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ABSTRACT

The aim of this investigation was to determine the effect of vermicompost, vermiwash, and phosphate rock on plant, total phenols, flavonoids, and anti-oxidant activity in *Piper auritum* Kunth leaves. *P. auritum* plants were obtained from cuttings and were planted according to the Box-Behnken experimental design with three repetitions at the central point. The factors and levels were vermicompost (10, 20, and 30 g plant^{-1}), vermiwash (5, 10, and 15 mL plant^{-1}), and phosphate rock (1, 2, and 3 g plant^{-1}). Plant growth parameters (plant height, stem diameter, leaves number) and chlorophyll content were measured 1 month after treatment applications. Total phenols, total flavonoids, and 1,1-diphenyl-2-picryl-hydrazyl radical scavenging activity was measured after 4 months. Vermicompost, vermiwash, and phosphate rock had no statistically significant effect on plant growth. Plant height, stem diameter, leaves number, chlorophyll, innermost number, fresh weight stem, fresh weight leaves, fresh weight root, dry weight stem, dry weight leaves, and dry weight root were not different among treatments. Total phenolic compounds were statistically affected for both vermiwash and phosphoric rock ($p < 0.05$) and the anti-oxidant activity decreased by vermicompost addition. The application of 15 mL plant^{-1} vermiwash, 1 g phosphate rock, and 20 g vermicompost plant^{-1} increased the total phenol content.

Introduction

Piper auritum Kunth is a small shrub that grows in the tropical area of Central America; it belongs to the *Piperaceae* family. Depending on the culture and region where it grows, *P. auritum* is commonly referred to as "hoja santa," "yerba santa," "anisillo," "acuyo," or "momo" in Mexico and Central America. In the United States, this herb is commonly referred to as "pepperleaf," "eared pepper," or "root beer plant." *P. aurium* leaves could be a source of phytochemicals, such as phenolic compounds (Conde-Hernández and Guerrero-Beltrán 2014). Many of these chemical compounds having antioxidant activity are used in food products and a number of medical treatments (Li et al. 2009). Some studies have shown that the content of phenolic compounds is higher in organic products, whereas other studies have found similar or lower contents of phenolic compounds in organic products (Huber et al. 2011; Vinkovic et al. 2011). Vermicompost utilized in organic culture can be obtained from different feedstocks (Warman and AngLopez 2010). It has been demonstrated consistently that vermicompost has beneficial effects on plant growth. Vermicompost stimulates mycorrhizal colonization of roots (Gutiérrez-Miceli et al. 2008) and promotes microbial activities, such as phosphate-solubilizing bacteria (PSB) and nitrogen-fixing bacteria (NFB) (Pramanik, Ghosh, and Chung 2010; Yu et al. 2012). Phosphate rock could be used to circumvent phosphorus deficiency; however, their effectiveness depends to a great extent on phosphate-solubilizing microorganisms (PSM) activity (Khan, Zaidi, and Wani 2007). Vermicompost may increase the activity of PSM that convert insoluble phosphate

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to plant available forms. Consequently, vermicompost amended with phosphate rock could stimulate plant growth and metabolites production in *P. auritum*. The demand for natural additives, including antioxidants, has grown worldwide in recent years. This requirement is coupled with the development of the food industry and the introduction of new technologies to meet the people's requirements worldwide. Therefore, it would be worthwhile to find new local sources of natural antioxidants, and try to introduce these new alternatives to the food industry (Conde-Hernández and Guerrero-Beltrán 2014). The aim of this investigation was to study the effect of vermicompost and vermiwash amended with phosphate rock on growth of *Piper auritum* Kunt and on total phenols, flavonoids content, and anti-oxidant activity in leaves.

Materials and Methods

Plant Material

Piper auritum Kunth plants were obtained from cuttings. Plants were cultivated in a nursery for 4 months and treatments were specified according to a three-level-three-factor Box-Behnken design with three repetitions at the central point. The variables and their levels selected were vermicompost (10, 20, and 30 g plant^{-1}), vermiwash (5, 10, and 15 mL plant $^{-1}$), and phosphate rock (1, 2, and 3 g plant^{-1}) (table 1). A total of 15 treatments were obtained and repeated for four times so that a total of 60 plants were used (table 2). Cow manure vermicompost and vermiwash was obtained from the Luanda organic farm located in Ocozocoautla-Villaflores (Chiapas, México). Further details of the vermicomposting process can be found in Gutiérrez-Miceli et al. (2007). The vermicompost had an organic C content of 233 g kg^{-1}, an EC of 8 mS cm^{-1}, a 1.7 humic-to-fulvic acid ratio (HA/FA), a total N content of 11.8 g kg^{-1}, a CEC of 43 cmolc kg^{-1}, and a respiration rate of 152 mg CO_2–C kg^{-1} compost-C day^{-1}. The concentration of NO_3^- was 234 mg kg^{-1}, NO_2^-, 2.17 mg kg^{-1}, and NH_4^+ 9.14 mg kg^{-1}, while the P content was 1.9 mg kg^{-1}. Vermiwash obtained from each vermicomposting bed was collected in 200-L storage tanks and analyzed 28 days after according to Contreras-Blancas et al. (2014). Vermiwash had an EC of 11.6 mS cm^{-1}, pH of 8.9, total nitrogen of 81 mg L^{-1}, total carbon of 1.8 g L^{-1}, inorganic carbon of 0.76 g L^{-1}, a concentration of NO_2^- of 0.44 mg L^{-1}, NO_3^- of 3.85 mg L^{-1}, and NH_4^+ 0.51 mg L^{-1}. Phosphate rock was obtained from 'Calizas Industrializadas de Hidalgo' (Pachuca, Hidalgo, México).

Table 1. Actual and coded levels of the experimental design parameters.

	Levels		
Factors	Low (−1)	Central (0)	High (+1)
(X^1) vermicompost (g plant^{-1})	10	20	30
(X_2) vermiwash (mL plant^{-1})	5	10	15
(X_3) Phosphoric rock (g plant^{-1})	1	2	3

Plant growth parameters (plant height, stem diameter, leaves number) and chlorophyll content were measured 1 month after vermicompost, vermiwash, and phosphate rock applications, while total phenols, total flavonoids, and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity was measured after 4 months. Leaf chlorophyll content was measured with a SPAD-502 meter (Minolta Corporation, Ramsey, NJ, USA) and expressed as SPAD units. Each reported value was the mean average of six readings, i.e., three measurements in each side of a leaf midrib. Leaves were air-dried, ground in an electric mill to 20 mesh (0.84 mm diameter), and analyzed.

Plant Extract Preparation and Phenolic, Flavonoids, and Free Radical Scavenging Activity

Crude extracts were obtained from 1 g dry plant material maceration in 20 mL of methanol, and sonicated at 25°C for 2 h. The extract was centrifuged at 4000 rpm and 4°C for 10 min to remove solids. The methanol supernatant was evaporated under vacuum at 40°C. The residue was re-suspended in 2 mL of methanol and stored at −20°C in the dark until analyzed. Phenolic content was determined with the Folin-Ciocalteau reagent, Total flavonoids content was determined by the aluminum trichloride colorimetric method as described by Chang et al. (2002). The free radical scavenging activity of all of the extracts was determined as described by Shen et al. (2010). Further details of these methods can be found in Luján-Hidalgo et al. (2015).

Statistical Analysis

The effect of treatment was determined by analysis of variance with a significance level of 5% and the mean test was conducted by Media Significant Difference

Table 2. Treatments implemented with a Box-Behnken surface response experimental design and results of plant growth in *Piper auritum* Kunth plants cultivated with vermicompost (VC), vermiwash (VW), and phosphate rock (PR).

	Factors							
	VC	PR	VW	Plant height	Stem diameter	Plant growth parameters		Chlorophyll
Treatment	g plant^{-1}		mL plant^{-1}	cm	mm	Leaves number	Internodes number	SPAD units
1	20	2	10	6.1 bc	10.4 ab	1.9 bc	5.8 a	29.4 a
2	10	2	5	5.3 bc	9.7 ab	1.8 bc	5.5 ab	35.1 a
3	30	2	5	5.2 bc	10.1 ab	2.0 bc	5.3 ab	32.7 a
4	10	2	15	4.0 c	10.6 ab	2.0 bc	5.8 a	29.8 a
5	30	2	15	7.7 ab	6.7 c	3.0 a	4.3 b	29.5 a
6	10	1	10	4.3 c	10.8 ab	3.0 a	5.0 ab	30.4 a
7	30	1	10	6.5 abc	10.9 ab	2.3 abc	5.8 a	34.1 a
8	10	3	10	8.6 a	9.8 ab	2.5 ab	5.8 a	34.4 a
9	30	3	10	7.8 ab	12.1 a	2.0 bc	6.0 a	31.0 a
10	20	1	5	7.3 ab	10.0 ab	2.0 bc	5.6 ab	34.8 a
11	20	1	15	6.9 abc	9.0 bc	2.3 abc	6.0 a	35.1 a
12	20	3	5	5.5 bc	11.7 a	1.5 c	5.0 ab	27.9 a
13	20	3	15	7.3 ab	11.2 ab	2.0 bc	5.3 ab	35.3 a
LSD				2.44	2.07	0.73	1.10	6.4

Note. Design was repeated four times. Data were collected 30 days after plant germination, $n = 10$. Values with the same letter in a column are not significantly affected by treatments.

test. The quality of the fit of the model was expressed by the coefficient of determination R^2 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 result. Nonlinear regression was applied using Statgraphics plus software to plot surface responses. A polynomial equation [equation (1)] was used and R^2 gives the significance of lack of fitting of the regression:

$$y = a + b(\times_1) + c(\times_2) + d(\times_3) + e(\times_1)(\times_2)(\times_3) + f(\times_1)(\times_2) + g(\times_1)(\times_3)) + h\ (\times_2)(\times_3)) + i\ (\times_1^2) + j\ (X_2^2) + k\ (\times_3^2), \quad (1)$$

where y is the response and x_1, x_2, and x_3 are the factors (vermicompost, vermiwash, and phosphate rock, respectively) and a, b, c, d, e, f, g, h, i, j, and k were the parameters to identify.

Results

The plant height varied in the range of 4.0 to 8.6 cm, stem diameter was 6.7 to 12.1 mm, the leaves number in the plant was between 1.5 to 3, the number of internodes was from 4.3 to 6.0, and chlorophyll was 27.9 to 35.3 spad unit in the different treatments (table 2). The fresh weight of stem, leaf, and root was of 6.4 to 12.1, 5.3 to 8.0, and 3.7 to 8.1 g plant^{-1}, respectively. While for the dry weight of stems, leaves and roots were 1.7 to 3.5, 0.6 to 1., and 0.6 to 2.2 g plant^{-1}, respectively (table 3). Vermicompost, vermiwash, and phosphate rock had no statistically significant effect on the parameters of plant growth, such as plant height, stem diameter, leaves number, chlorophyll, innermost number, fresh weight stem, fresh weight leaves, fresh weight root, dry weight stem, dry weight , and dry weight root, whereas the total phenolic compounds (TPC) were affected for both vermiwash and phosphoric rock ($p < 0.05$) (table 4).

The optimal maximum values obtained were: plant height of 8.7 cm, stem diameter of 12.50 mm, with an average number of 2.8 leaves, internodes 5.9, and 37.7 spad units of chlorophyll. Stem, leaves, and root fresh weight were 12.5, 8.6, and 8.2 g plant^{-1}, respectively. Stem, leaves, and root dry weight were 1.4, 3.2, and 1.8 g plant^{-1}, respectively. TPC was 20.5 mg DW plant^{-1}, whereas TFC was 5.2 mg DW plant^{-1} (table 5). These optimal maximum values could be obtained with 30 g plant^{-1} vermicompost and 15 ml plant^{-1} vermiwash; however, phosphate rock promoted a differential effect, higher concentration for plant growth, while a lesser concentration for metabolites production (TPC and TFC) (table 5). The application of 15 mL plant^{-1} vermiwash, 1 g phosphate rock, and 20 g vermicompost plant^{-1} increased the total phenol content with 19.4 mg g^{-1} dry weight compared with 9.9 to the fertilized plants with 3 g phosphate rock (table 3). The highest concentration of flavonoids was 5.6 mg g^{-1} dry weight obtained by

Table 3. Treatments implemented with a surface response experimental design Box-Behnken and results of plant growth and total phenols compounds and total flavonoid compounds in *Piper auritum* Kunth plants cultivated with vermicompost (VC), vermiwash (VW), and phosphate rock (PR).

	Fresh weight[a]			Dry weight[a]				
	g plant^{-1}						mg g^{-1} plant	
Treatment	Stem	Leaves	Root	Stem	Leaves	Root	TPC[b]	TFC[c]
1	11.0 ab	6.4 a	5.7 bc	2.2 ab	0.8 a	1.2 cd	11.7 bc	3.7 c
2	6.4 c	5.3 a	5.8 abcd	2.3 ab	0.9 a	1.5 abc	13.4 b	5.3 ab
3	7.2 c	6.4 a	5.5 bcd	1.8 b	0.8 a	1.4 bcd	12.1 bc	4.1 c
4	8.8 abc	6.8 a	3.7 d	2.1 ab	0.7 a	0.6 d	12.4 bc	3.9 c
5	7.8 abc	7.9 a	5.9 abcd	2.3 ab	1.1 a	1.0 cd	18.2 a	3.8 c
6	8.9 abc	7.1 a	5.3 bcd	2.1 ab	0.9 a	1.0 cd	11.4 bc	3.9 c
7	9.8 abc	7.8 a	4.8 bcd	2.0 ab	0.8 a	1.1 cd	12.7 bc	4.5 bc
8	9.1 abc	7.1 a	5.4 bcd	1.7 b	0.9 a	1.1 cd	13.2 b	4.1 c
9	12.1 a	7.5 a	6.0 abcd	2.5 ab	0.9 a	0.9 cd	9.9 c	3.9 c
10	12.1 a	8.0 a	6.7 ab	2.4 ab	1.0 a	1.3 bcd	17.2 a	4.2 c
11	7.5 bc	8.0 a	4.3 cd	2.2 ab	0.6 a	0.9 cd	19.4 a	5.6 a
12	10.3 abc	6.2 a	6.5 abc	3.5 a	0.9 a	1.9 ab	12.1 bc	3.8 c
13	10.9 abc	7.4 a	8.1 a	3.1 ab	1.2 a	2.2 a	13.5 b	4.3 bc
LSD	3.76	3.77	1.94	1.32	0.53	0.64	2.46	0.86

Note. Design was repeated four time. Data were collected 30 days after plant germination. Values with the same letter in a column are not significantly affected by treatments.
[a]Mean of four replicates.
[b]Total phenolic compounds (TPC) expressed as gallic acid equivalents (GAE mg g^{-1} plant).
[c]Total flavonoids compunds (TFC) expressed as quercetin equivalents (QE mg g^{-1} plant).

applying 1 g plant^{-1} of phosphate rock compared to treatment with 3 g plant^{-1} phosphate rock, which produced 3.9 mg g^{-1} dry weight. The fertilization with vermiwash and phosphate rock had a statistically significant effect on the production of total phenols in the leaves ($p < 0.05$) (table 4). The synthesis of flavonoids and phenolic compounds decreased by application of phosphate rock.

The antioxidant activity values ranged from 3.02 ± 0.45 to 4.86 ± 0.96 g ml^{-1} reported as EC_{50}. Significant differences ($p < 0.05$) were observed for the antioxidant activity in the treatments (table 6). Anti-oxidant activity decreased by vermicompost addition.

Discussion

In our study, the parameters of plant growth were positively influenced by the addition of phosphate rock and vermicompost. Phosphorus influenced photosynthesis, biosynthesis of protein and phospholipids, nucleic acid synthesis, membrane transports, energy transformation, and cell division of the plant system. The greater uptake of nutrients led to increased root and shoot development, nodulation, plant height, branching, and dry matter accumulation. The regulatory function of phosphorus in photosynthesis and carbohydrate metabolism of leaves can be considered to be one of the major factors limiting growth, particularly during the reproductive phase. The favorable influence of phosphorus on plant growth parameters could be attributed to the overall improvement in crop growth as reflected by plant height, number of branches, dry matter accumulation, and root nodulation (Das et al. 2013).

Vermiwash and vermicompost improved the trace element content of the soil. However, the combination of these biofertilizers is more effective in improving soil

Table 4. *p*-values for plant growth parameters and total phenolics compounds and total flavonoids from *Piper auritum* leaves cultivated with vermicompost, vermiwash, and phosphate rock.

	Factors *p*-Values												
	PH	SD	LN	CC	IN	LFW	SFW	RFW	LDW	SDW	RDW	TPC	TFC
Vermicompost	0.2825	0.7319	0.0958	0.7838	0.6446	0.7022	0.1039	0.2105	0.9395	0.3776	0.1341	0.5030	0.7135
Vermiwash	0.6191	0.1204	0.0731	0.5014	0.3796	0.5983	0.2143	0.4487	0.7921	0.4674	0.0662	0.0000	0.0975
Phosphate rock	0.1101	0.0935	0.1675	0.1950	0.9640	0.3907	0.3845	0.0867	0.6687	0.2231	0.0926	0.0042	0.1083

Note. PH, plant height; SD, stem diameter; LN, leaves number; CC, chlorophyll contents; IN, innermost number; LFW, leaves fresh weight; SFW, stem fresh weight; RFW, root fresh weight; LDW, leaves dry weight; SDW, stem dry weight; RDW, root dry weight; TPC, total phenolics compounds; TFC, total flavonoids compounds.

Table 5. Optimal values of vermicompost, vermiwash, and phosphate rock for maximize plant growth parameters, chlorophyll, total phenolic compounds (TPC), and total flavonoids compounds (TFC) in *Piper auritum* Kuhn plants.

	Optimal values		
	g plant^{-1}		mL plant^{-1}
Maximum value for	Vermicompost	Phosphate rock	Vermiwash
Plant height (8.7 cm)	30	3	15
Stem diameter (12.5 mm)	30	3	5
Leaves number (2.8)	30	1	14
Internode number (5.9)	10	3	12
Chlorophyll (37.7 SPAD)	30	1	5
Stem FW (12.5 g plant^{-1})	22	3	12
Leaves FW (8.6 g plant^{-1})	30	1	15
Root FW (8.2 g plant^{-1})	28	3	15
Stem DW (1.4 g plant^{-1})	30	3	15
Leaves DW (3.2 g plant^{-1})	21	3	5
Root DW (1.8 g plant^{-1})	18	3	5
TPC (20.5 mg DW plant^{-1})	30	1	15
TFC (5.2 mg DW plant^{-1})	30	1	15

Note. Fresh weight (FW), Dry weight (DW), Total phenolic compounds (TPC) expressed as gallic acid equivalents (GAE mg g^{-1} plant). Total flavonoids compounds (TFC) expressed as quercetin equivalents (QE mg g^{-1} plant).

micronutrients content. Bio-fertilizers (vermicompost and vermiwash) contribute with macronutrients and micronutrients in the amount that is required by plants (Ansari and Kumar 2010). Applications of organic fertilizers have an emphatic effect on plant growth and production (Tejada and Benítez 2014). The soil enriched with vermicompost provides additional substances that are not found in chemical fertilizers (Ansari and Ismail 2008). The effect of vermiwash and vermicompost on the plant growth may be attributed to the presence of promoters like humic and fulvic acids (Arancon et al. 2006). The optimal total phenols compounds were 20.5 GAE mg g^{-1} dry plant. This value was higher than that reported by Conde-Hernández and Guerrero-Beltrán (2014), who reported that total phenolic compounds on ethanol extracts of *Piper auritum* dry leaves was 8.72 GAE mg g^{-1} dry plant. These differences could be due to the fact that they do not use an organic system and the solvent used to obtain the extract.

Table 6. Vermicompost, vermiwash, and phosphate rock effects on antiradical activity of *Piper auritum* Kunth extracts.

Treatment	Vermicompost g planta^{-1}	Vermiwash mL planta^{-1}	Phosphate rock g planta^{-1}	EC50[a,b] μg mL^{-1}
1	20	10	2	4.86 ± 0.96 b
10	30	10	3	3.02 ± 0.45 a
12	20	15	1	4.22 ± 0.85 ab

[a]Mean of four repetitions.
[b]Values with the same letter in a column are not significantly affected by changes in the concentration of the considered factors.

The type of organic fertilizer evaluated had a statistically significant effect on the production of total phenols. The concentration of phenolic compounds of methanol extracts of *P. auritum* leaves were affected positively by the addition of vermiwash. Lalitha, Fathima, and Ismail (2000) reported that the vermicompost and vermiwash are also enriched in certain metabolites and vitamins that belong to the B group or provitamin D, which also helps to enhance plant growth. Phenolic compounds present in humic and fulvic acids may also be precursors of the synthesis of endogenous plant phenolics. Nardi et al. (2000) found that the humic fractions exhibiting a greater amount of phenolic and carboxylic groups showed the best metabolic effect in *Pinun sylvestris* and *Picea abies*. Supplementation experiments with the amino acid phenylalanine, which is the precursor of all phenylpropanoids including flavonoids, induced resistance of apple to scab by the fungus *Venturia inaequalis* (Leser and Treutter 2004). Taie, El-Mergawi, and Radwan (2008) explained that the use of bioorganic fertilizers help plants to fix nitrogen from air and utilize it to produce phytohormones and other growth-promoting compounds, such as phenols. Konopka et al. (2012) confirmed the higher accumulation of total polyphenols in grain from organic cultivation.

The phosphate rock had a negative effect on phenol total compounds. The increase on the concentration of phosphate rock decreases the synthesis of phenols and flavonoids in the plant. Studies have also indicated that P, B, and Mn deficiency induce flavonoid accumulation (Lillo, Lea, and Ruoff 2007), while Co and Ni supplementation increased flavonoid production and antioxidant enzyme activity (Jayakumar, Bhaskaran, and Tsushima 2007). Phenol accumulation in B and P deficiency has been considered to occur due to increased activity of phenylalanine ammonia lyase, a key enzyme in the phenolic pathway, but also enhanced oxidation of phenols to generate reactive oxygen species, which cause oxidative stress in plants (Lehto, Ruuhola, and Dell 2010).

The accumulation of these various secondary metabolites has been shown to be influenced by interactions between plant genotype (species and variety within species) and environmental factors, including cultivation technique, season, abiotic and biotic stress, and nutrient status (Downey et al. 2013). Flavonoids, such as quercetin and kaempferol are phenolic compounds, which can be synthesized by plants as a

response to the attack of pathogens. The level of phenolic compounds in plants depends on their maturity stage, variety, storage, and genetic factors among others and may be present at different levels in the same plant grown under different soil conditions. Antioxidant phenolic compounds, especially flavonoids, neutralize reactive oxygen species before they can cause damage to cells and the cultivation practice may interfere with the amount of these compounds (Upadhyaya, Khatiwora, and Lakhi 2010).

Moure et al. (2000) pointed out that the antioxidant activity of phenolic compounds may depend on factors, such as growing conditions, quality, and origin (geographical location), of plants as well as the extraction and purification methods (type and polarity of solvents and extraction conditions) used to determine the antioxidant activity (Usaquén-Castro et al. 2006). It has been reported that the antioxidant activity of plants is well correlated with phenolic compounds in some plants (Chizzola, Michitsch, and Franz 2008).

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