

Use of Biosolids to Enhance Tomato Growth and Tolerance to *Fusarium Oxysporum* f. sp. *Radicis-Lycopersici*

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Research Article

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Abstract

Aims: A biosolid made from municipal sludge, acting as an organic fertilizer that enhanced plant growth and crop productivity, was evaluated for its effect on tomato growth and tolerance enhancement against the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl).

Methods: Peat and/or two soil types were amended with mixtures of this biosolid (0, 80 and 160 tn/ha) in order to study the growth of tomato plants and their response to Forl, either under controlled conditions or outdoors in a net protected area.

Results: The results showed that biosolid addition increased tolerance of tomato plants against the disease. There was also an increase of tomato fresh weight, root weight, stem height and leaf number compared to the Forl-inoculated control soils. Forl, at 5 weeks after tomato transplanting, caused higher disease index on plants grown outdoors in biosolid plus clay soil than in biosolid plus sandy soil, while the opposite occurred under laboratory conditions where higher disease index was recorded on plants grown in peat plus sandy soil than in peat plus clay soil.

Conclusions: The findings strongly support the evidence that this biosolid may act as an organic fertilizer and as a possible stimulant of tomato tolerance against Forl. Therefore, this type of biosolid, previously proven to be minimal ecotoxicological impact, should be considered for its possible use in agriculture according to the principles of circular economy and waste minimization.

Introduction

The main disposal method of sludge produced by wastewater treatment plants (WWTP), has been landfilling due to its relatively low cost. However, this method has been abandoned in many countries because of strict environmental regulations (Collivignarelli et al. 2019; Council Directive 1999/31/EC; Joo et al. 2015; H. Wang et al. 2008). Therefore, other methods such as the use of treated sludge in the form of biosolids have been applied, aiming to minimize waste. This recirculation process of raw materials reduces waste, preserves valuable resources and protects the environment (Papastergiadis et al. 2014; Rufí-Salís et al. 2020; Svarnas et al. 2020; Xue et al. 2015). Field application of biosolids in particular enhances organic matter, as well as soil density and structure (Fischer et al. 2020). Moreover, biosolids provide nitrogen and phosphorus to the plants, restore soil fertility and improve water circulation (Brown et al. 2020; Giannakis et al. 2020a). Nevertheless, precautions should be taken when using sludge-based biosolids as soil amendments, in order to avoid soil pollution with toxic metals and toxic organic compounds or soil contamination with dangerous pathogens (Clarke et al. 2016; Gonzalez-Ollauri et al. 2020). For this reason, European legislation has set limit values for metals of toxicological concern when sludge is used for fertilization (Council Directive 86/278/EEC).

Crop production is strongly affected by several persisting diseases caused by fungi, such as tomato *fusarium* foot and root rot (TFFR) caused by the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl). TFFR is a serious disease that infects tomato crop grown both under

greenhouse and field conditions (Lagopodi et al. 2002). It is difficult to combat the disease with synthetic fungicides, and for this reason alternative to chemical control methods are used (Kamou et al. 2015). Among these methods, organic soil amendments such as green waste compost mixtures were found effective against diverse plant diseases and especially against soil-borne pathogens (Tubieleh and Stephenson 2020; Milinković et al. 2019). Other compost mixtures made from sewage sludge were also able to suppress plant diseases caused by *F. oxysporum* f. sp. *lycopersici* race 1, *F. oxysporum* f. sp. *basilica*, *Pythium ultimum* and *F. oxysporum* f. sp. *radicis-cucumerinum* (Cotxarrera et al. 2002; Ferrara et al. 1996; Markakis et al. 2016).

Since most of the soils in Greece exhibit low organic matter (Kouloubis and Tsantilas 2008), composts and biosolids could well be used to enhance soil fertility, improve plant growth and suppress plant diseases (Khaleel et al. 1981; Stewart-Wade 2019; Pascual et al. 2009). This use of biosolids agrees with the principles of circular economy and of sustainable development because organic waste is transformed into a product with economic and environmental benefits. As such, the present work was conducted to investigate the possible use of a biosolid as a medium that improves soil properties, enhances tomato growth and increases tomato tolerance to Forl, suggesting its potential use as a soil amendment in agricultural land. Based on the results reported by Giannakis et al. (2020b), this biosolid, made from municipal sludge produced in a WWTP of Thessaloniki, Greece, showed negligible ecotoxicological concerns.

Materials And Methods

Culture of Fusarium oxysporum f. sp. *radicis-lycopersici* and preparation of inocula

A virulent strain of Forl from the collection of the Plant Pathology Laboratory, Aristotle University of Thessaloniki (Kamou et al. 2015) was used for artificial inoculations of tomato plants. The fungus, which was kept on potato dextrose agar (PDA; Lab M, Lancashire, UK), On Petri dishes at 4°C, was inoculated on surface-sterilized tomato fruit and re-isolated on PDA. For the production of inoculum used in artificial inoculations, the fungus was grown in liquid cultures, as described in Kamou et al. (2015) and Lagopodi et al. (2002). Conidia were separated from mycelium by filtering through Miracloth (Calbiochem, USA), washed with sterile distilled water by centrifugation, at 5,000 rpm, for 10 min (RC5C; Sorvall Instruments, Waltham, USA), and adjusted to 10⁶ spores per mL, using a haemocytometer (Thoma, Blaubrand GmbH, Germany). Furthermore, a mixture of colony-forming units (cfu) consisting of fine hyphal fragments and conidia was prepared by centrifugation of the whole culture, followed by maceration in a blender. The culture was then passed through four layers of cheesecloth and inoculum was adjusted to 10⁶ cfu per mL using a haemocytometer. Conidia inoculum was used in experiments performed under controlled conditions while for experiments carried out outdoors, the mixture of hyphal fragments and conidia was used.

Pot experiment under controlled conditions

Six different substrates prepared from biosolid, peat and two soil types (Table 1) were used for tomato growth. In particular, the substrate treatments consisted of two levels of biosolid (-, +), two levels of inoculum (-, +), and 3 peat treatments (peat, peat plus clay soil, peat plus sandy soil). The biosolid properties were described in Giannakis et al. (2020b), the clay soil had silt 40%, 30% clay, 30% sand, 2.5% matter, pH 7.9, and the sandy soil had 71% sand, 20% silt, 9% clay, 1.15% organic matter, and pH 8.1. Biosolid, peat and soil mixture of each substrate was inoculated with *Forl* conidia at a concentration of 10^4 spores/g of the substrate, as described above.

Each substrate (M1-M3B) was used to fill 100 ml pots where one tomato seedling at the two-leaf stage of cv. 'ACE 55' was transplanted. All plants were placed in the same growth chamber and they were scored for disease severity at 4 weeks after transplanting, applying the 1-5 disease index used in Kamou et al. (2015) slightly modified. In particular, the scale index was as follows: (1) apparently healthy plant, no visible symptoms, (2) symptomless aboveground part, root and crown slightly symptomatic, with one or two light brown lesions, (3) symptomless aboveground part, severe symptoms on the crown and root, with extended dark brown lesions, (4) advanced foot and root rot and wilting of the aboveground part of the plant, (5) dead or almost dead plant. In addition, fresh weight, root weight, stem height and number of tomato plant leaves were recorded. A 2 x 2 x 3 [2 levels of biosolid (-, +) by 2 levels of inoculum (-, +) by 3 peat treatments (peat, peat plus clay soil, peat plus sandy soil)] factorial experiment was used. This experiment was carried out three times using a completely randomized design (CRD) with 15, 15, 10 replications for each treatment in the first, second and third repetition, respectively.

Pot experiment under natural conditions

The biosolid was mixed with sandy or clay soil, at rates of 0%, 2% or 4% w/w, corresponding to 0, 80 or 160 tn/ha, respectively. After thorough mixing in a big container, each substrate was used to fill 3 L pots where two uniform tomato seedlings at the two-leaf stage of cv. 'ACE 55' were transplanted. *Forl* was applied during transplanting, as soil drenching, using 30 ml of cfu inoculum, prepared as described above, and poured in the vicinity of each plant's roots. Tomato seedlings transplanted in each substrate without fungal inoculum were used as controls. A 2 x 3 x 2 factorial experiment in a split-plot arrangement was used, where the two soil types were the main plots, the three biosolid rates were the sub-plots, and the two inoculation levels were the sub-sub plots. A Randomized Complete Block Design (RCBD) was used with four replicate pots (two plants per pot) for each combined treatment. After transplanting, all pots were placed outdoors in a net protected area at the farm of the School of Agriculture. Half of the plants (one plant from each pot) were scored for disease severity at 5 weeks after transplanting (WAT), using the scale described above. Isolations from diseased and apparently diseased plants were done on PDA in order to confirm the presence of the pathogen. Growth parameters, namely fresh weight, root weight, stem height, number of leaves and number of flowers were also recorded. Disease severity scoring and growth parameters were also determined to the other half of the plants at 7 WAT. The experiment was repeated simultaneously at two different sites of the farm.

Statistical analyses

A combined over three experiments analysis of variance (ANOVA) for the parameters: disease index, fresh weight, root weight, stem height and number leaves data obtained from tomato plants grown under controlled conditions was performed using a 2 x 3 x 3 (x 10-15) [2 levels of biosolid (-, +) by 2 levels of inoculum (-, +) by 3 levels of peat mixture (peat, peat and clay soil, peat and sandy soil) with 9 replications per combined treatment] factorial experiment. Differences among treatment means were compared at the 5% level of significance ($P \leq 0.05$) using the Least Significant Difference (LSD) criterion.

A combined over two experiments analysis of variance (ANOVA) for the parameters: disease index, fresh weight, root weight, stem height, number of leaves, and number of flowers of tomato plants grown outdoors was performed using a 2 x 3 x 2 (x 4) (2 soil types by 3 biosolid concentrations by 2 levels of inoculum with 4 replications per combined treatment) factorial experiment based on the RCBD in a split plot arrangement. Differences among treatment means were compared at the 5% level of significance using the Least Significant Difference (LSD) criterion. All statistical analyses were performed using SPSS v.25 (IBM, USA).

Results

Pot experiment under controlled conditions

For the data obtained from tomato plants grown under controlled conditions, ANOVA results showed that the biosolid levels, inoculum levels and peat mixtures along with the interactions “biosolid by inoculum” and “inoculum by peat mixture” had a statistically significant effect, at $P \leq 0.05$, on the parameters disease index, fresh weight, root weight, stem height and number of leaves. In particular, inoculum (averaged over peat treatments), caused a lower disease index in the presence of biosolid. Consequently, it decreased less the parameters fresh weight, root weight, stem height, and leaf number when compared with no biosolid (Fig. 1).

Inoculum (averaged over biosolid levels) caused lower disease index in peat plus clay soil substrate as compared with peat or peat plus sandy soil substrates, and thus resulted in more fresh weight, stem height, and number of leaves in peat plus clay soil substrate than in peat or peat plus sandy soil (Fig. 2). However, root weight of plants grown in peat plus clay soil substrate was lower than in peat substrate.

Pot experiment under natural conditions

For the data obtained from tomato plants grown under natural conditions at 5 weeks after transplanting, ANOVA results showed that biosolid levels, inoculum levels and soil types along with the interactions “biosolid by inoculum” and “inoculum by soil type” had a statistically significant effect, at $P \leq 0.05$, on disease index, fresh weight, root weight, stem height, number of leaves and number of flowers. More specifically, inoculum (averaged over clay and sandy soil) at 4% of biosolid caused lower disease index on tomato plants than in 2% biosolid (Fig. 3), but all parameters were reduced less at both 2% and 4% of biosolid as compared with no biosolid.

Inoculum (averaged over biosolid levels) caused a lower disease index on tomato plants grown in the sandy soil as compared to the clay soil at 5 weeks after transplanting (Fig. 4). In addition, it caused a lower reduction on all parameters of tomato plants grown in the sandy soil than in the clay soil, whereas, in the absence of inoculum, all growth parameters of tomato plants were higher in the sandy soil than in the clay soil.

For the data obtained from tomato plants grown under natural conditions at 7 weeks after transplanting, ANOVA results showed that biosolid levels, inoculum levels and soil type along with the interactions “biosolid by inoculum” and “inoculum by soil type” had a statistically significant effect, at $P \leq 0.05$, on disease index, fresh weight, root weight, stem height, number of leaves and number of flowers. In particular, disease index (averaged over the two soil types) at 7 WAT was slightly higher on tomato grown in the presence of both 2% and 4% biosolid (Fig. 5), as compared to 5 WAT (Fig. 3). However, regardless the higher disease index at 7 WAT, all growth parameters were similar or higher in the presence of 2% and 4% of biosolid, as compared to no biosolid and to the non-inoculated controls.

Although Forl at 7 WAT caused similar disease index (averaged over biosolid levels) in both soils, all growth parameters of plants grown in the sandy soil were higher than in the clay soil, and the same growth response was observed in the absence of inoculum.

Discussion

The observed reduction in fresh weight, root weight, stem height, and leaf number of tomato plants grown on substrates inoculated with Forl, under both controlled and outdoor conditions, as compared to non-inoculated substrates, is the result of the disease caused by the fungus (Agrios 2005). The lower disease index on tomato plants grown outdoors, for 5 weeks after transplanting, in the presence of biosolid, as compared to no biosolid could be attributed to increased crop tolerance because of the indirect beneficial effect of biosolid on biotic and abiotic factors (Brown et al., 2020). Hence, fresh weight, root weight, stem height, and leaf number of tomato plants grown on biosolid substrates were reduced less by Forl inoculum as compared to the absence of biosolid. Most growth parameters of tomato plants grown outdoors in inoculated soil mixtures, in the presence of biosolid were similar to the respective parameters in non-inoculated mixtures, at 7 WAT, suggesting enhanced recovery and increased tolerance of the plants to fungus due to biosolid amendment.

These findings may be attributed to biosolids acting as organic solid amendments that restore or increase slightly organic matter of the soil (Brown et al. 2020; Pascual et al. 2008), a very important component that provides nutrients, favors plant growth and promotes soil biological diversity (Abawi and Widmer 2000). The beneficial edaphic microorganisms, according to Hoitink and Boehm (1999), are stimulated in soils rich with organic matter and show enhanced biological control mechanisms for suppression of pathogen activity. Moreover, improved plant nutrition due to biosolid amendment enhances crop tolerance to disease (Noble and Coventry 2005). Similar results were reported by Cotxarrera et al. (2002) who found that compost, prepared from vegetable and animal wastes, sewage

sludge and yard waste, suppressed tomato wilt caused by *F. oxysporum* f. sp. *lycopersici* race 1, at the early stages of plant growth. Also, Pinto et al. (2013) found that composted sewage sludge, incorporated into pine bark substrate, significantly reduced chrysanthemum wilting caused by *F. oxysporum* f. sp. *crysanthemi*.

It was here shown that tomato growth was increased in mixtures with biosolid as compared to those without biosolid, regardless of the presence or the absence of Forl inoculum. This finding strongly supports the evidence of the beneficial effects of the biosolid. In addition, increased crop tolerance to Forl along with increasing biosolid amendment could be attributed to dose-dependent beneficial effects, such as improvement of organic matter, density and structure of soil, nitrogen and phosphorus supply to the plants, restoration of soil fertility and improvement of water circulation (Fischer et al. 2020; Zaman et al. 2019). Similar results were reported by X. Wang et al. (2008) who found that *Zoysia japonica* biomass was increased by 64–316% in biosolid treatments (15, 30, 60, 120 and 150 tn/ha) as compared to the control soil. Singh and Agrawal (2010) also found that stem height, leaf area, root length, number of nodules and total biomass of bean plants increased, in soil amended with sludge-based biosolid at rates of 6-9 kg/m² (1 kg/m² corresponds to 10 tn/ha), as compared to unamended soil.

The higher disease index on tomato plants grown in the laboratory than outdoors could be attributed to conditions favoring the disease. Hibar et al. (2006) reported that the 25°C temperature prevailing in the growth chamber (controlled conditions) is the optimum temperature for the mycelial growth of Forl. Higher growth parameters of tomato plants grown under laboratory conditions and in inoculated peat plus clay soil were recorded in comparison to peat plus sandy soil. However, this is in contrast with the results found outdoors, where the growth parameters of tomato plants grown in inoculated biosolid plus clay soil were lower than those grown in biosolid plus sandy soil. This difference could be attributed to environmental conditions prevailing, size of pots used and different density of soil substrates.

The results of this study showed clearly that the amendment of clay and sandy soils with biosolids made from municipal wastewater sludge enhanced tomato growth and its tolerance to the fungus Forl. This biosolid has been proven to be of negligible ecotoxicological concern since leachates from biosolid-amended soil did not show toxicity on aquatic organisms. Furthermore, biosolid application did not significantly enrich these leachates with metals of toxicological concern, in relation to leachates from soils that had received this fertilization one year ago (Giannakis et al. 2020a, b). Therefore, these findings strongly support the use of this biosolid as a natural and environmentally acceptable amendment, since it improved soil fertility, tomato growth, and crop tolerance to the pathogenic fungus. The use of 80 and 160 tn biosolid /ha in the present study is similar to that reported by Sharma et al. (2017), and as such this is a realistic field scenario.

Conclusions

This study has shown that the addition of biosolids made from municipal wastewater sludge on clay and sandy soils, improved soil fertility, tomato growth and crop tolerance to Forl as compared to inoculated

soils that had not received biosolid amendment. Forl, at 5 weeks after tomato transplanting, caused higher disease index on tomato plants grown outdoors in biosolid plus clay soil substrate than in biosolid plus sandy soil, while the opposite occurred under laboratory conditions where higher disease index was recorded on plants grown in peat plus sandy soil than in peat plus clay soil. These findings strongly support the evidence that this biosolid acts as an organic fertilizer and possible stimulant of tomato tolerance against fungus disease. Therefore, this type of biosolid, which also has a minimal ecotoxicological impact as confirmed in a previous study, should be considered for its possible use in agriculture according to the principles of circular economy and waste minimization.

Declarations

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Conflicts of interest/Competing interests

The authors declare no conflicts of interest or competing interests

Availability of data and material

Data will be available upon request

Code availability

Not applicable

Authors' contributions

I. Giannakis: principal investigation, data curation, writing-original draft

C. Manitsas: investigation, data curation

I. Eleftherohorinos: methodology, validation, formal analysis, reviewing and editing

G. Menexes: formal analysis

C. Emmanouil: conceptualization, funding aquisition, reviewing and editing

A. Kungolos: funding aquisition, project administration

A. L. Lagopodi: supervision, methodology, resources, validation, writing, reviewing and editing

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Tables

Table 1 Peat/biosolid/soil mixtures used as substrates for tomato growth under controlled conditions

Treatments	Substrates	Inoculum
M1	Peat	-, +
M2	Peat plus clay soil at 1/1 ratio (w/w)	-, +
M3	Peat plus sandy soil at 1/2 ratio (w/w)	-, +
M1B	The M1, M2 and M3 treatments with the addition of wet biosolid (B) equal to 2% (w/w), corresponding to 80 tn/ha	-, +
M2B		-, +
M3B		-, +

Figures

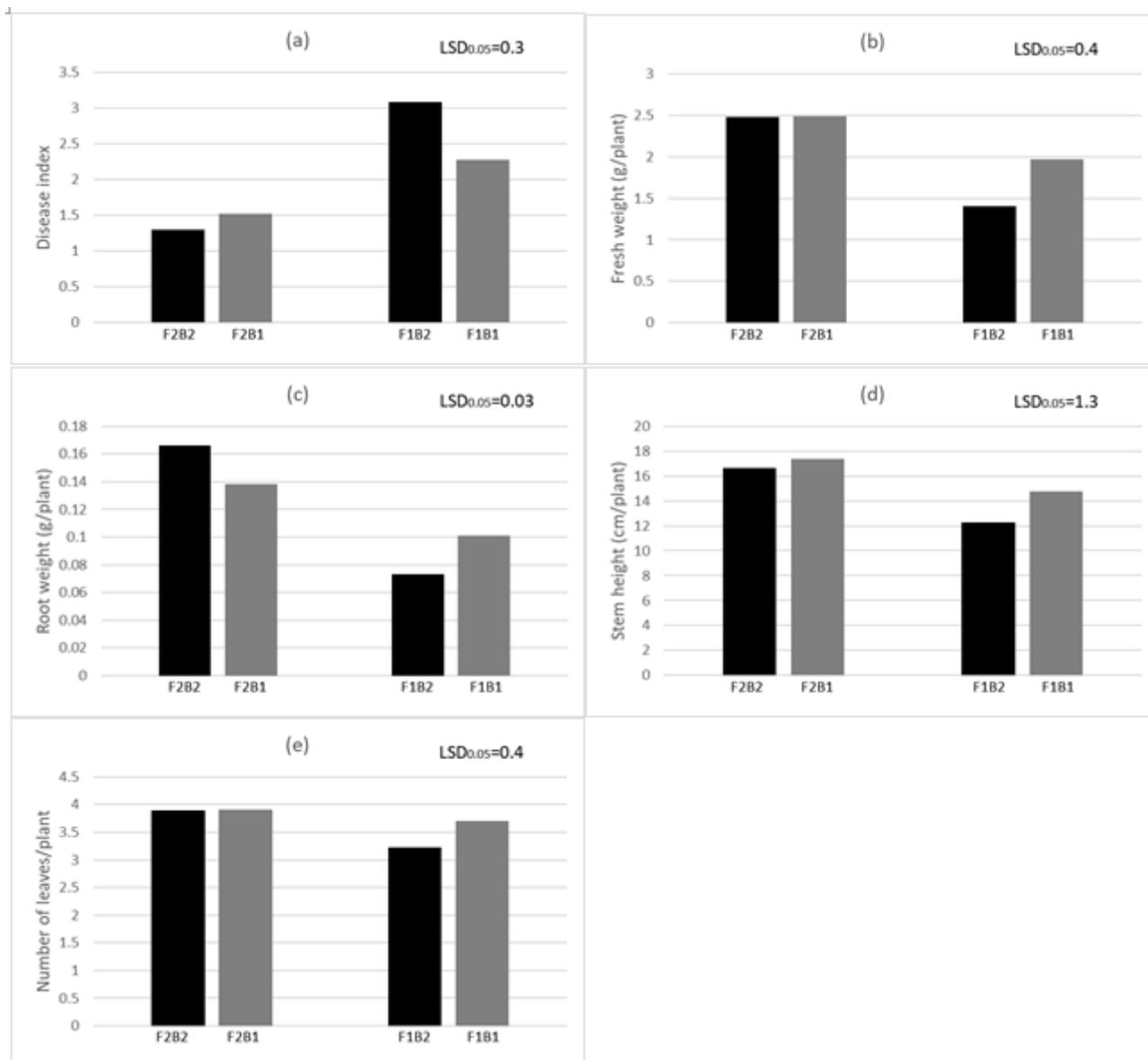


Figure 1

Disease index (a), fresh weight (b), root weight (c), stem height (d), and number of leaves (e) of tomato plants as affected by Forl inoculum (F1=presence, F2=absence) and biosolid levels (B1=with, B2=without). Treatment means are averaged over the three peat mixtures

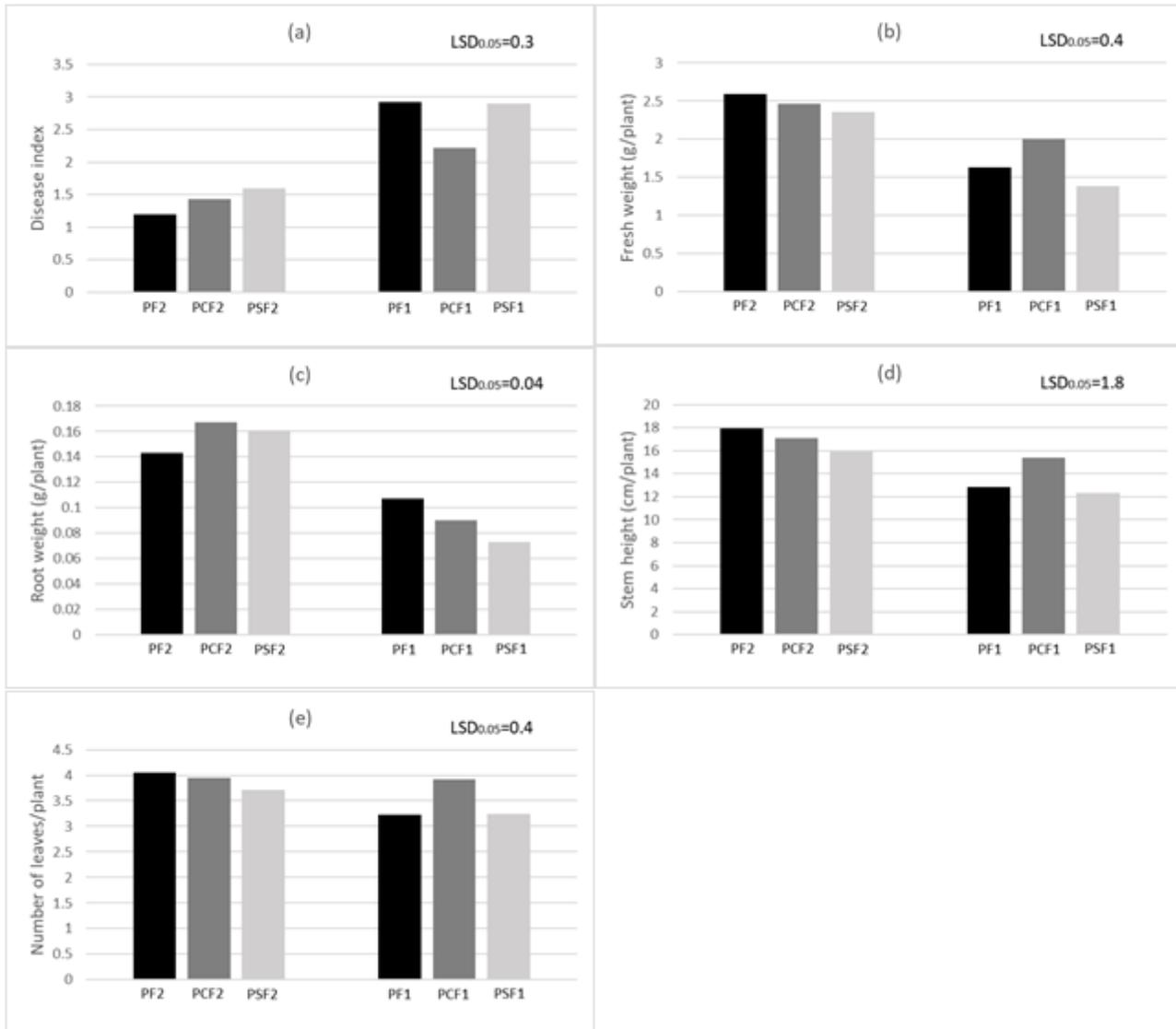


Figure 2

Disease index (a), fresh weight (b), root weight (c), stem height (d), and number of leaves (e) of tomato plants as affected by Forl inoculum (F1=presence, F2=absence) and peat mixture P=peat, PC=peat plus clay soil, PS=peat plus sandy soil). Treatment means are averaged over the biosolid levels

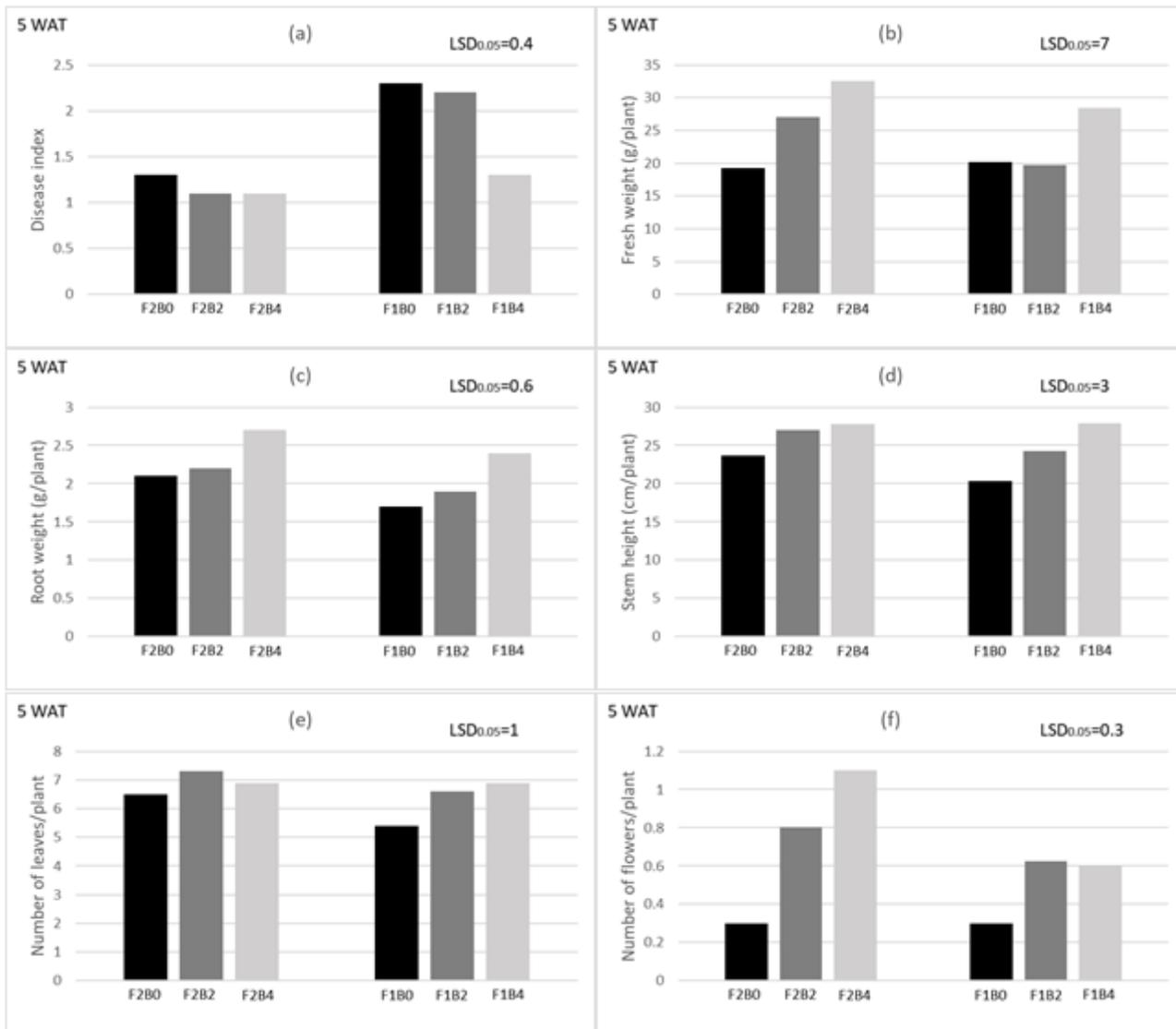


Figure 3

Disease index (a) fresh weight (b), root weight (c), stem height (d), number of leaves (e), and number of flowers (f) of tomato plants as affected by Forl inoculum (F1=presence, F2=absence) and biosolid levels (B0=0%, B2=2%, B4=4%) at 5 weeks after transplanting. Treatment means are averaged over the two soil types

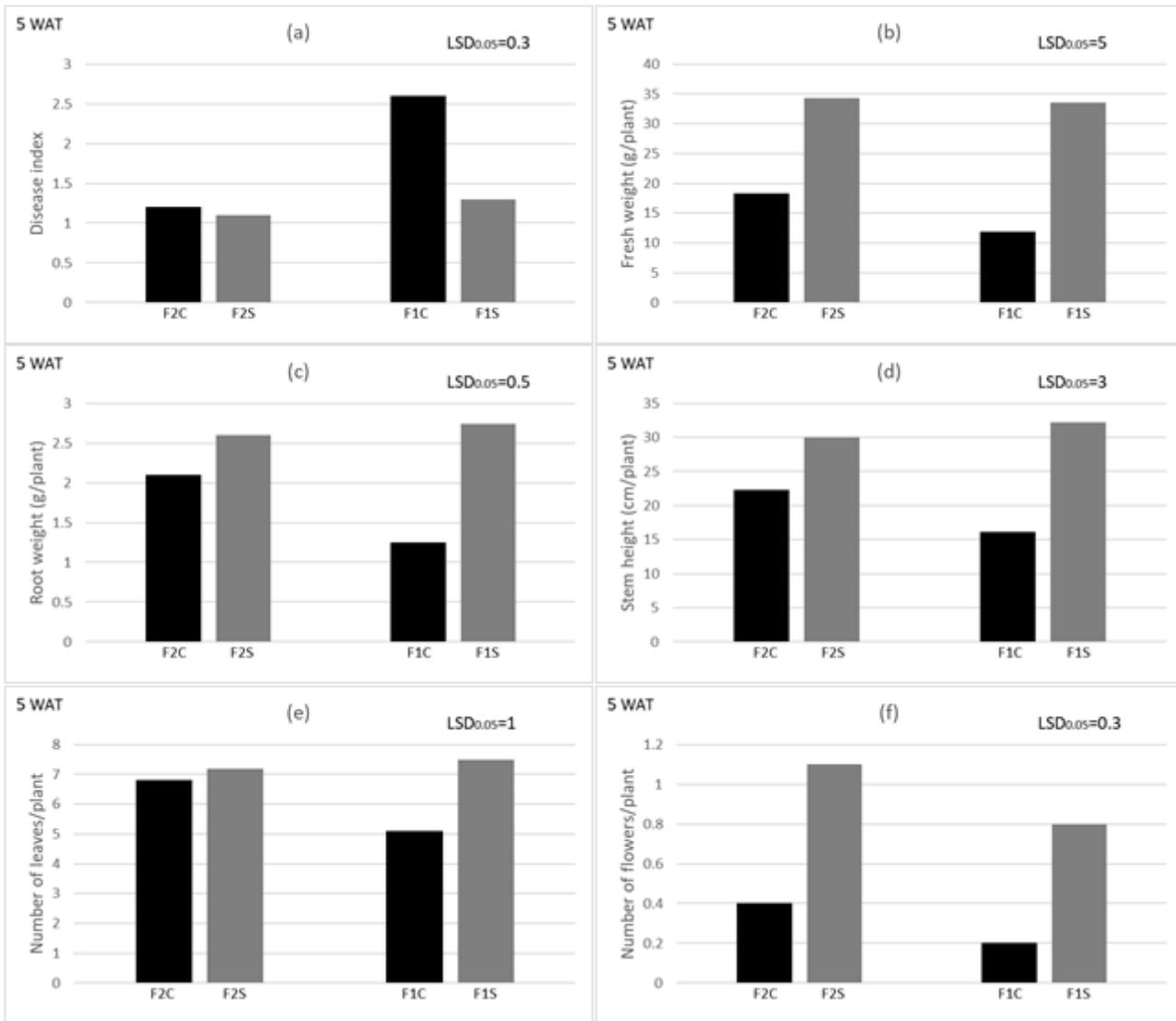


Figure 4

Disease index (a) fresh weight (b), root weight (c), stem height (d), number of leaves (e), and number of flowers (f) of tomato plants as affected by Forl inoculum (F1=presence, F2=absence) and soil type (C=clay, S=sandy) at 5 weeks after transplanting. Treatment means are averaged over three biosolid levels (B0=0%, B2=2%, B4=4%)

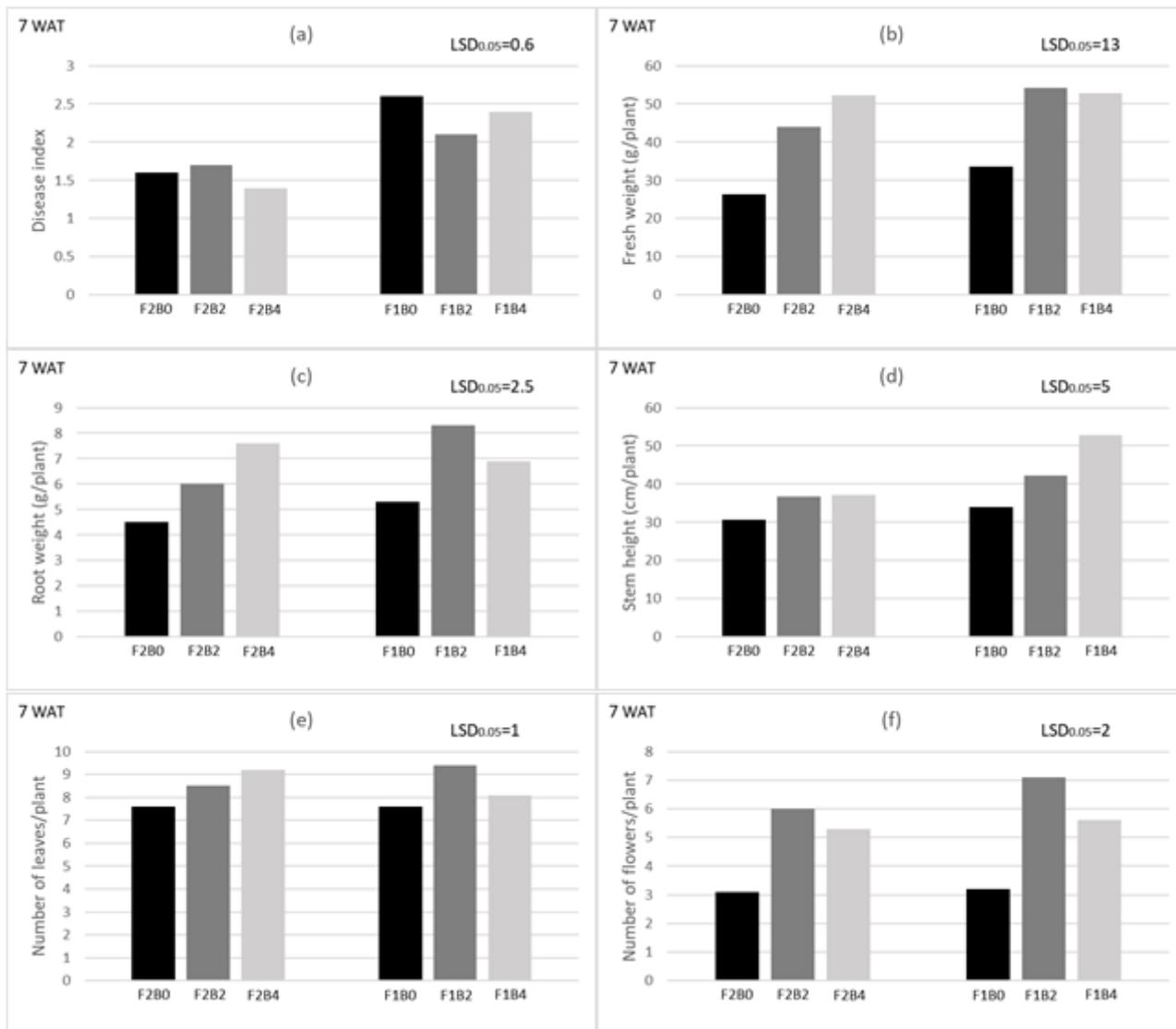


Figure 5

Disease index (a) fresh weight (b), root weight (c), stem height (d), number of leaves (e), and number of flowers (f) of tomato plants as affected by Forl inoculum (F1=presence, F2=absence) and biosolid levels (B0=0%, B2=2%, B4=4%) at 7 weeks after transplanting. Treatment means are averaged over the two soil types

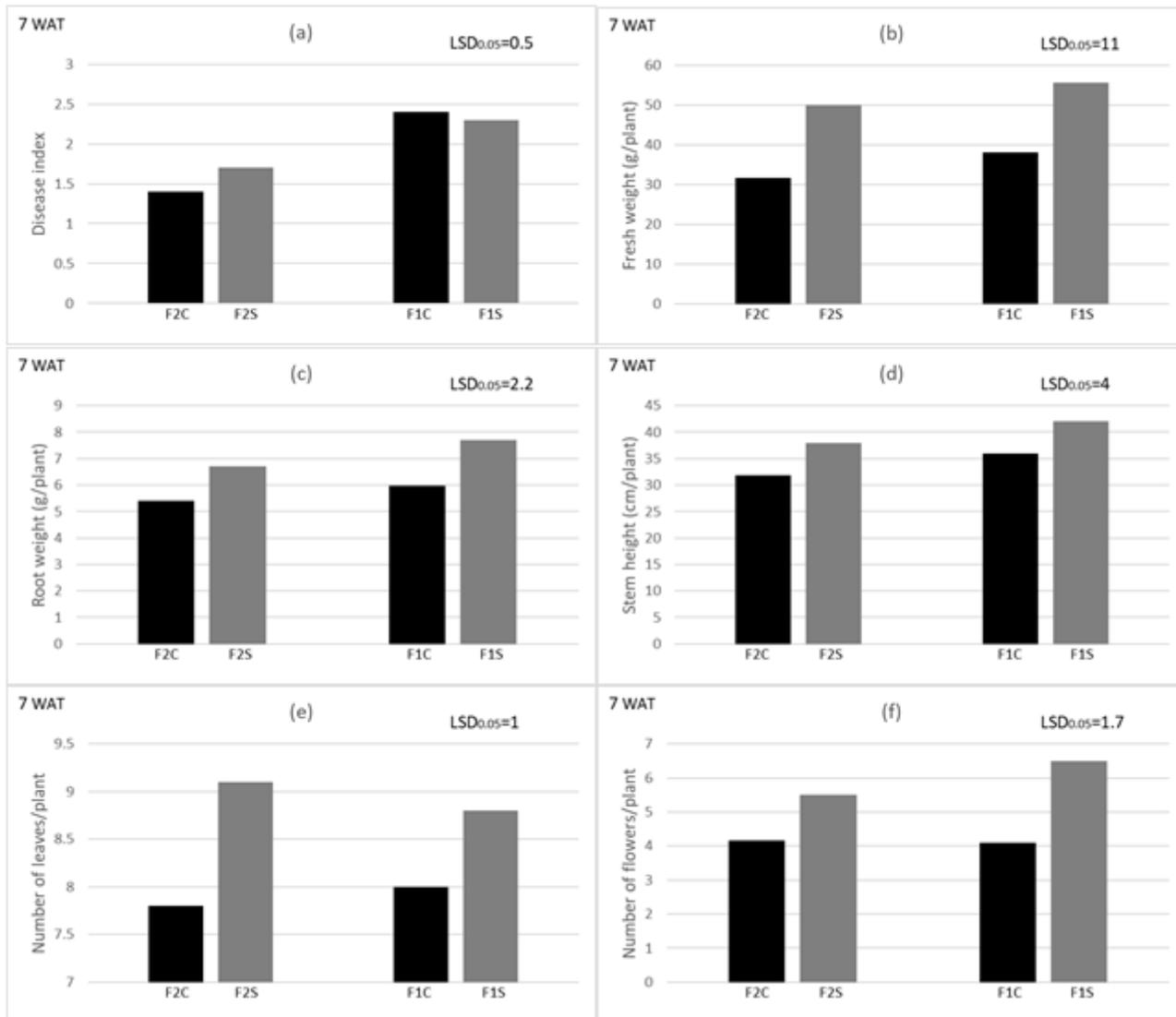


Figure 6

Disease index (a) fresh weight (b), root weight (c), stem height (d), number of leaves (e), and number of flowers (f) of tomato plants as affected by Forl inoculum (F1=presence, F2=absence) and soil type (C=clay, S=sandy) at 7 weeks after transplanting. Treatment means are averaged over the three biosolid levels (B0=0%, B2=2%, B4=4%)