

Optimization of worm-bed leachate for culturing of tomato (*Lycopersicon esculentum* Mill) inoculated with *Glomus fasciculatum* and *Pseudomonas fluorescens*

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Abbreviations: FAWBL: frequency of application of worm-bed leachate
RSM: response surface methodologies
WBL: worm-bed leachate

A response surface technique was used to analyze the effect of *Glomus fasciculatum*, *Pseudomonas fluorescens* and worm-bed leachate (WBL) on growth, yield and characteristics of tomato (*Lycopersicon esculentum* Mill). The treatments combined inoculation with or without *P. fluorescens* or *G. fasciculatum* and the application of WBL at 20% (v/v) each day or every three days. Plant height, number of leaves and yield of tomato fruits was not affected by the factors studied. However, plants with foliar application of WBL each

day developed wider stems than those with an application every three days. The pH of the fruits was lower when WBL was applied every three days compared to a daily application. The soluble solids content of the fruits was higher when WBL was applied daily compared to those sprayed every three days. Plant development was not affected by addition of *P. fluorescens*, *G. fasciculatum* or WBL, but WBL changed fruit characteristics.

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Tomato (*Lycopersicon esculentum* Mill.) originates from the Andean region of South America. It was probably the Aztecs, living in modern day México, who domesticated the crop over 1,500 years ago. This crop is one of the most popular and widely grown vegetables in the world. Leader tomato producing countries include China, USA, Turkey, Russia, Italy, India, Spain and México. In 1999, the global tomato production was 9.4×10^9 kg cultivated on 5.5 million ha, but production and land cultivated with them is still increasing. Fresh tomatoes are key ingredients in cooking all around the world and processed tomatoes are used to make soup, juice, ketchup, puree and other products. The vast majority of tomatoes are still produced with conventional production systems, *i.e.*, the use of large amounts of fertilizer and pesticides (Hasna et al. 2007). The importance of tomato in many diets and the large amounts of pesticide used to cultivation raises concern about the health and safety of workers, small scale farmers and consumers (Cavagnaro et al. 2006). In today's health conscious society, there is increasing demand for organic agricultural products. Therefore, growers increasingly prefer to cultivate tomatoes 'organically' for a variety of reasons. One of the advantages of organic cultivated crops is that they often give a 10-30% premium on the market (Cavagnaro et al. 2006). Other benefits are the possibility of reduced production costs, improved farm safety, reduced environmental impact and sustainable agro-ecosystems (Tu et al. 2006). Organic agriculture is an alternative to conventional production system, and it can contribute to socio-economic and ecologically sustainable development, especially in poorer countries. The use of vermicompost in the organic production of tomato in the greenhouse might decrease costs, increase yields, improve the fruit composition and reduce negative effects on the environment (Atiyeh et al. 2000). For instance, improvement in phytonutrients in tomatoes can be achieved by cultivar selection, environmental factors and agronomic practices (Dorais et al. 2008).

In the vermicomposting process, beds filled with composted waste, earthworms and bedding material are fitted with a drainage and collection system. Leachate derived from vermicomposting often called 'worm tea' or 'worm-bed leachate' (WBL) can be used as a liquid fertiliser as it contains large amounts of plant nutrients (Warburton and Pillai-McGarry, 2002). Apart from its large nutrient content, WBL might contribute to plant growth as it contains humic acids (Arancon et al. 2005). Humic acids regulate processes involved in plant development, such as macro and micro nutrient uptake (Atiyeh et al. 2002).

Berta et al. (2005) reported that *Glomus mosseae* in combination with *Pseudomonas fluorescens* suppresses the soilborne disease *Rhizoctonia solani* root-rot in tomato, this result indicates that AM fungi can be used as biocontrol agents of tomato. Mycorrhizal tomato plants had significantly less *Alternaria solani* symptoms than non-mycorrhizal plants (Fritz et al. 2006). Poulton et al. (2002) reported that *Glomus etunicatum*, a mycorrhizal fungus,

significantly increased above-ground dry mass, root length, phosphorus (P) content and yield of tomato under P limited conditions. Mena-Violante et al. (2006) evaluated the effect of *G. fasciculatum* on chile ancho (*Capsicum annum* L.) and found that fruit fresh weight was 25% greater in comparison to non-inoculated plants. In a greenhouse experiment, the weight of maize plants cultivated in peat moss amended with vermicompost increased when supplemented with *G. fasciculatum* (Gutiérrez-Miceli et al. 2008). However, host plant responsiveness to mycorrhizal colonization is highly variable (Poulton et al. 2002). The effect of these fungi on growth is not only different between plant species, but also between genotypes of the same species.

Colonization by *Pseudomonas fluorescens* affects root development in a complex way, causing an early stimulation of the primary root growth in tomato plants (Gamalero et al. 2005). *P. fluorescens* also acts as elicitor of plant defence reactions and can therefore be used to control pests in organic farmed crops (Diby and Sarma, 2006).

Worm-bed leachate is increasingly being used in Mexico, but little is known how it could be used in 'organic' culturing of tomato. We investigated how worm-bed leachate, added with *P. fluorescens* and *G. fasciculatum* affected growth, yield and fruits characteristics, *i.e.*, soluble solids and pH, of tomato plants cultivated in a commercial nursery.

MATERIALS AND METHODS

Worm-bed leachate

Eight beds (1.5 m by 6.6 m and 1 m deep) were used to obtain worm-bed leachate. Each bed was covered with a plastic sheet to protect the vermicompost against sun and rain. A total bed area of 79 m² was available. Cow manure was composted thermophilically for two months while mechanically being turned over every 15 days. The composted cow manure, adjusted to 80% moisture content, was placed in the beds to a depth of 0.5 m and earthworms (*Eisenia fetida*) were added at a rate of 25 g earthworms/kg cow manure or 2.5 kg earthworms m⁻² bed. The mixture was left to vermicompost for two months.

Each bed contained a leachate drainage and collection system. Leachate from each bed was collected in a separate 200 l tank, pumped into a central collection 1500 l tank and characterized.

Characteristics of the worm-bed leachate

The germination index for tomato seed (*L. lycopersicum* Mill) cv Sunn 7,705 was determined by placing a filter paper in a Petri dish and submerging it with worm-bed leachate. Seeds of tomato were placed on the filter paper and the Petri dish was covered with a larger filter paper. The number of seeds germinating was measured after

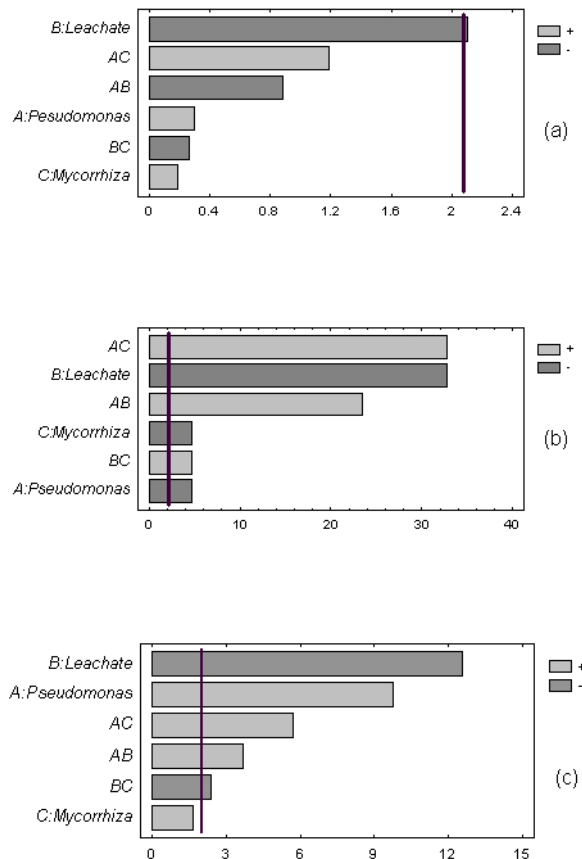


Figure 1. Standardized Pareto chart to investigate the effect of *Pseudomonas fluorescens*, *Glomus fasciculatum* and frequency of worm bed leachate application on stem diameter (cm, a), pH of the fruits (units, b) and soluble solids of the fruits (g/kg, c) of tomato plants.

incubating the covered Petri dishes in the dark at 28°C for 8 days (Alvarez and Grigera, 2005).

Details of the methods used to characterize the chemical composition of WBL can be found in Contreras-Ramos et al. (2004) and Sánchez-Monedero et al. (1996).

The worm-bed leachate was analyzed for total and faecal coliforms (*Escherichia coli*), *Salmonella sp.* and *Shigella spp.* (USEPA, 1999). *Salmonella* and *Shigella* were determined by serial dilution. A sub-sample of 10 ml worm-bed leachate was added to 90 ml 1% peptone solution under sterile conditions and 10^{-1} , 10^{-2} and 10^{-3} dilutions were made with sterile 0.8% NaCl solution. A 100 µl aliquot was plated on two selective media *Salmonella-Shigella* agar and sulphite-bismuth agar. The second medium is highly specific for *Salmonella*. The colonies were identified by form and colour (USEPA, 1999). For the measurement of total and faecal coliforms (*E. coli*), a 100 µl aliquot of each serial dilution was incubated in lactose broth for at 35°C for 24 hrs and total coliforms were counted. The faecal coliforms were differentiated from the

rest of the coliforms by incubating a 100 µl aliquot of each serial dilution in *E. coli* medium at 44°C. Gas production in each assay was considered as positive after 48 hrs. Results were confirmed by plating on eosin methylene blue (EMB) agar, incubating for 24 hrs, and examining for typical coliform colonies (USEPA, 1999).

The worm-bed leachate (mean of three samples collected every 8 weeks) had pH 7.8 ± 0.1 , electrolytic conductivity 2.6 ± 0.2 dS/m, 128.3 ± 42.2 mg suspended solids/l; 46 ± 12 mg Na^+ /l, no detectable NH_4^+ , 834 ± 71 mg K^+ /l, 59 ± 9 mg Mg^{2+} /l, 84 ± 13 mg Ca^{2+} /l, 130 ± 2 mg Cl^- /l, 247 ± 43 mg NO_3^- /l, 168 ± 11 mg PO_4^{3-} /l, 47 ± 13 mg SO_4^{2-} /l. The fulvic acid concentration was 1.5% and the humic acid 2.4% of the total C content of the worm-bed leachate. As such, the humic to fulvic acid ratio was 1.6. The electrolytic conductivity of the vermicomposting leachate was low indicating low concentrations of dissolved salts while it contained large amounts of K^+ , NO_3^- and PO_4^{3-} . Considering the above mentioned characteristics, the vermicomposting leachate could easily be used as a fertilizer.

The germination index for tomato was $55 \pm 5\%$. A germination index $> 50\%$ indicates that the worm-bed leachate is mature (Alvarez and Grigera, 2005). The worm-bed leachate was free of pathogens, *i.e.* coliforms (*Escherichia coli*), *Salmonella sp.* and *Shigella spp.* The reduction of pathogens in the worm-bed leachate could be due at physical and biological factors during vermicomposting (Li et al. 2008).

Glomus fasciculatum* and *Pseudomonas fluorescens

G. fasciculatum and *P. fluorescens* were obtained from the Cinvstav-Irapuato microbial collection (Mexico). Tomato plants were inoculated with or without *P. fluorescens* at 3×10^8 cell/ml (Gamalero et al. 2005) and *G. fasciculatum* at 2,250 spores/plant (Mena-Violante et al. 2006).

Cultivation of tomatoes and growth parameters

Three tomato seeds (*L. lycopersicum* Mill.) of the Sunn 7,705 cultivar were placed in small PVC containers filled with 50 g Canadian sphagnum peat moss potting material. The Sunn 7,705 cultivar is characterized by continuous growth and the production of flowers at every third internode, but it needs staking and pruning. After sowing, the pots were placed in a greenhouse until the seeds germinated. After germination, 320 tomato plantlets were transplanted to soil with pH 8.4, organic matter content 47 g/kg, total N content 2.3 g/kg, P-Olsen 22.2 mg/kg, and a particle size distribution of 640 g clay/kg, 100 g sand/kg and 260 g silt/kg amended with 10 g vermicompost. Plants were grown under 60% black knitted shade cloth without temperature control and grouped in plots 7 m long and 2.1 m wide each containing 10 plants spaced 70 cm apart. As such 32 groups of ten plants were obtained, *i.e.* eight

treatments with four replicates (Table 1). Each group of ten plants was separated 1 m from each other. Plants were secured to vertical nylon strings hung from horizontal ceiling wires 3 m above the greenhouse floor. The ten plants were monitored for growth and harvested. All treatments were drip irrigated with tap water ranging from 1 to 2 dm³ per plant per day depending of soil water content and crop maturity. Worm-bed leachate was applied by spraying the underside of the leaves until run-off using a single nozzle 18 l backpack sprayer with a 1 mm spray orifice and an application pressure of between 68.9 and 137 KPa (Hudson Industrial Sprayer # 65,010) around 6 pm.

The plant height, stem diameter and number of leaves were measured 70 days after transplanting (DAT). At first harvest, the number of fruits and their weight was determined for each plant. Fruits were separated into marketable and non-marketable, *i.e.* cracked, damaged and infected, and only marketable ones were used to calculate yields (Atiyeh et al. 2000). The results reported here are the mean of ten plants and four replicates for each of the eight treatments. A total of 320 plants were thus monitored and harvested.

Chemical analyses of the tomato fruits

All marketable tomato fruits were cut into small parts and mixed. Mixing was done manually to homogenize the samples. A 10 g sub-sample was pressed through cheese cloth to extract the juice, which was analysed for pH and soluble solids. A 5 ml aliquot of the tomato juice was added to 100 ml distilled water and the pH was measured using a 130 Conductronic pH meter (Conductronic S.A. 72,470 Puebla, México) fitted with a glass electrode. Soluble solids of the fruits were determined with a portable refractometer 300,003 (Sper Scientific Ltd., Scottsdale, Arizona, USA) standardized with distilled water.

Statistical analyses

Growth, fruit characteristics and yield of the tomato plants were subjected to a one-way analysis of variance to test for significant difference between the treatments (SAS Institute, 1989). The Statgraphic Plus Software (1999) was used for the regression analysis of the experimental data obtained. The quality of the fit of the model was expressed by the coefficient of determination R² and its statistical significance checked by an F-test. The significance of the regression coefficient was tested by a t-test. The level of significance was $P < 0.05$. A differential calculation method was then used to predict the optimum.

RESULTS

Growth and yield parameters

A factorial experimental design with eight treatments in four blocks with different combinations of *P. fluorescens*, frequency of application of worm-bed leachate (FAWBL) and *G. fasciculatum* was used to obtain optimal conditions

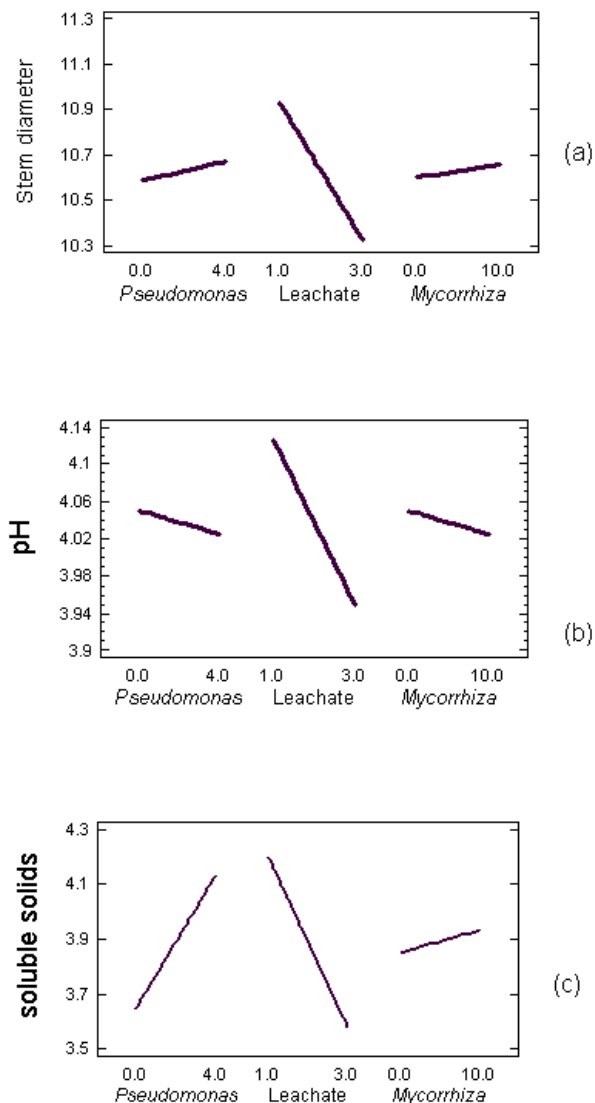


Figure 2. Main effects of *Pseudomonas fluorescens*, *Glomus fasciculatum* and frequency of worm bed leachate application on stem diameter of tomato plants (cm, a), pH (units, b) and soluble solids of the fruits (g/kg, c).

for tomato growth and fruit characteristics (Table 1). Plant height ranged from 121 to 134 cm, but was not significantly different between the treatments. Tomato yields and number of leaves per plant ranged from 2.8 to 3.8 kg from 115 to 123, respectively but treatments had no significant effect on them. Stem diameter varied from 1.0 cm to 1.1 cm and the frequency of leachate application affected the stem diameter significantly ($P < 0.05$) (Figure 1a). Plants with foliar application of WBL each day were significantly broader than those with an application every three days ($P < 0.05$) (Figure 2a). The equation that fitted the data was:

$$\text{Stem diameter} = 11.0 + 0.0619 \times A - 0.137 \times \text{FAWBL} - 0.0133 \times B - 0.0628 \times A \times \text{FAWBL} + 0.0169 \times A \times B - 0.00763 \times \text{FAWBL} \times B,$$

Table 1. Experimental design to the study the effect of *Pseudomonas fluorescens* (3×10^8 cell/(ml plant)), frequency of worm-bed leachate application and *Glomus fasciculatum* (2,250 spores/(g plant)) on plant growth and fruits characteristics of tomato.

		FWBL ^a		Height	SD ^b		Yield	pH	SS ^d
Treatment	<i>P. fluorescens</i>	(days)	<i>G. fasciculatum</i>	(cm)		LN ^c	(kg/plant)	fruit	(g/kg)
1	Control	1	Control	131 a	1.1 a	118 a	2.8 a	4.3 a	4.0 b
2	Control	1	Inoculated	127 a	1.1 a	118 a	3.0 a	4.1 b	4.0 b
3	Control	3	Control	132 a	1.1 a	116 a	3.3 a	4.0 bc	4.0 b
4	Control ^e	3	Inoculated	127 a	1.1 a	119 a	3.0 a	3.9 c	3.0 c
5	Inoculated	1	Control	134 a	1.1 a	115 a	3.3 a	4.0 bc	4.0 b
6	Inoculated	1	Inoculated	131 a	1.1 a	123 a	3.8 a	4.1 b	5.0 a
7	Inoculated	3	Control	121 a	1.0 a	115 a	3.0 a	3.9 c	4.0 b
8	Inoculated	3	Inoculated	131 a	1.0 a	118 a	3.3 a	4.1 b	4.3 b
LSD ^f ($P < 0.05$)				18	0.1	11	1.2	0.1	0.3

^aFWBL: Frequency of worm-bed leachate application; ^bSD: Stem diameter; ^cLN: Number of leaves; ^dSS: Soluble solids in the fruits, ^eControl: Tomato plants that were not inoculated, ^fLSD: Least significant difference.

$$R^2 = 46.5$$

With A: *P. fluorescens*, B: *G. fasciculatum*.

The broadest plant stems were obtained with 10 g *G. fasciculatum*/plant and 4 ml *P. fluorescens* applied to soil while spraying the worm-bed leachate every day (Figure 3a). With these values, the predicted maximum stem diameter was 1.13 cm.

pH and soluble solids of tomato fruits

The pH of tomato fruits varied from 3.9 to 4.3 (Table 1) and was significantly affected by FAWBL ($P < 0.05$) (Figure 1b). The pH was lower when WBL was applied every three days compared to a daily application (Figure 2b). The equation that fitted the data was:

$$\text{pH} = 3.63 - 0.059 \times A + 0.222 \times \text{FAWBL} - 0.00125 \times B + 0.0141 \times A \times \text{FAWBL} + 0.00406 \times A \times B - 0.00188 \times \text{FAWBL} \times B$$

$$R^2 = 99.2$$

Tomato fruits with the lowest pH were obtained without *G. fasciculatum* and *P. fluorescens* while spraying the tomato plant each day (Figure 3b). The model predicted a minimum pH of 3.6.

Soluble solids in tomato fruits varied from 3.0 to 5.0 mg/kg (Table 1). The FAWBL and interaction between FAWBL and *G. fasciculatum* had a significant effect on soluble solids in tomato fruits ($P < 0.05$) (Figure 1c). The soluble solids content was higher when the WBL was applied daily compared to those sprayed every three days (Figure 2c). The equation that fitted the data was:

$$\text{Soluble solids} = 4.88 - 0.0281 \times A - 0.522 \times \text{FAWBL} + 0.0175 \times B + 0.0109 \times A \times \text{FAWBL} + 0.00406 \times A \times B - 0.0169 \times \text{FAWBL} \times B$$

$$R^2 = 93.4$$

The response surface plot indicated that higher soluble solids in the tomato fruits were obtained without *G. fasciculatum* and *P. fluorescens*, but spraying the worm-bed leachate each day (Figure 3c). Under these conditions, the model predicted 44.6 mg/kg soluble solids in tomato fruit.

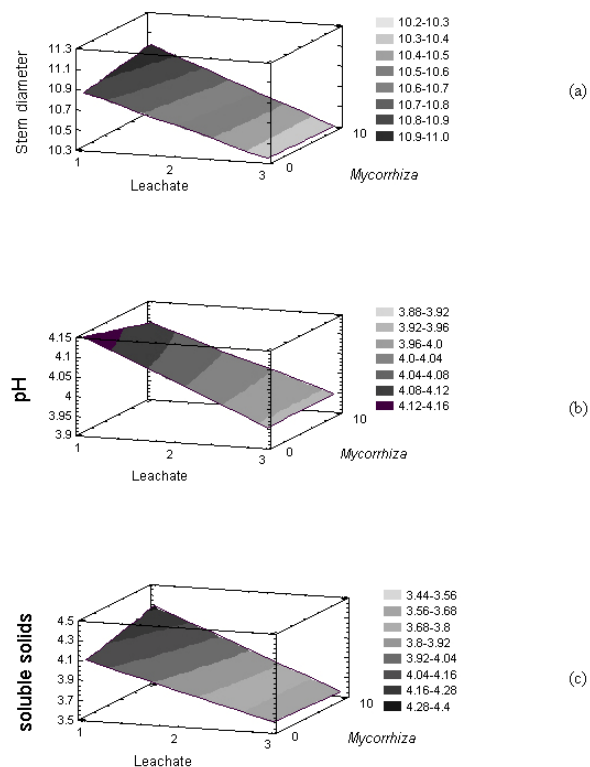


Figure 3. Response surface plots showing the relative effect of *Glomus fasciculatum* and frequency of worm bed leachate application on stem diameter of tomato plants (cm, a), pH (units, b) and soluble solids of the fruits (g/kg, c).

DISCUSSION

Response surface methodologies (RSM) have been used to optimize culture conditions in diverse biotechnology processes. It has been used to optimize the medium for lipopeptide iturin production (Mizumoto and Shoda, 2007), to enhance acetoin production (Xiao et al. 2007), and to optimize inactivation of endospores of *Bacillus cereus* (Huang et al. 2007). RSM includes different statistical techniques related to experimental design, model development, and evaluation of factors, identifying optimum conditions and also to study interactions between variables (Lin et al. 2007; Liu and Wang, 2007). In our work, we used RSM to evaluate how the application rate of vermicompost leachate and the addition of two microorganisms, *P. fluorescens* and *G. fasciculatum* affected growth, yield, soluble solids and pH of tomato plants cultivated in a commercial nursery.

It has been reported that foliar application of aqueous vermicompost extracts onto three field-grown tomato varieties had no significant effects on plant growth, biomass and nutrient allocation (Zaller, 2006). In the experiment reported here foliar application of aqueous vermicompost extracts together with *G. fasciculatum* and *P. fluorescens* increased stem diameter. It has been

demonstrated that the mycorrhizal fungus, but not the bacterial strains, promoted plant growth, however bacteria induced synergistic effects on plant biomass and the results suggest a key role of the bacteria producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme that modulate plant ethylene levels (Gamalero et al. 2008). Additionally, some fruit characteristics were affected. The pH of the fruits was lower when WBL was applied every three days compared to a daily application. The soluble solids content of the fruits, however, was higher when WBL was applied daily compared to those sprayed every three days. Zaller (2006) reported that vermicompost spraying consistently increased fruit size as well as N content, but decreased L-ascorbic acid compared with water sprayed fruits. It can be assumed that microorganisms in the leachate or humic substances in the extracts affected fruit quality. It has been shown that mycorrhizal fungi and the level of soil phosphorus increased fruit and seed development (Poulton et al. 2002). Plants with higher concentrations of P produce more organic acids so that pH of the fruits is lower (Poulton et al. 2002). Gutiérrez-Miceli et al. (2007) reported that the addition of vermicompost increased soluble and insoluble solids in tomatoes compared to untreated ones. Azarmi et al. (2008) demonstrated that addition of vermicompost had significant positive effects on growth, yield and elementary content of tomato plants as compared to control. It might be that vermicompost leachate changed the physicochemical, microbial and biological characteristics of the soil thereby improving the nutrient status of the soil or certain substances in the WBL directly affected fruit characteristics (Atiyeh et al. 2000).

CONCLUDING REMARKS

In conclusion, this study showed that yield, height and number of leaves of the tomato plant were not affected by the factors studied. However, worm-bed leachate added with *P. fluorescens* and *G. fasciculatum* affected stem diameter, and the soluble solids and pH of the tomato fruits. Daily applications of worm-bed leachate increased stem diameter, while pH of the fruits was lower when worm-bed leachate was applied every three days. The soluble solids content of the tomato fruits was higher when the worm bed leachate was applied daily.

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