

Potential of *Fusarium* wilt-inducing chlamydospores, in vitro behaviour in root exudates and physiology of tomato in biochar and compost amended soil

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Abstract

Background and aims Biochars are recognised for their ability to improve soil functions and to stimulate plant defense mechanisms. We evaluated the response of *Fusarium oxysporum* f. sp. *lycopersici* chlamydospores to tomato plants grown in biochar and compost amended soil to get a deeper insight into the tomato-*Fusarium* pathosystem.

Methods Wood chips and green waste biochar in combination with compost ('WCB_{comp}' and 'GWB_{comp}' respectively) were studied for their ability to suppress the *Fusarium* chlamydospores infectivity. Plant growth parameters and in vitro effects on chlamydospores were determined.

Results The 'GWB_{comp}' soil amendment stimulated plants growth and gaseous exchange rates and had a suppressive effect on the chlamydospore infectivity in comparison with the 'WCB_{comp}' treatment and the treatment containing compost only. The germination rate of

chlamydospores was unaffected by the source of root exudates, whereas the mycelial growth was significantly higher in root exudates from chlamydospore inoculated plants grown in 'WCB_{comp}' amended soil unlike to 'GWB_{comp}' amended soil.

Conclusion Overall, our findings indicate that both biochars had a variable effect on chlamydospores. We conclude that soil amendment with garden waste biochar and compost exhibit a great potential in suppressing *Fusarium* chlamydospore infectivity and alleviating pathogen-induced physiological stress in tomato plants.

Keywords Soil amendments · *Solanum lycopersicum* · *Fusarium oxysporum* · Germination assay · Fungal growth

Introduction

Plant pathogenic *Fusarium* species are remarkably diverse with a broad host range including economic important crops such as tomato, cotton and banana (Smith 2007). According to a recent survey, the scientific community ranked *Fusarium oxysporum* fifth most important pathogen among the list of top ten plant pathogens (Dean et al. 2012). *Fusarium* wilt of tomato (*Solanum lycopersicum* L.) caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hansen, a soil-inhabitant fungus was first described by Masee (1895) in England. This wilt-inducing fungus is a major devastating factor in tomato cultivation worldwide (McGovern 2015). Tomato is ranked second amongst

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the economic important vegetable crops worldwide, with an annual production of 164 million tonnes from 4.73 million hectares of area under cultivation in 2013 (FAOSTAT 2015).

The tomato pathogen *F. oxysporum* f. sp. *lycopersici* (*Fol*) induces blockage of the xylem vessels and, thus, interferes mainly with the water transport causing wilting, yellowing of older leaves, stunted growth and even plant death (Beckman 1987). Depending on the host availability and prevailing environmental conditions, *F. oxysporum* can produce three types of asexual spores, (I) microconidia (II) macroconidia and (III) chlamydospores, whereas the sexual or teleomorphic stage is unknown. *F. oxysporum* can survive saprophytically in soil and on plant debris in the absence of a host either as mycelium or in all of the spore types. However, in the regions with cooler climates, *F. oxysporum* overwinters as chlamydospores (Agrios 2005). In the field chlamydospores are the main survival structures of *F. oxysporum*. DeCal et al. (1997) reported that the chlamydospores of *Fol* cause a more severe disease development in tomato than microconidia do. Chlamydospores were also found to be more infectious on other crop plants. Couteaudier and Alabouvette (1990) reported enhanced disease severity in flax (*Linum usitatissimum*), when inoculated with *F. oxysporum* f. sp. *lini* chlamydospores than with microconidia.

As disease incidence and yield losses due to *Fusarium* induced-wilt are increasing in both open-field and greenhouse production, it is important to develop promising disease management strategies (McGovern 2015). Chemical control of wilt diseases is neither satisfactory nor environment-friendly (Fravel et al. 2003). Furthermore, the emergence of resistance breaking pathogenic strains emphasizes the necessity for alternative disease management strategies (Takken and Rep 2010). One alternative disease management strategy involves the use of soil organic amendments such as compost and biochar (Smith and Collins 2007; Sohi et al. 2010; Ruano-Rosa and Mercado-Blanco 2015). Composts are known to improve the soil health and to suppress various soil-borne diseases caused by fungal pathogens belonging to diverse genera such as *Fusarium*, *Pythium*, *Rhizoctonia* and *Phytophthora* (Larkin 2015; Mehta et al. 2014). The suppressiveness of compost may be attributed to a beneficial microbial community, an improvement in plant growth and vigour, an increased nutrient availability, the induction of systemic resistance

or to the stronger fungistatic abilities of the compost amendments (Bonanomi et al. 2007).

Biochar is a pyrolytic porous solid coproduct of organic waste material such as wood chips, garden waste material and others (Kammann et al. 2015). Biochar application not only improves crop productivity by modifying the soil structure, pH, cation exchange capacity, porosity, water holding capacity and by increasing the nutrient retention and availability but also stimulates the plant defence system through biochar-borne elicitor chemicals (Sohi et al. 2010; Elad et al. 2010). However, in some studies different biochar types (depending on feedstock and pyrolysis temperature) have been reported to negatively affect crop yield, soil properties and beneficial soil microbiota (Mukherjee and Lal 2014). Previously, biochar has been shown to induce resistance in plants against a variety of aerial as well as soil-borne phytopathogenic fungi and bacteria (Graber et al. 2014; Harel et al. 2012). Jaiswal et al. (2014, 2015) demonstrated the dependency of the disease suppressing ability of different biochars on the feedstock and production parameters. Biochar-induced systemic resistance was documented against gray mold (*Botrytis cinerea*) and powdery mildew (*Oidiopsis sicula*) on pepper and tomato (Elad et al. 2010). Recently, Jaiswal et al. (2015) reported that the biochar application suppresses damping off (*Rhizoctonia solani*) on common bean (*Phaseolus vulgaris* L.) seedlings. A synergistic effect of biochar and compost has been reported to improve soil fertility, plant growth and beneficial microbial activity in the rhizosphere (Aegehehu et al. 2015; Fischer and Glaser 2012). Only recently, it has been shown that tomato plants growth response and *Fol* development in plants inoculated with microconidia is dependent on the raw material used for the production of biochars (Akhter et al. 2015).

However, previous studies involving soil-borne fungal pathogens did not evaluate the effectiveness of biochar against the fungal resting spores which have the potential to initiate disease epidemics. To date, no study has assessed the infectivity potential of chlamydospores in biochar-amended soils.

In the presence of a suitable host and favourable environmental conditions such as root exudates and nutrients, chlamydospores germinate to infect plants or to produce more chlamydospores (Kommedahl 1966; Smith and Snyder 1972). Root exudates of tomato plants are already known to stimulate *Fol* microconidia germination (Steinkellner et al. 2005). Akhter et al.

(2015) observed an alteration in the pattern of microconidia germination in response to root exudates from tomato plants grown in soil with compost and biochars. However, the information is scarce regarding the *Fusarium* chlamydospores response to the plant root exudates grown in soil containing biochar and compost. Therefore, it is imperative to study the effect of soil organic amendments such as biochar and compost on the tomato root exudate-chlamydospore interaction to better understand the onset and progression of tomato wilt together with the development of effective disease management strategies.

In our study, tomato plants were grown in different potting media compositions including biochar and compost, infested with chlamydospores to reproduce natural *Fusarium* infested field conditions. The prime objective of this study was to evaluate the effectiveness of biochar and compost application in suppressing the infectivity of chlamydospores and to analyse the *in vitro* growth and development of the main *Fol* inoculum sources, microconidia, chlamydospores and mycelium in the root exudates. To our knowledge, the results presented here for the first time highlight the potential of biochar and compost application as an soil organic amendment in suppressing the infectivity of chlamydospores and wilt development in tomatoes.

Materials and methods

Fungal inoculum production

In order to produce microconidia, *F. oxysporum* f. sp. *lycopersici* (isolate 007; kindly provided by BJ Cornelissen, University of Amsterdam, The Netherlands) was cultivated for two weeks on Czapek Dox (CZD) agar (Duchefa Biochemie, Haarlem, The Netherlands) in dark at 24 °C. In order to harvest the microconidia, the fungal culture plates were flooded with autoclaved, distilled water and gently rubbed with a Drigalski spatula under aseptic conditions. The liquid fraction was filtered through three layers of fleece cloth filters (150 µm). The concentration of microconidial suspension was adjusted according to Steinkellner et al. (2008).

Chlamydospores were obtained by slight modification of the method described by Goyal et al. (1973) and Bennett and Davis (2013). Soil broth was prepared by using sterilised soil (Aussaaterde, Gramoflor GmbH & Co. KG, Vechta, Germany) and distilled autoclaved

water in the ratio of 1:4 (w/v); 250 g of soil in 1000 ml water were placed on an orbital shaker for 1 h at 90 rpm. Afterwards, the suspension was sieved through 1 mm sieve to remove large soil particles followed by filtering through 8 layers of fleece cloth filters (150 µm). Before autoclaving, the soil broth was separated into aliquots of 50 ml and glucose was added to each aliquot at a concentration of 500 µg/ml. A second autoclaving was done on the following day and the remaining sediments in the broth were allowed to settle down for 3 to 4 h, and then 50 ml of soil broth was decanted into sterilised 125 ml flasks. Aliquots of 50 ml of soil broth were inoculated with 300 µl of spore suspension (1×10^7 microconidia/ml) and placed near a window under natural light conditions for 7 days at 24 °C without shaking. Afterwards the contents of the flasks were homogenized with a blender and chlamydospores were quantified with a haemocytometer (Bennett and Davis 2013).

Soil preparation and experimental setup

The substrate used for this experiment sterilized in an autoclave at 121 °C for 20 min, comprised a mixture of sand (Quarzsand 0–3 mm, Quarzwerke Österreich GmbH, Melk, Austria), leca (Liapor fit Blähton 1–4 mm, Lias Österreich GmbH, Fehring, Austria) and soil (Aussaaterde, Gramoflor GmbH & Co. KG, Vechta, Germany), in the ratio of 1:1:1 (v/v/v). This substrate was used as an un-amended soil (US) and in combination with compost (Comp) at 20 % and/or biochar at 3 % (v/v). Two types of biochars, namely wood chips biochar (WCB) and green waste biochar (GWB), made from beech wood chips and garden waste residues, respectively, at a pyrolysis temperature of 500 °C were used in this study. GWB has a comparatively higher pH, higher ash contents and cation exchange capacity. GWB was sieved with a 2 mm sieve before mixing with potting medium. The compost of quality A+ according to the Austrian compost regulation (BGBl. II Nr. 929/2001) was obtained from the municipal compost works in Klosterneuburg, Austria. The compost had a high percentage (22.7 %) of degradable organic matter contents with a C/N ratio of 10. The detailed characteristics of compost and biochars are available from previous publications of Akhter et al. (2015). Table 1 summarizes the basic properties of the potting-mixture.

For practical applications biochars are usually recommended to be used in combination with compost or with other additional source of fertilization because of

Table 1 Basic properties of soil, compost, wood chip biochar and green waste biochar

Substrate	Organic carbon (%)	pH	Electrical conductivity (mS/cm)	Density (kg/L)
Soil	–	5.6 ^a	0.23	0.96
Compost	27.00	7.10 ^a	1.40	0.77
Wood chips biochar	80.30	8.78 ^b	0.54	0.36
Green waste biochar	79.78	9.03 ^b	1.67	0.34

– Not specified

^a pH was measured in CaCl₂

^b pH was measured in de-ionized water

potential negative effects associated with soil amendment with biochar only (Kammann et al. 2015). The experimental setup comprised the following treatments: (i) Comp (ii) WCB with compost (WCB_{comp}) (iii) GWB with compost (GWB_{comp}), and (iv) US. Treatments were either inoculated with chlamydo spores (+Chl) or remained pathogen free (–Chl). The experiment was conducted with five replicates in each treatment and each replicate represented a pot with one tomato plant. All the experiments were repeated thrice.

Chlamydo spores were mixed with the soil to obtain the final concentration of 5000 chlamydo spores/g of dry soil of the potting mix (Rush and Kraft 1986). Soil samples were taken to quantify the fungal inoculum density in the potting mix, both at the beginning and end of the experiment according to Steinkellner and Langer (2004). Air dried soil subsamples of 50 g each were mixed thoroughly with 100 ml of water agar (0.15 %) and further diluted by taking 1 ml of suspension in a 9 ml of water agar (0.15 %). The serial dilutions were made to ten-fold thrice (10⁻³) and 1 ml from the dilution was spread uniformly on petri dishes containing Nash-Snyder medium (Nash and Snyder 1962). The petri dishes were incubated for 5–7 days at 22 °C under UV-light with a photoperiod of 16-h light/8-h dark. Afterwards *Fol* colonies were counted and represented as colony-forming units per g of dried soil (CFU/g of dry soil).

Plant material and growth conditions

Tomato cultivar ‘Kremser Perle’ seeds were surface-sterilised by immersing them in the disinfecting solution [50 % household bleach (‘Dan Clorix’, 3.8 % NaOCl)] for 10 min. Afterwards the seeds were rinsed thoroughly with autoclaved distilled water for three times. The surface-sterilised seeds were cultivated in pots filled with autoclaved perlite (Granuperl S 3–6, Knauf Perlite

GmbH, Vienna, Austria) and incubated in a growth chamber (Rumed, Rubarth Apparate GmbH, Germany) with a 16-h light/8-h dark photoperiod (photosynthetic active radiation 296 μmol/m²/s) at 22 °C. The perlite was irrigated with tap water. After 4 weeks, the tomato seedlings were uprooted gently from the perlite and transplanted to separate pots according to the experimental setup. The tomato plants were grown in the greenhouse for 8 weeks, under long day conditions and watered regularly with tap water to sustain optimum moisture conditions for tomato growth (Steinkellner et al. 2005).

Agronomic traits, physiological measurements and extraction of root exudates

Gas exchange measurements [net photosynthetic rate or CO₂ assimilation rate at light saturation (Asat)] and stomatal conductance (gs) were measured with a portable photosynthesis system (Li-Cor, Inc. model 6400, Lincoln, Nebraska, USA). The youngest mature leaves of a plant were used for the measurements. All the measurements were made 2 days before harvesting between 10:00 and 14:00 h. After 8 weeks, the data regarding tomato plant phenological growth stage was recorded and plants were harvested by washing the roots with tap water. From each treatment 5 plants were pooled together and roots were dipped for 6 h in acetate buffer (25 mM, pH = 5.5) for the extraction of root exudates. The sterile filters (0.22 μm, Steriflip, Millipore, Bedford, USA) were used for filtration of root exudates (Hage-Ahmed et al. 2013). For further analysis, the root exudates were adjusted to the concentration of 20 ml/g of root fresh weight with acetate buffer and stored at –80 °C. After that, the roots were separated from the base of shoot. Measurements were made for root fresh weight, root length, shoot fresh

weight and shoot height. In order to obtain the dry weights, roots and shoots were placed in an oven at 60 °C until weight was unchanged.

Disease incidence and severity assessment

The *Fol* incidence and severity assessment were made according to Akhter et al. (2015). Briefly, plants were assessed for disease incidence both visually and by incubating a surface-sterilized segment of the shoot base (0.5 cm) on potato dextrose agar [(PDA), Carl Roth GmbH + Co. KG, Germany] plates at 24 °C in the dark. Disease incidence was calculated for each batch of five plants separately by using the following formula:

$$\text{Disease incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

In order to assess the disease severity, the length of the xylem vessel with brownish discoloration was estimated. The plants were assigned to a category on a scale of 1–5 (c1 = 1–5 %, c2 = 5–15 %, c3 = 15–35 %, c4 = 35–67.5 %, c5 = 67.5–100 %) according to the length of xylem vessel discoloration. Disease severity was calculated for a batch of five plants in each treatment by the following formula:

Disease severity

$$= \frac{5 \times (\text{nc1} + 2\text{nc2} + 5\text{nc3} + 10\text{nc4} + 20\text{nc5})}{\text{number of infected plants}}$$

In vitro effect of compost and biochar to chlamydo-spores

The effect of compost and the two types of biochar on the mycelial growth and hyphal development of *Fol* was evaluated on PDA plates amended with antibiotics (10 mg/l of each Streptomycinsulfat and Chloramphenicol) to prevent bacterial contamination. The compost and biochars were sieved through 100 µm sieves before mixing with growth media. The PDA was amended with compost (20 %) alone and in combination with WCB/or GWB (3 %, w/v) before autoclaving (Jaiswal et al. 2015). The amended PDA was poured into petri dishes (diameter = 9 cm). After solidification of the media, sterilised filter paper segments (diameter = 6 mm) were placed in the centre of petri dishes. For inoculation with

chlamydo-spores, 5 µl of the suspension (1×10^5 chlamydo-spores/ml) was applied directly on the filter paper and the dishes were incubated under dark conditions at 24 °C for 5 days. At the end of the incubation period, the mycelial growth was determined by measuring the fungal colony diameter (cm). The data were collected from six replicates arranged randomly. The inhibition percentage of the mycelial radial growth in the compost and biochar amended media (A_{med}) and in the un-amended control (UA_{cont}) was measured with the following formula:

$$\text{Inhibition}(\%) = \left[\frac{(UA_{\text{cont}} - A_{\text{med}})}{UA_{\text{cont}}} \right] \times 100$$

In vitro fungal growth assays (microconidia germination assay, chlamydo-spore germination assay and mycelial development in tomato root exudates)

The fungal growth assays were performed in 96-well plates (NUNCLONTM D Surface, F96 MicroWellTM Plates, NUNCTM Brand Products, Roskilde, Denmark). The root exudates were analysed in triplicates. In order to evaluate the microconidia germination rate, 35 µl of a conidial spore suspension (1×10^7 microconida/ml) was added into 175 µl of root exudates into each well of 96-well plate incubated for 20 h at 24 °C. Likewise, for chlamydo-spore germination assay, aliquots of 175 µl of root exudates were mixed with 35 µl of chlamydo-spore suspension (1×10^5 chlamydo-spore/ml) and incubated at 24 °C in dark on a rotary shaker at 200 rpm for 10 h. The microconidia germination rate (%) was determined by counting 200 spores/well and chlamydo-spore germination rate was recorded by counting 100 chlamydo-spores/well microscopically. The 96-well plates for the mycelial growth assay were prepared as described for the chlamydo-spore germination assay. The mycelial growth and development was assessed by evaluating the optical density at 600 nm for a total period of five successive days after regular intervals, with a spectrophotometer (FLUOstar Omega, BMG LABTECH GmbH, Ortenberg, Germany) according to Steinkellner and Mammerler (2007). The CZD broth and acetate buffer served as a positive and negative control, respectively, for all fungal growth assays.

Statistical analysis

A two-way general linear model analysis of variance (ANOVA) was used to compare the significant effects of

main factors [treatment (different soil substrate compositions) and chlamyospore] and their interaction (treatment x chlamyospore) on root and shoot dry biomass, root length, shoot height, net photosynthetic rate and stomatal conductance, in vitro mycelial growth, microconidia and chlamyospore germination rate. A one-way ANOVA was performed on disease incidence, disease severity, CFU of *Fol*/g of dry soil and on in vitro toxicity of compost and biochars since the data from ‘-Chl’ treatments was zero. The data were log-transformed for the CFU of *Fol*/g of dry soil. All the replicates were analysed together with experimental repeats. Percentage data was transformed before analysis (Jaiswal et al. 2015). Levene’s test was used to check the homogeneity of variances. The individual treatment means were compared using Tukey’s honestly significant difference (HSD) test, with a significant level at $P \leq 0.05$. All statistical tests were performed using PASW Statistics 18 (Version 18.0.0, IBM, Armonk, NY, USA) software.

Results

Effect of compost and biochar on disease incidence, severity and chlamyospore survival

Disease incidence and severity was determined 8 weeks after transplanting tomato plants to the chlamyospores-infested potting media (Table 2). There was no significant difference in disease incidence between compost (Comp + Chl; 60 %) and wood chips biochar (WCB_{comp} + Chl; 66 %) amended treatments. However, in the potting media comprising green waste biochar (‘GWB_{comp} + Chl’) the disease incidence (20 %) was significantly reduced. Likewise, we also observed a

significant ($P < 0.001$) reduction of disease severity in the plants grown in ‘GWB_{comp}’ amended soil substrate (5.7 ± 0.94) as compared to the plants grown in ‘Comp + Chl’ and ‘WCB_{comp} + Chl’, whereas un-amended soil control (‘US + Chl’) has depicted maximum *Fusarium* incidence (100 %) and severity (87 ± 5.20). The un-infested soil treatments did not show any *Fusarium* symptoms.

The colony forming units (CFUs) of *Fol*/g of dry soil decreased significantly in the treatments containing compost alone and compost-biochar mixture in comparison with soil without any organic amendment (US + Chl; 6.78×10^4). Among compost and biochar amended treatments, green waste biochar (GWB_{comp}) comprising soil substrate has shown the lowest (0.30×10^4) CFU/g of dry soil, whereas no significant difference in *Fol*-chlamyospore survival was recorded in ‘Comp’ and ‘WCB_{comp}’ amended treatments (Table 2). No *Fusarium* colony was observed in chlamyospore-free treatments.

Effect of compost and biochar on fungal growth

The response of *Fusarium* chlamyospores in PDA media amended with compost alone and in association with biochar (‘WCB_{comp}’ or ‘GWB_{comp}’) as well as un-amended control (UA_{cont}) is summarized in Table 3. There was almost similar radial growth of *Fol* in media amended with ‘Comp’ and ‘WCB_{comp}’ with an inhibition of 8.27 % and 12.84 % of mycelium radial growth, respectively. On the other hand, it was observed that the addition of ‘GWB_{comp}’ in PDA has efficiently suppressed the *Fusarium* radial growth. There was a significantly higher ($P < 0.01$) inhibition (31.27 %) of *Fusarium* radial growth in ‘GWB_{comp}’ amended fungal growth media as compared to the un-amended control.

Table 2 Effect of different soil substrate compositions comprising compost, compost with wood chips biochar (WCB_{comp}) and green waste biochar (GWB_{comp}) and un-amended soil inoculated with

chlamyospores (+Chl), on the disease incidence, disease severity and on colony forming units (CFU) of *Fol*/g of dry soil, 8 weeks after transplanting

Treatments	Comp + Chl	WCB _{comp} + Chl	GWB _{comp} + Chl	US + Chl
Disease incidence (%)	60.00 ± 0.00 ^b	66.67 ± 11.54 ^b	20.00 ± 0.00 ^c	100 ± 0.00 ^a
Disease severity ¹	14.45 ± 2.55 ^b	16.39 ± 4.27 ^b	5.67 ± 0.94 ^c	87.00 ± 5.20 ^a
CFU/g dry soil ² ($\times 10^4$)	0.44 ± 0.12 ^b	0.47 ± 0.12 ^b	0.30 ± 0.08 ^c	6.78 ± 1.24 ^a

¹ Data are mean values ± standard deviation followed by different letters in the superscript within each row indicate significant differences according to Tukey’s HSD test ($P < 0.05$)

² Data represent mean values ± standard deviation followed by different letters in the superscript within each row indicates significant differences according to Tukey’s HSD test ($P < 0.05$). For statistical analysis data has been transformed (log)

Table 3 In vitro evaluation of fungal radial growth (cm) and inhibition (%) to the direct toxicity of compost (Comp) alone and in combination with wood chips and green waste biochar (WCB_{comp} and GWB_{comp} respectively) compared to the un-amended control (UA_{cont})

Treatments	Radial growth (cm)	Inhibition (%)
UA_{cont}	7.82 ± 0.25^a	
Comp	7.22 ± 0.15^b	8.27 ± 2.32^b
WCB_{comp}	6.93 ± 0.17^b	12.84 ± 2.71^b
GWB_{comp}	5.96 ± 0.71^c	31.27 ± 5.52^a

Data are mean values \pm standard deviation followed by different letters in the superscript within a column denote significant differences according to Tukey's HSD test ($P < 0.05$)

Effect of compost, biochar and chlamydo spores on plant growth and gas exchange parameters

In the presence of compost, green waste biochar has promoted plant growth both in chlamydo spore inoculated (+Chl) and un-inoculated (-Chl) tomato plants (Fig. 1a, b). Among all the treatments, tomato plants grown in ' GWB_{comp} ' amended soil substrate had higher root (0.71 g) and shoot dry biomass (2.67 g) yield. Moreover, the plants in ' GWB ' amended substrate has sustained the disease stress more effectively than ' $Comp + Chl$, $WCB_{comp} + Chl$ and $US + Chl$ '

treatments. There was a substantial decrease in root and shoot dry biomass of '+Chl' plants grown in 'Comp' and ' WCB_{comp} ' treatments as compared to their respective un-inoculated counterparts. The maximum *Fusarium* chlamydo spore induced reduction in root dry biomass was recorded in ' $WCB_{comp} + Chl$ ' (0.36 g) as compared to its respective un-inoculated control (' $WCB_{comp}-Chl$ '; 0.55 g). The significant reduction in root dry biomass in all of the inoculated treatments were ranked as (from minimum to maximum reduction) ' $GWB_{comp} + Chl$ Comp+Chl $WCB_{comp} + Chl$ US+Chl' (Fig. 1a), whereas in case of the shoot dry biomass treatments were ordered as ' $GWB_{comp} + Chl$ Comp + Chl \approx $WCB_{comp} + Chl$ US + Chl' (Fig. 1b).

The chlamydo spore inoculated tomato plants grown in 'Comp' and ' WCB_{comp} ' containing soil substrate remained shorter in stature (21.14 cm and 18.16 cm respectively), when compared with the plants grown in ' $GWB_{comp} + Chl$ ' (31.03 cm) (Fig. 2b). However, we did not observe any significant differences in root length between compost and biochar amended treatments, although there was approximately 2.5 to 3 fold increase in root length as compared to +Chl plants grown in 'US' (Fig. 2a).

Significant effects of main factors [treatment (different soil substrate compositions) and chlamydo spore] and their interactions (treatment \times chlamydo spore) are

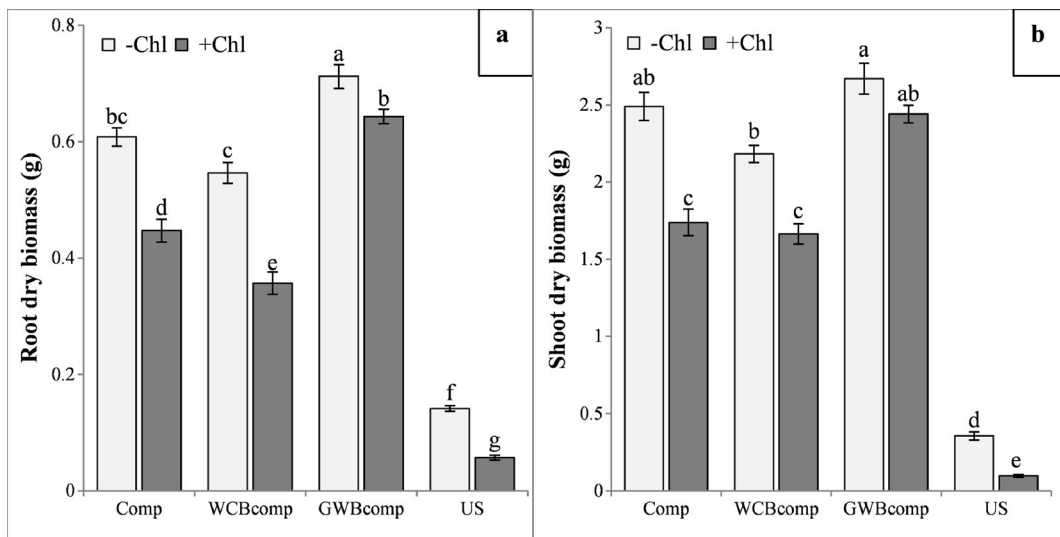


Fig 1 Root (panel a) and shoot dry biomass (panel b) of tomato plants either inoculated with chlamydo spores (+Chl; dark bars) or un-inoculated (-Chl; light bars) grown in different soil substrates comprising compost (Comp), compost and wood chips biochar (WCB_{comp}), compost and green waste biochar (GWB_{comp}) or in

un-amended soil (US). Data were collected 8 weeks after transplanting. Data are mean values \pm standard error; different letters above bars denote significant differences according to Tukey's HSD test ($P < 0.05$)

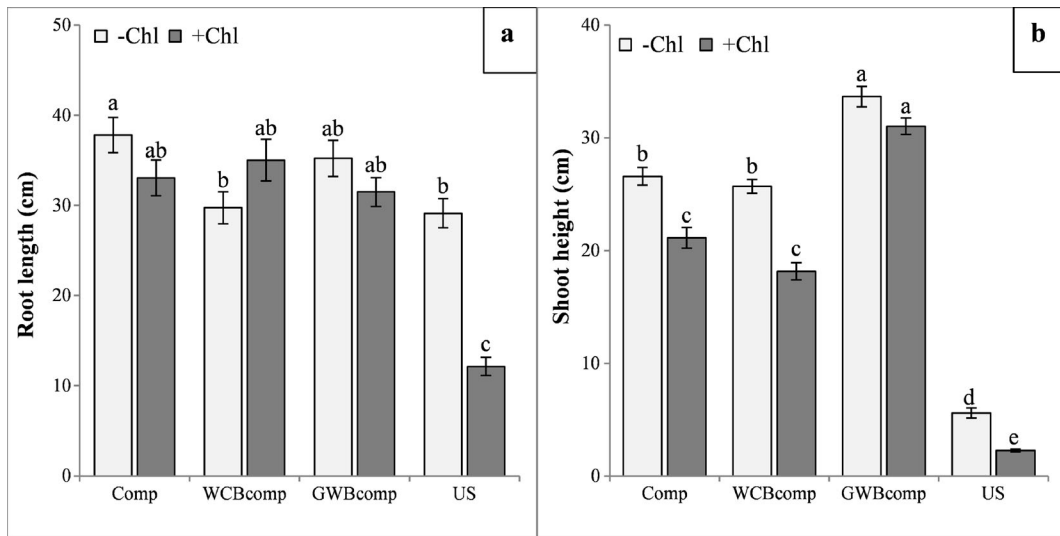


Fig 2 Root length (panel a) and shoot height (panel b) of tomato plants either inoculated with chlamydo spores (+Chl; dark bars) or un-inoculated (-Chl; light bars) grown in different soil substrates comprising compost (Comp), compost and wood chips biochar (WCB_{comp}),

compost and green waste biochar (GWB_{comp}) or in un-amended soil (US). Data were collected 8 weeks after transplanting. Data are mean values ± standard error; different letters above bars denote significant differences according to Tukey's HSD test ($P < 0.05$)

summarized in Table 4. Overall, significant interactive effect of main factors treatment and chlamydo spore was observed in the tomato plants growth and physiological parameters including root and shoot dry biomass, root length, shoot height and stomatal conductance.

However, in case of photosynthetic rate or net- CO_2 assimilation rate (Asat) only a significant effect of main factors treatment and chlamydo spore was documented.

Amongst the 'Comp' and biochar amended treatments, chlamydo spore inoculation significantly reduced

Table 4 Statistical results of the two-way ANOVA presented as F values and level of significance (P) on plant growth and physiological parameters and on in vitro fungal growth and development

Variables	Main effects		Interaction effects
	Treatment ^a	Chlamydo spore ^b	Treatment × Chlamydo spore
Agronomic and physiological parameters			
Root dry biomass	92.86**	84.29**	6.32*
Shoot dry biomass	32.69**	59.75**	5.20*
Root length	1.28 ^{NS}	0.46 ^{NS}	4.01*
Shoot height	96.67**	63.70**	4.72*
Net photosynthetic rate	44.14**	81.38**	3.07 ^{NS}
Stomatal conductance	16.49**	325.54**	27.77**
Fungal growth parameters			
Microconidia germination	73.45**	65.72**	0.59 ^{NS}
Chlamydo spore germination	0.49 ^{NS}	0.00 ^{NS}	0.48 ^{NS}
Mycelial growth assay	115.79**	466.57**	215.30**

^{NS} not significant

* $P < 0.05$

** $P < 0.001$

^a Factor treatment represents different soil substrate compositions

^b Factor chlamydo spore represents inoculated or un-inoculated plants

net-CO₂ assimilation rate in ‘Comp + Chl’ (12.67) and ‘WCB_{comp}’ (13.18) treatments as compared to ‘GWB_{comp}’ (Table 5). In the absence of chlamydo-spores, the plants grown in ‘GWB’ amended substrate (‘GWB_{comp}’) showed maximum (17.78) rate of photosynthesis and remained in higher ranges among the plants under *Fusarium* chlamydo-spore stress (‘Comp + Chl’; 15.67). The differences in stomatal conductance (gs) in the absence of chlamydo-spores were not significant between the treatments. However, a reduction in ‘gs’ was observed in the treatment ‘Comp + Chl’ (0.105) and ‘WCB_{comp} + Chl’ (0.109), whereas the plants grown in ‘GWB_{comp} + Chl’ had maintained higher level (0.117) of ‘gs’ upon exposure to *Fusarium* chlamydo-spore stress (Table 5).

Influence of root exudates on in vitro fungal growth and development of *Fusarium oxysporum* f. sp. *lycopersici* spores and mycelium

I. Microconidia germination in root exudates

Depending on the composition of potting media and the inoculum source for *Fol* inoculation, the microconidia germination rate was between 16 and 63.2 % in the tomato root exudates (Fig. 3). The pH values of root exudates ranged between 5.82 and 6.00 (data not shown). The main factor treatment and chlamydo-spore had significant ($P < 0.001$) effects on the microconidia germination (Table 4). The minimum germination rate (5.5 %) was observed in the acetate buffer (negative control), whereas maximum germination rate (68.44 %) corresponded to the CZD broth which served as a positive control of the germination assay. Overall, the germination rate was higher in root exudates from chlamydo-spore inoculated treatments as

compared to their respective un-inoculated counterparts, except in case of root exudates of plants from treatment ‘US’. The *Fol* microconidia germination rate was highest in root exudates from plants grown in treatments ‘Comp + Chl’ (63.22 %) and ‘Comp–Chl’ (56.39 %). However, the germination rate was reduced in root exudates of plants from biochar amended treatments, with the minimum in ‘GWB_{comp}–Chl’ (42.28 %). No significant differences in germination rate were observed between ‘WCB_{comp} + Chl’ (53.72 %) and ‘GWB_{comp} + Chl’ (51.05 %) treatments.

II. Chlamydo-spore germination in root exudates

The experiments to assess the chlamydo-spore germination rate indicated that the chlamydo-spores germinated efficiently in the root exudates and reached up to 98 % of germination after 10 h of incubation period (Fig. 4). The lowest germination rate was recorded in acetate buffer (17.56 %) and the maximum in root exudates from treatments ‘WCB_{comp} + Chl’ (98.44 %) and ‘Comp + Chl’ (98.33 %). We did not observe any significant difference in the chlamydo-spore germination rate in the root exudates from compost and biochar amended treatments either inoculated or un-inoculated with chlamydo-spores except in case of the exudates from ‘un-amended soil where the germination rate was reduced to 90 % and 92.2 % in ‘–Chl’ and ‘+Chl’ treatments, respectively, as well as in the CZD broth control (90 %).

III. Mycelial growth and development in root exudates

Mycelial growth and development from chlamydo-spores was assessed in the root exudates of the different

Table 5 Net photosynthetic rate and stomatal conductance (gs) of tomato leaves as affected by different soil substrate compositions [compost (Comp), combination of compost with wood chips

Treatments	Net photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)		Stomatal conductance (gs) ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	
	–Chl	+Chl	–Chl	+Chl
Comp	15.89 ± 1.07 ^b	12.67 ± 1.36 ^c	0.124 ± 0.004 ^a	0.105 ± 0.005 ^c
WCB _{comp}	14.87 ± 0.81 ^b	13.18 ± 1.46 ^c	0.127 ± 0.002 ^a	0.109 ± 0.003 ^c
GWB _{comp}	17.78 ± 1.36 ^a	15.67 ± 1.21 ^b	0.123 ± 0.004 ^{ab}	0.117 ± 0.004 ^b
US	09.23 ± 0.94 ^d	04.76 ± 0.88 ^e	0.083 ± 0.001 ^d	0.042 ± 0.002 ^e

Data are mean values ± standard deviation followed by different letters in the superscript under the same parameter denote significant differences according to Tukey’s HSD test at $P < 0.05$

biochar (WCB_{comp})/or with green waste biochar (GWB_{comp}) or un-amended soil (US)] either inoculated (+Chl) with wilt-inducing chlamydo-spores or un-inoculated (–Chl)

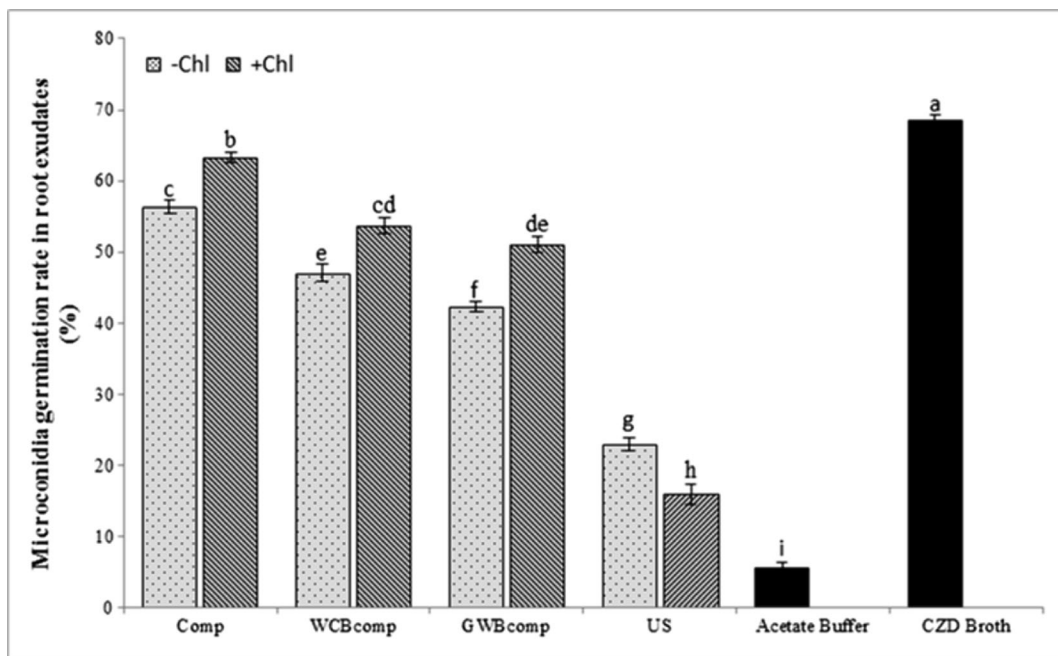


Fig 3 Differences in microconidia germination rate of *Fusarium oxysporum* f. sp. *lycopersici* in root exudates from tomato plants either inoculated with chlamydo spores (+Chl) or un-inoculated (-Chl) grown in different soil substrates comprising compost (Comp), compost and wood chips biochar (WCB_{comp}), compost and green waste biochar (GWB_{comp}) or in un-amended soil (US)

after 20 h at 24 °C. Bars with solid filling represent microconidia germination rate in acetate buffer (negative control) and Czapek dox medium (Positive control; CZD). Data are mean values \pm standard error with different letters above bars that denote significant differences according to Tukey's HSD test ($P < 0.05$)

treatments for five consecutive days (Fig. 5). The CZD broth produced maximum optical density (2.89 ± 0.04), whereas the minimum optical density (0.148 ± 0.01) was observed in the acetate buffer (Supplementary Table 1). Different soil substrate compositions either inoculated or un-inoculated with chlamydo spores had a varying influence on the tomato root exudates which in turn altered the mycelial growth and development from chlamydo spores. There was a significant ($P < 0.001$) interaction effect of treatment and chlamydo spores on the mycelial growth and development (Table 4). Among the biochar amended treatments in the absence of chlamydo spores inoculation (-Chl), 'WCB_{comp}' has produced the lowest optical density (0.381 ± 0.01), while the maximum (0.525 ± 0.01) was in root exudates from 'GWB_{comp}' treatment. Contrarily, greatest stimulation of the mycelial growth was observed in 'WCB_{comp} + Chl' (47.51 %). 'Comp + Chl' and 'US + Chl' increased mycelial growth by 8.8 and 9.1 %, respectively. However, there was no significant difference observed in mycelial growth and development in the root exudates of plants grown in 'GWB_{comp}' amended treatment either inoculated or un-inoculated with chlamydo spores.

Discussion

During the recent years, biochar induced plant protection has been demonstrated against several foliar and soil-borne phytopathogenic bacteria and fungi (Graber et al. 2014; Jaiswal et al. 2015). The plant growth response of tomato plants inoculated with *Fol* microconidia has been studied well (Akhter et al. 2015). However, as chlamydo spores are the main source of inoculum, their effect on root exudation and subsequent disease suppression phenomena is of great interest to the growers as well as to phytopathologists. In our study, we have shown the capability of biochar and compost in the plant growth medium to reduce the infectivity potential of wilt-inducing chlamydo spores.

Generally, the disease causing potential of chlamydo spores of *Fol* is ranked higher than microconidia (DeCal et al. 1997). Silva-Muniz et al. (1991) observed a greater infectivity of *F. oxysporum* f. sp. *phaseoli* chlamydo spores than microconidia either because of the increased ability of germ tubes to penetrate roots or due to the release of a higher amount of toxins harmful to the plants.

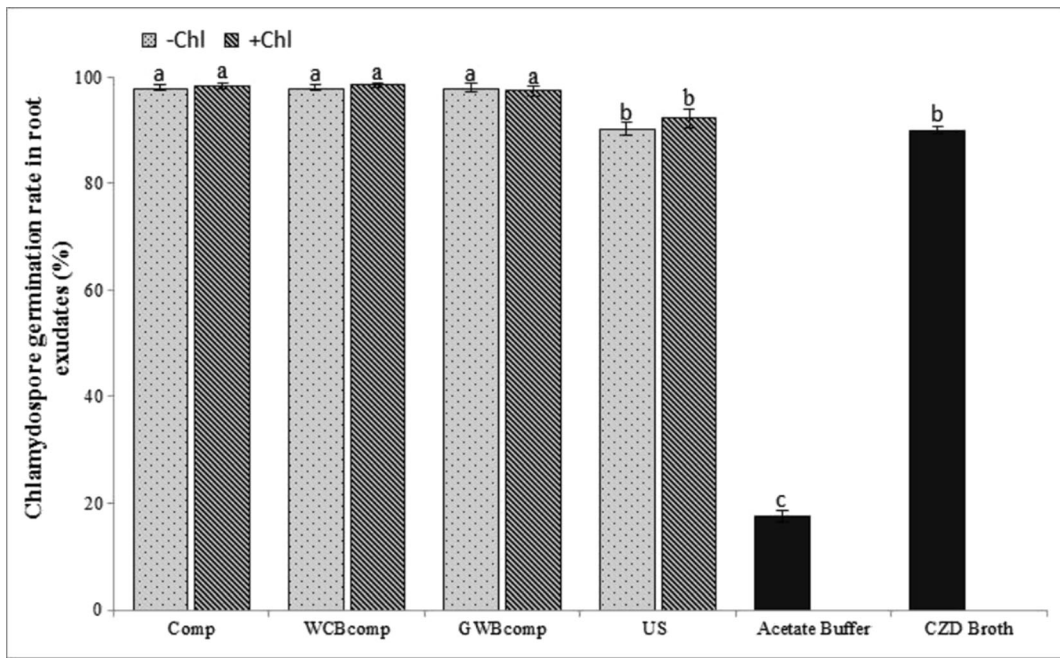


Fig 4 Chlamydospore germination rate in root exudates from tomato plants either inoculated with chlamydo-spores (+Chl) or un-inoculated (-Chl) grown in different soil substrates comprising compost (Comp), compost and wood chips biochar (WCB_{comp}), compost and green waste biochar (GWB_{comp}) or in un-amended soil

(US) after 10 h at 24 °C. Bars with solid filling represent chlamydo-spore germination rate in acetate buffer (negative control) and Czapek dox medium (Positive control; CZD). Data are mean values ± standard error with different letters above bars that denote significant differences according to Tukey's HSD test ($P < 0.05$)

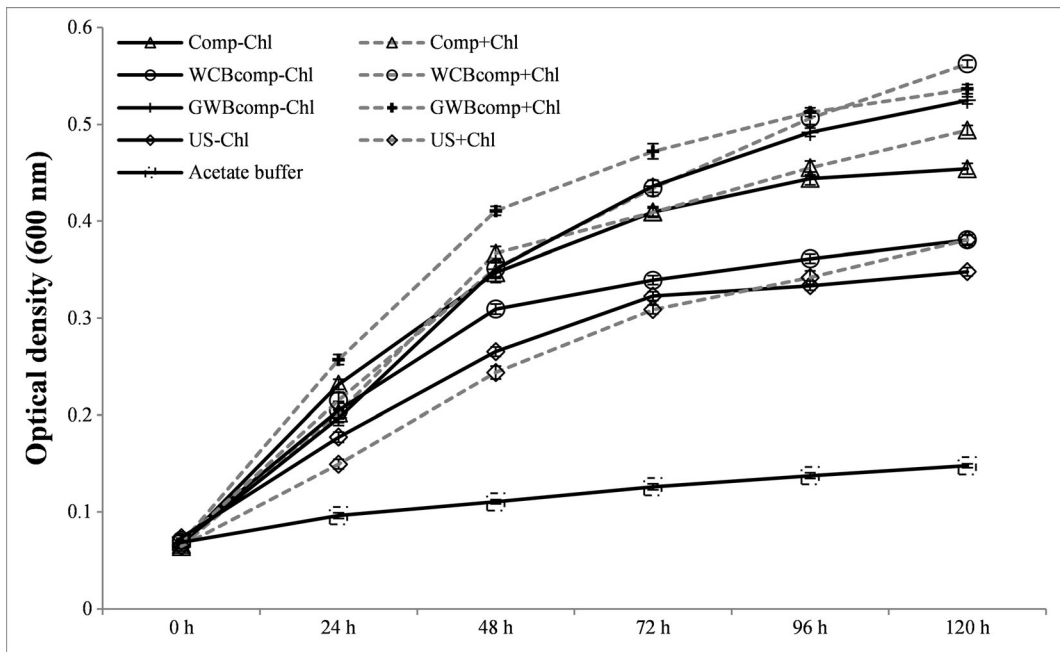


Fig 5 Differences in mycelial growth and development from chlamydo-spores in the root exudates of tomato plants either inoculated with chlamydo-spores (+Chl) or un-inoculated (-Chl) grown in compost (Comp), compost and wood chips biochar (WCB_{comp}),

compost and green waste biochar (GWB_{comp}) or in un-amended soil (US) monitored for a total period of five days at 24 °C after regular intervals

We found that ‘GWB’ along with compost (GWB_{comp}) not only stimulates plant growth but also has a suppressive effect on the survival and infectivity of chlamydospores as compared to ‘WCB_{comp}’ and ‘Comp’ treatments. Previous studies have shown that an increase in soil pH not only suppressed the incidence of *Fusarium* wilt but also enhanced the tomato yield. This was because of reduced availability of nutrients such as iron and zinc to *Fol*, thus, hampering its survival and development of *Fusarium* wilt (Jones and Woltz 1968, 1970). Similarly, higher ash contents of ‘GWB’ with greater ability to increase the soil pH (Kloss et al. 2014) plausibly contributed to enhanced plant growth and suppression of *Fusarium* wilt. In spite of the lower inoculum recovery from ‘GWB_{comp}’ treatment at the plant harvesting time, the level of inoculum (0.30×10^4 CFU/g of dry soil) in soil is high enough to cause severe disease (Jaiswal et al. 2015; Silva-Muniz et al. 1991). The ability of inoculum to survive in the soil is reliant on the soil characteristics and organic matter contents. However, the phenomena of inoculum survival and soil suppressiveness are not necessarily linked together (Couteaudier and Alabouvette 1990; Amir and Alabouvette 1993). Elmer and Pignatello (2011) showed the reduction of *F. oxysporum* f. sp. *asparagii* development in asparagus without any significant effect on the pathogen survival in biochar amended potting media. In addition to that, the characteristics of the raw material used for the biochar production have a strong influence on its disease suppressing ability. Jaiswal et al. (2014, 2015) have recently reported that the biochar produced from different feedstocks had a varying effect on damping-off incidence and other disease parameters in cucumber and beans.

In order to better understand the mechanism of disease suppression, we examined the direct effect of compost and compost-biochar mixtures on the chlamydospores of *Fusarium* wilt. In many reports, compost associated suppressive ability to soil-borne pathogens has been eliminated after sterilisation (Larkin 2015; Yogeve et al. 2006). As a consequence, poor inhibition of fungal radial growth was observed in compost amended PDA plates. Moreover, this also indicates the biotic nature of the compost associated suppression of soil-borne pathogens (Alabouvette et al. 2006; Bonanomi et al. 2007). Comparatively, a higher fungal radial growth inhibition was observed in ‘GWB_{comp}’ treatment. A recent study also documented the in vitro ability of greenhouse waste biochar to inhibit 50 % or

higher radial growth of *Rhizoctonia solani* in comparison with eucalyptus wood biochar (Jaiswal et al. 2015). However, in the present work none of the compost and compost-biochar mixtures resulted into a fungal growth inhibition of 50 % or higher. In this scenario, resistance of the plants towards infection could be the result of physical barriers or physiological changes occurring in response to biochar and compost. These alterations may have a role in avoiding the establishment of the pathogen or by confining its spread within host plant (Whalley and Taylor 1973).

The reduction in gas exchange rate and stomatal conductance has been attributed to the pathogen infection in higher plants (Wang et al. 2015). The blockage of the vascular system of plants because of *Fusarium* infection increases resistance to water movement which may induce symptoms similar to drought stress in plants. Therefore, stomatal conductance as well as net photosynthetic activity was reduced in tomato plants grown in chlamydospores infested soil which is in accordance with the previous studies as well (Duniway and Slatyer 1971; Nogués et al. 2002).

Our findings revealed that the plants grown in soil amended with ‘GWB_{comp}’ resisted to *Fusarium* wilt-induced reduction to net photosynthetic activity as well as stomatal conductance in comparison to ‘WCB_{comp}’ and ‘Comp’ treatments. This might be due to the increased water availability as well as to resistance of the plants to *Fol* colonization of xylem vessel in ‘GWB_{comp}’ containing soil substrate. Asai et al. (2009) and Haider et al. (2014) reported an increase in sap flow from xylem and water use efficiency in rice and maize plants, respectively, grown in biochar amended soil. Akhter et al. (2015) also suggested that the addition of biochar has positive influence on soil moisture contents which ultimately improves the growth of *Fol* microconidia inoculated tomato plants.

In our study, the combination of biochar and compost had both stimulating and suppressive effects on tomato plant growth. The plant growth was suppressed in the ‘WCB_{comp}’ treatment whereas a positive plant growth response was observed in soil amended with ‘GWB_{comp}’, irrespective of the presence and absence of chlamydospores. In general, the available literature documents an enhanced plant growth response under biochar application in combination with balance nutrient supply (Steiner et al. 2007; Agegnehu et al. 2015). However, there are reports suggesting negative effects of different biochar types on plant growth as well if

biochar competes with nutrient supply for plant roots (Rajkovich et al. 2012; Chan et al. 2008). Another study showed that biochar-compost soil amendments had a non-significant effect on grapevine growth in alkaline, temperate soil (Schmidt et al. 2014). Conclusively, the response of the plants towards biochar with reference to growth stimulation and disease suppression is influenced by the type of feedstock, pyrolysis conditions, application rate of biochar, soil characteristics and nutrient availability (Akhter et al. 2015; Jaiswal et al. 2015; Graber et al. 2014).

Altered nutrient availability from biochar amended potting media can also influence the plant growth. Biochars prepared from woody feedstocks are generally lower in nutrient status and ash contents (Brewer et al. 2009; Singh et al. 2010). In contrast, biochar made from leaf-containing plant materials are richer in readily available nutrients to plants (Mukherjee and Zimmerman 2013). Besides, the phenomenon of ‘hormesis’ where biochar borne small quantities of toxic elicitor compounds such as phenolics and organic acids stimulating plant growth should also be considered (Graber et al. 2014). In addition to biochar, the response of the plants may also vary with the host, pathogen type and inoculum source. However, it is hard to determine a single factor responsible for the possible effects of biochar on plant growth and pathogen impact because of the interactions between them.

Root architecture is not only influenced by the genetics but also by the environmental conditions (i.e. organic amendments of the soil, bulk density, nutrient and water availability) have their role in modulating of root development and exudation (Caffaro et al. 2011; Hodge 2004). We observed a slight impact on tomato plants root length grown in chlamyospore infested biochar and compost amended soil. It appears that the incorporation of compost in the soil substrate has an inhibitory effect on *Fol* induced changes in the root morphology. Recently, Morauf and Steinkellner (2015) have also shown the stabilizing effect of compost against the *Fol* induced changes in tomato root traits. Soil additives not only effect root morphology but also have a strong influence on quality and composition of root exudates. Substrate dependent root exudation has previously been described in *Lupinus albus* and *Brassica napus* (Mimmo et al. 2011) and in tomato plants (Akhter et al. 2015). Recently Neumann et al. (2014) has also shown the strong influence of different soil types on the root exudates of *Lactuca sativa*. Principally,

root exudates play an important role in the establishment of diseases caused by soil-borne pathogenic fungi (Haichar et al. 2014). However, little is known about the interaction between root exudates and wilt-inducing *Fusarium* chlamyospores.

To test the direct effect of tomato root exudates obtained from plants grown in different soil substrate compositions on the chlamyospores germination and mycelial growth, chlamyospores were directly added to the extracted root exudates. Interestingly, a very high germination rate of chlamyospores was observed in the root exudates from all the treatments, unlike to the diverse pattern of microconidia germination. This observation correlates with the findings of Schippers and Voetberg (1969) that no specific activating or suppressing substances were released by the roots of susceptible and resistant pea cultivars which could influence *F. oxysporum* f. sp. *pisi* chlamyospore germination. Moreover, the higher germination rate of chlamyospores was associated with the root exudates containing ninhydrin positive substances and reducing sugars. Whalley and Taylor (1973) also noticed higher germination rates of chlamyospores of *F. oxysporum* and *F. solani* f. sp. *pisi* incubated in root exudates under sterile conditions irrespective to the origin of the exudates. However, microconidia germination is vulnerable to alterations in response to the lower concentrations of chlorogenic acid and citrate in root exudates (Hage-Ahmed et al. 2013). In our previous study (Akhter et al. 2015), we also found variations in the *Fol* microconidia germination responsiveness to the root exudates of plants grown in biochar and compost amended soil. The reduction in microconidia germination rate was shown in tomato plant root exudates taken from 50 to 60 days old plants because plant age induced alterations in sugars and organic acid contents of the root exudates (Steinkellner et al. 2005). In contrast, our study shows higher microconidia germination rate in compost and biochar amended treatments indicating substrate-induced qualitative or quantitative changes in the root exudates.

Contrary to the uniform chlamyospore germination response, the differences in mycelial growth and development became prominent when chlamyospores were exposed to the root exudates for longer period of time. We found that the root exudates influence mycelial growth and development differently to microconidia or chlamyospore germination. Mycelial growth was augmented in root exudates from inoculated (+Chl) plants

grown in 'WCB_{comp}' treatments and interestingly lowest mycelial growth was also in the exudates from plants 'WCB_{comp}' treatment though un-inoculated (–Chl), along with 'US' treatment. On the other hand, non-significant differences were observed in mycelial growth in exudates from plants grown in 'GWB_{comp}' treatment either inoculated or un-inoculated. Together with biochar and compost tomato roots upon infection with *F. oxysporum* influenced the composition of root exudates probably because of exudation of new compounds or by altering the composition of root exudates (Akhter et al. 2015; Steinkellner et al. 2008; Benhamou 1991). The level of sugar contents and secondary metabolites such as flavonoids in the root exudates varies during tomato-*Fol* interaction which ultimately plays a role in stimulating or suppressing the mycelial growth and development (Hage-Ahmed et al. 2013; Steinkellner and Mammerler 2007). However, the extent of alteration of root exudates induced by *Fol* might also depend upon severity of infection.

The different inoculum sources of *Fol* react in a specific way to the root exudates indicating the responsiveness of fungal microconidia, chlamyospore and mycelial growth to different fractions of the root exudates. Additional studies are required to better understand the mechanism involved in alterations of the exudates in response to soil organic amendments and pathogen infection. Disease severity in the field is not only influenced by root exudate mediated chlamyospore germination or mycelial growth (Smith 1977). The fate of plants either being resistant or susceptible to pathogenic *Fusarium* might be decided on or within the roots (Steinberg et al. 1999). Thus, soil substrate induced changes in plant physiology and microbial behaviour have to be considered (Akhter et al. 2015; Amir and Alabouvette 1993).

To summarize, our study demonstrates an enhanced plant growth and suppression of wilt-inducing chlamyospores in green waste biochar amended soil. The combined application of biochar produced from different feedstocks and compost has a variable effect on the infectivity potential of chlamyospores. The influence of the two different biochars on plant performance and *Fusarium*-wilt suppression is dependent on the physical and chemical properties of the biochar. The present study, to our knowledge is the first to highlight the great potential of green waste biochar and compost combination as a soil organic amendment in positively stimulating tomato plant growth and suppressing the disease

causing ability of *Fusarium* wilt-inducing chlamyospores. Apart from the direct or indirect effects of the biochar on the nutrient availability and *Fol*, alternative mechanisms encompassing biochar-borne chemicals together with shift in soil associated microbial communities plausibly contributed to elicit plant growth and defense response. There is a great scope of biochar in disease management programs; however, further work is needed to elucidate the mechanisms involved and to evaluate the plant protection potential of biochars.

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