

Endophytes of *Lippia citriodora* (Syn. *Aloysia triphylla*) enhance its growth and antioxidant activity

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Abstract Endophytes of medicinal plants are valuable resources for plant growth promotion and lead drug discovery. Lemon verbena, *Lippia citriodora* Kunth. (Verbenaceae), is an ethnomedicinal shrub. Here, the endophytic bacterium *Sphingomonas paucimobilis* and the endophytic fungus *Aspergillus* sp. isolated from *L. citriodora* were used for plant interaction studies. Foliar spraying and soil drenching methods of endophyte's inocula application were used for *in planta* assays. The results showed that both fungal and bacterial endophytes increased the growth parameters of *L. citriodora* including plant height, leaf number, fresh weight and dry weight of shoot, root and leaf. Indeed, soil drenching of *S. paucimobilis* increased the root weight, but its foliar spray increased the plant height. Also, soil drenching of *Aspergillus* sp. increased the leaves dry weight, while its foliar spray increased the number of branches, leaves, and the leaves fresh weight. Soil drenching of either of both endophytes increased

the antioxidant activity of *L. citriodora*'s foliage, but foliar sprays yielded lower increases. Endophytes had no apparent effects on the phenolics and flavonoids at the time of sampling, i.e. 30 days post-inoculation. Our findings indicate the enhancing effects of endophyte application on the growth and antioxidant property of *L. citriodora*.

Keywords Lemon verbena · *Lippia citriodora* · Endophyte · Antioxidant · Growth promotion · *Sphingomonas paucimobilis* · *Aspergillus*

Introduction

The plant genus *Lippia* (Verbenaceae) includes approximately 200 species that are indigenous to South and Central America and Africa (Pascual et al. 2001). Lemon verbena, *Lippia citriodora* H.B.K. (Syn: *Aloysia triphylla*) is a medicinal and ornamental shrub being cultivated mainly due to the lemon-like aroma emitted from its leaves. The main medicinal part of lemon verbena is its leaves which include 0.9%–1.5% of the total essential oil, principal components of which are geranial, neral and limonene (Argyropoulou et al. 2007). Essential oil of lemon verbena is used for perfumery and cosmetic preparations and has antimicrobial and insecticidal activities (Bangou et al. 2012; Funes et al. 2009; Khani et al. 2012; Duarte et al. 2005). The antioxidant activity of lemon verbena's leaf extracts are of high interest in the food industry (Abderrahim et al. 2011; Nemat Shahi et al. 2014)

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Medicinal properties of plants are due to their chemical compositions which are in high demand for pharmaceutical, industrial and agricultural applications (Strobel and Daisy 2003). One of the novel sources of natural bioactive compounds are endophytic microorganisms (Hosseyini Moghaddam et al. 2013; Hosseyini Moghaddam and Soltani 2014a, b; Soltani and Hosseyini Moghaddam 2014, 2015; Soltani et al. 2016; Pakvaz and Soltani 2016). Endophytes are microorganisms that in whole or part of their life cycle colonize the healthy tissues of plants, but do not cause any symptoms of disease (Bacon and White 2000). It is speculated that the majority of endophytes are beneficial to their host plants through synthesizing bioactive compounds inside plants (Owen and Hundley 2004). Such bioactive compounds can be used by plants for defense against pathogens, insects, nematodes and herbivorous animals (Rodriguez et al. 2004). Also, endophytes employ a range of mechanisms to facilitate plant growth (Glick 2015). For example, some endophytes produce plant hormones, thus positively affect plant growth and its tolerance to biotic and abiotic stresses (Hoffman et al. 2013; Rodriguez et al. 2004). Also, endophytic microorganisms increase antioxidants of the host plants, especially under abiotic stress (Baltruschat et al. 2008; Rouhier et al. 2008; Sun et al. 2010).

To the best of our knowledge, there are no studies on the endophytes of *Lippia citriodora* and their possible link to the plant growth promotion and production of beneficial compounds to plants and humans. Therefore, growth improvement and antioxidant activity of lemon verbena in interaction with its endophytic microorganisms are investigated here.

Materials and methods

Plant material and growth condition

One hundred and nine healthy plants of 12 months-old *Lippia citriodora* cv. Nirmal, each of 5 mm diameter and 30 cm length were initially used in this study. The plants were first cultivated in pots and incubated at 27 ± 1 °C under greenhouse condition. In the summer time (July–September) the plants were translocated to an open field, in the

greenhouse location of the Bu-Ali Sina University, Hamedan. The potting mixture included soil: rotted manure (2:1) and was sterilized for 3 h at 121 °C. The plants were irrigated every 5 days by ca. 1 l water.

Endophytic microorganisms

To recover endophytic fungi and bacteria from lemon verbena, leaves, stems and roots of ten randomly selected plants (out of 109) were surface sterilized according to de Siqueira et al. 2011 and da Silva et al. 2013. Subsequently, the samples were cut into 0.5 cm² pieces and aseptically transferred to Petri plates containing Potato Dextrose Agar (PDA; Merck Millipore, Germany) for the growth of fungi, and Nutrient Agar (NA; Merck Millipore, Germany) for the growth of bacteria. The 48 emerged colonies of fungi and bacteria were purified and pre-identified by microscopy (fungi) and Gram test (bacteria) (Supplementary Table 1). The identity of *Sphingomonas paucimobilis* was confirmed using DNA extraction and 16S rDNA sequencing (data not shown).

Using an antifungal assay against the phytopathogens *Botrytis cinerea* and *Rhizoctonia* sp. (data not shown), two superior isolates out of 48 endophytes, i.e. the bacterium *Sphingomonas paucimobilis* B10 (isolated from lemon verbena's roots) and the fungus *Aspergillus* sp. F14 (isolated from lemon verbena's stems) were selected for plant interaction assays.

Experimental design

A total of 96 fully flourished 27 months-old plants were employed for plant-endophyte interaction assays. Based on endophyte inocula treatment methods, i.e. foliar spraying and soil drenching, two groups of 48 plants were considered. In each group, plants were divided to four subgroups each containing 12 plants for the designed treatments which consisted of the control (no treatment), fungal endophyte application, bacterial endophyte application, and finally a combination of fungal and bacterial endophytes application. A factorial experiment in a completely randomized design with three replications was conducted to investigate growth improvement and antioxidant activity of lemon verbena in interaction with its endophytic microorganisms. Each unit consisted of four pots, each with a one-year old plant.

*In planta assays**Endophyte inoculum preparation*

Inocula of endophytic fungus and bacterium were prepared according to Tiwari et al. (2013) with some minor modifications. Briefly, bacteria were grown in Nutrient Broth (NB; Merck Millipore, Germany), at 25 °C for 48 h at 200 rpm. The bacterial cells were harvested by centrifuging at 4000 rpm for 6 min, and diluted to a CFU of $\sim 10^8$ mL⁻¹. The fungal endophyte was grown on PDA at 28 °C for 7 days. The fungal conidia were harvested by sterile distilled water, and diluted to a CFU of 1×10^6 mL⁻¹.

Plant inoculation methods

The plants were inoculated at the evening of the 1st August, 2013, at the age of 27 months-old. Two methods, i.e. foliar spraying and soil drenching by CFUs suspensions of each endophyte, were used for plant inoculations. For foliar tissue spraying, each *L. citriodora* plant was completely sprayed by an aliquot of 15 mL CFUs suspension from each endophytic microorganism. For soil drenching, 30 mL aliquots of the CFUs suspensions were poured onto the soil surrounding the crown of the plant. Sterilized distilled water was applied as the control. The morphometric analyses were performed 30 days post-inoculation.

Establishment of endophytic microorganisms in inoculated plants

To ensure the establishment of endophytes in plants, tissue samples (root, crown, stem and leaf) from eight indicative plants according to the experimental design were collected, 20 days post-inoculation. Isolation of endophytic microorganisms was performed according to the same references (de Siqueira et al. 2011, da Silva et al. 2013). The identities of the recovered fungus and the bacterium were confirmed as mentioned.

*Morphometric analyses**Growth parameter measurements*

Growth parameters of the inoculated plants were measured before and after 30 days of inoculations. Plant

height, leaf number, leaf surface area, number of branches per plant, fresh and dry weights of leaves, stems and roots were measured. Leaf images were analyzed by Image J software.

Determination of total phenolics of the leaves

The total soluble phenolic content of the leaves was determined using the Folin-Ciocalteu (F-C) colorimetric assay (McDonald et al. 2001). Gallic acid was used as the phenolic standard. Two mL sodium carbonate (1 M) was added to 250 μ L of the leaves methanol extract, and the mixture was vortexed briefly. Subsequently, 2.5 mL F-C reagent (10%) was added, and the mixture was left in a dark place at room temperature for 15 min. The absorbance of the solution was measured at 765 nm. Gallic acid (1.0 mg mL⁻¹) was used as the standard. Using the Gallic acid standard curve and taking into account the dilution ratio, total phenolic content was measured and expressed as mg Gallic acid per g of the dry weight.

Determination of total flavonoid content of the leaves

The total flavonoids of the leaves was determined using colorimetry according to Chang et al. 2002. Briefly, 100 μ L of 10% aluminum chloride and 100 μ L of 1 M potassium acetate were mixed. Subsequently, 2.8 mL distilled water and 500 μ L of leaves methanol extract were added to the mixture and mixed well. The solution was incubated at room temperature for 30 min. The absorbance of reaction mixture was measured at 415 nm. Quercetin was used to calculate the standard curve. A concentration vs. absorbance curve was plotted and the slope value was obtained. The total flavonoid content was expressed as mg Quercetin per gr of the dry weight.

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay

Sample leaf extracts at different concentrations (1, 2, 4, 6, 8 and 1 V/V) were used to determine the ability to scavenge DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radicals according to Stojichevich et al. 2008. Thus, 5 mL methanol (85%) was added to 0.05 g of leaf and shook 24 h to a complete dissolution. The absorbance of different concentrations of the obtained methanol extracts (as blanks) were measured at 517 nm. Then, a

2.5 mL aliquot from each extract (blank) was mixed with 1 mL of DPPH (0.0029 g / 25 mL of 85% methanol) and incubated for 15 min at room temperature in darkness. The absorbance of each sample was then measured at 517 nm. As the control, a mixture of 1 mL methanol (85%) and 2.5 mL DPPH was used. The Antioxidant activity was determined as the inhibition (%) of free radical generation by the sample, and calculated using the formula:

DPPH radical scavenging (%)

$$= [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100.$$

A_{blank} was the absorbance of the blank, and A_{sample} was the absorbance of the sample extract at 517 nm.

Statistical analyses

Data were subjected to analyses of variance. Where the main effects were significant ($P < 0.05$), differences among means were evaluated by Duncan's multiple range test using the SAS software.

Results and discussion

Several studies have shown the plant growth promotion activities (Marks and Clay 1990; Varma et al. 1999; Redman et al. 2001) and *in planta* antioxidant enhancement of endophytes (Baltruschat et al. 2008; Rouhier et al. 2008; Sun et al. 2010). Currently, there is no evidence on endophytic communities of *L. citriodora* or their effects on plant growth and biochemical contents. Here, we isolated and screened the antifungal activity of 48 endophytes of *L. citriodora* (data not published) and analyzed the effect of their application on plant growth and phenolic, flavonoid and antioxidant contents.

Establishment of endophytic microorganisms in inoculated *Lippia citriodora* plants

Twenty days after inoculation the random samplings of the infected plants were performed. Recovery methods isolated the endophytic fungus and bacterium from the infected tissues. The microscopy and molecular means confirmed the identity of the recovered microorganisms. Although the colonization rate or percentage of endophyte presence were not investigated, the observed

phenomena, in comparison to the controls, could be attributed to the applied endophytes.

Growth parameters of endophyte infected *L. citriodora*

Effect of endophyte application

In total, endophyte application promoted lemon verbena's above- and/or under-ground growth. Application of either *Aspergillus* sp. or *S. paucimobilis*, by soil drenching or by foliar spray, affected plant height, the number of branches, the number of leaves, leaves fresh and dry weights, aerial fresh weight vs. root fresh weight and leaves fresh weight vs. total fresh weight at $P < 0.01$ (Table 1). Aerial dry weight vs. root dry weight was affected at $P < 0.05$. However, neither leaf area nor leaves dry weight vs. total dry weight were affected.

Effect of plant inoculation method

Data indicate that endophyte application, either by foliar spraying or soil drenching, promoted plant health. Moreover, the choice of endophyte application method affected the plant height, the number of branches and aerial fresh weight vs. root fresh weight at $P < 0.01$ (Table 1). Also, leaves fresh weight, root fresh and dry weights, aerial dry weight vs. root dry weight, and leaves fresh weight vs. total fresh weight were affected at $P < 0.05$. However, the number of leaves, leaf area, leaves dry weight and leaves dry weight vs. total dry weight were not affected.

Effect of interaction between endophyte and inoculation method

The number of branches and leaves ($P < 0.01$) and leaf area ($P < 0.05$) were influenced by the interaction between endophyte and inoculation method (Table 1).

Lippia citriodora's growth enhancement by endophyte application

Tables 2 and 3 represent the comparisons among the averages data obtained for the effect of endophytes and inoculation methods on the growth parameters of *L. citriodora*.

As seen in total, application of *Aspergillus* sp. or *S. paucimobilis*, either by soil drenching or foliar spray, increased plant height, the number of leaves, leaves fresh and dry weights, and roots fresh and dry weight

Table 1 Analysis of variance of endophytic inoculation effects on growth parameters of *Lippia citriodora* plants

Sources of changes	Endophytes inoculation	Method of inoculation	Interaction between treatments	Experimental error	Coefficient of variation (CV.)
Parameters					
Plant height (mm)	19,494.56 **	17,753.44 **	1450.99 ns	993.73	14.53
Number of branches	33.46 **	227.38 **	10.76 **	0.47	12.02
Number of leaves	15,875.31 **	1249.29 ns	2478.17 *	516.48	16.29
Leaf area (mm ²)	1087.96 ns	911.66 ns	1436.59 *	408.36	14.08
Leaves fresh weight (g)	45.26 **	6.28 *	1.53 ns	1.00	5.16
Leaves dry weight (g)	20.13 **	1.07 ns	1.42 ns	0.81	7.97
Root fresh weight (g)	34.26 **	17.58 *	4.78 ns	2.40	12.41
Root dry weight (g)	8.25 **	2.77 *	0.08 ns	0.49	12.96
Aerial fresh weight/ Root fresh weight	1.51 **	2.07 **	0.31 ns	0.15	11.97
Aerial dry weight/ root dry weight	1.30 *	2.36 *	0.14 ns	0.39	13.95
Leaves fresh weight/total fresh weight	0.0032 **	0.0021 *	0.0012 ns	0.0004	5.11
Leaves dry weight/total dry weight	0.0030 ns	0.0002 ns	0.0008 ns	0.0014	9.59

ns not significantly different, * significantly different at P 5% level, ** significantly different at P 1% level

(Tables 1, 2 & 3). In this respect, the most significant effects of *Aspergillus* sp. were the increases in the number of leaves and root fresh weight by foliar spray (Table 1). The most significant effects of *S. paucimobilis* were the increases in plant height, and the number of leaves by soil drenching; and root dry weight by foliar spray (Tables 1, 2 & 3).

Also, *Aspergillus* sp. application either by soil drenching or foliar spray, increased the number of plant branches, but application of *S. paucimobilis* had no (by soil drenching) or negative effect (by foliar spray) on this parameter. Co-application of *Aspergillus* × *Sphingomonas* further decreased the number of plant branches (Tables 1, 2 & 3).

Table 2 Comparison of the averages for the effect of endophytes and “foliar spraying” inoculation method on the growth parameters of *Lippia citriodora* (cv. Nirmal) plants

Parameters	Treatments				A × S*	Control	
	<i>Aspergillus</i> sp.		<i>Sphingomonas paucimobilis</i>				
Height (mm)	176.33 cd	28.94%	238.50 b	74.40%	207.33 bc	51.61%	136.75 ^d
Number of branches	13.12 a	26.27%	6.17 ^c	−40.61%	5.37 ^c	−48.31%	10.39 b
Number of leaves	248.00 a	175.83%	130.63 cd	45.28%	118.25 cd	31.52%	89.91 ^d
Leaves fresh weight (g)	23.3450 a	33.03%	20.1500 b	14.82%	18.5633 bc	5.78%	17.5483 cd
Leaves dry weight (g)	12.7100 ab	48.22%	11.9133 ab	38.93%	11.0733 b	29.13%	8.5750 c
Root fresh weight (g)	13.195 b	60.32%	13.250 b	60.99%	11.775 bc	43.07%	8.230 ^d
Root dry weight (g)	4.9533 cd	39.52%	6.4500 ab	81.69%	5.3133 bcd	49.67%	3.5500 e
Aerial fresh weight/ Root fresh weight	3.5967 ab	−13.72%	3.1877 bc	−23.53%	3.1197 bc	−25.16%	4.1690 a
Aerial dry weight/ root dry weight	5.0753 ab	−4.28%	4.1863 abc	−21.04%	4.5653 abc	−13.89%	5.3023 a
Leaves fresh weight/total fresh weight	0.3891 ab	−6.24%	0.3682 b	−11.27%	0.3826 ab	−7.80%	0.4150 a

Values denoted by same letter are not significantly different at P 5% level

* A × S means the co-application of fungal and bacterial endophytes

Table 3 Comparison of the averages for the effect of endophytes and “soil drenching” inoculation methods on the growth parameters of *Lippia citriodora* (cv. Nirmal) plants

Parameters	Treatments						
	<i>Aspergillus</i> sp.		<i>Sphingomonas paucimobilis</i>		A × S*		Control
Height (mm)	239.00 ^b	51.98%	331.75 ^a	110.96%	248.50 ^b	58.02%	157.25 ^{cd}
Number of branches	4.06 ^d	54.96%	2.62 ^e	0%	1.12 ^f	−57.25%	2.62 ^e
Number of leaves	173.25 ^b	95.63%	143.75 ^{bc}	62.31%	123.50 ^{cd}	39.453%	88.56 ^d
Leaves fresh weight (g)	23.23 ^a	43.30%	17.8400 ^{cd}	10.05%	18.2350 ^c	12.49%	16.2100 ^d
Leaves dry weight (g)	12.90 ^a	48.78%	11.4933 ^{ab}	32.56%	12.9000 ^a	48.78%	8.6700 ^c
Root fresh weight (g)	12.44 ^{bc}	21.30%	16.783 ^a	63.65%	13.810 ^b	34.66%	10.255 ^{cd}
Root dry weight (g)	5.6733 ^{abc}	36.15%	6.8700 ^a	64.87%	6.2767 ^{ab}	50.63%	4.1667 ^{de}
Aerial fresh weight/ Root fresh weight	3.6320 ^{ab}	12.59%	2.2757 ^d	−29.45%	2.5887 ^{cd}	−19.74%	3.2257 ^{bc}
Aerial dry weight/ root dry weight	4.7433 ^{abc}	11.63%	3.6030 ^c	−15.20%	4.0237 ^{bc}	−5.30%	4.2490 ^{abc}
Leaves fresh weight/total fresh weight	0.4054 ^{ab}	8.25%	0.3256 ^c	−13.05%	0.3748 ^b	0.08%	0.3745 ^b

Values denoted by same letter are not significantly different at P 5% level

* A × S means the co-application of fungal and bacterial endophytes

However, aerial fresh weight vs. root fresh weight, aerial dry weight vs. root dry weight, and leaves fresh weight vs. total fresh weight were increased by soil drenching of *Aspergillus* sp., but were decreased by its foliar spray (Tables 2 and 3). Application of *S. paucimobilis* decreased those parameters in total, and co-application of *Aspergillus* × *Sphingomonas* showed almost a similar pattern (Tables 2 and 3).

As seen in Table 2, the maximum increase in the number of branches (26%), and the number of leaves (176%) (i.e. Statistical A category) were achieved by foliar spray of *Aspergillus* sp. conidia. Plant colonization by fungal endophytes depends on several factors, including tissue type (Hardoim et al. 2015). *Aspergillus* sp. was initially isolated from the *L. citriodora* stem. Thus, it seems that it was more capable of foliage growth promotion in lemon verbena. However, as seen in Table 3, the maximum increase in leaves dry weight (50%), and leaves fresh weight (43.3%) (i.e. Statistical A category) were obtained by soil drenching of *Aspergillus* sp. conidia. It was shown in other studies that the endophytic fungi *Piriformospora indica* (Waller et al. 2005), *Acremonium strictum* (Hol et al. 2007) and *Stagonospora* spp. (Ernst et al. 2003) were able to promote the plant growth. Moreover, the ascomycetous endophyte PGP-HSF isolated from *Mentha piperita* increased plant height, leaf dry matter and root dry matter when applied on the host plant (Mucciarelli et al. 2003). In addition, the endophytic bacterium

Pseudomonas sp. increased the plant growth by a dense colonization of the endorhizosphere (van Peer and Schippers, 1989), and *Sporosarcina aquimarina* isolated from the mangrove plant *Avicennia marina* promoted the growth of inoculated plants (Janarthine and Eganathan 2012). Thus, our findings are in accordance with such similar findings on other host plants showing the growth-promoting effects of endophyte application on plants.

Furthermore, as seen in Table 3, the maximum increase in plant height (111%), root fresh weight (64%), and root dry weight (65%) (i.e. Statistical A category) were achieved by soil drenching of *S. paucimobilis*. The bacterium *S. paucimobilis* has been found in various environments, including terrestrial and aqueous habitats, plant rhizosphere, and clinical specimens (White et al. 1996). Here, *S. paucimobilis* was initially isolated from the internal tissues of *L. citriodora*'s roots. This plant root inhabitation trait may explain its significant root growth promotion activity. Several other root and foliage colonizing endophytic bacteria have shown plant growth promotion activities (Aswathy et al. 2013; Janarthine and Eganathan 2012; Kim et al. 2012; Luo et al. 2012; Sun et al. 2009; Tiwari et al. 2013). Increasing evidence suggests that endophytic bacteria produce a wide range of described and undescribed metabolites, e.g. plant hormones and their analogues, and a scintillating array of secondary metabolites which promote plant health (reviewed in Brader et al. 2014). Also, it is shown that the endophytic

bacteria colonize plant vascular systems as the main transport system to colonize plant tissues (James et al. 2002). Although, the involvement of this phenomena in our *in planta* assays are yet to be discovered, our *in planta* finding is in accordance with similar findings and suggests the potential applications of endophytes in growth promotion of *L. citriodora*.

Despite the above described effects of endophyte application on *L. citriodora*, it should be noted that, compared to the control treatments, endophyte application decreased the ratios of aerial fresh weight vs. root fresh weight, aerial dry weight vs. root dry weight, and leaves fresh weight vs. total fresh weight. This may be due to an increase in root system growth and also non-leaves organ growth, which is in total a good phenomenon for plant's health.

Endophytes enter leaves, stems and roots through stomata (Roos and Hattingh 1983), lenticels (Scott et al. 1996) and germinating radicles (Gagné et al. 1987). Endophytes commonly enter into plant root tissues through the cracks and wounds occurring during the plant growth (Agarwal and Shende 1987; Sprent and de Faria 1998; Sørensen and Sessitsch 2015). Plant metabolite leakage at these sites attracts endophytes (Hallmann et al. 1997). Differences seen in the effects of inoculum application methods on plant growth parameters could be partly attributed to the mode of entry of endophytes into plant tissues.

Effect of endophyte application on biochemical parameters of *L. citriodora*

Analysis of endophyte's effect on biochemical parameters

Data represented in Tables 4 and 5 indicate that endophytes, inoculation method, and interaction of them exerted significant effects ($P < 0.01$) on antioxidant

activity of *L. citriodora* leaf extracts (Table 3), but had no apparent effects on the content of phenolics and flavonoids at the time of sampling, i.e. 30 days post-inoculation (Table 5).

Increase in antioxidant activity by endophyte application

Table 5 represents the comparisons of the averages for the effect of endophytes on biochemical parameters of *L. citriodora*. As seen, the inoculated *L. citriodora* plants showed higher antioxidant activity than the controls. Soil drenching of either of both endophytes increased the antioxidant activity of *L. citriodora*, but foliar sprays resulted in less increases. Application of *S. paucimobilis* increased the antioxidant activity of leaf extracts up to 86 and 22% using soil drenching and foliar spray assays, respectively. Also, application of *Aspergillus* sp. conidia increased the antioxidant activity of leaf extracts up to 78 and 21% using soil drenching and foliar spray assays, respectively. Different studies have indicated an increase in plant's antioxidant activity upon endophyte application. Indeed, it was shown that root colonization of Chinese cabbage (*Brassica campestris* L. ssp. *Chinensis*) by the endophytic fungus *Piriformospora indica* stimulates antioxidant enzyme activities in the leaves (Sun et al. 2010). Moreover, root colonization of barely and rice plants by *P. indica* increased plants growth and their antioxidant activities under salinity stress (Bagheri et al. 2013; Baltruschat et al. 2008). Also, the endophytic bacterium *Pseudomonas psychrotolerans* TPs-04 isolated from tomato increased the plant growth and its antioxidant activity under chilling stress (Chen et al. 2014). It was suggested that enhanced antioxidant production by endophyte colonized plants may be the result of the production of reactive oxygen species by the plants or endophytes (White and Torres 2010). Our findings are in accordance with such findings highlighting the role of endophytes in

Table 4 Analysis of variance of endophytic inoculation effects on biochemical parameters of *Lippia citriodora* plants

Sources of changes	Endophytes inoculation	Method of inoculation	Interaction between treatments	Experimental error	Coefficient of variation (CV.)
Parameters					
Total phenolic content	0.09 ^{ns}	0.02 ^{ns}	0.36 ^{ns}	0.34	7.14
Total flavonoid content	0.0001 ^{ns}	0.0005 ^{ns}	0.0004 ^{ns}	0.0002	5.40
Antioxidant activity	186.12 ^{**}	45.22 ^{**}	37.71 ^{**}	2.16	4.38

^{ns} not significantly different, * significantly different at P 5% level, ** significantly different at P 1% level

Table 5 Comparison of the averages for the effect of endophytes on biochemical parameters of *Lippia citriodora* plants

Treatments	<i>Aspergillus</i> sp.		<i>Sphingomonas paucimobilis</i>		A × S*		Control	
	drenching	spray	drenching	spray	drenching	spray	drenching	spray
Parameters								
Antioxidant activity ^a	36.86 ^a	37.30 ^a	38.62 ^a	37.53 ^a	32.74 ^{bc}	34.29 ^b	20.75 ^d	30.84 ^c
Total phenolic ^b	8.46 ^a	7.96 ^a	8.28 ^a	8.32 ^a	7.70 ^a	8.40 ^a	8.09 ^a	8.05 ^a
Total flavonoid ^c	0.26 ^a	0.29 ^a	0.27 ^a	0.29 ^a	0.28 ^a	0.28 ^a	0.29 ^a	0.28 ^a

- Values denoted by the same letters are not significantly different at P 5% level

- * A × S is a mixture of fungal and bacterial endophytes inoculants

- a: Antioxidant activity is expressed as DPPH radical scavenging (%)

- b: The total flavonoid content is expressed as mg quercetin per gr of the dry weight

- c: Total phenolic content is expressed as mg Gallic acid per gr of the dry weight

enhancing plant antioxidant activity. However, the finding that soil drenching of endophytes was more enhancing than foliar spray on antioxidant activity needs to be investigated at the cellular and molecular levels, studying the modes of root and foliage colonization and patterns of induced gene expression by those endophytes.

Moreover, the phenolic and flavonoid contents of the endophyte-infected plants were not enhanced at the time of tissue sampling i.e. 30 days post-inoculation. These plant compounds are or could be part of the plant's chemical defense against invading pathogens. Our findings may implicate the symbiotic lifestyle of the endophytes in that they do not trigger plant host's defense mechanisms. Alternatively, earlier times of sampling e.g. 1–7 week after inoculation may affect the content of such plant compounds. This needs to be investigated in future experiments.

Taking altogether, here, we show for the first time that the bioactive endophytic bacteria and fungi isolated from *L. citriodora* could enhance its growth and antioxidant activity. Plant growth promotion activities of such endophytes could find application in agriculture especially in ethnomedicinal plant farming. Moreover, because of a high demand for leaves extracts with antioxidant activity (Abderrahim et al. 2011; Nemat Shahi et al. 2014), the antioxidant increasing activity of such endophytes would be of high value in ethnomedicine. However, the molecular and physiological bases behind the observed phenotypes remains to be investigated.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

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