



# Dark septate endophytic fungi mitigate the effects of salt stress on cowpea plants

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## Abstract

The association of plant with microorganisms, such as dark septate endophytic fungi, has mitigated the harmful effects of chemical, physical, and biological agents on the host. The objective of this work was to evaluate the interaction of the dark septate endophytic fungi with cowpea plants under salt stress. Endophytic fungi were isolated from *Vochysia divergens* root system, and molecular identification of fungi was performed by sequencing the ITS region. We selected and identified Sordariomycetes sp1-B'2 and *Melanconiella elegans*-21W2 for their ability to infect *V. divergens* root in vitro with development of typical dark septate fungi structures. Cowpea plants— inoculated or not inoculated with Sordariomycetes sp1-B'2 and *M. elegans* 21W2—were cultivated in 5-L pots under greenhouse conditions and submitted to four different electrical conductivities of irrigation water (1.2, 2.2, 3.6, and 5.0 dS m<sup>-1</sup>). The salinity caused decrease in leaf concentration of K and increased leaf concentration of calcium, sodium, and chlorine; and no influence of dark septate endophytic fungi was observed in these responses. On the other hand, root colonization with Sordariomycetes sp1-B'2 and *M. elegans* 21W2 resulted in improved nutrition with N and P in cowpea under salt stress, favoring the growth and rate of liquid photosynthesis. However, such positive responses were evident only at moderate levels of salinity.

**Keywords** *Vigna unguiculata* L. · Salinity · Plant-fungi interaction · Photosynthesis · Mineral nutrition

## Introduction

The water crises observed in different regions of the planet, and particularly in semi-arid regions, have led to the increasing interest in the use of lower-quality water, such as brackish water. However, indiscriminate use of these water sources can

reduce crop yield and cause soil salinization. Nonetheless, there are several management strategies for the soil-water-plant system that can minimize the impacts of using water with moderate salinity, which can favor the sustainability of agricultural systems in the semi-arid environments [1]. In addition to these techniques, the use of microorganisms, such as mycorrhizal and endophytic fungi, as salt stress-mitigating agents, has been proposed [2–5].

Endophytes are microorganisms capable of colonizing internal tissues of plant organs, at least in some period of their life cycle, without causing visible symptoms of disease or morphological alterations [6]. The diversity of the endophytic community varies with plant species [7], host genotype [8], type of tissue analyzed [9], host age [10], climatic factors [11], and geographic distribution [8, 9].

Dark septate endophytic fungi (DSE) are important root endophytes involved with host resistance to environmental stress. These fungi are characterized by the presence of brown to black mycelium with septate and melanized hyphae, capable of infecting the intercellular and intracellular spaces of root tissues where it differentiates structures of resistance called microsclerotia inside the cells within a wide range of plant species [12–14].

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DSE can occur from the tropics to the Arctic and are particularly common in stressful environments, such as cold, nutritionally poor soils, alpine or subalpine ecosystems, environments with high salinity, and polar regions [15–21]. The abundance and disseminated occurrence of DSE in different biomes, colonizing more than 600 species of hosts distributed in 320 genera and 114 families, suggests lack of specificity of the host and an important role of these microorganisms in natural ecosystems [15].

In addition to natural systems, DSE are present in agroforestry systems associated with plant species of economic interest such as soybean [20, 22], rice [23], tomato [24], orchids [25], and forest species [26]. To the best of our knowledge, we found no reports of the DSE-cowpea plants association.

Cowpea is an important crop cultivated in different regions of the world, being an important source of vegetal protein for human consumption in semi-arid regions, such as the population of Brazilian Northeast [27]. This species is widely cultivated in intercropping systems and crop rotation in this region, contributing to the improvement of soil properties due to symbiotic interaction with nitrogen-fixing bacteria [28] and also with mycorrhizal fungi [29].

Dark septate fungi act as symbiont and promote host growth, facilitating the absorption of nutrients, such as nitrogen, phosphorus, and micronutrients [21, 30–35]. In plants under abiotic stress, the association of roots with DSE fungi also promotes changes in physiological and biochemical responses, resulting in improved host performance [36, 37]. For example, the presence of DSE increase tolerance of rice to water stress, reducing the oxidative stress, indicating that the beneficial effects of DSE is not due only to nutrient uptake [23]. In maize under heavy metal stress, the improvement of efficiency of photosynthesis and the decrease of translocation factor of Pb, caused by DSE fungi colonization, were efficient strategies to improve Pb tolerance of host plants [36].

The above results show that DSE fungi acts as a mitigating agent in plants under abiotic stress, and this benefic effect can be related to changes in physiological, biochemical, and nutritional responses. Since salinity is a mineral stress, our hypothesis is that the beneficial effect of DSE fungi on cowpea plants under salt stress can be related to the control on the acquisition of essential nutrients and potentially toxic ions, especially  $\text{Na}^+$  and  $\text{Cl}^-$ . In this context, the objective was to evaluate the responses of cowpea plants associated with DSE fungi, when irrigated with water of different salinity levels. In this study, we selected DSE from *Vochysia divergens* roots, an invasive species in the Brazilian Pantanal, to evaluate the functional role of these fungi in plant of agricultural interest under salt stress conditions.

## Material and methods

### Isolation of root endophytes from *Vochysia divergens* Pohl

The fungi were obtained from the roots of *Vochysia divergens* Pohl (Vochysiaceae). This tree is native to the Brazilian Cerrado, popularly known in Brazil as ‘Cambará’, and is considered an invasive species in the Pantanal, preferentially occurring in open areas and alluvial floodplains forming monospecific stands due to its rapid and extensive propagation [38, 39]. This species was chosen to obtain root endophytes because of its resistance to the long drought and flooding periods of the Pantanal [39]. *V. divergens* roots are associated with dark septate endophytic fungi, reaching root colonization rates close to 50% [40], and the functional roles of these fungi associated with *V. divergens* are not known. The association between *V. divergens* and root endophytes interferes in the metabolic profile of the host, contributing to qualitative changes in its phytochemical profile depending on the fungal species associated [41].

Plant material was collected in the area (16° 35' 22.9" S; 56° 47' 83.4" W) in the period of low soil moisture (September–dry period). Endophytes were isolated from the roots of *V. divergens* using sodium hypochlorite for superficial disinfection of the roots [42]. Root fragments were incubated in potato dextrose agar medium. The isolates were purified and stored in flasks containing PDA medium under refrigeration.

Part of the roots collected was previously fixed in FAA (formaldehyde–acetic acid–alcohol) for observation of fungal structures after the bleaching of plant tissues [43]. The segments were diaphonized with 2.5% KOH heated in a water bath for approximately 30 min in boiling water. Subsequently, KOH was drained and the roots were rinsed with tap water. The pH was acidified with 1% HCl for 5 min in water bath at 65 °C. The acid was drained from the fragments and then they were stained with 0.05% trypan blue in lactoglycerol (1:1:1, lactic acid:glycerol:water) for 15 min. The dye solution was drained and the fragments were preserved in lactoglycerol. Fungal structures were classified according to specialized literature [44]. The frequency of colonization of these structures was calculated according to the gridline intersection method [45], observing 100 squares under  $\times 400$  magnification.

### Selection of dark septate strains

Potentially dark septate strains were selected based on morphological characteristics of the mycelium and capacity to infect seedlings of the host. Strains showing brown or dark mycelium with septate and melanized hyphae were activated in PDA medium at 28 °C for 7 days. Mycelium fragments were inoculated close to the radicles of *V. divergens* seedlings

obtained according to [40]. The plates were kept under natural illumination at 28 °C. After 2 weeks, the radicles were processed according to [43] and observed under the microscope. The presence of microsclerotia and brown septate hyphae were considered indicative of dark septate.

### Identification of dark septate strains

Molecular identification was carried out only for strains capable of infecting *V. divergens* radicles in vitro with microsclerotial differentiation. The total DNA of the strains 21W2 and B'2 was obtained with the extraction kit (Norgen Biotek Corp., Canada) and the ITS region was amplified with the primers ITS1 and ITS4 [46]. The amplicons were purified using polyethylene glycol 6000 (PEG) and sequenced by the Sanger method (BigDye Terminator Cycle Sequencing). The sequences were aligned (MEGA 7) and compared with sequences obtained from the GenBank database through the nBLAST tool (<http://www.ncbi.nlm.nih.gov/>) and deposited in GenBank (Access Number): MK680501 (*Melanconiella elegans*-21W2) and MK680499 (*Sordariomycetes* sp-B'2).

### Inoculation of cowpea roots under salt stress

The experiment was carried out in a greenhouse at the Agricultural Engineering Department, located in the experimental area of the Agrometeorological Station of the Federal University of Ceará (UFC), Brazil, with coordinates 3° 45' S, 38° 33' W and average altitude of 20 m.

A completely randomized 4 × 3 factorial design was used, represented by four saline concentrations of irrigation water (1.2; 2.2; 3.6; and 5.0 dS m<sup>-1</sup>) and three treatments of inoculation of plants with dark septate endophytic fungi (without inoculation and inoculation with two different strains). The experiment was conducted with 10 replicates, totaling 120 experimental plots, each plot represented by a pot with one plant.

The experiment used seeds of cowpea (*Vigna unguiculata* (L.) Walp.), cultivar 'Setentão', obtained from the seed bank of the Plant Science Department–UFC. A pre-germination was performed on a tray with sterilized river sand to select uniform plants for the experiment. Five days after germination, the seedlings were transferred to pots containing 5.0 L of substrate, composed of river sand sterilized by sodium hypochlorite, mixed with vermiculite at 1:1 proportion, with the following characteristics: 763 g kg<sup>-1</sup> of coarse sand, 201 g kg<sup>-1</sup> of fine sand, 20 g kg<sup>-1</sup> of silt, 16 g kg<sup>-1</sup> of clay, 14 g kg<sup>-1</sup> of natural clay, sand textural class, degree of flocculation of 8.0 g kg<sup>-1</sup>, bulk density 1.54 g cm<sup>-3</sup>, particle density of 2.59 g cm<sup>-3</sup>, pH in water of 7.5, EC of 0.30 dS m<sup>-1</sup>, Na<sup>+</sup> of 0.11 cmol<sub>c</sub> kg<sup>-1</sup>, C/N ratio of 11 g kg<sup>-1</sup>, and OM of 1.34 g kg<sup>-1</sup>.

The strains *Sordariomycetes* sp1-B'2 and *M. elegans* 21W2 were used for inoculation, with the following treatments: without inoculation (NI), plants inoculated with 21W2 strain, and plants inoculated with B'2 strain. Endophytic fungi were activated on PDA plates (infusion of 200 g potato, 20 g dextrose, 15 g agar, and water-q.s. 1000 mL) for 7 days at room temperature.

Fungal inoculation was performed 5 days after germination, at the moment of transferring cowpea seedlings to the 5.0-L pots. The inoculation was performed by adding in the planting hole two fragments of culture medium (4 × 2 cm) containing mycelium of the strains used. In the control treatment, the same procedure was performed using fragments of sterile culture medium.

Fertilization began at 7 days after germination and consisted of weekly application of 250 mL per pot of Hoagland nutrient solution. Irrigation with water of different salinity levels began at 20 days after planting and continued until the end of the experiment. The highest levels of irrigation water salinity were obtained by adding in well water (1.2 dS m<sup>-1</sup>) the salts: sodium chloride (NaCl), calcium chloride (CaCl<sub>2</sub>·2H<sub>2</sub>O), and magnesium chloride (MgCl<sub>2</sub>·6H<sub>2</sub>O), dissolved at 7:2:1 ratio (proportion commonly observed in waters of Northeast Brazil), assuming the relationship between the irrigation water electrical conductivity and their concentrations (mmol<sub>c</sub> L<sup>-1</sup> = EC × 10). Irrigation was performed daily, in a localized manner, with a leaching fraction of 0.15.

### Plant measurements

At 57 days after planting, the rates of net carbon assimilation (A), transpiration (E), and stomatal conductance (gs) were measured in the first completely mature leaf from the apex, using an infrared gas analyzer (IRGA, LI-6400XT, Licor, USA). Readings were taken between 8 and 10 h, under saturating light (1500 μmol m<sup>-2</sup> s<sup>-1</sup>) and under natural conditions of temperature and CO<sub>2</sub> concentration.

Plants were then collected and their leaf area was determined (LI-3100, Area Meter, Li-Cor., In. Lincoln, NE, USA). Shoot and root materials were dried in a forced air circulation oven at 60 °C, to obtain the dry biomass. Samples of leaves, previously dried in the oven, were ground in a Wiley-type mill and used for extraction and determination of Na<sup>+</sup>, Ca<sup>2+</sup>, K<sup>+</sup>, N, P, and Cl<sup>-</sup> contents.

Root samples were separately collected to evaluate the colonization by dark septate endophytic fungi. Roots that were thinner and at least 1 cm long were selected for this purpose. Then, these roots were washed in tap water using a sieve and subjected to clearing [47]. In order to determine the frequency of root system colonization, 40 root fragments were separated and fixed on slides for observation under light microscope with × 40 magnification. The percentage of colonization by the endophytic dark septate fungus was determined using the

method of [48]. On the slides, all the root/grid intersection points were visualized and considered positive when the structures typical of the dark septate endophytic fungi were observed. Colonization percentage was calculated based on three replicates, considering the number of typical structures observed in the 40 root fragments in each slide.

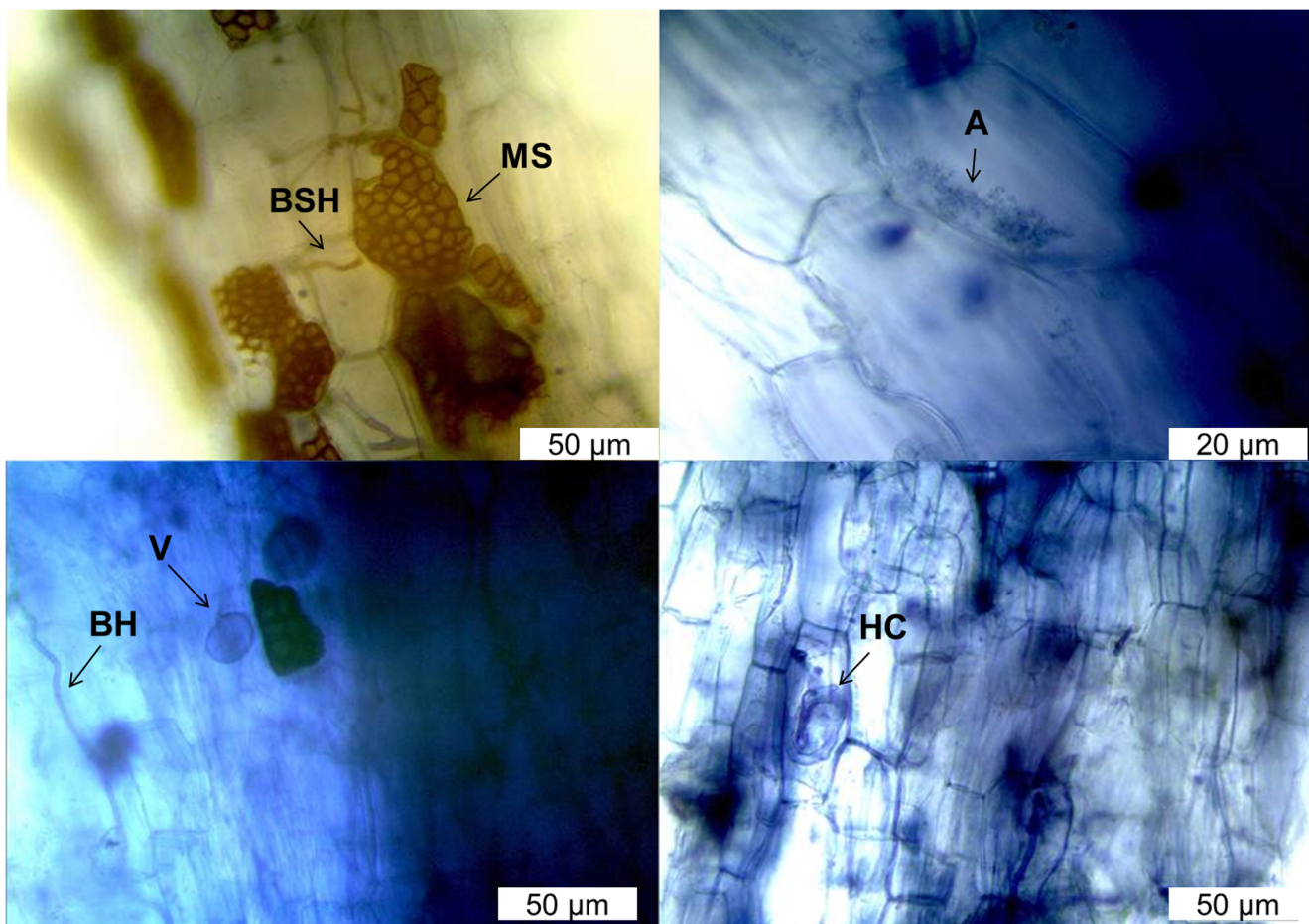
### Statistical analyses

Initially, the Shapiro-Wilk test was used to verify the normality of the data. As the data followed normal distribution, the analysis of variance was then performed by the *F* test. Regression analysis was performed to show the effects of salinity and the interaction between salinity and DSE. Tukey's test was used to compare the means of inoculated treatments within the same salinity level. For all evaluations, significance levels of 0.05 and 0.01 were used. The statistical procedures were conducted using the ASSISTAT 7.5 BETA program [49].

## Results

### Isolation, selection, and identification of root endophytes of *Vochysia divergens* Pohl

The preparations showed that *V. divergens* internally have a set of structures characteristic of dark septate endophytic and arbuscular mycorrhizal fungi (Fig. 1; Table 1). Dark septate fungi were characterized by brown septate hyphae (BSH) growing inter- and intracellularly in the root cortex. Another characteristic typical of this endophytic group was the differentiation of structures called microsclerotium (MS) intracellularly. These structures were characterized by intracellular masses of hyphae in the plant cell. Besides the colonization by dark septate fungi, *V. divergens* roots showed interaction with arbuscular endomycorrhizal fungi. Different structures, differentiated by this group of fungus, were observed, such as hyphal coil (HC), vesicles (V), and arbuscules (A) located intracellularly in the host tissue (Fig. 1; Table 1).



**Fig. 1** Morphological structural differences observed in *V. divergens* roots: microsclerotium (MS), vesicle (V), blue hypha (BH), brown septate hypha (BSH), arbuscule (A), and hyphal coil (HC)

**Table 1** Frequency of colonization (%) of microsclerotia (MS), brown septate hyphae (BSH), blue hypha (BH), vesicle (V), arbuscule (A), and hyphal coil (HC)

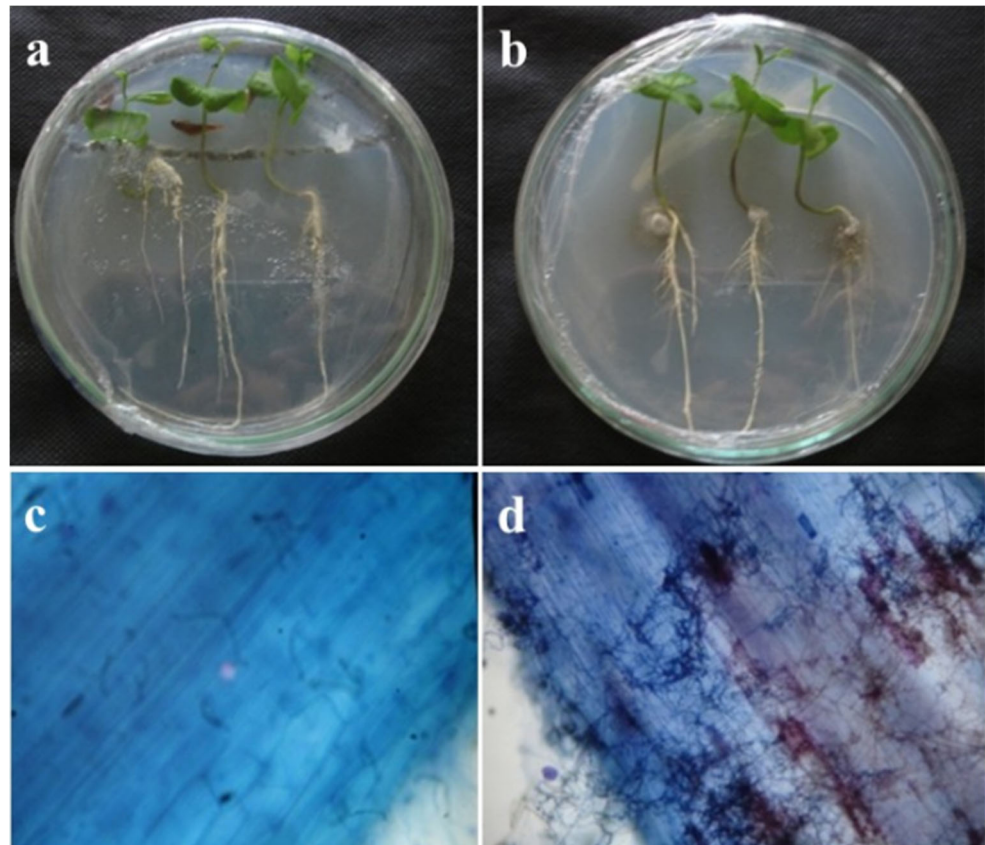
MS	BSH	BH	V	A	HC
67.7* ± 5.7	72.7 ± 5.6	84.7 ± 2.3	47.7 ± 5.2	81.7 ± 2.6	27.7 ± 1.7

\*Values represent the mean ± standard error of the mean.  $n = 3$

The observations indicate the existence of 10 strains with morphological characteristics of dark septate (brown to black and septate hyphae), but only two strains (21W2 and B'2) were able to infect *V. divergens* roots kept in vitro (Fig. 2). These two strains colonized the inter- and intracellular spaces of the root tissue, with dark septate hyphae and differentiation of microsclerotium.

Molecular identification of the strain 21W2 indicated high identity (> 98% and 100% coverage) with the ITS sequence of the fungus *Melanconiella elegans* (KJ173701.1) (hereinafter referred to as 21W2). Differently, the ITS sequence of the strain B'2 does not have sufficient identity capable of identifying at species level (> 97%), allowing only its identification as a representative of the class Sordariomycetes (96% of identity and 100% of coverage with ITS sequence of the fungus *Sordariomycetes* sp. V19-540–KJ439202.1) (hereinafter referred to as B'2).

**Fig. 2** *V. divergens* associated with the strain B'2 in in-vitro conditions. Seedlings were cultivated without (a) and with inoculation (b) on Petri dishes containing mineral medium. Radicles of seedlings not inoculated (c) and associated with the fungus B'2 (d) were observed under the optical microscope with × 100 magnification



## Colonization rate and cowpea growth

Colonization rate, growth variables (leaf area, shoot dry biomass, and root dry biomass), leaf gas exchanges (photosynthesis and stomatal conductance rates), and N and P contents were influenced by the interaction between salinity and inoculation with endophytic fungi ( $p < 0.01$  or  $p < 0.05$ ) (Table 2). On the other hand, transpiration rate and Na, Cl, Ca, and K contents were influenced only by the salinity factor ( $p < 0.01$  or  $p < 0.05$ ).

The rates of root colonization by dark septate fungi, measured at the end of the experiment, were significant for both 21W2 and B'2 (Fig. 3a). On average, the isolate B'2 had higher colonization rate, being inferior to 21W2 only in the treatment with 3.6 dS m<sup>-1</sup>. Although it was not possible to fit a mathematical model, it is clear that salinity did not negatively influence the rate of root colonization.

Leaf area (Fig. 3b), shoot dry mass (Fig. 3c), and root dry mass (Fig. 3d) data decreased as the concentration of salts in the irrigation water increased, following linear or quadratic models. In general, treatments that were inoculated showed higher means compared with the control for the same level of irrigation water salinity, with significant differences for the three variables studied.

The stress linearly reduced cowpea leaf area in the three treatments of inoculation, and the differences between them were significant at the highest salinity level. The increase in

**Table 2** Summary values of variance analysis for colonization rate (CR), photosynthetic rate (A), stomatal conductance (gs), leaf area, shoot dry mass (SDM), roots dry mass (RDM), and leaf concentration

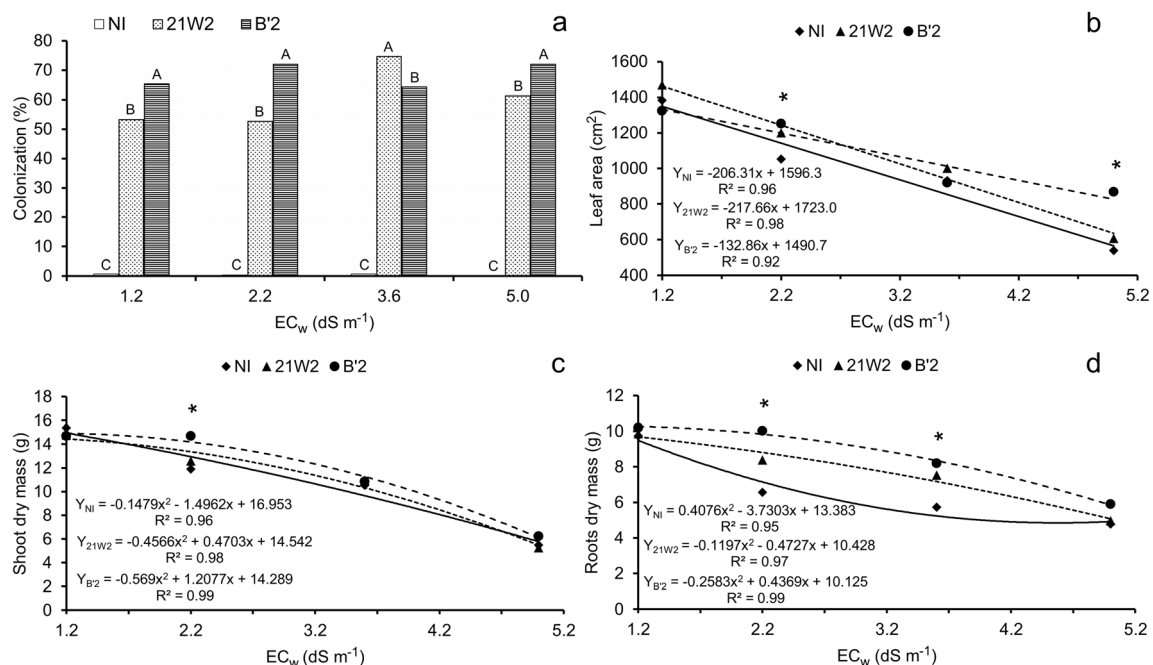
Mean square								
Source of variation	DF	CR	A	gs	DF	Leaf area	SDM	RDM
Salinity (A)	3	80.52**	25.838**	0.140**	3	2849611.87**	4858541.42**	1189254.78**
Fungi (B)	2	16593.36**	13.364**	0.021**	2	148290.158**	72432.70*	353454.15**
A × B	6	142.87**	10.813**	0.011**	6	112424.358**	58591.38*	40449.86*
Error	24	8.58	1.519	0.003	108	23101.869	22511.45	19468.09
CV%	-	6.80	10.00	20.94	-	14.52	13.48	18.23
Source of variation	DF	N	P	Ca	K	Na	Cl	
Salinity (A)	3	251.22**	2.26**	30.42**	77.48**	3.50**	26721.18**	
Fungi (B)	2	40.90**	7.81**	0.40 <sup>ns</sup>	1.55 <sup>ns</sup>	0.01 <sup>ns</sup>	24.77 <sup>ns</sup>	
A × B	6	4.33**	1.01**	0.20 <sup>ns</sup>	5.07 <sup>ns</sup>	0.01 <sup>ns</sup>	6.74 <sup>ns</sup>	
Error	48	0.87	0.20	0.50	3.87	0.04	70.12	
CV%	-	5.13	9.26	6.77	9.17	17.87	7.92	

\*\* \*, and <sup>ns</sup> Significant at 0.01 and 0.05 probability, and not significant by the *F* test, respectively. *DF* degrees of freedom, *CV* coefficient of variation

electrical conductivity from 1.2 to 5.0 dS m<sup>-1</sup> caused reductions of 58.2%, 56.6%, and 38.0% in leaf area for the treatments NI, 21W2, and B'2, respectively, and this last-mentioned isolate stood out at this level of severe stress (Fig. 3b).

In terms of root biomass production, the differences between treatments with and without inoculation were higher at the intermediate levels of salinity (2.2 and 3.6 dS m<sup>-1</sup>). For these salinity levels, the degree of reduction in the

treatment without inoculation was 2.4 and 4.0 times higher than that observed in the treatments 21W2 and B'2, respectively. The reductions observed in the treatment of 5.0 dS m<sup>-1</sup>, compared with the lowest salinity level (1.2 dS m<sup>-1</sup>), were similar between treatments with and without inoculation, reaching values of 48.2, 45.4, and 43.0%, respectively, in the treatments NI, 21W2, and B'2 (Fig. 3d). Similar results were found for shoot biomass production (Fig. 3c), but the



**Fig. 3** Root colonization (a), leaf area (b), shoot dry mass (c), and root dry mass (d) of cowpea plants irrigated with saline water and inoculated with dark septate endophytic fungi. NI = without inoculation; 21W2 = roots inoculated with strain 21W2; and B'2 = roots inoculated with strain

B'2. Fig. 3a: bars with the same letter do not differ by Tukey's test ( $p < 0.01$ ),  $n = 3$ . Fig. 3 b, c, and d: \*significant differences by Tukey's test ( $p < 0.05$ ),  $n = 10$

differences between treatments with and without inoculants were much lower than those observed for root biomass (Fig. 3d), even at the intermediate levels of salinity.

### Leaf gas exchanges

Increasing irrigation water salinity from 1.2 dS m<sup>-1</sup> to 5.0 dS m<sup>-1</sup> caused reductions in the photosynthetic rate of 40.0, 0.6, and 22.4%, respectively, in the treatments NI, 21W2, and B'2 (Fig. 4a). In the treatment with the fungus 21W2, a quadratic response was observed, with reduction in the photosynthetic rate from the salinity level of 3.8 dS m<sup>-1</sup>. Significant differences between the treatments with inoculation were observed from the salinity level of 3.6 dS m<sup>-1</sup>, when the NI treatment showed lower means compared with the inoculated treatments, demonstrating that the plant-dark septate fungus association improved the photosynthetic rate of cowpea plants. Improvement in photosynthetic rate seems to be related to the maintenance of higher values of stomatal conductance (Fig. 4b), and higher values were found in inoculated plants, especially with the strain 21W2.

### Mineral contents

Leaf contents of nitrogen decreased with increasing levels of irrigation water salinity (Fig. 5a). However, plants inoculated with dark septate endophytic fungi showed higher values, except at the highest level of salinity. Inoculated plants also had higher leaf contents of P (Fig. 5b), especially at low and intermediate levels of salinity.

Salinity caused reduction in potassium contents and increase in calcium, sodium, and chloride contents (Fig. 6), and these responses were not affected by the inoculation of dark septate endophytic fungi. Increase in irrigation water salinity from 1.2 dS m<sup>-1</sup> to 5.0 dS m<sup>-1</sup> caused increment of more than 200% in the sodium content of cowpea leaves. It is worth emphasizing that, for the chloride content, the increment in salinity from 1.2 dS m<sup>-1</sup> to 2.2 dS m<sup>-1</sup> was already significant and the increment from 1.2 dS m<sup>-1</sup> to 5.0 dS m<sup>-1</sup> led to an increase of more than 140%.

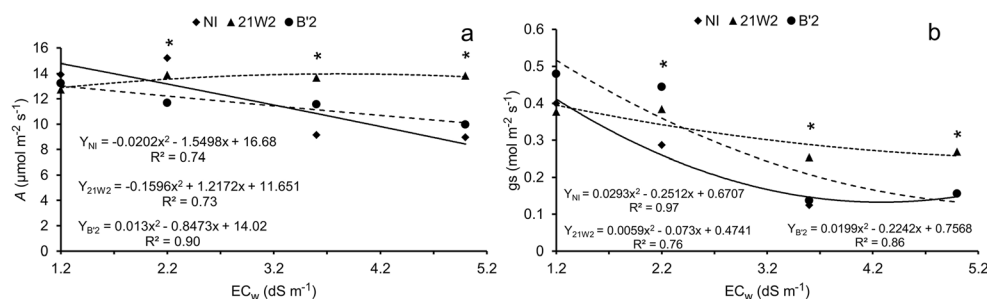
## Discussion

The strains *Melanconiella elegans*-21W2 and *Sordariomycetes* sp.-B'2 infected the root system and showed characteristics of dark septate in the roots of both *V. divergens* and cowpea plants. *Sordariomycetes* is a class commonly reported in the communities of root endophytic fungi of different hosts in Brazilian biomes [8, 42, 50] and is one of the representative classes of dark septate fungi [51, 52].

The colonization rates of DSE in cowpea roots were high under control conditions and remained high even at high levels of salinity (Fig. 3a), demonstrating the capacity of resistance of endophytic fungi under salt stress conditions [17–20, 23].

Such ease of plant root colonization by DSE has been observed under different natural conditions, from regions of Antarctica to areas of temperate, tropical, and arid and semi-arid climates [8, 15, 53, 54]. For instance, the characteristic structures of dark septate fungi (MS and BSH) in *V. divergens* roots (Fig. 1; Table 1) have also been reported in other plant species of the Brazilian Pantanal, including representatives of the families Combretaceae [42], Lamiaceae [50], Vochysiaceae, and Poaceae [40], and in various hosts of other humid areas, such as *Serratula tinctoria* L., *Betonica officinalis*, *Lycopodiella inundata* (L.) Holub, and *Carex sp* [55]. On the other hand, [56] observed colonization of endomycorrhizal and dark septate endophytic fungi in different grass species in southern India. According to [57], myelination of the hypha causes DSE to be more resistant and may be related to the defense mechanism of the fungus under more severe environmental conditions.

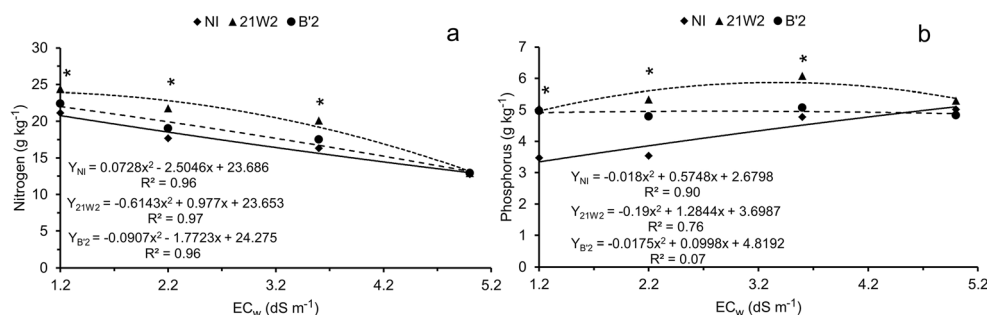
Dark septate endophytes fungi were shown to increase the tolerance of rice plants to stress caused by water deficiency [23]. Cowpea growth data (Fig. 3) demonstrate that plants inoculated with the fungal isolates were significantly more tolerant to the negative effects of salinity than non-inoculated plants, and the responses were more evident at the intermediate levels of salinity, notably for the biomass data. These results are similar to those observed in other crops, such as cucumber [58] and soybean [2, 59], where plants



**Fig. 4** Net photosynthetic rate-A (a) and stomatal conductance-gs (b) of cowpea plants irrigated with saline water and inoculated with dark septate endophytic fungi. NI = without inoculation; 21W2 = roots inoculated

with strain 21 W2; and B'2 = roots inoculated with strain B'2. \*significant differences by Tukey's test ( $p < 0.05$ ).  $n = 3$

**Fig. 5** Leaf concentrations of nitrogen (a) and phosphorus (b) of cowpea plants irrigated with saline water and inoculated with dark septate endophytic fungi. NI = without inoculation; 21W2 = roots inoculated with strain 21W2; and B'2 = roots inoculated with strain B'2. \*significant differences by Tukey's test ( $p < 0.05$ ).  $n = 5$



inoculated with endophytic fungi also had higher values of biometric variables than non-inoculated plants under salt stress conditions. Such behavior can be explained by the capacity of endophytic fungi to produce growth-inducing phytohormones, especially indoleacetic acid and gibberellins [2, 59, 60].

Inoculated plants were also superior in terms of photosynthesis and stomatal conductance (Fig. 4), showing better physiological performance at the end of the experiment. However, the instantaneous measurements of photosynthesis were not similar to those of the growth data (Fig. 3), which reflect the whole response of the plant along the entire experimental period. In relation to that, it was observed that the inoculation of B'2 caused the best response in terms of growth whereas the inoculation of 21W2 led to the best physiological response of leaves at the end of the experiment. It becomes clear, however, that root colonization with dark septate endophytic fungi results in benefits to plants, compared with not inoculated ones. Such beneficial effect of endophytic fungi on C assimilation rate has also been observed in maize plants under conditions of stress by heavy metals [36, 61].

DSE-rice plants association increasing the tillering and nutrients uptake, especially N (due to an enhanced affinity for N uptake) and P [33]. Plants associated with the fungus 21W2 showed higher contents of N and P than the plants of the other treatments (Fig. 5). This result is consistent with the evaluations of photosynthetic rate, which was higher in plants inoculated with this strain (Fig. 4), indicating that their leaves

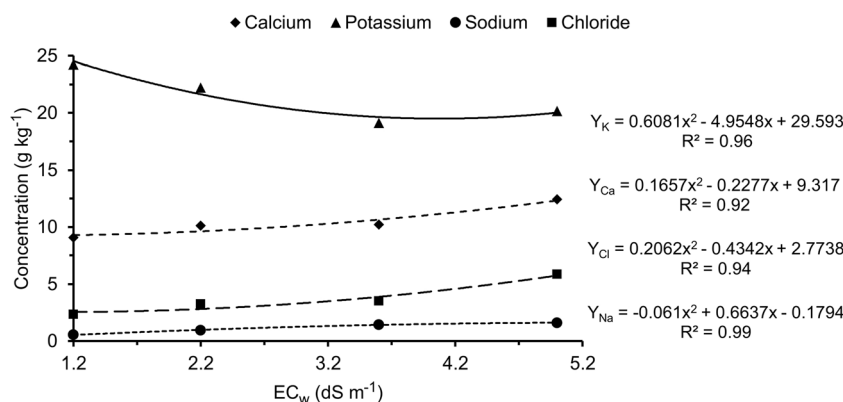
showed better nutritional status at the end of the experiment, resulting in higher capacity for C assimilation.

Lower contents of nutrients in plants under salt stress are mainly due to the osmotic effects and predominance of some ionic species in saline media, which act by competing with essential nutrients for absorption sites in the root cell membranes [62]. In the case of N, the reduction caused by salt stress has been attributed to the excess of chloride, which causes decrease in nitrate absorption due to the direct competition between these ions for the same transporter, or to reduced water flow caused by the excess of salts in the root zone [1, 63].

Dark septate endophytic fungi facilitate the absorption of nutrients such as N and P [21, 30, 32, 33, and]. Therefore, the higher contents of P and N in the leaves of cowpea inoculated with dark septate endophytic fungus (Fig. 5) can be justified by the ability of the fungus to produce extraradical hyphae which explore soil regions that roots cannot reach, allowing higher absorption of nutrients necessary for plant growth. It should be pointed out that dark septate endophytic fungi are able to solubilize P that is unavailable to plants, favoring their nutrition and growth in stressful environments [64–66].

Inoculation of cowpea roots with dark septate endophytic fungi did not influence the acquisition of K, Ca, Na, and Cl by cowpea plants, under conditions of high and low salinity (Fig. 6). The increase in irrigation water salinity from 1.2 dS m<sup>-1</sup> to 5.0 dS m<sup>-1</sup> caused an increase of 37%, on average, in leaf Ca content. Such increment in Ca content in the leaves can be explained by

**Fig. 6** Leaf concentrations of calcium, potassium, sodium, and chloride of cowpea plants as a function of levels of irrigation water salinity.  $n = 5$





the high concentration of Ca in the irrigation water, notably in the treatments of highest salinity, since CaCl<sub>2</sub> was used as source of water salinization.

The contents of sodium and chloride, ions considered potentially toxic [67], increased as water salinity increased, regardless of the inoculation treatments (Fig. 6). The accumulation of sodium in the root environment, due to salinity, causes reduction in the leaf contents of K<sup>+</sup>, as observed in the present study (Fig. 6). According to [62], the lower K<sup>+</sup> absorption is caused by the competition with Na<sup>+</sup> ions for the same sites of the absorption system in the plasma membrane of root cells.

The differences of growth in plants under salt stress can be explained, at least in part, by the lower accumulation of potentially toxic ions or by the maintenance of K nutrition [67]. In the present study, however, the differences observed in the growth (Fig. 3) and photosynthetic rate (Fig. 4) of plants under salt stress, comparing plants inoculated and not inoculated, seem to be associated with the beneficial effect of the fungus on the acquisition of N and P (Fig. 5) and not with the control over Na, Cl, and K absorption (Fig. 6). The capacity of dark septate endophytic fungi to reduce the absorption and translocation of potentially toxic ions has been reported for conditions of stress by heavy metals [36, 68, 69]. Nonetheless, heavy metals are normally found at concentrations that are much lower than those observed for cations and anions in saline environments, especially Na<sup>+</sup> and Cl<sup>-</sup>. Therefore, our results indicate that endophytic fungi were not able to limit the absorption and transport of these potentially toxic ions present at high concentrations in the irrigation water.

## Conclusions

Cowpea plants associated with DSE Sordariomycetes sp1-B'2 and *Melanconiella elegans*-21W2 fungi, isolated from the *V. divergens* root system, and this association improved nutrition with N and P in cowpea under salt stress, favoring growth, and net photosynthetic rate. Therefore, endophytic fungi were not able to limit the absorption and transport of potentially toxic ions (Na<sup>+</sup> and Cl<sup>-</sup>) present at high concentrations in the irrigation water.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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