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REGULAR ARTICLE

Growth, yield and physiology of *Verticillium*-inoculated pepper plants treated with ATAD and composted sewage sludge

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Abstract A greenhouse experiment was conducted to investigate the impact of sanitized sewage sludges, ATAD (*aerobic thermophilic autothermic digestion*) and composted, on *Verticillium*-induced wilt in pepper plants (*Capsicum annuum* L. cv. Piquillo). Two doses of ATAD (15 and 30% v/v) and three of composted sludge (15, 30 and 45% v/v) were applied to a peat-based potting mix. Unamended substrate was included as control. Half of the plants were inoculated with *V. dahliae*, whereas the other half remained non-inoculated. Result showed that ATAD and composted sludge increased growth and yield of non-inoculated plants. *V. dahliae* reduced net photosynthesis (P_n), mainly as a consequence of stomatal closure, 5 weeks after pathogen inoculation. The actual photosystem II efficiency was also reduced and consequently the electron transport rate (ETR). No photoinhibitory damage was observed at this time in diseased plants. At the end of the experiment, diseased plants showed lower plant biomass and fruit yield. ATAD sludge had little effect on the disease. Compost slightly alleviated *Verticillium*-induced wilt when applied at lower doses (15% v/v), which resulted in increased P_n and ETR, and higher plant biomass and fruit yield. By contrast, higher doses of compost (45% v/v) enhanced the effect of the pathogen, which was related to the high substrate salinity in this treatment.

Keywords ATAD · Compost · Sewage sludge · Pepper (*Capsicum annuum* L.) · *Verticillium dahliae* · photosynthesis

Introduction

The increasing production of sewage sludge from wastewater treatment plants creates significant pressure concerning the optimal management and disposal of this by-product. Land application is the best recycling option since most sewage wastes contain valuable nutrients and organic matter that can be used to improve soil fertility. Sewage sludge can also be used to control soil-borne plant pathogens, practice that could help growers save money, reduce the use of pesticides, and conserve natural resources. Some aspects of the use of sewage sludge are well documented, but not other. There is a wealth of information on the nutritional effect of this waste on

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plant performance (Antolín et al. 2005; Barzegar et al. 2002; Casado-Vela et al. 2007; Pascual et al. 2008). However, there is a lack of data worldwide on their effect on plant diseases, which calls for further studies.

In addition, the literature contains contradictory reports regarding the benefits of sewage sludge on the incidence of plant diseases. The sludge application to soil or potting mixtures reduced the severity of *Pythium ultimum* and *Phytophthora* sp. in pea and pepper (García et al. 2004), Fusarium wilt of tomato (Cotxarrera et al. 2002), *Phytophthora nicotianae* in citrus (Leoni and Ghini 2006), *Rhizoctonia solani* in radish, *Ralstonia solanacearum* in tomato, *Sclerotium rolfsii* in bean (Dos Santos and Bettiol 2003; Ghini et al. 2007), *Pythium myriotylum* in bean, *Fusarium oxysporum* sp. *melonis* in melon and *Phytophthora capsici* in pepper (Lumsden et al. 1983). On the other hand, Kim et al. (1997) reported that sewage sludge did not interfere with the incidence of root and crown rot caused by *Phytophthora capsici* in pepper. Similar results were obtained by Ghini et al. (2007) with *Fusarium oxysporum* in tomato and Lumsden et al. (1983) with *Pythium* in bean and pea. Finally, there are also reports of increased diseases as a consequence of sewage sludge incorporation, for example those caused by *Fusarium* spp. in corn (Bettiol 2004), *Pythium* spp. in cucumber, *Sclerotinia sclerotium* in tomato (Ghini et al. 2007) and *Fusarium solani* in pea (Lumsden et al. 1983).

The European Union (EU) through the Proposal for a Directive on Sludge (European Union 2003) asserted that sludge should be appropriately treated to satisfy specific microbial standards before its application to land. In this respect, the Proposal distinguishes two types of treatments: those that fully sanitize sludge, recognized as “advanced” treatments, and those that do not meet the degree of sanitation established in the above-mentioned Proposal, regarded as “conventional” treatments. The EU has specified use restrictions for those sludges treated with conventional processes, as a result, there is considerable interest in sludge treatment processes that operate at temperatures of 55°C or higher. Several thermophilic processes are used to stabilize sewage sludge in order to reduce pathogens, eliminate odours and reduce volume. One of the most widely employed technologies is composting. Although composting includes many benefits, it requires careful control of process parameters to ensure complete pathogen destruction and minimal odour production. Innovative methods of thermophilic stabilization include aerobic thermophilic autothermic digestion (ATAD), commonly referred as “liquid composting”. ATAD is one of the most promising technologies, which achieves a high sludge treatment rate and stabilization, and a high level of disinfection (Epstein 2003; Juteau 2006). It can also be referred to as a pasteurization process, because the sludge can achieve a sustained temperature of 70°C. In addition, the ATAD process has several environmental advantages, such as a high volatile solids reduction capability (between 38–50%) and reduced emissions of methane. The desired end product is a re-usable, high-quality biosolid that can be applied to land without further treatment.

Verticillium spp. is a soilborne pathogen that causes vascular wilt in over 160 agronomically important plant species worldwide, including vegetable, field, tree and ornamental crops (Schnathorst 1981). In Navarra, Northern Spain, Verticillium wilt is one of the most common diseases that affects pepper, and drastically decreases yield (Goicoechea et al. 2001). The control of *V. dahliae* is especially difficult due to the long viability of the resting structures, such as microsclerotia (Fradin and Thoma 2006). Therefore, many studies have been focused on different strategies in order to eradicate or minimise its persistence in soils. Restrictions on chemical products, the lack of genetic resistance and the failure of cultural methods for controlling Verticillium wilt in pepper induce to look for other alternatives (Palazón 1985). Concerning the use of organic amendments, several cases of controlling this pathogen have been reported in the literature. Tenuta and Lazarovits (2002) demonstrated that ammonia and nitrous acid from liquid swine manure and nitrogenous amendments inhibited microsclerotia germination. Goicoechea et al. (2004) suggested that organic amendments could stimulate defence mechanisms, providing an ecological and efficient means for the control of pepper wilt caused by *V. dahliae*. Other studies have revealed that organic wastes can alleviate the symptoms caused by *V. dahliae* (LaMondia et al. 1999; Paplomatas et al. 2005, the last one using biosolids). By contrast, Lazarovits et al. (1997) observed an increased infection index in tomato plants after conifer sawdust application, and more recently, Termorshuizen et al. (2006) have reported a disease stimulating effect of yard waste on eggplant.

In the literature, many of these papers deal with the suppressive effect of the amendments and their mechanisms, but few have focused on the physiological response of the plant. Knowledge of plant physiological processes and the way they are affected by a pathogen may be used to analyse and predict the effect of the disease on crop growth and yield (Bastiaans 1993).

The aim of the present work was, therefore, to investigate the effect of two sanitized sewage sludges, treated with ATAD and composting technologies, on the *Verticillium*-induced wilt of pepper plants, focusing on growth, yield and the photosynthetic response to the pathogen. For this purpose, a long-term experiment was carried out with plants grown in a peat-based potting mix until fruit ripening in controlled environmental greenhouse.

Material and methods

Organic amendments

Two sanitized sewage sludges treated with "advanced" technologies (European Union 2003) were employed in the experiment: ATAD and composting. ATAD is an exothermic process in which sludge is subjected to temperatures greater than 55°C with a hydraulic retention time of 6–15 days. Organic solids are degraded and heat released during the microbial degradation maintains thermophilic temperatures. ATAD and composted sludge were obtained from Tudela and Pamplona (Navarra, Spain) wastewater plants, respectively. The main properties of the sludges are shown in Table 1.

Table 1 Sewage sludge properties

	ATAD	Compost
dry matter (%)	7.34	47.44
pH	6.4	5.1
EC[1] (dS m^{-1})	6.3	4.5
TOC[2] (%)	37.58	27.63
$N_{Kjeldahl}$	1.18	2.65
C/N	28	10
P_2O_5 (%)	3.88	5.40
K_2O (%)	0.62	1.43
CaO (%)	11.39	14.44
MgO (%)	1.21	2.15
SO_3 (%)	3.46	2.59
Na_2O (%)	0.56	0.20
Fe (%)	0.99	1.19
Mn (mg kg^{-1})	200	230
B (mg kg^{-1})	60	70
Cd (mg kg^{-1})	0.7	0.6
Cu (mg kg^{-1})	103	117.5
Ni (mg kg^{-1})	7	10.8
Pb (mg kg^{-1})	42.3	48.1
Zn (mg kg^{-1})	523	498.9
Hg (mg kg^{-1})	0.47	0.61
Cr (mg kg^{-1})	17	28

[1] EC: electric conductivity

[2] TOC: total organic carbon

Biological material, growth conditions and experimental design

A peat-based commercial container medium mixed with perlite and sand (4:1:1, v/v/v) were packed into pots with a capacity of 2 L. ATAD sludge was added to this substrate at two doses: 15 and 30% (v/v) (A1 and A2, respectively). Composted sludge was added at doses of 15, 30 and 45% (v/v) (CP1, CP2 and CP3, respectively). Pots containing substrate without the addition of sludge were included as a control (C) group. The sludges were added to the substrate 1 month before transplanting. This period of time allows the level of phytotoxic substances (e.g. excess ammonium) to decrease, as well as the mixture to homogenize microbiologically. Then, half of the pots from each treatment (five pots per treatment) were inoculated with *V. dahliae*. The other half of the pots remained uninoculated (-V). *Verticillium* was isolated from diseased pepper plants grown under field conditions, and cultured in Petri dishes for 10 days on Messiaen culture medium at 25°C in the dark (Hoyos et al. 1993). Conidia from the surface of several plates were carefully harvested by adding sterile distilled water to the plates and gently rubbing the surface of the colony with a sterile bent glass rod. The conidial suspension was filtered through a double layer of sterile cheesecloth. Conidial concentration was determined with a Neubauer chamber and the suspension was adjusted to obtain a concentration of 5×10^5 conidia per mL. Substrate was inoculated with 200 mL of this suspension to obtain a concentration of 5×10^4 conidia per mL of substrate. Therefore, twelve treatments were assayed: C, A1, A2, CP1, CP2, CP3 inoculated (+V) or not (-V) with *V. dahliae*.

One pepper seedling (*Capsicum annuum* L. cv Piquillo) (2 or 3-leaf stage) was transplanted into each pot. Plants were grown in a controlled environment greenhouse maintained at 25/15°C day/night and received natural daylight supplemented with irradiation from halogen lamps Son-T-Agro (Philips Nederland B.V., Eindhove) during 14 h photoperiod. Plants were irrigated daily with deionised water and once a week with full strength Hoagland nutrient solution. During fruit set and ripening, irrigation with Hoagland was increased from one to two times a week. Growth media samples were taken before transplanting for physicochemical and microbial analyses. Leaf gas exchange, chlorophyll fluorescence, leaf photosynthetic pigment concentrations and relative water content (RWC) were determined at the end of the vegetative stage (5 weeks after inoculation). Plants were harvested at maturity stage (plants with red fruits) for the determination of growth and yield parameters. The experiment was repeated twice with similar results. Data from both repetitions were analyzed pooled (total of ten plants per treatment).

Disease assessment

Disease incidence and severity were calculated weekly along the growth period. Disease incidence (I) was estimated as the percentage of *Verticillium*-inoculated plants with visible symptoms (chlorotic, wilted or abscised leaves) related to total plants per treatment. Disease severity (S) was non-destructively estimated as the sum of chlorotic, wilted and abscised leaves related to the total leaves per plant, expressed as percentage (Goicoechea et al. 2001). Incidence and severity values were used to calculate a disease index (DI) calculated as follows: (S x I)/the maximum severity scale (100%) (Luo et al. 2000). In order to estimate statistical significant differences between treatments, the area under the disease progress curve (AUDPC) was calculated by the trapezoidal integration method (Campbell and Madden 1990).

Plant growth and water status

Plant height and total leaf number were measured before harvest. Leaf, shoot and root dry matter (DM) was determined after drying at 80°C for 2 days. Fruit DM was calculated after drying at 60°C for 45 days. Leaf area was measured with a leaf area meter (LI-300, Li-Cor). Relative water content (RWC) was estimated by a modification of Wheatherley's method (1950) on youngest fully mature leaves (Goicoechea et al. 2004).

Gas exchange and chlorophyll fluorescence

One day prior to measurements, the plants were transferred to a controlled environmental chamber with a day/night regime of 25/15°C, 60/80% relative humidity and 310 μmol photons m^{-2} s^{-1} PAR with a photoperiod of 14 h. Gas exchange and chlorophyll fluorescence measurements were conducted in asymptomatic fully expanded leaves of the same physiological stage (3rd or 4th node from the top), using a portable photosynthesis system (GFS-3000, Walz) with a 3 cm^2 cuvette. Dark respiration (R_D) measurements were performed 3 h before the beginning of the light period with the CO_2 concentration set at 350 ppm, the temperature in the measurement chamber at 25°C, and 60% relative humidity. Gas exchange characteristics in illuminated leaves were measured 3 h after the beginning of the light period under a photon flux density of 1600 μmol photons m^{-2} s^{-1}, 350 ppm CO_2, 25°C, and 60% relative humidity. Dark respiration, net photosynthesis (P_n), stomatal conductance (g_s), transpiration rate (T_r) and sub-stomatal CO_2 concentration (C_i) were calculated according to von Caemmerer and Farquhar (1981). Chlorophyll fluorescence was measured immediately after gas exchange measurements (in the dark and in the light) with a fluorescence module (PAM-fluorometer 3055-FL, Walz) attached to the photosynthesis equipment. The minimal and maximal fluorescence (Fo and Fm, respectively) were measured in dark-adapted leaves, whereas Fo' and Fm' were measured at mid-morning in the same leaves with a photon flux density of 1600 μmol m^{-2} s^{-1}, also measuring steady-state fluorescence signal (Fs). The maximum potential PSII efficiency was calculated as Fv/Fm, where Fv is Fm-Fo (Abadía et al. 1999; Morales et al. 1991). Actual (Φ_{PSII}) and intrinsic ($\Phi_{exc.}$) PSII efficiency were calculated as (Fm'-Fs)/Fm' and Fv'/Fm' (where Fv' is Fm'-Fo'), respectively. Photochemical quenching (qP) was calculated as (Fm'-Fs)/Fv', and non-photochemical quenching (NPQ) as (Fm/Fm')-1 (Larbi et al. 2006; Morales et al. 1998, 2000). Electron transport rate (ETR) was calculated according to Krall and Edwards (1992) as Φ_{PSII} × PPFD × 0.84×0.5, where PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 was used as the fraction of excitation energy distributed to PSII and 0.84 as the fractional light absorbance. Light respiration (R_L) was estimated as 1/12 (ETR – 4 x (P_n + R_D)) (Valentini et al. 1995).

Photosynthetic pigments

Leaf disks, harvested immediately after gas exchange and chlorophyll fluorescence measurements, were cut with a calibrated cork borer, wrapped in aluminium foil and immediately plunged into liquid nitrogen. Leaf photosynthetic pigments were extracted with acetone in the presence of Na ascorbate and stored as described by Abadía et al. (1999). Pigment extracts were thawed on ice, filtered through a 0.45-µm filter, and analysed by an isocratic HPLC method based on that developed by De las Rivas et al. (1989) with some modifications (Larbi et al. 2004). Two steps, were used: mobile phase A (acetonitrile: methanol, 7:1, v/v) was pumped for 3.5 min, and then mobile phase B (acetonitrile:methanol:water:ethyl acetate, 7:0.96:0.04:8 by volume) was pumped for 4.5 min. To both solvents, 0.7% (v:v) of the modified triethylamine (TEA) was added (Hill and Kind 1993) to improve pigment stability during separation. The analysis time for each sample was 13 min, including equilibration time.

Physicochemical and microbial properties of soil

Substrate pH and electrical conductivity (EC) were analysed in water extracts (1:2.5 and 1:5 w/v, respectively). Total N was measured by Kjeldahl's method. Available N ($N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$) was extracted with 1M KCl and determined spectrophotometrically in the filtered extracts as described by Pascual et al. (2007). Soil microbial activity was assessed by measuring soil respiration in hermetically sealed flasks, in which a 30-g soil sample was kept in the dark at 28°C and 60% of its water holding for 33 days. The CO_2 emitted was measured daily with an infrared gas analyser (IRGA) (HCM-100, Walz) (Pascual et al. 2008).

Statistical analysis

Two factor analysis of variance (ANOVA) was performed in order to partition the variance into the main effects and the interaction between the two factors: amendment and V. dahliae. Means ± standard errors were calculated, and when the F ratio was significant, least significant difference (LSD) test was applied as available in the SPSS statistical package version 12.0 programs for Windows XP.

Results

Plant growth

The application of the highest doses of ATAD (A2) and composted sludge (CP2 and CP3) to non-inoculated plants increased leaf number and area, leaf, shoot and fruit dry matter (DM) per plant, compared to control (Table 2). There was a clear effect of the sludge application dose on growth parameters, especially on leaf area and fruit dry matter production. *V. dahliae* significantly decreased plant growth and yield. However, this decline was less relevant in the plants amended with the lowest dose of compost (CP1 +V), which showed significantly higher height, leaf number and area, leaf and shoot dry matter, as well as fruit yield than control inoculated plants (C +V). On the contrary, when compost was applied at 45% (CP3 +V) plant growth was severely affected by the pathogen. ATAD sludge did not have any effect on plant growth when plants were infected with the pathogen. A significant interaction between factors (amendment and *V. dahliae*) was observed for all the growth parameters measured, therefore we did not take into account the effect of these two individual factors.

Disease assessment

Symptoms of the disease in inoculated ATAD plants (A1 +V and A2 +V) appeared on day 15 and 16 after inoculation, respectively, 6 days before than in C +V plants (Fig. 1). The disease incidence (I) in A2 +V was higher compared to C +V until day 35, but such differences disappeared at the end of the plant life cycle. Disease severity (S) of ATAD plants was similar to that of C +V, along the whole growth period (Fig. 1). The Area Under Disease Progress Curve (AUDPC) of ATAD plants did not differ significantly from C +V (Fig. 2). In pepper plants treated with 15% of compost (CP1 +V) first wilting

Table 2 Plant height, leaf number and area and leaf, stem, root and fruit dry matter (DM) of plants grown in unamended substrate (C) and substrate amended with varying rates of ATAD (A1, A2) and composted (CP1, CP2 and CP3) sewage sludge, inoculated (+V) or not (-V) with *V. dahliae*

Treatments	Height (cm)	Leaf number (number plant^{-1})	Leaf area (dm^2 plant^{-1})	Leaf DM (g plant^{-1})	Shoot DM (g plant^{-1})	Root DM (g plant^{-1})	Fruit DM (g plant^{-1})
C -V	79.97 ab[1]	118.3 cd	6.90 d	12.75 c	13.20 c	12.92 b	25.06 e
A1 -V	71.75 b	100.0 d	9.85 c	14.92 bc	18.23 bc	13.98 b	32.17 d
A2 -V	80.38 ab	147.3 bc	15.05 b	17.81 bc	24.48 ab	22.06 ab	38.56 bc
CP1 -V	77.30 ab	111.3 d	14.31 b	17.45 bc	20.46 bc	15.09 ab	35.88 cd
CP2 -V	90.55 a	186.0 a	21.24 a	22.30 a	30.59 a	26.05 a	43.94 a
CP3 -V	88.53 ab	173.2 ab	22.71 a	24.18 a	30.36 a	22.70 ab	42.22 ab
C +V	34.11 d	7.0 f	0.47 fg	2.20 ef	4.13 ef	3.47 cd	3.28 gh
A1 +V	25.07 de	15.3 f	0.32 g	2.47 ef	3.47 ef	2.60 ef	3.00 gh
A2 +V	22.38 ef	22.1 ef	0.79 g	2.59 ef	3.36 ef	2.02 ef	1.73 gh
CP1 +V	52.37 c	42.0 e	0.77 e	4.84 d	9.02 d	4.95 c	7.97 f
CP2 +V	35.30 d	44.0 e	0.70 ef	4.12 de	7.07 de	3.68 cd	5.37 fg
CP3 +V	12.76 ef	4.3 f	1.52 g	0.88 f	0.73 f	0.62 f	0.21 h
Interaction	**	**	**	**	**	*	**

[1] Within each column, values followed by different letters are significantly different based on LSD test ($P<0.05$).

Symbols used in the two-ways ANOVA: * and ** significant differences at 1 and 0.1%, respectively.

A1 (15% v/v), A2 (30% v/v), CP1 (15% v/v), CP2 (30% v/v) and CP3 (45% v/v). Values are means of ten plants

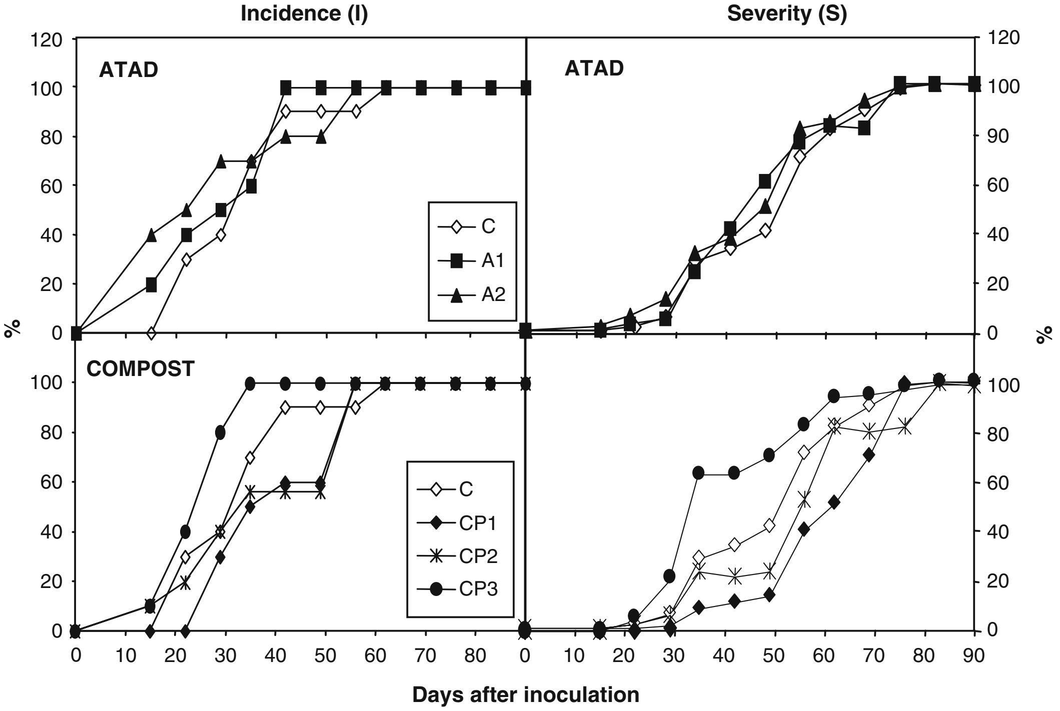

Fig. 1 Disease incidence (I) and severity (S) of plants grown in unamended substrate (C) and substrate amended with varying rates of ATAD (A1, A2) and composted (CP1, CP2 and CP3) sewage sludge, inoculated (+V) with *V. dahliae*. A1 (15% v/v), A2 (30% v/v), CP1 (15% v/v), CP2 (30% v/v) and CP3 (45% v/v). Each point represents the mean of ten plants

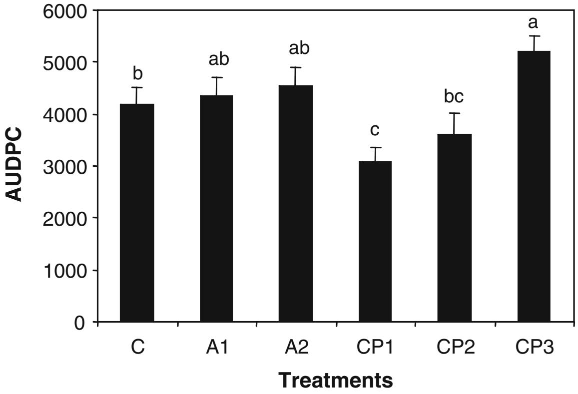

Fig. 2 Area Under Disease Progress Curve (AUDPC) of plants grown in unamended substrate (C) and substrate amended with varying rates of ATAD (A1, A2; 15 and 30% v/v, respectively) and composted (CP1, CP2 and CP3; 15, 30 and 45% v/v, respectively) sewage sludge, inoculated (+V) with *V. dahliae*. Each bar represents the mean of ten plants. The different letters indicate significant differences between treatments based on LSD test ($P<0.05$)

symptoms were detected ca. 28 days after inoculation, 6 days after C +V. In addition, these plants showed lower incidence and severity values, as well as a significantly lower AUDPC than C +V (Figs. 1 and 2). By contrast, the highest dose of compost (CP3 +V) led to an increase in I, S and AUDPC compared to control plants. Non-inoculated plants always remained symptomless.

Gas exchange, chlorophyll fluorescence and photosynthetic pigments

In general, the inoculation with *V. dahliae* affected neither R_D nor R_L rates (Figs. 3 a and b). Only CP1 showed higher R_L values in +V plants compared to -V. The net photosynthetic rate, transpiration and stomatal conductance of leaves decreased dramatically in *V. dahliae* inoculated plants (Fig. 3 c, d and e). However, a lower decline was observed in CP1 +V, which exhibited the highest P_n, g_s and T_r values among +V treatments. The sub-stomatal CO_2 concentration (C_i) increased in inoculated plants, compared to those healthy (Fig. 3 f), although differences were statistically significant only in A1, CP2 and CP3 +V plants.

In most cases, no differences between healthy and diseased plants were observed for Fv/Fm, $\Phi_{exc.}$, and NPQ (Fig. 4 a, d and e). On the contrary, *V. dahliae* decreased ETR, Φ_{PSII} and qP in all the treatments (Fig. 4 b, c and f). However, CP1 +V plants maintained higher values compared to other +V treatments. CP1 plants showed a significant increase in this parameter after pathogen inoculation, in line with their lowest $\Phi_{exc.}$ values (Fig. 4 d). Finally, *Verticillium*-induced wilt increased significantly $ETR/(P_n+R_D+R_L)$ ratio in all the treatments assayed (Fig. 5).

In general, inoculation with *V. dahliae* slightly decreased the total leaf chlorophyll concentration, although significant differences between healthy and diseased plants were only observed in A2, CP1, CP2 and CP3 treatments (Table 3). No differences between -V and +V were observed for lutein and antheraxanthin (A). However, +V plants showed lower β-carotene and violaxanthin (V) concentrations, and higher zeaxanthin (Z) in most treatments. Despite the high coefficients of variation, the sum of Z + A and the de-epoxidation state $(DPS=(Z+A)/(V+A+Z))$ tended to increase in diseased plants compared to those healthy (Table 3).

Plant water status

In general, *V. dahliae* inoculation did not modify significantly the leaf relative water content (RWC) 5 weeks after inoculation (Fig. 6 a). Only A2 +V showed significantly lower RWC values compared to A2 -V. Clearly, the correlation between P_n and RWC, as well as g_s and RWC, presented a very large scattering in the highest values of RWC, revealing that plants with similar RWC had quite different photosynthetic rates (Fig. 6 b and c). When comparing plants with similar and high RWC (between 80–95%), it can be noted that +V plants showed always lower P_n and g_s than -V.

Physicochemical and microbial properties of soil

The addition of both ATAD and composted sewage sludge to soil decreased pH and increased EC, total N, N-NH_4^+ and N-NO_3^- at the beginning of the experiment, specially with the highest doses of compost (CP2 and CP3) (Table 4). N-NO_2^- decreased significantly in the amended soils compared to control. Sewage sludge significantly increased soil respiration as the application dose increased (Table 4).

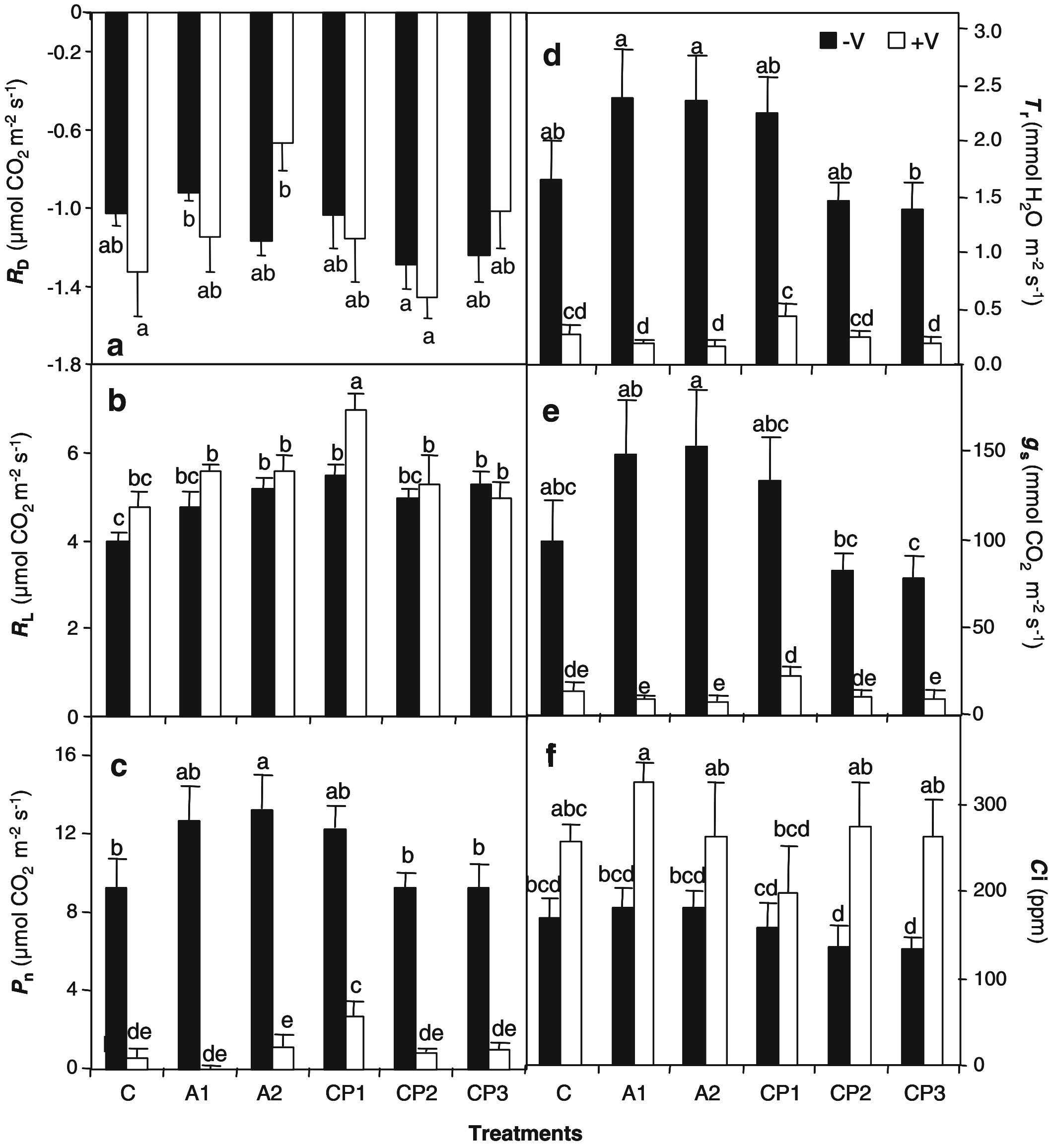

Fig. 3 Dark respiration (R_D, a), light respiration (R_L, b), net photosynthesis (P_n, c), transpiration rate (T_r, d), stomatal conductance (g_s, e), and sub-stomatal CO$_2$ concentration (C_i, f) of plants grown in unamended substrate (C) and substrate amended with varying rates of ATAD (A1, A2; 15 and 30% v/v) and composted (CP1, CP2 and CP3; 15, 30 and 45% v/v) sewage sludge, inoculated (+V) or not (-V) with *V. dahliae*. Bars represent the mean ± SE of ten plants. The different letters indicate significant differences between treatments based on LSD test ($P<0.05$)

Discussion

Verticillium dahliae reduced growth and yield in pepper plants. In particular, it caused defoliation, reduced shoot height and leaf number, and decreased total biomass as reported by Goicoechea et al. (2001, 2004). Significant correlations (not shown) were found between disease severity and total dry matter production (0.62***) and the number of days until the first wilting symptoms appeared (−0.56***), revealing a clear relationship between disease severity and plant growth. In non-inoculated plants, application of sewage sludge increased plant growth (21–203%) and fruit yield (28–75%), in line with previous reports (Arancon et al. 2004; Casado-Vela et al. 2007; Pascual et al. 2008). Within these ranges, the lowest and highest increases corresponded to application of 15% ATAD and 30% composted sewage sludge, respectively. From

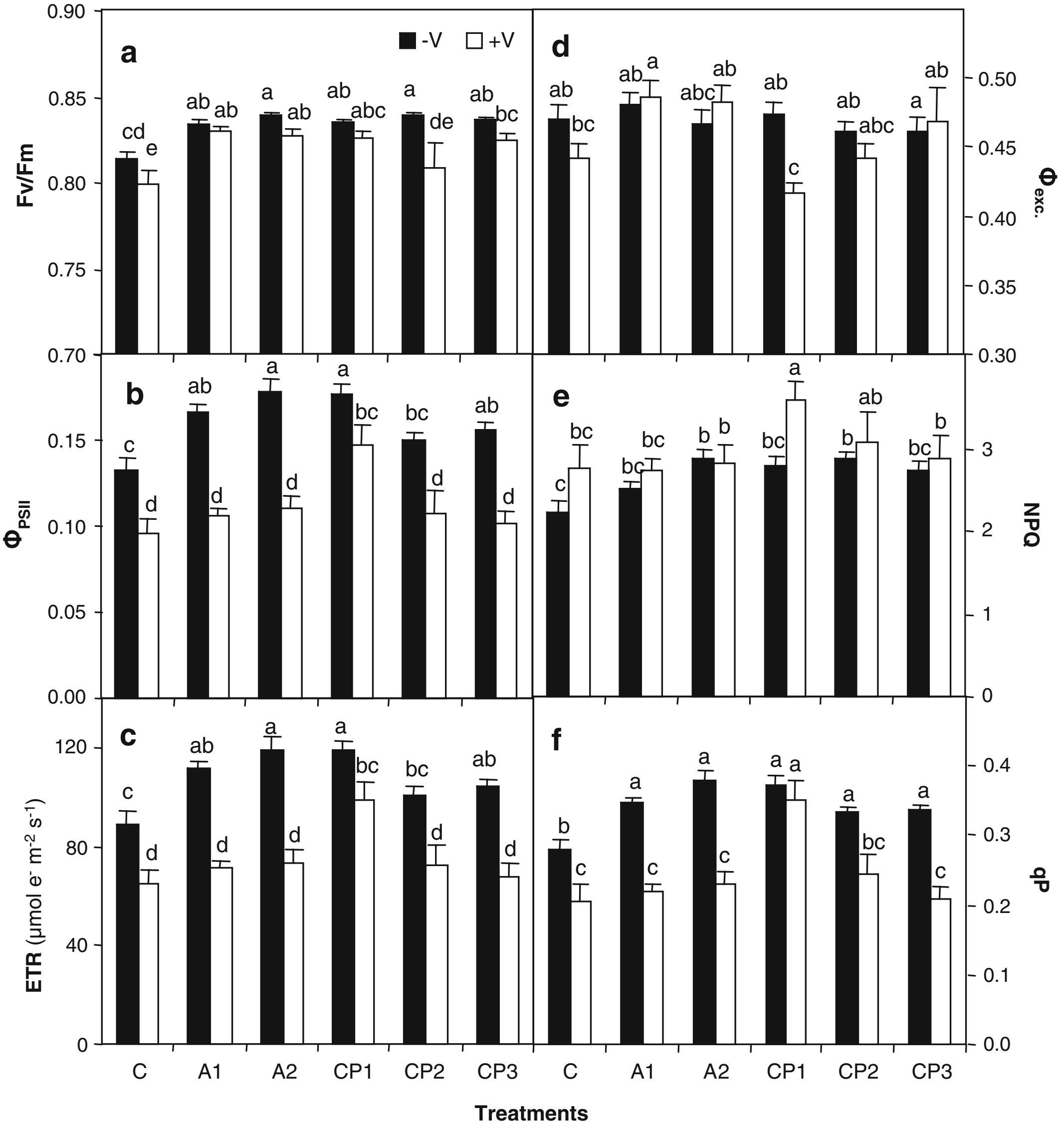

Fig. 4 Maximum potential PSII efficiency (Fv/Fm, a), actual (Φ_{PSII}, b) and intrinsic (Φ_{exc}, d) PSII efficiency, electron transport rate (ETR, c), non-photochemical quenching (NPQ, e) and photochemical quenching (qP, f) of plants grown in unamended substrate (C) and substrate amended with varying rates of ATAD (A1, A2; 15 and 30% v/v) and composted (CP1, CP2 and CP3; 15, 30 and 45% v/v) sewage sludge, inoculated (+V) or not (-V) with *V. dahliae*. Bars represent the mean ± SE of ten plants. The different letters indicate significant differences between treatments based on LSD test ($P<0.05$)

these data, it can be concluded that composted wastes improve vegetative growth more than growth of reproductive organs (fruit yield). The increased growth and yield have been previously attributed directly to nutrient availability (Pascual et al. 2008) and presence of humic acids (HA) (Sun et al. 2006), and indirectly to increased soil rhizosphere microorganisms activity (Pascual et al. 2008), through the production of growth-stimulating plant hormones (Frankenberger and Arshad 1995). In this report, we have shown increased sewage sludge-mediated soil respiration, indicative of higher microorganisms activity.

Effect of wastes on the disease was dependent on the type of sewage sludge treatment, and on the dose applied. ATAD sludge had only slightly negative effect at the beginning of the experiment, increasing the disease incidence of *V. dahliae*. This fact led to smaller plants at the end of the growth period (especially at

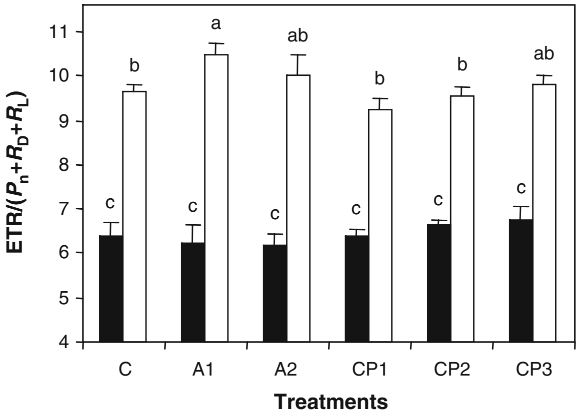

Fig. 5 Ratio of electron transport rate (ETR) to the sum of net CO_2 assimilation plus dark and light respiration of plants grown in unamended substrate (C) and substrate amended with varying rates of ATAD (A1, A2; 15 and 30% v/v) and composted (CP1, CP2 and CP3; 15, 30 and 45% v/v) sewage sludge, inoculated (+V) or not (-V) with *V. dahliae*. Bars represent the mean ± SE of ten plants. The different letters indicate significant differences between treatments based on LSD test ($P<0.05$)

30% ATAD), but did not cause differences neither in dry matter production (except roots) nor in yield. Compost applied at a dose of 15% (v/v) attenuated the decrease of plant growth (from 75–52%) and yield (from 87–68%) induced by *V. dahliae*, due to a reduced disease incidence and severity. Among the possible factors responsible for such disease attenuation, several authors have pointed out the alteration of the soil physico-chemical properties (electric conductivity, pH or level of different N forms) caused by the application of the sewage sludge. Leoni and Ghini (2006) and Dos Santos and Bettiol (2003) established negative correlations between EC and the incidence of *Phytophthora nicotianae* in citrus and *Sclerotioum rolfsii* in bean, respectively. Tenuta and Lazarovits (2002) observed a decrease in the viability of *Verticillium* microsclerotia as a consequence of the accumulation of nitrogenous compounds. In the present study, sludge application decreased soil pH and increased EC, as well as the concentration of N-NO_3^-. However, these values were similar in the substrate treated with 15% compost and that treated with ATAD at dose 30%, which did not show any significant effect on the disease. Consequently, sludge-induced changes in these physico-chemical parameters would not explain the disease attenuation in CP1. Among the biological factors, although Termorshuizen et al. (2006) reported a negative correlation between respiration and Verticillium wilt suppression in a compost/peat mix, in general, the increase in the soil microbial activity and the microbiota itself comprised in the organic material has been related to the reduction of plant severity in several pathosystems (Craft and Nelson 1996; Dos Santos and Bettiol 2003; Downer et al. 2001; Leoni and Ghini 2006). Nevertheless, as was observed for the physico-chemical properties, the stimulation of the microbial activity after the addition of 15% composted sewage sludge was similar to that observed in the ATAD treatments. Therefore, we also have to rule out this factor as responsible for the attenuation of the disease observed in this treatment (15% of compost). We may only hypothesize that the presence of antagonistic microorganisms in the compost, as well as the compost-mediated changes in the soil microorganisms population could produce this effect. Such factor may also explain the different biocontrol capacity of ATAD and composted sewage sludge. García et al. (2004) also observed a higher biopesticide effect of composted sewage sludge compared to an anaerobically digested sludge against *Pythium ultimum* and *Phytophthora* sp., suggesting that the incorporation in the composting process of a bulking agent, rich in lignin and cellulose, as well as the composting process itself may have led to changes in the microbial community of the sludge, increasing the population of microorganisms with biocontrol capacity.

The addition of compost at doses of 45% enhanced the deleterious effect of *V. dahliae* on plant growth (from 75–94%) and yield (from 87–99%). Such result could be related to the toxicity observed in the early establishment of seedlings (first 2 weeks after transplanting) in this treatment, with plants significantly smaller than controls (6.9 and 10.1 cm, respectively) and with fewer expanded leaves (4.8 and 6.5, respectively) (data not shown). Such toxicity could have weakened the plants and made them more susceptible to the pathogen. Leoni and Ghini (2006) and Ghini et al. (2007) reported a temporary phytotoxicity when large volumes of sewage sludge were incorporated to the soil, which were attributed to heavy metals and salinity. Analyses of the sewage sludge used in this work revealed the presence of both heavy metals and soluble salts. Calculations of heavy metals concentrations (considering the amount of water available in the pots at field capacity, not

Table 3 Chlorophyll (Chl) and carotenoids concentrations and de-epoxidation state (DPS) of plants grown in unamended substrate (C) and substrate amended with varying rates of ATAD (A1, A2) and composted (CP1, CP2 and CP3) sewage sludge, inoculated (+V) or not (-V) with *V. dahliae*

Treatments	Chl a + b (μmol m^{-2})	β-Carotene	Neoxanthin	Lutein	Violaxanthin (V)	Antheraxanthin (A)	Zeaxanthin (Z)	Z + A	V + A + Z	$DPS(Z+A)/(V+A+Z)$
		(mmol mol^{-1} Chl)								
C -V	103.4 f[1]	92.1 a	49.4 a	124.8 a	48.7 a	2.91 a	1.50 c	4.41 b	53.15 a	0.08 b
A1 -V	146.3 cd	86.3 a	43.2 ab	106.5 bc	39.5 b	3.11 a	3.11 bc	6.22 ab	45.71 ab	0.13 abc
A2 -V	173.1 a	76.9 cd	38.5 bc	99.7 c	34.8 bc	1.84 a	0.59 c	2.43 b	37.23 bc	0.06 c
CP1 -V	157.2 abc	77.4 bcd	38.1 bc	105.1 bc	34.9 bc	3.20 a	0.55 c	3.75 b	38.63 bc	0.10 abc
CP2 -V	170.1 ab	81.1 bc	39.2 b	98.4 c	32.4 bc	1.68 a	3.04 bc	4.72 ab	37.15 bc	0.13 abc
CP3 -V	156.9 abc	79.9 bcd	41.1 ab	100.2 bc	32.2 bc	2.48 a	2.78 c	5.26 ab	37.60 bc	0.13 abc
C +V	97.7 f	77.9 bcd	43.2 ab	114.1 ab	31.4 c	4.51 a	8.66 ab	13.17 a	44.55 bc	0.28 a
A1 +V	151.2 cd	76.4 cd	40.6 b	104.1 bc	32.4 bc	0.74 a	1.78 c	2.52 b	34.89 c	0.06 c
A2 +V	154.6 bc	75.0 cde	40.1 b	103.8 bc	23.7 d	3.60 a	4.51 abc	8.10 ab	31.82 c	0.26 ab
CP1 +V	129.8 e	78.2 bcd	41.2 ab	108.6 bc	33.4 bc	2.02 a	2.56 c	4.58 ab	38.00 bc	0.12 abc
CP2 +V	136.0 de	66.7 e	29.4 c	100.1 c	18.5 d	3.32 a	9.98 a	13.30 a	31.77 c	0.28 ab
CP3 +V	145.2 de	71.9 de	35.8 bc	99.3 c	23.8 d	3.17 a	3.99 bc	7.15 ab	30.93 c	0.21 abc
Amendment	***	*	*	***	***	ns	ns	*	**	ns
V. dahliae	***	**	*	ns	***	ns	**	*	***	*
Interaction	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

[1] Within each column, values followed by different letters are significantly different based on LSD test ($P<0.05$).

Symbols used in the two-ways ANOVA: ns, no significant difference; *, ** and *** significant differences at 5, 1 and 0.1%, respectively.

A1 (15% v/v), A2 (30% v/v), CP1 (15% v/v), CP2 (30% v/v) and CP3 (45% v/v). Values are means of ten plants

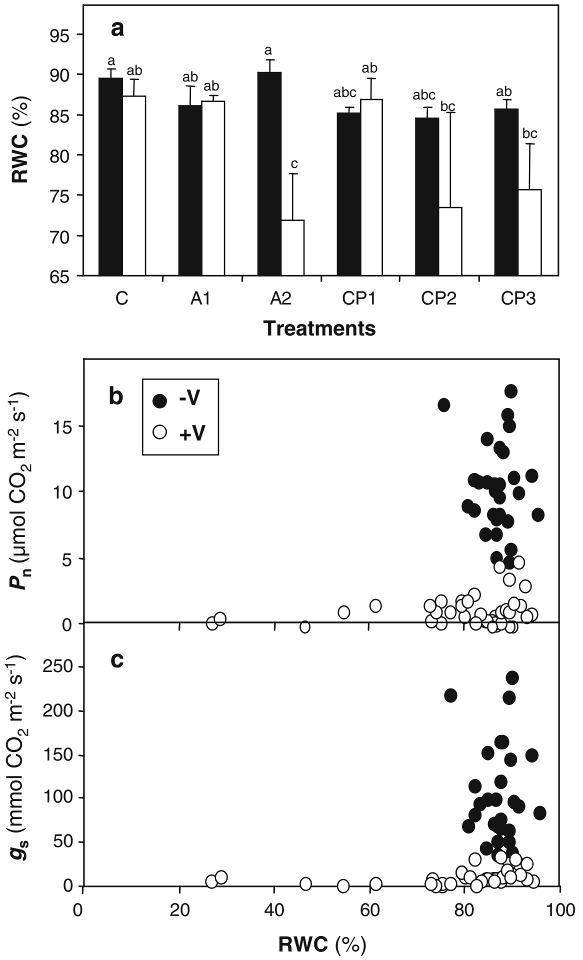

Fig. 6 Relative water content (RWC) and correlation between P_n, g_s and RWC of plants grown in unamended substrate (C) and substrate amended with varying rates of ATAD (A1, A2; 15 and 30% v/v) and composted (CP1, CP2 and CP3; 15, 30 and 45% v/v) sewage sludge, inoculated (+V) or not (-V) with *V. dahliae*. Bars represent the mean ± SE of ten plants. The different letters indicate significant differences between treatments based on LSD test ($P<0.05$)

shown) revealed concentrations far below those inducing symptoms in plants (see Fodor et al. 2005 and references therein; Larbi et al. 2002), and plants did not develop heavy metals-related symptoms during the whole experiment. In line with this, in a separate experiment using ATAD as amendment, leaf concentrations of heavy metals were similar in presence or absence of the sewage sludge (Pascual et al. 2008). Electric conductivity of the substrate amended with 45% composted sewage sludge reached 3.17 dS m^{-1} (see Table 4), which points out salinity as a putative cause for the enhancement of the deleterious effect of *V. dahliae*. Recent works have demonstrated that salinity increases fungal root and shoot colonization by *V. dahliae* and, as a consequence, disease severity (Levin et al. 2007; Saadatmand et al. 2008). Dickerson (1996) also proposed salinity as the main cause for losses found with *Phytophthora capsici* in a field experiment with pepper.

Verticillium dahliae reduced photosynthetic rates in pepper plants, mainly due to stomatal closure (reflected in lower leaf conductance and transpiration rates) (Bowden et al. 1990; Haverkort et al. 1990; this work). This result agrees with data reported for other plant species (Bowden et al. 1990; Goicoechea et al. 2001; Haverkort et al. 1990; Lorenzini et al. 1997; Pennypacker et al. 1990; Sadras et al. 2000; Saeed et al. 1999). Vascular wilt pathogens decrease plant hydraulic conductance due to plugging of the xylem vessels by the fungus, which may lead to leaf water deficits (Adams et al. 1987). Accelerated senescence, presumably caused by hormonal changes (Tzeng and DeVay 1985), and a possible involvement of toxins and ethylene (Aguirreolea et al. 1995) have also been proposed as responsible for stomatal closure in pathogen-wilted plants. The fact that photosynthesis in diseased plants showed a strong reduction compared to that observed in non-inoculated ones at similar and high RWC (80–95%) (see Fig. 6) suggests that pepper plants detect the presence of the fungus in the xylem and close stomata, avoiding water losses during the first stages of fungus colonization.

The impaired photosynthesis observed in Verticillium wilted pepper plants is a consequence of the regulatory response of the plant to the fungus attack, not reflecting damage to the photosynthetic apparatus. On one hand, inoculated plants compensated the decreases in photosynthesis with a fairly unchanged dark and light respiration, processes that consume electrons generated in the photosynthetic electron transport chain (maintaining or increasing C_i, the sub-stomatal CO_2 concentration). Thus, the ratio of electrons generated to electrons consumed (see Fig. 5) increased after fungus attack from ca. 6–7 to 9–10, which can be considered negligible from a physiological point of view (Morales et al. 2006). Data indicate therefore that there is not an excess of electrons available to react with oxygen, generating reactive oxygen species and oxidative damage to biomolecules. Photosynthetic pigments are one of the

Table 4 pH, electric conductivity (EC), $N_{Kjeldahl}$, N-NH_4^+, N-NO_3^-, N-NO_2^- and soil respiration in unamended substrate (C) and substrate amended with varying rates of ATAD (A1, A2) and composted (CP1, CP2 and CP3) sewage sludge, at the beginning of the experiment

Treatments	pH	EC (dS m^{-1})	$N_{Kjeldahl}$ (g 100 g^{-1})	N-NH_4^+ (mg kg^{-1})	N-NO_3^- (mg kg^{-1})	N-NO_2^- (mg kg^{-1})	Soil respiration (mg C-CO_2 kg^{-1} d^{-1})
C	6.97 a[1]	0.90 e	0.32 e	158.0 bc	454.7 c	1.83 a	15.07 d
A1	6.37 b	1.33 d	0.45 cd	208.4 ab	672.7 c	0.37 b	24.28 c
A2	6.27 c	1.60 c	0.41 de	49.0 c	833.3 c	0.68 b	27.04 b
CP1	6.27 c	1.67 c	0.53 c	142.0 bc	577.3 c	0.37 b	26.81 bc
CP2	6.13 d	2.37 b	0.80 b	157.6 bc	1358.7 b	0.78 b	35.15 a
CP3	6.10 d	3.17 a	1.00 a	316.6 a	2133.3 a	0.70 b	36.07 a

[1] Within each column, values followed by different letters are significantly different based on LSD test ($P<0.05$)

A1 (15% v/v), A2 (30% v/v), CP1 (15% v/v), CP2 (30% v/v) and CP3 (45% v/v). Values are means of ten plants

main targets of oxidative damage in plants, especially chlorophylls. In line with the previously mentioned data about electrons consumption, loss of chlorophyll (and therefore leaf yellowing) was not observed in inoculated plants. Other photosynthetic parameters, indicative of an increased photoprotection under stress, like thermal energy dissipation mediated by zeaxanthin and antheraxanthin (see Table 3) and reflected in increased NPQ and decreased intrinsic PSII efficiency ($\Phi_{exc.}$) values (see Fig. 4), were not markedly affected in Verticillium wilted plants. Among the amendments investigated, only 15% composted sewage sludge slightly increased photosynthetic and electron transport rates in inoculated plants (Figs. 3 and 4).

On the other hand, one of the most classical methods to detect damage to the photosynthetic apparatus is to estimate the maximum potential PSII efficiency using the dark-adapted Fv/Fm chlorophyll fluorescence ratio (Abadía et al. 1999; Morales et al. 1991). In the present study, Fv/Fm ratios were almost unaffected by the pathogen, revealing the absence of permanent photoinhibition and the subsequent photodamage. It must be noted that Fv/Fm ratio was measured in the early morning after the natural night period, so diurnal decreases of Fv/Fm as a consequence of a dynamic photoinhibition cannot be discarded. In fact, some down-regulation of PSII activity occurred in response to the pathogen infection. The actual PSII efficiency (Φ_{PSII}) decreased in wilted plants, through a decrease in qP (the photochemical quenching), related to the redox state of the primary electron acceptor of PSII (Morales et al. 1998, 2000; Rosenqvist and van Kooten 2003) and without remarkable changes in Φ_{exc}.

Gas exchange and chlorophyll fluorescence measurements were conducted 5 weeks after pathogen inoculation, when disease severity was not maximal. It is very likely that, in more advanced disease stages, plants might suffer a sharp decline in fluorescence variables. This contention is based on results from Pomar et al. (2004), who reported strongly damaged photochemical processes in Verticillium wilted pepper plants. In addition, we used in this work asymptomatic leaves for the physiological measurements, in order to minimize variability due to physiological imbalances caused by the distance from the point of colonization (Lorenzini et al. 1997). Such factor may also contribute to explain our results when compared to those of Pomar and co-workers. Probably, all these variables were more affected on leaves with symptoms than without symptoms.

Our experiment has been carried out with a peat-based potting mix and under controlled conditions, therefore it is difficult to extrapolate the results to other type of soils or to field conditions, and determine their significance for sewage sludge-treated, field-grown pepper plants. However, in order to compare our results with those obtained under field conditions, we have estimated that the dose of 15% composted sludge would correspond to an application of ca. 100 t/Ha, considering that our pots had a capacity of 2 L of soil and 1 Ha of soil may have on average 3 millions Kg per Ha. These calculations agree reasonably well with previous results from field experiments. Dickerson (1996) found that the lowest doses of compost (10–20 t/acre, equivalent to 25–50 t/Ha) suppressed *Phytophthora capsici*-mediated chile wilt. In the same experiment, applications of 30–50 t/acre (equivalent to

75–125 t/Ha) led to the highest yield losses, which was attributed to the high content of soluble salt in the compost (6.5 dS m^{-1}). The compost used in the present work had 4.5 dS m^{-1} salinity (see Table 1), which may explain differences between our calculations and the doses used by Dickerson. Anyway, from a physiological point of view, salinity seems to be the main factor modulating the disease severity in presence of sewage sludge amendments. It is therefore recommended to analyse the composted amendments for salinity, in order to calculate doses of application in tons per Ha.

Conclusions

The effect of sewage sludge on *Verticillium*-induced wilt in pepper plants was dependent on the type of sludge treatment and on the dose applied. The use of ATAD sludge did not have biopesticide effect. Composted sludge caused a decrease in Verticillium wilt when applied at lower doses to a peat-based potting mix. Further research is needed in order to elucidate the mechanism(s) of this suppression. By contrast, the highest dose of compost enhanced the disease, probably due to the high content of soluble salts in the compost. The application of sludge treated with ATAD and composting processes to peat-based potting mixtures seems to be a valuable recycling technique that increases substrate fertility and contributes to an alleviation of the current waste disposal demand. In addition, both technologies meet the future European Directive, which will restrict the use of sludge with a lower level of sanitation. However, the dose of application should be taken into consideration in order to prevent the possible negative effect of salinity on plant growth and yield, and the enhancement of plant diseases. Finally, further research involving different types of growing media and soils is needed before adapting sewage sludge amendments to practical field applications.

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