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Essential title page information

Physiological effects of the induction of resistance by compost or *Trichoderma asperellum* strain T34 against *Botrytis cinerea* in tomato

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Abstract

Certain types of compost used as growth media can induce resistance to foliar pathogens in above-ground parts of a plant. The induction of resistance can sometimes be associated with growth impairment and yield reduction. The objective of this study was to establish whether plants grown in olive marc compost had enhanced resistance against *Botrytis cinerea* at the cost of growth or physiological performance.

Tomato plants grown in mature olive marc compost had approximately 60% less disease severity than plants grown in perlite. As a reference, plants grown in perlite enriched with the known inducer of resistance *Trichoderma asperellum* strain T34 (T34) had 35 % less disease severity than plants grown in perlite. The salicylic acid (SA) pathway/ abscisic acid (ABA) is involved in compost induced systemic resistance. Instead, perlite enriched with T34 is not linked to SA pathway/ABA. Physiological measures of water status, root/shoot ratio, stable isotopes of C and chlorophyll fluorescence showed that the plants grown in compost were close to a stress situation. However, growth measured as biomass and plant height of plants grown in compost was higher than in plants grown in perlite suggesting that plants in compost were not grown in a stress situation, but in a eustress. Tomato plants grown in perlite enriched with T34 had better growth, measured as total leaf area, biomass, height and nutrient uptake, than plants grown in perlite. Physiological measures showed that plants grown either in perlite or perlite enriched with T34 did not show any abiotic stress situation.

Keywords:

Eustress; Induced plant disease resistance; Olive marc compost; *Solanum lycopersicum*; Stable isotopes

1. Introduction

Botrytis cinerea Persoon: Fries, teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel is a plant pathogenic fungus of economic relevance, since it can infect over 200 plant species. This fungus is also known as grey mold and is one of the most extensively studied necrotrophic fungal pathogens. According to Dean et al. (2012), this pathogen was rated the second most important in an international survey of fungal pathologists. Specific fungicide (botryticide) applications and broad spectrum fungicides are the most common method to control this disease. However, fungicide resistance is becoming an important problem (De Ward et al., 2006; Leroch et al., 2011). Moreover, Directive 2009/128/EC will implement integrated pest and disease management in Europe by 2014.

The tomato (*Lycopersicon esculentum* [Miller 1768], *Solanum lycopersicum* [Linné 1753]) crop is the eighth largest in the world in terms of food and agricultural commodities production, according to the Food and Agricultural Organization (FAO) (**http://faostat.fao.org/site/339/default.aspx**). Spain occupies ninth position in the world in value and production of tomato. This crop is susceptible to *B. cinerea*, which leads to losses during production (greenhouse) and post-harvest. The tomato crop was also selected for this study because it is classified as moderately tolerant to salinity (yield decline at a threshold value of 2.5 mS cm-1) (Maas and Hoffman, 1977). The growth medium evaluated in this study is alperujo compost characterized by basic pH and high electrical conductivity (EC) that may not be optimal for plant cultivation. Therefore, some compost requires formulation with other materials like peat, perlite, coconut fiber, etc. (Cotxarrera et al., 2002). Spain generates a large amount of horticultural residues, such as alperujo, which is waste from the olive oil industry. Alperujo is highly contaminating, acidic, and rich in nutrients (K and N) and lignocellulosic organic matter and has a high fat; carbohydrate and water-soluble phenol content (Albuquerque et al., 2004). Alperujo is a mixture of dregs (liquid that emerges from

the olive paste) and marc (pits, skins and pulp). Alperujo can be composted by the addition of olive tree leaves and manure and can be used in agriculture as growth media, amendments or fertilizer. Composts can promote plant growth by nutrition improvement (Bugbee and Finck, 1989; Gallardo-Lara and Nogales, 1987; Zhang et al., 2012).

Hoitink et al. (1997) proposed that some types of compost naturally suppress certain plant pathogens. The first studies showed that microorganisms played an important role, with antagonistic interactions (competition, hyperparasitism and antibiosis) between the pathogens and the beneficial microorganisms (Cotxarrera et al., 2002; Hoitink et al., 1997; Hoitink and Boehm, 1999; Sant et al., 2010). Moreover, some authors have claimed that certain types of compost used as growth media can induce resistance to foliar pathogens in above-ground parts of a plant, as the microbial populations of the compost are spatially separated (Abbasi et al., 2002; Horst et al., 2005; Kavroulakis et al., 2005; Yogev et al., 2010). This spatially separation is an indirect evidence of the induction of plant resistance and also could be attributed to improved nutrition in plants (Abbasi et al., 2002; De Meyer et al., 1998; Horst et al., 2005; Segarra et al., 2007b; Yogev et al., 2010). A significant increase in peroxidase activity was observed in plants grown in compost vs. plants grown in peat (Zhang et al., 1998), which suggests that compost affects a plant's defense mechanisms. Knowledge about the induction of systemic resistance has been accumulating in recent years, mostly in microbials (plant growth promoting rhizobacteria [PGPR]- and fungal biological control agents) (Durrant and Dong, 2004; Mathys et al., 2012; Pieterse et al., 1996; Ryals et al., 1996; Segarra et al., 2009; Van Loon, 1997; Van Loon et al., 1998). In this study, we used *Trichoderma asperellum* strain T34 as a reference, as it is known to induce priming and systemic resistance (ISR) against other bacterial and fungal diseases, also in other necrotrophic fungi in Arabidopsis plants (Segarra et al., 2009). Moreover, the induction of

89 resistance by T34 is a concentration depending phenomena and plants use the route of ISR or
90 SAR (Segarra et al., 2006, 2007a).

91 The main phytohormones involved in plants defense mechanisms are salicylic acid (SA),
92 jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) (Pieterse et al., 2012; Vos et al.,
93 2013). Mainly, SA signaling pathway is related with plant resistance against biotrophic and
94 hemibiotrophic pathogens (Vos et al., 2013). However, plant resistance against necrotrophic
95 pathogens as *B.* cinerea, is related with JA signaling pathway (Birkenbihl and Somssich,
96 2011; Vos et al., 2013).Recently, we showed that mature compost from olive oil residues
97 induced resistance against *B. cinerea* in *Arabidopsis thaliana* and that the enhanced resistance
98 was mainly related to processes mediated by SA and ABA, with responses similar to systemic
99 acquired resistance (SAR) and abiotic stress responses (Segarra et al., 2013b). Various studies
100 suggest that SAR can be associated with growth impairment and yield reduction (Denancé et
101 al., 2013; Walters and Heil, 2007). ABA has recently been reported to cross talk with SA and
102 JA in plant disease and defense (Robert-Seilaniatz et al., 2011). ABA has been regarded more
103 as a modulator rather than a primary hormone in plant defense (Ton et al, 2009). The role of
104 ABA is still unclear, because it sometimes appears to promote disease, while at other times it
105 does the opposite.
106 The objective of this study was to establish whether tomato plants grown in compost had
107 enhanced resistance against *B. cinerea* at the cost of growth or physiological performance.

2. Materials and methods

2.1. Growth media

The growth media used were: mature olive marc compost (CM) produced at the University of Seville; perlite (P), an inert mineral growth medium obtained from Europerlite was used as a standard substrate; perlite enriched with the biological control agent *T. asperellum* strain T34 (P+T34) was used as a positive control of resistance induction.

CM compost was selected from five different olive marc composts from Andalucía (South Spain) because in a previous study it was demonstrated that did not require formulation to obtain similar germination of *S. lycopersicum* cv. Roma to the perlite control. Compost CM was produced by turned piles and was mature (2 years of stabilization). The composition of CM was olive marc 47% and leaf residues 53% and had a pH of 7.74 and an EC of 0.37 mS cm^{-1}. Perlite was composed of SiO_2 (73%) and Al_2O_3 (13%) and had a pH of 6.69 and an EC of 0.12 mS cm^{-1}. To prepare P+T34, perlite was inoculated with *T. asperellum* strain T34 at a concentration of 10^4 CFU mL^{-1} growth media by dilution on nutrient solution of the concentrated commercial product at 10^9 CFU g^{-1} growth media. After the incubation period the concentration of T34 was about 10 times higher. It was then incubated at a water tension of 1000 Pa (adjusted by weight) at 25°C for 14 days. In order to standardize initial conditions of microbial biomass, P and 4°C stored CM were incubated for the same time and in the same conditions as P+T34.

2.2. Plant growth studies

Tomato plants (*S. lycopersicum* cv. Roma) were sown in 15 mL multipots for 15 days. Subsequently, the plants were transplanted to 250 mL pots in each growth media for 20 days (the end of the experiment). Multipots and pots were placed in a growth chamber (25 ± 2°C, 16 hours [h] of light at 180–210 µmol m^{-2} s^{-1} photosynthetic photon flux density [PPFD] and

60-80% relative humidity [RH]). Plants were hand irrigated on the media as needed (50 and 100 mL solution mL^{-1} medium day^{-1} in the multipot and pot period, respectively) with the following nutrient solution: 0.5 g L^{-1} Peter's Foliar Feed 27-15-12 (Scotts), 0.22 g L^{-1} $CaCl_2$ and 0.25 g L^{-1} $MgSO_4$ 7 H_2O.

2.2.1. Plant analysis

The plant biomass analysis was performed twice in two separate studies and 4 replicates per treatment were used in each study. Shoots, leaves and roots were separated and dried (forced air oven) at 60°C for 48 h. We determined the dry weight of shoots and leaves (DWA), the dry weight of whole plant (DWT), the plant height (H), the root/shoot ratio, and the percentage of water in each plant.

We determined the leaf mineral composition analysis according to Segarra et al. (2007b), using dried leaves from two separate studies and 3 replicates per treatment in each study. An aliquot of 50 mg per sample was digested with 2 mL of concentrated HNO_3 and 2mL of H_2O_2 in a Teflon container at 90°C for 3 days. Analyses of Ca, K, Si, Mg, Fe, P and S were performed by inductively coupled plasma optical emission spectrometry (ICP-OES) using Optima-3200RL (Perkin Elmer). Analyses of Ni, Mo, B, Cu, Zn and Mn were performed by inductively coupled plasma mass spectrometry (ICP-MS) using Elan 6000 (Perkin Elmer). The carbon and nitrogen percentage and the stable isotope ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) for leaves and roots were determined using an elemental analyzer (EA1108, Series 1, Carlo Erba Instruments) coupled to an isotopic ratio mass spectrometer (IRMS, Delta C, Finnigan MAT). Three leaves and roots per treatments were used from the last study. Leaves and roots were ground separately (to pass through a 1 mm sieve) and aliquots of 0.50 mg were weighed in tin cups and analyzed by the EA-IRMS. The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios were expressed as δ notation ($\delta^{13}C$ and $\delta^{15}N$ respectively), as described by Coplen (2008): $\delta = [(\text{Isotope Ratio}_{\text{Sample}}/\text{Isotope Ratio}_{\text{Standard}})-1] \times 1000$ (‰). The standard

158 used to calculate $\delta^{13}C$ was Vienna Pee Dee Belemnite (VPDB) calcium carbonate, and to
159 calculate $\delta^{15}N$ was N_2 in air. In both measurements, international isotope secondary standards
160 were used to obtain an analytical precision of 0.1‰.

161 Leaf gas exchange and fluorescence analysis was performed on attached tomato leaves.
162 The youngest fully expanded leaves were used (adaxial side). Four plants per treatment were
163 used from the last study for each measure. An infrared gas analyzer (LI-6400, Li-Cor Inc.)
164 was used to measure net CO_2 assimilation rates (A) and stomatal conductance (Gs), using
165 equations developed by Von Caemmerer and Farquhar (1981). Plants were exposed to
166 decreasing PPFD at 1200, 900, 300 and 85 μmol m^{-2} s^{-1} at 25°C and 400 μmol CO_2 mol^{-1}.
167 Chlorophyll fluorescence was analyzed with an Imaging-PAM fluorometer (Walz). Plants
168 were first dark-adapted for 20 minutes and a saturating light pulse was applied to determine
169 the maximum quantum efficiency of Photosystem II (PSII) (Fv/Fm) (Fv, variable
170 fluorescence; Fm, maximum fluorescence yield in the dark-adapted state). Later, every leaf
171 was adapted for 5 minutes to an actinic light of PPFD at 228 μmol m^{-2} s^{-1} (similar to the mean
172 growth PPFD). Then, a second saturating light pulse was used to calculate: the relative
173 quantum efficiency of PSII electron transport (Φ_{PSII}) estimated from $\Phi_{PSII}=(Fm'-F)/Fm'$ (Fm',
174 maximum fluorescence yield in the light-adapted state; F, fluorescence yield) according to
175 Genty et al. (1989); the coefficient of photochemical quenching (qP) estimated from
176 $qP=(Fm'-F)/(Fm'-F_0')$ (F_0', minimum fluorescence yield in the light-adapted state); and the
177 non-photochemical coefficient (qN) estimated from $qN=(Fm-Fm')/(Fm-F_0')$ (Andrews et al.,
178 1993). The parameter F_0' was estimated using an approximation by Oxborough and Baker
179 (1997).

180 Total leaf area (TLA), specific leaf weight (SLW) and relative water content (RWC) were
181 used from leaves after monitoring gas exchange measures. TLA was determined using a
182 scanner, Image leaf area measurement software (University of Sheffield, 2003), and dry

183 weight. SLW was calculated from SLW = DW/TLA and the RWC was determined according
184 to Turner (1981).
185
186 2.3. Plant disease studies
187 Tomato plants were grown in the same conditions as mentioned for the plant growth
188 studies up to day 15 of transplantation to 250 mL pots. On that day, plants were placed in
189 mini-tunnels (inside the growth chamber) to establish the best conditions for *B. cinerea*
190 disease. The growth chamber was modified to obtain the following conditions inside the mini-
191 tunnels: 24°C (day) and 20°C (night), 16 h light and near 100% RH. Plants were adapted to
192 the new environmental conditions one day before inoculation with the pathogen. The number
193 of plants in each of the three growth media was 14: 5 (controls) without pathogen inoculation,
194 and 9 with pathogen-inoculated leaves. The control plants were placed in separate mini-
195 tunnels to the inoculated plants, each of which was grown in a different mini tunnel. The
196 experiment was repeated three times.
197 2.3.1. Pathogen inoculation
198 A virulent strain of *B. cinerea* isolated from tomato-infected leaves and stored in silica gel
199 crystals at 4°C was cultivated in mixed vegetable solid medium for 21 days at 20°C, 7 days
200 under dark conditions, and 14 days at PPFD 85 µmol m^{-2} s^{-1} and 16 h light. The mixed
201 vegetable medium was prepared by cooking 500 g of a commercial frozen mix of potato,
202 carrot and beans in water. The boiled vegetables and cooking water were homogenized with a
203 kitchen blender, the volume was brought to 1 L and 150 mL of the mixture plus 7.5 g of agar
204 were used to prepare 500 mL of mixed vegetable medium. Conidia were collected from the
205 plates in an inoculation buffer containing 0.5 mg mL^{-1}glucose and 0.5 mg mL^{-1} KH_2PO_4 (De
206 Meyer et al., 1998). Twelve mL of the buffer was used per plate; the resulting suspension was
207 filtered through two cotton gauzes. The concentration of *B. cinerea* was adjusted to 10^5

208 conidia mL^{-1} by hemocytometer counting. Finally, a drop of Tween 20 was added to the
209 inoculum (0.005%) to promote uniform dispersion of the inoculum on plants leaves. Two
210 expanded leaves from each plant were sprayed with approximately 550 μL per leaf with a low
211 pressure plastic hand sprayer.

212 2.3.2. Assessment of disease

213 The severity and incidence of disease was examined 7, 10 and 14 days post-inoculation.
214 Severity was evaluated using the following score for each leaf: 0 (asymptomatic), 1 (chlorotic
215 spots), 2 (necrotic specks), 3 (necrotic spots), 4 (dead leaf). The area under the disease
216 progress curve (AUDPC) per leaf was calculated by disease severity as described by Shaner
217 and Finney (1977). The AUDPC was standardized by dividing with the total area of the graph
218 (total days of observing disease symptoms multiplied by the maximum degree of disease).
219 Disease incidence was evaluated as: 0, healthy; 1, infected. Disease incidence was calculated
220 as the percentage of diseased leaves.

221 2.3.3. Plant hormone analysis

222 The plant hormones SA, ABA and JA were quantified as follows. The first fully expanded
223 tomato leaf of each plant was sampled on day 0, 1, 3 and 5 post-pathogen inoculation. Three
224 plants per treatment were used from the last study. The leaf from each plant was collected
225 separately and quick-frozen in liquid N_2. Frozen samples were ground under liquid N_2 with a
226 mortar and pestle. A total of 50 mg of the resulting powder was first extracted and twice re-
227 extracted with methanol: isopropanol: acetic acid (20:79:1, v/v/v) (Müller and Munné-Bosh,
228 2011). The extracted samples were quantified according to Segarra et al. (2006), using the
229 following transitions: 137/93, 263/153 and 209/59 for SA, ABA and JA respectively.

230

231 2.4. Experimental design and statistical analysis

Data were analyzed by IBM SPSS Statistics 19 version statistical software. Data from plant growth studies that were performed twice (DWA, DWT, Root/Shoot, H, Total H_2O, mineral composition) were analyzed using a multifactorial ANOVA ($p<0.05$). The factor experiment and the interaction with the factor treatment were not significant, so data from the various experiments were pooled. Hence, data was analyzed with a unifactorial ANOVA to study the factor treatment for each growth and physiological parameter analyzed (DWA, DWT, root/shoot, H, Total H_2O, mineral composition, C/N ratio, $\delta^{13}C$, $\delta^{15}N$, A, Gs Fv/Fm, φ_{PSII}, qP, qN, TLA, SLW and RWC). When significant differences were observed ($p<0.05$), the Duncan's multiple range test was performed ($p<0.05$). Data from disease assessments (DS, DI and AUDPC) that were performed three times were also analyzed using a multifactorial ANOVA ($p<0.05$). The factor experiment and the interaction with the factor treatment were not significant. Therefore, data from the three experiments were also pooled. Consequently, data from disease assessments (DS, DI and AUDPC) and plant hormone analyses (SA, ABA and JA) were analyzed with a unifactorial ANOVA to study the factor treatment over the evaluation days. When significant differences were observed ($p<0.05$), the Duncan's multiple range test was performed ($p<0.05$). In cases in which normal distribution and homogeneity of variances were not found, the Kruskal-Wallis test was performed ($p<0.05$). Relationships between DS, DI and AUDPC and plant physiological parameters and plant hormone content were analyzed with the Pearson correlation coefficient ($p<0.05$).

3. Results

3.1. Effect of substrate on plant growth and physiological performance

The overall plant growth measured as DWA and DWT was higher in plants grown in P+T34 and in CM than in P alone (Table 1). The root/shoot ratio was higher in P and in P+T34 than in CM. Plants grown in P+T34 developed a higher TLA than in the other treatments, and the SLW (g m^{-2}) was the same for all leaves (data not shown). The lowest plant height was found in plants grown in P, followed by P+T34. Plants grown in CM were the highest. Leaf water status, measured as RWC, was higher in leaves grown on P and P+T34 than in CM. A similar pattern was obtained when the water content was measured in the whole plant (roots, shoots and leaves) (Table 1).

The Ca composition in leaves was higher in plants grown in CM than in the rest of the growth media (Table 2). The overall mineral composition of Mg, P, B and Cu was higher in plants grown in P+T34, followed by P, whilst the lowest values were found in plants grown in CM. Plants grown in P+T34 and P had the highest Fe, Mn and Mo levels in leaves. The lowest Fe, Mn and Mo levels were obtained in plants grown in CM No significant differences among treatments were observed in K, S, Si and Zn levels in leaves (Table 2). The ratio C/N in the leaves was higher in plants grown in CM than in P+T34 and P. The ratio C/N in the roots was the same for plants in all plant growth media (Table 2).

The $\delta^{13}C$ of leaves of plants grown in CM was less negative than in plants grown in P+T34 and P (Table 3). The $\delta^{13}C$ of roots was also less negative in CM, followed by P+T34, whilst the most negative values were found in P. The $\delta^{15}N$ of leaves was higher in plants grown in CM than in the rest of the growth media. The $\delta^{15}N$ of roots was higher in plants grown in P+T34, followed by P and the lowest values were found in compost CM (Table 3).

Leaves of tomato plants grown in the different growth media showed an increase in the net CO_2 assimilation rate (A), according to an increase in PPFD from 85 to saturation levels 1200

277 µmol m^{-2} s^{-1}. No significant differences among treatments were observed below 1200 µmol m^{-2} s^{-1} PPFD. Plants grown in CM showed the highest rate (Figure 1). The results for A are in agreement with the Gs. The highest Gs and A values were observed in plants grown in CM. The lowest levels of Gs and A were observed in plants grown in P and P+T34. At 1200 µmol m^{-2} s^{-1} of PPFD plants grown in P+T34 showed a significant decrease measured in both A and Gs (Figure 1).

The fluorescence analysis showed that the highest values of Fv/Fm were observed in plants grown in P+T34, followed by plants grown in P alone, whilst the lowest values were observed in plants grown in CM (Table 4). No significant differences among treatments were observed in Φ_{PSII} and qP. The highest values of qN were in plants grown in P and P+T34, being the lowest values in CM plants (Table 4).

3.2. Effect of substrate on *Botrytis* disease control

Tomato plants grown on compost CM showed the lowest levels of disease, measured as disease severity (DS) (lower than 1, on a scale from 0 to 4), disease incidence (DI) (from 57,5-72,5%) from 7 to 14 days and AUDPC (0.20±0.03) (Table 5). Plants growing in P showed a DS from 2.07 to 3.36, a DI of around 100% and a AUDPC of 0.72±0.02. P+T34 improved the suppression to levels between those of CM and P, as shown in the values attained for DS and AUDPC (Table 5). Negative correlations ($p<0.05$) were attained for disease (DS on day 14 and AUDPC) and plant growth parameters such as the DWA, H, C/N shoots, $\delta^{13}C$ of roots and Gs at 1200 and 900 µmol m^{-2} s^{-1} of PPFD. Moreover, negative correlations ($p<0.05$) were attained for DI on day 14 and C/N shoots. Positive correlations ($p<0.05$) were attained for disease (DS on day 14 and AUDPC) and plant growth parameters such as root/shoot ratio and qN. Moreover, positive correlations ($p<0.05$) were attained for DI on day 14 and root/shoot ratio. The hormone quantification of SA, ABA and JA did not show

302 significant differences between treatments on day 0 (previous to inoculation of the leaves with
303 the pathogen) (Table 6). The SA concentration of infected leaves of plants grown in compost
304 CM significantly increased on day 1 post-inoculation, and was higher than in plants grown in
305 P and P+T34. On days 3 and 5 post-inoculation, the SA levels of CM still were higher than in
306 the rest of treatments. On day 5, leaves of plants grown in P and P+T34 decreased similarly or
307 below the levels of day 0. The ABA concentration in leaves of plants grown in compost CM
308 on day 1 was higher than the rest of treatments, followed by P, whilst the lowest values were
309 found in leaves of plants grown in P+T34. On days 3 and 5, ABA levels of all treatments
310 decreased similarly or below the levels of day 0 and there were no differences among
311 treatments (Table 6). The JA concentration was the same along the days of the study and for
312 all treatments (Table 6). Negative correlations ($p<0.05$) were attained for disease (DS and DI
313 on day 14 and AUDPC) and plant hormone status, such as SA on days 1 and 3 and ABA on
314 day 1. Furthermore, negative correlations ($p<0.05$) were attained for DI on day 14 and plant
315 ABA status on day 5.
316

4. Discussion

The beneficial effect of composts on plant growth is well-documented (Arthur et al., 2012; Gallardo-Lara and Nogales, 1987; Zhang et al., 2012) and was also observed in this study on tomato plants grown in compost, compared to those grown in perlite. The high pH (7.7) of CM could explain the lower levels of several mineral elements (Mg, P, Fe, Mn, Zn and Cu) in the plants. No difference was observed in the leaf nutrients when the same compost CM was used in *A. thaliana* plants (Segarra et al., 2013a). The highest levels of Ca in compost CM could explain, in part, the involvement of this element in the reduction of gray mold disease caused by *B. cinerea*. Indeed, high levels of Ca in leaves have been associated with foliar disease resistance (Wójcik and Lewandowski, 2003). In several studies, Ca is involved in biotic and abiotic stress responses (Segarra et al., 2007b), callose synthesis (Trillas et al., 2000), pectin binding molecules (Carpita and McCann, 2000), SA (Schneider-Müller et al,. 1994) and phytoalexin synthesis (Vögeli et al., 1992).

The lower water status (RWC and total water) of plants grown in compost could be due to the EC of compost, which might have an inhibitory effect on Botrytis development. According to Mayak et al. (2004), RWC is an adequate indicator of water status in plants and is characterized by a decrease in stress conditions (drought and salinity). The pH and EC of olive marc compost were similar to those of other kinds of compost and other samples of the same type (Borrero et al., 2004; Cotxarrera et al., 2002; Segarra et al., 2007b). In one study, the RWC of tomato plants grown with high irradiation in a high EC solution was reduced to 81.5% (Claussen, 2005).

All measures of plant biomass were higher for tomato plants grown in P+T34 than in P alone; and the results were similar to those of plants grown in compost. The use of beneficial microorganisms in the rhizosphere (bacteria and fungus) can facilitate solubility, enhance the availability of nutrients to plants from the nutrient solution (Altomare et al., 1999), and

342 protect against biotic stress. The most relevant characteristic of plants grown in P+T34 was
343 the greater investment in leaf area. This could explain the greater accumulation of most of the
344 elements, especially Mg, P, B and Cu in the leaves. In particular, Mg, P and Cu are involved
345 in key reactions in leaves energetic processes. The increased uptake of Mg^{2+} and P was also
346 observed in tomato shoots and roots grown in soil amended with *T. harzianum* strain T447
347 (Azarmi et al., 2011). Whereas in a study with *T. harzianum* strain T-203 increased uptake of
348 Cu was observed in roots and not leaves of cucumber plants (Yedidia et al., 2001). Moreover,
349 the principal function of B is a structural role related to the stability of the cell wall (O'Neill
350 et al., 2004). Accordingly greater amount of B could improve plant resistance to *B. cinerea*
351 attack.

352 The highest C/N ratio of plants grown in compost showed the lowest content of nitrogen in
353 leaves, which would make the leaves more resistant to attack by the pathogen. Conversely, in
354 a study with tomato plants with high C/N ratio makes plants more susceptible to the primary
355 lesions formation caused by *B. cinerea* (Hoffland et al., 1999). The role of host nitrogen
356 content in the susceptibility to *B. cinerea* is still unclear because there are other factors
357 involved as N source and amount and *B. cinerea* isolates virulence (Lecompte et al., 2010).

358 Our study of stable isotopes of $\delta^{13}C$ and $\delta^{15}N$ clearly distinguishes the roots of plants
359 grown in P from those grown on P+T34. This suggests that the same C assimilation occurs,
360 but the post-photosynthetic fractionation of stable carbone isotopes between leaves and roots
361 and N assimilation by roots in contact with T34 differs. Makarov (2009) have observed that
362 mycorrhizal fungi are involved in determining the plant $\delta^{15}N$. In a similar way, T34 could be
363 involved in determining tomato plants $\delta^{15}N$ probably by improving the availability of this
364 element to plants. Several studies have shown that $\delta^{13}C$ increase or carbon isotope
365 discrimination decrease in water deficit conditions (Condon et al., 2002; Ehleringer and
366 Cooper, 1988; Farquhar et al., 1982; Yousfi et al., 2009). Similarly, in our study, plants grown

in compost were characterized by the lowest water status and also showed the highest $\delta^{13}C$, probably related to a certain degree of stress due to compost EC. In contrast, the effect of water status in $\delta^{15}N$ differs between studies (Handley et al., 1997; Lopes and Araus, 2006). The diverse composition of the growth media could explain the lowest $\delta^{15}N$ in CM. Despite this, according to Mariotti et al. (1982) the $\delta^{15}N$ signature of the source of N is not the only factor determining plant $\delta^{15}N$.

The data for photosynthesis and chlorophyll fluorescence were similar for tomato plants grown in a growth chamber at a similar age (Nogués et al., 2002). Measures of photosynthesis showed that plants grown in CM behave as plants grown at high light intensity (measure carried out at 1200 μmol $m^{-2}s^{-1}$ PPFD). This value contrasts with that found for plants grown in all other treatments, which were saturated at this light level. Measures of chlorophyll fluorescence were similar among treatments and the Fv/Fm ratios in all treatments were over 0.75, which is considered the limit for photoinhibition (Björkman and Demmig, 1987).

All these data confirm that plants were grown properly in all growth media, although plants grown in CM were near to the limits of stress conditions (lowest root/shoot ratio, RWC, total water, Fv/Fm and highest $\delta^{13}C$). Growth parameters and physiological measures of plants grown in CM suggests that the plants had grown in a eustress situation. According to Hideg et al. (2012), eustress is considered mild and acclimative stress that improves growth and health. It is the opposite of distress or severe stress, which exceeds tolerance limits and leads to the death of plants.

Our results showed the suppressive capacity of mature olive marc compost CM in reducing the severity and incidence of *B. cinerea* and in inducing systemic resistance. The induction of systemic resistance was also linked to SA (which only increased after pathogen exposure). Although *B. cinerea* is a necrotrophic pathogen and triggers mainly the JA signaling pathway and not the SA signaling pathway (Birkenbihl and Somssich, 2011; Vos et al., 2013). Indirect

evidence of the induction of resistance has been observed previously in composted cannery wastes against anthracnose tomato rot disease (Abbasi et al., 2002); in composted cow manure against *B. cinerea* in *Begonia hiemalis* (Horst et al., 2005); and in grape marc compost, olive marc-cotton gin trash (1:1, v:v) compost, cork compost, municipal organic and yard waste compost and spent mushroom compost against *B. cinerea* in *Cucumis sativus* (Segarra et al., 2007b). In another study, disease reduction was also induced by SAR using composted pine bark mix inoculated with *Trichoderma hamatum* 382 and *Flavobacterium balustinum* 299 and compost water extract against anthracnose and against bacterial speck in cucumber plants (Zhang et al., 1998). The spatial separation between the biological control agent (T34) and the pathogen and the reduction of *Botrytis* disease showed the involvement of the induction of plant resistance, even though an increase in JA levels could not be detected by T34 in the evaluated days of this study. Indeed, T34 applied to the roots has been shown to prime for induced resistance independently of SA (Segarra et al., 2009; Trillas and Segarra, 2009). However, SA increases have only been found when high concentrations (laboratory levels, not field levels) of T34 are applied (10^7 CFU mL^{-1}) (Segarra et al., 2007a).

Various studies suggest that ABA is involved in disease reduction or increase, according to the type of pathogen (Robert-Seilaniatz et al., 2011). ABA-deficient *A. thaliana* mutants were more resistant to *B. cinerea*, but more susceptible to *Pythium irregulare* (Adie et al., 2007). Similarly, ABA-deficient tomato mutants were more resistant to *B. cinerea* (Asselbergh et al., 2007). Ton et al. (2009) describes the role of ABA in plant disease defense more as a modulator rather than a primary hormone. Other studies suggested that the levels of SA, ABA and JA prior to contact with the pathogen had a determining impact on the interaction dynamics between these hormones (Robert-Seilaniatz et al., 2011). Interestingly, ABA levels of plants grown in CM increased after *B. cinerea* exposure. Recent studies from our group corroborate the involvement of both SAR and ABA-dependent/independent abiotic stress

417 responses in *A. thaliana* plants grown in olive marc composts or perlite exposed to *B. cinerea*

418 (Segarra et al., 2013a, 2013b).

419

5. Conclusions

In conclusion, physiological parameters measured in tomato plants grown in mature olive marc compost showed no negative influence on plant biomass, CO_2 assimilation rate or chlorophyll fluorescence measurements, plants grew in a eustress situation that might have had a positive influence on disease resistance. The compost induction of systemic resistance was also linked to SA pathway/ABA.

Tomato plants growing in perlite enriched with *T. asperellum* strain T34 had better nutrient uptake, better C allocation and N assimilation in roots leading to an improvement in dry weight, height and total leaf area than plants grown in perlite alone. Plants grown in perlite enriched with T34 had no stress effects, measured by the overall physiological parameters. The induction of disease resistance observed in perlite enriched with T34 is not linked to the SA pathway/ABA.

By describing the positive effects and the diverse responses of plants grown in compost and perlite enriched with T34, we are contributing to understanding the role of compost and beneficial organisms that help plants to growth healthier and improve their innate resistance to foliar pathogen attacks.

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642

643 **Table 1**

644 Effect of growth medium (P, perlite; P+T34, perlite enriched with *Trichoderma asperellum* strain T34 at a
645 concentration of 10^4 CFU mL^{-1}; CM, olive marc compost) on various physiological parameters of tomato plants
646
647 [a] Aerial dry weight (leaves and shoots)
648 [b] Total dry weight
649 [c] Total leaf area
650 [d] Plant height
651 [e] Relative water content
652 Values of DWA, DWT, Root/Shoot and Total H_2O are means ± standard error of 8 plants per treatment
653 collected from two separate studies (4 replicates each study). Values of TLA, H and RWC are means ±
654 standard error of 4 plants per treatment collected from one of the studies. Different letters show significant
655 differences $p<0.05$ on Duncan's multiple range test
656
657
658
659 **Table 2**
660 Effect of growth medium (P, perlite; P+T34, perlite enriched with *Trichoderma asperellum* strain T34 at a
661 concentration of 10^4 CFU mL^{-1}; CM, olive marc compost) on mineral composition of fully expanded tomato
662 leaves
663
664 Values of macronutrients and micronutrients are means ± standard error of 6 leaves per treatment collected from
665 two separated studies (3 replicates per treatment in each study). Values of C/N are means ± standard error of 3
666 leaves and roots per treatment collected from one of the studies. Different letters show significant differences
667 $p<0.05$ on a Duncan's multiple range test
668
669
670
671

672 **Table 3**

673 Effect of growth medium (P, perlite; P+T34, perlite enriched with *Trichoderma asperellum* strain T34 at a
674 concentration of 10^4 CFU mL^{-1}; CM, olive marc compost) on stable isotope ratios of carbon ($^{13}C/^{12}C$, expressed
675 as $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$ express as $\delta^{15}N$) of leaves and roots of tomato plants
676
677 Values are means ± standard error of 3 leaves and roots per treatment collected from one of the studies. Different
678 letters show significant differences $p<0.05$ on a Duncan's multiple range test
679
680
681
682 **Table 4**
683 Effect of growth medium (P, perlite; P+T34, perlite enriched with *Trichoderma asperellum* strain T34 at a
684 concentration of 10^4 CFU mL^{-1}; CM, olive marc compost) on chorophyll fluorescence of fully expanded tomato
685 leaves
686
687 [a] Maximum quantum efficiency of Photosystem II (PSII)
688 [b] Relative quantum efficiency of PSII
689 [c] Coefficient of photochemical quenching
690 [d] Coefficient of non photochemical quenching
691 Values are means ± standard error of 4 leaves per treatment collected from one of the studies. Different letters
692 show significant differences $p<0.05$ on a Duncan's multiple range test
693
694
695
696 **Table 5**
697 Disease severity (DS), disease incidence (DI) (%) and area under disease progress curve (AUDPC) based on DS
698 caused by the pathogen *Botrytis cinerea* (10^5 CFU mL^{-1}) in tomato plants in three independent bioassays. Plants
699 were grown in three growth media: P, perlite; P+T34, perlite enriched with *Trichoderma asperellum* strain T34

700 at a concentration of 10^4 CFU mL^{-1}; CM, olive marc compost. Disease was evaluated at 7, 10 and 14 days post-
701 inoculation (dpi)

702

703 [a] DS evaluated with a scale of five grades: 0, asymptomatic; 1, chlorotic leaf; 2, necrotic specks; 3, necrotic spot;
704 4, dead leaf

705 [b] DI was evaluated as: 0, healthy; 1, infected. DI was calculated as the percentage of diseased leaves

706 [c] AUDPC was standardized by dividing with the total area of the graph (total days of observing disease
707 symptoms per maximum degree of disease)

708 Values are means ± standard error of 35-54 leaves per treatment collected from three separated studies (6-18
709 replicates per treatment in each study). Different lower case letters show significant differences between
710 treatments and different capital letters show significant difference between days (dpi) within treatments ($p<0.05$)
711 on an Duncan's multiple range test.

712

713

714

715 **Table 6**

716 Effect of growth medium (P, perlite; P+T34, perlite enriched with *Trichoderma asperellum* strain T34 at a
717 concentration of 10^4 CFU mL^{-1}; CM, olive marc compost) on hormone (SA, salicylic acid, ABA, abscisic acid
718 and JA, jasmonic acid) content in leaves of tomato plants that were not inoculated (0) or inoculated (1, 3, 5 days
719 post-inoculation) with *Botrytis cinerea* (10^5 CFU mL^{-1})

720

721 Values are means ± standard error of 2-3 leaves per treatment collected from one of the studies. Different lower
722 case letters show significant differences between treatments and different capital letters show significant
723 difference between days within treatments for each plant hormone ($p<0.05$) on a Duncan's multiple range test

724

725

726

727 **Figure 1**

728 Photosynthesis response curves to light in fully expanded tomato plant leaves. Measurements were made on 20
729 days post-seeding, in plants grown on three growth media: perlite (P), perlite + *Trichoderma asperellum* strain

730 T34 (P+T34) (10^4 CFU mL^{-1}) and compost (CM). Curves were performed at 25°C, 400 µmol mol^{-1} CO_2 and at a
731 decreasing photosynthetic photon flux density (PPFD) of 1200, 900, 300 and 85µmol photon m^{-2} s^{-1}. (A) Net
732 CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}) (B) stomatal conductance (Gs, mmol H_2O $m^{-2}s^{-1}$). Values are means
733 ± standard error of 4 leaves per treatment collected from one of the studies. Different lower case letters show
734 significant differences between treatments and different capital letters show significant difference between days
735 within treatments ($p<0.05$) on an Duncan's multiple range test.
736

Table 1

Growth Medium	DWA[a] (g)	DWT[b] (g)	Root/Shoot	TLA[c] (cm^2)	H[d] (cm)	RWC[e]	Total H_2O (%)
P	0.14 ± 0.009 a	0.17 ± 0.011 a	0.23 ± 0.010 b	112.52 ± 9.31 a	7.66 ± 0.36 a	90.57 ± 0.88 b	95.21 ± 0.05 b
P+T34	0.25 ± 0.010 b	0.31 ± 0.011 c	0.23 ± 0.018 b	181.52 ± 9.13 b	9.62 ± 0.30 b	89.55 ± 1.64 b	95.07 ± 0.10 b
CM	0.23 ± 0.016 b	0.26 ± 0.020 b	0.12 ± 0.009 a	99.65 ± 9.49 a	10.60 ± 0.28 c	81.66 ± 3.41 a	94.10 ± 0.28 a

Table 2

Growth Medium	Macronutrient (mg planta^{-1})														
	K		Ca		Mg		P		S		Si		Fe		
P	7.75 ± 0.90	a	4.89 ± 0.27	a	0.92 ± 0.02	b	1.95 ± 0.05	b	1.01 ± 0.10	a	0.08 ± 0.01	a	0.03 ± 0.00	b	
P+T34	9.84 ± 1.33	a	6.12 ± 0.56	a	1.21 ± 0.08	c	2.52 ± 0.08	c	1.25 ± 0.13	a	0.09 ± 0.03	a	0.04 ± 0.00	b	
CM	8.10 ± 0.62	a	7.81 ± 0.33	b	0.76 ± 0.02	a	1.21 ± 0.05	a	1.32 ± 0.08	a	0.03 ± 0.01	a	0.02 ± 0.00	a	
Growth Medium	**Micronutrient (µg planta^{-1})**										**C/N**				
	B		**Mn**		**Zn**		**Cu**		**Mo**		**Leaves**		**Roots**		
P	13.53 ± 0.29	b	30.92 ± 3.88	b	12.20 ± 2.04	a	5.73 ± 0.20	b	0.53 ± 0.02	b	5.79 ± 0.14	a	7.93 ± 0.10	a	
P+T34	18.42 ± 1.74	c	40.06 ± 6.82	b	16.55 ± 1.93	a	7.89 ± 0.39	c	0.60 ± 0.04	b	6.31 ± 0.19	a	8.05 ± 0.37	a	
CM	6.90 ± 0.33	a	14.81 ± 2.00	a	13.81 ± 0.94	a	3.46 ± 0.34	a	0.24 ± 0.02	a	7.19 ± 0.16	b	7.27 ± 0.23	a	

Table 3

Growth Medium	$\delta^{13}C$ (‰)		$\delta^{15}N$ (‰)	
	Leaves	Roots	Leaves	Roots
P	-35.30 ± 0.17 a	-34.53 ± 0.10 a	-3.14 ± 0.12 a	1.94 ± 0.37 b
P +T34	-34.97 ± 0.15 a	-34.14 ± 0.08 b	-2.71 ± 0.21 a	3.39 ± 0.26 c
CM	-34.28 ± 0.11 b	-33.41 ± 0.13 c	1.22 ± 0.21 b	0.34 ± 0.40 a

Table 4

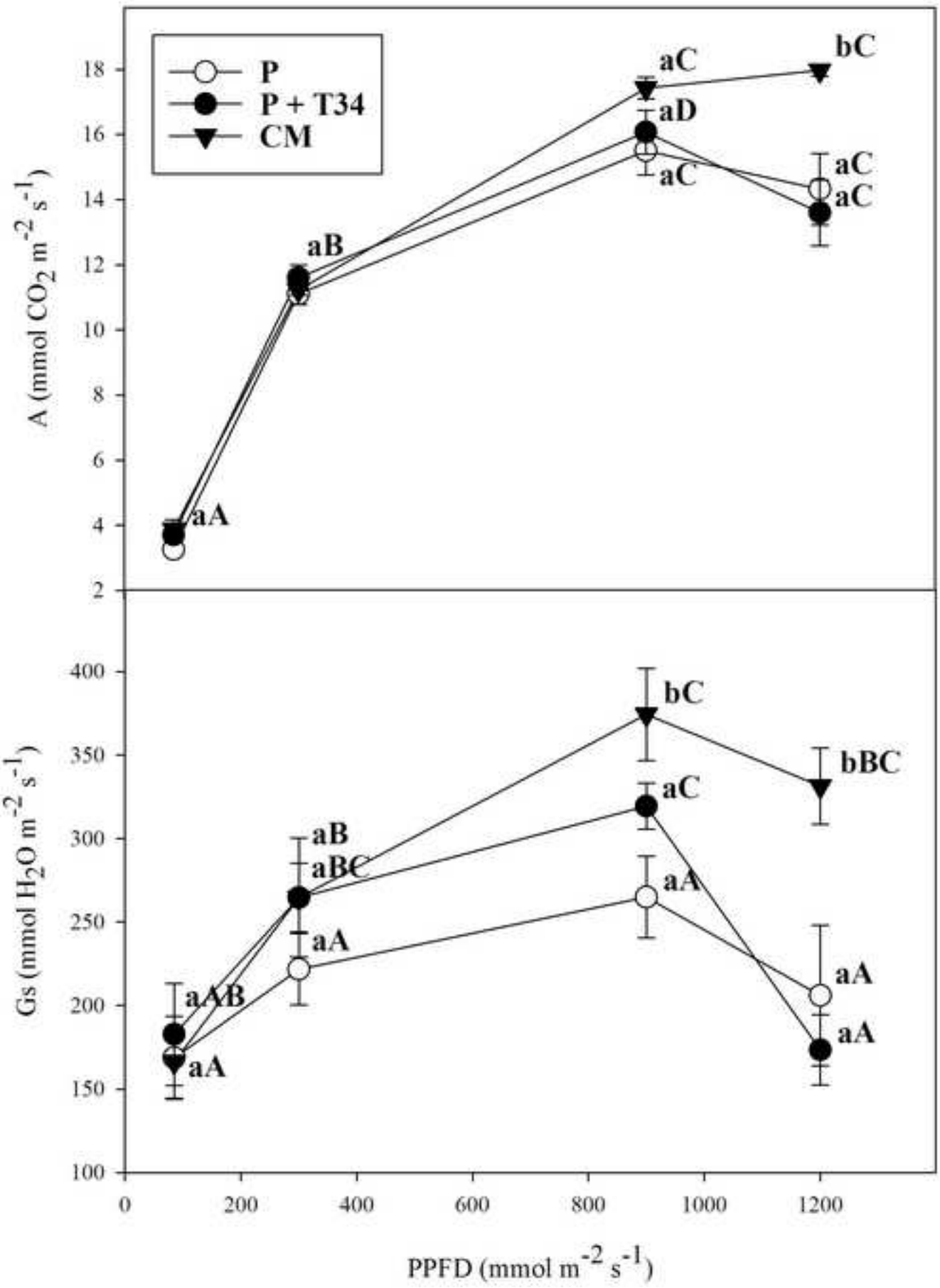
Growth Medium	Fv/Fm[a]	Φ_{PSII}[b]	qP[c]	qN[d]
P	0.808 ± 0.001 b	0.497 ± 0.016 a	0.703 ± 0.013 a	0.507 ± 0.034 b
P+T34	0.820 ± 0.005 c	0.514 ± 0.003 a	0.710 ± 0.007 a	0.502 ± 0.010 b
CM	0.793 ± 0.002 a	0.533 ± 0.009 a	0.740 ± 0.013 a	0.402 ± 0.013 a

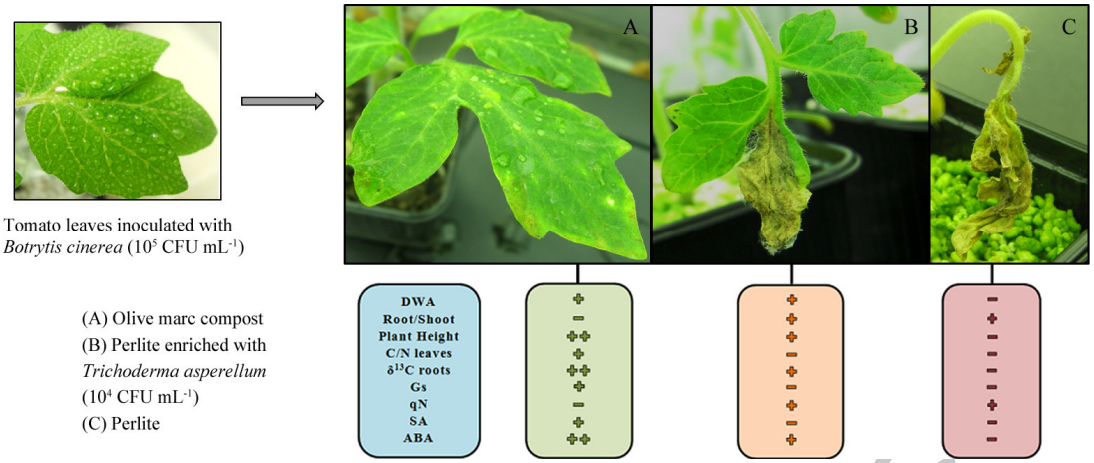
Table 5

Growth Medium	DS[a]			DI (%)[b]			AUDPC[c]
	7 dpi	10 dpi	14 dpi	7 dpi	10 dpi	14 dpi	
P	2.07 ± 0.08 cA	2.93 ± 0.12 cB	3.36 ± 0.11 cC	100.0 ± 0.00 bA	100.0 ± 0.00 bA	100.0 ± 0.00 bA	0.72 ± 0.02 c
P+T34	1.46 ± 0.08 bA	1.77 ± 0.09 bB	1.94 ± 0.11 bB	97.1 ± 2.86 bA	97.1 ± 2.86 bA	97.1 ± 2.86 bA	0.44 ± 0.02 b
CM	0.62 ± 0.12 aA	0.78 ± 0.13 aA	0.94 ± 0.15 aA	57.5 ± 7.91 aA	65.0 ± 7.64 aA	72.5 ± 7.15 aA	0.20 ± 0.03 a

Table 6

Hormone	Growth Medium	Days post-inoculation			
		0	1	3	5
SA (ng g^{-1} FW)	**P**	39.50 ± 2.65 aBC	47.53 ± 4.53 aC	33.83 ± 3.37 aB	22.53 ± 1.03 aA
	P+T34	47.10 ± 6.82 aBC	52.53 ± 3.92 aC	33.57 ± 5.70 aAB	25.10 ± 0.59 aA
	CM	49.83 ± 6.20 aAB	103.75 ± 5.35 bC	65.90 ± 10.31 bBC	35.87 ± 4.23 bA
ABA (ng g^{-1} FW)	**P**	161.90 ± 12.49 aC	142.37 ± 2.52 aC	94.17 ± 12.15 aB	58.63 ± 5.42 aA
	P+T34	151.93 ± 11.31 aC	107.30 ± 4.29 bB	79.27 ± 18.41 aAB	51.97 ± 10.94 aA
	CM	251.50 ± 40.55 aB	345.25 ± 1.05 cC	130.50 ± 6.57 aA	115.37 ± 8.47 bA
JA (ng g^{-1} FW)	**P**	1.00 ± 0.10 aA	0.40 ± 0.06 aA	1.03 ± 0.34 aA	0.57 ± 0.12 aA
	P+T34	0.83 ± 0.58 aA	1.50 ± 0.58 aA	0.80 ± 0.11 aA	0.77 ± 0.47 aA
	CM	1.60 ± 0.55 aA	0.90 ± 0.10 aA	0.67 ± 0.12 aA	0.73 ± 0.14 aA





Tomato leaves inoculated with *Botrytis cinerea* (10^5 CFU mL^{-1})

(A) Olive marc compost
(B) Perlite enriched with *Trichoderma asperellum* (10^4 CFU mL^{-1})
(C) Perlite

737

738

Highlights

- Compost triggered eustress in tomato plants, improving growth and health
- Compost induced systemic resistance linked to SA pathway/ABA
- *Trichoderma*-enriched perlite improved plant growth and innate disease resistance
- Different mechanisms of induced resistance are involved in compost and *Trichoderma*