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Abstract

Lettuce Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lactucae*, represents a major problem in most lettuce production areas worldwide. In the present study, a number of resistance inducers, organic amendments and biocontrol agents were applied in a preventative way, in experimental and commercial situations, to soils artificially or naturally infested with race 1 of the pathogen, and to moderately susceptible lettuce cultivars. Potassium phosphite, acibenzolar-S-methyl, green composts, and *Bacillus subtilis* Qst713, *Trichoderma asperellum* + *T. gamsii* and *Pseudomonas* strains achieved the most consistent disease control under the experimental conditions. Moreover, potassium phosphite, green compost, *Bacillus subtilis* Qst713 and *T. asperellum* + *T. gamsii*, also showed a positive effect on plant development. In general, the results of the different treatments in naturally infested soil were similar to those observed in glasshouse trials under artificial

inoculation. Potassium phosphite provided a consistent disease reduction (48 to 62% in artificially infested soil and 60 to 75% in naturally infested soil). The effects of adding 10% compost to a peat growing medium in the nursery, followed by a soil mixing application when lettuce was transplanted, significantly reduced the severity of Fusarium wilt (50-59% efficacy) and increased fresh biomass production. Compost enrichment with *Trichoderma* TW2 generally further increased its efficacy. When tested under field conditions, the commercially available *Trichoderma* spp. and *Bacillus subtilis,* together with experimental strains of *Pseudomonas* and *Trichoderma* spp., applied at the nursery level, provided a disease reduction of 30 to 78%. Early application of the different control measures under nursery conditions and at lettuce transplant is noteworthy because it was carried out at a more localized level, with reduced amounts of products. Their use in practice should be integrated with other control strategies.

Key words: *Fusarium oxysporum* f. sp. *lactucae*, vegetables, preventative treatments, organic farming

1 INTRODUCTION

Approximately 20,000 ha of lettuce is grown in Italy (ISTAT, 2017) in greenhouses or plastic tunnels and in open field, in specialized farms as a principal crop or in rotation with cucurbits and solanaceous crops. Because it is a short seasonal product, most leaf lettuce is transplanted to reach harvest in 55-60 days and 28-40 days in spring and summer plantings, respectively. Leaf lettuces harvested for whole heads include Batavian, Romaine and Butterhead types, often sold in local markets or in supermarkets.

Fusarium wilt of lettuce, caused by *Fusarium oxysporum* f. sp. *lactucae*, is at present the most serious disease of this crop, and causes significant losses throughout the world (Matheron & Gullino, 2012; Gilardi et al., 2017; Subbarao et al., 2017). This pathogen, which was first reported in Taiwan (Matuo & Motohashi, 1967) and in Japan, and was discovered in the United States in 1990, where it was named *Fusarium oxysporum* f. sp. *lactucum* (Hubbard & Gerik, 1993), and was later observed for the first time in Europe, in Italy, in 2002 (Garibaldi et al., 2002). The pathogen spreads easily and quickly, in part due to the fact that it is seed-borne (Garibaldi et al., 2004a; Mbofung & Pryor, 2010); such a feature affects its prevention and management.

In the case of severe disease pressure, which is often present under favourable environmental and cultural conditions, its control is complicated by several factors, including the reduced availability of chemical fumigants for soil disinfestation (Colla et al., 2014; Gordon & Koike, 2015). Although resistant genotypes are recommended to manage the disease (Garibaldi et al., 2004 b; Matheron et al., 2005; Scott et al., 2010; Gilardi et al., 2014b), the development and release of new resistant cultivars is complicated by the high variability of the pathogen, which has already differentiated at least four races (Matheron & Gullino, 2012; Fujinaga et al., 2003; Gilardi et al., 2017). Registered, effective fungicides for post-planting soil applications are limited to conventional cultivation and are prohibited in organic farming, thus providing one more reason for the need of environmentally friendly control methods, including the use of resistance inducers, organic amendments and biological control agents.

Over the past few years, a number of products with different chemical structures and of organisms from different origins that share the ability to induce resistance in the host have been tested and have been applied in practice in some cases (Walters & Fountaine, 2009; Deliopoulos et al., 2010; Walters et al., 2013; Alexandersoon et al., 2016). The increasing interest in their use depends on their broad spectrum of activity, since they generally act on the host instead of on the pathogen, as well as on the possibility of reducing, through their use, the number of fungicide sprays, due to their long-lasting action. In general, resistance inducers are able to activate the inducible signaling

pathways of the host, thus strengthening the plant's defence (Shoresh et al., 2010; Walters et al., 2005, 2013). However, their efficacy is rarely complete (Walters & Fountaine, 2009), because it is generally influenced by several factors, such as the target pathogen (i.e. biotrophic or necrotrophic), the plant genotype and its development stage, the environmental conditions (temperature, relative humidity, disease pressure), the timing, and the formulation and type of application (Walters et al., 2011; 2013).

Other methods under evaluation against Fusarium wilt diseases in horticultural crops (basil, melon, tomato, lettuce, cucumber…) include the use of organic soil amendments, such as compost (Ros et al., 2005; Bonanomi et al., 2007; Borrero et al., 2008; Pugliese et al., 2015; Akhter et al., 2016; Gilardi et al., 2016 a, b; Raza et al., 2017), biochar (Bonanomi et al., 2015; Akhter et al., 2016; Frenkel et al., 2017), and *Brassica* green manure and defeated dried pellet, together with soil solarization (Garibaldi et al. 2010; Gilardi et al., 2014a; Gilardi et al. 2016 a, b). Several examples of success against *Fusarium* wilt agents have been registered using biocontrol agents (BCAs), such as saprophytic *Fusarium oxysporum* (Fravel et al., 2003; Spadaro & Gullino 2005), different *Trichoderma* species (Chet 1997; Yedidia et al., 2003; Harman, 2006; El-Komy et al., 2016) and plant growth-promoting rhizobacteria, including *Pseudomonas* spp. and several strains of *Bacillus* species (Devendra et al., 2009). Additionally, biostimulants, which consist of different substances taken from a broad range of source materials and microorganisms that are able to enhance plant growth, have received increasing attention (Calvo et al., 2014; Le Mire et al., 2016).

Fusarium wilt management relies on a combination of different control measures, which need to be adapted to the different cultural practices adopted in the different cropping systems, and exploited in larger scale experiments, under practical conditions (Katan, 2017).

In the present study, a number of resistance inducers, including phosphite salts, phosethyl aluminum and acibenzolar-S-methyl, organic amendments (compost and biochar), and several biocontrol agents (*Bacillus subtilis; Trichoderma asperellum* + *T. gamsii*; *Tricoderma* spp.; *Pseudomonas putida;* non-pathogenic *Fusarium oxysporum*) were used. The tested products were

applied in a preventative way and tested against Fusarium wilt of lettuce (Race 1) in a greenhouse under controlled conditions, in the presence of artificial inoculation, and on commercial farms using Batavian and Butterhead lettuce types transplanted in naturally infested soil. Their efficacy was compared with that of a chemical fungicide (azoxystrobin).

2 MATERIALS AND METHODS

2.1 Plant material and experimental layout

Ten trials were carried out in 2015, 2016 and 2017 under glasshouse and field conditions in order to test the efficacy of different products, resistant inducers, organic amendments and microorganisms against the Fusarium wilt of lettuce.

A first set of four trials was carried out in an experimental glasshouse in 2015 and in 2016 at the Centre of Competence Agroinnova of the University of Torino, at Grugliasco, in a peat substrate (Tecno 2, 70% white peat and 30% clay, pH 5.5-6, N 110-190 mg L^{-1}, P_2O_5 140-230 mg L^{-1}, K_2O 170-280 mg L^{-1},Turco Silvestro terricci, Bastia d'Albenga, SV, Italy) artificially infested with *Fusarium oxysporum* f.sp. *lactucae* (Table 1).

Lettuce varieties belonging to different types that are grown extensively on commercial farms because of their marketable value were selected. The 'Volare' of the butterhead type, 'Novelsky' and 'Gentilina' varieties belonging to the Batavian type were selected as they are moderately susceptible to Fusarium wilt (Gilardi et al. 2014; 2017). Lettuce seeds (cvs. Gentilina, Maraldi sementi, Volare, Enza Zaden and Novelski, RijkZwaan) were sown in 100-plug trays (2.5 cm Ø per pot, 4-L of soil capacity) filled with a steamed (90°C for 30 minutes) peat mix substrate (blond peat: black peat 15:85, pH 5.5-6.0, 1,100 g m–3 of N:P:K and traces of molybdenum, Turco, Savona, Italy).

On the basis of the preliminary results obtained in glasshouse trials, six trials were carried out using the Novelsky and Volare lettuce cultivars during 2016 and in 2017 under plastic tunnels (Table 2), on an experimental farm, in artificially infested silk-loamy soil (sand : silt : loam 68.16 : 10.7 :21.1

soil, pH 8.2 and 0.94% of organic material) (Trials 5 and 6) and in a sandy-loamy, naturally infested soil (sand : silt : loam 56 : 19 : 25 soil, pH 7.12% and 1.37% of organic material) on a commercial farm (Trials 7- 10), with a history of lettuce cultivation with several crop cycles prior to the beginning of this study, with approximately 45 to 60% of plants affected by Fusarium wilt. Isolations from affected plants revealed that the soil in this farm was naturally infested with race 1 of *F. oxysporum* f. sp. *lactucae* (Tables 2, 7 and 8).

Ten plants/pot, which represented the experimental unit, were kept in a greenhouse on benches with air temperatures ranging from 26 to 32°C during the day and from 20 to 24°C during the night. Pots were arranged in a completely randomized block design in each trial, with four replicated trials, as reported in Table 1.

During the trials carried out under plastic tunnels, 12-16 plants/plot for each cultivars, which represented the experimental unit, were arranged in a complete randomized block design in each trial (Table 2). The lettuce plants were grown according to standard commercial practices (Table 2).

2.2 The pathogen and artificial inoculation

A highly virulent strain of *F. oxysporum* f. sp. *lactucae*, coded Mya3040, belonging to race 1, was used in all trials carried out under the glasshouse (Tables 1, 3 and 4) as well as in trials 5 and 6 conducted on the experimental farms (Tables 2, 5, 6).

The treated seedlings were transplanted into the artificially infested peat soil substrate in 12-L plastic pots using a highly virulent strain of *F. oxysporum* f. sp. *lactucae* (ATCC MYA 3040), isolated in 2002 from infected lettuce plants in north-west Italy. The pathogen inoculum formulated in talc to stimulate chlamydospore development (Locke & Colhoun, 1974) was mixed with the steamed substrate to achieve a final concentration of $1x10^5$ chlamydospores ml^{-1} of substrate. A non-infested substrate was used as a control (Table 1).

For the field trials carried out in the experimental farm, pathogen inoculum was prepared by adding the Mya3040 strain cultured in PDA to wheat kernels previously sterilized for 30 minutes at 121°C.

In order to achieve a uniform soil infestation and high disease incidence, the fungal biomass obtained after 15-days of incubation at 23°C was incorporated in the soil at 30 g/m^2 by rototilling at a depth of 1–20 cm, seven days before transplanting (Table 2).

2.3 Types and timing of treatments

Products known as resistance inducers, organic amendments, organic fertilizer, experimental and commercial biocontrol agents (BCAs) and fungicides were tested (Tables 1 and 2).

Among the resistance inducer products, a mineral fertilizer, based on potassium phosphite (Alexin 95PS, P_2O_5 52%, K_2O 42%, Massò, Spain), and two chemicals, that is, acibenzolar-S-methyl (Bion 50WG, 50% a.i., Syngenta Crop Protection, Italy) and phosethyl-Al (Alliette, 80% a.i, Bayer Crop Science, Italy), were tested.

Among the organic amendments, a green waste compost from a static aerated composting process (Compost 214, Profikomp, Hungary, pH = 8.1, moisture= 27%, Organic C = 17% d. m., Organic N = 1.0% d.m., P_2O_5 = 0.3% d.m., K_2O = 0.7% d.m), a sieved < 20 mm green compost from a dynamic aerated composting process (Ant's Compost 2015V, AgriNewTech, Torino, Italy, pH = 8, moisture= 40%, Organic C = 22% d. m., Organic N = 1.1% d.m., P_2O_5 = 0.7% d.m., K_2O = 1.0% d.m), a sieved < 10 mm green waste compost from a dynamic aerated composting process (Ant's Compost V, AgriNewTech, Torino, Italy, pH = 7.5, moisture= 48%, Organic C = 20% d. m., Organic N = 1.2% d.m., P_2O_5 = 0.8% d.m., K_2O = 1.1% d.m.), a sieved < 10 mm green compost from a dynamic aerated composting process, enriched with the *Trichoderma* strain TW2 (Ant's Compost M, AgriNewTech, Torino, Italy, pH = 7.7, moisture= 48%, Organic C = 20% d. m., Organic N = 1.2% d.m., P_2O_5 = 0.8% d.m., K_2O = 1.1% d.m.), an organic fertilizer based on 26% soluble humic and fulvic acids (K-Humixol, AgriNewTech, Torino, Italy) and an NP organic fertilizer (Help Plus. AgriNewTech, Torino, Italy) were tested. A animal bone biochar, produced, according to the methods described in Someus and Pugliese (2018), from food grade animal bones

with 92% calcium-phosphate (30% $P2O_5$) (Biochar ABC, Terra Humana Ltd, Hungary) was also used.

BCAs included: Serenade WP (15.6 % of *Bacillus subtilis* QST 713, Bayer Crop Science, Italy) and Remedier WP (2+2% of *Trichoderma asperellum* +*T. gamsii*, Isagro Ricerca, Milano, Italy) which are commercially available, the bacterial isolates of *Pseudomonas putida* FC7B (EU836173), *P. putida* FC8B (EU836174); *Pseudomonas* sp. FC9B and the antagonistic *Fusarium oxysporum* MSA35 belonging to the Agroinnova collection (Gilardi et al., 2005; 2007; Clematis et al., 2009) and *Trichoderma* sp. (*Trichoderma* TW2), isolated from composted biowaste, according to the methodology described in Pugliese et al. 2008, which was provided by AgriNewTech (Torino, Italy).

An azoxystrobin fungicide (Ortiva, 23.2% a. i., Syngenta Crop Protection, Italy) was used as the chemical reference.

All the tested resistance inducers and biocontrol agents were applied four times at a nursery level by spraying the soil in each plug tray with a high volume of water (1,800 L ha^{-1}), using a 1 L capacity hand sprayer. The lettuce seedlings from different cultivars grown in each tray were treated by means of a soil treatment with four applications at 5 day intervals, according to the experimental protocols. Azoxystrobin was only applied once, that is, immediately before transplanting.

The first treatment (T0) with the tested resistance inducers and biocontrol agents was carried out on seedlings at the germination stage, while the other three treatments were applied at 5 day intervals.

The products based on compost (Ant's Compost V and Ant's Compost M) were applied using 4 L of substrate (10% v/v) per plug tray at T0, while Ant's Composts 2015V and 214 were mixed in the soil immediately before transplanting at 1 kg/m^2, twenty days after sowing (T20) (Tables 1 and 2). The formulation based on animal bone biochar was mixed with the substrate used to fill the 4 L plug tray at T0 (20 days before transplanting). The organic fertilizer, K-Humixol, was applied in the nursery at T0, while 0.5% of Help Plus was applied at T7 and at T14 in the nursery, and then by root dipping at 2% during transplanting of the lettuce at T21.

203 The timing and the application dosages of the tested products, were based on the manufacturer's
204 suggestions (Tables 3 - 8).
205
206 **2.4 Data collection and analysis**
207 The data were recorded by the appearance of first symptoms (such as yellow leaf and reduced
208 growth) that were observed 10-14 days after the lettuce had been transplanted into infested substrate
209 or naturally infested soil. The number of infected plants showing wilting, stem necrosis and totally
210 wilted (dead) plants was counted to assess disease incidence. The final disease rating was made
211 four-six weeks after transplanting by dissecting each plant. The disease severity (DS) index was: 0
212 = healthy plant, 25 = initial leaf chlorosis, 50 = severe leaf chlorosis and initial symptoms of wilting
213 during the hottest hours of the day, 75 = severe wilting and severe symptoms of leaf chlorosis; 100
214 = plant totally wilted, leaves completely necrotic. At the end of the trials, the total biomass of 10 to
215 16 plants/treatment was weighed in order to evaluate the effect of the tested treatments on fresh
216 weight. All data were subjected to one-way analysis of variance (ANOVA) using SPSS 24 software,
217 and the significance of mean differences was assessed using Tukey's multiple comparison test
218 ($p \leq 0.05$).
219
220 **3. RESULTS**
221 **3.1 Glasshouse trials**
222 The artificial inoculation method adopted in the trials carried out in the greenhouse in peat soil
223 (Trials 1,2,3,4) led to disease severity rating in the control plots, ranging from 54.4 to 81.3 (Table
224 3), thus permitting a good evaluation and comparison of the different treatments. Among the three
225 tested cultivars, cv. Volare was the least susceptible to the pathogen, when artificially inoculated
226 (Table 3).

227 Where plants were artificially inoculated with *F. oxysporum* f.sp. *lactucae*, the best control was
228 provided by azoxystrobin (55 to 93% *DS* reduction), followed by the two chemicals that act by
229 inducing host resistance, that is, phosethyl Al (52 to 81% *DS* reduction) and acibenzolar-S-methyl
230 (43 to 65% *DS* reduction) (Table 3). Disease severity rating was significantly reduced using
231 potassium phosphite (41 to 74% *DS* reduction), the 214 and Ant's 2015V composts at 10% (55 to
232 72% and 62 to 82% *DS* reduction, respectively) and the animal bone biochar (42 to 83% *DS*
233 reduction). The four tested biocontrol agents provided similar results, with *Fusarium oxysporum*
234 MSA 35 and *Bacillus subtilis* QST 713 being slightly more effective (30 to 62% and 32 to 70% *DS*
235 reduction, respectively) (Table 3). The biomass of the plants grown in peat treated with the
236 composts 214 and 2015V, mixed with the substrate used to fill the plug tray or mixed into the soil
237 immediately before transplanting, was significantly improved in comparison to that of the untreated
238 control (+ 222 to 487 g/pot for 'Novelsky', +161 to 288 g/pot for 'Volare'; + 77 to 216 g/pot for
239 'Gentilina'). Potassium phosphite, acibenzolar-S-methyl and the biocontrol agents provided similar
240 increases in lettuce biomass to azoxystrobin (+ 111 to 172 g/pot for 'Novelsky', +47 to 139 g/pot
241 for 'Volare'; + 66 to 84 g/pot for 'Gentilina) (Table 4).

242

243 **3.2 Field trials**

244 Under field conditions, in artificially infested soil (Trials 5 and 6), cv. Volare was confirmed to be
245 less susceptible to Fusarium wilt than the Novelski cv. The tested commercial biocontrol agents
246 (*Bacillus subtilis* and *Trichoderma gamsii+T asperellum*) provided better disease control on the
247 Novelski cv. (disease reduction from 58 to 78%) than on 'Volare' (disease reduction from 44 to
248 49%) (Table 5). The tested experimental BCAs, *Trichoderma* TW2 and the mixture of
249 *Pseudomonas*, both provided variable results (35 to 54% DS reduction and 13 to 55% DS
250 reduction, respectively). The green ANT's V compost, mixed in the plug tray (10% v/v) and
251 applied once immediately before transplanting, significantly reduced severity of Fusarium wilt (51

252 to 60% DS reduction), while the green ANT's M compost provided a partial disease reduction,
253 with statistically different results from those of the inoculated and untreated control (36 to 51%,
254 disease reduction). Azoxystrobin was confirmed to be efficacious (47 to 66% disease reduction),
255 even under field conditions (Table 5). The highest fresh weight of the lettuce plants generally
256 reflected the disease reduction provided by the phosphite-based products, *Bacillus subtilis*, and
257 compost V, with statistically similar results to those of azoxystrobin (Figure 1).
258 Under high disease pressure in the control plots in naturally-infested soil (Trials 7 - 10; Table 6), cv.
259 Volare (DS from 32 to 45) was again less susceptible than cv. Novelsky (DS from 42 to 62). The
260 commercial biocontrol agent, *B. subtilis,* provided up to 50% of disease control on the Novelski cv.
261 (disease reduction from 51 to 60%), while it caused a disease reduction of 30 to 53% on 'Volare'
262 (Table 6). Four treatments, based on the mixture of *Trichoderma gamsii+T asperellum,* showed
263 inconsistent results on the Novelski cv. (disease reduction from 35 to 62%) at the nursery level,
264 while it caused a disease reduction of 35 to 52% on 'Volare' (Table 6). The tested experimental
265 BCAs, *Trichoderma* TW2 and the mixture of *Pseudomonas* also provided variable results (35 to
266 56% DS reduction and 39 to 67% DS reduction, respectively on cv. Novelski and 36 to 51% DS
267 reduction and 40 to 50% DS reduction on cv. Volare).
268 The green ANT's V compost provided statistically similar results to those of the ANT's M
269 compost, which was enriched with *Trichoderma* TW2 (47 to 79% disease reduction on Novelsky
270 and 48 to 78% disease reduction on 'Volare') (Table 6). The best results, in terms of disease
271 severity reduction, was provided by the phosphite-based fertilizers (61 to 75 DS reduction on cv.
272 Novelski and 65 to 71% DS reduction), with statistically similar results to those observed in the
273 azoxystrobin-treated plots. The average lettuce fresh weight ranged from 2.4 to 4.2 kg/m^2 and 1.9 to
274 4.5 kg/m^2 for 'Novelski' and 'Volare' treated with azoxystrobin (Table 7), respectively. The
275 highest fresh weight was provided by the composts (2.7 to 4.7 kg/m^2 for 'Novelsky'and 2.1 to 4.5
276 kg/m^2 for 'Volare') and potassium phosphite (3.2 to 4.3 kg/m^2 for 'Novelsky' and 2.5 to 4.7 kg/m^2
277 for 'Volare'). The BCAs also showed results, in terms of fresh biomass (Table 7): *Bacillus subtilis*

278 significantly improved the fresh biomass (from 2.9 to 3.4 kg /m^2 for Novelski and 2.3 to 4.0 kg/m^2
279 for Volare), and the commercially available *Trichoderma* mixture provided statistically similar
280 results (2.8 to 3.6 kg/m^2 for Novelski and 2.1 to 3.6 kg /m^2 for Volare) to those observed in the
281 azoxystrobin-treated plots.
282

283 **4. DISCUSSION**

284 In recent years, the demand of consumers for more healthful and nutritious food has stimulated
285 increases in vegetable consumption, and changes in the cultivation methods. Such changes have
286 also led to new challenges as far as disease and pest management in lettuce production is
287 concerned (Barrière et al., 2014; Subbarao et al., 2017). Among the various diseases, Fusarium wilt
288 still represent a major problem in most lettuce production areas, and the adoption of a combination
289 of control measures is thus required. Several products alternative to conventional fungicides were
290 tested against *F. oxysporum* f.sp *lactucae,* under controlled conditions, in soil artificially infested
291 with the pathogen. Different methods of application were examined, including soaking seeds in
292 biocontrol agents or plant growth promoting bacteria (Lopez et al., 2014), root immersion of
293 lettuce seedlings in liquid suspensions of selected non-pathogenic *Fusarium oxysporum* (Gilardi et
294 al., 2007), and preventative treatments in a nursery with liquid suspensions of *Bacillus subtilis*
295 Qst713 and potassium phosphite applied on soil surfaces (Gilardi et al., 2016 a), resulting in
296 Fusarium wilt control similar to the reference fungicide used in different studies.
297 In the present study, a number of resistance inducers, organic amendments and biocontrol agents
298 were applied in a preventative way, in experimental and commercial situations, in soils artificially
299 or naturally infested with *F. oxysporum* f. sp. *lactucae* race 1, on lettuce cultivars moderately
300 susceptible to race 1 of the pathogen, in a crop system where lettuce transplanting is a standard
301 practice.

In the first set of trials, it was shown that nursery treatments provided a significant reduction in lettuce Fusarium wilt severity. Overall, potassium phosphite, acibenzolar-S-methyl, green composts, animal bone biochar and the BCAs *Bacillus subtilis* Qst713, *T. asperellum + T. gamsii* and *Pseudomonas* strains achieved the most consistent disease control. Moreover, potassium phosphite, green compost (ANT's V), *Bacillus subtilis* Qst713 and *T. asperellum + T. gamsii* also had a positive effect on plant development and increased the lettuce fresh weight to an equal or even significantly higher level than the healthy control. The best treatments were selected for a second set of trials, which were carried out in a sandy-loam and loamy soil naturally or artificially infested with *F. oxysporum* f.sp. *lactucae* race1. In general, the results provided by the different treatments in the naturally infested soil were similar to those observed in the glasshouse trials in the presence of artificial inoculation with the pathogen.

All the tested treatments significantly reduced the Fusarium wilt symptoms on both lettuce cultivars. Potassium phosphite provided a consistent disease reduction (48 to 62% in the artificially infested soil and 60 to 75% in the naturally infested soil). These findings suggest that treatment in practice at the early stages of cultivation may serve to provide long-term protection.

In the present study, the effects of adding 10% compost to a peat growing medium in the nursery, followed by a soil mixing application at lettuce transplanting, significantly reduced severity of Fusarium wilt (50-59% efficacy) and increased fresh weight of lettuce, compared to the untreated control. This result is in agreement with a previous research in which the addition of compost to sandy soil reduced the disease caused by *Fusarium oxysporum* f. sp. *lactucae* by almost 40% (Franceschini et al., 2016). According to the same research, the rhizosphere of plants grown with compost had a higher total enzymatic activity, a higher total fungal concentration and a greater diversity in the fungal community than that of bacteria, thus suggesting a possible role mediated by fungi.

A further decrease in disease severity, from 10 to 25.9 %, was observed after compost enrichment with *Trichoderma* TW2 in three out of four of the trials carried out in naturally infested soil, using

328 the Novelski lettuce cultivar. However, populations of *Trichoderma* spp. are often abundant in
329 composts (Hoitink & Boehm1999; Pugliese et al., 2008). The here presented results are in
330 agreement with other results that show that an enrichment of composts with *Trichoderma*
331 *asperellum* or *T. harzianum* may enhance their suppressive capacity against Fusarium wilt and
332 other soil-borne pathogens (Cotxarrera et al., 2002; Borrero et al., 2004; Trillas et al., 2006;
333 Pugliese et al., 2011; Blaya et al., 2013). Thus, *Trichoderma* TW2 could be a good candidate for
334 compost enrichment as it can provide a more consistent control of the Fusarium wilt of lettuce,
335 probably because it has already been isolated from compost and is more suitable for use in a
336 substrate based compost. The suppression of *Fusarium* disease through the use of mature composts
337 has mainly been attributed to biotic and abiotic factors (Hoitink & Fahy 1986; Borrero et al., 2004).
338 However, the type of lettuce cultivar and the type of soil can influence its effect.
339 Several studies have shown that the composts used in the nursery industry seem to enhance the level
340 of resistance against foliar and soil-borne pathogens under laboratory conditions (Stone et al., 2003;
341 Veeken et al., 2005; Trillas et al., 2006). *Fusarium oxysporum* f. sp. *lini* pathogen is reorted to be
342 suppressed more by composts than other soil-borne pathogens (*Rhizoctonia solani, Verticillium*
343 *dahliae* and *Phytophthora* spp. (Termorshuizen et al., 2006).

344 The results of this study provide evidence on how to improve the efficacy of a compost in
345 preventative nursery treatments against lettuce Fusarium wilt through the introduction of a selected
346 *Trichoderma* strain, and show the possibility of their adoption on commercial farms.

347 The difficulties encountered in the registration and commercialization of new chemicals, together
348 with the increasing limitations to the use of the already available ones, make resistance inducers
349 and organic amendments a more attractive option. More studies on how products known to induce
350 host plant defense and compost can be applied (e.g., season, frequency and dose) to achieve the
351 maximum efficacy in practical field conditions against specific diseases are necessary.

352 The Fusarium wilt severity reduction using animal bone biochar has been demonstrated in this
353 work under controlled experimental conditions, and further studies are necessary under commercial
354 field conditions. However, the use of animal bone biochar as a constituent of growing mixtures has
355 been shown for the first time. Our results agree with those of other researches, where quest and
356 coconut charcoal, wood and green waste biochars reduced disease caused by *Fusarium oxysporum*
357 f.sp. *asparagi* (Matsubara et al., 2002; Elmer & Pignatello 2011) and *F. oxysporum* f.sp.
358 *lycopersici* (Akhter et al., 2016). Previous studies reported that biochar induced a plant diseases
359 reduction when applied at lower concentrations (≤1% v:v), whereas it has been found to be
360 ineffective on disease at higher concentrations (>3% v:v) (Frenkel et al., 2017). These results were
361 confirmed in our study, in which animal bone biochar was applied at 1% and provided a reduction
362 in the Fusarium wilt of lettuce. Again according to Frenkel et al., 2017, a positive influences on
363 plant growth when biochar is used instead of peat media is mostly observed when the biochar is
364 applied in higher concentrations than 25% v:v, and this has also been confirmed in our lettuce
365 experiment, where animal bone biochar increased the fresh weight of lettuce, compared to the
366 untreated control, but not compared to healthy control and other treatments based compost and
367 potassium.
368 Humic acids + NP fertilizers were only used in the second set of trials, and they reduced disease
369 severity in 3 out of 4 trials in naturally infested soil. These treatments also increased lettuce fresh
370 weight in 1 out of 2 trials in artificially infested soil. Similar results have been reported by Afifi et
371 al. (2017), who found that humic and fluvic acids reduced the disease severity of
372 *Fusarium oxysporum* f. sp. *cucumerinum* and improved the growth of cucumber plants in a
373 greenhouse. However, our results are in contrast with those of Yigit and Dikilitas (2008), who
374 found that humic and fluvic acids caused tomato plants to be more susceptible to the root-rot
375 diseases caused by *Fusarium* spp. The positive results we observed may be explained by the
376 formation of complexes with humic substances, which inhibit the growth of *F. oxysporum* f. sp.

lactucae (Oshima et al., 2015). Further work is needed to better understand the interactions that take place at the rhizosphere level.

In the present study, commercially available *Trichoderma* spp. and *Bacillus subtilis*, together with experimental strains of *Pseudomonas* and *Trichoderma* spp., applied at the nursery level, provided a disease reduction of 30 to 78%, when tested under field conditions. Moreover, *Bacillus subtilis* and the *Trichoderma* mixture were able to significantly improve the fresh biomass of lettuce. The improved nutrient status of the plant as a result of solubilization of phosphates has also been reported as a growth promotion mechanism of *Bacillus* spp. *Pseudomonas*, and *Trichoderma* (Babalola, 2010). Such a capability has been explained, in part, in the case of the Fusarum wilt of tomato, by the production of lipopeptide antibiotics by *B. subtilis*, which are known for their strong antifungal potential towards necrotrophic phytpathogenic fungi, and/or the induction of host defense through the involvement of chitinase genes (Ramyabharathi & Raguchander 2014; Abdallah et al., 2017). Unfortunately, such a level of protection and the positive effect on yield are not so interesting for growers, in part due to the lack of consistency. The formulation and the application method are probably among the most critical parameters that determine the efficiency of biocontrol products (Bashan et al., 2014). The use of preventative treatments in the nursery will lead to a more efficient use of biocontrol agents. Furthermore, the reproducibility of the effects of microbial inoculants needs to be tested across a wider range of soil types and local environmental conditions (soil temperature and agronomic practices) (Kloepper, 1996; Vallad & Goodman 2004; Walters et al., 2011). Their use in practice should be integrated with other control strategies, involving host plant resistance and pesticides.

ACKNOWLEDGEMENTS

401 The present study has received funding from the European Union's Horizon 2020 Research and
402 Innovation Programme EUCLID EU-CHINA Lever for IPM Demonstration. Grant agreement no.
403 633999 and from the European Union Seventh Framework Programme (FP7/2007- 2013) under
404 grant agreement n° 289785 (Collaborative project REFERTIL: Reducing mineral fertilisers and
405 chemicals use in agriculture by recycling treated organic waste as compost and biochar products).
406 The authors kindly thank Marguerite Jones for the language revision.

407

408 Conflict of interest

409 Massimo Pugliese declares he has a financial interest (shareholder) in the company AgriNewTech
410 that provided some of the products tested in this paper.

411

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Table 1. General information and date (day) of the activities conducted in the first set of trials in a glasshouse in peat soil artificially infested with *Fusarium oxysporum* f. sp. *lactucae*.

Activity	Trial 1	Trial 2	Trial 3	Trial 4
Plug-tray treatment	09/10/15	21/01/16	06/05/16	07/09/16
	14/10/15	26/01/16	11/05/16	12/09/16
	20/10/15	02/02/16	16/05/16	16/09/16
	26/10/15	7/02/16	20/05/16	21/09/16
Compost treatment in the nursery	1/10/15	11/01/16	27/05/16	29/08/16
Compost treatment in the pot	26/10/15	7/02/16	20/05/16	21/09/16
Azoxystrobin	26/10/15	7/02/16	20/05/16	21/09/16
Artificial inoculation of the peat substrate	14/10/15	26/01/16	11/05/16	12/09/16
Transplanting	26/10/15	07/02/16	20/05/16	21/09/16
End of the trial	15/12/15	024/03/16	24/06/16	14/09/16

631 **Table 2.** General information date (day) of the activities carried out in the in the second set of trials
632 to validate the nursery treatments on commercial and experimental farms.

Trial	5	6	7	8	9	10
Site	Carmagnola (TO)	Carmagnola (TO)	Moretta (CN)	Moretta (CN)	Moretta (CN)	Moretta (CN)
Soil infestation with FOL	Artificial	Artificial	Natural	Natural	Natural	Natural
Lettuce cultivar	Novelski, Volare	Novelski, Volare	Novelski, Volare	Novelski	Novelski, Volare	Novelski, Volare
Sowing	02/06/16	18/08/17	30/05/16	02/06/16	23/05/17	18/08/17
Plug-tray treatment	09/06/16; 13/06/16; 17/06/16; 21/06/16	21/8/17; 25/8/17; 30/8/17; 4/09/17	02/06/16; 06/06/16; 10/06/16; 15/06/16;	09/06/16; 13/06/16; 17/06/16; 21/06/16	26/05/17; 30/05/17; 5/06/17; 9/06/17	21/08/17; 25/08/17; 30/08/17; 04/09/17
Compost treatment in the nursery on a	2/06/16	18/08/17	-	02/06/16	23/05/17	18/08/17
Compost treatment in the field	22/06/16	05/09/17	15/06/16	22/06/16	09/06/17	04/09/17
Azoxystrobin treatment	21/06/16	4/09/17	15/06/16	21/06/16	09/06/17	04/09/17
Transplanting	22/06/2016	05/09/17	15/06/16	22/06/16	09/06/17	04/09/17
End of the trial	03/08/2016	23/10/17	25/07/16	04/08/16	27/07/17	27/10/17

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Table 3. Effect of the nursery treatments on Fusarium wilt on cvs. Volare, Novelski and Gentilina grown in an artificially infested peat substrate. The data are expressed as disease severity 0-100 at the end of the greenhouse trials.

Treatment	Formulation	Dosage a.i.	cv. Novelski				cv. Volare				cv. Gentilina			
			Trial 1		Trial 2		Trial 3		Trial4		Trial 3		Trial 4	
Inoculated untreated control	-	-	64.4	f[a]	81.3	e	54.4	c	56.3	d	77.5	b	72.5	f
Non-inoculated control	-	-	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a
Bacillus subtilis QST713	Serenade Max	0.63 g/L	29.4	de	41.9	cd	23.8	b	38.1	b-d	23.1	b	30.0	b-e
Trichoderma asperellum + T. gamsii	Remedier	0.04 g/L	31.3	de	41.3	cd	25.0	bc	40.0	cd	45.6	b	41.9	c-e
Pseudomonas 9FC	Agroinnova'*Pseudomonas*	1x10^7 CFU/ml	33.1	e	52.5	de	21.9	b	36.9	b-d	36.3	b	48.1	ef
Fusarium oxysporum MSA 35	Agroinnova' *F.oxysporum*	1x10^7 CFU/ml	28.8	c-e	46.9	cd	20.6	b	39.4	b-d	41.3	b	41.9	de
Acibenzolar-S-methyl	Bion 50 WG	0.025 g/L	26.3	c-e	30.0	b-d	17.5	b	31.9	bc	29.4	b	25.6	b-e
Potassium phosphite P:K 52:42	Alexin	1.3+1.06 g/L	16.9	bc	35.6	cd	16.3	ab	33.1	bc	31.3	b	30.6	b-e
Phosethyl-Al	Aliette	1.6 g/L	12.5	b	26.3	bc	12.5	ab	28.1	bc	37.5	b	29.4	b-e
Green compost [b]	Ant's compost 2015 V	10%	19.4	b-d	14.4	b	13.8	ab	19.4	b	29.4	b	20.0	b-d
Green compost[b]	Ant's compost 2015 V	1 kg/m^2	25.0	c-e	26.9	bc	16.3	ab	23.1	bc	41.3	b	19.4	bc
Green compost [b]	Compost 214	10%	28.1	c-e	33.1	cd	15.0	ab	24.4	bc	33.3	b	32.5	b-e
Animal bone biochar[b]	ABC	1%	25.0	c-e	36.3	cd	19.4	b	27.5	bc	46.7	b	41.9	de
Azoxystrobin	Ortiva	0.19 g/L	4.4	a	30.0	bcd	5.6	a	25.6	bc	31.3	b	15.0	b

[a] Values in the same column followed by the same letter are not significantly different, according to Tukey's HSD test (p=0.05)

[b] Applied at sowing at 1 or 10% or by mixing in the soil before transplanting at 1 kg/m^2.

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646 **Table 4.** Effect of the nursery treatments on the biomass of cvs. Volare, Novelsky, and Gentilina grown in
647 an artificially infested peat substrate. The data are expressed as fresh weight (g/m^2) at the end of the trials.

Treatment	Formulation	Dosage a.i.	cv. Novelski				cv. Volare				cv. Gentilina			
			Trial 1		Trial 2		Trial 3		Trial 4		Trial 3		Trial 4	
Inoculated Untreated Control	-	-	144.5	d[a]	32.5	f	124.7	e	86.2	e	43.4	b	52.6	c
Non-Inoculated Control	-	-	377.2	b	205.4	bc	344.6	ab	225.5	ab	135.6	a	227.9	a
Bacillus Subtilis Qst713	Serenade Max	0.63 g/L	361.4	b	131.4	de	302.8	a-c	143.9	c-e	118.4	a	154.7	b
Trichoderma asperellum + *T. gamsii*	Remedier	0.04 g/L	362.8	b	150.5	c-e	358.2	ab	126.0	c-e	102.2	a	150.4	b
Pseudomonas FC7B+ FC8B +FC9	Agroinnova'*Pseudomonas*	1x10^7 CFU/ml	243.8	b-d	120.0	de	266.9	b-e	147.5	cd	111.0	a	96.0	bc
Fusarium oxysporum Msa 35	Agroinnova' *F.oxysporum*	1x10^7 CFU/ml	256.1	b-d	121.0	de	187.7	c-e	153.1	cd	108.2	a	113.3	bc
Acibenzolar-S-Methyl	Bion 50 Wg	0.025g/l	283.9	bc	95.1	e	170.9	c-e	119.6	de	114.8	a	137.1	b
Potassium Phosphite P:K 52:42	Alexin	1.3+1.06 g/L	357.6	b	172.2	cd	288.3	a-d	125.1	de	123.2	a	143.9	b
Phosethyl-Al	Aliette	1.6 g/L	228.9	cd	146.7	c-e	243.7	b-e	184.6	bc	107.4	a	125.5	b
Green compost [b]	Ant's compost 2015 V	10%	525.4	a	281.2	a	434.5	a	254.7	a	130.8	a	268.8	a
Green compost[b]	Ant's compost 2015 V	1 kg/m^2	631.8	a	254.9	ab	383.0	ab	265.6	a	120.0	a	245.4	a
Green compost [b]	Compost 214	10%	360.6	b	119.7	de	285.5	b-d	228.8	ab	127.8	a	227.7	a
Animal bone biochar[b]	ABC	1%	197.4	cd	115.9	de	254.6	b-e	99.6	de	98.6	a	130.5	b
Azoxystrobin	Ortiva	0.19g/L	316.9	bc	143.5	de	264.1	b-e	133.3	c-e	109.0	a	136.2	b

648 [a] Values in the same column followed by the same letter are not significantly different, according to Tukey's
649 HSD test (p=0.05)
650 [b] Applied at sowing at 1-10% or by mixing in the soil before transplanting at 1 kg/m^2.
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654 **Table 5.** Effect of the nursery treatments on Fusarium wilt on cvs. Novelsky and Volare. The data are
655 expressed as disease severity (0-100) at the end of the experimental farm trials, in artificially inoculated soil.

Treatment	Formulation	Dosage a.i.	cv. Novelski		cv. Volare	
			Trial 5	Trial 6	Trial 5	Trial 6
Untreated control	-	-	67.2 d[a]	49.0 c	53.1 b	41.3 c
Bacillus subtilis	Serenade max	0.63 g/L	14.8 a	17.3 a	32.0 a	22.7 ab
Trichoderma asperellum +	Remedier	0.04 g/L	43.0 b-	27.1 a-	39.8 ab	20.8 ab
Pseudomonas FC7B+	Agroinnova'*Pseudomonas*	1x10^7 CFU /ml	30.3 a-	34.4 bc	35.2 a	35.4 bc
Trichoderma TW2	ANT'S *Trichoderma*	1x10^7 CFU/ml	44.5 b-	28.3 a-	38.4 ab	18.8 ab
Potassium phosphite 52-42	Alexin	1.3+1.06 g/L	25.4 a-	20.6 ab	25.8 a	20.8 ab
Green Compost +	ANT'S Compost M[b]	10% +1kg/m^2	28.5 a-	20.4 ab	35.5 ab	19.8 ab
Green Compost	ANT'S Compost V[b]	10% +1kg/m^2	27.2 a-	19.8 ab	33.6 a	16.7 a
Humic acids + NP fertilizers	K-Humixol and Help	0.5% and 0.5% -	56.3 cd	24.5 a-	44.1 ab	19.8 ab
Azoxystrobin	Ortiva	0.19g/L	22.7 ab	19.0 ab	33.0 a	21.7 ab

656 [a] Values in the same column followed by the same letter are not significantly different, according to Tukey's
657 HSD test (p=0.05)
658 [b]Applied at sowing at 10% and by mixing in the soil before transplanting at 1 kg/m^2.
659 [c] K-Humixol was applied at sowing at 0.5%, followed by Help Plus used at T7 (0.5%) and at T14 (2%) in
660 the nursery and by root deeping at T21 (2%).
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Table 6. Effect of the nursery treatments on Fusarium wilt on cvs. Novelsky and Volare. The data are expressed as disease severity (0-100) at the end of the commercial farm trials, in naturally infested soil.

Treatment	Formulation	Dosage a.i.	cv. Novelski				cv. Volare		
			Trial 7	Trial 8	Trial 9	Trial 10	Trial 7	Trial 9	Trial 10
Untreated control	-	-	41.8 b[a]	58.4 b	52.7 c	61.7 c	44.1 b	32.4 c	44.5 c
Bacillus subtilis	Serenade max	0.63 g/L	20.0 a	23.3 a	25.8 ab	22.1 a-c	20.6 a	22.7 a-c	24.0 a-c
T. asperellum + *T gamsii*	Remedier	0.04 g/L	27.2 ab	23.0 a	27.0 ab	29.5 a-c	24.4 a	15.6 ab	23.5 a-c
Pseudomonas FC7B+ FC8B +FC9B	Agroinnova'*Pseudomonas*	1x10^7 Cell/ml	25.4 ab	34.4 a	17.6 a	35.2 a-c	21.9 a	16.4 ab	26.5 a-c
Trichoderma TW2	ANT'S *Trichoderma*	1x10^7 CFU/ml	24.2 ab	30.8 a	23.4 ab	36.4 a-c	25.8 a	15.9 ab	28.4 a-c
Potassium phosphite 52-42	Alexin	1.3+1.06 g/L	16.3 a	19.8 a	21.1 ab	15.2 ab	15.5 a	9.4 a	14.8 ab
Green compost + *Trichoderma* TW 2	ANT'S Compost M[b]	10% +1kg/m^2	16.0 a	23.4 a	28.2 ab	13.0 a	19.2 a	16.8 ab	10.0 a
Green compost	ANT'S Compost V[b]	10% +1kg/m^2	23.2 ab	29.5 a	19.9 a	29.0 a-c	23.9 a	12.7 ab	18.0 a-c
Humic acids + NP fertilizers	K-Humixol and Help Plus [c]	0.5% and 0.5% - 2%	26.0 ab	27.0 a	20.3 ab	39.8 a-c	21.9 a	14.1 ab	27.3 a-c
Azoxystrobin	Ortiva	0.19g/L	15.6 a	22.2 a	22.3 ab	19.8 ab	24.9 a	12.1 ab	23.3 a-c

[a] Values in the same column followed by the same letter are not significantly different, according to Tukey's HSD test (p=0.05)

[b] Applied at sowing at 10% and by mixing in the soil before transplanting at 1 kg/m^2.

[c] K-Humixol was applied at sowing at 0.5%, followed by Help Plus used at T7 (0.5%) and at T14 (2%) in the nursery and by root deeping at T21 (2%).

Table 7. Effect of the nursery treatments on the yield of cvs. Novelsky and Volare. The data are expressed as fresh weight (g/m^2) at the end the of commercial farm trials, in naturally infested soil.

Treatment	Formulation	Dosage a.i.	cv. Novelski								cv. Volare					
			Trial 7		Trial 8		Trial 9		Trial 10		Trial 7		Trial 9		Trial 10	
Untreated control	-	-	1557.6	b[a]	2021.8	c	987.5	b	963.5	e	1562.1	d	1947.0	b	1489.1	e
Bacillus subtilis	Serenade max	0.63 g/L	3026.0	a	2935.9	a-c	2929.5	a	3409.1	a-c	2321.7	ab	4014.0	a	3204.0	a-c
T. asperellum + T gamsii	Remedier	0.04 g/L	2938.1	a	2788.5	a-c	2809.5	a	3585.5	a-c	2170.2	bc	3673.5	a	3078.0	b-d
Pseudomonas FC7B+ FC8B +FC9B	Agroinnova'*Pseu domonas*	1x10^7 CFU/ml	3229.5	a	2253.9	bc	2829.5	a	1910.0	de	2308.1	ab	3730.5	a	2308.1	c-e
Trichoderma TW2	ANT'S *Trichoderma*	1x10^7 CFU/ml	1750.8	b	2258.3	bc	2943.8	a	2397.0	b-e	1691.3	cd	4221.0	a	2801.6	b-e
Potassium phosphite 52-42	Alexin	1.3+1.06 g/L	3298.2	a	4270.2	a	3937.4	a	4377.0	a	2482.5	ab	4722.0	a	3561.5	a-c
Green compost + *Trichoderma* TW 2	ANT'S Compost M[b]	10% +1kg/m^2	2716.1	ab	4338.4	a	2859.3	a	4707.5	a	2726.7	a	3565.5	ab	4459.5	a
Green compost	ANT'S Compost V[b]	10% +1kg/m^2	3133.5	a	3845.6	ab	2905.5	a	4684.5	a	2102.3	b-d	3730.5	a	3926.6	ab
Humic acids + NP fertilizers	K-Humixol and Help Plus [c]	0.5% and 0.5% - 2%	1695.0	b	3466.1	a-c	3937.4	a	3965.6	a-c	1742.0	cd	3961.5	a	2851.1	b-d
Azoxystrobin	Ortiva	0.19g/L	2601.0	ab	3053.6	a-c	4138.5	a	4200.5	ab	1939.7	b-d	4552.5	a	3157.1	a-c

[a] Values in the same column followed by the same letter are not significantly different, according to Tukey's HSD test (p=0.05)

[b]Applied at sowing at 10% and by mixing in the soil before transplanting at 1 kg/m^2.

[c] K-Humixol was applied at sowing at 0.5%, followed by Help Plus used at T7 (0.5%) and at T14 (2%) in nursery and by root deeping at T21 (2%).

Fig.1. Effect of the nursery treatments on the yield of cvs. Novelsky and Volare. The data are expressed as fresh weight (g/m^2) at the end of experimental trials 5 and 6, in artificially inoculated soil. Values with the same letter are not significantly different, according to Tukey's Test (p<0.05).