Microbiological features associated with the suppression of Fusarium wilt of lettuce studied by applying a single selected compost.

Microbiological features of compost-mediated suppression to *Fusarium* wilt of lettuce

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Abstract

The effect of compost suppressiveness toward the *Fusarium* wilt of lettuce was studied using a polyphasic approach. Bioassays were carried out in controlled conditions to allow a standardization of the environment in order to minimize disturbances and variability of soil microbial communities. Compost addition demonstrated significant efficacy in controlling the disease. Microbial activities, bacterial and fungal loads were quantified and correlated in a Principal Component Analysis in order to clarify the correlation between original variables. The samples were well distinguished between the substrates where the plants were grown and the rhizosphere samples.

DGGE was used to track microbial communities along the bioassays. The approach demonstrated to be useful to detect either bacterial or fungal species that preferentially associated with roots of seedlings, and hence involved in the compost-mediated suppression, with a high probability to be vital microorganisms because not detected in the substrate where the plants were grown at the start of the experiment.

Introduction

Fusarium oxysporum f. sp. *lactucae* is an economical important pathogen of *Lactuca sativa*. The pathogen was reported for the first time in Japan in 1955, then in California in 1990. Elsewhere it is present in Iran and Taiwan. It was first reported in Europe by Garibaldi (*et al*. 2002) but is was found also in Latin America (Ventura and Costa 2008). The pathogen can be seedborne allowing therefore an easy movement between continents (Garibaldi *et al*. 2004a).

Disease control is normally carried out through crop rotation and genetic resistance (Scott *et al*. 2011). Among preventive measure, seed dressing have been proved to be successful in pathogen control (Gilardi *et al*. 2005). The use of biological control agents is still in the experimental stage (Fera 2011).

Compost, improving directly and indirectly soil properties, represents one of the most important and cost-effective tool to control soil-borne plant pathogens and it is indeed one of the alternative to be assayed in the control of *Fusarium* wilt of lettuce. In literature there are many examples of

containment of soilborne diseases resulting from the application of compost (Noble and Coventry 2005; Hadar and Papadopoulou 2012). Compost amendment acting either on the size and the composition of soil microbial communities (Saison *et al*. 2006) can lead to control of plant diseases. Compost suppressiveness varies depending on different pathosystems and compost applied (Termorshuizen *et al*. 2006; Yogev *et al*. 2006) but it is almost agreed in the scientific community that the phenomenon of suppressiveness fully involves microbial communities. Among compost bacteria the groups that majorly influence the development and suppressive activity of soil microbial communities are *Bacillus* and *Pseudomonas* (Zaccardelli *et al*. 2013), but soil and rhizophere microbial associations could underlie to different shifts either in composition or size in suppressive soils or substrates (Hagn *et al*. 2008; Meng *et al*. 2012; Klein *et al*. 2012).

Due to the complexity of the ecological framework in which soil microorganisms and plants interact, polyphasic approaches should be adopted to study the mechanisms that drive the suppressiveness of the disease (Garbeva *et al*. 2004; Boutler-Bitzer *et al*. 2006). The importance to look in the rhizophere to detect significant microbial associations and novel microorganisms involved in disease control has been also pointed out recently (Huang *et al*. 2013; Mendes *et al*. 2013).

Polyphasic approaches applied to study soil and compost microbial population structures and assemblages usually combine different biochemical, cultural and molecular assays with different aims and resolutions (Saison *et al*. 2006; Nocker *et al*. 2007; Gardner *et al*. 2011; Gao *et al*. 2012 Suàrez–Estrella *et al*. 2012).

Among biochemical assays fluorescein diacetate (FDA) can be hydrolysed by many enzymes (lipases, proteses and esterases) and organisms providing a broad-spectrum indicator of soil and compost biological activity (Schnürer and Rosswall 1982; Adam and Duncan 2001; Ryckeboer *et al*. 2003; Komilis *et al*. 2011) but β-glucosidase is also considered a good enzymatic indicator (Abellan *et al*. 2011; Gardner *et al*. 2011).

Among community-level molecular techniques applied in microbial ecology, DGGE is, together with T-RFLP one of the most widely used PCR-based method for the study of fungal and bacterial assemblages (Marshall *et al*. 2003; Cherif *et al*. 2008; Danon *et al*. 2010; Maeda *et al*. 2010; Ferrocino *et al*. 2013).

The aim of the present study was: 1) to assay the applicability of municipal waste-based compost against *Fusarium oxysporum* f. ap. *lactucae*; 2) to typify the microbiological features that characterize suppressive assays.

To reach those objectives experimental trials, in presence and absence of compost, have been conducted in growth chamber and microbiological parameters have been monitored along the experiment in order to detect differences that could be attributed to the observed suppressive effect of compost. DGGE profiles have been obtained for either the substrates at the beginning of the experiments or for the rhizophere samples in order to detect the active microflora involved in the process.

The combined approach adopted in this study demonstrates to be effective to typify suppressive substrate on the base of microbiological features while DGGE suggests the involvement of either fungi, especially *Simplicillium lamellicola*, or bacteria, especially *Pseudomonas*, in the observed control of the disease.

Materials and methods

Experimetal approach

Compost used in this study was prepared from green wastes, organic domestic wastes and urban sludges by ACEA Pinerolese SpA (Pinerolo, Torino, Italy). This compost was chosen because this product showed a good suppressive activity in previous trials (Pugliese et al. 2007).

To assess the effect of compost amendment on disease development of *Fusarium* wilt of lettuce and on the rhizosphere microbial communities, the experimental design included the presence and the absence of the pathogen and the application of ACM compost at 0 and 1% concentration (v/v).

Experiments have been carried out in a climatic chamber at optimum temperature for pathogen development (27±2°C). Disease progress was checked weekly.

Sampling were carried out at the time of plants transplant (Ti) and at the end of the disease suppression experiment after 35 days (Tf).

At each sampling time following parameters were evaluated: a) enzymatic activities through the Fluorescein Diacetate (FDA) hydrolysis and the β-glucosidase assays; b) estimate of the fungal and bacteria total load; c) molecular fingerprinting of the bacterial and fungal population through the application of DGGE technique.

Growing media and inoculum preparation

The isolate of *Fusarium oxysporum* f.sp. *lactucae* MYA30/40 was used in bioassays. The fungus was grown in Potato Dextrose Broth. After two weeks the mycelia was collected through centrifugation at 15000g for twenty minutes. A chlamydospore powder for inoculation was produced adding talc in the ratio 4:1 respect to the fresh weight of the mycelia. The powder was allowed to dry for further two weeks at room temperature and mixed thoroughly to obtain a fine and homogenous inoculum source.

The concentration of the powder was assessed through dilution plating in Potato Dextrose Agar amended with 60mg/L of streptomycin. The inoculum was added to the substrates to reach the final concentration of 5×10^4. Growing media consisted in steamed sandy soil to which the compost was added at a rate of 100ml per liter. Control thesis did not contain the pathogen.

One month old lettuce seedlings of “crispilla bianca” variety were root washed to remove any peat debris from the root and transplanted in 1 lt pots in number of 10 per thesis.

In vivo experiments were conducted twice in a climatic chamber at an optimum temperature for growth of the pathogen and humidity-controlled (27 ° C, 80-90 % HR).

Disease progress

Plant disease assessment was carried out weekly following a disease scale of 5 points from 0 (healthy plant) to 4 (dead plant). The Area Under Disease progress Curve (AUDPC) and the Suppressive Capacity (SC%) Termorshuizen *et al*. (2006).

Sampling and microbiological parameters estimate

Samples analyzed were collected at the time zero (Ti=Tinitial) of the experiment and at the end of the experiment (Tf=Tfinal) either for inoculated or not inoculated theses. Ti samples were representative of the mixtures used for setting the experiment whether at the Tf samples were collected from the rhizophere of plants (amended and not amended; inoculated and not) through shaking the root apparatus in sterile petri dishes after carefully digging out the plant from the pot. Single samples were composed of a bulk of the rhizophere sample from the same thesis. The single unit for all the microbiological and molecular assays was 0.5g.

Enzymatic activity was measured through FDA following the protocols of Ryckeboer *et al*. (2003) and Adam *et al*. (2001) and β-glucosidase was carried out according to Andres Abellan *et al*. (2011).

The total fungal and bacterial load were determined using the dilution plating technique. Briefly 0.5g of sample was suspended in 50ml of ¼ strength Ringer (Merk®, Germany) solution and two drops of tween (Sigma Aldrich®, Germany) and incubated in a rotary shaker for one hour. Serial dilution were spread on Potato Dextrose Agar amended with 60mg/L of streptomycin for fungi and Luria Bertani Agar for bacteria.

DGGE

153 DNA was extracted from each sample unit using NUCLEO SPIN SOIL KIT (Macherey-Nagel GmbH &
154 Co. KG) following manufacturers' instructions, with a final elution step in MilliQ autoclaved water
155 as for fungal DNA.
156 Fungal DNA was amplified using NS1/GCFung primers according to the protocol of Gao *et al*.
157 (2012) while for bacteria 1070F/1392R as developed by Nocker *et al*. (2007) was used.
158 Each PCR mixture contained 10 ng of DNA, 2µl of primer 10 mM, 4µl 2.5mM DNTPS, 2.5µl of
159 $MgCl_2$, 5 µl of 10XBuffer, and water up to 50µl of reaction volume.
160 The amplicons (20µl), were loaded in 8% acrilammide, 30-70% gradient, gels for bacteria and in
161 6% acrilammide, 25-45% gradient, gels for fungi. In both cases the gels were run at 60°C for 16 h
162 at 75V. Images were acquired with Geldoc BIORAD®. DGGE bands were excised, re-amplified
163 and sequenced using the forward primer either for bacteria (1070F) or for fungi (NS1) to determine
164 the identity of dominant taxa.
165
166 *Statistical Analysis*
167 All datasets were assessed for the normality of variance (Shapiro Wilk W test) assuming 0.05 as α-
168 value. The data of the two experiments were pooled together after the verification of the
169 homogeneity of variances according to the Levene's test ($P>0.05$). Unless otherwise stated,
170 ANOVA and Tukey's post hoc test was used to verify the statistical significance of differences at a
171 $P<0.05$, unless otherwise specified. All statistical analyses were carried out with SPSS (IBM SPSS
172 Statistics 21, NY, US) except PCA that was elaborated with PAST (Hammer *et al*. 2001)
173
174 ***Results and Discussion***
175 *Compost suppressive capacity*
176 Mean disease severity in the plants inoculated with *Fusarium oxysporum* f. sp. *lactucae* was 2.85 ±
177 0.37 for the not amended thesis and 1.55 ± 0.45 for the compost amended thesis. Level of disease
178 in untreated plants agreed with the susceptible behavior typical of the cultivar crispilla (Garibaldi *et*

179 *al*., 2004b). The addition of 1% compost lead to a suppressive capacity of 41% with significant
180 differences between either the mean disease severity and the AUDPC values (Figure 1). No disease
181 were observed in control theses.
182
183 *Microbiological parameters*
184 All microbiological parameters were higher at the Tf (Table 1 and 2), confirming a highest
185 microbial activity around the roots of plants. As expected in general enzymatic activities
186 significantly higher in the 1% theses confirming the ability of the assay in detecting microbial
187 activity changes among samples. Specifically FDA and total fungal load were highest in the
188 rhizophere of inoculated plants while beta-glucosidase and bacterial load was highest in the healthy
189 plants.
190 PCA analysis (Figure 2) allowed to resolve three main groups: a groups embodied by the samples
191 of substrates taken at the beginning of the experiment (1-4) and another group of samples embodied
192 by the rhizophere samples (5-8). Among this last group samples were further subdivided in not
193 inoculated (6,7) and inoculated (7,8).
194 While the higher enzymatic activity (FDA) around the root is likely to be consequence of the
195 activation of microbial population that followed plant inoculation (Caravaca *et al*. 2006), the
196 highest concentration of beta-glucosidase enzyme in healthy plants might be probably the
197 consequence of the enhanced carbon and nutrient allocation into the rhizosphere due to better
198 growth conditions (Tscherko *et al*. 2003).
199
200 *DGGE profiling*
201 Bacterial population was composed mainly by *Flavobacteria* (Figure 3) either at the start or at the
202 end of the experiment. This is consistent with the recent findings of Gardner *et al*. (2011) who,
203 investigating the microbial communities in five different farming systems, discovered that
204 *Flavobacteria* are among the predominant bacterial phyla in the first 0-10 cm of soil, including

lettuce. DGGE profile of bacterial population suggest that *Pseudomonas* sp., alone or in association with *Flavobacterium* sp., could be involved in the process of suppression. In fact *Pseudomonas* was found only in the rhizophere of 1% only while *Flavobacterium* spp. were present in all thesis (see arrows in figure 3). This result is in agreement with the proved affectivity of *Pseudomonas* in controlling wilt pathogens (Srinivasan *et al*. 2009; Karimi *et al*. 2012; Mansoori *et al*. 2013).

ITS primers chosen in this study amplified DNA from deuteromycetes (*Verticillium, Fusarium, Simplicillium*) but also other organisms such as the yeast *Metschinkowia chrysoperlae,* the lichen *Dyctionema sericeum,* other the ascomycete *Peziza basisiofusca*.

The highest number of fungal taxa was recorded for lettuce plants inoculated and grown in soil amended with 1% of compost. According to DGGE profiling *Verticillium* (Figure 4) is fungal genus that preferentially associates to the roots of lettuce plants.

Among other fungal taxa *Simplicillium lamellicola* was the only species detected in the rhizophere of plants challenged with the pathogen and treated with 1% compost, suggesting that it also may specifically contribute to the process of disease suppression.

As expected not all DGGE bands resulted in known organisms. In details one band in the bacterial (2) and two in the fungal fingerprints (2 and 3) resulted in uncultured bacterium, uncultured fungi to an ascomycete respectively. This is possible because also unknown and uncultured organisms could be detected with the use of molecular techniques.

Summary

ACM compost added to the percentage of 1% to steamed sandy soil allowed to obtain around 40% disease control of *Fusarium oxysporum* f.sp. *lactucae* “in vivo” demonstrating that ACM compost can be considered as alternative or integrative of resistant cultivars for controlling the disease. PCA analysis of microbiological features showed that all samples taken from the rhizophere of plants at the end of the experiment were more active than the respective samples taken as representative of the substrates only. Samples obtained from the rhizosphere of plants grown in suppressive media

were characterized by highest total enzymatic activity and highest load of total fungi. The comparison of DGGE profiles of microbial populations of the thesis has revealed a greater diversity to the fungal community than that of bacteria. *Pseudomonas* sp., among bacteria, and *Simplicillum lamellicola*, among deuteromycetes, were detected only in the rhizophere of plants treated with 1% compost indicating that they may play an active role disease control. Further studies are needed in order to clarify the involvement of these two species in the compost-mediated disease suppression of *Fusarium* wilt of lettuce.

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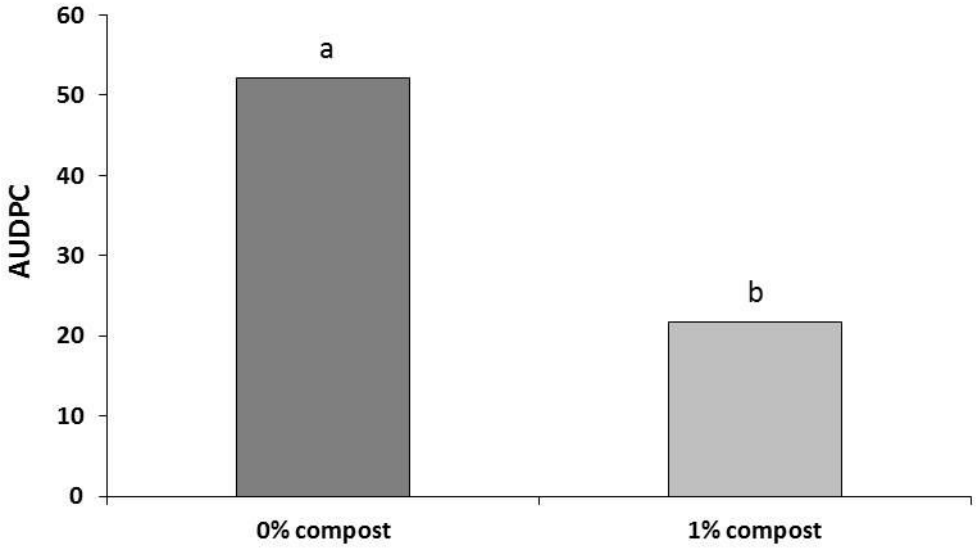
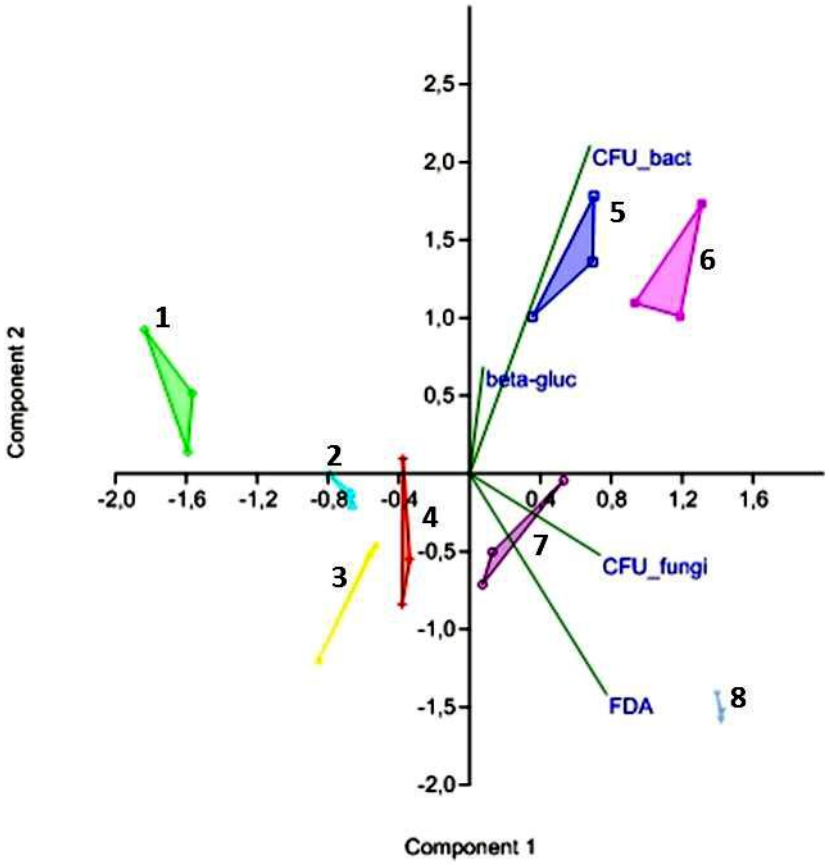


FIGURE 1. Mean AUDPC values for plants inoculated and grown in soil non amended (0%) and amended with 1% of ACM compost. Different letters above the column indicate significant ($P<0.02$) differences between groups of values (Mann Whitney test).



367

368 FIGURE 2. Principal Component Analysis scattered plot of the microbiological parameters assessed
369 for soil and rhizosphere samples during suppressive assays on lettuce plants challenged with
370 *Fusarium oxysporum* f.sp. *lactucae*. Numbers correspond to treatments/samples: 1) Ti-not
371 inoculated-0% compost-no plant; 2) Ti-not inoculated-1% compost-no plant; 3) Ti-inoculated-0%
372 compost-no plant; 4) Ti-inoculated-1% compost-no plant; 5) Tf-not inoculated-0% compost-
373 rhizophere; 6) Tf-not inoculated-1% compost-rhizophere; 7) Tf-inoculated-0% compost-rhizophere;
374 8) Tf-inoculated 1%-compost-rhizophere.

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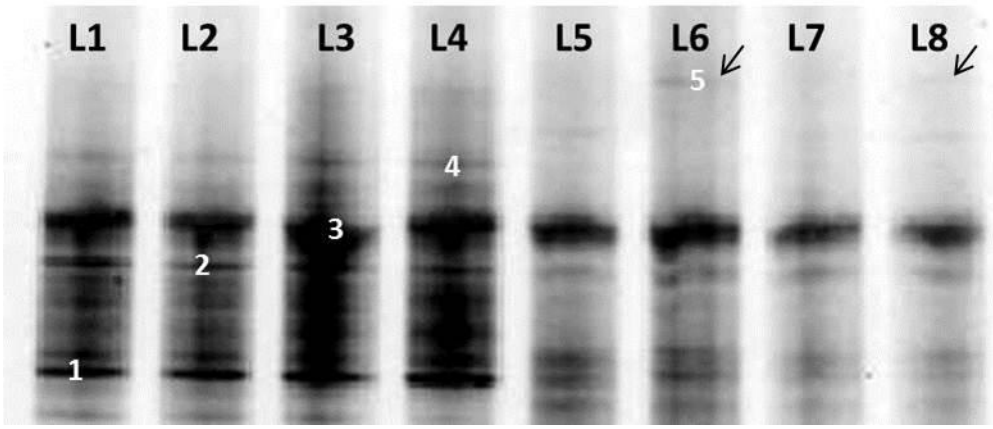

FIGURE 3. DGGE profiles of bacterial community at the Time zero (Ti) lines 1 to 4 and at the end of the experiment (Tf) lines 5 to 8. L1) Ti-not inoculated-0% compost-no plant; L2) Ti-not inoculated-1% compost-no plant; L3) Ti-inoculated-0% compost-no plant; L4) Ti-inoculated-1% compost-no plant; L5) Tf-not inoculated-0% compost-rhizophere; L6) Tf-not inoculated-1% compost-rhizophere; L7) Tf-inoculated-0% compost-rhizophere; L8) Tf-inoculated 1%-compost-rhizophere. 1) *Flavobacterium tiangeerense*; 2) Uncultured bacterium; 3) *Flavobacterium xinjangense*; 4) *Flavobacterium* sp. 5) *Pseudomonas* sp..

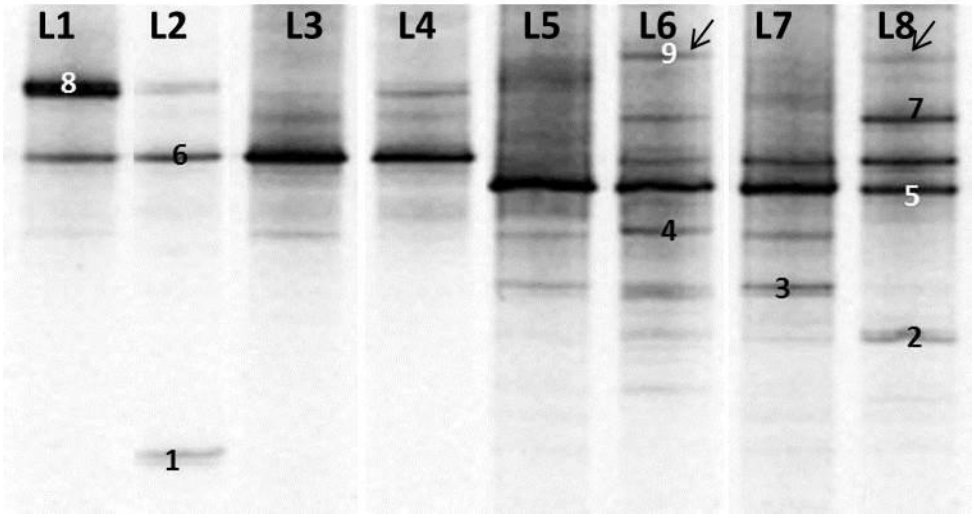

FIGURE 4. DGGE profiles of fungal community at the Time zero (Ti) lines 1 to 4 and at the end of the experiment (Tf) lines 5 to 8. L1) Ti-not inoculated-0% compost-no plant; L2) Ti-not inoculated-1% compost-no plant; L3) Ti-inoculated-0% compost-no plant; L4) Ti-inoculated-1% compost-no plant; L5) Tf-not inoculated-0% compost-rhizophere; L6) Tf-not inoculated-1% compost-rhizophere; L7) Tf-inoculated-0% compost-rhizophere; L8) Tf-inoculated 1%-compost-rhizophere. 1) *Metschinkowia chrysoperlae*; 2) Uncultured fungus; 3) Ascomycete; 4) *Verticillium* sp.; 5) *Verticillium* sp; 6) *Fusarium oxysporum*; 7) *Peziza basidiofusca*; 8) *Dyctionema sericeum*; 9) *Simplicillium lamellicola*.

TABLE 1. Statistical differences in the mean enzymatic activities (±SD) as assessed through the FDA hydrolysis and betaglucosidase assays at the time zero (Ti) and at the end of the experiment (Tf). Different letters represent statistical differences at P<0.05 (ANOVA-Tukey's honestly post-hoc test).

			FDA (µg/mlh^{-1})		Beta-glucosidase (µg/mlh^{-1})	
		compost	**Ti**	**Tf**	**Ti**	**Tf**
(Ti) substrate	**control**	0%	7.30±0.02 e	-	0.22±0.01 ef	-
		1%	7.67±0.03 c	-	0.35±0.01 b	-
	inoc	0%	7.76± 0.05 c	-	0.27±0.04 d	-
		1%	7.72±0.03 c	-	0.28±0.02 cd	-
(Tf) plant rhizospere	**control**	0%	-	8.13±0.04 b	-	0.75±0.02 a
		1%	-	8.17±0.07 b	-	0.34±0.02 bc
	inoc	0%	-	8.09±0.03 b	-	0.20±0.01 f
		1%	-	9.22± 0.01 a	-	0.28±0.04 cd

TABLE 2. Statistical differences in the mean CFU of fungi and bacteria at the time zero (Ti) and at the end of the experiment (Tf). Different letters represent statistical differences at P<0.05 (ANOVA- Tukey's honestly post-hoc test).

			Total bacteria (CFU/gfw)		Total fungi (CFU/gfw)	
		compost	**Ti**	**Tf**	**Ti**	**Tf**
(Ti) substrate	**control**	0%	6.58±0.11 de	-	2.64±0.30 d	-
		1%	6.69±0.01 de	-	3.43±0.08 d	-
	inoc	0%	6.72±0.20 de	-	3.78±0.25 bcd	-
		1%	6.50±0.20 e	-	3.65±0.09 cd	-
(Tf) plant rhizosphere	**control**	0%	-	7.66±0.20 ab	-	3.77±0.07 bcd
		1%	-	7.91±0.18 a	-	4.31±0.12 a
	inoc	0%	-	7.02±0.21 cd	-	4.00±0.13 abc
		1%	-	7.27±0.04 bc	-	4.16±0.05 ab