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Original Article

# Biostimulation on cotton growth and nutrient accumulation through inoculants based on endophytic fungi[1]

## Bioestimulação no crescimento e acúmulo de nutrientes em algodão através de inoculantes à base de fungos endofíticos

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*HIGHLIGHTS:*
*Identification of promising inoculants from endophytic fungi able to stimulate the cotton growth and production.*
*The inoculants favor the N and P accumulation during the flowering and square formation, increasing fruit production.*
*Identification of a specific inoculant that expressively elevates the accumulation of calcium, benefiting fruit quality.*

**ABSTRACT:** Dark septate fungi (DSF) are endophytic microorganisms characterized by the formation of detached and melanized hyphae that confer adaptive advantages, including benefits in plant development. This study aimed to evaluate the nutrient accumulations, growth, and production of cotton plants inoculated with different DSF accessions. The experiment was carried out in a greenhouse in a completely randomized design with eight replicates. Cotton plants were grown in plastic bags containing commercial substrate and watered daily. Data were collected over the cycle of plants. Promising results were found in all inoculants; however, those obtained from ERR 26 and ERR 42 accessions provided broad benefits for cotton plants, improving the accumulation of N and P and improving plant growth and production. It is worth highlighting the significant contribution of the ERR 42 inoculant to the accumulation of calcium, which brings additional benefits to the formation and resistance of the fruit cell wall. This information is unprecedented and elevates the usage prospects of this inoculant for the biofertilization of cotton plants. However, further studies should be deepened to attest to its biostimulant advantages in cotton management.

**Key words:** *Gossypium hirsutum*, dark septate microorganism, fiber production

**RESUMO:** Fungos de micélio escuro (DSF) são microrganismos endofíticos caracterizados pela formação de hifas destacadas e melanizadas que conferem vantagem adaptativa, incluindo benefícios no desenvolvimento das plantas. O objetivo deste estudo foi avaliar o acúmulo de nutrientes, o crescimento e a produção em plantas de algodão inoculadas com diferentes acessos de DSF. O ensaio foi realizado em casa de vegetação, em delineamento inteiramente casualizado, com oito repetições. As plantas foram cultivadas em sacos plásticos contendo substrato comercial e regadas diariamente. Os dados foram coletados durante todo ciclo das plantas. Resultados promissores foram encontrados para todos os inoculantes, porém aqueles obtidos a partir dos acessos ERR 26 e ERR 42 forneceram maiores benefícios para as plantas do algodoeiro, melhorando o acúmulo de N e P, o que contribuiu para melhoria do crescimento e produção das plantas. Cabe destacar a significativa contribuição do inoculante ERR 42 no acúmulo de cálcio, elemento que traz benefícios adicionais à formação e resistência da parede celular dos frutos. Esta informação é inédita e eleva as perspectivas de utilização deste inoculante para biofertilização do algodoeiro. Entretanto, novos estudos deverão ser aprofundados para atestar as vantagens do bioestimulante no manejo do algodão.

**Palavras-chave:** *Gossypium hirsutum*, microrganismo de micélio escuro, produção de fibras

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## Introduction

Several endophytic microorganisms have been reported to improve the management of commercial crops through growth biostimulation and mitigation of environmental stresses (Zhang et al., 2017; Shen et al., 2020; Liu et al., 2021; Elnahal et al., 2022). Among them are the dark septate fungi (DSF), which, like some endophytic bacteria, contribute to enhancing the agronomic performance of crops, as they act by solubilizing nutrients, making them available to the plants. In addition, they increase the root absorption area, allowing better use of water and nutrients and, therefore, minimizing the deleterious effects of water scarcity (Vergara et al., 2018; Fors et al., 2020; Gehring et al., 2020; Yang et al., 2021).

Additionally, these fungi are characterized by the formation of extended and melanized hyphae that confer adaptive advantages since they are found in the cell wall or secreted in the extracellular environment, stimulating plant development (Yu et al., 2015; Yuan et al., 2016). Several species of DSF benefit the plants by increasing the root absorption area, allowing greater use of water and nutrients, in addition to contributing to tolerance to abiotic stresses, such as heavy metals, drought, and salinity, biotic stresses, reducing the oxidative stress, improvement of efficiency of photosynthesis, and mineralize organic nitrogen into inorganic nitrogen (Yihui et al., 2017; Liu et al., 2021).

Recent studies have demonstrated that inoculants from DSF could play an important role in modern sustainable agriculture systems, helping to provide nutrients to the plants and preserve soil microbiota (Santos et al., 2021; Vergara et al., 2023). Based on this information, it is possible that bioactives derived from them could become an attractive opportunity to boost regenerative agriculture practices. This study aimed to evaluate the nutrient accumulations, growth, and production of cotton plants (*Gossypium hirsutum*) inoculated with different DSF accessions.

## Material and Methods

### Germplasm origin and inoculant preparation

The experiment was carried out in a greenhouse (Campina Grande, PB, Brazil, 7° 13' 51" S and 35° 52' 54" W, and 594 m of altitude), during the dry season, with temperature means varying from 21.9 to 39.2 °C, relative air humidity ranging from 31.4 to 56.4%, and photoperiod 12:12 (light:dark). Four inoculants prepared from DSF accessions (Table 1), originating from the Brazilian Amazon biome, plus a treatment control (no inoculant) were used in this study. The fungus accessions were provided by a microbial collection belonging to Embrapa Agrobiology (Rio de Janeiro state, Brazil).

The inoculum preparation followed the procedures reported in Vergara et al. (2018) with few modifications. The isolates were grown in a 250 mL Erlenmeyer flask containing 200 mL of potato dextrose medium for 20 days under 70 rpm shaking at 28 °C. The fresh mycelium was filtered and washed twice with autoclaved distilled water. Then, they were transferred to Falcon tube (50 mL), resuspended with autoclaved distilled water (40 mL) for 1 min, and centrifuged at 6000 × g for 10 min (SPLabor - model SP-HCL-4). This procedure was repeated three times. After the last washing, distilled water (180 mL) was added to the fungal mass for liquefication for 10 s, using a blender (Walitta) at low speed. Next, the samples were centrifuged (6000 x g for 10 min), discarding the supernatant. Each inoculant was resuspended in 50 mL of distilled water for further use in inoculation of the seeds.

### Experimental procedures

Cotton seeds FM 966 (Fibermax 966, BASF) previously disinfected (Vergara et al., 2018) were immersed in the inoculant of each accession (180 μL of inoculant per seed) for 40 min and sown in plastic bags containing seven liters of commercial substrate (MecPlant, Brazil). For control treatment (no inoculant), the seeds were immersed in autoclaved distilled water for the same period. A randomized complete design was adopted with eight replicates.

Before sowing, the moisture of the substrate was raised to the level of maximum water retention capacity through capillary saturation followed by drainage. After seedling emergence, watering was daily. The water volumes in each irrigation event were determined by the water balance of the plants, according to Eq. 1:

$$VI = \left(Va - Vd\right) + LF \qquad (1)$$

where:

VI - volume of water to be applied in the next irrigation event (mL);

Va - volume of water applied to plants in the previous irrigation event (mL);

Vd - volume of water drained in the previous irrigation event (mL); and,

LF - leaching fraction of 10%, applied fortnightly.

From 30 days after emergence (DAE) on, data of nutrient accumulation in leaves, and plant growth were collected. For the first, the accumulation was estimated by nitrogen (N), phosphorus (P), potassium (K), and calcium (Ca) carried out in dehydrated leaves (100 mg to N, P, and K, and 500 mg to Ca), at 30, 45 and 60 DAE, following the methodology described in Silva (2009). N, P, and K were determined by sulfuric digestion (5 mL of concentrated $Na_2SO_4$ + catalysts: 50 mg $Na_2SO_4$ and

**Table 1.** Passport data of dark septate fungi (DSF) used in the present study. Accessions deposited at the Johanna Dobereiner Biological Resources Center (CRB-JD)

| Code | Isolate | Genus | Origin | Coordinates |
|---|---|---|---|---|
| ERR 16 | A 103 | *Pleosporales* | Bonfim, RR | 3°21'25" N; 59°49'60" W; 59 m |
| ERR 26 | A 104 | *Pleosporales* | Bonfim, RR | 3°21'25" N; 59°49'60" W; 59 m |
| ERR 31 | A 107 | *Pleosporales* | Bonfim, RR | 3°21'25" N; 59°49'60" W; 59 m |
| ERR 42 | A 105 | *Pleosporales* | Bonfim, RR | 3°21'25" N; 59°49'60" W; 59 m |

0.5 mL 5% $CuSO_4$). To estimate N, it was added to a final volume of 50 mL the follows solutions: extract (1 mL) of each treatment, 10% NaOH (1 mL), 20% $Na_2SiO_3$ (1 mL) and Nessler's reagent (2 mL); to determine P, it was added to 5 mL of the extract: $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$ (10 mL) and $C_6H_8O_6$ (50 mg). N and P were read at 410 and 660 nm, respectively (UV-VIS Spectrometer, Thermo Scientific, Mod. Biomat 3, USA). Potassium was determined by flame photometry (Analyzer, Mod. 910M, Brazil) using 5 mL of sulfuric digestion, and Ca by nitric-perchloric digestion (2:1), determined by titration with standard EDTA solution (0.02 N), using murexide ($NH_4C_8H_4N_5O_6$) as the indicator. All analyses were performed in biological and experimental triplicates.

For plant growth, the following traits were evaluated: Height of the main stem at 30, 45, and 60 DAE; number of leaves at 30 and 45 DAE; fruit production at 60 and 100 DAE, counting all reproductive structures, i.e., flower buds, squares, and mature bolls present on the plants. The harvest was at 128 DAE; on that occasion, data of roots length and dry weight, mature bolls, lint weight, and seeds number and weight were collected in each treatment.

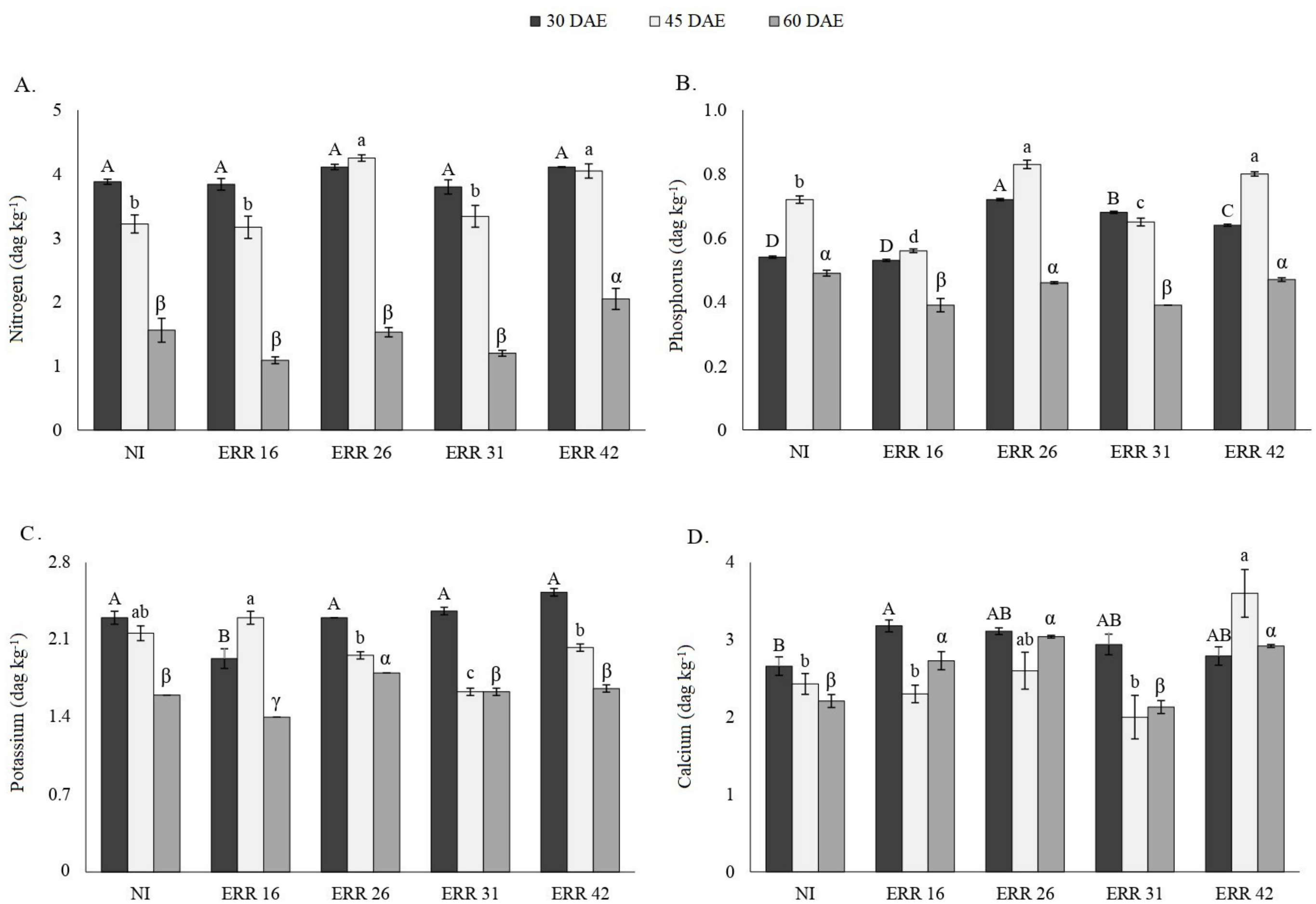
### Statistical analysis

Data were submitted to normality analysis of error distribution using the Shapiro-Wilk test (1965); subsequently, analysis of variance (F test, $p \leq 0.01$) and mean comparison test (Tukey test) were performed using SISVAR software version 5.8 (Ferreira, 2019).

## Results and Discussion

Statistical differences (F test, $p \leq 0.05$) were found in most traits of nutrient accumulation and growth in cotton plants inoculated with endophytic fungus. The supply of nutrients provided by DSF inoculants during plant growth is displayed in Figure 1. The ERR 26 and ERR 42 accessions provided inputs of 31 and 28% of N, respectively, at 45 DAE concerning the control (Figure 1A), whose phase is the beginning of the bud performing. As the cotton plant has indeterminate growth, this supplementation contributes to the canopy extension and the emission of floral meristems (Vasconcelos et al., 2020; Dias et al., 2023). These same accessions also contributed to P supplementation, starting at 30 DAE (33% for ERR 26 and 11% for ERR 42) until 45 DAE, with an average of 19% to both, decreasing from that date onwards (Figure 1B).

Regarding K (Figure 1C), no relevant result was found, except for ERR 26 accession, which provided a plus of 12% at the initial development of bolls (60 DAE). In a study carried out by Vergara et al. (2018) using DSF inoculants, authors reported that this accession provided a significant contribution to NPK accumulation in shoots of rice (*Oryza sativa*), with increases of 30, 49 and 33%, respectively, concerning the control treatment;




NI - No inoculant. Uppercase, lowercase, and Greek letters compare treatments at 30, 45, and 60 DAE (days after emergence), respectively. Means followed by the same letter do not differ according to the Tukey test ($p \leq 0.05$)

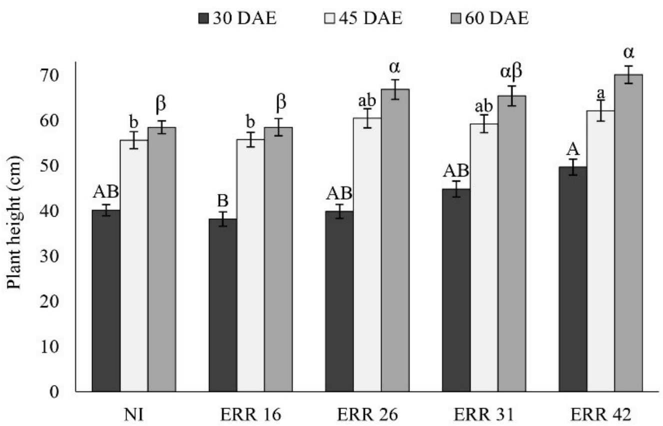
**Figure 1.** Accumulation of nitrogen (A), phosphorus (B), potassium (C), and calcium (D) in cotton leaves grown with dark septate fungi (DSF) inoculants

the ERR 42 (A105) contributed to the accumulation of P (20%) and K (11%), compared to the control. In a study with tomato (*Solanum lycopersicum*), Vergara et al. (2023) found an elevation of 26% to P and K accumulations in plants inoculated with ERR 42 (A105) concerning the control. According to these authors, the inoculation with DSF accessions in rice and tomato leads to greater nutrient absorption, plant growth and activation of genes related to proton pumps, creating conditions for plants to absorb more nutrients.

Although the benefits of DSF depend on each genotype, the contribution is generally positive. It largely depends on the interaction between signal receptors released by the plant's root system and the fungus. Based on the results in the literature, it is believed that ERR 42 has wide interaction with several plant species, offering different nutritional benefits compared to other accessions. As shown in Figure 1D, this accession provided an exceptional increase of 48% in Ca accumulation in cotton plants at 45 DAE concerning the control. No result like that involving DSF inoculants was found in the literature.

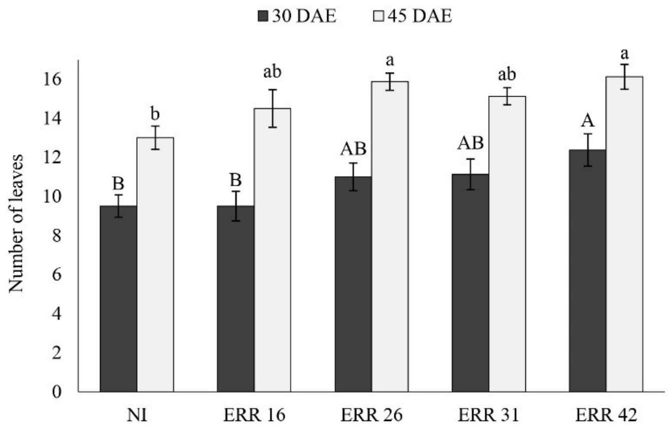
Without disregarding the relevance of other macroelements, the results with calcium are encouraging because it is a major component of the middle lamella (calcium pectate), and its supply in cells plays a critical role in cotton growth, number of bolls, and fiber elongation (Guo et al., 2017). An adequate supply of Ca may inhibit square and boll abscission (Sawan et al., 1997).

The contribution of the macroelements provided by DSF inoculants directly affected the growth and production of cotton plants. In general, the benefits were found especially with those obtained from ERR 26, ERR 31, and ERR 42 accessions; this latter, by the way, contributed with inputs of 10 cm (25%), 7 cm (13%), and 12 cm (21%) in plant height at 30, 45, and 60 DAE (Figure 2), respectively, and 23% in number of leaves, at 45 DAE (Figure 3), compared to control treatment. As can be seen in Figure 1, the inoculant from ERR 42 provided 31% of N and 19% of P at 45 DAE. This phase corresponds to initial reproductive events, with simultaneous occurrences of young buds, sympodial branches, and new leaves, which will contribute directly to mature boll production and both lint and seed yield (Baloch et al., 2014; Haq et al., 2017; Rehman et al., 2020). Considering the entire canopy, the data found here represent additional benefits to plant growth due to favor the light interception rate and, consequently, the photosynthetic rate by plants, both positively correlated with the number of leaves (Wu et al., 2023).




NI - No inoculant. Uppercase, lowercase, and Greek letters compare treatments at 30, 45, and 60 DAE (days after emergence), respectively. Means followed by the same letter do not differ according to the Tukey test (p ≤ 0.05)

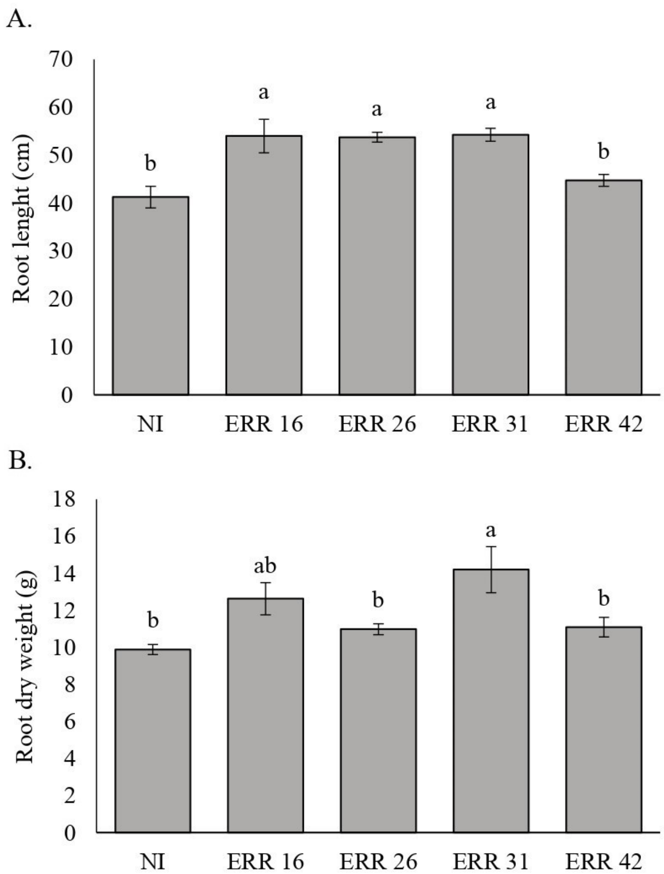
**Figure 2.** Height of cotton plants grown in the presence of dark septate fungi (DSF) inoculants




NI - No inoculant. Uppercase and lowercase letters compare treatments at 30 and 45 DAE (days after emergence), respectively. Means followed by the same letter do not differ according to the Tukey test (p ≤ 0.05)

**Figure 3.** Number of leaves of cotton plants grown in the presence of dark septate fungi (DSF) inoculants

Regarding root system (length and weight), the best response was found to inoculant obtained from ERR 31, which provided 34 and 40% increases, respectively, compared to control plants (Figure 4). These are expressive results and represent an additional contribution to the uptake of nutrients by plants. Although ERR 31 did not outperform the other inoculants in aerial traits, it is possible to hypothesize that




NI - No inoculant. Means followed by the same letter do not differ according to the Tukey test (p ≤ 0.05)

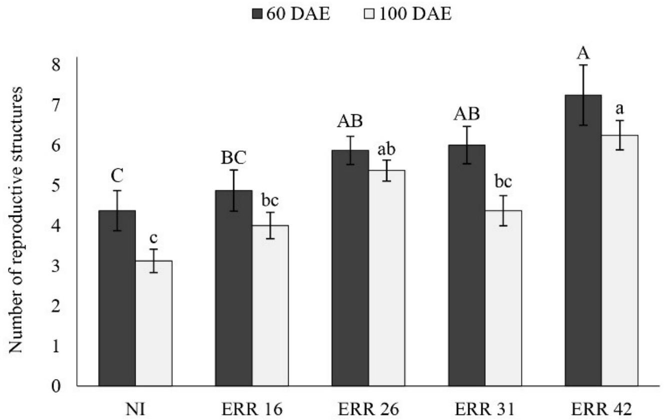
**Figure 4.** Root length (A) and root dry weight (B) of cotton plants grown in the presence of dark septate fungi (DSF) inoculants

this inoculant could favor plant growth in water restriction situations. This premise is based on differentiated responses of DSF inoculants, which may not be responsive to plant growth but may be widely useful to mitigate environmental stresses.

Santos et al. (2023) carried out a prospective study with DSF-inoculants aiming to identify accessions able to mitigate the deleterious effect of water stress in cotton plants, using growth and physiological traits. The authors found one inoculant (ERR 01) that protected all plants submitted to seven days of water suppression, starting at blooming. Root length was increased by 15%, contributing to an increase in the capacity of water absorption in plants and the tissue turgor, even under water stress. According to these authors, the rate of electron transport and the relative water content in inoculated plants did not differ between irrigated and stressed plants, indicating that the energy produced in the photochemical phase of photosynthesis in inoculated plants was not altered. In another study, Vergara et al. (2018) induced water stress in rice (*Oryza glumaepatula*) plants using polyethylene glycol (PEG) and found that plants inoculated with ERR 01 promoted growth both in the presence and in the absence of water deficit.

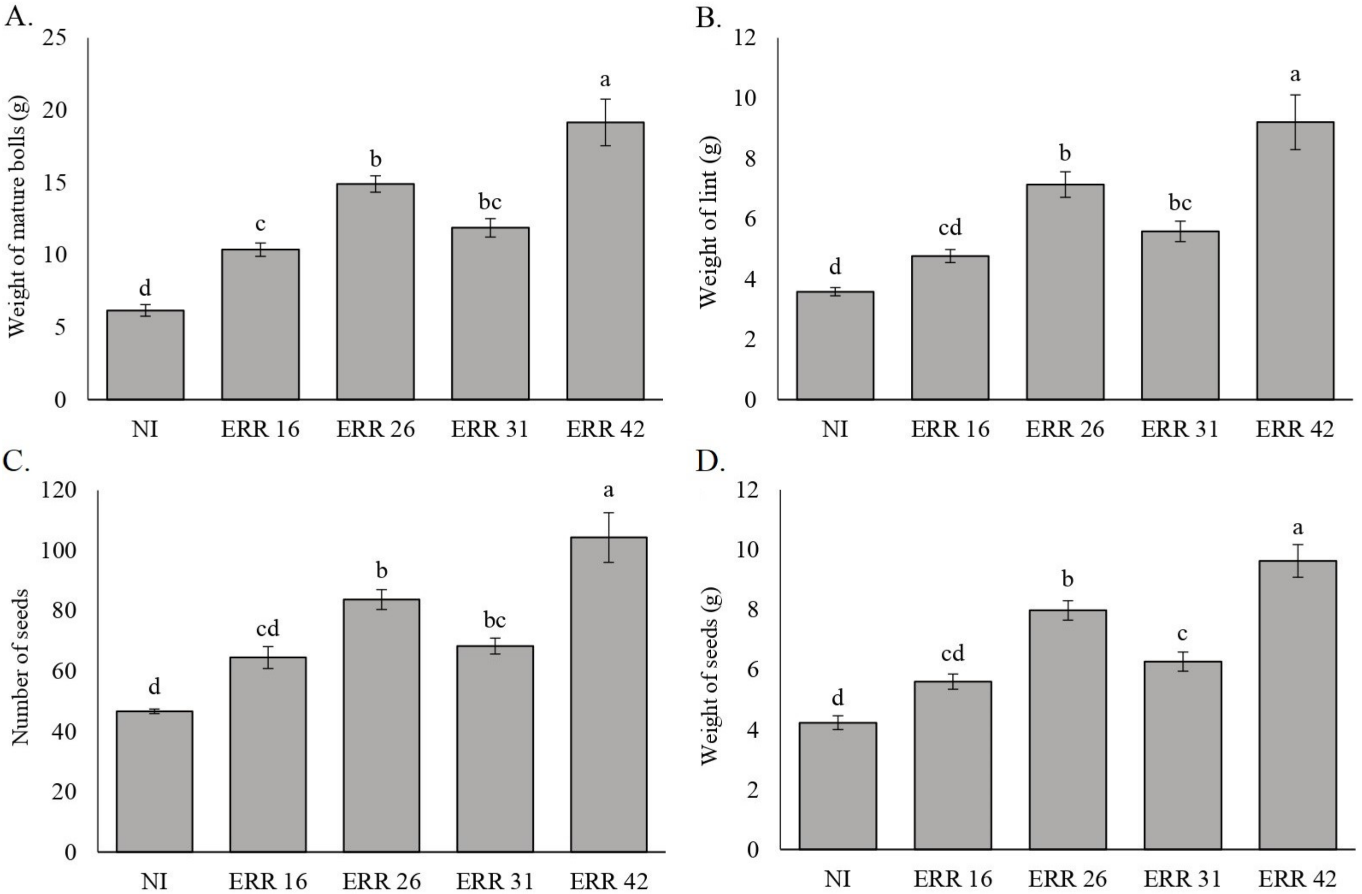
Additionally, decreased oxidative stress of plants in response to inoculation was found, contributing to an increase in the tolerance of rice plants to stress caused by water deficiency. In control plants (no water stress), authors reported that rice plants inoculated with ERR 16 increased the dry mass from roots, sheathes, leaves, and shoots to 44, 43, 25, and 32%, respectively, compared to no inoculated plants. Additionally, the shoot height and the number of tillers were increased by 8 and 103%. The authors did not find effective benefits in rice growth with the other accessions, except ERR 42, which provided an increase of 72% in the number of tillers. As there is specificity in the interaction between plant and fungal signals, prospective trials are important to identify contributory inoculants able to mitigate environmental stresses.

The production data from cotton plants grown in the presence of different DSF inoculants are shown in Figures 5 and 6. Data were collected at 60 and 100 DAE, considering




NI - No inoculant. Uppercase and lowercase letters compare treatments at 60 and 100 DAE (days after emergence), respectively. Means followed by the same letter do not differ according to the Tukey test ($p \leq 0.05$)

**Figure 5.** Number of reproductive structures of cotton plants grown in the presence of dark septate fungi (DSF) inoculants




NI - No inoculant. Means followed by the same letter do not differ according to the Tukey test ($p \leq 0.05$)

**Figure 6.** Weight of mature bolls (A), weight of lint (B), number of seeds (C), and weight of seeds (D) collected at cotton harvest from plants grown in the presence of dark septate fungi (DSF) inoculants

squares and bolls present in plants during each period. In both cases, the inoculant from ERR 42 stood out, showing inputs of 59 and 100% at 60 and 100 DAE, respectively, concerning the control (Figure 5). This increase positively impacted the weights of mature bolls (Figure 6A) and lint (Figure 6B), with increases of 246 and 126% over the control, respectively.

As cotton seeds have a large commercial value due to their derivatives (oil and by-products), the number (Figure 6C) and weight (Figure 6D) of seeds from different treatments were reported, and the wide contribution of ERR 42 inoculant was again verified, revealing expressive increases of 108 and 137%, respectively, concerning control.

To understand what these values could represent in an aspect of cotton management, the production gains here presented by the use of inoculant from ERR 42 accession far exceed the prediction expected in traditional breeding works, which ranges between 25 to 30%, depending on the parents and its ability to generate desirable progenies to meet market demands (Vidal Neto & Freire, 2013; Vasconcelos et al., 2020). This leads to the conclusion that the management with an improved cultivar, additionally inoculated with a promising DSF-inoculant, could significantly boost the crop yield due to the several benefits provided by the plant-fungus symbiosis.

Regarding ERR 42-inoculant, studies in the literature have reported the properties of growth activating in rice and tomato, but information during the reproductive phase is not available, until now (Vergara et al., 2018). This limits a comparative analysis with the results presented here. To the authors' knowledge, this is the first report of DSF inoculants focusing on nutrient accumulation and cotton growth. The benefits of the ready absorption of nutrients provided by inoculants are well demonstrated here in the growth and production of cotton plants. Further studies must be encouraged, especially because positive, neutral, or negative interactions between the DSF and other organisms may arise and interfere with the plant's response.

## Conclusions

1. ERR 42 and ERR 26 inoculants contribute to N and P accumulation in cotton plants, benefiting growth and production.

2. As the inoculant from ERR 42 favors high calcium accumulation, it is a promising accession for further studies involving the biostimulation of plants under natural conditions.

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