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Laboratory Bioassay and Greenhouse Evaluation of a Pine Tree Substrate Used as a Container Substrate

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Summary

Bioassays with tomato and lettuce seeds and three plant experiments with marigold (*Tagetes erecta* L.) 'Inca Gold' were conducted in the laboratory and greenhouse, respectively, in order to investigate possibilities of determining and mitigating the effects of phytotoxins in pine tree substrate (PTS). PTS is produced by grinding loblolly pine tree (*Pinus taeda* L.) logs, and using as horticultural growing substrate. PTS extracts obtained with cold and hot water reduced the germination rate and radicle growth of tomato and lettuce. After the washing, an improvement was monitored for radicle length of both species. The germination index supported the data of radicle length showing a better performance after the substrate washing. Radicle growth of tomato in extracts from PTS used in a plant growth experiment was improved after leaching PTS six times.

The performance of marigold plants was similar to bioassays with tomatoes and lettuce. Marigold growth was improved by pretreatments of PTS. Some of these pretreatments could be recommended for use in the manufacturing process for PTS or from growers before planting. However, the best results were shown in a commercial peat-lite substrate.

Key words. germination – lettuce – marigold – phytotoxins – phytotoxicity – *Pinus taeda* L. – radicle growth – tomato

Introduction

The use of forestry products (barks, sawdust, wood chips) as container substrates can involve problems of phytotoxicity. Phytotoxicity depends on the chemical composition of the substrate, which in turn can cause salinity, nutritional disorders and enzymatic or hormonal metabolic alterations (Ortega et al. 1996). High potassium and manganese contents (Maher and Thomson 1991), high C/N ratios (Gruda et al. 2000), and the presence of phenolic compounds (Ortega et al. 1996), terpenes, organic acids and fatty acids (Morel and Guillemain 2004) could be the cause of such problems.

Pine tree substrate (PTS) made from grinding loblolly pine (*Pinus taeda* L.) has been shown to be a potential substitute for peat moss and bark as a horticultural container growing medium (Wright and Browder 2005; Wright et al. 2006). However, before the product can be commercialized there are some inconsistencies in the substrate that need to be addressed such as phytotoxicity.

In several studies using hardwood sawdust as a growing medium, it was found that wood contained phytotoxins that affected the growth of plants (Worrall 1976, 1981; Maas and Adamson 1982; Rau et al. 2006). Indeed, these compounds have a protection effect and defend woods against insects or infections and are therefore toxic to other organism, such as plants. Methods to evaluate phytotoxicity may consist of analytic techniques to detect and quantify phytotoxic molecules, or rapid bioassays, with low technical requirements using species sensitive to the toxic elements (Ortega et al. 1996). Allison (1965) used for instance, a bioassay of pea seedlings to determinate the phytotoxicity of 28 kinds of woods and barks. Still et al. (1976) used a bioassay of cucumber seedlings and mung bean cuttings in bark extracts. Yazaki and Nichols (1978) applied methods based on bioassays using radish and turnip seedlings, whereas Nichols (1981) investigated bark extracts in a bioassay with radish seedlings to determine the rate at which the phytotoxicity of the fresh bark disappeared. A more common bioassay used by the growing media industry is the germination rate of Chinese cabbage. However, Ortega et al. (1996) carried out bioassays with eight different vegetables that included Chinese cabbage and found that tomato and lettuce are the most sensitive species, both in germination and radicle growth when grown in substrates with phenolic compounds.

Methods such as composting, aging, leaching, washing, mixtures and fertilization have been used to reduce or eliminate phytotoxicity properties (Estaun et al. 1985; Ortega et al. 1996; Gruda et al. 2000). Recently, several authors reported that the growth of fungi on woody tissues in solid-state cultures on pine chips fermentations decreased their toxicity (Gutierrez et al. 1999; Dorado et al. 2000; Linares et al. 2003).

The purpose of this study was to determine if extracts from PTS were toxic to plants and to examine the possi-

bilities of avoiding or mitigating these effects with above-mentioned substrate-pretreatment.

Material and Methods

The experiments were carried out in laboratory and glasshouse at „Virgina Tech, Blacksburg, VA". The PTS was prepared by grinding coarse (2.5 x 2.5 x 0.6 cm) pine chips from loblolly pine logs harvested April 15 in a hammer mill fitted with a 2.38 mm screen. The particle size range of the substrate was as follows: 52.9 % (w/w) in the 0.5–2 mm range, 47.1 % (w/w) in the 0.5 mm and less range. The peat-lite (PL) substrate composed of 45 % peat, 15 % perlite, 15 % vermiculite, and 25 % bark (Fafard, Anderson, S.C.) was used for comparative purposes.

Bioassays with extracts from fresh PTS

A germination bioassay of two vegetable species, lettuce (*Lactuca sativa* L.) and tomato (*Lycopersicon esculentum* L.) was carried out in accordance with the method described by Ortega et al. (1996). Aqueous extracts were prepared by stirring PTS for 2 h in either cold water (PTS-CW) 15 °C or hot water (PTS-HW) 70 °C (1:1 substrate/water ratio (v/v)). They were then strained through cheese cloth using hand pressure (treatments 1 and 2). After this, the substrates were washed only with distilled cold water at 15 °C, stirred for 2 h and then re-extracted (WPTS-CW and WPTS-HW, respectively treatment 3 and 4). A treatment with distilled water (DW) served as a control.

5 mL of the extracts from its respective treatment were placed on 6 cm square Anchor seed germination paper with a thickness of 0.6 mm (Anchor Paper Co., Minnesota) in square style Petri dishes. An additional 1 mL of each solution was added 2 and 3 d after the sowing (DAS), respectively for lettuce and tomatoes. Ten seeds were sown in each dish.

After sowing, the dishes were placed in an incubator, maintaining temperatures of 15 and 25 °C, respectively for lettuce and tomato (Aosa 1988). On the last day lettuce temperature was switched to 20 °C. The germinated seeds (G) were counted 2 and 3 DAS for lettuce and 3 and 4 DAS for tomato, respectively. The radicle growth (L) was measured with a caliper. The germination index (GI) was calculated according to Zucconi et al. (1985) and Ortega et al. (1996) in GI = G/Go x L/Lo x 100, where Go and Lo are respectively the germination percentage and radicle growth of the control (DW).

This bioassay was conducted twice under the same conditions and setup in completely randomized design with respectively 10 and 6 replicates per treatment and plant species.

Plant growth experiments

Three plant growth experiments PG 1, PG 2, and PG 3 were conducted with marigold (*Tagetes erecta* L.) 'Inca Gold' in the greenhouse. The cultivation data are presented in Table 1. Each treatment received 0.6 kg m^{-3} $CaSO_4$ and the PL received 3.56 kg m^{-3} of dolomite lime for the first and second trial. Dolomite lime was not used for the third experiment.

Marigold plants were fertilized at each watering (150–250 mL beaker-applied) according to growth stage with a 300 ppm N solution from a 20 N-4.4 P-16.6 K fertilizer (20-10-20 Peat Lite Special, Scotts Company, Marysville, OH) three times a week and grown for two weeks. Six randomized sequences were used for all experiments. Growth index ([height + width1 + width2] / 3 (cm)) was documented at harvest for the 2nd and 3rd experiment. After the harvesting fresh mass (g $plant^{-1}$), and after the drying for 3 d at 70 °C, dry mass (g $plant^{-1}$) were determined. For the 1st experiment only fresh mass was recorded. Substrate pH and EC (electric conductivity) were monitored throughout the course of the experiments using the pour through method (Wright 1986).

A description of the treatments and setup of three marigold plant growth experiments follow:

PG 1. There were four treatments for this experiment: PTS leached, PTS soaked, PTS (control 1), and PL (control 2). For the leached treatment, each pot was leached once daily for 6 d with 1 L water before potting. PTS for the soaked treatment was inundated in water for 24 h, drained, inundated for another 24 h, drained and then used as a substrate.

PG 2. In addition to the treatments of experiment 1, a fungi inoculated treatment was investigated in this experiment. Cartapip 97 (*Ceratocystis pilifer* Fries), from Center for Forest Mycology Research, USDA, Forest Service, Madison, WI, USA was chosen since it was shown to remove a high percentage of resinous extractives from loblolly pine chips (Croan 2004). The fungi was grown on 1.5 % Malt MEA agar plates and incubated at 25 °C for two weeks in the dark. Spore counts at the end of two weeks were approximately $2x10^9$ spores per plate. Un-ground chips (100 g) were placed into a 500 mL Erlenmeyer flasks, and then brought to 60 % moisture content. Each was then inoculated with the spore solution from one plate. The flasks were then incubated at 25 °C in the dark for 2 weeks, at which time fungi were seen to be growing on the chips. Chips from the four flasks were mixed with 71 L of coarse pine chips and incubated for two weeks in greenhouse. Chips were then ground in a hammermill as above and used in plant growth experiments (PG) (PG 2 and PG 3).

PG 3. In addition to the treatments of experiment 2, PTS pretreated with cold and hot water were used (PTS-CW and PTS-HW, respectively). Fungi spore solution concen-

Table 1. Cultivation data.

Operation	PG[z] 1	PG 2	PG 3
harvest of loblolly pines	February, 19	February, 19	April, 9
chipped	February, 20	February, 20	April, 10
Grinded	February, 20	March, 19	April, 12
seed sowing	February, 2	March, 12	April, 18
Potted in 1 L containers	March, 02	March, 28	May, 02
Harvested	March, 16	April, 13	May, 16

[z]PG = Plant Growth

tration for the inoculation was approximately $1.3x10^{10}$ spores per 700 mL in this experiment.

The experiments were set up in a completely randomized design with six replicates.

Bioassay with extracts from substrates used in a plant growth experiment (PG3)

The germination of tomato seeds was tested in aqueous extracts obtained from all substrates used in the tomato plant experiment 3 (see Table 4). For the leached treatment, each container was leached once daily by apply 1 L of distilled water to 1 L container filled with PTS for 6 d. Leachates from PTS on the 1st, 3rd and 6th day (L1, L3 and L6), respectively, were tested.

The extracts from PTS-CW and PTS-HW were prepared and collected as described in the bioassays with extracts from fresh substrates.

PTS for the soaked treatment was inundated in water for 24 h. Drained water after soaking was used as an extract (S1). The substrate was inundated for another 24 h, and the drained water was also tested (S2).

Each 1 L pot filled with this substrate was leached once with 1 L distilled water before potting and the leachate tested in bioassay (PTS-fungi inoculated).

In addition 1 L containers filled with PTS and without plants were watered with the same amount of distilled water as the nutrient solution in containers with plants (150–250 mL beaker-applied, according to growth stage) three times a week except on day 0, 7, and 14 when 1 L of distilled water was applied and leachates collected (PTS0, PTS7, and PTS14), respectively. These leachates were tested in this bioassay. A distilled water treatment and PL were also used as controls.

6 mL of each extract were applied once at beginning of the experiment. Ten seeds were sown in each dish with

Table 2. Germination rate (%), radicle growth (mm) and germination index for tomatoes and lettuce in bioassays with aqueous extracts of PTS before and after washing with distilled water.

Treatments	Tomato		Lettuce	
	3 DAS	4 DAS	2 DAS	3 DAS
PTS-cold water	68.1 a	71.2 b	78.1 b	88.7 b
PTS-hot water	70.6 a	76.2 ab	75.0 b	90.0 ab
WPTS-cold water	71.2 a	77.5 ab	95.0 ab	93.1 ab
WPTS-hot water	75.0 a	80.6 ab	81.3 ab	94.3 ab
Distilled water	76.2 a	85.0 a	91.8 a	96.9 a
	Radicle growth (mm)	Germination index	Radicle growth (mm)	Germination index
PTS-cold water	13.4 c	40.5 b	4.7 c	61.5 b
PTS-hot water	12.9 c	42.7 b	4.3 c	57.5 b
WPTS-cold water	16.9 bc	57.4 ab	6.0 b	81.5 a
WPTS-hot water	19.2 b	66.4 a	5.8 b	78.8 a
Distilled water	30.2 a		7.4 a	

PTS = pine tree substrate, WPTS = washed PTS, DAS = days after the sowing. Means followed by the same letter(s) within each column are not significantly different. Tukey's test at $P<0.05$. $n_{(seeds/dishes)} = 10$; $n_{(dishes)} = 10$ and 6, respectively for the 1st and 2nd test. Germination index = G/Go x L/Lo x 100, where Go and Lo are respectively the germination percentage and radicle growth of the control (DW).

Table 3. Yield of marigold cultivated direct in pine tree substrate (PTS) and after different pretreatments in comparison to a peat lite substrate (PL), two weeks after the planting.

Treatments	PG 1	PG 2		PG 3	
	Dry mass (g)	Fresh mass (g)	Dry mass (g)	Fresh mass (g)	Dry mass (g)
PTS-leached (6x)	0.44 ab	10.57 a	1.06 ab	7.03 b	0.70 b
PTS-soaked (2x)	0.40 ab	10.17 a	1.03 ab	6.97 bc	0.69 b
PTS-fungi inoculated		9.69 a	1.02 ab	5.93 d	0.60 b
PTS-cold water				6.46 bcd	0.62 b
PTS-hot water				6.08 cd	0.63 b
PTS (control 1)	0.30 b	8.84 a	0.88 b	5.93 d	0.61 b
PL (control 2)	0.48 a	10.98 a	1.20 a	8.15 a	0.83 a

PTS = pine chips, PL = peat-lite, PG = plant growth experiment. Means followed by the same letter(s) within each column are not significantly different. Tukey's test at $P<0.05$. $n = 6$.

Table 4. Germination rate (%), radicle growth (mm) and germination index of tomatoes 4 DAS in the bioassay with aqueous extracts of substrates used in PG3.

Treatments Extracts from:	Parameters		
	Germination rate (%)	Radicle growth (mm)	Germination index
PTS-leached L1	90.00 a	17.25 bcd	77.17 a
PTS-leached L3	86.00 a	16.56 cd	70.70 a
PTS-leached L6	98.33 a	23.36 abc	112.28 a
PTS-cold water	81.67 a	16.84 dc	67.83 a
PTS-hot water	93.33 a	16.03 d	72.93 a
PTS-soaked S1	93.33 a	19.56 abcd	89.32 a
PTS-soaked S2	88.33 a	21.67 abcd	93.70 a
PTS-fungi inoculated	91.67 a	21.71 abcd	97.37 a
PTS0	85.00 a	18.98 abcd	82.57 a
PTS7	90.00 a	20.65 abcd	92.44 a
PTS14	98.33 a	24.34 ab	117.83 a
PL	95.00 a	25.02 a	116.93 a
Distilled water	93.33 a	22.63 abcd	

PG = plant growth experiment; PTS = pine tree substrate; L1, L3, and L6 = 1st, 3rd and 6th leaching before the planting, respectively; S1 and S2 = 1st and 2nd soaking, respectively; PTS0, PTS7, and PTS14 = extract taken on day 0, 7 and 14 after the planting, respectively, in container without plants: PL = peat-lite. Means followed by the same letter(s) within each column are not significantly different. Tukey's test at $P<0.05$. $n_{(seeds/dishes)} = 10$; $n_{(dishes)} = 6$. Germination index = G/Go x L/Lo x 100, where Go and Lo are respectively the germination percentage and radicle growth of the control (DW).

tomatoes. After sowing, the dishes were placed in an incubator, maintaining temperatures of 25 °C. The germinated seeds (G) were counted four DAS. The radicle growth (L) was measured and the germination index (GI) was calculated as in bioassay 1. The experiment was set up in a completely randomized design with six replicates.

Statistical analyses.

All the data were subjected to statistical analysis. One-way analysis of variance (ANOVA) was used to determine the significance of variation among the treatments using SAS (SAS Inistitute, Cary, NC, version 9.1.3). Means separation was accomplished by Tukey's multiple range test ($P<0.05$). Since the same trends were drawn from the bioassays with extracts from fresh PTS the data are presented as mean values of 16 replicates across the two experiments. The correlation analyses between the average EC of the solution during the vegetation and plant parameters were calculated using Pearson's correlation coefficient ($P<0.05$).

Results and Discussion

Bioassays with extracts from fresh PTS

Extracts were characterized by slight colour, acidic pH (4.38–4.56), and low EC-values (0.13–0.17 dS m^{-1}). The cold water extracts inhibited germination compared to DW control on day 4 for tomato and for day 2 and 3 for lettuce (Table 2). Also the hot water extracts inhibited germination on day 2 for lettuce. However, germination rate in the WPTS (cold or hot) was not different from DW.

The same trend was shown for radicle growth (Table 2). Radicle growth was greater in the extract of washed growing media (WPTS-HW) than in the unwashed for tomato and for both CW and HW for lettuce. This demonstrates that the levels of toxins were reduced by the aqueous washing.

The best radicle growth was achieved with distilled water, in agreement with Linares et al. (2003). The authors reported that aqueous extracts from pine chips inhibited the germination rate and shoot growth in alfalfa, maize, and particularly in tomato and rape. An inhibition of radicle growth or no differences between the treatment after the washing and distilled water for different vegetable species was shown by Ortega et al. (1996).

The GI which takes into account germination and radicle growth is shown in Table 2. According to Zucconi et al. (1985), the organic materials are phytotoxic when the germination index is below 60. However, Ortega et al. (1996) considered this value to be to low for plant substrates. With respect to extracts obtained with cold and hot water (PTS-CW, PTS-HW) for tomatoes as well as PTS-HW for lettuce the GI were lower than 60. The GI for PTS-CW of lettuce was with 61.5 slightly higher than 60, however no significant differences were shown between PTS-CW and PTS-HW. For both species, the GI was higher for extracts after the washing in comparison to first extract, with exception of WPTS-CW for tomato. These results support the radicle growth results (Table 2).

Plant growth

Generally dry mass was reduced in all three experiments for marigold directly cultivated in PTS compared to the control PL, a commercial substrate (Table 3). According

to FARMER (1998), resin acids, fatty acids and phenols are the extractives most linked to toxicity. Phenolic compounds are found in softwood trees but are more prevalent in hardwood. While they occur throughout the tree, phenols are primarily located in bark and cambial tissues (FARMER 1998). With regard to literature, the percentage of bark in a mixture could influence the extent of the phytotoxicity (NICHOLS 1981). However, according to foresters and our calculations whole tree wood chips from *Pinus taeda* L. contains 5–10 % bark. This is similar to the amount of bark in wood fibre substrate produced in Europe (GRUDA and SCHNITZLER 2004) where no plant growth inhibition has been reported. To be noted are, however, the different tree species and the production procedure: wood chippings from the woodworking industry, mainly from Norway spruce (*Picea abies* [L.] Karst.), are shredded under frictional pressure and impregnated with fertilizer (GRUDA and SCHNITZLER 2004). RAU et al. (2006) demonstrate that *Pinus taeda* L. is comparatively lower in polyphenolics and thus a preferred species for production of a wood-based container substrate compared to a number of other softwood and hardwood species.

Taking into account that the chemical properties of PTS before and after the pretreatments are similar (ORTEGA et al. 1996), the different plant growth can be attributed to the reduction of phytotoxins.

Pretreatments improved plant growth and resulted in dry mass not different than PL for PG 1 and PG 2. No differences were shown between PTS and pre-treatment in PG 3. Pine tree logs for PG 1 and PG 2 were harvest on February, while the material for PG 3 was harvest on April 2007 (Table 1). However, in the PG 3, in respect to fresh mass a positive effect was noticed in PTS after six times leaching with 7.03 g plant^{-1}, follows by soaked PTS with 6.97 g plant^{-1}, as well as washed PTS. Several authors reported that the growth of fungi on woody tissues in solid-state cultures on PTS fermentations decreased their toxicity (GUTIERREZ et al. 1999; DORADO et al. 2000; LINARES et al. 2003). LINARES et al. (2003) found that solid-state cultures of *Phanerochate flavido-alba* on pine chips reduce the phytotoxic effects in fermented substrates. In our experiments Cartapip97 was used to mitigate the phytotoxins in the substrate. With a dry mass of 1.02 g plant^{-1} (experiment 2) a positive trend for fungi inoculated substrate was shown in comparison to PTS, however the differences were not significant and were not confirmed in 3rd experiment.

However, generally a better yield was recorded in a commercial substrate. Pour-through analyses during the course of the study shows that the substrate solution pH and EC of the PTS including pretreatments were always lower than for PL (Fig. 1). The solution pH of PTS with their pre-treatments 2 d after the potting (DAP) was between 4.02 and 4.76. These low initial ph values may affect the plant growth at the first growth period. This indication should be an object of further investigations. However, during the experiment (14 DAP) the pH increased, so that at the end of the experiment, with exception of PTS-leached, pH values from 5.42 to 6.1 were recorded. These values were similar to solution pH of PL (pH = 5.72). A pH increase during the culture was earlier observed using wood fibre substrates (GRUDA 2005). Wood substrates posses a buffering effect which is positive, since the pH value does not fluctuate as strongly as in substrates without buffering (GRUDA 2005) and are therefore successfully used to neutralize low pH levels of the nutrient solution.

The lower EC of the PTS (<1.75 dS m^{-1}) could be explained with low CEC, high leaching of nutrients during irrigation (WRIGHT and BROWDER 2005) due to a high hydraulic conductivity of wood substrate (GRUDA and SCHNITZLER 2004), and N-immobilization (WRIGHT et al. 2006) due to a large C/N ratio in these materials (GRUDA et al. 2000). Correlation coefficient of r = 0.59 and r = 0.56, respectively for fresh and dry mass of the marigold at the end of presented study, result between the average EC of the solution during the vegetation and plant parameters. The highest EC were recorded for PL, a commercial substrate which shows also the best results for plant growth.

However, due to a combination of pretreatments and fertilizer supply an improvement of plant growth and quality were achieved (Table 3). YAZAKI and NICHOLS (1978) reported that phytotoxicity of bark and sawdust was eliminated more rapidly if the materials were amended with fertilizers. In accordance with these re-

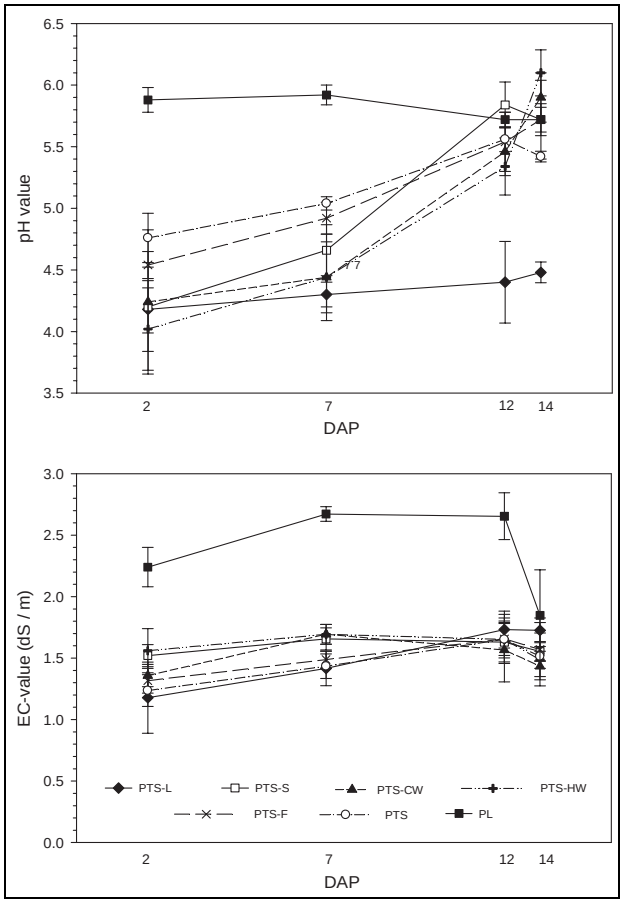


Fig. 1. Substrate solution pH and EC, obtained due to pour through analyses 2, 7, 12 and 14 d after potting (DAP) (PG 3). PG = plant growth experiment, PTS = pine chips, L = PTS after 6x leaching, S = PTS after 2x soaking, CW and HW = cold and hot water, respectively, F = fungi inoculated, PL = peat-lite, a commercial substrate, EC = electric conductivity. Bars indicate the standard errors (SE) of the means for each treatment and measurement day; n = 6.

sults it would be expected, therefore, that the PTS would be established better in containers by an appropriate fertilizing. More research is needed to address fertility issues.

Bioassay with extracts from substrates used in a plant growth experiment

In general, in L6 and PTS14, a positive trend was recorded with regard to germination rate of tomatoes 4 DAS (Table 4). Radicle length tended to be higher in L6 compared to L3 and L1, and not different from PL and DW water extracts. Likewise radicle growth in leachates obtained from PTS in containers without plants, tended to increase from PTS0 to PTS7 and PTS14. No significant differences were showed between PTS14 and either DW or PL (Table 4).

In accordance with results of ORTEGA et al. (1996), the values of the germination index of tomatoes in this bioassay were higher than 60. However, the differences between the treatments were not significant. By taking into consideration of all parameters, a tendency for better results were shown by L6, and PTS14. Both these treatments, L6 and PTS14 (three times a week x 2 weeks), were obtained after a leaching six times. Probably, after 14 d in culture toxins had been leached from containers and were no longer a problem.

In respect to a comparison between laboratory and greenhouse experiments the conclusion as follows could be stated: although it is difficult to extrapolate the results of the bioassays to real growing conditions due to a number of variables involved, and especially interference with nutrients (VAUGHAN and ORD 1990) and different plant species, the performance of marigold plants in leached substrates was similar to bioassay performance.

Some of pretreatments such as leaching and soaking could be recommended being used in the manufacture production process or by growers, in order to reduce the phytotoxicity of PTS.

More research is needed to address fertility issues as well as incubation tests to analyze the N-immobilization in PTS. These results will contribute to the introduction of PTS as a growing medium in horticultural industry.

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