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SCIENTIFIC ARTICLE
Plant Health

Antagonistic microorganisms and nitrogen fertilization in control of tomato southern blight

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ABSTRACT
The present study assessed the efficacy of formulated biocontrol agents and nitrogen fertilization on southern blight control. Antagonism test *in vitro* was performed to assess the inhibitory activity of *Bacillus methylotrophicus* and *Trichoderma asperellum* against the growth of *Sclerotium rolfsii*. Tomato seedlings were transplanted into the substrate added with ammonium nitrate doses and inoculated with the formulated biocontrol agents Ônix (*B. methylotrophicus*) or Quality (*T. asperellum*). Subsequently, seedlings were inoculated with *S. rolfsii*. Plant mortality, shoot and root weight were assessed 11 days after the last inoculation. Agents had effective inhibitory activity against S. rolfsii; thus, they could reduce southern blight severity when combined with ammonium nitrate. However, plant mortality was not reduced by them.

Keywords: *Bacillus methylotrophicus*; *Sclerotium rolfsii*; *Solanum lycopersicum*; *Trichoderma asperellum*; ammonium nitrate.

INTRODUCTION

Sclerotium rolfsii, the pathogen of southern blight, causes root and crown rot, wilt and damping off in plants. This fungus can infect over 500 plant species due to its low host specificity (PUNJA, 1985).

Sclerotium rolfsii colonizes crop residues and produces sclerotium (resistance mechanism) to survive environmental extremes, such as lack of food sources for long periods-of-time (BENÍTEZ et al., 2004). The sclerotium of *S. rolfsii* has a melanized outer layer, cortex and a medulla consisting of thread-like hyphae. It is round-shaped and its size ranges from 0.5 to 3 mm in diameter. The secretion of oxalic acid triggers the infectious process and reacts to calcium in the cell wall, which first produces calcium oxalate, then the pathogen secrets pectinase and endopolygalacturonase (enzymes). Therefore, it enables plant tissue penetration by fungal mycelium (PUNJA, 1985; SARMA et al., 2002; SERRA; SILVA, 2005).

Southern blight is difficult to manage due to the low efficiency of the chemical control (RISTAINO et al., 1991; ROSE et al., 2003) and the low host specificity of its pathogen impairs the adoption of appropriate crop rotation and using genetic resistance (PUNJA, 1985). Soil fertility (GHINI, et al., 2001) and inoculation with antagonistic biocontrol agents (BROŽOVÁ, 2004; MORETINI; MELO, 2007) are alternatives to southern blight management.

Soil fertility helps to suppress soilborne pathogens. Nitrogen may be associated with disease severity mitigation in plants, as it is a vital nutrient for their survival. Several nitrogen sources, such as ammonium nitrate, were effective in suppressing soilborne pathogens; consequently, nitrogen was able to minimize the severity of *S. rolfsii* (CRUZ et al., 2013; GHINI et al., 2001; MORI, 2015; PACHECO, 2012).

Biological control has been considered an alternative solution to plant disease management (BROŽOVÁ, 2004), since it has helped mitigating root disease severity (BARAKAT et al., 2005) — mainly when combined to crop management (DIMKIĆ et al., 2013). *Trichoderma fungi* and *Bacillus* are able to control southern blight due to their antimicrobial action against *Sclerotium* spp. (BONTEMPO, 2016; MCLEAN; STEWART, 2000; MELERO-VARA et al., 2000; PACHECO,

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2012; VILLALTA et al., 2012). In addition, these microorganisms can be easily cultivated *en masse* (BONTEMPO, 2016) and have already been grown for commercial purposes. Therefore, the aim of this study was to assess the effectiveness of formulated biocontrol agents and nitrogen fertilization in southern blight management.

MATERIAL AND METHODS

Experiments were carried out *in vitro* and *in vivo* to assess the efficacy of antagonistic microorganisms and nitrogen fertilization in southern blight management. Experiments *in vitro* assessed the inhibitory activity of *Bacillus methylotrophicus* and *Trichoderma asperellum* against mycelial growth of *S. rolfsii* in culture medium. Experiments in vivo assessed the efficacy of antagonistic microorganisms amended with ammonium nitrate doses in reducing southern blight severity in tomato plants.

Isolated *S. rolfsii* was collected from bean plant roots (*Phaseolus vulgaris* L.) presenting damping off symptoms and grown in pure cultures, performed at Mato Grosso do Sul State University (UEMS) (20º27' S, 55º40' W). Uncontaminated sclerotia were collected and stored in plastic microtubes at 4 °C for further use.

Effect of biocontrol agents on *S. rolfsii*

The inhibitory activity of *B. methylotrophicus* against *S. rolfsii* was assessed through the cross-streak method (FUGA et al., 2011). The microbial strain Ônix (Farroupilha Laboratory: Patos de Minas City, MG) (*B. methylotrophicus* 16 $g.L^{-1}$) was selected for the experiment. Yeast extract-dextrose-$CaCO_3$ (YDC) agar plates (CHUNG et al., 2015) were added with bacterial suspension (0.5 mL) and were incubated at 30 ± 2 °C (PALOP et al., 1999) under a 12-hour photoperiod for 24 h (PACHECO, 2012). The bacterium was subsequently inoculated in potato-dextrose-agar (PDA) by a single streak in the center of the dish. Two mycelial pieces (4 × 4 mm) were placed 1 cm apart from the edges of the plates and later grown in a biological oxygen demand (BOD) growth chamber at 27 ± 2 °C under a 12-hour photoperiod for 2 days. Finally, the diameter of *S. rolfsii* colonies was measured.

The efficacy in vitro of the inhibitory activity and the action of volatile and nonvolatile metabolites of *T. asperellum* against *S. rolfsii* were assessed using commercial biocontrol agent Quality (*T. asperellum* 280 $g.L^{-1}$) (Farroupilha Laboratory: Patos de Minas City, MG), whose granules were washed and placed in PDA agar plates added with streptomycin sulfate (300 $mg.L^{-1}$). The plates were incubated at 25 ± 2 °C under a 12-hour photoperiod for 7 days (CARVALHO et al., 2014). Mycelial pieces were placed on the opposite end of PDA agar plates added with streptomycin sulfate (300 $mg.L^{-1}$) and arranged in parallel to the mycelial pieces. Colonies were measured 3 days after incubation at 25 ± 2 °C under a 12-hour photoperiod.

The assessment of nonvolatile metabolites was carried out based on agar disk diffusion method. First, a cellophane disc (10 cm in diameter) was placed on the surface of PDA agar added with streptomycin sulfate (300 $mg.L^{-1}$). A mycelial disc of *T. asperellum* fungus was placed in the center of the cellophane, incubated at 25 ± 2 °C under a 12-hour photoperiod for 3 days, for growth and metabolite production purposes. The cellophane paper was discarded and the plate lid was disinfected by flaming to a Bunsen burner flame to reduce contamination. One mycelial disc of *S. rolfsii* was placed in the center of each agar plate, incubated at 25 ± 2 °C under a 12-hour photoperiod. Incubation period ended when *S. rolfsii* had grown over the agar surface on plates without metabolites of T. asperellum. (DENNIS; WEBSTER, 1971; MARTINS-CORDER; MELO, 1998).

Trichoderma asperellum volatile metabolites antagonistic to *S. rolfsii* was assessed as follows: mycelial pieces of *S. rolfsii* and *T. asperellum* were individually placed in the center of PDA agar plates, which were separately incubated at 25 ± 2 °C under a 12-hour photoperiod for 24 h. After incubation, the lid of the plates containing *T. asperellum* was replaced by the plates containing *S. rolfsii*. The two half-plates were taped together for later incubation, which kept on going until *S. rolfsii* colonies without volatile metabolites completely colonized the agar. The diameter of *S. rolfsii* colonies was measured (CARVALHO et al., 2014; DENNIS; WEBSTER, 1971).

Diameters of *S. rolfsii* colonies (DMsr) and their nonantagonistic controls (DM_{WB}) were measured in all tests *in vitro*. Inhibition (expressed in percentage) was calculated through the following (Eq. 1):

$$\%\text{Inhibition} = \left(\frac{DM_{WB} - DM_{SR}}{DM_{WB}} \right) \text{x } 100 \quad (1)$$

Inhibition was graded based on the scale by BELL et al. (1982) (BARAKAT et al., 2005), which classifies five grades of antagonistic degrees: grade 1 is full control; grade 2 is 75% control; grade 3 is 50% control; grade 4 is up to 25% control; grade 5 is no control. Experiments *in vitro* followed a completely randomized design with 10 replications. Tests were seen as separate experiments.

Effect of biocontrol agents and ammonium nitrate on tomato southern blight

Sclerotium rolfsii inoculum was prepared in polished rice, based on the method by SERRA; SILVA (2005), with modifications. Polished rice grains were immersed in distilled water for 2 h and sterilized in an autoclave. Subsequently, five mycelial pieces were inoculated in flasks added with 50 g of rice, incubated at 27 ± 2 °C under a 12-hour photoperiod for 7 days.

Tomato seedlings (*Solanum lycopersicum* L. 'Santa Cruz Kada Gigante') were grown in a greenhouse using 128 cells polystyrene trays filled with Carolina substrate (Soil do Brasil). The seedlings were irrigated by micro sprinklers for 1 h, daily, for 30 days. Seedlings were transplanted to pots filled with 1.5 L of substrate (Carolina Soil do Brasil) amended with the following ammonium nitrate doses: 0, 50, 100, 200 or 400 $mg.L^{-1}$.

The experiment was carried out in a randomized design, following a 5×3+1 factorial design (5 doses of ammonium nitrate × 3 biocontrol agents + additional treatment without ammonium nitrate, antagonistic agents and *S. rolfsii*), with 3 replicates (6 plants each).

Biocontrol agents Ônix (*B. methylotrophicus*) and Quality (*T. asperellum*) were inoculated through Pasteur pipette right after transplantation. The applied doses were proportional to the recommended ones: 3 mL of Ônix or 2 mL of Quality inoculated into 100 kg of seeds. Five days after seedling transplantation, *S. rolfsii* was inoculated with 8 g of colonized rice per liter of substrate.

Plant mortality was assessed when the tomato plants inoculated with *S. rolfsii* started presenting the symptoms. The assessment was carried out daily for 10 days. Subsequently, plants were removed from substrate and sectioned to assess the following variables: shoot fresh weight (SFW) and root fresh weight (RFW); shoot dry weight (SDW) and root dry weight (RDW).

Statistical analysis

Colony diameters measured *in vitro* were subjected to t-test at 5% significance level with 10 replications. The resulting variables were subjected to variance analysis, Bartlett's test for homogeneity of variance. Contrasts of interest were performed by applying the Scheffé test ($p < 0.05$) as follows: not inoculated × (*B. methylotrophicus* + without biocontrol + *T. asperellum*); without biocontrol × (*B. methylotrophicus* + *T. asperellum*); *B. methylotrophicus* × *T. asperellum*. Plant weight data were transformed through square root ($\sqrt{x}$), whereas mortality was transformed through arcsine square root ($\sin^{-1}\sqrt{x}$). Statistical analyses were performed using SAS 9.1 software (SAS Institute Inc. Cary, NC, USA).

RESULTS AND DISCUSSION

Bacillus methylotrophicus and *T. asperellum* (antagonistic microorganisms) were effective in reducing mycelial growth of *S. rolfsii* during tests of parallel growing and metabolites antagonism assessment in vitro ($p < 0.001$). Treatments without biological control were also not colonized by *Trichoderma* and/or *Bacillus*. Thus, cross-contamination between treatments and replications was disregarded.

Bacillus methylotrophicus reduced the mycelial growth of *S. rolfsii* by 32%, classified as grade 3 antagonism (Fig. 1). Genus *Bacillus* produced substances such as fengycin, iturin and surfactin (BAIS et al., 2004; CHUNG et al., 2015; CRANE et al., 2013; MCLEAN; STEWART, 2000) with fungicidal ability to inhibit pathogen growth (FUGA et al., 2011; STEIN, 2005). Therefore, this inhibition is assumingly induced by antibiosis.

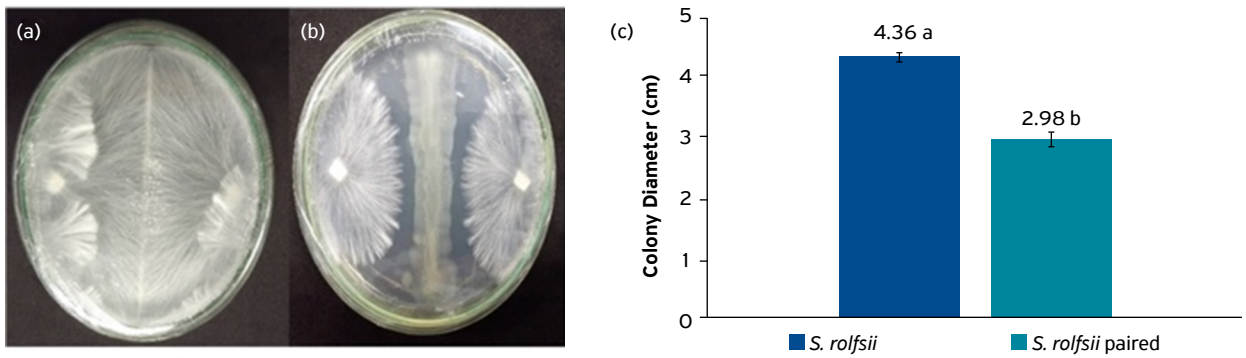

Figure 1. Inhibitory activity of *B. methylotrophicus* against *S. rolfsii* 2 days after incubation. (a) *S. rolfsii*; (b) paired *S. rolfsii*; (c) colony diameters. Means followed by the same letter were not significantly different at $p < 0.0001$ in the t-test. Bars represent mean standard error. Growth inhibition = 32%.

Trichoderma asperellum reduced mycelial growth of *S. rolfsii* by 14% when they were paired together. This rate was classified as grade 4 antagonism (Fig. 2). ROCHA et al. (2016) found that *T. asperellum* isolates inoculated in Quality showed decreased growth against high toxin-producing *Fusarium solani* isolates, which could suggest limiting factors to Quality dose for soilborne pathogen control.

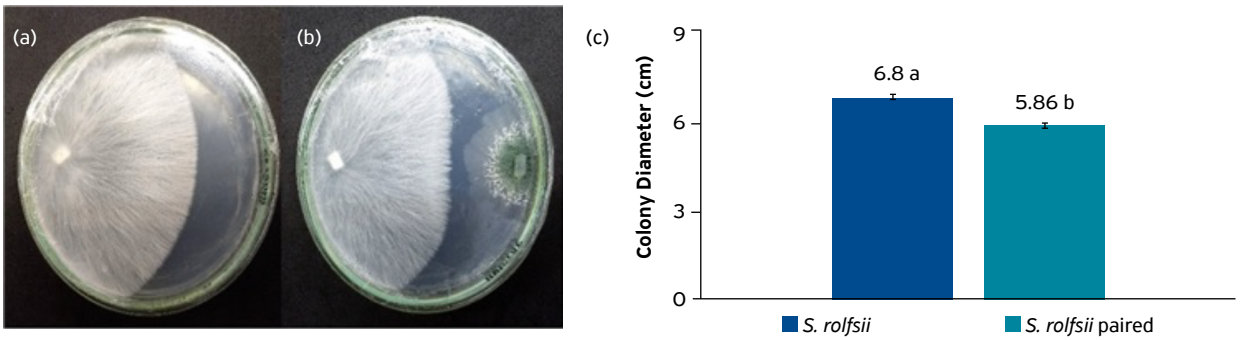

Figure 2. Mycelial growth of *S. rolfsii* paired with *T. asperellum* 3 days after incubation. (a) *S. rolfsii*; (b) paired *S. rolfsii*; (c) colony diameters. Means followed by the same letter were not significantly different in the t-test. Bars represent mean standard error. Growth inhibition = 13.8%.

The sensitivity of *S. rolfsii* to metabolites secreted by *T. asperellum* has shown that nonvolatile metabolites reduced the mycelial growth of *S. rolfsii* by approximately 57% (Fig. 3), whereas volatile metabolites reduced it by approximately 40% (Fig. 4). These rates were classified as grade 3 and 4 antagonisms, respectively. Data indicated that the secretion of toxic antifungal metabolites was the most effective biocontrol response of *T. asperellum* against *S. rolfsii*. Although *Trichoderma* species are associated with antibiosis through mycoparasitism — colonization and degradation of hyphae and pathogen resistance structures (BENÍTEZ et al., 2004; BONTEMPO, 2016) — their key mechanism against some fungal species and isolates is the secretion of toxic metabolites, as the case of *T. asperellum*.

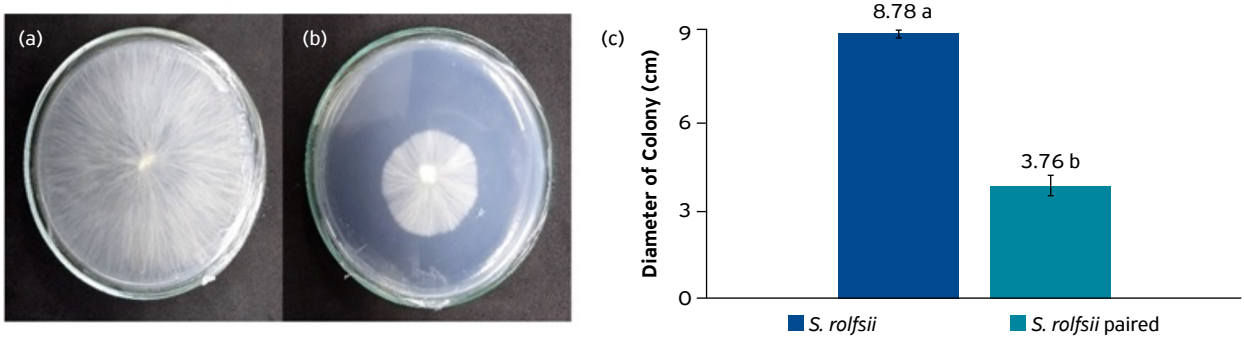

Figure 3. Mycelial growth of *S. rolfsii* under antibiosis against *T. asperellum*'s nonvolatile metabolites 2 days after incubation. (a) *S. rolfsii*; (b) *S. rolfsii* under antibiosis; (c) t-test. Different letters indicate different values in the t test ($p < 0.0001$). Bars represent mean standard error. Growth inhibition = 57%.

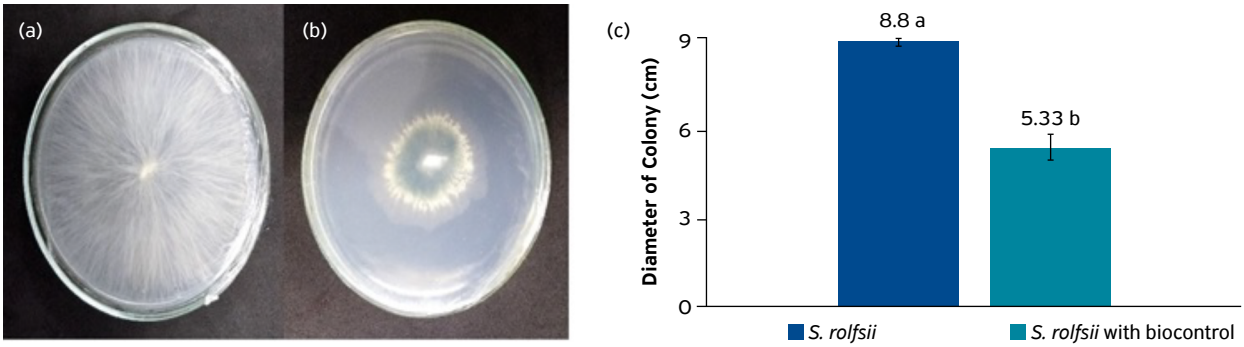

Figure 4. Mycelial growth of *S. rolfsii* under antibiosis against *T. asperellum*'s volatile metabolites 2 days after incubation. (a) *S. rolfsii*; (b) Antagonized *S. rolfsii*; (c) Diameter of *S. rolfsii* colonies. Different letters indicate statistically different values in the t test ($p < 0.0001$). Bars represent mean standard error. Growth inhibition = 39.5%.

Biocontrol agents had no effect on tomato plant mortality 11 days after inoculation with *S. rolfsii* (Fig. 5), according to the F test (p = 0.1550). Ammonium nitrate doses did not reduce tomato plant mortality (Fig. 5) in the F test (p = 0.5228).

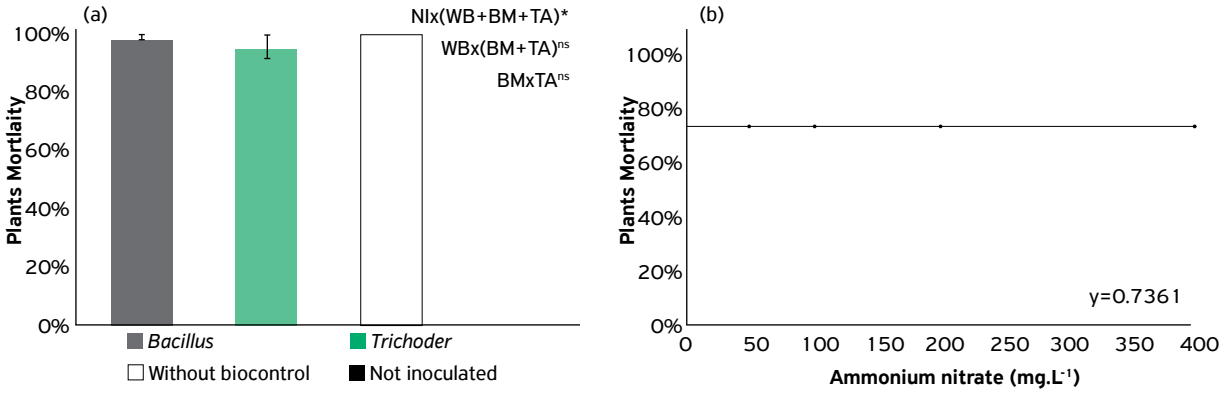

Figure 5. Mortality of tomato plants 11 days after inoculation with *S. rolfsii*: (a) by different biocontrol agents and (b) under ammonium nitrate doses. Bars represent mean standard error; ns: not significant at 5% in the Scheffé's test; *: significant at 5% in the Scheffé's test. *Bacillus methylotrophicus* (BM); *T. asperellum* (TA); without biocontrol (SB); not inoculated (TT).

Although the genus *Trichoderma* is acknowledged as a soilborne biocontrol agent, it was not effective against *S. rolfsii*. This inefficiency could be caused by variations on type and amount of toxic secondary metabolites among *Trichoderma* species and even isolates (MARTINS-CORDER; MELO, 1998). These variations cause biocontrol effectiveness to vary by up to 95% (ROSA; HERRERA, 2009). Nevertheless, *Trichoderma* spp. efficacy in soilborne pathogen biocontrol still depends on critical factors, such as mycelial growth speed (SERRA; SILVA, 2005), ability to compete for nutrients and high sensitivity to environmental extremes (ETHUR et al., 2005). Experiments *in vivo* showed that competitors can reduce the inhibitory activity of *Trichoderma* sp. against *Sclerotinia sclerotiorum* in cucumber (ETHUR et al., 2005).

Root dry weight was affected by biocontrol agent's inoculation (p < 0.05) (Fig. 6) and nitrogen doses were only effective for shoot dry biomass (Fig. 7). However, the data did not suggest suppressiveness, the higher biomass values could be attributed only to nitrogen fertilization (CARDOSO; USTULIN FILHO, 2013).

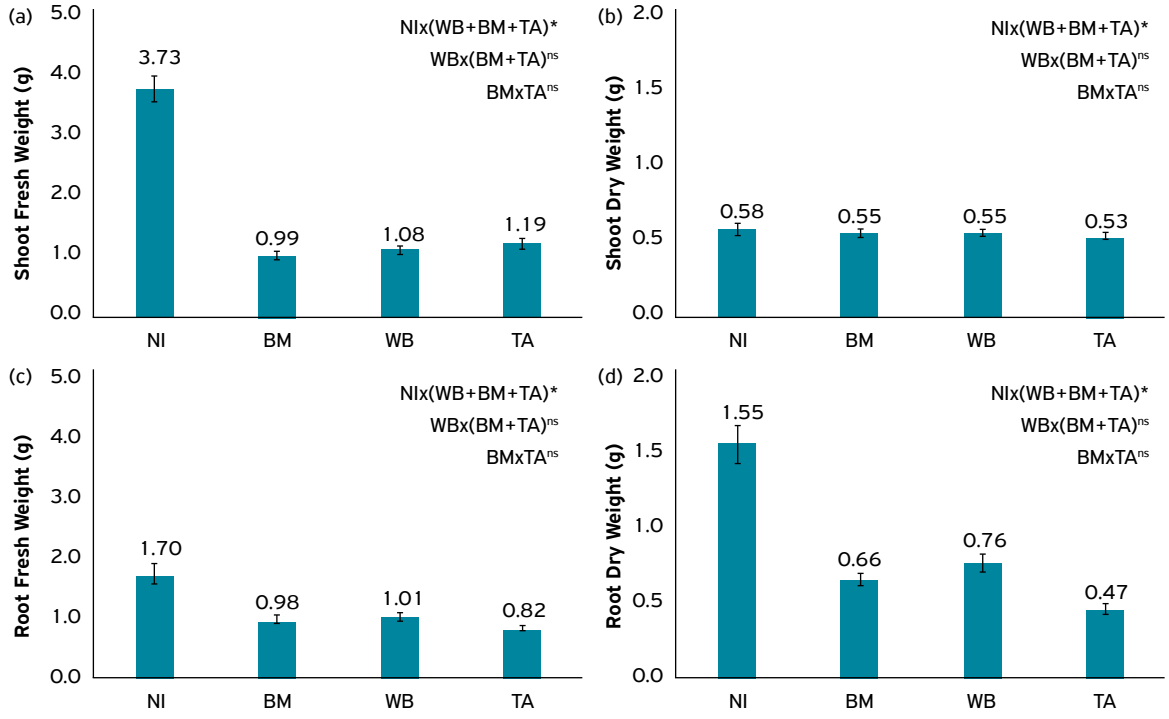

Figure 6. Variables above-ground Fresh (a) and dry (b) biomass; variables fresh (c) and dry (d) weight of tomato plant roots inoculated with *S. rolfsii* based on the following classifications: not inoculated, *B. methylotrophicus*, without biocontrol and *T. asperellum.* Bars represent mean standard error; ns: not significant at 5% in the Scheffé's test; *: significant at 5% in the Scheffé's test. *Bacillus methylotrophicus* (BM); *T. asperellum* (TA); without biocontrol (SB); not inoculated (TT).

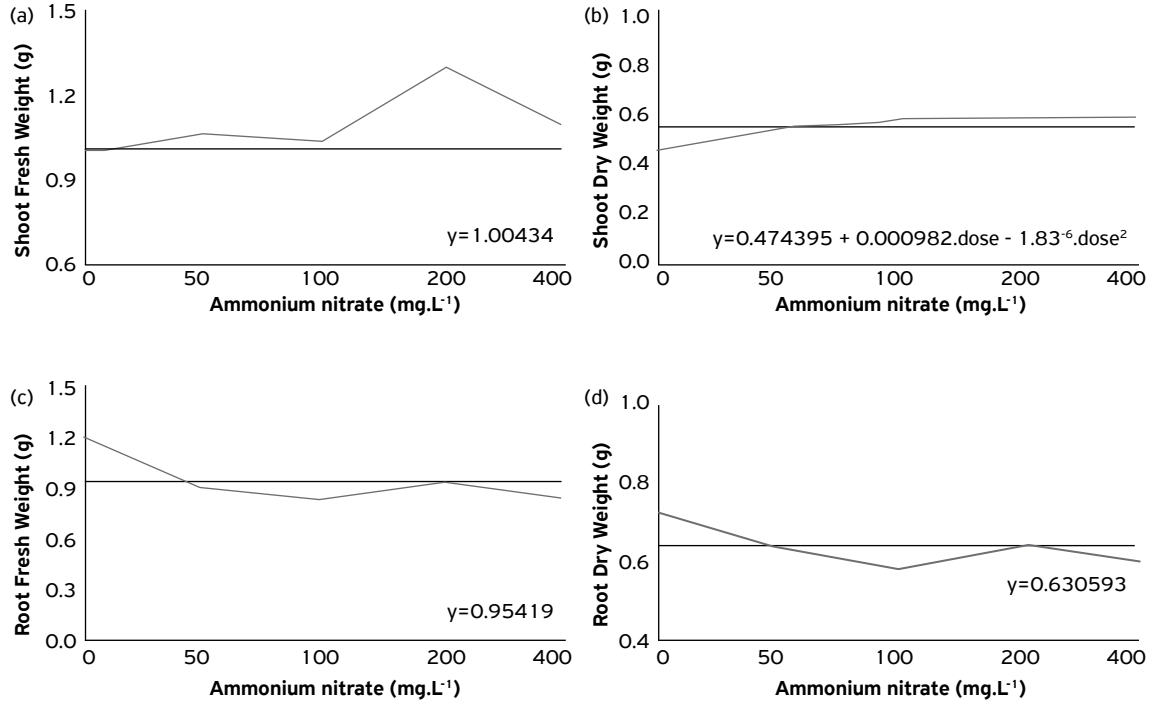

Figure 7. Regression adjustment of above-ground fresh (a) and dry (b) weight; regression adjustment of fresh (c) and dry (d) weight of tomato plant roots inoculated with *S. rolfsii* in agar added with different ammonium nitrate doses.

Root dry weight recorded higher values when tomato plants were treated with *B. methylotrophicus*, this finding suggests an inhibitory activity of these bacteria against *S. rolfsii*. On the other hand, *B. methylotrophicus* had no effect on shoot weight of tomato plants, which indicates that it does not promote plant growth, but produces antifungal metabolites or competes for nutrients (antibiosis) as biocontrol response (ALABOUVETTE et al., 2009; CHUNG et al., 2015). Moreover, plants inoculated with antagonistic *Bacillus* species may develop the ability to inhibit oxidative stress and to reduce disease severity (HASHEM et al., 2017), this reaction must be further studied on tomato southern blight.

Although bacteria belonging to the genus *Bacillus* have biocontrol ability (CHUNG et al., 2015), tomato plants mortality was not reduced by *B. metylotrophicus*. Biocontrol efficacy could have been impaired by other factors, such as bacterial population density, specific bacterial activity and application method (ABDALLA et al., 2014). Experiments *in vivo* carried out with *Bacillus* sp. demonstrated that the inoculation of this species in the soil reduced charcoal rot (*Macrophomina* sp.) severity in strawberry plants by 10%. On the other hand, when it was inoculated straight into their roots, disease severity was reduced by 30% (PASTRANA et al., 2016). Moreover, *Bacillus* spp. applied as seed treatment presented up to 93% reduction in root rot of mustard seeds inoculated with *S. sclerotiorum* (MORETINI; MELO, 2007). Even though *Bacillus* has several action mechanisms for plant protection, research results suggested that it is more effective when inoculated straight into plant tissue, rather than just in the soil.

CONCLUSIONS

Data has shown that biocontrol agents assessed *in vitro* have inhibitory activity against *S. rolfsii* growth. Biocontrol helped to mitigate southern blight severity when it was combined with nitrogen fertilization. Nevertheless, the tested biocontrol agents, when combined with ammonium nitrate, did not control southern blight. Hence, further research must be carried out on the following: biocontrol application methods; the association of biocontrol agents with alternative management measures; inhibitory activity in vivo of different *Bacillus* and/or *Trichoderma* species and isolates against *S. rolfsii*.

AUTHORS' CONTRIBUTIONS

Conceptualization: Graichen, F.A.S.; Blanco, N.H.M. **Methodology:** Graichen, F.A.S.; Blanco, N.H.M. **Formal Analysis:** Graichen, F.A.S.; Blanco, N.H.M. **Visualization:** Graichen, F.A.S.; Blanco, N.H.M. **Investigation:** Blanco, N.H.M.; Barbosa, D.F.R. **Writing – original draft:** Blanco, N.H.M.; Barbosa, D.F.R.; Graichen, F.A.S. **Writing – review & editing:** Blanco, N.H.M.; Graichen, F.A.S. **Supervision:** Graichen, F.A.S.

AVAILABILITY OF DATA AND MATERIAL

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CONFLICTS OF INTEREST

All authors declare that they have no conflict of interest.

ETHICAL APPROVAL

Not applicable.

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