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Effect of endophytic fungus *Serendipita indica* and vermicompost water extraction plant growth and development of *Stevia rebaudiana* Bertoni

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ABSTRACT

Because it generates the zero-calorie sweeteners steviol glycosides, *Stevia rebaudiana* is a valuable medicinal plant that offers herbal care for diabetic patients. The effects of applying vermicompost water extract and inoculating *S. rebaudiana* with *Serendipita indica* on the growth of the plant were investigated in this study using an in vitro experimental design that was completely randomized. The application of vermicompost water extract and inoculation with S. indica had a significant impact on the majority of the plant's growth parameters, as indicated by the results. *S. rebaudiana* inoculated with S. indica exhibited the greatest root length, chlorophyll content, and dry weight of aerial parts. Additionally, when inoculated with vermicompost water extract, *S. rebaudiana* demonstrated the maximum chlorophyll content and the ratio of fresh weight of roots to fresh weight of aerial parts. Large-scale application of the findings from this study is possible in the tissue culture of medicinal plants. Conversely, in sustainable agriculture, the utilization of water extract vermicompost and S. indica fungus may lead to enhanced vegetative products and, consequently, secondary metabolites in medicinal plants.

Highlights

- The study employed a completely randomized in vitro experimental design to investigate the effects of vermicompost water extract and inoculation with *Serendipita indica*.
- Results showed a significant improvement in most growth parameters of *S. rebaudiana* when treated with vermicompost water extract and inoculated with S. indica.
- Plants inoculated with S. indica had increased root length, higher chlorophyll content, and greater dry weight of aerial parts.
- The maximum chlorophyll content and an improved ratio of fresh weight of roots to aerial parts were observed in plants treated with vermicompost water extract.
- The study suggests that these treatments could be scaled for large-scale tissue culture of medicinal plants and could enhance vegetative growth and secondary metabolites in sustainable agriculture.

1. Introduction

Stevia rebaudiana Bertoni is a perennial sweet herb that belongs to the Asteraceae family. It produces zero- calories of diterpene glycoside in its leaves, which can be used as a substitute for sucrose around the world (Kalpana et al., 2009). It has been demonstrated that the compounds obtained from the leaves of *S. rebaudiana* have no side effects on humans and can be used by both healthy people and diabetic patients (Kalpana et al., 2009). The major limiting factor for large -scale cultivation of *S. rebaudiana* is its low seed germination percentage. Nowadays, the use of *in vitro* culture techniques is an appropriate method for increasing plant production in a shorter timeframe (Debnath, 2008). The micropropagation method can mediate rapid multiplication of plants, and it may overcome many of the limitations associated with conventional

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methods, such as the instability of the produced plantlets through stem cutting plantation (Kumar et al., 2014).

Serendipita indica is an endophytic fungus that belongs to the order Sebacinales (Varma et al., 2014). The trait of being axenically cultivable with a wide range of hosts opened a new vision of a symbiotic relationship between *S. indica* and plants. *S. indica* can be cultivated *in vitro* under controlled conditions for studying its potential effects on the plant's morphogenesis and on the production of secondary metabolites. The biotechnological application of *S. indica,* by using cultivable mycelium, stimulated the host plant's growth (Kari Dolatabadi et al., 2011b). In a study, inoculation of barley roots (*Hordeum vulgare* L.) with *S. indica* enhanced biomass (Deshmukh et al., 2006). Furthermore, it has been demonstrated that inoculation of *Coleus forskohlii*'s roots with *S. indica* induced more aerial biomass, chlorophyll contents, and phosphorus absorption (Das et al., 2012). Organic fertilizers, especially vermicompost, can improve soil properties and essential oil yield (Heidarzadeh et al., 2021). Vermicompost is a process of degradation and detoxification of organic matter through the interactions of earthworms with other microorganisms, which eventually becomes the required product for agricultural purposes (Sartaj et al., 2019). As an organic fertilizer, it contains nutrients in the available forms and has some characteristics such as higher porosity, water holding capacity, aeration, and drainage, which may affect plants's growth (Arancon et al., 2004; Nadi et al., 2011).

Considering the medicinal importance of *S. rebaudiana*, the present study was designed to investigate the effects of *S. indica* and vermicompost water extracts on the morphological traits of *S. rebaudiana,* which were not well investigated previously, especially in *in vitro* culture.

2. Materials and methods

The effects of vermicompost water extract and *Serendipita indica* on *the* morphological traits of *Stevia rebaudiana* were investigated in an *in vitro* culture experiment in a completely randomized design with three replications. The experiment was conducted at the Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran, in 2018.

2.1. Plant material

Tissue cultures of plants were bought from the Sari Khazar Abad institute, Mazandaran, Iran, and placed in a growth chamber at 25 ºC under cool white fluorescent lamps in a 16L/8D photoperiod regime.

2.2. Fungal culture (*Serendipita indica*)

For preparing the fungal culture, 48 g of agar malt extract was brought to a volume of 1 liter, autoclaved at 121 ºC for 20 minutes, and poured into the sterile Petri Dishes. After 48 h, the dishes were inoculated with 5mm mycelia disks of *S. indica* from 10 days old agar under quite sterile conditions. Plates were incubated at 25±1 ºC for 10 days for the multiplying and re-growing of spores, so dense mycelia suspensions were generated. Then, the fungal culture was used in the *in vitro* culture experiment.

2.3. Vermicompost water extract preparation

Vermicompost was prepared by Professor Mohammadi Goltapeh on the campus of Tarbiat Modares University from poultry manure and *Eisenia fetida,* the earthworm species. In order to prepare the vermicompost water extract, 1 kg of vermicompost was dissolved in 1 liter of water and kept in stable condition for 24 hours. Then, the solution was filtered with muslin fabric.

2.4. *In vitro* plant inoculation

In this study, three different treatments, including vermicompost water extract, *S. indica*, and control, were used. The vermicompost water extract was injected near the plants' roots using a syringe. For the treatments of *S. indica,* 30 days -old established micropropagated plants in transparent glass culture were inoculated at the center with a 5 mm -diameter mycelia disc of 10 days -old *S. indica* culture. An effort was made to keep the root system in direct contact with the inoculum materials. The control plants were not treated. Sixty days after inoculation, the micropropagated plants grown in glass bottles were removed from the bed and analyzed for the growth parameters of *S. rebaudiana.*

2.5. Statistical analysis

Data analysis was performed by SAS 9.4 software. The Least Significant Differences (LSD) test was applied for the means comparison at $P< 0.05$. Two orthogonal contrasts were determined, including contrast 1: control versus treatments (*S. indica* inoculation and vermicompost water extract application) and contrast 2: *S. indica* inoculation versus vermicompost water extract application.

3. Results

The influence of vermicompost water extract application and *S. indica* inoculation on the morphology of the micropropagated plants in the *in vitro* cultures were assessed 60 days after inoculation. Analysis of variance showed that except plant height, other traits: root length, chlorophyll content (a+b), aerial parts' fresh weight (APFW), aerial parts' dry weight (APDW), root fresh weight (RFW), root dry weight (RDW), and the ratio of root fresh weight/aerial parts' fresh weight (RFW/APFW) were significantly affected by the tested treatments (Table 1).

The orthogonal contrasts showed that all indices except plant height, RFW, and RDW were significantly different in control versus treatments (*S. indica* inoculation and vermicompost water extract application) (Table 1). In addition, comparison between *S. indica* and vermicompost water extract treatments showed significant differences in all indices except plant height and the ratio of RFW/APFW (Table 1).

Based on the results, the highest root length (7.10 cm) was recorded in plants inoculated with *S. indica*. The chlorophyll contents were highest in both vermicompost water extract and *S. indica* treatments and lowest in control plants (Table 2). The aerial parts fresh weight was highest in control. Furthermore, the highest aerial parts' dry weights was obtained in *S.indica* treatment, The root fresh and dry weights ranged from 1.27 to 1.69 g and 0.07 to 0.11 g, respectively on different treatments, which the highest values of these parameters were obtained in control plants (Table 2).The results of this study showed that application of vermicompost water extract induced the highest ratio of RFW/APFW (Table 2).

Table 1. Analysis of variance and orthogonal contrasts for plant growth parameters in *Stevia rebaudiana* in the in vitro culture experiment

S.O.V	df	Mean square							
		Plant height (cm)	Root length (cm)	Chlorophyll content (a+b) ($\mu g.ml^{-1}$)	APFW*** (g)	APDW*** (g)	RFW***(g)	RDW*** (g)	RFW / APFW (g)
Treatments	2	3.29^{ns}	4.82^{**}	74.39^{**}	1.43^{**}	0.09^{**}	0.13^{**}	0.0015^{**}	0.026^{**}
Contrast 1	1	1.32^{ns}	5.15^{**}	27.07^{**}	2.03^{**}	0.016^{**}	0.004^{ns}	0.00002^{ns}	0.05^{**}
Contrast 2	1	5.26^{ns}	4.49^{**}	121.72^{**}	0.84^{**}	0.16^{**}	0.26^{**}	0.002^{**}	0.001^{ns}
Error	6	1.09	0.055	1.76	0.01	0.0003	0.004	0.00003	0.0004
Total	8								
CV		4.88	4.14	4.90	4.56	3.96	4.70	6.40	4.47

ns: non significance,* : significant at $P< 0.05$ and,** : significant at $P< 0.01$

***APFW: Aerial Parts' Fresh Weight; APDW: Aerial Parts' Dry Weight; RFW: Root Fresh Weight and RDW: Root Dry Weight

Table 2. Growth parameters (mean ± SE) of *Stevia rebaudiana* treated with vermicompost water extract and *Serendipita indica*

Treatments	Plant height (cm)	Root length (cm)	Chlorophyll content (a+b) ($\mu g.ml^{-1}$)	APFW* (g)	APDW* (g)	RFW*(g)	RDW* (g)	RFW / APFW (g)
Vermicompost water extract	22.00 ± 0.76^{a}	4.63 ± 0.07^{c}	29.50 ± 0.88^{a}	2.39 ± 0.03^{c}	0.41 ± 0.008^{b}	1.43 ± 0.03^{b}	0.09 ± 0.005^{b}	0.59 ± 0.02^{a}
S. indica	20.25 ± 0.43^{a}	7.10 ± 0.06^{a}	30.32 ± 0.30^{a}	3.02 ± 0.09^{b}	0.66 ± 0.01^{a}	1.27 ± 0.03^{c}	0.07 ± 0.0008^{c}	0.41 ± 0.002^{b}
Control	22.12 ± 0.57^{a}	5.37 ± 0.21^{b}	21.31 ± 0.94^{b}	3.77 ± 0.09^{a}	0.33 ± 0.008^{c}	1.69 ± 0.04^{a}	0.11 ± 0.003^{a}	0.44 ± 0.004^{b}

Means followed by different letters in each column are significantly different ($P< 0.05$, LSD Test).

*APFW: Aerial Parts Fresh Weight; APDW: Aerial Parts Dry Weight; RFW: Root Fresh Weight and RDW: Root Dry Weight

4. Discussion

In the current study, the highest root length, chlorophyll content and aerial parts' dry weight were observed in plants inoculated with *S. indica*. Similar to our results, *S. indica* stimulated growth of other medicinal plants like *Artemisia annua, Curcuma longa*, *Stevia rebaudiana,* and *Bacopamonniera* in the *in vitro* experiments (Varma et al., 2014). Barley root growth was also stimulated by inoculation of *S. indica* 2 or 3 weeks after inoculation (Achatz et al., 2010). Numerous factors are reported to have influence on the success of *in vitro* propagation of different medicinal plants. The effect of plant growth regulators and their interaction on micropropagation of different plant species have been discussed in detail by Rout and colleagues (Rout et al., 2000).

The heavy root proliferation in inoculated plants has been attributed to the synthesis of phytohormones. It has been reported that *S. indica* produces auxins and cytokinin (Kari Dolatabadi et al., 2012). Several researches reported that auxin and cytokinin influence micropropagation of medicinal and aromatic plants (Al-Sulaiman and Barakat, 2010; Meena et al., 2010; Kari Dolatabadi et al., 2011b).Chen et al. showed that cytokinin play the most critical role in the micropropagation of many medicinal plants (Chen et al., 1995). It has been demonstrated that *S. indica* produces the low amounts of auxins, but relatively high levels of cytokinins. So, cytokinins may be higher in treated roots compared to untreated plants (control) (Vadassery et al., 2008). *S. indica* was also reported to produce IAA (indole acetic acid) in liquid culture that promote root growth in plants (Sirrenberg et al., 2007).

The increased growth parameters of *S. rebaudiana* inoculated with *S. indica* were probably due to greater water and nutrients absorption due to extensive colonization of roots by S. *indica.* It seems that the stimulation of *S. rebaudiana*'s root system extension by *S. indica* almost caused the promotion of above ground growth in our experiment.

Some other advantageous of Arbuscular mycorrhizal fungi (AMF) inoculation are enhancing plant growth by increasing the availability of nutrients, water uptake and exploring soil volume nearly 100 times greater, preventing pathogenic infection, and improving soil structure (Kari Dolatabadi et al., 2011a). It has been also reported that *S. indica* not only stimulated faster growth of aerial parts of plant, but also induced higher chlorophyll a content ($mg.\ g^{-1}$ fresh weight) in *Coleus forskohlii* than in non-colonized plants (Das et al., 2012). It could be confirmed by the hypothesis that plants beneficially affected from the mutualistic interaction with fungi and delivery of phosphorus to their roots by the fungal hyphae through a phosphate transporter (PiPT).Varma et al. reported the increased chlorophyll content in plants treated with *S. indica* (Varma et al., 2014). Increase in chlorophyll levels, photosynthetic potential and leaf area could stimulate higher carbon absorption in plant colonized with *S. indica*, which consequently induce more biomass production (Jurkiewicz et al., 2010; Varma et al., 2014).

Based on the results of present study, the beneficial effect of *S. indica* was confined to the aerial parts of plants whereas the underground biomass was decreased by fungal inoculation. Plants colonized by AMF have shown lower root/ shoot ratios. The reason for this may be related to taking the absorbed nutrients over by hyphae (Tsang and Maun, 1999). Inoculation of Arabidopsis with *Trichoderma* induced more lateral root which were attributed to auxin production, even though there were no significant effects on primary root growth with the inoculation of *T. atroviride* or *T. virens* (Contreras-Cornejo et al., 2009). However, reduction in growth of hairy roots of the medicinal plant *Linum album* was observed soon after inoculation with live fungal cells (Varma et al., 2014).The increased number of secondary roots and root length may be associated with auxin, since it plays a pivotal role in plant and fungal symbiosis (Das et al., 2012). The pattern of root morphogenesis and development is also modified in mycorrhizal plants. Plants forming mycorrhizae tend to have a greater above ground biomass, since less energy is directed to root formation so their root/shoot (R/S) ratio would be lower (Lovato et al., 1996).

Root thickness was reduced in *C. forskohlii* plants treated with *S. indica* as they became fibrous, but more lateral roots were recorded (Das et al., 2012). The symbiotic interaction of *C. forskohlii* (*Plectranthus barbatus*) with *S. indica* under field conditions prompted more aerial part biomass production including flower development. The plant aerial parts are important source for metabolites which have medicinal characteristics. Therefore, the usage of the root endophyte fungus (*S. indica*) in sustainable agriculture would be beneficial in higher production of active in gredients (Das et al., 2012).

The results of this study showed that application of vermicopost water extract induced the highest chlorophyll content and ratio of RFW/APFW in treated plants. Bijeh keshavarzi et al. showed that among different kind of bio-fertilizers and chemical fertilizers, the highest chlorophyll content was obtained in vermicompost treated plants (Bijeh Keshavarzi et al., 2012). Several studies have stated that vermicompost contain hormones such as gibberellins, auxins and cytokinins and plant-growth regulating materials like humic acid which could increase plant's growth and production (Nadi et al., 2011; Tomati et al., 1990). In the present study, application of vermicompost water extract only affected the above-mentioned parameters of *S. rebaudiana.*

5. Conclusion

The results of this research showed inoculation of micropropagated *S. rebaudiana* plants with *S. indica* positively influenced some growth parameters of *S. rebaudiana*, which could assure the success of tissue culture technique for propagation of this medicinal plant. Also, the apply water extract vermicompost and *S. indica* fungus in sustainable agriculture may be improved vegetative items and, as a result, secondary metabolites in medicinal plants.

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