

***Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore IN SOYBEAN SEEDS: OCCURRENCE, PATHOGENICITY, AND ANTAGONISTIC POTENTIAL OF *Bacillus* spp.**

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ABSTRACT:

Soybean (*Glycine max* (L.) Merr.) is an important global crop that is often affected by phytopathogenic fungi. These fungi compromise seed germination and early seedling development, resulting in significant yield losses. This study examined the fungi naturally associated with soybean seeds, the physiological performance of various seed lots, the impact of *Bacillus* spp. treatments on the incidence of fungi associated with seeds and seed germination, and the ability of *Bacillus* isolates to antagonize *Colletotrichum truncatum*. Four soybean cultivars (OLIMPO, 82i78, NR8383RR, and SYN16871PRO) were evaluated using assays of seed health, germination, emergence, pathogenicity, seed treatment, and *in vitro* antagonism. The most prevalent fungi identified were *Aspergillus* sp. (5.5%), *Cladosporium* sp. (7.5%), and *Colletotrichum truncatum* (0.25%). The pathogenicity of the *C. truncatum* isolate was confirmed by symptom development and subsequent re-isolation of the pathogen from inoculated plants. However, cultivar 82i78 could not be evaluated due to the absence of plant development. The incidence of fungi associated with seeds and germination varied depending on the treatment with *Bacillus* species, cultivar, and surface disinfection conditions. *In vitro* confrontation assays demonstrated strong antagonistic activity against *C. truncatum*. All *Bacillus* isolates showed high mycelial growth inhibition, and isolate MOL27 (*Bacillus* sp.) reached the highest inhibition value (84%). Overall, these results demonstrate that *Bacillus* spp. can influence the incidence of fungi naturally associated with soybean seeds and exhibit strong antagonistic activity against *C. truncatum in vitro*. This supports further investigation of these isolates as potential biological control agents.

Keywords: Biological control; Seed health; Anthracnose.

***Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore EM SEMENTES DE SOJA: OCORRÊNCIA, PATOGENICIDADE E POTENCIAL ANTAGÔNICO DE *Bacillus* spp.**

RESUMO:

A soja (*Glycine max* (L.) Merr.) é uma cultura mundialmente importante, mas é frequentemente afetada por fungos fitopatogênicos. Estes, por sua vez, afetam a germinação das sementes e o desenvolvimento inicial das plântulas, causando perdas significativas na produção. O presente estudo teve como objetivo examinar os fungos naturalmente associados às sementes de soja, avaliar o desempenho fisiológico de vários lotes de sementes e determinar o impacto dos tratamentos com *Bacillus* spp. na incidência de fungos associados às

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sementes e na sua germinação, bem como avaliar a capacidade dos isolados de *Bacillus* de antagonizar o *Colletotrichum truncatum*. Foram avaliadas quatro cultivares de soja (OLIMPO, 82i78, NR8383RR e SYN16871PRO) por meio de ensaios de sanidade das sementes, germinação, emergência, patogenicidade, tratamento das sementes e de antagonismo *in vitro*. Identificou-se que os fungos mais prevalentes foram o *Aspergillus* sp. (5,5%), o *Cladosporium* sp. (7,5%) e o *Colletotrichum truncatum* (0,25%). A patogenicidade do isolado de *C. truncatum* foi confirmada pelo desenvolvimento de sintomas e pelo subsequente reisolamento do patógeno a partir de plantas inoculadas. No entanto, não foi possível avaliar a cultivar 82i78, devido à falta de desenvolvimento das plantas. A incidência de fungos associados às sementes e a germinação variaram entre os tratamentos com *Bacillus* spp., as cultivares e as condições de desinfestação superficial. Os ensaios de confronto *in vitro* demonstraram uma forte atividade antagônica ao *C. truncatum*. Todos os isolados de *Bacillus* inibiram significativamente o crescimento micelial, sendo que o isolado MOL27 (*Bacillus* sp.) obteve a maior taxa de inibição (84%). Em suma, os resultados mostram que as espécies de *Bacillus* podem influenciar a incidência de fungos naturalmente associados às sementes de soja e que têm uma forte atividade antagônica ao *C. truncatum in vitro*. Isto justifica uma investigação mais aprofundada desses isolados como potenciais agentes de controle biológico.

Palavras-chave: Controle biológico; Saúde das sementes; Antracnose.

INTRODUCTION

Glycine max (L.) Merr., commonly known as soybean, is an oilseed crop of great global importance, valued for its high protein content and as one of the main sources of vegetable oil (Anderson *et al.*, 2019). Phytopathogenic fungi, however, pose a significant threat to the production of this crop. The genera *Alternaria*, *Colletotrichum*, *Diaporthe*, *Fusarium*, and *Phoma* are among the main causes of disease, resulting in serious economic losses (Batzner and Mueller, 2020; Huilgol *et al.*, 2020). In more severe cases, species such as *Fusarium* can cause plant death during germination, which affects the entire crop cycle (Zhao *et al.*, 2022).

The main agent of anthracnose in soybeans is *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore, which is part of the genus *Colletotrichum*. It is a seed-borne pathogen capable of compromising the entire development of the plant. In addition to compromising seed vigor, infected seeds constitute the main source of inoculum for new growing areas, directly contributing to the spread of the disease. Significant reductions in productivity have been associated with areas contaminated by this pathogen, particularly when high humidity favors the development of the disease (Silva Júnior *et al.*, 2025).

In this context, the study of biocontrol strategies is a fundamental parameter for minimizing the impact of the disease and ensuring soybean productivity. Among the known strategies, microorganisms are increasingly recognized as promising tools for controlling plant pathogens because of their sustainability. These microorganisms promote resistance to both biotic and abiotic stresses, favor nutrient supply, and play an essential role in interacting with plants, especially in the rhizosphere, which is crucial for plant health. Strains of *Bacillus* sp. act directly by producing phytohormones (indole-3-acetic acid, cytokinins, and gibberellins), siderophores, and by promoting nutrient solubilization. In addition, they act indirectly through mechanisms such as competition, antibiosis, and induction of systemic resistance (Andrić *et al.*, 2020; Khan *et al.*, 2023).

Several studies have demonstrated the potential of these bacteria in managing soybean diseases. *Bacillus subtilis*, for example, has been shown to be effective against *Fusarium oxysporum*, mainly due to the production of antifungal metabolites that destabilize the pathogen's hyphae

(Santos *et al.*, 2022). Similarly, other *Bacillus* strains reduced the progression of white mold and stimulated soybean growth, increasing aboveground biomass and plant resistance (Putri *et al.*, 2023). These results reinforce the viability of using *Bacillus* sp. as a sustainable biocontrol agent aligned with integrated disease management.

Considering the economic importance of soybeans and the impact of threats posed by seed-borne fungi such as *C. truncatum*, it is essential to understand their incidence, transmissibility, and impact on the physiological quality of cultivars. Therefore, this study aimed to: (i) characterize the occurrence and incidence of fungi associated with seeds of four soybean cultivars; (ii) assess the pathogenicity of the *C. truncatum* isolate recovered from soybean seeds; and (iii) evaluate the antagonistic activity of *Bacillus* spp. isolates against *C. truncatum* *in vitro*, as well as their effects on overall fungal incidence and soybean seed germination.

METHODOLOGY

Microorganisms used in the study

The experiments were conducted in the Molecular Biology and Plant Pathology laboratories at the Federal University of Tocantins, Gurupi Campus (UFT). Seeds from four soybean cultivars were used: OLIMPO and 82i78, originating from the municipality of Gurupi, Tocantins (1st harvest of 2024; 11°43'48" S, 49°04'08" W; altitude of 287 m), and NR8383RR and SYN16871PRO, from the municipality of Formoso, Tocantins (1st harvest of 2024; 11°47'48" S, 49°31'44" W; altitude of 240 m).

The bacterial strains used in this study belonged to the *Bacillus* collection of the Molecular Biology Laboratory at the Federal University of Tocantins (UFT), Gurupi campus, and were originally isolated from rhizosphere soils. One isolate was molecularly identified as *Bacillus velezensis* (GenBank accession number OQ725688.1), whereas the remaining isolates were classified as *Bacillus* spp. based on morphological and phenotypic characteristics. These strains were kept at 5°C in cryopreservation tubes containing Luria-Bertani (LB) culture medium, composed of 5 g L⁻¹ of yeast extract, 10 g L⁻¹ of tryptone, 10 g L⁻¹ of NaCl, and 20 g L⁻¹ of agar, according to the methodology described by Silva *et al.* (2017). For the experiments, the strains

were reactivated in Petri dishes with LB culture medium.

Detection of fungal pathogens in soybean seeds

The phytosanitary analysis of the seeds was conducted using the Blotter method with deep freezing, in accordance with the International Rules for Seed Testing (ISTA, 2024), with the aim of detecting the presence of fungal pathogens associated with seeds. To this end, the treatments were organized into two experimental conditions: (i) seeds without surface disinfestation; and (ii) seeds subjected to surface disinfestation. In the surface disinfestation treatment, the seeds were immersed in 50% ethyl alcohol for 30 seconds, followed by immersion in 1% sodium hypochlorite for 40 seconds. Next, three sequential washes were performed with autoclaved distilled water (Kuldybayev *et al.*, 2023). Subsequently, the seeds were distributed evenly on moistened Germitest paper substrate and arranged in Gerbox-type boxes. The process was carried out with four replicates consisting of 25 seeds per treatment, totaling 100 seeds per experimental condition.

The Gerbox boxes were incubated in a growth chamber with a 12-hour photoperiod and a constant temperature of 25 °C for 24 hours. They were then frozen at -20 °C for 24 hours and subsequently returned to the initial incubation conditions for another five days, totaling seven days of testing (Goulart, 1997). At the end of the incubation period, each seed was individually evaluated for the presence of fungal structures under a stereoscopic microscope, with pathogens identified based on morphological characteristics according to the criteria of Barnett and Hunter (1972).

Initial seed performance: germination and emergence

The germination test was performed according to the methodology described by BRASIL (2009). The seeds were sown at a depth of 1.0 cm in autoclaved medium sand substrate, using 100 seeds per treatment. The trays were kept in a greenhouse with daily manual irrigation, which was carried out in the morning and late afternoon to ensure the necessary humidity for germination and seedling emergence.

Germination was evaluated daily, and seeds were considered germinated when the cotyledons were fully open. Germination percentage (G%) was calculated according to the following equation: $G\% =$

$(N/A) * 100$, where G% represents the germination percentage, N is the number of germinated seeds, and A is the total number of seeds sown (Borghetti and Ferreira, 2004).

To determine the Emergence Speed Index (ESI), seedlings with cotyledons visible above the soil surface were counted daily until the 21st day after sowing. The IVE was calculated using the following equation:

$$ESI = (E1/N1) + (E2/N2) + \dots + (En/Nn)$$

where E corresponds to the number of seedlings that emerged in each count and N corresponds to the number of days from sowing to each assessment (Maguire, 1962).

Assessment of the natural seed-to-seedling transmission of seed-associated fungi

The potential seed-to-seedling transmission of fungi naturally associated with soybean seeds was evaluated according to the methodology adapted from Sales *et al.* (2018). A total of 200 seeds from each soybean cultivar (OLIMPO, 82i78, NR8383RR, and SYN16871PRO) were evaluated under their naturally occurring phytosanitary condition, without artificial fungal inoculation. Two experimental conditions were considered: non-surface-disinfested seeds and surface-disinfested seeds, with 100 seeds per condition.

Seedlings were monitored at 5, 10, 15, and 20 days after sowing for the development of visible symptoms potentially associated with fungal infection. Symptomatic seedlings were collected and examined under a stereomicroscope, and fungi associated with the affected tissues were isolated on potato dextrose agar (PDA) and identified based on their morphological characteristics. This approach was designed to investigate the potential transmission of naturally occurring seed-associated fungi from seeds to seedlings rather than transmission following artificial inoculation.

Pathogenicity test of *Colletotrichum truncatum* on soybeans

The pathogenicity test was conducted by artificially inoculating *C. truncatum* onto soybean plants 30 days after emergence (DAE), following a modified version of the methodology described by Sales *et al.* (2018). The plants were grown in plastic bags (25 × 30 cm) containing a 1:1:1 mixture of sand,

soil, and commercial substrate, enriched with 10 g of NPK 5-25-15 fertilizer. At 30 DAE, a conidial suspension of *C. truncatum* (1×10^6 conidia mL⁻¹) was sprayed uniformly over the aerial parts using a manual sprayer. After inoculation, plants were maintained in a humid, dark chamber for 48 h. Disease development was assessed based on symptoms compatible with soybean anthracnose. To complete Koch's postulates for *C. truncatum*, the fungus was re-isolated from symptomatic tissues and cultured on PDA, and its morphological characteristics were compared with those of the original isolate (Alfenas and Mafia, 2007). The experiment was conducted with three replicates, with non-inoculated healthy plants serving as the control group.

Antifungal potential of *Bacillus* spp. and its effects on soybean seed germination

The effects of *Bacillus* isolates on seed-borne fungal incidence and soybean seed germination were evaluated according to a modified version of the methodology described by Cavaglieri *et al.* (2005). *Bacillus velezensis* (OQ725688.1) and six additional *Bacillus* spp. isolates (626, MOL02, MOL11, MOL22, MOL23, and MOL27) were cultivated in liquid Luria-Bertani (LB) medium under constant agitation (200 rpm) at 28 ± 2 °C for 48 h. After incubation, 5 mL of each bacterial suspension adjusted to 1×10^9 CFU mL⁻¹ was applied to surface-disinfested and non-disinfested seeds of the soybean cultivars OLIMPO, 82i78, NR8383RR, and SYN16871PRO. The treated seeds were incubated for 12 h at 25 °C and subsequently transferred to Gerbox boxes containing a moistened substrate. After eight days of incubation, two response variables were assessed: (i) the incidence of fungi associated with the seeds and (ii) seed germination percentage.

In vitro* tests of *Bacillus* spp. against *Colletotrichum truncatum

The methodology was based on the direct confrontation assay, adapted from Rangeshwaran and Prasad (2000) and Zambrano *et al.* (2022). *Bacillus velezensis* (OQ725688.1) and six additional *Bacillus* spp. isolates (626, MOL02, MOL11, MOL22, MOL23, and MOL27) were cultured as previously described until reaching a concentration of 1×10^9

CFU mL⁻¹. For the assay, a 5 mm mycelial plug obtained from the actively growing margin of a *Colletotrichum truncatum* colony was placed on one side of a Petri dish containing PDA medium. Each bacterial isolate was inoculated on the opposite side of the plate, maintaining a distance of 30 mm between the inoculation points and the edge of the dish. The plates were incubated at 28 °C in a BOD chamber, and mycelial growth inhibition was assessed every 24 h for eight days (24, 48, 72, 96, 120, 144, 168, and 192 h). The radial growth inhibition percentage (RGIP) was calculated according to the equation proposed by Forkema (1976): $RGIP (\%) = [(R1 - R2) / R1] \times 100$, where R1 represents the radial growth of the fungus away from the antagonist and R2 represents the radial growth towards the antagonist microorganism. As a control, the phytopathogen was grown alone in PDA medium under the same experimental conditions.

Statistical analysis

All assays were performed in triplicate. GraphPad Prism software version 10.4 was used for statistical analysis of the data. Means were subjected to analysis of variance (ANOVA) and comparison of means using Tukey's test ($p < 0.05$).

RESULTS

Main fungi found in soybean seeds

The seed health test showed that the overall incidence of seed-associated fungi was higher in non-surface-disinfested seeds than in surface-disinfested seeds. Three fungal groups were identified morphologically: *Cladosporium* sp. (7.5%), *Aspergillus* sp. (5.5%), and *Colletotrichum truncatum* (0.25%). In the context of soybean seed health, *C. truncatum* was considered a phytopathogenic fungus detected in the assay, whereas *Aspergillus* sp. was treated primarily as a storage-associated fungus and *Cladosporium* sp. as a saprophytic/contaminant fungus. Surface disinfestation markedly reduced the detection of *Cladosporium* sp. (100%) and partially reduced *Aspergillus* sp. (24%) (Figure 1A-B). The very low natural incidence of *C. truncatum* precluded inference about treatment effects on this pathogen from the seed-health assay alone (Figure 1C).

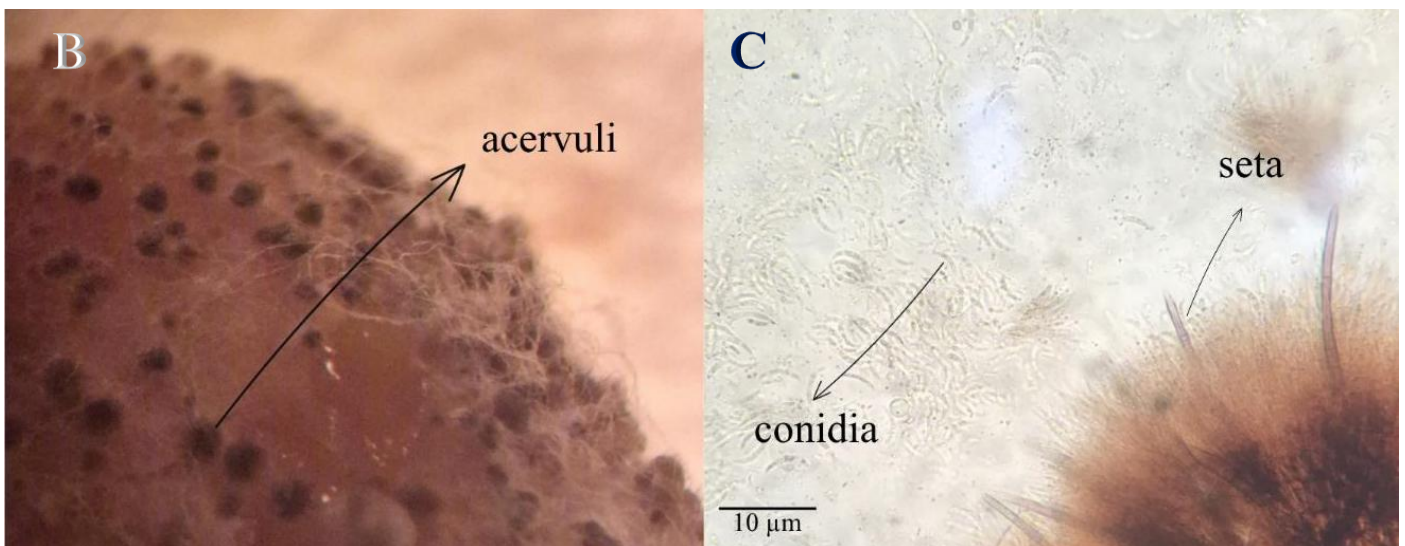
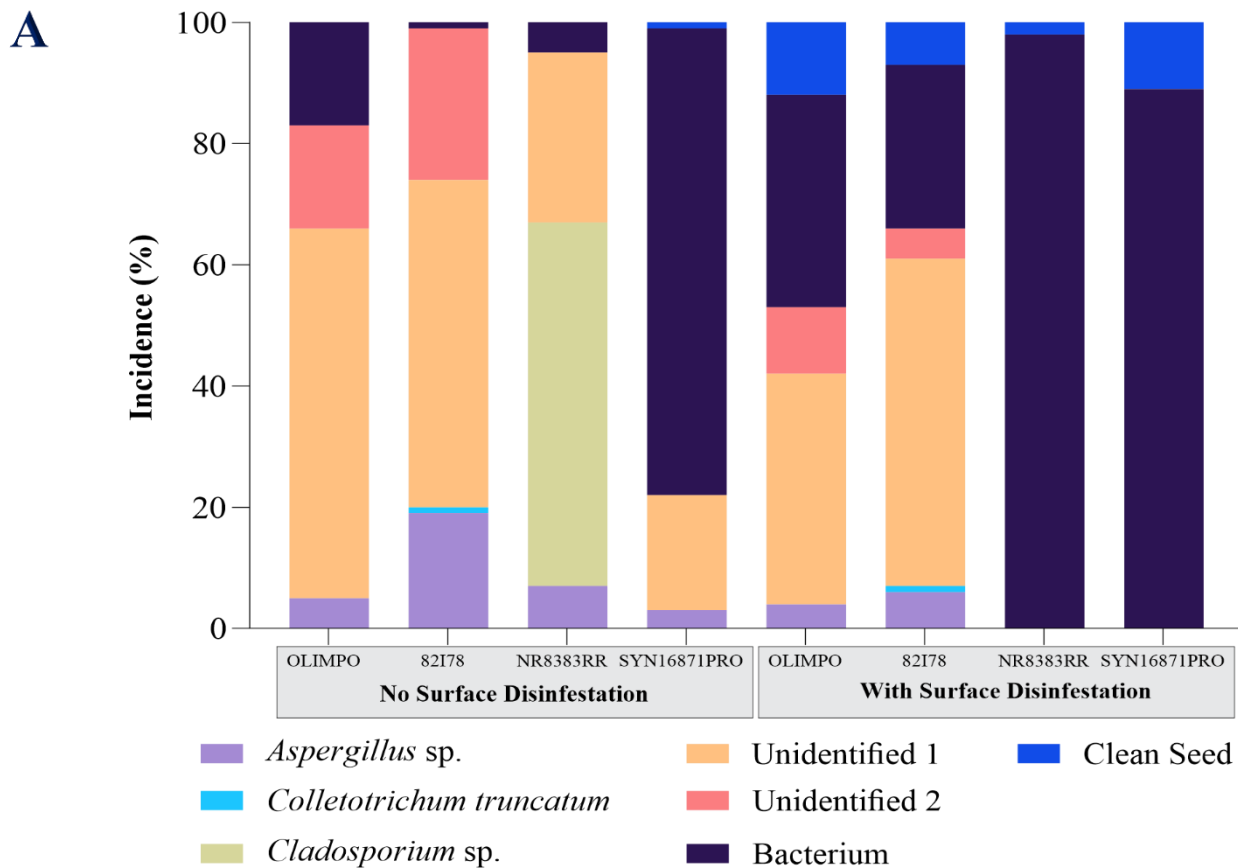


Figure 1. (A) Incidence of fungi associated with seeds of cultivars OLIMPO, 82i78, NR8383RR, and SYN16871PRO, according to treatments with and without surface disinfection (distilled water, 1% hypochlorite, and 50% alcohol). N1: unidentified species 1; N2: unidentified species 2. (B) Details of acervuli on soybean seeds under a magnifying glass belonging to the species *Colletotrichum truncatum*. (C) Optical microscopy (40x magnification) of *C. truncatum* conidia and arrows.

Two unidentified fungal isolates, named N1 and N2, were also found (Figure 1A). Morphological analysis did not reveal the presence of sporulated structures, showing only hyphae, which hindered their taxonomic characterization. Additionally, bacterial colonies were observed in the seeds of all

cultivars. The number of colonies was significantly higher in treated than in untreated seeds.

Germination and emergence

In the germination assessment, significant differences were observed among soybean cultivars within each surface-disinfestation condition (Figure 2). In the absence of surface disinfestation, NR8383RR and SYN16871PRO showed the highest germination performance and did not differ significantly from each other, whereas OLIMPO and 82i78 exhibited significantly lower values. Under the surface-disinfestation condition, NR8383RR showed the highest germination performance, followed by SYN16871PRO, while OLIMPO and 82i78 presented the lowest values and did not differ significantly from each other. Overall, OLIMPO and 82i78 exhibited markedly lower germination than NR8383RR and SYN16871PRO.

Analysis of the Emergence Speed Index (ESI) revealed that cultivars NR8383RR and SYN16871PRO performed best in the treatment without surface disinfestation, achieving 100%

efficiency. In contrast, cultivars 82i78 (in both treatments) and OLIMPO (in the treatment with disinfestation) presented rates below 50%. Even under the asepsis treatment, the NR8383RR cultivar stood out, maintaining 72.2% efficiency, followed by SYN16871PRO, with 55.6%. In contrast, 82i78 and OLIMPO showed very low values (5.6%), suggesting lower initial vigor under these conditions.

Seedlings derived from naturally infected seeds were monitored for symptoms during the cultivation period. Overall, visible lesions were infrequent. Occasional symptoms were observed in cultivar SYN16871PRO at 10 and 15 days after sowing, and *Aspergillus* sp. was recovered from some symptomatic tissues. However, because pathogenicity testing and Koch's postulates were not performed on the *Aspergillus* isolate, a causal relationship between this fungus and the observed symptoms cannot be established. In addition, the low natural incidence of *C. truncatum* in the seed lots did not provide a sufficient number of infected seeds for a robust quantitative assessment of seed-to-seedling transmission.

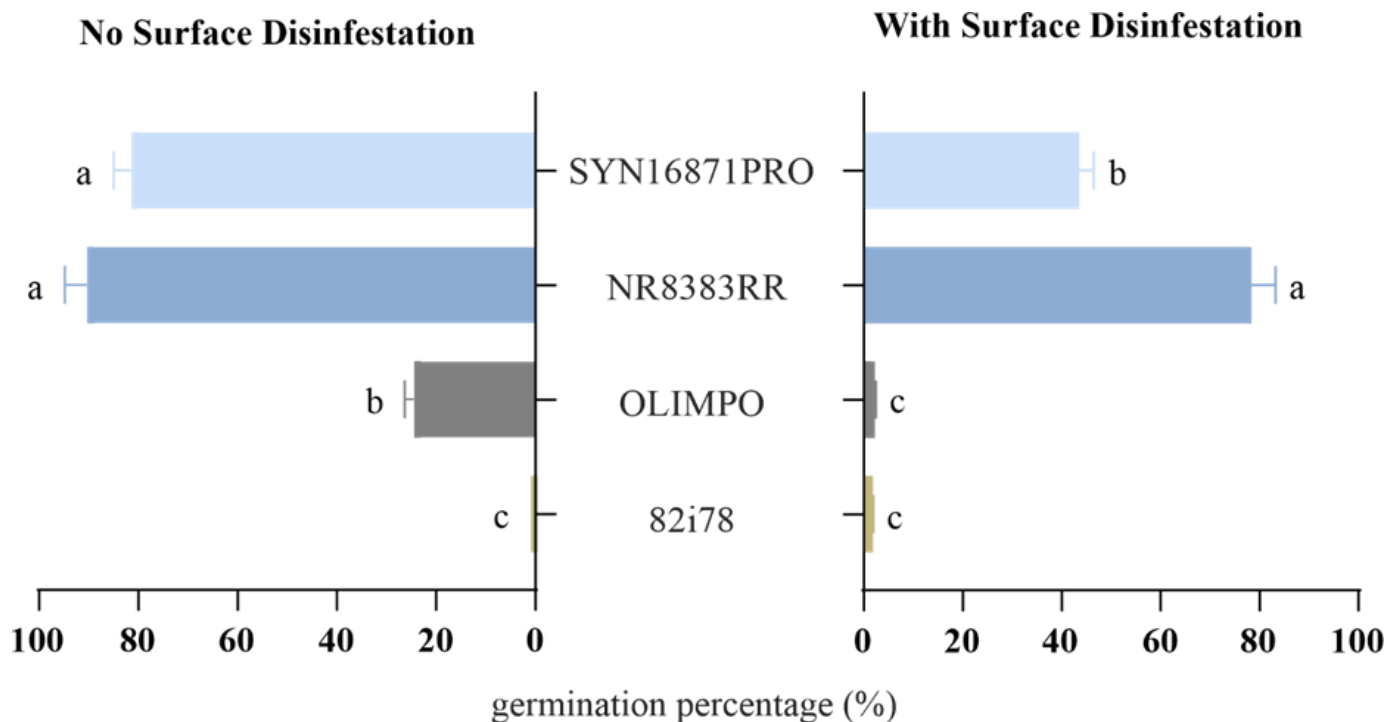


Figure 2. Final germination percentage recorded on the 21st day after sowing for the soybean cultivars OLIMPO, 82i78, NR8383RR, and SYN16871PRO under conditions with and without surface disinfestation. Within each surface-disinfestation condition, cultivars followed by the same lowercase letter do not differ significantly according to one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test ($p < 0.05$).

Pathogenicity test of *Colletotrichum truncatum*

Pathogenicity tests were performed on healthy plants 30 days after sowing using the *C. truncatum* isolate recovered from soybean seeds. At 48 h after artificial inoculation, symptoms developed in 100% of the inoculated plants of cultivars OLIMPO, NR8383RR, and SYN16871PRO (Figure 3A-C), whereas non-inoculated control plants remained asymptomatic. Symptoms included necrotic areas and leaf lesions compatible with anthracnose. Cultivar 82i78 could not be evaluated in this assay because the seed lot did not produce plants with sufficient development for inoculation.

C. truncatum was re-isolated from symptomatic tissues of the inoculated plants. Its morphology was consistent with that of the original isolate, completing the re-isolation step of Koch's postulates (Figure 3D-F). These results support the pathogenicity of the recovered *C. truncatum* isolate under the experimental inoculation conditions. The absence of adequate plant development in cultivar 82i78 was interpreted as a limitation related to the poor physiological performance of that seed lot and was not attributed specifically to *C. truncatum*.

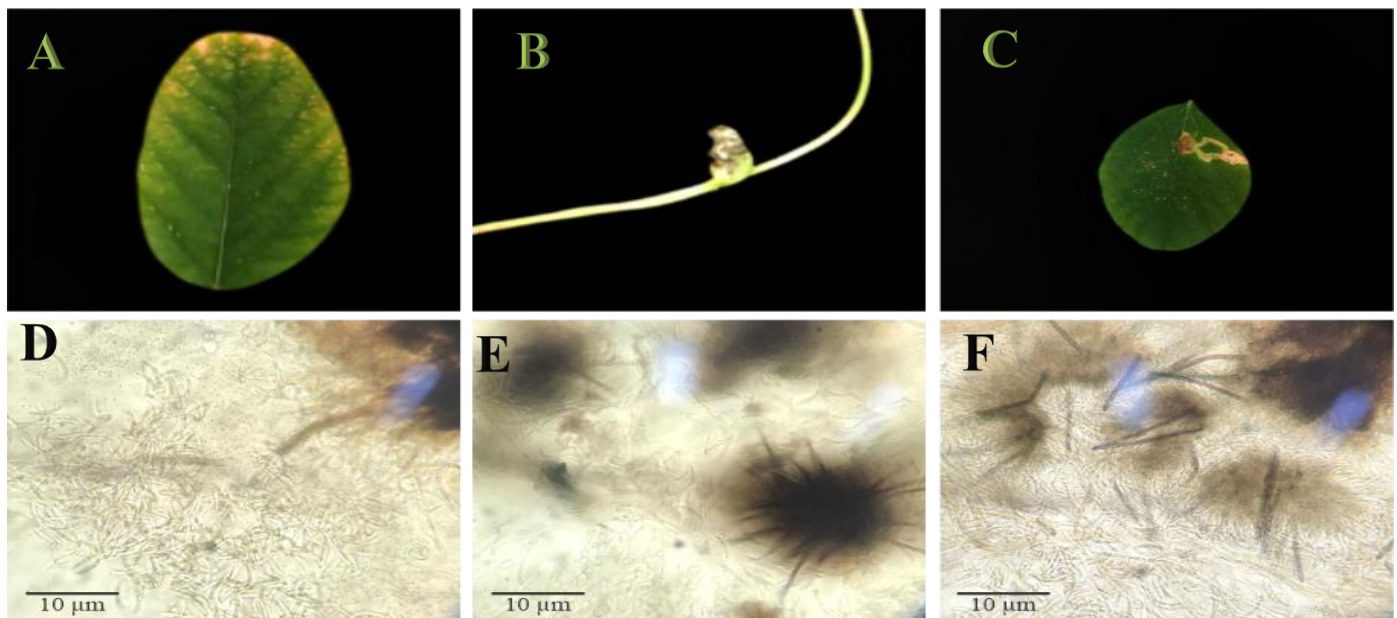


Figure 3. Pathogenicity test of *C. truncatum* on soybean plants (cultivars OLIMPO, NR8383RR, and SYN16871PRO) 48 hours after inoculation with the fungus. (A, B, and C) Symptoms of *C. truncatum* on soybean leaves of cultivars OLIMPO, SYN16871PRO, and NR8383RR, respectively. (D, E, and F) Re-isolation of *C. truncatum* on leaves with symptoms of cultivars OLIMPO, SYN16871PRO, and NR8383RR, respectively.

Effect of *Bacillus* spp. on soybean seeds

The incidence of seed-associated fungi varied according to the surface-disinfestation condition and *Bacillus* treatment (Figure 4). Overall fungal incidence was lower in surface-disinfested seeds (122%) than in non-disinfested seeds (386%). Among the fungi detected, *Aspergillus* sp., a genus commonly associated with seed deterioration during storage, showed a lower incidence under the surface-

disinfestation condition. *C. truncatum* was detected at a low frequency only in the non-disinfested control and was not detected following treatment with the *Bacillus* isolates. However, because of the low natural incidence of *C. truncatum* in the evaluated seed lots, its absence after bacterial treatment should be interpreted only descriptively and not as evidence of specific biocontrol efficacy against this pathogen.

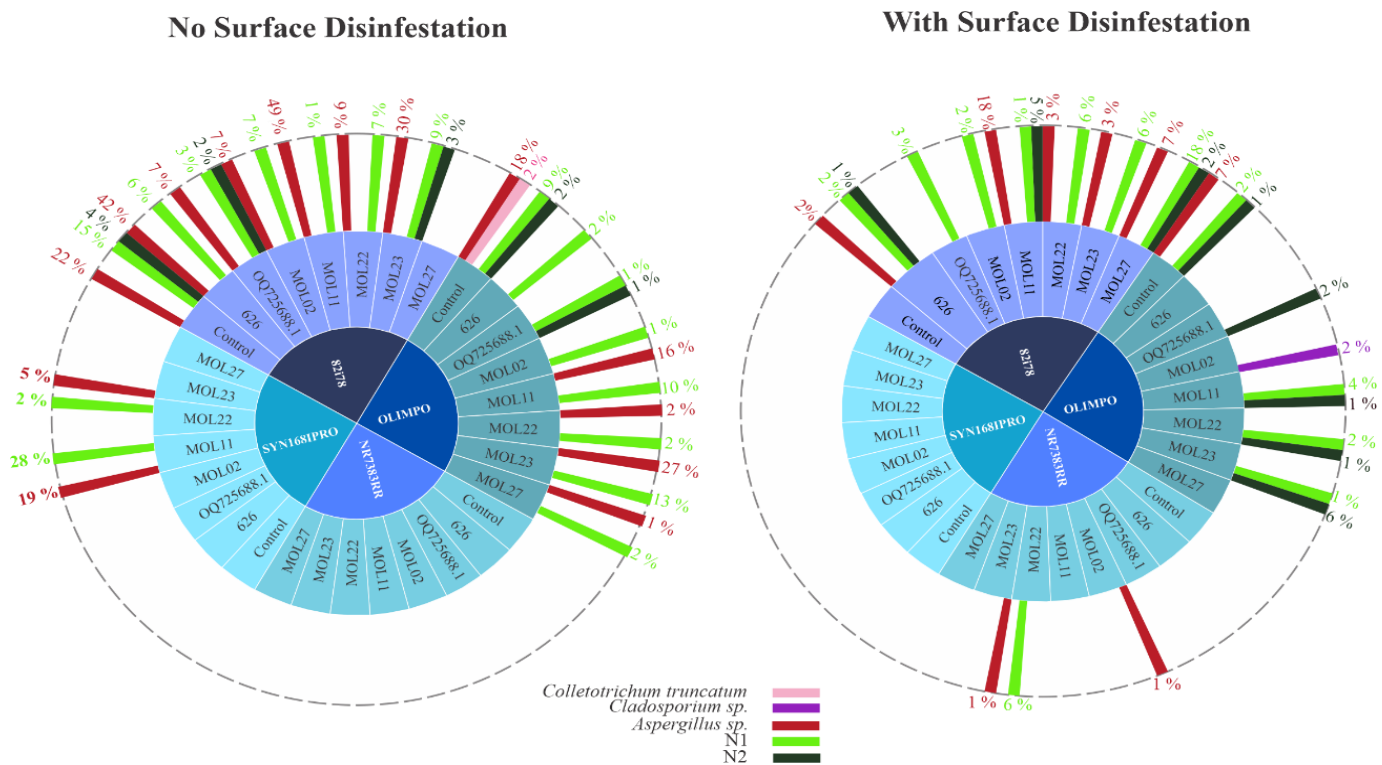


Figure 4. Incidence of fungi associated with soybean seeds from different cultivars (OLIMPO, 82i78, NR8383RR, and SYN16871PRO) subjected to two conditions: without surface disinfestation and with surface disinfestation, after treatment with *Bacillus* spp. isolates. (A) Treatment without surface disinfestation. (B) Treatment with surface disinfestation.

Differences in overall fungal incidence were also observed among the *Bacillus* treatments. Under the surface-disinfestation condition, *Bacillus velezensis* (QO725688.1) and *Bacillus* spp. isolates 626 and MOL02 were associated with the lowest overall fungal incidences (5% each), compared with 20% in the corresponding control. In non-disinfested seeds, the overall fungal incidences associated with these same treatments were 14%, 50%, and 15%, respectively. The occurrence of seed-associated fungi also varied among soybean cultivars. SYN16871PRO showed no detectable fungi under the surface-disinfestation condition, whereas no fungi were detected in NR8383RR under the non-disinfested condition. The unidentified fungal isolates N1 and N2 occurred under both experimental conditions. Isolate N1 was particularly frequent in cultivar 82i78, with incidences of 24% in surface-disinfested seeds and 48% in non-disinfested seeds. Overall, these results demonstrate that the incidence of naturally occurring seed-associated fungi varied according to seed surface disinfestation, *Bacillus* isolate, and soybean cultivar.

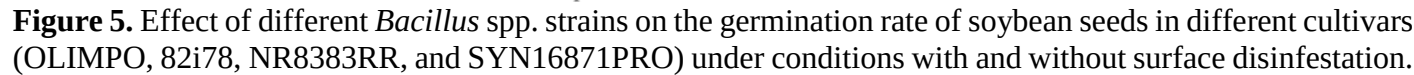
Germination of seeds treated with *Bacillus* spp.

In addition to fungal incidence, the effects of *Bacillus* treatments on seed germination were evaluated to determine whether the bacterial isolates influenced the physiological performance of soybean seeds. Germination responses varied considerably among cultivars, surface-disinfestation conditions, and *Bacillus* isolates, indicating a cultivar- and isolate-dependent response (Figure 5). Overall, surface disinfestation alone did not result in a consistent effect on germination across cultivars. Similarly, the treatment with *Bacillus* spp. did not consistently improve germination, with responses varying according to the specific cultivar-isolate combination. Cultivar 82i78 exhibited particularly low germination under both experimental conditions. Nevertheless, in surface-disinfested seeds of this cultivar, the treatments with isolates MOL02 and MOL22 resulted in 2% germination, whereas germination was not observed in the corresponding control. Although this response indicates that germination occurred following treatment with these isolates, the low germination percentage does not

In vitro antifungal activity of *Bacillus* spp. on *Colletotrichum truncatum*

truncatum. All isolates showed inhibition percentages greater than 80% (Figure 6A).

Antifungal activity became evident after 96 hours of incubation, when all strains exhibited inhibition rates greater than 50%. After 192 hours, the *Bacillus* spp. isolates MOL11, MOL22, MOL23, and MOL27 showed the highest inhibition percentages (82%, 81%, 82%, and 84%, respectively), demonstrating high antagonistic activity against *C. truncatum* (Figure 6B).



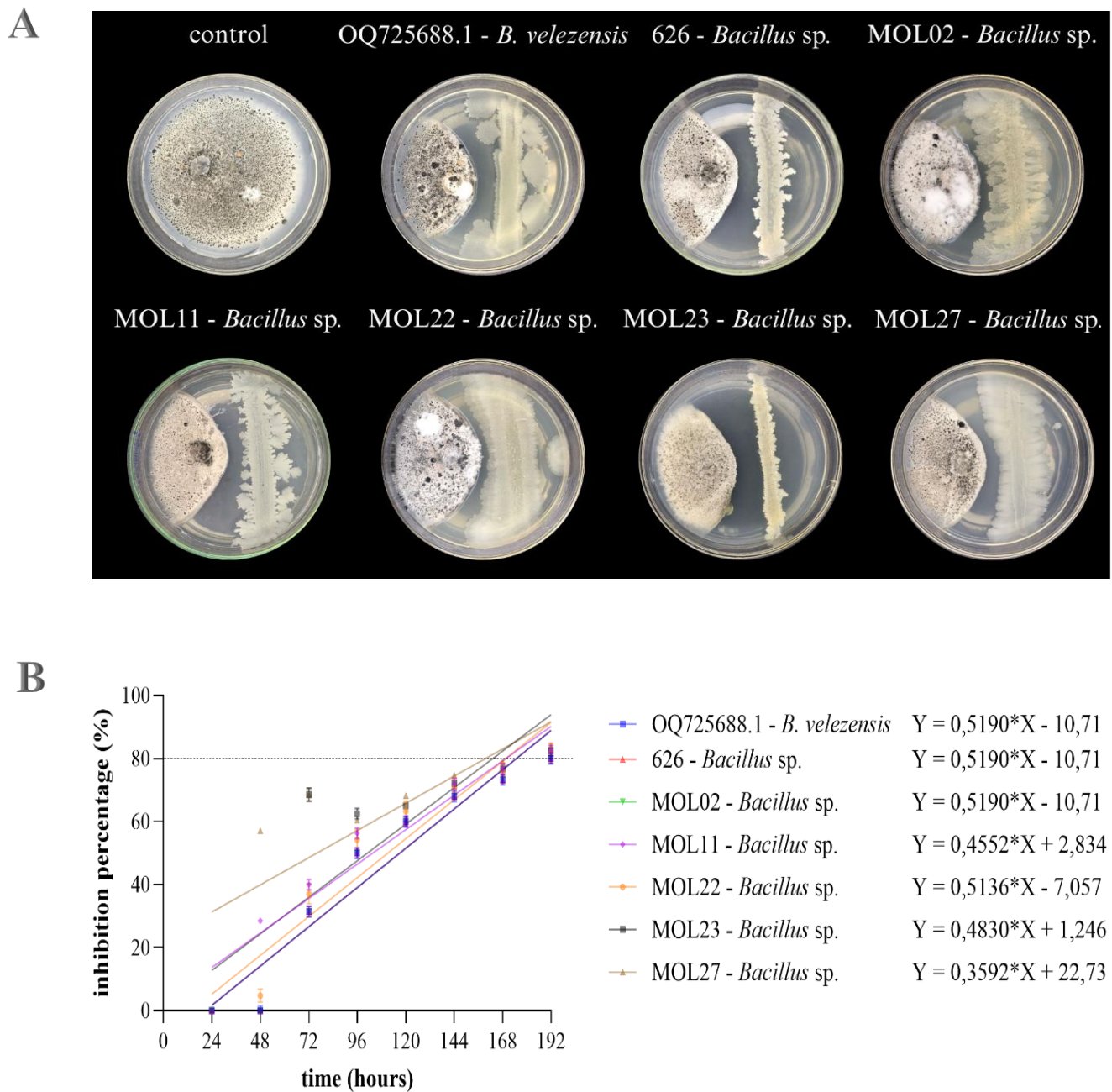


Figure 6. Antagonistic activity of *Bacillus* spp. isolates in direct confrontation against *C. truncatum*. (A) *In vitro* inhibition on PDA medium up to 192 hours after inoculation. (B) Percentage inhibition of *C. truncatum* mycelial growth, assessed at 24-hour intervals.

DISCUSSION

The presence of *Colletotrichum truncatum* in soybean seeds is a significant phytosanitary concern because infected seeds can facilitate the survival and spread of the pathogen. This study investigated the presence of *C. truncatum* and other fungi associated with different soybean cultivars, as well as seed germination and the effects of treating seeds with *Bacillus* spp. Distinct phytosanitary and

physiological responses were observed among the evaluated seed lots and under the different surface-disinfestation conditions. Although *C. truncatum* occurred less frequently than *Aspergillus* and *Cladosporium*, its detection is significant because infected seeds are a major source of inoculum for soybean anthracnose.

The analyzed seeds were associated with various fungal genera, reflecting the microbiological complexity of the soybean production chain. Fungi

associated with the seeds, such as *Aspergillus* sp. and *Cladosporium* sp., were widely detected. This finding is consistent with the results reported by Soesanto *et al.* (2020) and reinforces the prevalence of these fungi in stored seeds. While not primary pathogens, these fungi negatively impact seed lot quality due to their ability to cause spoilage, as previously reported by Goulart (2004) and Kobayashi *et al.* (2011). Under surface disinfection, we observed a predominance of *Aspergillus* sp. in almost all cultivars except for SYN16871PRO and NR8383RR. *Cladosporium* sp. showed a limited but significant occurrence in the NR8383RR cultivar with an incidence of 60%. These results align with those of Olszaka-Przybyś and Korbecka-Glinka (2024), who identified *Aspergillus* species as the most prevalent fungal group in soybeans, with an incidence rate of 24.2%.

The exclusive detection of *C. truncatum* in cultivar 82i78 under both surface-disinfested and non-surface-disinfested conditions demonstrates the presence of the pathogen in this seed lot and underscores its phytosanitary significance. Confirmation of pathogenicity through Koch's postulates further supports the isolate's ability to cause anthracnose, a disease of considerable economic importance under warm and humid conditions (EMBRAPA, 2003; Henning *et al.*, 2014). *C. truncatum* occurred at a lower frequency than the other seed-associated fungi detected in this study. However, its presence is phytosanitarily significant because infected seeds can serve as a source of primary inoculum. This can contribute to the dissemination of the pathogen and the establishment of anthracnose in production areas (Rogério *et al.*, 2017; Dias *et al.*, 2019).

An analysis of germination performance revealed distinct responses among soybean cultivars following surface disinfection. NR8383RR exhibited the greatest physiological stability, maintaining 99% germination with or without surface disinfection. In contrast, SYN16871PRO and OLIMPO exhibited significant reductions in germination rates after disinfection, decreasing from 88% to 56% and from 32% to 3%, respectively. These results differ from those reported by Bortolini and Fortes (2005), who observed germination rates above 90% after sodium hypochlorite treatment regardless of immersion time or exudate release. Cultivar 82i78 exhibited the lowest germination percentages under both conditions, indicating the poor physiological performance of this seed lot.

Galli *et al.* (2005) reported that *C. truncatum* infection negatively affects soybean seed physiological quality, including reduced germination, demonstrating the potential relationship between seed infection and impaired physiological performance. In the present study, however, the observed response for 82i78 may involve multiple factors, including the physiological quality of the seed lot, genotype-specific characteristics, and the presence of seed-associated fungi. Therefore, the contrasting responses among cultivars indicate that the effects associated with surface disinfection depend on the genetic, structural, physiological, and phytosanitary characteristics of each seed lot.

When subjected to treatment with *Bacillus* strains, the germination responses followed similar trends. Cultivar 82i78 did not germinate in the control treatment; however, residual germination rates were observed in some treatments, reaching up to 6% with isolate MOL23 (*Bacillus* sp.) in seeds without surface disinfection and 2% with isolates MOL02 and MOL23 (*Bacillus* sp.) in seeds subjected to surface disinfection. These results suggest a slight beneficial effect of biocontrol on seed physiological vigor in the present study. OLIMPO also demonstrated high sensitivity, with a reduction from 12% in the control (without surface disinfection) to 0–4% with the strains and 0–2% under the treatment with surface disinfection.

In contrast, NR8383RR maintained high germination rates, ranging from 66% to 92%, even after the biological treatments, which reinforces its physiological robustness despite interference from external agents. The SYN16871PRO cultivar, despite exhibiting high germination rates in the control treatment (88% for seeds without surface disinfection and 86% for seeds subjected to surface disinfection), showed variations in germination depending on the *Bacillus* strain used, with complete inhibition observed in the treatment with isolate MOL11 (*Bacillus* sp.) in seeds without surface disinfection.

Although the potential of *Bacillus* species is well known in relation to seed maintenance and physiological quality (de Andrade Bezerra *et al.*, 2013), the diversity of germination responses among cultivars treated with *Bacillus* sp. underscores that the success of biocontrol in seeds depends on multiple factors, such as the intensity of the initial infection, the location of the pathogen, the genetic susceptibility of the cultivars, and possible phytotoxic interactions

with the metabolites produced by the strains (Abdelfattah *et al.*, 2023; Wu and Chang, 2024).

Despite the variations observed *in vivo*, the results obtained *in vitro* confirmed the high antifungal potential of the evaluated bacteria. The *Bacillus* strains exhibited inhibition rates greater than 80% against *C. truncatum* under laboratory conditions, demonstrating their high antagonistic activity. Among the isolates evaluated, MOL27 (*Bacillus* sp.) exhibited the highest percentage of inhibition (84%), followed by MOL11 and MOL23 (*Bacillus* sp.) at 82%. These rates exceeded those reported by Santos *et al.* (2026), who observed reductions in the growth of *C. truncatum* of 41.1% for *Bacillus megaterium* BC003, 37.0% for *Bacillus velezensis* BC004, and 34.2% for *Bacillus velezensis* BC005, and those of Duré *et al.* (2022), whose strains showed less than 10% efficacy against *Colletotrichum* spp. Similar findings were reported by Miljaković *et al.* (2022) and Patel and Math (2024), who demonstrated the antifungal activity of *Bacillus* sp. against various plant-pathogenic species and their positive effects on germination. The observed effects may be related to compounds such as lipopeptides, siderophores, and hydrolytic enzymes, which are responsible for fungal suppression (Richter *et al.*, 2024).

CONCLUSION

Colletotrichum truncatum was detected at a low natural incidence in the evaluated soybean seed lots, whereas *Aspergillus* sp. and *Cladosporium* sp. occurred more frequently. The latter were primarily interpreted as storage-associated and saprophytic/contaminant fungi, respectively. Due to the low natural incidence of *C. truncatum*, a robust assessment of seed-to-seedling transmission and the efficacy of *Bacillus* treatments against the pathogen in naturally infected seeds was not possible. Nevertheless, the pathogenicity of the recovered *C. truncatum* isolate was demonstrated through symptom development after artificial inoculation and re-isolation from symptomatic soybean tissues. *In vitro*, all evaluated *Bacillus* isolates exhibited strong antagonistic activity against *C. truncatum*. Mycelial growth inhibition exceeded 80% after 192 hours, reaching 84% for isolate MOL27. Germination responses varied among cultivars and bacterial treatments and could not be attributed exclusively to fungal infection due to differences in the initial physiological quality of the seed lots. Thus, the

present results support the antagonistic potential of *Bacillus* spp. against *C. truncatum* under *in vitro* conditions. However, experiments using artificially inoculated seeds or seed lots with a higher natural incidence are necessary to determine the efficacy of seed treatments and pathogen transmission under controlled conditions.

REFERENCES

- Abdelfattah, A.; Tack, A. J.; Lobato, C.; Wassermann, B. & Berg, G. (2023). From seed to seed: the role of microbial inheritance in the assembly of the plant microbiome. **Trends in Microbiology**, 31(4): 346-355. <https://doi.org/10.1016/j.tim.2022.10.009>
- Alfenas, A. C. & Mafia, R. G. (2007). **Métodos em fitopatologia**. ed. 2. Viçosa, MG: Ed. UFV. p. 382.
- Anderson, E. J.; Kantar, M. B. & Diers, B. W. (2019). Soybean [*Glycine max* (L.) Merr.] breeding: history, improvement, production and future opportunities. In: Al-Khayri, J.; Jain, S.; Johnson, D. (eds) **Advances in Plant Breeding Strategies: Legumes**. Springer, Cham. https://doi.org/10.1007/978-3-030-23400-3_12
- Andrić, S.; Meyer, T. & Ongena, M. (2020). *Bacillus* responses to plant-associated fungal and bacterial communities. **Frontiers in Microbiology**, 11: 1350. <https://doi.org/10.3389/fmicb.2020.01350>
- Barnett, H. L. & Hunter, B. B. (1972). **Illustrated genera of imperfect fungi**. 3. ed. Minneapolis: Burgess Publishing Company. 241 p.
- Batzer, J. C. & Mueller, D. S. (2020). Soybean fungal endophytes *Alternaria* and *Diaporthe* spp. are differentially impacted by fungicide application. **Plant disease**, 104(1): 52-59. <https://doi.org/10.1094/PDIS-05-19-1001-RE>
- Andrade Bezerra, G. de; Macedo, D.A.; Oliveira Nascimento, I. de; Sousa, T.P. de; Costa, N.B. & Aquino Sousa, L.F.R. de (2013). Uso de *Bacillus* spp. no controle de fitopatógenos em sementes de soja variedade BRS Valiosa RR. **Revista Agroecossistemas** 5(1): 68-73. <https://doi.org/10.18542/ragros.v5i1.1414>

- Borghetti, F. & Ferreira, A. G. (2004). Interpretação de resultados de germinação. In: Borghetti, F., & Ferreira, A. G. **Germinação: do básico ao aplicado**. Porto Alegre: Artmed. 209-222 p.
- Bortolini, M. F. & Fortes, A. M. T. (2005). Efeitos alelopáticos sobre a germinação de sementes de soja (*Glycine max* L. Merrill). **Semina: Ciências Agrárias**, 26(1): 5-10.
- BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. (2009). **Regras para análise de sementes**. Brasília, DF: MAPA/ACS SNDA/DNDV/CLAV. 399 p.
- Cavaglieri, L.; Orlando, J. R. M. I.; Rodriguez, M. I.; Chulze, S. & Etcheverry, M. (2005). Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides* in vitro and at the maize root level. **Research in microbiology**, 156(5-6): 748-754. <https://doi.org/10.1016/j.resmic.2005.03.001>.
- Dias, M. D.; Dias-Neto, J. J.; Santos, M. D.; Formento, A. N.; Bizerra, L. V.; Fonseca, M. E. N.; ... & Café-Filho, A. C. (2019). Current status of soybean anthracnose associated with *Colletotrichum truncatum* in Brazil and Argentina. **Plants**, 8(11): 459. <https://doi.org/10.3390/plants8110459>.
- Duré, L. M. M.; Galeano, R. M. S.; Viana, T. F. C.; Roque, C. G.; Matias, R.; Paggi, G. M.; ... & da Silva Brasil, M. (2022). *Bacillus* strains with potential for growth promotion and control of white mold in soybean. **Biologia**, 77(11): 3305-3317. <https://doi.org/10.1007/s11756-022-01186-5>.
- EMBRAPA. Empresa Brasileira de Pesquisa Agropecuária. (2003). **Tecnologia de produção de soja: Região Central do Brasil 2004**. Londrina: Embrapa Soja. 237 p.
- Forkema, N. J. (1976). Antagonism between fungal saprophytes and pathogens on aerial plant surfaces. In: Dickinson, C. H.; Preece, T. F. (ed). **Microbiology of aerial plant surfaces**. London: Academic Press. 487-506 p.
- Galli, J. A.; Panizzi, R. D. C.; Fessel, S. A.; Simoni, F. D. & Ito, M. F. (2005). Efeito de *Colletotrichum dematium* var. *truncata* e *Cercospora kikuchii* na germinação de sementes de soja. **Revista Brasileira de Sementes**, 27(2): 182-187. <https://doi.org/10.1590/S0101-31222005000200026>.
- Goulart, A. C. P. (1997). **Fungos em sementes de soja: detecção e importância**. Dourados: Embrapa Agropecuária Oeste.
- Goulart, A. C. P. (2004) **Fungos em sementes de soja: detecção, importância e controle**. 2. ed. Dourados: Embrapa Agropecuária Oeste.
- Henning, A. A.; Almeida, A. M. R.; Godoy, C. V.; Seixas, C. D. S.; Yorinori, J. T.; Costamilan, L. M.; Ferreira, L. P.; Meyer, M. C.; Soares, R. M. & Dias, W. P. (2014). **Manual de identificação de doenças de soja**. 1. ed. Brasília, DF: Embrapa.
- Huilgol, S. N.; Patil, S. H. & Bhat, R. S. (2020). First record of target leaf spot disease of soybean and progression of other fungal foliar spots in Karnataka. **Journal of Pharmacognosy and Phytochemistry**, 9(4): 3203-3205.
- INTERNATIONAL SEED TESTING ASSOCIATION (ISTA). (2024). **International rules for seed testing**. Bassersdorf, Switzerland: ISTA.
- Khan, A. R.; Ali, Q.; Ayaz, M.; Bilal, M. S.; Sheikh, T. M. M.; Gu, Q.; ... & Gao, X. (2023). Plant-microbes interaction: exploring the impact of cold-tolerant *Bacillus* strains RJGP41 and GBAC46 volatiles on tomato growth promotion through different mechanisms. **Biology**, 12(7): 940. <https://doi.org/10.3390/biology12070940>.
- Kobayashi, L.; Adoriam, A. I.; de Paiva Neto, V. B.; Alves, C. Z. & Zuffo, M. C. R. (2011). Incidência de fungos em sementes de pinhão-mansão. **Pesquisa Agropecuária Tropical**, 41: 385-390. <https://doi.org/10.5216/pat.v41i3.10948>.
- Kuldybayev, N., Dutbayev, Y., Konstantinova, O., Borodulin, D., Yessimbekova, M., Daugaliyeva, S., Toishimanov, M., Yesserkenov, A., Bastaubaeva, S., & Temreshev, I. (2023). Identification and pathogenicity of the soybean root rot pathogen in arid conditions. **Online Journal of Biological Sciences**, 23(2): 202-209. <https://doi.org/10.3844/ojbsci.2023.202.209>.

- Maguire, J. D. (1962). Speed of germination — Aid in selection and evaluation for seedling emergence and vigor. **Crop Science**, 2(1): 176-177. <https://doi.org/10.2135/cropsci1962.0011183X000200020033x>.
- Miljaković, D.; Marinković, J. & Jošić, D. (2022). Effect of plant growth promoting *Bacillus* spp. on germination and seedling growth of soybean. **Legume Research – An International Journal**, 45(4). <https://doi.org/10.18805/LRF-665>.
- Olszak-przybyś, H. & Korbecka-glinka, G. (2024). The diversity of seed-borne fungi associated with soybean grown in southern Poland. **Pathogens**, 13(9): 769. <https://doi.org/10.3390/pathogens13090769>.
- Patel, M. K. & Math, R. (2024). Efficacy of organic inputs and bio-agents against *Colletotrichum truncatum* causing anthracnose of black gram (*Vigna mungo* L. Hepper). **Plant Archives**, 24(2). <https://doi.org/10.51470/PLANTARCHIVES.2024.v24.no.2.046>.
- Putri, R. E.; Kurniawan, F. & Santoso, A. (2023). Antifungal substances produced by *Bacillus subtilis* strain W3.15 inhibit *Fusarium oxysporum* and trigger cellular damage. **HAYATI Journal of Biosciences**, 30(5): 843-854. <https://doi.org/10.4308/hjb.30.5.843-854>.
- Rangeshwaran, R. & Prasad, R. D. (2000). Biological control of *Sclerotium* rot of sunflower. **Indian Phytopathology**, 53(4): 444-449.
- Richter, A.; Blei, F.; Hu, G.; Schwitalla, J. W.; Lozano-Andrade, C. N.; Xie, J.; ... & Kovács, Á. T. (2024). Enhanced surface colonisation and competition during bacterial adaptation to a fungus. **Nature Communications**, 15(1): 4486. <https://doi.org/10.1038/s41467-024-48812-1>.
- Rogério, F.; Ciampi-Guillardi, M.; Barbieri, M. C. G.; Bragança, C. A. D.; Seixas, C. D. S.; Almeida, A. M. R. & Massola Jr, N. S. (2017). Phylogeny and variability of *Colletotrichum truncatum* associated with soybean anthracnose in Brazil. **Journal of Applied Microbiology**, 122(2): 402-415. <https://doi.org/10.1111/jam.13346>.
- Sales, N. I. S.; Alves, F. S. & Pereira, R. J. (2018). Patogenicidade e transmissão de fungos associados às sementes de *Tectona grandis* Lf. **Ciência Florestal**, 28: 970-978. <https://doi.org/10.5902/1980509833369>.
- Santos, F. M.; Silva, A. L. & Lima, C. A. (2022). Integrating a *Bacillus*-based product with fungicides by foliar application to protect soybean: a sustainable approach to avoid exclusive use of chemicals. **Pest Management Science**, 78(11): 4832-4840. <https://doi.org/10.1002/ps.7104>.
- Silva, A. O.; Gomes, E.; Oliveira-paiva, C. A.; Valicente, F.; de Oliveira, M. C. & Fernandes, T. (2017). Preservação de linhagens de *Bacillus thuringiensis* por ultracongelamento e incorporação ao acervo da Coleção de Microrganismos Multifuncionais e Fitopatogênicos da Embrapa Milho e Sorgo.
- Silva Júnior, M. B.; de Resende, M. L. V.; Pozza, E. A.; de Resende, A. R. M.; Silveira, G. C. D.; da Veiga, J. D.; ... & da Silva, A. C. (2025). Phosphite compounds suppress anthracnose in soybean seeds infected by *Colletotrichum truncatum* and stimulate growth and defense mechanisms. **Plants**, 14(10): 1494. <https://doi.org/10.3390/plants14101494>.
- Soesanto, L.; Hartono, A. R. R.; Mugiastuti, E. & Widarta, H. (2020). Seed-borne pathogenic fungi on some soybean varieties. **Biodiversitas**, 21(9): 4010-4015. <https://doi.org/10.13057/biodiv/d210911>.
- Wu, P. H. & Chang, H. X. (2024). Colonization compatibility with *Bacillus altitudinis* confers soybean seed rot resistance. **The ISME Journal**, 18(1). <https://doi.org/10.1093/ismejo/wrae142>.
- Zambrano, M. A. R.; Castanharo, G. R. P.; Aguiar, R. W. D. S. & Chagas Junior, A. F. (2022). *In vitro* antagonistic activity of *Sclerotium* sp. by *Bacillus* sp. **Society and Development**, 11(13). <https://doi.org/10.33448/rsd-v11i13.35351>.
- Zhao, L.; Wei, X.; Zheng, T.; Gou, Y. N.; Wang, J.; Deng, J. X. & Li, M. J. (2022). Evaluation of pathogenic *Fusarium* spp. associated with soybean seed (*Glycine max*) in Hubei Province, China. **Plant Disease**, 106(12): 3178-3186. <https://doi.org/10.1094/PDIS-12-21-2793-RE>