilution on PDA + streptomycin sulphate plates.

Seeds of basil (*Ocimum basilicum*) cv Genovese Gigante were used for this study. A testing seed lot, containing 50 % of inoculated seeds, was produced to reproduce reliable Fusarium wilt symptoms on seedlings and plants in a greenhouse. Fifty percent of the basil seeds were inoculated through the addition of the previously described *F. oxysporum* f. sp. *basilici* infested talc, until a concentration of 1×10^6 chlamydospores per gram of seed was reached; these seeds were then mixed thoroughly with the remaining non-inoculated seeds. The chlamydospore presence on the inoculated seeds was confirmed through a Washing Test, which was performed according to ISTA methods (Mathur and Kongsdal 2003).

Dry hot air treatments

A preliminary *in vitro* and *in vivo* screening was performed in order to define an appropriate duration and temperature combination for the heat treatment (data not shown). Four-gram batches of inoculated seeds were deposited in Petri dishes and placed in a pre-conditioned Venti-line VL 53 oven with forced convection (VWR International, Leuven, Belgium) at 65°C for 10 minutes. Then, the Petri dishes containing the basil seeds were placed in a vertical-laminar flow chamber until room temperature was reached.

Chemical seed treatments

The inoculated seeds were treated with either a resistance inductor (acibenzolar-S-methyl: Bion 50, 50.0% a.i., Syngenta Crop Protection) or a fungicide (prochloraz: Octave, 50.0% a.i., Bayer Crop Science) as the chemical reference. The chemical products were applied to the inoculated seeds in a Hege 11 seed treater (Wintersteiger, Ried, Switzerland) at a concentration of 0.1 g a.i. per Kg of seeds for acibenzolar-S-methyl and of 1.0 g a.i. per Kg of seeds for prochloraz.

Essential oil treatments

The savory (*Satureja montana*) and thyme (*Thymus vulgaris*) essential oils were available as commercial preparations and were purchased from Soave (Turin, Italy). Analysis of the essential oil composition was carried out by means of GC/MS, as in our previous studies (Lopez-Reyes *et al.* 2015). A 10% emulsion (10% essential oil, 88% sterilized water and 2% Tween20; Merck) was prepared from each essential oil. In order to conduct the savory essential oil treatments, one gram of inoculated seeds was deposited in a quadrant Petri dish and covered with filter paper (No. 1, Whatman) containing one milliliter of the essential oil emulsion (for the savory + thyme essential oil treatments, the seeds were covered with filter paper containing 0.5 ml of each emulsion). The Petri dishes were then sealed with Parafilm and stored at room temperature for 24 hours.

Treatments with biocontrol agents

FC7B (*Pseudomonas* sp., EU836173), FC8B (*Pseudomonas putida*, EU836174), and FC9B (*Pseudomonas* sp., EU836171) strains, isolated by Clematis *et al.* (2009) from recycled substrates of tomato soilless crops in northern Italy, were used in this study. The bacterial strains were maintained on LBA dishes and slants (Sigma-Aldrich / Fluka) at 8°C, then grown in shaken cultures for 24 h on LB (Sigma-Aldrich / Fluka) at 25°C. The cell suspension was centrifuged and the pellet re-suspended in sterile deionized water. Concentrations of the resulting dilutions were determined at OD600 in a Lambda 35 UV/ VIS spectrophotometer (PerkinElmer, Monza, Italy). Basil seeds were treated by spraying each bacterial suspension (when applied as a strain mix, a 1:1:1 proportion of each suspension was used) in a Hege 11 seed treater (Wintersteiger, Ried, Switzerland) using a container modified to treat seeds of this size; a concentration of 1×10^7 bacterial cells per gram of seed was reached. The treated seeds were air-dried in a vertical-laminar flow chamber for at least 1 hour.

Antagonistic *F. oxysporum* strains 251/2 and MSA 35 (Agroinnova, the University of Torino), which were isolated from Fusarium-suppressive soils, were tested. The fungal strains were grown in shaken cultures for 10 days on PDB at 25°C under 12 h/day fluorescent light. The culture suspensions were centrifuged (20 minutes at 17000 RCF), and the pellet was mixed with talc (1:2 w/w), distributed on clean paper, and stored at 25°C for ten days to dry and induce chlamydospore production. The chlamydospore concentration of the inoculated talc was determined as previously specified. Basil seeds were treated by adding the inoculated talc of both fungal

strains in a 1:1 mix, until a concentration of 1×10^7 chlamydospores per gram of seed was reached.

In vivo effects of the treatments on inoculated seeds

The treated seeds were sown in plastic boxes containing ten litres of peat-perlite substrate (1:3 v/v; Perlite Agrilit3, Perlite Italiana, Milano, Italy; Peat Tecno2, Turco, Italy);125 seeds were sown in each box. The boxes were then placed in a randomised design on greenhouse benches for 30 days at temperatures of between 26 and 28°C, and an RH of between 85 and 95%; the boxes were watered daily. Non-inoculated / non-treated seeds and inoculated / non-treated seeds were also sown as controls. Four replications (one per box) were used for each treatment and for each control.

The germination rate was calculated by counting the number of plants that had emerged ten days after sowing. Surveys were carried out weekly to establish the number of dead plants, and the fresh biomass from each replication was determined at the end of the experiment. At the end of each experiment, a 0–100 disease index was calculated for each replication by rating each plant from 0 up to 4, where 0 was a healthy plant, 1 was a plant with very slight Fusarium wilt symptoms, 2 was a plant with evident wilt symptoms, where all the leaves were still green, 3 was a plant with evident wilt symptoms, where at least one leaf was dead, and 4 was a dead plant. The following formula was used:

$$\text{Disease index } 0{-}100 = [(a \times 25) + (b \times 50) + (c \times 75) + (d \times 100)]/e$$

where "a" is the number of plants rated "1", "b" the number of plants rated "2", "c" the number of plants rated "3", "d" the number of plants rated "4", and "e" the number of emerged plants. Each trial was performed at least five times.

Once the values of pathogen control were determined, the relationship between each treatment combination with the dry air treatment was compared to its Colby's "expected" level of control (Colby, 1967) and considered as synergistic (if greater than the "expected" level of control), antagonistic (if less than), or additive (if equal to). The "expected" level of control (E) was calculated using the following formula:

$$E = (a \times b)/100$$

Where "a" is the percentage of pathogen control of treatment "a", and "b" is the percentage of pathogen control of treatment "b".

Statistical analysis

The data from all the experiments were pooled together, a statistical analysis was performed by one-way analysis of variance (ANOVA), using SPSS-WIN software, and Duncan's multiple range test was employed; $p<0.05$ was considered as the significance level.

Results

In vivo effects of the treatments on inoculated seeds

In the present experiments, the germination rate of the non-inoculated / non treated seeds was generally better than those of any of the inoculated seeds (treated or not), and the tested treatments did not affect the germination rate of treated seeds. The *Pseudomonas* sp. FC9B strain treatment applied alone and the treatment with acibenzolar-S-methyl + the bacterial strain mix, were the only treatments that statistically reached the same germination level as the non-inoculated / non-treated seeds without the dry hot air pre-treatment (Figure 1). The statistical behavior of the germination rates from the treated seeds was statistically quite similar. The germination rates of the seeds treated with the acibenzolar-S-methyl, FC7B strain alone, the prochloraz + acibenzolar-S-methyl + bacterial strain mix, and the acibenzolar-S-methyl + bacterial strain mix, were statistically similar to that of the non-inoculated / non-treated control, but only when the dry hot air treatment was performed first; after these treatments, no reductions on germination rate due to the heat pre-treatment were detected statistically.

The cumulative results pertaining to the infection rate and disease index of the plants from the inoculated basil seeds showed a similar pattern (Tables 1 and 2). The plants from the seeds treated with savory essential oil (applied alone or combined with thyme essential oil) showed a statistically similar infection rate and disease index to both of the chemical references. The plants from the seeds treated with prochloraz + acibenzolar-S-methyl, with the prochloraz + acibenzolar-S-methyl +

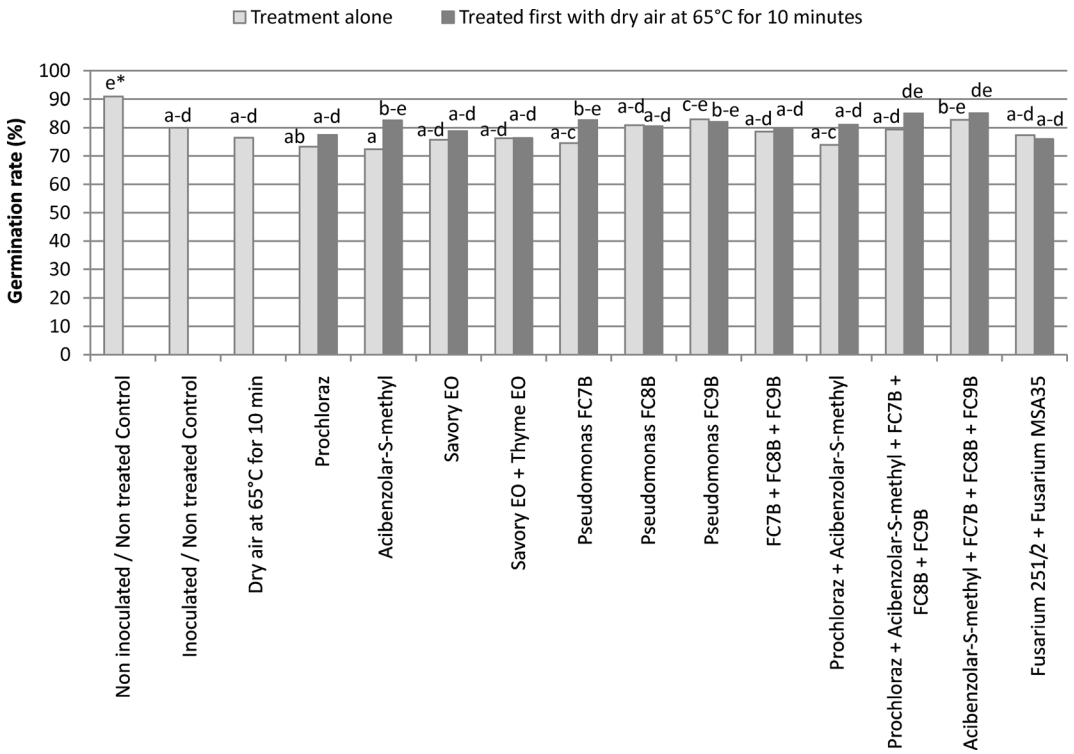

Fig 1 *In vivo* germination rate (%) of treated basil cv Genovese Gigante seeds, previously inoculated with *Fusarium oxysporum* f. sp. *basilici*, 10 days after sowing. The bars represent the mean of five trials, each trial consisted in 4 replications and each replication consisted in 125 seeds, for a total of 500 per treatment per trial. *Bars followed by the same letter are not statistically different (Duncan's multiple range test, $p<0.05$)

bacterial strain mix, and with the acibenzolar-S-methyl + bacterial strain mix also showed a statistically similar infection rate and disease index to those obtained with the chemical products on their own. The heat pre-treatment did not significantly influence the effect of the above-mentioned treatments, but it showed a clear effect on the infection rate and disease index of plants from seeds treated with the bacterial strains applied alone or in a mix. If the effect of the seed treatments with the bacterial strains alone is compared with the effect of these seed treatments combined with the dry hot air treatment, the pooled infection rate for the plants from seeds treated with the *Pseudomonas* sp. strain FC9B was reduced about 30%, about 38% when treated with the *Pseudomonas putida* strain FC8B, about41% when treated with the *Pseudomonas* sp. strain FC7B, and about54% when the seeds were treated with the bacterial strain mix. A similar reduction was also observed for the pooled disease index evaluated on the same plants. The dry heat treatment alone was able to reduce the infection rate from 30.3% in trial 3 to 52.1% in trial 1, and to reduce the disease index from 39.8% in trial 2 to 55.1% in trial 4. Only in trial 5, did the plants from the seeds treated with the *Pseudomonas* sp. strain FC7B show a reduction of 86.9% of the infected plants and 88.1% of the disease index when the seeds were treated with dry hot air before the treatment with bio-control agents. The infection rate and disease index of the plants from the seeds treated with the antagonistic *F. oxysporum* strains 251/2 and MSA 35 were very variable, and even showed a marked increase when combined with the heat treatment in trial 2 and trial 4.Overall, the pooled data on the infection rate and disease index of the plants from the inoculated seeds treated with only bacterial or fungal antagonistic strains were statistically different from the chemical references. Using the Colby approach, all treatments tested were synergistic when combined with the dry heat treatment (Table 3). The obtained values of pathogen control with the treatments including essential oils, procloraz, and acibenzolar-S-methyl where way higher than the "expected" ones.

The mean fresh biomass of the plants obtained from the inoculated and non-inoculated basil seeds ranged

Table 1 Infection rate (%) on plants from treated basil cv Genovese Gigante seeds, previously inoculated with *Fusarium oxysporum* f. sp. *basilici*, 30 days after sowing. The values in the "Trial" columns are the mean of the four replications in each trial(each replication consisted in 125 seeds, for a total of 500 per treatment per trial)

Treatment	Treatment alone						Treated first with dry air at 65°C for 10 minutes					
	Trial					Mean	Trial					Mean
	1	2	3	4	5		1	2	3	4	5	
Non inoculated / Non treated Control	0.0	0.0	0.0	0.0	0.0	0.0 + 0.0 a*	-	-	-	-	-	-
Inoculated / Non treated Control	44.5	27.8	14.2	32.5	30.1	29.8 + 10.8 i	-	-	-	-	-	-
Dry air at 65°C for 10 min	21.3	15.5	9.9	16.2	15.7	15.7 + 4.0 fg	-	-	-	-	-	-
Prochloraz	7.2	1.8	0.9	2.0	3.0	3.0 + 2.5 ab	0.8	3.0	2.3	3.2	4.7	2.8 + 1.4 ab
Acibenzolar-S-methyl	4.4	4.1	7.4	3.2	3.5	4.5 + 1.7 abc	4.7	6.3	8.5	3.0	5.2	5.5 + 2.0 abc
Savory EO	8.0	3.3	2.6	3.3	4.4	4.3 + 2.2 abc	2.3	1.2	1.8	1.7	4.5	2.3 + 1.3 a
Savory EO + Thyme EO	8.7	1.8	2.1	2.8	5.1	4.1 ± 2.9 abc	5.5	2.5	3.0	1.1	1.8	2.8 ± 1.7 ab
Pseudomonas FC7B	13.3	16.7	4.8	11.1	29.8	15.1 ± 9.3 efg	10.5	18.1	3.0	8.8	3.9	8.9 ± 6.1 b–e
Pseudomonas FC8B	16.2	19.1	27.9	19.7	14.0	19.4 + 5.3 gh	13.2	16.6	5.0	12.6	12.7	12.0 + 4.3 def
Pseudomonas FC9B	18.0	19.6	5.4	14.2	11.4	13.7 + 5.6 efg	9.2	13.9	4.3	12.4	8.1	9.6 + 3.8 c–f
FC7B + FC8B + FC9B	20.5	17.9	26.6	26.8	21.2	22.6 + 3.9 h	9.1	17.4	5.9	10.8	8.2	10.3 + 4.4 c–f
Prochloraz + Acibenzolar-S-methyl	0.4	0.0	3.6	1.2	2.7	1.6 + 1.5 a	0.7	2.0	0.5	1.7	4.6	1.9 + 1.6 a
Prochloraz + Acibenzolar-S-methyl + FC7B + FC8B + FC9B	0.9	4.3	0.6	2.3	2.5	2.1 + 1.5 a	0.0	2.2	1.8	0.9	2.8	1.5 + 1.1 a
Acibenzolar-S-methyl + FC7B + FC8B + FC9B	5.5	4.8	9.8	3.3	10.0	6.7 + 3.1 a–d	10.6	5.5	8.7	4.8	4.1	6.7 + 2.8 a–d
Fusarium 251/2 + Fusarium MSA35	19.0	12.5	9.6	8.5	17.3	13.4 ± 4.6 efg	8.2	20.3	7.1	22.8	12.7	14.2 ± 7.1 efg

* Values followed by the same letter are not statistically different (Duncan's multiple range test, $p<0.05$)

Table 2 Disease index (0–100) on plants from treated basil cv Genovese Gigante seeds, previously inoculated with *Fusarium oxysporum* f. sp. *basilici*, 30 days after sowing. The values in the "Trial" columns are the mean of the four replications in each trial (each replication consisted in 125 seeds, for a total of 500 per treatment per trial)

Treatment	Treatment alone						Treated first with dry air at 65°C for 10 minutes					
	Trial					Mean	Trial					Mean
	1	2	3	4	5		1	2	3	4	5	
Non inoculated / Non treated Control	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0 a*	-	-	-	-	-	-
Inoculated / Non treated Control	28.1	21.1	11.4	29.7	27.3	23.5 ± 7.1 j	-	-	-	-	-	-
Dry air at 65°C for 10 min	14.2	12.7	6.7	13.3	13.6	12.1 ± 2.8 ghi	-	-	-	-	-	-
Prochloraz	4.2	1.7	0.6	1.1	2.1	1.9 ± 0.6 abc	0.4	2.2	1.5	1.8	2.3	1.7 ± 0.4 ab
Acibenzolar-S-methyl	2.4	3.5	6.1	1.7	2.6	3.3 ± 1.6 a–d	5.4	3.7	5.4	2.1	4.3	4.2 ± 1.2 a–e
Savory EO	4.6	2.8	2.1	1.9	2.8	2.8 ± 0.5 a–d	1.4	0.9	1.6	1.2	3.8	1.8 ± 1.1 ab
Savory EO ± Thyme EO	5.2	1.3	1.4	1.6	4.1	2.7 ± 1.2 ad	6.3	3.8	2.0	2.6	1.2	3.2 ± 1.0 a–d
Pseudomonas FC7B	8.1	13.8	2.9	9.0	27.0	12.2 ± 8.9 ghi	6.5	15.1	1.8	6.9	3.3	6.7 ± 5.2 b–g
Pseudomonas FC8B	9.9	16.3	23.7	16.6	12.3	15.7 ± 4.2 i	9.1	13.5	2.8	10.5	11.7	9.5 ± 4.1 efg
Pseudomonas FC9B	11.4	16.9	3.2	11.7	9.7	10.6 ± 4.9 f–i	5.8	11.4	2.9	10.6	7.1	7.6 ± 3.4 c–g
FC7B ± FC8B ± FC9B	12.7	16.0	22.2	6.5	17.9	15.1 ± 5.7 hi	5.8	14.6	4.9	9.4	6.8	8.3 ± 3.7 d–g
Prochloraz ± Acibenzolar-S-methyl	6.0	0.2	3.2	0.6	2.5	2.5 ± 1.3 abc	0.7	0.0	1.7	0.2	1.0	0.7 ± 0.7 a
Prochloraz ± Acibenzolar-S-methyl ± FC7B ± FC8B ± FC9B	0.9	2.8	0.3	1.6	1.8	1.5 ± 0.9 ab	0.0	1.7	1.0	0.6	1.9	1.1 ± 0.5 ab
Acibenzolar-S-methyl ± FC7B ± FC8B ± FC9B	3.6	4.6	8.2	2.2	7.1	5.1 ± 2.3 a–f	6.4	4.8	7.5	3.8	2.8	5.1 ± 1.8 a–f
Fusarium 251/2 ± Fusarium MSA35	11.9	11.3	7.5	5.5	14.2	10.1 ± 3.4 fgh	5.4	16.1	5.9	19.6	10.3	11.5 ± 5.3 ghi

* Values followed by the same letter are not statistically different (Duncan's multiple range test, $p<0.05$

Table 3 Obtained and Colby's "expected" level of pathogen control (%) of each seed treatment combination with the dry heat treatment (dry air at 65°C for 10 min) on basil cv Genovese Gigante seeds, previously inoculated with *Fusarium oxysporum* f. sp. *basilici*

Treatment	Colby's "expected" level of pathogen control (%)	Obtained level of pathogen control (%)	Synergy of the treatment when combined with the dry heat treatment (65°C for 10 min)
Prochloraz	40,7*	89,2*	+
Acibenzolar-S-methyl	37,3	76,1	+
Savory EO	38,5	91,5	+
Savory EO + Thyme EO	38,9	89,6	+
Pseudomonas FC7B	21,6	70,0	+
Pseudomonas FC8B	12,6	58,9	+
Pseudomonas FC9B	24,2	66,8	+
FC7B + FC8B + FC9B	8,1	63,0	+
Prochloraz + Acibenzolar-S-methyl	42,1	93,4	+
Prochloraz + Acibenzolar-S-methyl + FC7B + FC8B + FC9B	41,6	93,5	+
Acibenzolar-S-methyl + FC7B + FC8B + FC9B	33,7	73,3	+
Fusarium 251/2 + Fusarium MSA35	24,3	49,2	+

*Mean of all trials

from 100 to 180 g (Figure 2). The values were quite variable, and no statistical difference was therefore observed for the fresh biomass from treated seeds throughout the whole study.

Discussion

In this study, the combined treatments tested on inoculated basil seeds have shown significant results on *F. oxysporum* f. sp. *basilici* control, and they did not affect the seed germination and the fresh biomass obtained from treated seeds. Prochloraz has been shown to be an efficient active compound against the Fusarium wilt of basil when applied as seed dressing, while treatments that involved prochloraz alone or in combination with acibenzolar-S-methyl and the bacterial strain mix have shown interesting results on both pathogen control and seed germination. The use of fungicides has been popular and adopted extensively in seed dressing for many decades (Gullino *et al.* 2014), and including prochloraz in the seed dressing of basil against *F. oxysporum* f. sp. *basilici* could therefore be a useful practice. In the present experiments, a positive effect has also been observed on plants from seeds treated with acibenzolar-S-methyl, as has also been pointed out by Munkvold *et al.* (2014).

The dry hot air treatment has been shown to improve the germination of inoculated seeds, when performed as a pre-treatment but, according to the obtained results, the effect on *F. oxysporum* f. sp. *basilici* control is more critical. It has been possible to observe important reductions in plant infection and the disease index on plants from seeds treated with antagonistic bacteria when heat treated first. The tested dry hot treatment therefore seems to be more compatible with the *Pseudomonas* seed dressing. However, the provided pathogen control of these combined treatments never reached the performance level of the chemical treatments considered as the reference. If the fresh biomass obtained from the seeds treated with the bacterial or fungal strains is considered, it can be observed that no clear growth promotion due to the treatments with biocontrol agents has been registered. The mechanism of action of the tested antagonistic bacterial strains has not been determined in this study, but considering that bacteria need to multiply and be metabolically active in order to contrast the pathogen, the *in vivo* conditions made it possible to attain a good evaluation of the bacterial performance against the Fusarium wilt of basil. The effect of the introduced bacteria may depend on the physiology of the plants and the agronomic conditions of the cultivation (Ugoji *et al.* 2006), therefore it may still be possible to improve the *Pseudomonas* treatments of basil seeds against *F. oxysporum* f. sp. *basilici*.

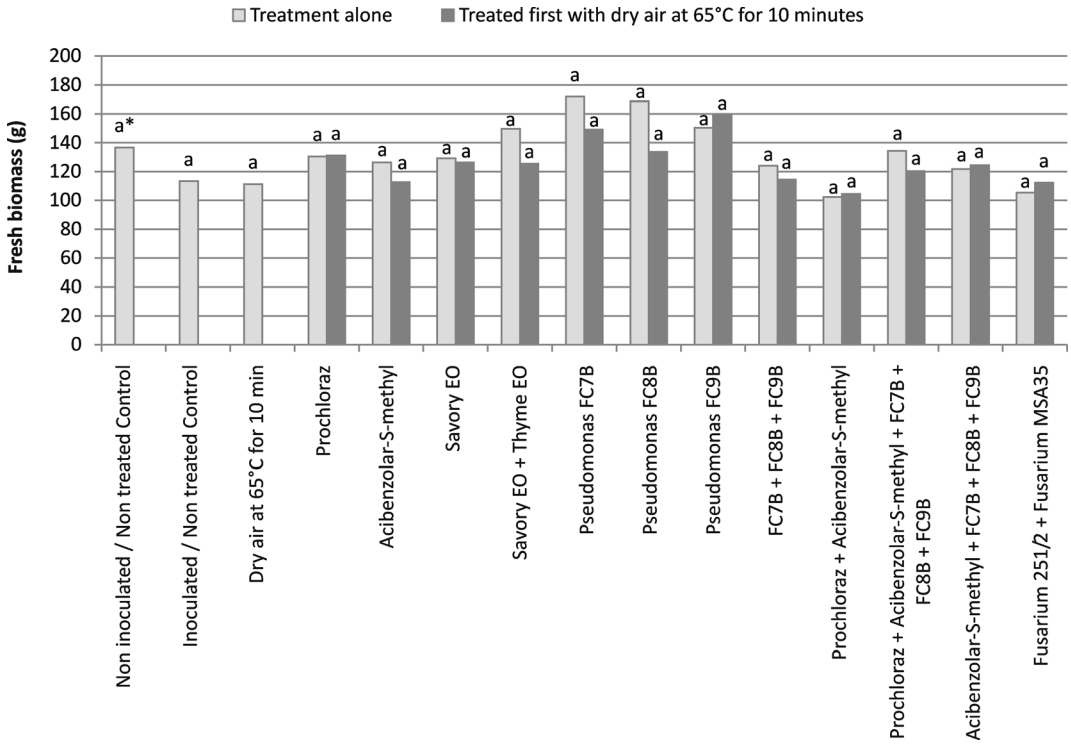

Fig. 2 Fresh biomass (g) of plants from treated basil cv Genovese Gigante seeds, previously inoculated with *Fusarium oxysporum* f. sp. *basilici*, 30 days after sowing. The bars represent the mean of five trials, each trial consisted in 4 replications and each replication consisted in 125 seeds, for a total of 500 per treatment per trial. *Bars followed by the same letter are not statistically different (Duncan's multiple range test, $p<0.05$)

Seed fumigation with savory and thyme essential oils has been confirmed to be active against *F. oxysporum* f. sp. *basilici* and to be safe, as far as seed germination is concerned, as pointed out in previous studies (Lopez-Reyes *et al.* 2015), because of the antifungal activity of the tested essential oils and because of the application method that has been used. The seed treatments with vapours from savory essential oil and from both essential oils applied in combination has not shown any significant difference on the germination rate or on pathogen control. The dry heat pre-treatment has not shown a critical effect on the performance of the essential oil treatments.

According to the analysis, the combinations of the tested treatments with the dry heat pre-treatment resulted as synergic on pathogen control, so dry heat can be safe and compatible with the evaluated strategies for seed disinfection. A two-step disinfection of propagation material including dry heat can be considered then, leading to a reduction of the usage of chemicals in seed treatments.

Seed infection by *F. oxysporum* f. sp. *basilici* has been considered in this study as external, this approach allowed a reliable wilt symptoms evaluation *in vivo*. It is possible to evaluate an internal seed infection, but it is still important to evaluate the performance of tested treatments on naturally contaminated seed lots. Treatments with essential oils and dry heat surely can reach the internal structures of the seed, so it would be interesting to see how efficient they are on pathogen control no matter the level of seed infection.

Treatments with dry hot air represent an interesting alternative for the disinfection of basil seeds, when performed for a short period of time, as in the present experiments. Nevertheless, its efficacy against *F. oxysporum* f. sp. *basilici*, when applied alone to basil seeds, has not been comparable with that obtained with chemical products. Since the advantage of physical treatments is that they can target a number of pathogens at the same time (Koch and Roberts 2014), it might be interesting to consider adopting dry heat seed treatments alone in the organic production of vegetables if the phytotoxicity is also limited. Dry hot air seed exposure

is still open to improvements regarding treatment precision and batch size, and the tested integrated seed disinfection methods could therefore become a feasible and competitive strategy for organic farming.

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