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Physiological changes in tomato colonized by dark septate endophytic fungi

Alterações fisiológicas em tomateiro colonizado por fungos endofíticos dark septate

Alteraciones fisiológicas en tomate colonizado por hongos endófitos septados oscuros

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Carlos Vergara
ORCID: https://orcid.org/0000-0001-9002-0934
Universidade Federal Rural do Rio de Janeiro, Brasil
E-mail: vergaramaputo93@gmail.com
Karla Emanuelle Campos Araujo
ORCID: https://orcid.org/0000-0001-8932-2775
Faculdade de Ciências Agrárias de Araripina, Brasil
E-mail: karlaeca@gmail.com
Jerri Édson Zilli
ORCID: https://orcid.org/0000-0003-2138-3488
Embrapa Agrobiologia, Brasil
E-mail: jerri.zilli@embrapa.br

Abstract
As is customary in mycorrhizae, the interaction between plant and dark septate endophytic (DSE) fungi can result in physiological changes in the host plant, which are still poorly understood. This study aimed to evaluate the physiological changes in tomato plants colonized by DSE fungi. Four DSE isolates previously identified through ITS phylogeny were inoculated on tomato seeds and compared to non-inoculated plants (control). Kinetic parameters (V_{max} and K_m) were calculated measuring the nitrate content in the nutrient solution. The contents of NO_3^--N, NH_4^+-N, amino-N, soluble sugars in the root, petiole, stem and leaf, and the contents of macronutrients in the shoot were determined. The plants inoculated with A101 and A105 exhibited (i) significant increases in the soluble sugar contents; (ii) increases in the contents of P, K, Mg and S; and (iii) increased dry biomass compared to control. The A103 inoculation was antagonistic when compared to the other treatments, leading to a higher influx of NO_3^--N in the plants, resulting in a higher amino-N and the lower soluble sugar content in the shoot. The physiological parameters of tomato varied depending on the inoculation, and the changes ranged from positive to negative depending on each isolate involved in the interaction.
Keywords: Root colonization; Tomato; Nitrogen fraction; Soluble sugars; pH.

Resumo
Assim como acontece nas micorrizas, a interação entre vegetais e fungos dark septate (DSE) pode resultar em alterações fisiológicas na planta-hospedeira, que ainda são pouco compreendidas. Este trabalho teve como objetivo avaliar alterações fisiológicas em tomateiro colonizado por fungos DSE. Quatro isolados DSE, previamente identificados filogeneticamente através de ITS, foram inoculados em sementes de tomate e comparados com plantas não-inoculadas (controle). Parâmetros cinéticos ($V_{máx}$ e K_m) foram calculados medindo o conteúdo de nitrato na solução nutritiva. Foram determinados os teores de N-NO_3^-, N-NH_4^+, N-amino livre e açúcares solúveis na raiz, pecíolo, caule e folha, bem como os teores de macronutrientes na parte aérea. As plantas inoculadas com A101 e A105 apresentaram (i) aumentos significativos nos conteúdos de açúcares solúveis; (ii) aumentos nos conteúdos de P, K, Mg e S; e (iii) aumento na biomassa seca em relação ao controle. A inoculação com A103 foi antagônica em relação aos demais tratamentos, levando a um maior influxo de N-NO_3^- nas plantas, que resultou em maior N-amino e menores teores de açúcares solúveis na parte aérea. Os parâmetros fisiológicos do tomateiro variaram em função da inoculação, e as alterações variaram de positivas a negativas dependendo de cada isolado envolvido na interação.
Palavras-chave: Colonização radicular; Tomate; Frações nitrogenadas; Açúcares solúveis; pH.

Resumen
Al igual que con las micorrizas, la interacción entre las plantas y los hongos septados oscuros (DSE) puede provocar alteraciones fisiológicas en la planta hospedera, que aún no se conocen bien. Este estudio tuvo como objetivo evaluar las alteraciones fisiológicas en plantas de tomate colonizadas por hongos DSE. Cuatro aislados de DSE, previamente identificados filogenéticamente a través de ITS, fueron inoculados en semillas de tomate y comparados con plantas no inoculadas (control). Los parámetros cinéticos ($V_{máx}$ y K_m) se calcularon midiendo el contenido de nitrato en la solución nutritiva. Se determinaron las concentraciones de N-NO_3^-, N-NH_4^+, N-amino libre, azúcares solubles en raíz, pecíolo, tallo y hoja, así como los contenidos de macronutrientes en la parte aérea. Las plantas inoculadas con A101 y A105 exhibieron (i) aumentos significativos en los contenidos de azúcar soluble; (ii) incrementos en los contenidos de

P, K, Mg y S; y (iii) mayor biomasa seca en comparación con el control. La inoculación de A103 fue antagónica en comparación con los otros tratamientos, lo que condujo a una mayor entrada de N-NO_3^- en las plantas, lo que resultó en un mayor contenido de N-amino y un menor contenido de azúcar soluble en la parte aérea. Los parámetros fisiológicos del tomate variaron dependiendo de la inoculación, y las alteraciones variaron de positivos a negativos dependiendo de cada aislado involucrado en la interacción.
Palabras clave: Colonización de raíces; Tomate; Fracciones nitrogenadas; Azúcares solubles; pH.

1. Introduction

The cultivated tomato *Solanum lycopersicum* (L.) (Tubiflorae: Solanaceae) is a cosmopolitan specie descended from the wild species *S. lycopersicum* var. *cerasiforme*, which produces cherry-type fruit (Lucini, 2013). The fruit of this plant is an important source of vitamins and minerals, flavonoids and carotenoids (Paixão et al., 2020), and the carotenoid lycopene is an anticancer substance (Filgueira, 2008).

The tomato crop has a high nutrient demand, primarily due to its production capacity, which has justified the adoption of massive fertilization efforts by farmers (Filgueira, 2008). In agrosystems, excessive N fertilization can intensify nitrous oxide (N_2O) emissions and NO_3^- leaching. N_2O has a global warming potential 298 times higher than that of CO_2 (Cerri et al., 2007), while NO_3^- leaching causes environmental contamination and public health problems (Alaburda & Nishihara, 1998). In this sense, the use of growth-promoting microorganisms, such as dark septate endophytic (DSE) fungi, has gained prominence given that they can improve the recovery efficiency of fertilizers by plants.

DSE fungi are a group of phylogenetically diverse endophytic fungi belonging to the class Ascomycetes, which are distributed all over the world and associated with a wide range of hosts. These fungi can be found in approximately 600 plant species belonging to 320 genera and 114 families, including *Solanaceae* (Addy et al., 2005; Jumpponen & Trappe, 1998). DSE fungi are characterized by dark pigmentation, septate hyphae and microsclerotia that colonize the root epidermis and cortex, both intercellularly and intracellularly, and are present in all parts of the healthy roots of various plants (Addy et al., 2005; Barrow & Aaltonen, 2001; Jumpponen & Trappe, 1998; Mandyam & Jumpponen, 2008; Peterson et al., 2008; Yu et al., 2001).

In general, the herbaceous plants/DSE fungi interaction shows that this group of fungi can improve the efficiency of nutrient use (Alberton et al., 2010; Vergara, et al., 2019) and plant growth through the absorption and transfer of nutrients, such as nitrogen, primarily from organic sources, when a mutual interaction is established (Mahmoud & Narisawa, 2013; Usuki & Narisawa, 2007; Vergara, et al., 2018). Other studies have shown the contribution of these fungi to (i) the protection of plants against biotic stresses (Andrade-Linares et al., 2011) (ii) increased chlorophyll content; (iii) the quantum efficiency of photosystem II in plants (Zhang et al., 2012); and (iv) glucose content in tomato fruits (Andrade-Linares et al., 2011).

In a previous study, we evaluated the effect of DSE fungi on tomato plants, growing under organic and inorganic sources, and observed positive effects of inoculation (Vergara et al., 2017). But what were the physiological changes promoted by the fungus-plant interaction? That is what we are trying to answer in this article; for this, we determined, under controlled conditions, nitrogen fractions (NO_3^--N, NH_4^+-N, amino-N), soluble sugars and kinetic parameters (V_{max} and K_m) of NO_3^- uptake (based on low concentrations of this anion in the external medium), nutrient solution pH, macro and micronutrients, and growth in the Santa Clara I-5300 tomato variety with and without inoculation with different isolates of dark septate fungi, previously identified through ITS phylogeny (Ribeiro, 2011). These experiments were performed to evaluate physiological aspects and nutrient accumulations in plants colonized by dark septate fungi.

2. Methodology

2.1 Fungal isolates

All the fungi isolates investigated here were obtained from *O. glumaepatula* and identified through ITS phylogeny (Ribeiro, 2011) and deposited in the CRB-JD (Centro de Recursos Biológicos Joanna Döbereiner at Embrapa Agrobiologia – www.embrapa.br/agrobiologia/crb-jd) (A101, A103, A104, and A105). The ITS region sequences are deposited in GenBank (KR817246 = A 101, KR817248 = A103, KR817249 = A104, and KR817250 = A105).

2.2 Experimental design, treatments, and conditions of the incubator room

This experiment followed the quantitative laboratory research methodology proposed by Pereira et al. (2018), being conducted in an incubator room at Embrapa Agrobiologia, in Seropédica, Rio de Janeiro state, Brazil. The experimental design was completely randomized, with tomato plant treatments grown without (control) and with the inoculation of DSE. Each treatment, including the control, had four replicates, and each replicate had four plants. The Santa Clara I-5300 variety was used, along with the fungal isolates A101, A103, A104 and A105 (Ribeiro, 2011). The Santa Clara I-5300 variety possesses indeterminate growth and belongs to the Santa Cruz group, which has been cultivated in the Brazilian Center-South since the 1940s (Filgueira, 2008). The plants were grown with a 13 h/11 h (light/dark) photoperiod, a luminosity of 384 μmol m^{-2} s^{-1} (photosynthetically active photon flux), a relative humidity of 50-60% and a temperature of 28 ºC/24 ºC (day/night).

2.3 Inoculation and growth conditions

The tomato plants were grown in disposable Petri plates containing sterilized agar-water (1%), adapted by Vergara, Araujo, Alves, et al. (2018) to allow for mycelial growth and development of the fungus. The fungal isolates were previously grown on potato-dextrose-agar (PDA) medium for seven days at 28 ºC. The tomato seeds were washed in 70% alcohol for three min and disinfected with 2.5% sodium hypochlorite for three min, followed by eight successive washes in autoclaved distilled water. The seeds were placed on the agar-water medium. Additionally, one disk of PDA medium (approximately 8 mm in diameter) containing mycelia was placed along side each seed. The Petri plates for the control plants were inoculated with PDA plugs without fungal mycelium. After sowing, the Petri plates were incubated for three days at 28 °C to allowed fungal germination and establishment.

After the seeds had germinated, four Petri plates with uniformly sized seedlings were selected for transfer to glass pots containing only autoclaved, distilled water (120 °C for 1 h, at 24 h intervals). After five days, the water contained in the pots was replaced with a nutrient solution that was formulated according to Hoagland and Arnon (1950) at 1/4 of ionic strength. This solution was modified with 1.5 mmol L^{-1} NO_3^--N (KNO_3 as the source). After three days, this solution was replaced by another solution at 1/2 of ionic strength, with 2.0 mmol L^{-1} NO_3^--N and 0.5 mmol L^{-1} NH_4^+-N, using $Ca(NO_3)_2$ and $(NH_4)_2SO_4$, respectively. This strategy was adopted to avoid causing salt stress in the plantlets (Furlani & Furlani, 1988). Thereafter, the 1/2 of ionic strength solution was replaced every three days.

Thirty-eight days after germination (DAG), the plants were deprived of nutrient solution NO_3^--N for a period of 72 h to increase the roots' capacity to uptake N, (Lee & Rudge, 1986) after which a nutrient solution containing 0.5 mmol L^{-1} NO_3^- (again using KNO_3 as the source) was supplied. The samples of the solution were then collected every 30 min by removing 0.5 mL aliquots from each pot to plot the depletion curves and determine the kinetic parameters V_{max} and K_m (Baptista et al., 2000; Santos et al., 2011). The samples were placed in microtubes, and the NO_3^- concentration was measured according to Miranda et al. (2001) as adjusted following Alves et al. (2016). The V_{max} and K_m values were determined using the mathematical graphing method proposed by Cometti et al. (2006). with the Cineticawin 1.0 program (Universidade Federal de Viçosa, Minas Gerais, Brazil). The pH of the nutrient solution was also measured at 30 min intervals, and the proton content was calculated.

2.4 Soluble fraction, colonization measurement and DSE structures

Following the collection of the last nutrient solution sample, the plants were cut and the heights from each plant were determined. The dry biomass of the root, petiole, stem, leaf and shoot were measured after being dried in a forced-air chamber at 65°C until reaching a constant weight.

The fresh samples (one gram of root, petiole, stem, and leaf tissue) were homogenized in 80% ethanol. After partitioning the samples with chloroform (Fernandes, 1984), the levels of NO_3^--N (Miranda et al., 2001), free amino-N (Yemm & Cocking, 1955), NH_4^+-N (Felker, 1977), and soluble sugars (Yemm & Willis, 1954) in the soluble fraction were measured. The remaining shoot material was used to determine the macronutrient concentrations (Tedesco, 1982). After removing the shoots, a fresh root was removed from each pot and kept in 50% alcohol to observe the colonized roots. The diaphonization and staining of the roots were performed according to Phillips and Hayman (1970). The root sections (approx. 1 cm) were placed in slides with glycerin and screened at high magnifications (400× and 1000×) using an Axioplan light microscope (Carl Zeiss, Jena, Germany) equipped with an Axiocam MRC5 digital camera (Carl Zeiss). The microsclerotia and intraradical hyphae were counted in 100 microscopic fields per root system under 200× magnification (Kohout et al., 2012; McGonigle et al., 1990).

2.5 Statistical analyses

The data were subjected to analysis of variance (ANOVA) and when significant differences were indicated by ANOVA, treatments means were separated using the minimum significant differences calculated by t-test at $p<0.05$ level with R software version 3.4.1 ("R Development Core Team," 2017).

3. Results

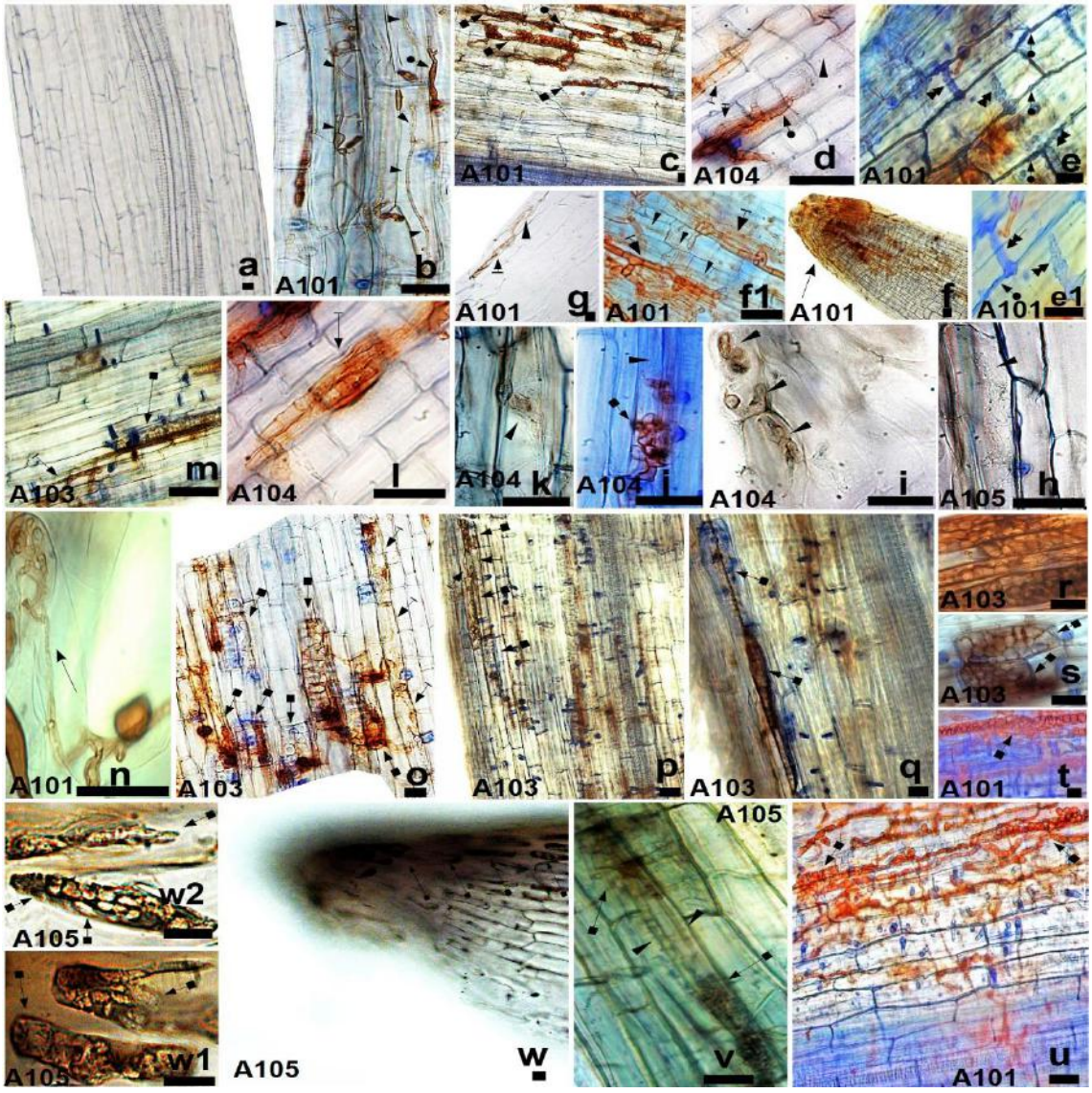
3.1 DSE structures and colonization

The non-inoculated plants (controls) did not exhibit any presence of fungi, indicating the absence of fungal colonization (Figure 1a). However, all of the roots of tomato plants inoculated with fungi A101 and A104 showed septate and melanized intercellular hyphae (Figure 1b, c, d). In the A101 inoculation treatment, intercellular branched hyphae within cortex cells stained with methyl blue were also observed (Figure 1e with details in e1). In all plants roots inoculated with fungi A101 and A104 and A105 intracellular melanized septate hyphae were present (Figure 1b, d and f, with details in f1, g, i, and v), which were stained with methyl blue (Figure 1e, h, j, k, and v) on the all inoculated plants.

The initial stages of microsclerotia development (Figure 1d and f with details in f1, g, l, m, o, and p) were observed in all plants inoculated with the fungi A101, A103 and A104. All of the inoculated plants exhibited fully developed microsclerotia in the root cells (Figure 1c, j, m, o, p, q, r, s, t, u, v, w with details in w1, w2). The intercellular septate hyphae predominated in the cortex cells (Figure 1b, c, d and e with detail in e1). In turn, the intracellular hyphae were located in the root hair (Figure 1n) in the cells of the root epidermis (Figure 1g and b), cortex (Figure 1d and e, with details in e1, g, h, j and k) and apex (Figure 1f with detail in f1 and i). The microsclerotia predominated in the cells of the root cortex (Figure 1c, j, m, p, q, r, s, t, u, and v), epidermis (Figure 1o) and apex (Figure 1w with details in w1 and w2).

Fungi root colonization, that was estimated using the point-intercept method, showed high values of fungus infection in all inoculated plants, varying from 53-79% (Figure 2a), corroborating with the fungi structures observed (Figure 1). The percentage of root colonization in the plants inoculated with the fungus A101, A103 and A105 was significantly higher than the fungus A104 (Figure 2a).

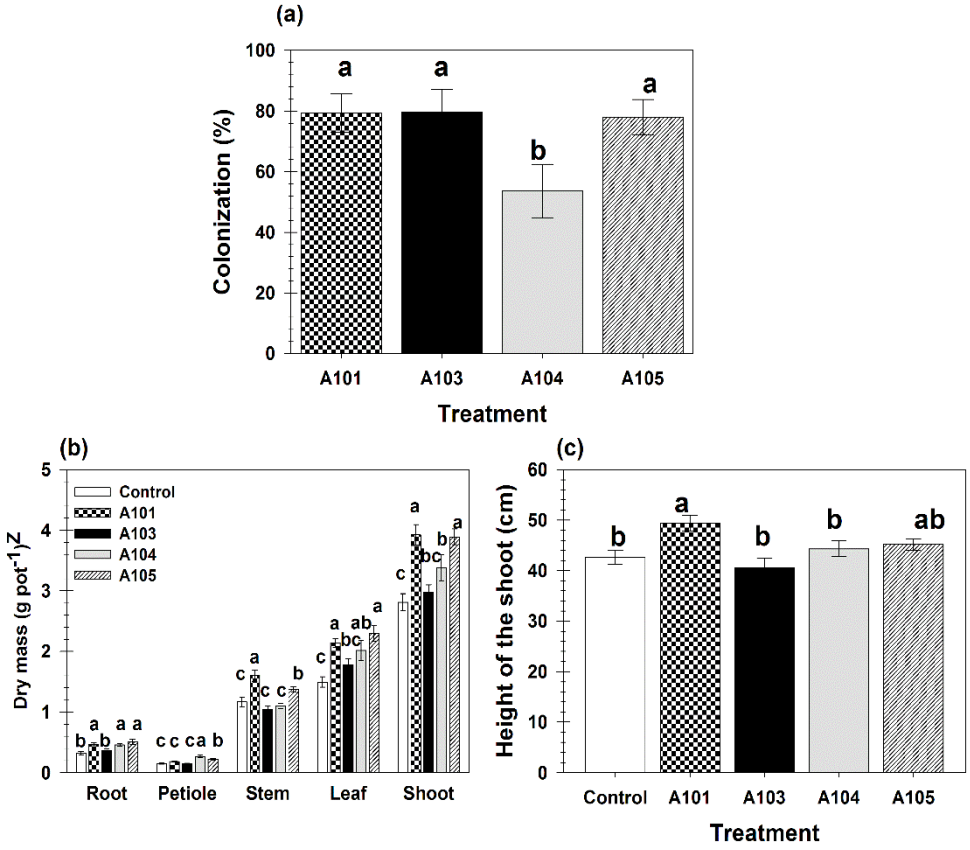
Figure 1 - Morphological aspects of tomato roots (Santa Clara I-5300 variety) at 38 DAG in control plants (non-inoculated) and inoculated with the fungi A101, A103, A104 and A105. Root cells in the control plants (**a**). Melanized (**b**, **c**, and **d**) and stained (**e** with details in **e**1) septate hyphae in the intercellular cortex cells (arrow with circle at the base). Intercellular stained septate hyphae (**e**, **h**, **j**, **k**, and **v**) in the cortex cells (arrowhead). Melanized intracellular hyphae (arrowhead) in the cells of the epidermis (**b** and **g** longitudinal section), the cells of the cortex (**d**, **g**) and the cells of the root apex (**f** with detail in **f**1 and **i**). Intercellular hyphae with intracellular lateral branches (double arrow) in the cells of the cortex (**e** with details in **e**1). Initial stage of microsclerotia development (arrow with transverse dash at the base) in cells of the epidermis (**o**), cortex (**d**, **g**, **l**, **m**, and **p**) and root apex (**f** with detail in **f**1). Fully developed microsclerotia (arrow with square base) in cells of the cortex (**c**, **j**, **m**, **p**, **q**, **r**, **s**, **t**, **u**, and **v**), epidermis(**o**) and root apex (**w** with details in **w**1 and **w**2). The samples were stained with 0.02% methyl blue. DAG (days after germination). Bar = 20 μm.



Source: Authors.

In this figure, it can be seen that all of the inoculated plants presented fully developed microsclerotia in the root cells, although the experiment was only carried out for 38 days.

Figure 1 - The fungal colonization percentage of tomato roots (**a**), dry biomass of the root, petiole, stem, leaf and shoot (**b**) and the height of the shoot (**c**) of the tomato plants (Santa Clara I-5300 variety) at 38 DAG without inoculation (white bar) and those inoculated with the following different dark septate fungal isolates: A101 (checkered bars), A103 (closed bars), A104 (grey bars) and A105 (striped bars). Different letters within a given plant tissue (root, petiole, stem, leaf or shoot) indicate significant differences among the treatment at $p < 0.05$ (t-test (LSD)). The error bars are standard error (n = 4). The means of four composed repetitions (each repetition had four plants per pot). The shoot biomass is the sum of the petiole, stem and leaves



Source: Authors.

All fungi colonized the tomato plants without causing pathological symptoms, increasing shoot dry mass and height in relation to the control.

3.2 Plant biomass, growth and nutrient accumulation

Among the treatments, the tomato inoculation with the fungi A101, A104 and A105 promoted greater accumulation of root, leaf and shoot dry biomass (with increases about of 40% for A101, 20% for A104 and 38% for A105). An increase in the petiole dry biomass was observed following inoculation with A101 and A105 and a higher stem dry biomass was observed for A104 inoculation compared to the control (Figure 2a). In addition, plants inoculated with the fungus A101 had shoot height identical to the plants treated with A105 and higher compared to the control treatment (Figure 2c). In contrast, inoculation with A103 did not affect tomato growth (Figure 2b).

In relation to macronutrient accumulation, none of the inoculation treatments affected the N and Ca content in the tomato shoot (Table 1), regardless of the trends of more than 20% increase for Ca content in plants inoculated with A101, A104 and A105. In another hand, in the plants inoculated with A101 and A105, there was greater accumulation of P, K, Mg and S (with increases of 26, 26, 27 and 44% for A101 and 34, 36 27 and 34% for A105, respectively). In the plants inoculated

with A104, there was higher accumulation of P and S (with increases of 20% and 32%, respectively), whereas plant inoculation with A103 did not affect nutrient accumulation compared to the control.

Table 1 - Macronutrients content (mg pot^{-1}) determined at 38 DAG in the shoot of tomato plants (Santa Clara I-5300 variety) with and without inoculation with different isolates of dark septate fungi.

Treatment	N	P	K	Ca	Mg	S
	mg $vaso^{-1}$					
Control	77.8±6.29	16.8±1.14 c	86.6±5.83 cd	51.5±2.37	11.7±0.55 b	14.2±0.92 d
A101	74.9±6.91	21.2±2.00 a	108.8±8.91 ab	65.4±6.46	14.9±1.21 a	20.4±1.46 a
A103	76.7±1.45	18.0±0.97 bc	81.3±3.21 d	62.2±4.05	12.7±0.82 ab	15.1±1.21 cd
A104	80.0±7.96	20.2±1.96 a	98.0±3.23 bc	61.8±11.19	14.1±1.81 ab	17.3±1.99 bc
A105	88.5±6.85	22.5±1.00 a	117.5±12.06 a	67.3±2.55	15.1±0.99 a	19.0±0.45 ab
CV (%)	11.24	10.69	10.75	14.35	11.95	10.81

Mean ± SE (n = 4) followed by the same lowercase letter in the column did not differ significantly (t-test (LSD), p <0.05). Means not followed by a letter were not significant according to the F-test at 5% probability. DAG (days after germination). Mean of four composite replicates (each replicate had four plants per pot). SE, standard error. Source: Authors.

The plants inoculated with A101 and A105 exhibited significant increases in the contents of P, K, Mg and S compared to control condition.

3.3 Kinetics of the N-uptake and H^+ concentration

The kinetic parameters of the NO_3^--N uptake were affected in tomato plants colonized by different DSE isolates. V_{max} varied slightly between the control plants and those inoculated with A101 and A104 (Table 2). By contrast, the inoculation with isolates A103 and A105 led to a higher V_{max}.

Table 2 - The kinetic parameters of NO_3^- uptake (K_m and V_{max}) as determined at 38 DAG in tomato plants (Santa Clara I-5300 variety) with and without inoculation with different isolates of dark septate fungi.

Treatment	V_{max} (µmol g^{-1} h^{-1})	K_m (µmol L^{-1})
Control	3.07±0.10 cd	111.32±12.41 b
A101	2.56±0.22 d	87.21±2.58 b
A103	4.51±0.04 a	166.01±21.32 a
A104	3.29±0.14 bc	92.71±4.86 b
A105	3.65±0.22 b	106.78±3.35 b
CV (%)	20.11	23.55

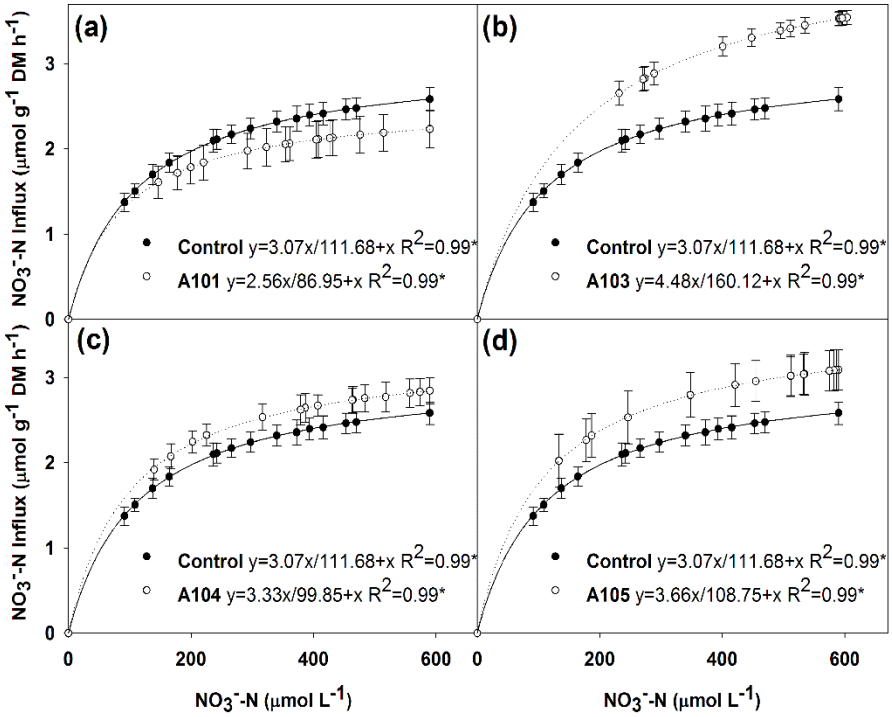
Mean ± SE (n = 4) followed by the same lowercase letter in the column did not differ significantly (t-test (LSD), p <0.05). Means not followed by a letter were not significant according to the F-test at 5% probability. DAG (days after germination). Means of four composite replicates (each replicate had four plants per pot). SE, standard error. Source: Authors.

In tomato plants inoculated with the fungi A103 and A105, the V_{max} was greater than in the control, an effect that may reflect higher levels of NO_3^--N transport proteins in plants colonized by these fungi.

All of the inoculation treatments had the same K_m as the control treatment, except for plants inoculated with the fungus A103, in which a higher K_m was observed (Table 2).

After determining the nutrient concentrations in the solution containing 0.5 mM NO_3^--N, the influx curves were determined for treatments without (control) and with inoculation by dark septate fungi (A101, A103, A104 and A105). At concentrations lower than 100 µM, the influx of NO_3^- of the inoculated plants was the same as the control treatment. However, as the concentration of NO_3^--N in the solution increased, the treatments with fungi A103 (Figure 3b) and A105 (Figure 3d) showed a higher influx, while the treatments with the remaining fungi remained the same as the control.

Figure 2 - Influx of NO_3^--N in the tomato plants (Santa Clara I-5300 variety) at 38 DAG without inoculation and those inoculated with the following different dark septate fungal isolates: A101 (**a**), A103 (**b**), A104 (**c**) and A105 (**d**), then subjected to 0.5 mM of NO_3^--N after 72 h of N deprivation. * Significant according to the F-test at 5% probability. The error bars are SE (n = 4). SE, standard error. DAG (days after germination).

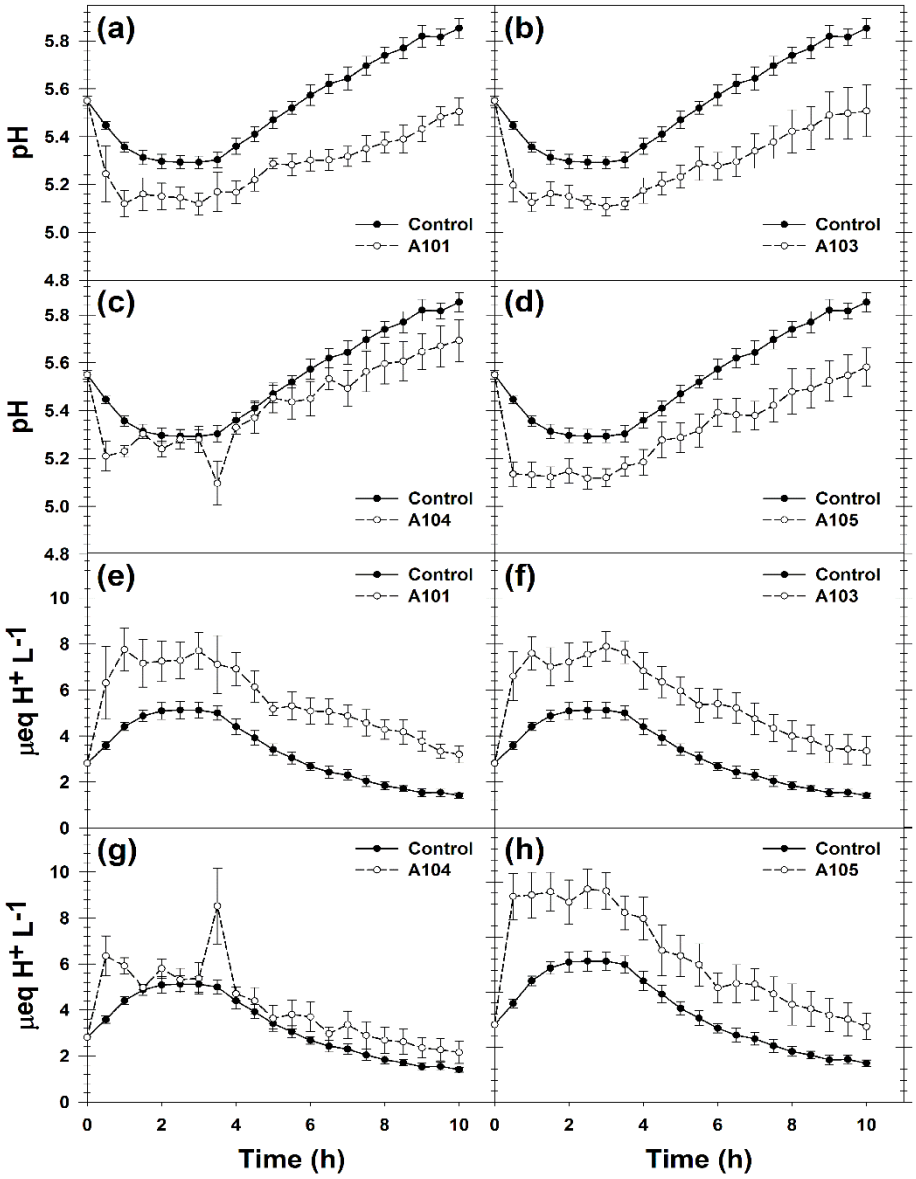


Source: Authors.

The influx of NO_3^--N in the plants inoculated with the fungi A103 and A105 was greater than in the control as the concentration of NO_3^--N in the solution increased.

After adding the nutrient solution containing 0.5 mM NO_3^--N, the pH was monitored every 30 min for 10 h. There was a decrease in pH in all treatments. In the first 30 min after adding the solution, the plants colonized by dark septate fungi promoted a higher acidification of the nutrient solution than the non-colonized control (Figure 4). Unlike the other treatments (Figure 4c), the pH increased 30 min after adding the solution in the condition with A104 inoculation, while an increase in pH was only observed after three hours in the other inoculation groups.

Figure 3 - pH changes (a-**d**) and the H^+ concentration (**e**-**h**) in a nutrient solution of tomato plants at 38 DAG (Santa Clara I-5300 variety) without inoculation and those inoculated with the following different dark septate fungal isolates: A101 (**a** and **e**), A103 (**b** and **f**), A104 (**c** and **g**) and A105 (**d** and **h**), then subjected to 0.5 mM of NO_3^--N after 72 h of N deprivation. The error bars are standard error (n = 4). DAG (days after germination).



Source: Authors.

In this figure, it can be seen that all plants colonized by the DSE fungi acidified the nutrient solution.

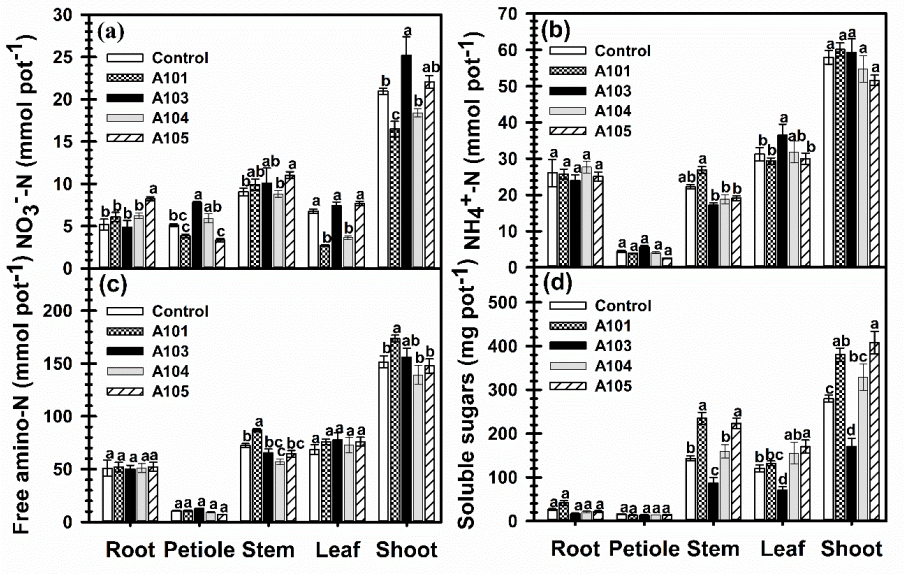
Between the first (0.5 h after adding the solution) and last collection (10 h after adding the solution), the plants colonized by the fungi A101 (Figure 4e), A103 (Figure 4f) and A105 (Figure 4h) showed higher H^+ concentrations in the nutrient solution than in the control treatment. In the plants colonized by the A104 fungus, the H^+ concentration in general was higher than in the control during the first hour of collection and in the last four hours; and the concentrations were the same as in control condition at all other times (Figure 4g). For the treatments with A101 and A103, there were increases of 76 and 84%, respectively, in the proton concentration at the first collection. At the last collection, these increases were 126 and 137%, respectively, compared to the control. In another hand, at the first collection for the A104 and A105 treatment groups, there

were increases in the proton concentration of 77 and 109%, respectively; at the last collection point, there were increases of 53 and 94%, respectively.

3.4 Nitrogen fraction and soluble sugars

Once the soluble fraction was obtained from the partitioning of the filtrate with chloroform, the nitrogen fractions (NO_3^--N, NH_4^+-N, free amino-N) and soluble sugar levels were determined. In general, the NO_3^--N contents were higher in the stem than in the other analyzed parts of the tomato plant. Differently from the other inoculation treatments, there was higher NO_3^--N content in the root with the A105 fungus compared to the control. With A103, a higher NO_3^--N content was observed in the petiole (Figure 5a). In addition, the treatments with A103 and A105 had the same NO_3^--N contents in the stem and shoot, being the NO_3^--N content in the shoot of treatment with the fungus A103 was greater than in the control condition (Figure 5a). By contrast, the NO_3^--N content in the root, petiole and stem of the plants inoculated with A101 and A104 was the same as in the control and in the smallest leaf. Regarding the content of NO_3^--N in the shoot, the plants treated with A104 had the same content as the control; with the A101 treatment, lower contents were observed.

Figure 4 - The contents of NO_3^--N (**a**), free NH_4^+-N (**b**), free amino-N (**c**), and soluble sugars (**d**) as determined at 38 DAG in tomato plants (Santa Clara I-5300 variety) without inoculation (white bar) and those inoculated with the following different dark septate fungal isolates: A101 (checkered bars), A103 (closed bars), A104 (grey bars) and A105 (striped bars). Different letters within a given plant tissue (root or stem or petiole or leaf or shoot) indicate significant differences among the treatment at $p < 0.05$ (t-test (LSD)). The error bars are standard error (n = 4). The means of four composed repetitions (each repetition had four plants per pot). The shoot biomass is the sum of the petiole, stem and leaves. DAG (days after germination).



Source: Authors.

In this figure, it can be seen that the plants inoculated with A101 and A105 had the highest levels of soluble sugars in the shoot. On the other hand, the tomato-A103 interaction showed the lowest soluble content. The A105 treatment also showed higher N-NO_3^- content in the root and stem when compared to the control.

Unlike what was observed for the NO_3^--N content, the NH_4^+-N contents in the root, petiole, stem and shoot were not affected by the fungi (Figure 5b). By contrast, A103 inoculation led to higher NH_4^+-N accumulation in the leaf compared to the

control, which did not differ from the A104 treatment. In general, the leaves showed a higher accumulation of NH_4^+-N (Figure 5b) and free amino-N (Figure 5c), and amino-N accumulation was also slightly higher in the stem compared to the other tomato plant compartments. The amino-N contents in the root, petiole and leaf were not influenced by the presence of fungi (Figure 5c). By contrast, in the stem and shoot, there were differences between the treatments. Specifically, the amino-N contents of the plants inoculated with the fungus A101 was the same as in the treatment with A103, exhibiting a higher accumulation of free amino-N (15% higher) in the stem and shoot compared to the control (Figure 5c). On the other hand, the plants inoculated with A104 presented the same amino-N content in the stem as in the treatments with A103 and A105, which exhibited a lower content than the control, representing a reduction of 8%. The other treatments had similar free amino-N contents.

In general, the soluble sugars accumulated preferentially in the stem than in the other parts of the plant (Figure 5d). Among the inoculation treatments, soluble sugars in the root and petiole were not influenced by the presence of fungi (Figure 5d). In the presence of the fungi A101 and A105, higher soluble sugars contents were observed in the stem and shoot compared to the control; higher contents were also observed in the leaves of plants inoculated with A105 (Figure 5d). By contrast, in the presence of A103, lower soluble sugars contents were detected in the stem, leaf and shoot compared to the control and the other inoculation treatments. The soluble sugars content of plants inoculated with A104 was the same as that for the control treatment. Plants inoculated with fungi A101 and A105 showed increases of 36 and 46%, respectively, in their soluble sugars content, whereas in the inoculation with A103, a 39% reduction was observed.

4. Discussion

Inoculation of tomato (Santa Clara I-5300 variety) with four different DSE isolates did not cause any symptoms of pathogenicity in the plants, indicating compatibility of the association. Other studies have also indicated that DSE fungi can colonize the plant cortex without causing any disease symptoms (Newsham, 2011).

The intensity of colonization by DSE fungi varies greatly between plant species and between individuals of the same species,(Zhang et al., 2013) and the morphology of the fungus that colonizes the root may change over time (Wilcox & Ganmore-Neumann, 1974), being primarily controlled by the host plant (Wilcox & Wang, 1987).

In this study, all of the inoculated plants presented fully developed microsclerotia in the root cells (Figure 1c, j, m, o, p, q, r, s, t, u, v and w, with detail in w1, and w2), although the experiment was only carried out for 38 days. In plants inoculated with fungi A101 and A104, intercellular (Figure 1b, c, d) and intracellular (Figure 1b, d and f with detail in f1 and g) melanized and septate hyphae were observed, reflecting a colonization rate of the tomato root system as high as 79% (Figure 2a). This observation confirmed the ability of the fungi to occupy the roots in a short period of time. Similarly, Lukešová et al. (2015) observed the formation of intracellular hyphae by the fungus *Acephala macrosclerotiorum* in birch roots. In this previous study, colonization rates ranged from 51 to 61% when the plants were inoculated with *Acephala applanata* and *Acephala macrosclerotiorum* and from 50 to 80% when blueberry plants were inoculated with *Phialocephala fortinii* s. l.-*Acephala applanata* species complex (PAC).

The fungi A101, A104 and A105 promoted a greater accumulation of root, leaf and shoot dry biomass (with increases of 40% for A101, 20% for A104 and 38% for A105), also confirming what has been observed in other studies with dark septate fungi. Greater dry biomass of tomato plants inoculated with *Scolecobasidium humicola* (Mahmoud & Narisawa, 2013) and *Leptodontidium orchidicola* (Andrade-Linares et al., 2011) was observed when plants were supplied with both organic and inorganic N sources. On the other hand, neutral effects and even reductions in dry biomass of several plant species have also been reported. For example, leek plants inoculated with fungus of the genus *Periconia* presented lower dry biomass than the control without inoculation, while plants inoculated with *Microdochium* sp. did not differ from the control (Mandyam et al.,

2010). The higher growth of the root system in the plants inoculated with fungi A101, A104 and A105 indicates higher nutrient uptake efficiency, which is a desirable characteristic, especially for cultivation under poor natural fertility conditions. Negative and neutral responses to inoculation with DSE fungi may be caused by the lack of specificity between the host plant and the fungus, since these interactions are being forced by laboratory conditions; under field conditions, however, these interactions would not occur because both fungi and host plants have a plethora of options to establish a symbiotic interaction. The results obtained in the present study indicate the contribution of the fungi A101, A104 and A105 to the growth of Santa Clara I-5300 tomato variety.

In general, in plants inoculated with DSE fungi, no changes have been observed in the N content of the shoot when the source is inorganic; however, when an organic source is supplied, significant increases in the N content of the shoot relative to the control are observed more frequently (Newsham, 2011; Upson et al., 2009; Vergara et al., 2017). In the present study, in which an inorganic source was also used, no significant increases in the N content were observed in any of the inoculation treatments when compared to the control treatment. By contrast, significant increases in the contents of P, K, Mg and S were observed in plants inoculated with A101 and A105 (26, 26, 27 and 44% increases for A101 and 34, 36, 27 and 34% increases for A105, respectively). These data indicate that DSE fungi can improve the efficiency of nutrient uptake and use in plants associated with these fungi. Increases in the P concentration of the shoot of plants inoculated with DSEs have been observed when plants are supplemented with inorganic P (Newsham, 1999, 2011; Upson et al., 2009). These increases can be attributed to the increase of the contact surface area of the roots, an effect that is promoted by the tomato-DSE fungus interaction (Mandyam & Jumpponen, 2005).

In tomato plants inoculated with the fungi A103 and A105, the V_{max} was greater than in the control, an effect that may reflect higher levels of NO_3^--N transport proteins in plants colonized by these fungi. This is a favorable characteristic because plants with a higher V_{max} have increased nutrient transport capacity (Alves et al., 2016; Santos et al., 2011).

Using the K_m value, it is possible to verify the affinity of the carriers for the nutrient being transported, and this is the most relevant parameter in the description of the transport systems (Zhang et al., 2009). Following inoculation with A103, the K_m values were higher than in the control condition, which represents a loss of affinity of carriers for the transport of NO_3^- present in solution.

The combination of high V_{max} and low K_m together with higher root growth are desirable characteristics in a species because they represent greater nutrient uptake efficiency (Li et al., 2007). In the present study, the plants colonized by A105 presented a higher V_{max}, root biomass and the same K_m as the control, indicating an increase in the uptake capacity of NO_3^--N from the external environment (Santos et al., 2011).

The influx of NO_3^--N in the plants inoculated with the fungi A103 and A105 was greater than in the control as the concentration of NO_3^--N in the solution increased. This result corroborates the kinetic parameters found in the A103 inoculation group, which showed a lower affinity for NO_3^--N. In turn, plants inoculated with A105 showed the same NO_3^--N affinity as the control, but with a higher V_{max} value for NO_3^--N transport (Table 2).

The acidification of the nutrient solution observed in this study, especially in the control group, has been attributed to the activation of PM-H^+-ATPases soon after the addition of NO_3^- (Santi et al., 1995; Santos et al., 2011; Vergara, et al., 2019). PM-H^+-ATPases use ATP to direct protons in the cytosol towards the apoplast (Sperandio et al., 2011), forming a proton gradient. The proton motive force then energizes the absorption of anions, such as NO_3^- (Gaxiola et al., 2007). The increase in the H^+ concentration of the nutrient solution of all of the inoculation treatments relative to the control suggests higher proton pumping and consumption in the inoculated plants for the absorption of anions, such as NO_3^-, $H_2PO_4^-$, and SO_4^{2-}.This observed increase also corroborates the greater phosphorus and sulfur accumulation observed in the groups inoculated with A101, A104 and A105 (Table 2) and the higher influx of NO_3^--N observed in plants inoculated with A103 and A105 (Figure 4b and d). The

pH increase observed after addition of the nutrient solution with 0.5 NO_3^--N is due to the absorption of anions, such as NO_3^-, that occurs in symport with two protons ($NO_3^-/2H^+$), leading to proton consumption (Santos et al., 2011).

Some plants, such as sunflower (Rocha et al., 2014) and rice (Santos et al., 2005; Santos et al., 2009; Santos et al., 2011) accumulate NO_3^--N preferentially in the stem over other parts of the plant. Similarly, the tomato plants accumulated NO_3^--N in the stem (Figure 5a), which suggests that the stem is the preferred compartment for the accumulation of NO_3^--N.

The higher accumulation of NO_3^--N in the shoots of the plants inoculated with the fungi A103 and A105 (Figure 5a) compared to the control reflects the marked reduction in pH (Figure 4), the increase in V_{max} (Table 2) and the influx of NO_3^- (Figure 3b and d) observed in these treatments.

The excess NO_3^- uptake by plants is transported and stored in the vacuoles, serving as a source of mineral N (Crawford & Glass, 1998; De Angeli et al., 2006). This strategy of storing N is interesting and of great value for future metabolic demands, especially those caused by the lack of nitrate, as usually happens in tropical soils.(Souza et al., 1998) The increased yield of corn genotypes is due to their ability to accumulate NO_3^- in the leaves during vegetative growth and efficient remobilization of this anion during grain filling, (Hirel et al., 2001) which can result in high crude protein content (Bucher, 2007; Santos, 2006).

The activity of nitrate reductase in the roots and shoots of plants colonized by arbuscular mycorrhizal fungi is generally higher than in non-colonized controls (Vergara, Araujo, Souza, et al., 2019). Thus, the higher accumulation of NH_4^+-N observed in the leaves of plants colonized by the fungi A103 and A104 suggest the presence of higher nitrate reductase activity in the leaves. This effect would lead to a greater reduction of NO_3^- to NH_4^+, resulting in greater accumulation of this nutrient in that compartment. The higher accumulation of NH_4^+-N is consistent with the higher accumulation of NO_3^--N in the shoot of the plants colonized with A103 (Figure 5a).

NH_4^+-N (Figure 5b) and free amino-N (Figure 5c) accumulated preferentially in the leaf over other compartments of the tomato plant, suggesting that this is the preferred organ for the accumulation of this nitrogen fraction. A similar effect has been observed in rice plants (Bucher, 2011; Santos et al., 2011; Sperandio, 2011; Vergara, et al., 2018).

The higher NO_3^--N uptake capacity by plants colonized by A103 was indicated by the higher values of V_{max} (Table 2), influx (Figure 3b), NO_3^--N content in the shoot (Figure 5a) and NH_4^+-N in the leaf (Figure 5b) compared to the control. These effects resulted in greater accumulation of free amino-N in the shoot in these plants. On the other hand, the marked reduction in the NO_3^--N content of 21% compared to the control (Figure 5a) explains the greater accumulation of amino-N observed in plants colonized with A101.

All of the treatments preferentially accumulated soluble sugars in the stem than in the other parts of the plant (Figure 5d). A similar effect was observed in sunflower (Alves et al., 2016; Rocha et al., 2014). Increases of 36 and 46% in the content of soluble sugars in the shoot of plants inoculated with A101 and A105, respectively, suggest a higher capacity of these plants to incorporate the absorbed N into C skeletons, thus affecting the synthesis of amino acids essential for plant development. It is known that soluble sugars can act as a source of readily available energy in plants for cellular metabolism and as a source of C skeletons, which are used in the synthesis of organic acids to assimilate absorbed N (Xu et al., 2012).

The high increases observed in sugar contents can be attributed to the greater efficiency of the photosynthetic activity of the plants inoculated with A101 and A105 given that inoculation with DSE fungi can increase the chlorophyll content and the quantum efficiency of photosystem II (Zhang et al., 2012). Other studies have reported increases in sugars in the tomato plant: for example, a 17% increase in the glucose content of fruits was observed in plants colonized by *Leptodontidium orchidicola* relative to the control treatment (Andrade-Linares et al., 2011).

In the plants inoculated with A105, the stem showed lower N-amino content (with a reduction of 8%) associated with increases of 46% in the content of soluble sugars (in the shoot) compared to the control; with A103 inoculation, the amino-N

and soluble sugar contents showed an inverse relationship, with a 39% reduction in soluble sugars and a slight increase in the amino-N content (Figures 5c and d) to compensate for the higher NO_3^--N uptake capacity of these plants (Table 2, Figure 3 and Figure 5a). Changes in the amino-N and soluble sugar contents are the result of the balance between C skeletons and N absorbed by the plant (Borges et al., 2006; Oliveira et al., 2011).

5. Conclusion

According to the results obtained, the fungi A101 and A105 are able to induce a greater accumulation of protons in the nutrient solution as well as a greater accumulation of nutrients, soluble sugars and dry matter in tomato plants. In addition, the presence of A105 in the tomato plant leads to an increase in the V_{max} of NO_3^--N uptake, an effect that is associated with the greater accumulation of this anion in the root and stem. The A103 fungus, on the other hand, is antagonistic: because it increases the ability of the colonized plants to uptake NO_3^--N in the solution but, in the same time, alters the metabolism of C and N, leading to an inadequate source of C skeletons for nutrient assimilation; therefore, among the dark septate endophytic fungi that were evaluated, only the fungi A101 and A105 exhibit clear potential for promoting tomato growth. However, other physiological parameters, such as nitrate reductase activity, still need to be investigated in plants colonized by dark septate endophytic fungi.

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