



Endophyte Inoculation Enhances Growth, Secondary Metabolites and Biological Activity of *Endostemon obtusifolius* Grown Under Drought Stress

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Abstract

There is a need to cultivate medicinal plants to meet the growing demand. Their cultivation is hampered by extreme environmental conditions such as drought that affect plant growth and its pharmacological potential. Application of stress-tolerant endophytic species may potentially attenuate these negative impacts. This study assessed the effects of individual and co-inoculation of two native endophytic species (bacterium *Paenibacillus polymyxa* and fungus *Fusarium oxysporum*) on growth, physiological responses, metabolite accumulation and therapeutic efficacy of *Endostemon obtusifolius* subjected to varying watering regimes (well watered, mild and severe stress) under greenhouse conditions. Drought stress negatively affected root and shoot biomass, carotenoid content, chlorophyll fluorescence and relative water content in *E. obtusifolius*. Electrolyte leakage and malondialdehyde and hydrogen peroxide accumulation increased with drought stress. Individual and co-inoculation endophyte treatments significantly improved growth and stress tolerance mechanisms via increased osmolyte production (soluble sugars, proline), up-regulation of the enzymatic antioxidant system (superoxide dismutase) and increased antioxidant metabolite content (total phenolics, flavonoids). Antioxidant (DPPH, FRAP) and in vitro α -glucosidase activity of ethyl acetate leaf extracts were negatively affected by water stress but significantly improved when plantlets were subjected to endophyte inoculation. The most active extracts were from plants subjected to mild water stress with co-inoculation. Thus severe drought stress negatively affected growth and therapeutic efficacy of *E. obtusifolius*. Inoculation with beneficial endophytes enhanced the biochemical responses, osmoregulatory network and improved the therapeutic efficacy of *E. obtusifolius*.

Keywords α -Glucosidase · *Fusarium oxysporum* · Medicinal plant · *Paenibacillus polymyxa* · Water stress

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Introduction

Wild populations of medicinal herbs are under pressure due to increased harvesting for research, trade, therapeutic and industrial benefits (Seile et al. 2022). This has led to a loss of biodiversity and habitat degradation (Nwafor et al. 2021). It is thus important to develop cultivation practices to meet the escalating demand for these plants with active principles and to restore their environment. Cultivation strategies need to focus on optimum cultivation conditions, ways to enhance *in-planta* biosynthesis of valuable bioactive compounds and to achieve high-quality product uniformity (Alamgir 2017). Adverse climatic conditions, diseases and pests, crop failure and the high cost of cultivation are some of the global problems hindering extensive cultivation of medicinal plants (Alamgir 2017).

Drought is a major global environmental threat affecting plant growth and yield. Water deficit effects can manifest at

different levels of plant organization and may impair processes such as water absorption, mineral nutrient uptake, photosynthesis, gaseous exchange, cell division and cellular respiration (Kapoor et al. 2020). This affects the health and efficiency of the plant (Caser et al. 2019). Tolerance to drought stress depends on the plant species and genotype, the timing and severity of the stress and the developmental stage of the plant (Kakar et al. 2022; Khan et al. 2018). Excessive reactive oxygen species (ROS) accumulate in plant tissues as a consequence of drought. This is lethal to cells as ROS peroxidizes membrane lipids, damages other macromolecules such as proteins and nucleic acids, inhibits enzymatic activities and ultimately leads to cell death (Khan et al. 2018). Plants activate a series of protective mechanisms in response to drought and oxidative stress. These include osmolyte (e.g. proline and soluble sugar) synthesis and accumulation, enhanced activities of antioxidant enzymes such as superoxide dismutase (SOD) and accumulation of non-enzymatic antioxidants (e.g. phenolic compounds and ascorbic acid) to scavenge toxic free radicals (Kapoor et al. 2020).

Resistance to drought and other stresses can be further enhanced by symbiont endophytes (fungi and bacteria) that colonize the plant's tissues (Rho et al. 2018; Salvi et al. 2022). These are typically mutualistic microbes that occur inter- and intra-cellularly in plant tissue without the plant showing any infection or adverse effects (Das et al. 2023; Khare et al. 2018). Under drought conditions, endophytes, in addition to their host's innate responses, promote growth, suppress diseases, assist in nutrient assimilation and induce drought tolerance via the synthesis of certain enzymes, exopolysaccharides, growth regulators and volatile compounds (Lu et al. 2021). They may also mediate stress-responsive genes, elicit anatomical and physiological responses and increase the *in planta* concentrations of antioxidants and osmolytes (Khare et al. 2018). They also promote the accumulation of bioactive compounds in medicinal plants (Yang et al. 2019).

Drought is one of the foremost environmental stress factors in arid and semi-arid regions and influences not only growth and development but also the curative properties of medicinal plants (Yue et al. 2022). *Endostemon obtusifolius* (E. Mey. ex Benth.) N. E. Br. of the Lamiaceae (mint) family, is a perennial shrub indigenous to southern Africa. It grows in semi-arid regions of South Africa and is thus perpetually exposed to some level of water deficit. The leaves of this aromatic plant are used traditionally as a culinary herb and by extension, as a medicinal plant (Sadashiva et al. 2014). It has neuroprotective, analgesic, anesthetic, anti-diabetic and antioxidant properties (Sadashiva et al. 2014; Ogbe et al. 2023a). Plant secondary metabolites with antioxidant properties are abundantly distributed in the Mint family (Tamokou et al. 2017) and play a protective role in

the pathogenesis of degenerative diseases including renal dysfunction, diabetes, obesity, high blood pressure and atherosclerosis (Ong et al. 2018).

One potential economically viable strategy to enhance the synthesis of valuable secondary metabolites is to deliberately subject cultivated medicinal plants to different abiotic stresses together with the application of exogenous substances and beneficial microorganisms (biotic elicitors) such as endophytic symbionts to improve their stress tolerance (Ouyang et al. 2023; Zhang et al. 2019) and accumulation of bioactive compounds (Yang et al. 2019). Previous studies on *E. obtusifolius* have mainly focused on its pharmacological value. Its morphological, biochemical and physiological responses as well as its pharmacological status under drought stress have not been studied. Previously, 26 endophytes (12 fungi and 14 bacteria) were isolated from the leaves and roots of *E. obtusifolius* and their growth-promoting characteristics assessed. The two most promising isolates were identified as the bacterium *Paenibacillus polymyxa* isolated from the roots and the fungus *Fusarium oxysporum* (EOLF-5) isolated from the leaves of *E. obtusifolius* (Ogbe et al. 2023b). The aim of the present study was to investigate the effects of individual and co-inoculations of the previously isolated *P. polymyxa* and *F. oxysporum* endophytes on the growth, drought tolerance and pharmacological activities of *E. obtusifolius* grown under different watering regimes.

Materials and Methods

Cultivation of *Endostemon obtusifolius* and Growth Conditions

Endostemon obtusifolius stem cuttings were prepared according to the procedure of Anith et al. (2018) with some modifications. Stem cuttings of approximately 10 cm long and 3 mm diameter with 3–4 nodes were harvested from young and disease-free lateral branches of a solitary mother *E. obtusifolius* plant growing in the University of KwaZulu-Natal Botanical Gardens, Pietermaritzburg Campus (latitude -29.625073 , longitude 30.403557). The cuttings were defoliated except for a flag leaf and placed in sterile distilled water until treated. The basal portion of the stem cuttings were dipped in a rooting hormone (Seradix B No.3, Bayer Crop Science, Germany) for 1 min and planted in disinfected trays (27×18 cm) containing sterile vermiculite. The trays were placed in a mist house with day and night temperatures of $28/15$ °C, 80–90% relative humidity and 10 s misting at 15 min intervals. After three weeks, the stem cuttings were transplanted into 7.5 cm diameter pots containing sterile palm peat and vermiculite (1:1) and placed in a greenhouse with 30–40% relative humidity and an average photosynthetic photon flux density of $450 \mu\text{mol m}^{-2} \text{s}^{-1}$. After three

weeks in the greenhouse, well-rooted stem cuttings were transplanted into 15 cm diameter pots containing 1:3 sterile vermiculite and twice autoclaved garden soil. The physiochemical properties of the medium were 11% moisture, 5 mg kg⁻¹ P, 1.7 mg kg⁻¹ K, 5 mg kg⁻¹ alkali hydrolysable N, 9 mg kg⁻¹ Ca, 40% field capacity (FC) and pH 7.80. These were placed under the same greenhouse conditions as above and irrigated as required with half-strength Hoagland's Solution (Hoagland and Snyder 1933) for four weeks.

Preparation and Application of Microbial Inoculants

Previously isolated and identified endophytes (*Paenibacillus polymyxa* and *Fusarium oxysporum*) from *E. obtusifolius* were used (Ogbe et al. 2023b). The bacterial endophyte, *P. polymyxa* inoculum was prepared by growing the isolate in Schott bottles containing 250 mL Mueller Hinton Broth and incubating at 28 °C on a shaking incubator at 180 rpm for two days. Subsequently, the cells in the broth were harvested by centrifugation (Avanti @ J-E Centrifuge Beckman Coulter, Ireland) at 5 000 × g for 10 min at 4 °C, washed and resuspended in sterile distilled water. The cell density was adjusted to 1 × 10⁷ CFU mL⁻¹ final concentration using sterile distilled water (Hussain et al. 2018). The fungal endophyte was maintained on potato dextrose agar plates for two weeks at 28 °C in an incubator. Thereafter, the plates were flooded with 10 mL sterilized distilled water containing 0.05% (v/v) Tween-20. The fungal colonies were then gently removed from the media surface using a sterile spatula and transferred to a sterilized beaker. The conidial suspension was made by filtering the harvested mycelial mass through a double layer of sterilized cheesecloth to remove the agar and mycelia debris. The final spore number was evaluated using a haemocytometer and adjusted to 7 × 10⁵ (Sadeghi et al. 2020).

A modified soil-drenching inoculation method of Ramakuwela et al. (2020) was employed where for single inoculation, either 20 mL bacterial suspension or 80 mL conidial suspension was poured around the root zone of the *E. obtusifolius* cuttings. A co-inoculation treatment was done by applying 20 mL bacterial suspension and 80 mL conidial suspension around the same region. An uninoculated control group was treated with 100 mL distilled water. To confirm the establishment of the inoculated endophytes in the stem cuttings, the roots from three pots of each sub-group were carefully harvested without disturbing the integrity of the root architecture five weeks after inoculation. The isolation procedure described in Ogbe et al. (2023b) was followed and the inoculated isolates were re-identified on plates using morphological characters.

Experimental Design and Treatments

The individual and combined effects of *P. polymyxa* and *F. oxysporum* inoculations on drought-stressed *E. obtusifolius* stem cuttings were investigated by setting up a factorial experiment with two combined factors: (1) inoculation with *P. polymyxa*, inoculation with *F. oxysporum*, co-inoculation with *P. polymyxa* and *F. oxysporum* and uninoculated control; (2) soil water content - well-watered with 100% FC, mild stress with 50% FC and severe stress with 25% FC. Each of the twelve treatments had ten replicates and the plants (ten-week-old stem cuttings) were arranged in a completely randomized fashion. Six weeks after inoculation (16 weeks post-stem cuttings), the plants were subjected to drought stress treatments for 8 weeks.

Determination of Dry Shoot and Root Weight

After 8 weeks drought treatment, three plants per treatment were randomly harvested, washed under running tap water and divided into root and shoot systems. The plants were oven-dried at 70 °C for 48 h until a constant weight was reached after which the shoot and dry weights (DW) were measured.

All the following measurements were carried out on leaves harvested from three representative pots per treatment (n = 3).

Photosynthetic Pigments

Total chlorophyll (*a* + *b*) and carotenoids of fully expanded leaves of *E. obtusifolius* plants were determined according to the method of Lichtenthaler (1987). Leaves were weighed and homogenized in 80% acetone. The extract was then centrifuged (1,000 × g for 10 min at 0 °C; Hettich Universal, Tuttlingen, Germany) and the absorbance of the samples measured at 470, 645 and 662 nm. The chlorophyll content was calculated (mg g⁻¹ fresh weight [FW]) using the formulae of Lichtenthaler (1987).

Photochemical Efficiency of Photosystem II (F_v/F_m)

Chlorophyll fluorescence of *E. obtusifolius* plants was assessed after 7 weeks of drought treatment using a portable chlorophyll fluorometer (OS-30p; Opti-Sciences, Inc., NH, USA) using leaves. Chlorophyll fluorescence measurements were carried out in the dark on the upper leaf (adaxial) surface on the youngest but mature leaf of each plant from three representative pots. After an initial 45 min dark adaptation, minimal fluorescence (F₀) was recorded by applying weak modulated light (0.4 μmol m⁻² s⁻¹). Maximal fluorescence (F_m) was subsequently measured after illumination with a saturating flashlight (8000 μmol m⁻² s⁻¹) for

0.8 s. Measurements were taken in triplicate from each leaf. Variable fluorescence (F_v) was computed by an in-built programme to determine the maximal photochemical efficiency of PSII (Khan et al. 2016).

Relative Water Content

The relative water content (RWC) of the leaves was estimated as per Zhang et al. (2019) with slight modifications. Fully developed leaves were selected and their FWs immediately recorded. The leaves were then immersed in 25 mL distilled water and incubated in the dark for 24 h at 4 °C. After drying the surface water with a paper towel, the turgid weight (TW) of each leaf was recorded. The samples were then oven-dried at 70 °C for 48 h and the DW measured. The water status was evaluated by estimating RWC using the formula:

$$\text{RWC (\%)} = \left[\frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \right] \times 100$$

Electrolyte Leakage

Electrolyte leakage was estimated using leaf discs (0.5 cm) obtained from leaf samples to measure cell membrane stability. Briefly, leaf discs were immersed in 10 mL distilled water for 4 h at room temperature. The electrical conductivity of the medium (EC1) was recorded using an electrical conductivity meter (Laborgerate GmbH). Thereafter, the sealed samples were autoclaved at 121 °C for 15 min to disrupt the cell membrane and fully release the electrolytes. After cooling at room temperature, the final electrical conductivity (EC2) was measured. Electrolyte leakage percentage was calculated using the formula (Kumar et al. 2018):

$$\text{EL (\%)} = (\text{EC1}/\text{EC2}) \times 100$$

Total Soluble Sugar Content

The total soluble sugar in *E. obtusifolius* leaves was estimated using the anthrone reagent method as described by Tyagi et al. (2017). Briefly, 200 mg freshly harvested leaves were hydrolyzed in 5 mL 2.5 N HCl before adding anthrone reagent. The absorbance was measured at 630 nm. The amount of total soluble sugar was estimated from a glucose concentration standard curve and expressed as $\mu\text{g g}^{-1}$ FW.

Proline Content

The proline content in the leaves of *E. obtusifolius* was determined following the Bibi et al. (2019) protocol. Briefly, 100 mg fresh leaf samples were homogenized in 5 mL 3% sulfosalicylic acid. After addition of a reaction mixture (250

μL 3% sulfosalicylic acid, 500 μL glacial acetic acid and 250 μL 2.5% acidic ninhydrin), toluene was added to separate the chromophore containing organic phase. The absorbance of the organic phase was measured at 520 nm. Proline concentration was calculated from a proline standard calibration curve and expressed as mg g^{-1} FW.

SOD Activity

SOD activity was quantified using the nitroblue tetrazolium (NBT) method described by Ye et al. (2021). Briefly, enzyme extract was prepared by homogenizing 200 mg FW leaf samples of *E. obtusifolius* in 2 mL extraction solution (100 mM phosphate buffer (pH 7.8) with 0.1 mM EDTA). The prepared enzyme extract was added to a reaction mixture (130 mM methionine, 750 μM NBT, 20 μM riboflavin and 1 mM EDTA Na_2) and incubated under light for 30 min at room temperature. SOD activity was quantified spectrophotometrically at 560 nm. One unit of SOD activity was calculated as the amount of enzyme that suppressed the photoreduction rate of NBT by 50% compared to tubes without enzyme extracts and was expressed as SOD U g^{-1} FW. Protein concentration was measured using bovine serum albumin as a standard (Bradford 1976).

Malondialdehyde (MDA) Content

MDA concentration in the leaves of *E. obtusifolius* were used to estimate the level of lipid peroxidation following a modified protocol of Tyagi et al. (2017). Briefly, leaf samples were homogenized in 2 mL 0.1% trichloroacetic acid and centrifuged at 11 000 $\times g$ for 10 min. Thereafter, 1 mL 0.25% thiobarbituric acid in 10% trichloroacetic acid was added to 1 mL supernatant. The reaction mixture was heated at 95 °C in a water bath for 30 min and then immediately cooled on ice. The absorbance of the reaction mixture was measured at 532 and 600 nm. MDA content was calculated using the extinction coefficient of MDA ($\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$) and expressed as nmol g^{-1} FW.

Histochemical Detection of Hydrogen Peroxide (H_2O_2)

Endogenously produced H_2O_2 was visualized using the 3',3'-diaminobenzidine (DAB) staining protocol described by Yang et al. (2013). Leaf samples of *E. obtusifolius* were submerged in 1 mg/mL DAB solution at room temperature, pH 3.8 for 8 h under illumination. After 8 h, the leaves were boiled in 95% (v/v) ethanol for 10 min to decolourize the leaves (remove pigments) except for the deep brown polymerization product of the DAB reaction with H_2O_2 . After cooling, the bleached leaves were preserved in 4:1 (v/v) ethanol-glycerol solution and kept at 4 °C until photographed.

Intense brown colouration indicated a higher concentration of H_2O_2 in the leaves.

Phytochemical Content

Total phenolic and flavonoid content in the leaves of *E. obtusifolius* were measured following the colourimetric methods described by Dragović-Uzelac et al. (2007). Briefly, ethyl acetate extracts were prepared from leaf samples. Total phenolic content was evaluated using Folin-Ciocalteu reagent. The optical density was measured at 725 nm and the total phenolic content expressed as $mg\ g^{-1}$ gallic acid equivalents. Flavonoid content was evaluated using aluminium chloride where the optical density was measured at 510 nm. Flavonoid content was expressed as $mg\ g^{-1}$ catechin equivalents.

1-1-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The radical scavenging activity of the leaves was measured following the method of Sridhar and Charles (2019). Briefly, crude ethyl acetate extracts were prepared from leaf samples and mixed with 0.1 mM DPPH solution prepared in methanol. Following 30 min incubation in the dark, the optical density was measured at 517 nm. The IC_{50} value of each extract was calculated using a non-linear regression curve of the % inhibition (scavenging activity) against the logarithm of concentrations. Butylated hydroxytoluene (BHT) was included as a standard.

Ferric-reducing Antioxidant Power (FRAP) Assay

The iron reducing power was measured following the method of Moyo et al. (2013). Ethyl acetate leaf extracts were serially diluted in microwell plates and a 1% (w/v) potassium ferricyanide reaction mixture added. Following incubation for 30 min at 50 °C, the mixture was acidified with trichloroacetic acid (1% in phosphate buffer) and then distilled water and ferric chloride (0.1% in phosphate buffer) added. Absorbance of the mixture was measured at 630 nm. OD values were plotted against their concentration gradient and slope values calculated. BHT was included as a standard.

α -Glucosidase Inhibitory Assay

The hypoglycemic activity of the *E. obtusifolius* leaves was measured as described by Rengasamy et al. (2013). Briefly, crude ethyl acetate leaf extracts were diluted in 5% dimethyl sulfoxide and 95% phosphate buffer (0.02 M, pH

6.9) containing 0.006 M NaCl to varying concentrations ($10\text{--}100\ \mu g\ mL^{-1}$). This was mixed with α -amylase enzyme solution ($2\ U\ mL^{-1}$) and incubated for 10 min at room temperature, after which 1% starch solution in phosphate buffer was added to initiate the reaction. The reaction was halted after 10 min by the addition of 3,5-dinitrosalicylic acid reagent and heating at 95 °C for 10 min. The optical density was measured at 540 nm. The enzyme inhibition percentage was calculated and IC_{50} values determined by extrapolation from the standard curve. The experiment was done in triplicate and acarbose included as a standard.

Statistical Analysis

The data was analyzed statistically based on the experimental design with a two-way analysis of variance using GraphPad Prism 7 (GraphPad Software, Inc. California USA). Mean comparison was done with the Bonferroni post hoc test at a 5% significance level ($p \leq 0.05$).

Results

Establishment of Inoculation Colonies

Endophytic species colonizing the roots of *E. obtusifolius* were isolated from the inoculated root segments. They were identified as *P. polymyxa* and *F. oxysporum* based on the morphological characters of the initial cultures. This indicated that the method was effective for the successful inoculation of *E. obtusifolius* stem cuttings with the two isolated endophytes.

Growth of *E. obtusifolius*

The main factors (watering regime and endophyte inoculation) and their synergistic effects significantly influenced the root and shoot biomass of *E. obtusifolius* plants (Table 1). The F-values and P-values were used to assess the level of significance of the factors and their interaction (Table 1). Probability (P) values less than 0.05 were considered significant. Control plants subjected to mild water stress (50% FC) had significantly higher shoot and root dry weights compared to the other watering regimes (Figs. 1 and 2). All endophyte inoculation treatments significantly increased growth of *E. obtusifolius* with the highest biomass accumulation in the well-watered (100% FC), endophyte-treated plants (Figs. 1 and 2). *F. oxysporum* and co-inoculation applications elicited the best response in the shoots (Fig. 2a) and roots (Fig. 2b) of well watered and mild water stressed plants. Under severe water stress, the co-inoculation treatments were the most effective in increasing shoot growth

Fig. 1 Growth of *Endostemon obtusifolius* stem cuttings subjected to various water stress levels for 8 weeks when inoculated with two endophytes. WW = well-watered (100% FC), MS = mild stress (50% FC), SS = severe stress (25% FC), a = uninoculated control, b = inoculated with *Paenibacillus polymyxa*, c = inoculated with *Fusarium oxysporum* and d = co-inoculated with *P. polymyxa* + *F. oxysporum*

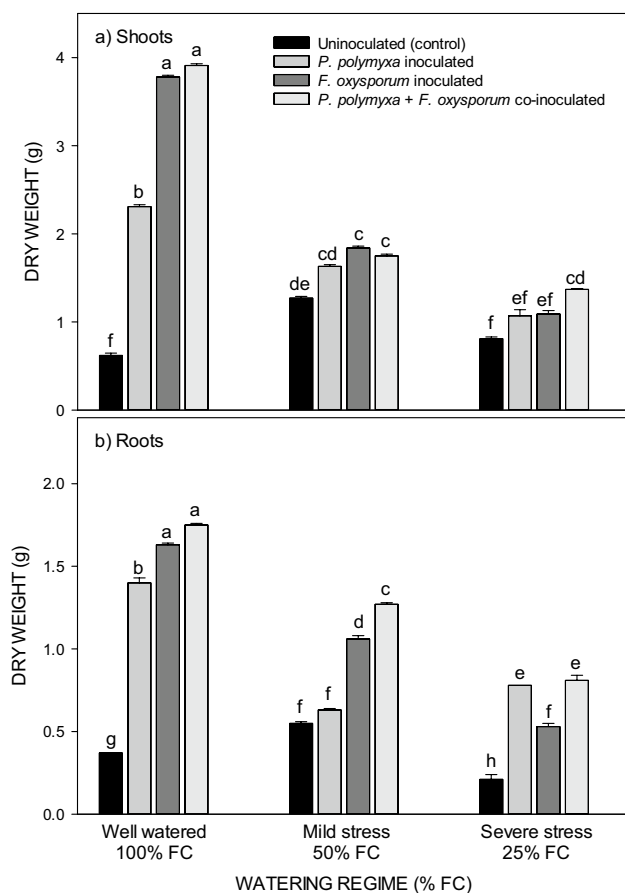
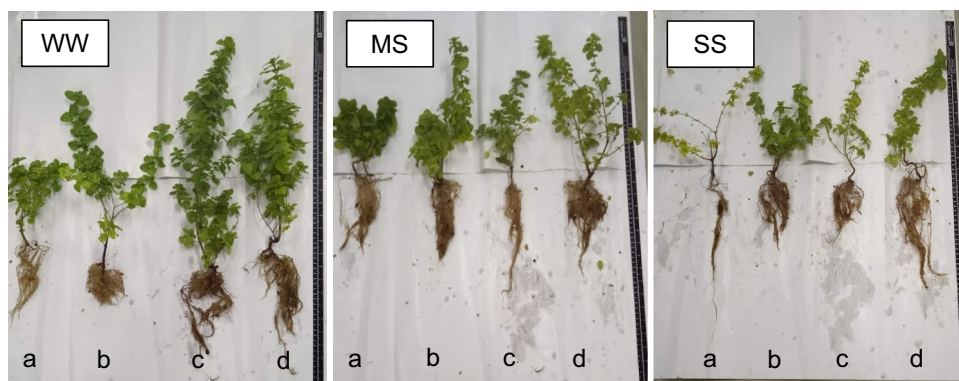


Fig. 2 a Shoot and b root dry weight of *Endostemon obtusifolius* stem cuttings subjected to various water stress levels for 8 weeks when inoculated with two endophytes. Results are presented as mean \pm standard error ($n=3$) where different letter(s) indicate a significant difference ($p < 0.05$) between treatments

(Fig. 2a). Co-inoculation treatments elicited the highest root DW across the three watering regimes.

Photosynthetic Pigments and Chlorophyll Fluorescence

The concentration of photosynthetic pigments in *E. obtusifolius* leaves were significantly affected by water stress, individual inoculation and co-inoculation with *P. polymyxa* and *F. oxysporum* (Table 1). The total chlorophyll content in *E. obtusifolius* increased significantly as the severity of water stress increased. Inoculation treatments had a positive effect with significantly higher chlorophyll content in the *F. oxysporum* (well-watered) and co-inoculation (mild and severe stressed) treated plants (Fig. 3a). Mild water stressed plants had a significantly higher carotenoid content compared to the well watered and severely water stressed plants. The carotenoid content decreased in the mild water stressed plants when treated with the inoculums. Inoculation, especially with *P. polymyxa* significantly increased the carotenoid content in well watered and severely water stressed plants (Fig. 3b).

Photochemical efficiency as measured by chlorophyll fluorescence (F_V/F_M) was significantly affected by water stress and inoculation treatments (Table 1). There was no difference between the F_V/F_M values of uninoculated plants under well watered and mild stress conditions but values decreased significantly in plants grown under severe water stress (Fig. 3c). Single inoculation treatments only increased chlorophyll fluorescence in the severe water stressed plants while the co-inoculation treatment (P + FI) increased chlorophyll fluorescence under all watering regimes (Fig. 3c).

RWC and Electrolyte Leakage

The severity of water stress, endophyte inoculation and their interactive effects significantly affected the RWC of *E. obtusifolius* leaves (Table 1). The RWC decreased in both untreated and inoculated *E. obtusifolius* plants as the severity of water stress increased (Fig. 4a). *F. oxysporum*

Table 1 Two-way analysis of variance for the effects of drought stress, endophyte inoculation and their interaction on growth parameters, metabolite content and bioactivities of *Endostemon obtusifolius*

Factor / Interaction	Drought (D)		Endophyte (E)		D+E	
	F-value	P-value	F-value	P-value	F-value	P-value
Shoot DW	371.01	0.00*	187.26	0.00*	85.26	0.00*
Root DW	1173.66	0.00*	1044.33	0.00*	168.23	0.00*
Total chlorophyll	32607.66	0.00*	28729.05	0.00*	5640.16	0.00*
Carotenoids	8.43	0.00*	364.06	0.00*	361.35	0.00*
Chlorophyll fluorescence	4279.11	0.00*	1250.44	0.00*	888.23	0.00*
Relative water content	90.31	0.00*	15.71	0.00*	3.16	0.02*
Electrolyte leakage	12.77	0.00*	16.18	0.00*	1.11	0.39 ^{ns}
Total soluble sugar	574.64	0.00*	4912.96	0.00*	2346.88	0.00*
Proline	13.67	0.00*	23.40	0.00*	22.33	0.00*
SOD	102.81	0.00*	40.68	0.00*	40.75	0.00*
MDA	41.66	0.00*	2.02	0.14 ^{ns}	0.72	0.64 ^{ns}
Total phenolics	34.94	0.00*	74.68	0.00*	22.93	0.00*
Flavonoids	7150.18	0.00*	2650.03	0.00*	1621.11	0.00*
DPPH IC ₅₀	267.52	0.00*	54.96	0.00*	40.49	0.00*
FRAP slope	286.70	0.00*	66.59	0.00*	72.57	0.00*
α-glucosidase IC ₅₀	300.14	0.00*	129.75	0.00*	27.44	0.00*

P-value probability value at $p \leq 0.05$, *F-value* the ratio of the variation between and within sample means, *ns* not significant at $p \leq 0.05$

*Significant at $p \leq 0.05$

inoculation was the most effective in improving the RWC of *E. obtusifolius* under well watered conditions and the co-inoculation treatment was the most effective under severe stress conditions. Endophyte inoculation treatments had no effect on the RWC on plants subjected to mild water stress (Fig. 4a).

The electrolyte leakage of *E. obtusifolius* was influenced by the individual factors although their synergistic effects were not statistically significant (Table 1). In general, there was an increase in electrolyte leakage as drought stress became more severe with the highest electrolyte leakage in the uninoculated plants under severe stress conditions (Fig. 4b). *F. oxysporum* and co-inoculation treatments significantly reduced electrolyte leakage with the lowest value in the well watered plants (Fig. 4b).

Osmolytes (Total Soluble Sugar and Proline Content)

Water stress, endophyte inoculation and their interactive effects significantly affected the total soluble sugar and proline contents in leaves of *E. obtusifolius* (Table 1). Uninoculated plants subjected to mild water stress had the highest total soluble sugar (Fig. 5a) and proline (Fig. 5b) content compared to well watered and severely stressed uninoculated plants. Generally, endophyte inoculation significantly promoted soluble sugar and proline accumulation across the

three watering regimes with the co-inoculation treatment consistently being the most effective (Fig. 5a and b).

Antioxidant Systems (SOD Activity, MDA Content and H₂O₂ Visualization)

Drought stress, endophyte inoculation and their interactive effects caused significant changes to SOD activity in *E. obtusifolius* (Table 1). Uninoculated plants subjected to mild water stress had higher SOD activity compared to uninoculated plants under well watered and severe stress conditions (Fig. 6a). Endophyte inoculation significantly increased SOD activity in plants grown under well watered (*F. oxysporum* inoculation) and severe stress conditions (co-inoculation treatment) but had no effect on plants grown under mild water stress (Fig. 6a).

While drought stress caused significant changes in the MDA content in *E. obtusifolius*, endophyte inoculation had no effect (Table 1). MDA content increased significantly in mild and severe water stressed plants compared to well watered plants (Fig. 6b). Endophyte inoculation reduced lipid peroxidation in mild and severe water stressed plants although the lower MDA content was not significantly different in the uninoculated plants. Endophyte inoculation had no effect on the MDA content in well watered plants (Fig. 6b).

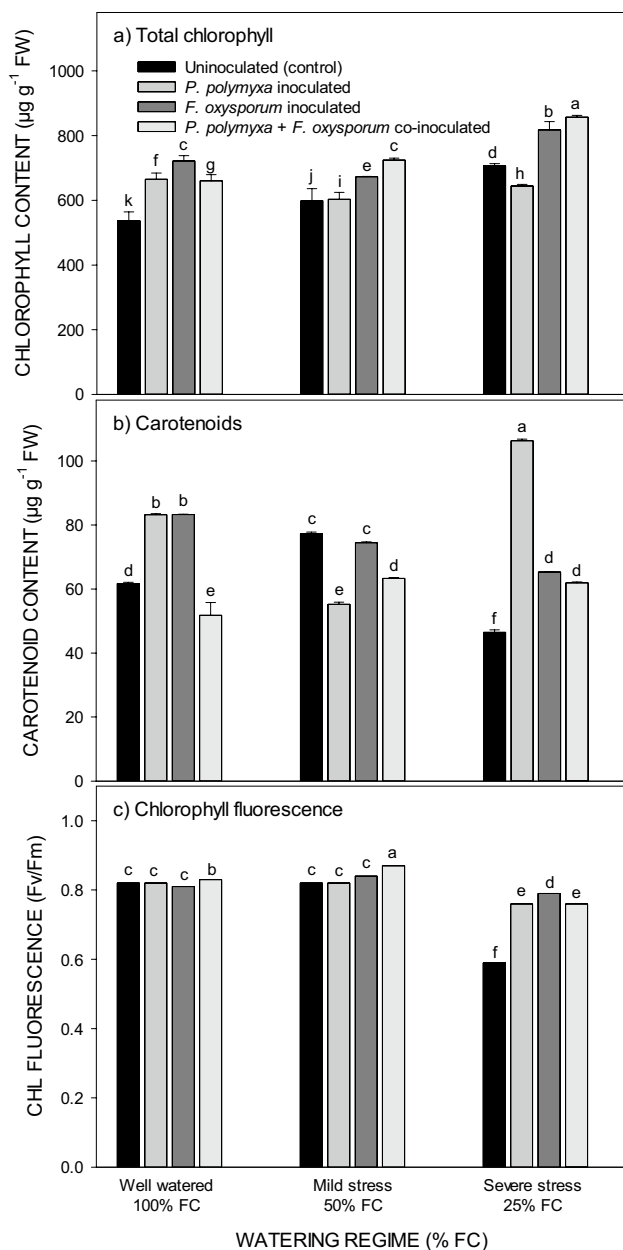


Fig. 3 **a** Chlorophyll and **b** carotenoid content and **c** chlorophyll fluorescence in leaves from *Endostemon obtusifolius* stem cuttings subjected to various water stress levels for 8 weeks when inoculated with two endophytes. Results are presented as mean \pm standard error ($n=3$) where different letter(s) indicate a significant difference ($p < 0.05$) between treatments

Histochemical visualization of H_2O_2 accumulation in the leaves of *E. obtusifolius* showed that DAB polymerization products increased under water stress (Fig. 7). Uninoculated plants grown under well watered (100% FC) and severe water stress (25% FC) conditions had higher amounts of DAB product compared to inoculated plants, indicating a reduction in H_2O_2 accumulation in inoculated plants. There was little or no visual differences between inoculated and

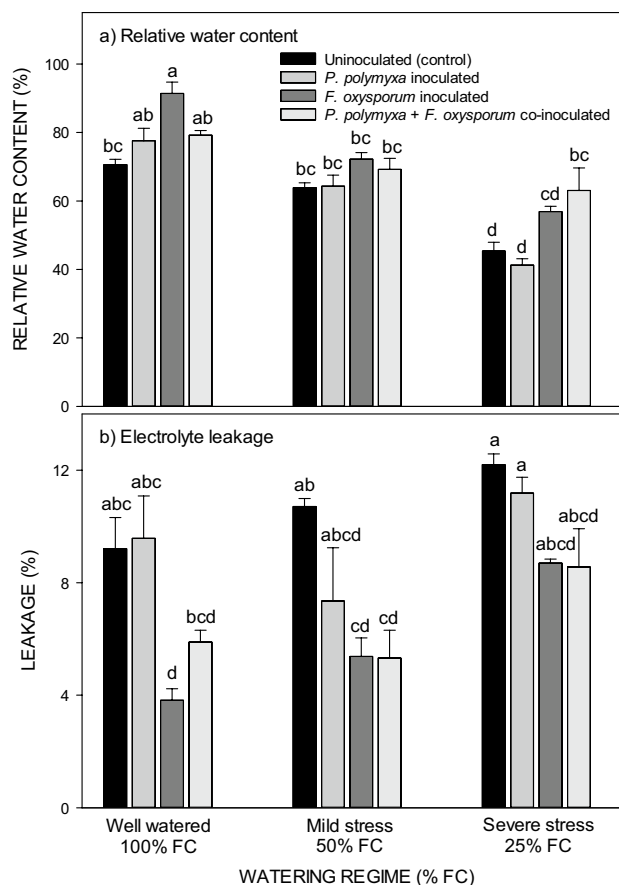


Fig. 4 **a** Relative water content and **b** electrolyte leakage in leaves of *Endostemon obtusifolius* stem cuttings subjected to various water stress levels for 8 weeks when inoculated with two endophytes. Results are presented as mean \pm standard error ($n=3$) where different letters indicate a significant difference ($p < 0.05$) between treatments

uninoculated plants grown under mild water stress (50% FC; Fig. 7).

Phytochemicals (Total Phenolic and Flavonoid Content)

The total phenolic and flavonoid contents of *E. obtusifolius* were significantly altered by the watering regime, endophyte inoculation and their interactive effects (Table 1). Uninoculated plants subjected to mild water stress had higher total phenolic (Fig. 8a) and flavonoid (Fig. 8b) content compared to uninoculated plants under well watered and severe stress conditions. The phytochemical content in the mild water stressed plants was not significantly affected by single endophyte inoculation but significantly increased with co-inoculation (Fig. 8). Both single and co-inoculation endophyte treatments significantly increased the phytochemical content in well watered and severe water stressed plants (Fig. 8).

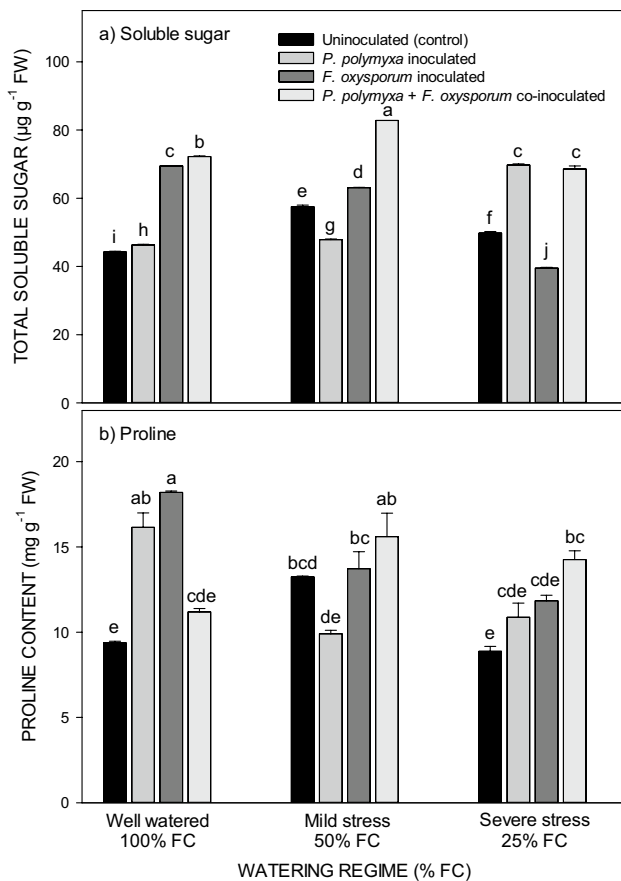


Fig. 5 **a** Total soluble sugar and **b** proline content in leaves of *Endostemon obtusifolius* stem cuttings subjected to various water stress levels for 8 weeks when inoculated with two endophytes. Results are presented as mean \pm standard error ($n=3$) where different letters indicate a significant difference ($p < 0.05$) between treatments

Antioxidant Activity (DPPH and FRAP Assays)

The radical scavenging capabilities of *E. obtusifolius* as measured in the DPPH and FRAP assays were significantly affected by the watering regimes, endophyte inoculation and their interactive effects (Table 1). Antioxidant activity of ethyl acetate extracts of leaf samples in the uninoculated treatments was similar in the DPPH and FRAP assays regardless of the watering regime (Fig. 9a and b). Endophyte inoculation significantly improved the radical scavenging abilities measured in the DPPH assay with co-inoculation treatments eliciting the lowest IC_{50} values. Co-inoculated plants subjected to mild water stress had the most potent DPPH activity (Fig. 9a). Co-inoculation had less effect on antioxidant activity as measured in the FRAP assay. While endophyte inoculation had no effect in the mild water stressed plants, extracts made from endophyte inoculated plants were more active (higher slope values) in the well watered and severe water stress treatments (Fig. 9b).

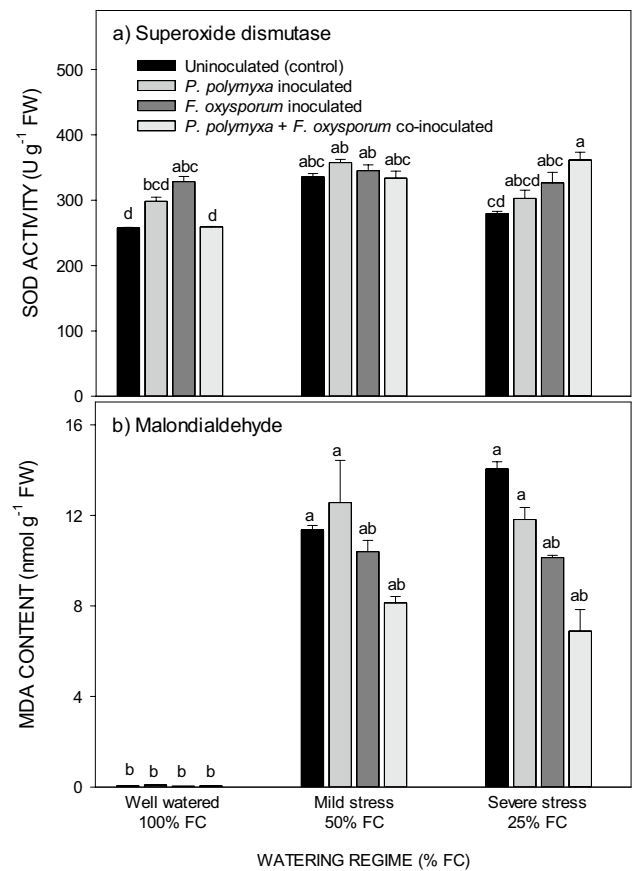


Fig. 6 Changes in **a** superoxide dismutase activity and **b** malondialdehyde content in *Endostemon obtusifolius* subjected to various water stress levels when inoculated with two endophytes. Results are presented as mean \pm standard error ($n=3$) where different letters indicate a significant difference ($p < 0.05$) between treatments

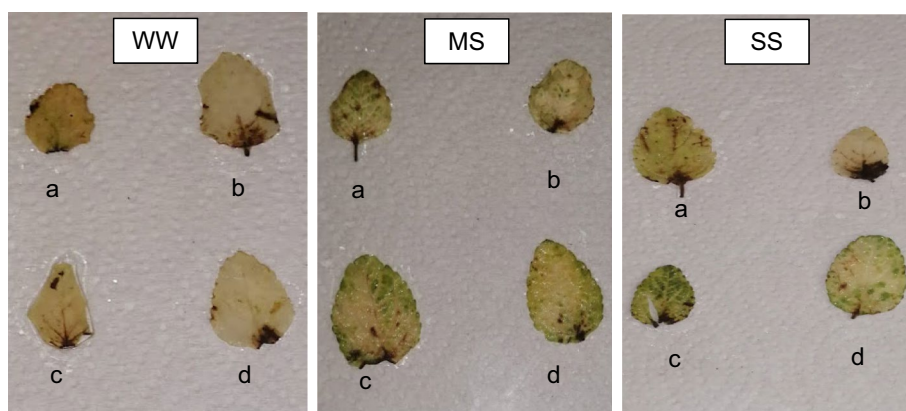
α -Glucosidase Inhibitory Activity

α -Glucosidase retardation activity of *E. obtusifolius* leaves was significantly altered by the water regimes, endophyte inoculation and their interactive effects (Table 1). The hypoglycemic properties of uninoculated *E. obtusifolius* plants significantly improved as water stress levels decreased (Fig. 9c). Endophyte inoculation significantly increased the inhibitory activity (lower IC_{50} values) in mild and severe water stressed plants. *F. oxysporum* and co-inoculated treatments were the most effective. Endophyte inoculation treatments had no effect in the well watered plants (Fig. 9c).

Discussion

The root tissues of *E. obtusifolius* used in the present study were successfully colonized by the bacterium *P. polymyxa* and fungus *F. oxysporum* when applied to the soil surrounding the plantlets. The attachment and subsequent

Fig. 7 Histochemical visualization of hydrogen peroxide accumulation in leaves of *Endostemon obtusifolius* stem cuttings grown under three watering regimes when inoculated with two endophytes. *WW* well-watered, *MS* mild stress, *SS* severe stress, a = uninoculated treatment, b = *P. polymyxa* inoculated, c = *F. oxysporum* inoculated and d = *P. polymyxa* and *F. oxysporum* co-inoculated



colonization of plants by endophytes depend on their rhizospheric presence, their motility rate towards the prospective host and their ability to break down plant cell walls (Mengistu 2020). The endophyte strains used in the present experiment were previously isolated from *E. obtusifolius* (Ogbe

et al. 2023b) and were thus predisposed towards colonizing the plantlets established for this study.

As *E. obtusifolius* occurs naturally in arid and semi-arid regions, it is adapted to grow under water deficit conditions. In the present study, uninoculated plantlets subjected to mild water stress had significantly higher growth compared to well watered and severe water stressed uninoculated plants. Individual and co-inoculations with *P. polymyxa* and *F. oxysporum* significantly improved plant growth under all watering regimes (Figs. 1 and 2). Similarly, growth was improved with microbial inoculations in some indigenous plants such as *Allium cepa* (Gana et al. 2022), *Glycyrrhiza uralensis* (Xie et al. 2019), *Astragalus mongholicus* (Sun et al. 2019) and *Piper nigrum* (Anith et al. 2018). Endophytes generally promote plant growth under normal and stressful conditions by improving the uptake of nutrients and water and through the regulation of growth and stress phytohormones (Afzal et al. 2019). Previously, 26 endophytes were isolated from *E. obtusifolius* and screened for growth-promoting characteristics. The bacteria *P. polymyxa* and fungus *F. oxysporum* isolates were selected as potential inoculi as they had good drought resistance capabilities combined with a number of beneficial plant-growth promoting characteristics. These included ammonia production, siderophore production, hydrogen cyanide, phosphate solubilization with *P. polymyxa* also having indole-3-acetic acid production and aminocyclopropane-1-carboxylate deaminase activity (Ogbe et al. 2023b).

Drought stress affects chlorophyll content differently in plants depending on plant tolerance and the severity of the stress factor. Generally, drought stress reduces photosynthetic pigments in plants (Qi and Yin 2023) but in *E. obtusifolius*, the total chlorophyll content significantly increased with drought stress severity (Fig. 3a). Similarly, drought stress induced higher chlorophyll concentrations in *Solanum tuberosum* (Ramírez et al. 2014), clones of *Eucalyptus grandii* × *E. robusta* (Michelozzi et al. 1995) and *Nicotiana tabacum* (Gubiš et al. 2007). Endophyte inoculation significantly increased the chlorophyll content in *E. obtusifolius* (Fig. 3a)

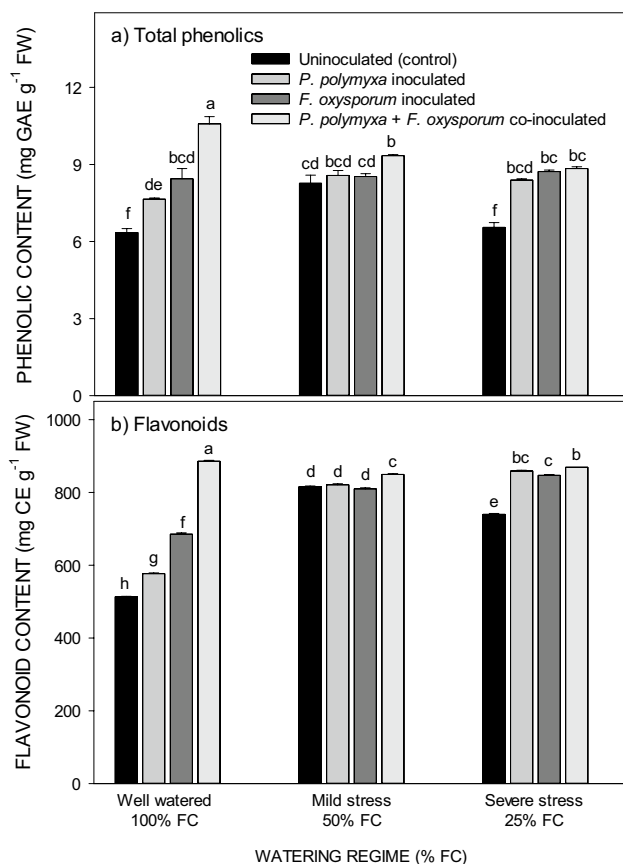


Fig. 8 Changes in **a** total phenolic and **b** flavonoid content in *Endostemon obtusifolius* subjected to various water stress levels when inoculated with two endophytes. Results are presented as mean ± standard error (n=3) where different letters indicate a significant difference (p < 0.05) between treatments

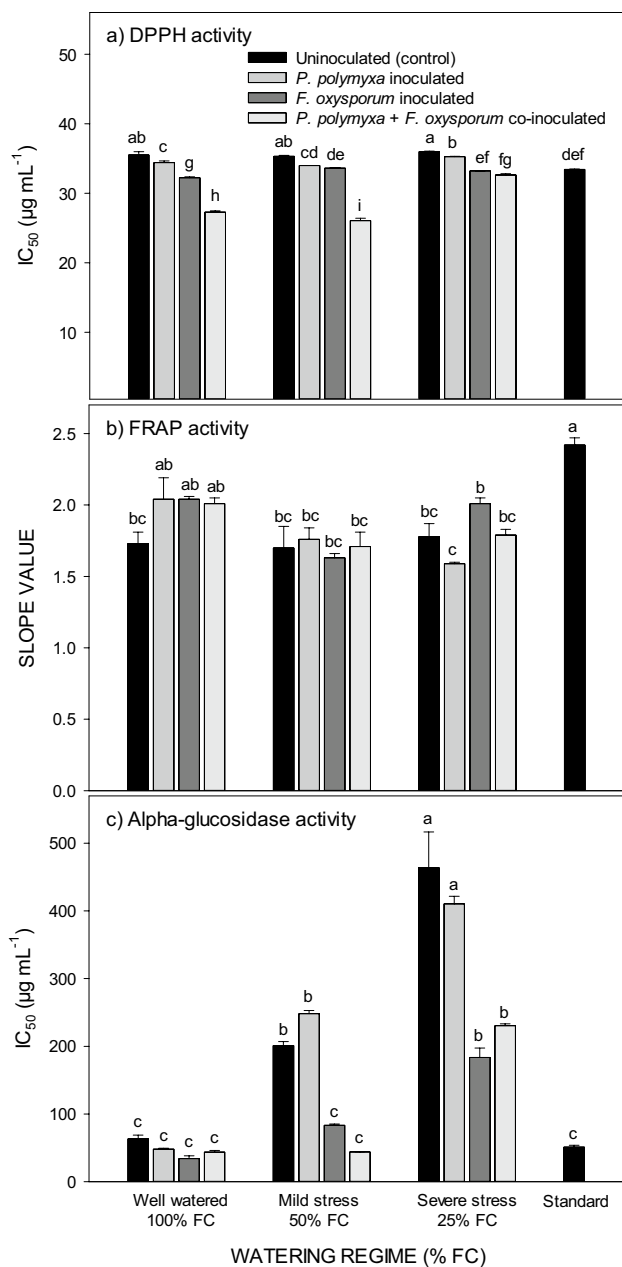


Fig. 9 **a** DPPH and **b** FRAP antioxidant activity and **c** α -glucosidase activity in *Endostemon obtusifolius* subjected to various water stress levels when inoculated with two endophytes. Activity of antioxidant butylated hydroxytoluene (a and b) and acarbose (c) standards are indicated. Results are presented as mean \pm standard error ($n=3$) where different letters indicate a significant difference ($p < 0.05$) between treatments

as in *Helianthus tuberosus* (Suebrasri et al. 2020) and *Citrus reticulata* (Sadeghi et al. 2020). Alteration in chlorophyll degradation during routine senescence occasioned by the “stay-green effect” may be responsible for the higher chlorophyll content in plants under drought stress (Ramírez et al. 2014). This can lead to a rise in ROS accumulation as

the chlorophyll continues to trap and transfer energy onto oxygen molecules (Hörtensteiner 2009). Carotenoid content was also enhanced with endophyte inoculation in well watered and severely water stressed *E. obtusifolius* plantlets (Fig. 3b). Similarly, carotenoid content increased in endophyte-treated *C. reticulata* (Sadeghi et al. 2020). Carotenoids may function as antioxidant protection to the photosynthetic system to mitigate the damage caused by excessive ROS accumulation (Al-Arjani et al. 2020).

Chlorophyll fluorescence is used as a stress marker where F_v/F_m ratio values less than 0.7 imply that the plant is not tolerant to prevailing conditions (Bu et al. 2012). *Endostemon obtusifolius* plantlets subjected to severe drought stress treatment had a decline in F_v/F_m ratios below 0.7 (Fig. 3c), indicating photoinhibition due to protein damage and subsequent disruptions in the photochemical activities at Photosystem II (PSII) reaction centers (Banks 2017). Endophyte treatments (individual and co-inoculation) improved the photochemical efficiency of PSII in both drought stressed and unstressed *E. obtusifolius* plants with the highest F_v/F_m ratio in the co-inoculated, mild water stressed plants (Fig. 3c). Endophytes had a positive influence on the maximum quantum yield in *Stevia rebaudiana* (Saravi et al. 2021) and *C. reticulata* (Sadeghi et al. 2020). Endophytes improve the photochemical efficiency of PSII via the stabilization of grana structure, synthesis of protective metabolites and the enhancement of nitrogen and potassium absorption (Al-Kahtani et al. 2020).

The leaf RWC decreased in *E. obtusifolius* grown under severe drought stress, however, it was significantly improved with endophyte treatments (Fig. 4a). RWC reflects the current physiological status of the plant and can be linked to enhanced water and nutrient transportation, stomatal regulation and efficient hydraulic conductivity in plants (Zhang et al. 2019). Endophyte inoculation led to a significant reduction in electrolyte leakage in *E. obtusifolius* grown under the three watering regimes (Fig. 4b). Drought stress would elicit higher accumulation of ROS, causing lipid peroxidation and a deterioration to cellular membrane integrity, thus leading to a rise in electrolyte leakage (Emami-Bistgani et al. 2017). Endophyte inoculation mitigated the effects of excessive ROS generation in drought stressed *E. obtusifolius* as indicated by lower oxidative stress markers such as MDA (Fig. 6b) and H_2O_2 (Fig. 7).

Endophyte inoculation influenced an array of stress tolerance mechanisms which resulted in improved drought tolerance in *E. obtusifolius*. This included enhanced accumulation of osmolytes such as soluble sugar and proline (Fig. 5). The accumulation of such metabolites under water shortage is a typical stress response (Dastogeer et al. 2018; Al-Arjani et al. 2020). Soluble sugars help maintain turgor pressure under drought stress (Emami-Bistgani et al. 2017). The amino acid

proline assists in maintaining the integrity and fluidity of cellular and molecular structures, photosynthetic apparatus (Abd-Allah et al. 2015), osmotic homeostasis (Al-Arjani et al. 2020) and scavenges free radicals (Chiappero et al. 2019). The enzymatic antioxidant system of *E. obtusifolius* was also enhanced with endophyte treatments where there was an increased SOD activity (Fig. 6a). Similarly, SOD activity was up-regulated in drought stressed *Bacillus pumilus* inoculated *G. uralensis* (Xie et al. 2019; Zhang et al. 2019). SOD is an antioxidant enzyme that converts O_2^- to hydrogen peroxide, which is further reduced to water and oxygen by the activities of other enzymatic and non-enzymatic antioxidants (Das and Roychoudhury 2014). Concentrations of antioxidant metabolites such as total phenolics and flavonoids were also increased in *E. obtusifolius* treated with endophyte inoculation (Fig. 8). The increase in these metabolites is an additional layer of defense to mitigate against the negative consequences of drought stress (Bettaieb et al. 2011). Other indigenous plants inoculated with microbial endophytes also have a higher phenolic and flavonoid content (Mona et al. 2017). Thus the endophyte inoculations used in the present study altered metabolite content and influenced some physiological processes in *E. obtusifolius*, thereby improving its growth and stress tolerance.

These physiological changes elicited by the endophytes resulted in increased antioxidant activity in leaf extracts of *E. obtusifolius*. Mild water stressed plantlets treated with co-inoculum had the highest antioxidant capacity in the DPPH assay. This activity was significantly better than the BHT standard (Fig. 9a). Endophyte inoculation significantly enhanced the antioxidant capacity of well watered plantlets in the FRAP assay to levels similar to the BHT standard (Fig. 9b). Similarly, antioxidant capacities of *Stevia rebaudiana* and *L. citriodora* increased with endophyte inoculation (Kilam et al. 2015; Golparyan et al. 2018). Application of these beneficial endophytes also enhanced the accumulation of other bioactive metabolites in *E. obtusifolius* where the in vitro α -glucosidase inhibitory potential of *E. obtusifolius* improved significantly in water stressed plants (Fig. 9c). Similarly, drought stress improved the antidiabetic activities of *Hibiscus sabdariffa* (Ifie et al. 2018) and endophytic bacteria inoculation was effective in enhancing antidiabetic activity in *Momordica charantia* (Pujianto and Ferniah 2017).

In conclusion, although *E. obtusifolius* grows in semi-arid and arid conditions, drought stress negatively affected the growth (root and shoot biomass), carotenoid content, chlorophyll fluorescence and relative water content. Under increasing water deficit conditions, *E. obtusifolius* generated more ROS which caused peroxidation of cellular molecules such as lipids and negatively affected cellular membrane integrity. Individual and co-inoculation treatments with *P. polymyxa* and *F. oxysporum* mitigated excessive ROS generation via increased osmolyte production (soluble sugars and proline), up-regulation of the enzymatic antioxidant system (SOD) and

increased antioxidant metabolite content (total phenolics and flavonoids). This suggests that *E. obtusifolius* has established a symbiotic relationship with these beneficial endophytes to enhance its stress mechanisms under drought stress conditions. The pharmacological potential (antioxidant and in vitro α -glucosidase inhibitory activities) of *E. obtusifolius* leaves was significantly improved with endophyte inoculation with the most active extracts made from leaves of mild water stressed plants. This study highlighted a potential strategy in the cultivation of medicinal plants where drought stress combined with beneficial endophyte isolates can be used to enhance the productivity and efficacy of the crop.

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Author Contributions AAO, SG and JvS conceptualized the study. The experiments and data collection was carried out by AAO and SG. AAO performed the data analysis. AAO wrote the first draft and WAS prepared the figures and reviewed and edited the manuscript. AAO was supervised by JvS and JFF. Resources and funding were provided by JvS and SG. All authors read and approved the final manuscript. WAS is an editor of this journal but was not involved in the evaluation of this manuscript.

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Data Availability The data is available upon request from the corresponding author (JvS).

Declarations

Conflict of interest There is no conflict of interests.

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