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ORIGINAL ARTICLE

The effect of glycine betaine and *Piriformospora indica* inoculation on some physiological traits and growth of *Stevia rebaudiana* under drought stress

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ABSTRACT
Stevia (Stevia rebaudiana Bertoni) is a perennial sweet herb belongs to Asteraceae family. In the present study, the effects of inoculation with symbiotic fungi, Piriformospora indica, on some physiological traits and photosynthetic pigments of stevia plants under four drought levels (irrigation intervals in 3, 6, 9 and 12 days) and three glycine betaine concentrations (0, 50 and 100 mM) was investigated. The results showed that drought stress had significant reduction effects on plant height, shoot fresh weight, chlorophyll a, chlorophyll b, total chlorophyll, proline and glycine betaine. P. indica inoculation improved growth parameters of stevia under drought stress. Due to increasing of glycine betaine, amounts of all traits were increased. In addition, P. indica significantly improved all traits under glycine betaine concentrations. Owing to amending the effect of glycine betaine, its high concentrations made less hazarding effects of drought on stevia plant. It was concluded that negative effects of drought stress on stevia plants were alleviated after P. indica inoculation and glycine betaine application, probably by improving physiological parameters such as photosynthesis and compatible solutes.
Keywords: *Stevia rebaudiana*, drought stress, glycine betaine, *Piriformospora indica*

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INTRODUCTION

In the recent years, an increasing demand has been noted for new natural substitute sweeteners such as sucrose or synthetic sweeteners. Leaves of stevia plant were used historically in northern of Paraguay as sweeteners and herbal remedy, and sweeteners contain compounds about 200 to 350 times higher than sucrose. It is a sweet gaining significance in different parts of the world because of the non-caloric sweeteners extracted from its leaves [1].

Water stress is one of the main abiotic stress factors, which leads to changes in morphological, physiological and biochemical responses of plants. Consequently, plant growth and crop production are negatively affected [2]. Most research to date has focused on studying the response to soil moisture variation for well-known crops, but these aspects have not been fully investigated in new or specialty crops. Understanding how plants respond to soil water limitation can play an important role in improving crop management and performance, especially since the climate-change scenarios suggest an increase in aridity in many areas of the globe [3].

Piriformospora indica, a root-colonizing endophytic fungus and a member of the basidiomycetous order *Sebacinales*, is originally isolated from the Indian Thar Desert. This fungus evolves mutualistic symbiosis with a wide variety of monocotyledonous and dicotyledonous plants. *P. indica* colonizes roots, forms pear-shaped spores called chlamydospores within the cortex, and does not enter vascular tissue and stems or leaves of plants [4]. Recent evidence suggests that plants benefit from this relationship through increased root and shoot, improved osmotic adjustment and nutrient uptake, early flowering, enhanced seed production, altered antioxidative capacities and higher resistance against various biotic and abiotic stresses. Unlike mycorrhizal fungi, which cannot be cultured axenically, *P. indica* can be easily grown on synthetic or complex media without a host [5]. Consequently, *P. indica* shows remarkable potential to be used as a biological agent for plant growth promotion and enhancement of plant tolerance against environment stress.

Glycine betaine (GB) is the low molecular weight organic compound which has been successfully applied to induce heat tolerance in various plant species. Ashraf and Foolad [6] reported that GB is an important compatible osmolyte in plants and accumulates in cytosol and chloroplast. GB can play an important role in effective protection against drought, high salt concentration and high temperature. Also, GB protects physiological processes such as photosynthesis by protecting the ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) enzyme and photosystem II under stresses condition. Moreover, plants treated with GB maintain higher antioxidative enzyme activities and minimize oxidative stress [7]. Yang and Lu [8] reported that, not all plants accumulate GB in sufficient amounts to help averting adverse effects of abiotic stresses. Thus, different approaches have been contemplated to increase the concentrations of these compounds in plants grown under stress conditions to increase their stress tolerance. Exogenous application of GB to low-accumulating or nonaccumulating plants may help reduce adverse effects of environmental stresses. Externally-applied GB can rapidly penetrate through leaves and be transported to other organs, where it would contribute to improved stress tolerance.

The target of this work was to study the effect of glycine betaine and inoculation with endophytic fungus *Piriformospora indica* on growth and productivity of stevia plant under drought stress.

MATERIAL AND METHODS

1. Plant and fungal culture and experimental conditions

A greenhouse experiment was carried out at Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari Agricultural and Natural Resources University using factorial under completely randomized block design with three replications, in 2016. Experimental factors included: drought stress in four levels, glycine betaine applicationin 3 levels and *P. indica* infection in two levels. The endophytic fungus *P. indica* was grown on Hill and Kaefer medium with 1 % agar [9] by transferring 8 mm disc (having spores and hyphae) from 1 week old culture plate. The plates were incubated in dark at 28 ± 2 °C for 7 days. Sterile distilled water was added to fresh cultures of *P. indica* plates and the plates were gently scraped to loosen the spores and mycelia. The suspension was collected, vortexed for 10 min and centrifuged at 10000 g for 5 min. The supernatant was discarded and the pellet was suspended in sterile distilled water. The fungal suspension was diluted to 5×10^5 spores/ml and plants were inoculated with 100 µl of this suspension placed near the base of 4 weeks old rooted plants of *S.rebaudiana*. Soil moisture was maintained near the field capacity for the first two weeks and then the irrigation treatments were applied at 3, 6, 9 and 12 day intervals. With respect to foliar spraying treatments, glycine betaine (MW 117.18) of Sigma Aldrich was used at 0, 50 & 100 mM as well as distilled water as control were applied as foliar application three days before drought stress induces.

2. Morphological analysis

The plants were harvested 62 days after transplanting in to pot. Leaves and stems were separated, weighted and used for further assays. Plant height was measured from the attachment point of root and stem up to the slip of uppermost fully opened leaf.

3. Leaf chlorophyll measurement

Total chlorophyll (Chlo T), chlorophyll a (Chlo a) and chlorophyll b (Chlo b) of fresh fully-expanded leaves were determined as described by Arnon [10]. Fresh leaf samples (0.5g) were homogenized in a mortar and extracted with 20 mL of 80% aceton solution using Whatman No. 42 filter paper. The absorbance of the extract recorded at 663 (A663) and 645 (A645) nm wavelengths using spectrophotometer.

4. Compatible solute content

Determination of proline (Pro) content was done by the method described by Bates ***et al***. [11]. Briefly, 0.5 g dried leaf sample was shaked and homogenized with sulfo-salicylic acid. Thereafter, the extract was exposed to react chemically with glacial acid and acid-ninhydrin for 1 h at 100 °C in a Bain-marie. Then, samples were extracted with toluene and the samples' absorbance were measured at 520 nm.

The concentration of glycine betaine (GB) was determined using the method of Grieve and Grattan [12]. 0.5 g dried leaf was homogenized with deionized water. Samples were mixed with sulfuric acid and potassium iodide-iodine (KI-I_2), and then centrifuged. To extract GB, the peridotite crystals which had formed were dissolved in 1,2-Dichloroethane (DCA), and then sample absorbance was measured at 365 nm.

5. Statistical analysis

Obtained data were analyzed by the analysis of variance (ANOVA) using Statistical Analysis System ver. 9.1 (SAS Institute Inc., Cary, NC., USA), and means were compared using Least Significant Difference (LSD) at 0.05 level.

RESULTS AND DISCUSION

1. Plant height and shoot fresh weight

Our results revealed that *P. indica* significantly increased stevia plant height and shoot fresh weight compared to non-inoculated plants (Table 3); Moreover, at different drought stress levels, *P. indica* inoculation significantly increased these traits in comparison with non-inoculated ones (Table 5). The largest plant height and shoot fresh weight were recorded in inoculated plants with 6-days irrigation interval treatment (Table 5). The results in Table 6 showed that the highest mean value of plant height were observed at 6-days irrigation interval and 100 mM of GB concentration. Enhancing GB content in plant tissues through exogenous GB application or genetic selection was considered a possible way to improve tolerance to abiotic stresses in cotton [13]. Concerning the shoot fresh weight, the treatment including 6-days irrigation interval and 100 mM of GB concentration gave the greatest values (50.45 cm).

2. Chlorophyll content

Our results show that chlo a, chlo b and chlo T were significantly decreased under drought stress. Under severe drought stress, chlo a and chlo b had 71 and 69% reduction compared to control, respectively (Table 2). The highest mean value of total chlorophyll content determined at 3–days irrigation interval by *P. indica*, while the lowest one was observed in the 12-days irrigation interval without *P. indica*. Interaction effect of drought stress and *P. indica* infection on chlo a, chlo b and chlo T was significant. The highest and lowest values of photosynthetic pigments were observed in colonized plants under control condition and in uncolonized plants under severe drought stress, respectively (Table 2). Similarly, Abadi and Sepehri [14] found that *P. indica* inoculation promoted the production of carotenoids, chlo a and chlo b in wheat under Zn deficiency conditions by 22, 10.7 and 24.5%, respectively. Jogawat et al. [15] reported that the photosynthetic pigment content was significantly higher in the colonized rice seedlings as compared with non-inoculated ones under salinity stress. The interactive effect of drought stress and *P. indica* inoculation was significant on shoot fresh weight and height of stevia (table 2). Increasing severity of drought stress significantly reduced the shoot fresh weight and plant height by 9% and 16%, respectively. Table 2 shows that shoot fresh weight of colonized plants were greater than their values in uncolonized ones by about 50 and 57% under moderate and severe drought stresses (i.e. 9 and 12-days irrigation intervals), respectively. *P. indica* was originally isolated in an arid sub-tropical soil (Indian Thar Desert, north-western Rajasthan). This implies that the fungus has evolved in a harsh ecosystem and can clearly affect plant stress tolerance under extreme environmental conditions. Similarly, Ghabooli and Mondani [16]studied the effect of *P. indica* on biomass and physiological responses of barley (*Hordeum vulgare* L.) under drought stress. They reported that under severe drought stress (maintaining soil water content at 25% of field capacity), shoot dry weight decreased up to 28% in inoculated plants, whereas the decrease in non-inoculated plants was 43%.

Under drought stress conditions, Chlo a, Chlo b and Chlo T contents significantly declined especially under severe drought stress. Moreover, free Pro concentration increased with increasing drought stress (Table 2). The tendency to decrease chlorophyll content under stress conditions might be due to synthesis of osmotic regulators such as Pro in the roots and hydrocyanic acid in the leaves which use large amount of nitrogen [17].

Data indicate that both drought stress and GB treatment significantly reduced height, shoot fresh weight, Chlo a and Chlo T of plants, with more reduction in drought stress (table 2). The highest reduction in height (16%) and fresh weight (25%) was observed at 12-days irrigation interval in comparison with control plant. GB treatment partly restored the reduction in shoot fresh weight in all irrigation interval levels. Results of chlorophylls are presented in Table 4. Chlo a and Chlo T contents significantly decreased in 9 and 12-days irrigation intervals. Chlorophylls showed no significant changes in GB with the exception of combination of 50 and 100 mM GB with 9 and 12-days irrigation intervals.

Drought stress resulted in no significant changes in Pro content (table 4). GB with drought stress treatments significantly increased proline content, especially in 100 mM of GB. The highest level of Pro was observed in combination of 100 mM of GB with 12-days irrigation interval, about 276% higher than that in control plant.

3. Leaf proline and glycine betaine concentrations

For any given dose of GB, plants exposed to 12-days irrigation interval had higher concentrations of GB than in the control plants. We are confident that the values given in table 4 represent the internal tissue concentrations of GB since the leaves were washed with 0.5 mM $CaSO_4$ prior to harvest for removing

residual GB from the leaf surface. The proline concentrations in all leaf tissues were 5–7 times higher than those of GB (table 4). GB applications had no effect on Pro concentration in leaves of plants without stress. Several research papers have been published during the last two decades which emphasize the supportive role of *P. indica* in mitigating the osmotic stress in different host plants. It has been reported that *P. indica* serves as a bioregulator, biofertilizer and bioprotector for many host plants. Regardless of the stress, *P. indica* has evolved to protect its food source-the plant. It activates different signaling, transport, metabolic and developmental programs in plants [18, 19, 20]. Saddique *et al*. [21] demonstrate that *P. indica* increased the rice seedling biomass, length of root and shoot, P and Zn concentration in root and shoot, Pro concentration, TAC, Fv/Fm and the expression of P5CS. Most of the previous
research articles elaborated the mechanisms that how *P. indica* ameliorates the drastic effects of salt, heavy metal and some biotic stresses in rice plant. It had been reported that this symbiotic fungus increases the concentration of Pro, antioxidants and decreases the concentration of malondialdehyde under these stresses [22, 23, 24]. Most of the yield damage in stevia is due to the water shortage. There is very less literature available explaining the stevia and *P. indica* symbiosis under osmotic stress. So, there is a need to investigate the role of *P. indica* for inducing drought tolerance in stevia. This research article highlighted the *P. indica* induced improvement in some of the important features of stevia plants under osmotic stress. We determined that *P. indica* significantly increased the stevia plant biomass and the length and weight of shoot under control and drought stress. Similar growth improvement was reported in Arabidopsis and many other field crops [25, 26] The supportive role of *P. indica* in increasing the synthesis of Pro was also evidenced by our experiment. The accumulation of Pro due to osmotic stress was more than double in inoculated plants compared to non-inoculated plants. We test the association of *P. indica* with stevia plant under osmotic stress and it was summarized that this fungus improved plant performance under water deficient environment as it is already reported for inducing tolerance in stevia under many other stresses.

Table 1. Variance analysis of drought stress, *Piriformospora indica* symbiosis and glycine betaine foliar spray on stevia

SOV	df	Plant height	Shoot fresh weight	Chlorophyll a	Chlorophyll b	Total chlorophyll	Proline	Glycine betaine
Block	2	1.52**	5.42**	0ns	0.002ns	0.005ns	0.042ns	0.012ns
Drought (D)	3	215.69**	142.06**	2.12**	0.24**	3.75**	272.52**	2.60**
Glycine betaine (G)	2	44.32**	35.17**	0.052**	0.002ns	0.072**	15.06**	1.93**
Fungi (F)	1	1241.18**	2303.05**	1.62**	0.41**	3.66**	104.44**	3.93**
D×G	6	3.87**	3.21*	0.013**	0.001ns	0.022**	1.89**	0.012*
D×F	3	2.64*	7.86**	0.015**	0.037**	0.098**	5.17**	0.14**
G×F	2	2.53*	5.81**	0.005*	0.0005ns	0.008ns	0.90**	0.12**
D×G×F	6	1.91*	2.09ns	0.003ns	0.0003ns	0.004ns	0.76**	0.018**
Error	46	0.79	1.02	0.001	0.0006	0.0028	0.12	0.0041
C.V (%)		1.95	3.82	2.55	4.95	2.62	4.61	4.36

ns, non-significant; *, significant at P≤0.05; **, significant at P≤0.01.

Table 2. The effect of drought stress on stevia

Drought (days)	Plant height (cm)	Shoot fresh weight (g)	Chlorophyll a (mg g^{-1}FW)	Chlorophyll b (mg g^{-1}FW)	Total chlorophyll (mg g^{-1}FW)	Proline (g $^{-1}$FW)	Glycine betaine (µmol g-
3	47.44b	28.35a	1.75a	0.61a	2.36a	3.59d	1.06d
6	48.78a	28.76a	1.74a	0.59b	2.34a	5.59c	1.26c
9	45.79c	25.88b	1.55b	0.48c	2.04b	8.85b	1.62b
12	40.86d	22.65c	1.02c	0.36d	1.38c	12.47a	1.92a

Means in a column followed by the same letter are not significantly different (P≤0.05).

Table 3. The effect of glycine betaine on stevia

Glycine betaine(mM)	Plant height (cm)	Shoot fresh weight (g)	Chlorophyll a (mg g-1FW)	Chlorophyll b (mg g-1FW)	Total chlorophyll (mg g-1FW)	Proline (g-1FW)
0	44.66c	25.21c	1.48c	1.98c	6.79c	1.16c
50	45.24b	26.38b	1.5b	2.02b	7.72b	1.52b
100	47.25a	27.63a	1.56a	2.09a	8.37a	1.71a

Means in a column followed by the same letter are not significantly different (P≤0.05).

Table 4. The effect of fungi inoculation on stevia

Fungi	Plant height (cm)	Shoot fresh weight (g)	Chlorophyll a (mg g-1FW)	Chlorophyll b (mg g-1FW)	Total chlorophyll (mg g-1FW)	Proline (g-1FW)	Glycine betaine (µmol g-1DW)
Non-inoculated	41.57b	20.75b	1.37b	0.50b	1.80b	6.42b	1.23b
inoculated	49.87a	32.07a	1.67a	0.59a	2.25a	8.83a	1.7a

Means in a column followed by the same letter are not significantly different (P≤0.05).

Table 5. The interaction effect of *Piriformospora indica* symbiosis and drought stress on stevia

Fungi	Drought	Plant height (cm)	Shoot fresh weight (g)	Chlorophyll a (mg g-1FW)	Chlorophyll b (mg g-1FW)	Total chlorophyll (mg g-1FW)	Proline (g-1FW)	Glycine betaine (µmol g-1DW)
Non-inoculated	3	43.11e	22.27d	1.63c	0.58b	2.21c	2.90h	0.90g
	6	44.23d	22.40d	1.61c	0.55c	2.16c	4.67f	1.04f
	9	42.13e	20.71e	1.38d	0.38e	1.75d	7.58d	1.43d
	12	36.80f	17.63f	0.85f	0.24f	1.09f	10.55b	1.55c
inoculated	3	51.79b	34.42a	1.87a	0.64a	2.51a	4.29g	1.22e
	6	53.32a	35.12a	1.87a	0.64a	2.51a	6.51e	1.47d
	9	49.45c	31.05b	1.73b	0.59b	2.33b	10.14c	1.82b
	12	44.92d	27.67c	1.19e	0.48d	1.67e	14.40a	2.28a

Means in a column followed by the same letter are not significantly different (P≤0.05).

Table 6. The interaction effect of drought stress and glycine betaine foliar spray on stevia

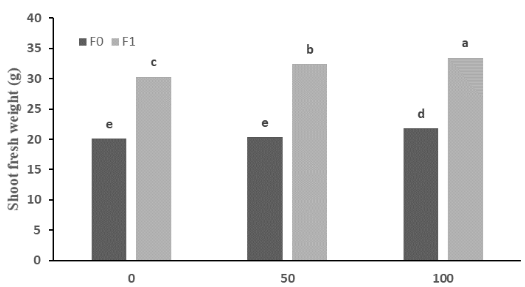
Drought	Glycine betaine	Plant height (cm)	Shoot fresh weight (g)	Chlorophyll a (mg g-1FW)	Total chlorophyll (mg g-1FW)	Proline (g-1FW)	Glycine betaine (µmol g-1DW)
3	0	46.64c	27.56c	1.75a	2.36a	3.54j	0.77i
	50	47.83b	28.96ab	1.75a	2.35a	3.60j	1.11g
	100	47.86b	28.52abc	1.75a	2.36a	3.62j	1.29f
6	0	47.74b	27.75bc	1.73a	2.33a	4.59i	0.96h
	50	48.14b	28.85abc	1.73a	2.33a	5.80h	1.34f
	100	50.45a	29.68a	1.75a	2.35a	6.39g	1.48e
9	0	44.55e	24.28d	1.49d	1.96c	8.03f	1.34f
	50	45.49d	25.54d	1.53c	2.01c	8.71e	1.68d
	100	47.34bc	27.81bc	1.64b	2.14b	9.82d	1.85c
12	0	39.73g	21.25e	0.95f	1.28f	11.01c	1.55e
	50	39.50g	22.20e	0.98f	1.34e	12.77b	1.96b
	100	43.35f	24.51d	1.13e	1.51d	13.64a	2.24a

Means in a column followed by the same letter are not significantly different (P≤0.05).

Table 7. The interaction effect of *Piriformospora indica* symbiosis, glycine betaine foliar spray and drought stress on stevia

Fungi	Glycine betaine	Drought	Plant height (cm)	Proline (g^{-1}FW)	Glycine betaine(µmol g^{-1}DW)
Non-inoculated	0	3	42.77jk	2.85m	0.66m
		6	42.97ijk	3.81l	0.78l
		9	40.96lm	6.65i	1.29i
		12	36.12n	8.40g	1.30hi
	50	3	42.58jk	2.82m	0.91k
		6	43.50ij	4.70k	1.09j
		9	42.7kl	7.51h	1.40gh
		12	34.61o	10.83e	1.59f
	100	3	43.97i	3.02m	1.12j
		6	46.22h	5.51j	1.27i
		9	43.27ijk	8.58g	1.62f
		12	39.67m	12.42c	1.77e
Inoculated	0	3	50.51e	4.24kl	0.89k
		6	52.51bcd	5.36j	1.14j
		9	48.13fg	9.42f	1.40gh
		12	47.03gh	13.62b	1.81e
	50	3	53.09b	4.39k	1.31hi
		6	52.78bc	6.91i	1.59f
		9	48.80f	9.92f	1.97d
		12	44.39i	14.71a	2.33b
	100	3	51.76bcde	4.24kl	1.47g
		6	54.67a	7.27h	1.68f
		9	51.42de	11.08d	2.09c
		12	43.34ijk	14.86a	2.70a

Means in a column followed by the same letter are not significantly different (P≤0.05).



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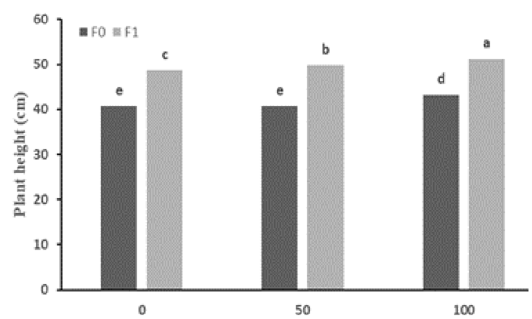

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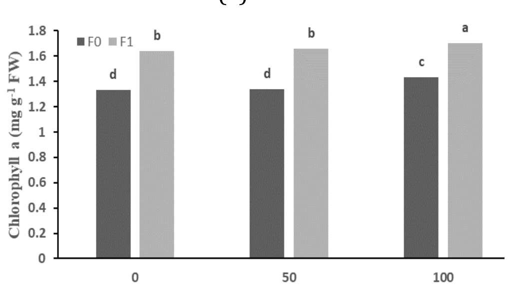

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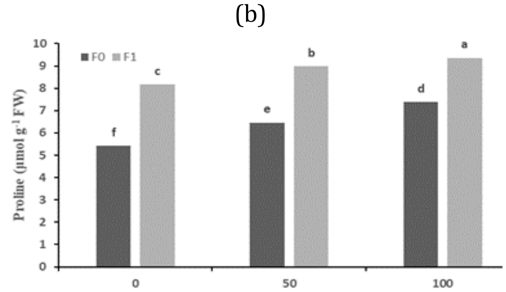

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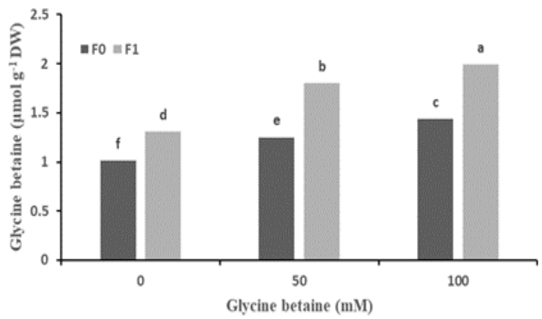

(e)

Fig1. Mean. comparisons of plant height (a), shoot fresh weight (b), chlorophyll a (c), proline (d) and glycine betaine (e) values as affected by the interactive effect of glycine betaine levels (0, 50 and 100 mM) and *P. indica* inoculation (F0: non-inoculated and F1: inoculated).
Means in a bar followed by the same letter are not significantly different (P≤0.05).

CONCLUSION

P. indica is known as a helper maintaining growth and yield in many field crops under drought stress, but the underlying mechanisms are not fully elucidated. In the present study, *P. indica* was found to improve morphological traits and protect the photosynthetic machinery in stevia plants submitted to osmotic stress. Moreover, the inoculation with *P. indica* increased the accumulation of Pro and GB. These results confirmed that the endophyte has a significant role in protecting the stevia plant against osmotic stress. Further investigation is needed, however, to validate these effects of the inoculation in stevia fields under various drought levels and use the inoculation with *P. indica* as an additional agronomical tool to improv estevia yield under drought-prone conditions.

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