Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Biological treatments with *Bacillus* and *Trichoderma* reduce cassava root rot under controlled conditions

Izis Katarina Santana Mello
Universidade Federal do Recôncavo da Bahia: Universidade Federal do Reconcavo da Bahia
Camila Santiago Hohenfeld
Serviço Social da Indústria: SESI-SP
Aline Brito Vaz
Instituto Federal Goiano - Campus Morrinhos
Maria Selma Alves Silva Diamantino
EMBRAPA Centro Nacional de Pesquisa de Mandioca e Fruticultura Tropical
Rafaela Vasquesdos Santos Oliveira
Universidade Federal do Recôncavo da Bahia: Universidade Federal do Reconcavo da Bahia
Diego Andrade Santos
Universidade Federal do Recôncavo da Bahia: Universidade Federal do Reconcavo da Bahia
Saulo Alves Santos de Oliveira
saulo.oliveira@embrapa.br

EMBRAPA Centro Nacional de Pesquisa de Mandioca e Fruticultura Tropical https://orcid.org/0000-0002-2992-1570

Research Article

Keywords: Manihot esculenta, Beneficial microorganisms, Plant growth promoters, Plant health, Soilborne diseases

Posted Date: September 23rd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10705859/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Cassava root rot diseases, caused by diverse soilborne fungi, significantly impair crop establishment and yield, challenging effective disease management. Biological control using native microbial antagonists offers a sustainable alternative but requires evaluation against multiple pathogens and cassava genotypes. Here we screened ten *Bacillus* and three *Trichoderma* isolates *in vitro* against fungi associated with dry and black root rots, identifying isolates with pathogen-specific antagonistic activity. Selected agents, including *Bacillus* mix 3 and *Trichoderma asperellum* TRI81, were evaluated *in planta* on two cassava cultivars, demonstrating improved germination, vigor, biomass, and reduced internal colonization under controlled conditions. *Bacillus* mix 3 and its sequential combination with TRI81 showed the most consistent benefits, particularly in cultivar BRS Formosa. These findings support the potential of native *Bacillus* and *Trichoderma* isolates as components of integrated cassava root rot management, while emphasizing the need for further validation under natural soil and field conditions.

Introduction

Cassava (*Manihot esculenta* Crantz) root rots comprise a major disease complex that reduces plant establishment, storage-root yield, and root quality. Management of these diseases is particularly challenging because multiple causal fungi can persist in soil, crop residues, and infected stem cuttings. Surveys in major cassava-producing regions have revealed substantial diversity among fungi recovered from symptomatic roots, demonstrating that cassava root rot is not attributable to a single pathogen (Bandyopadhyay et al. 2006; Vilas Boas et al. 2017).

Dry root rot is predominantly associated with species of *Fusarium*. Isolates belonging to the *F. oxysporum* and *F. solani* species complexes are frequently recovered from affected cassava roots, although other species may also occur depending on the geographical region. The high genetic and taxonomic diversity of *Fusarium* populations contributes to variation in pathogenicity and host response and may limit the broad effectiveness of resistant genotypes or management practices developed against individual isolates (Bandyopadhyay et al. 2006; Vilas Boas et al. 2017). Experimental inoculation studies have confirmed that *Fusarium* spp. can cause dry root rot and significantly reduce cassava agronomic performance (Teixeira et al. 2021).

Black root rot is mainly associated with fungi in the family Botryosphaeriaceae. In Brazil, *Lasiodiplodia theobromae* and *Neoscytalidium dimidiatum* have been repeatedly isolated from roots and stem cuttings showing black rot symptoms. Molecular identification and pathogenicity tests have confirmed their ability to reproduce disease symptoms in cassava, with *L. theobromae* among the most frequently recovered species (Brito et al. 2020). The occurrence of these pathogens in both storage roots and propagation material increases the risk of their introduction into new production areas through infected stem cuttings.

Control of cassava root rots relies primarily on preventive practices such as using healthy planting material, sanitation, crop management, and resistant or moderately resistant cultivars. However,

resistance varies among cassava genotypes and pathogen groups, and the diversity of fungi involved can reduce the reliability of host resistance (Teixeira et al. 2021). Chemical control is further limited by the belowground location and persistence of the pathogens, as well as the scarcity of products specifically registered for cassava. These challenges highlight the need for complementary strategies capable of suppressing multiple components of the root rot complex.

Species of *Trichoderma* are promising biological control agents because they can suppress soilborne fungi through competition, antibiosis, secretion of fungal cell-wall-degrading enzymes, and direct mycoparasitism. Their performance is nevertheless strain- and pathogen-dependent. In cassava, *Trichoderma* isolates have inhibited the growth of fungi associated with black root rot and reduced disease expression in planta, while also affecting host antioxidant enzyme activity, indicating that direct antagonism may be complemented by the activation of plant defense responses (da Silva et al. 2017).

Bacteria of the genus *Bacillus* are also relevant to biological control because of their capacity to colonize plant tissues and the rhizosphere, form stress-resistant endospores, and produce antifungal metabolites. Evidence in cassava has shown that bacterial inoculation can suppress *Fusarium* root rot while promoting shoot and root growth. In particular, stem inoculation with *Bacillus amyloliquefaciens* GB03 reduced disease symptoms caused by *Fusarium solani* and improved cassava growth under pathogen pressure (Freitas et al. 2019).

Despite this potential, comparative studies involving multiple native *Bacillus* and *Trichoderma* isolates against representative pathogens of both dry and black root rots remain limited. Evidence is also scarce on the performance of bacterial mixtures and their sequential combination with *Trichoderma* in cassava. Such combinations may integrate complementary mechanisms of pathogen suppression, but their efficacy depends on microbial compatibility, application sequence, cassava genotype, and the target pathogen.

Therefore, this study evaluated the in vitro antagonistic activity of ten native *Bacillus* isolates and three *Trichoderma* isolates against fungi associated with cassava dry and black root rots. Selected agents were subsequently evaluated in two independent in planta bioassays using ‘BRS Kiriris’ and ‘BRS Formosa’. The treatments comprised *Trichoderma asperellum* ‘TRI81’, three mixtures of *Bacillus* isolates, and sequential combinations of ‘TRI81’ with each bacterial mixture. Their effects on germination, plant height, vigor, internal colonization, and biomass were assessed to identify treatments with consistent potential for the biological management of cassava root rot.

Materials and methods

Experimental site and biological material

The experiments were conducted at the Plant Pathology Laboratory (LABFITO) and adjacent controlled-environment facilities of Embrapa Cassava and Fruits, Cruz das Almas, Bahia, Brazil. Ten native bacterial isolates belonging to *Bacillus* spp. and three fungal isolates of *Trichoderma* spp. were obtained from the

institutional microorganism collection. The fungal antagonists comprised *Trichoderma asperellum* 'TRI81', *T. asperellum* 'TRIBAHIA', and *T. harzianum* 'T. CANA'.

Seven *Fusarium* isolates associated with cassava dry root rot were used as target pathogens: *F. oxysporum* 'SERGIPE1' and 'FM09', *F. equiseti* 'C. CITRUS FM', *F. verticillioides* 'PR2', *F. lateritium* 'FM12-BR', *F. chlamydosporum* 'PR4-PR', and *F. solani* 'FM02'. Three isolates associated with black root rot were also evaluated: *Lasiodiplodia theobromae* 'COLO3' and *Neoscytalidium dimidiatum* 'SYM01' and 'SYM02'. The pathogen isolates were selected based on the aggressiveness classifications established by Santana and Oliveira (2017) and on historical collections characterized by Vilas Boas et al. (2015).

In vitro antagonistic bioassays

Fungal isolates were reactivated on potato dextrose agar (PDA) and incubated at 25 ± 2°C for 7 days under a 12-h photoperiod. *Bacillus* spp. isolates were reactivated on nutrient agar and incubated at 25 ± 2°C for 24 h. Bacterial colonies were subsequently transferred to Erlenmeyer flasks containing nutrient broth and incubated for 24 h at 25 ± 2°C under constant agitation at 120 rpm. The bacterial suspensions were adjusted to an optical density of 0.7 at 600 nm (OD_{600}). Viable cell density was confirmed by serial dilution and plate counting on nutrient agar, resulting in approximately $2x10^8$ CFU mL^{-1} across the bacterial isolates used in the diffusion assay. Plates were incubated at 25 ± 2°C for 24 h before colony-forming units were counted.

The antagonistic activity of the *Bacillus* isolates was evaluated using a solid-medium diffusion assay adapted from Dennis and Webster (1971) and Silva et al. (2017). A mycelial disc measuring 5 mm in diameter was excised from the actively growing margin of each pathogen colony and placed at the center of a 90 mm Petri dish containing PDA. Subsequently, 3 µL of the adjusted bacterial suspension was applied to sterile Whatman Grade 4 qualitative filter paper discs measuring 20 mm in diameter and previously sterilized by autoclaving. Four discs were positioned equidistantly, each 10 mm from the inner edge of the Petri dish.

The antagonistic activity of the *Trichoderma* isolates was evaluated using the dual-culture method described by Bell et al. (1982). A 5-mm-diameter mycelial disc of the pathogen and a 5-mm-diameter disc of the antagonist were placed on opposite sides of a PDA plate. For both assays, plates inoculated only with the respective pathogen served as controls.

The assays were conducted in a completely randomized design with 10 independent replicates for each pathogen–antagonist combination. The entire experiment was performed twice. Plates were incubated at 25 ± 2°C under a 12-h photoperiod until the pathogen colonies in the control plates reached the plate margins.

Antagonistic activity was quantified from digital images of the plates obtained under standardized lighting, camera position, and scale conditions. Pathogen colony boundaries were manually delineated

using ImageJ, including irregular margins adjacent to inhibition zones. In dual cultures, only the area occupied by the pathogen before overlap with *Trichoderma* was measured. Colony area was expressed in square centimeters, and all images were analyzed by a single evaluator using the same boundary criteria.

The percentage of mycelial growth inhibition was calculated relative to the pathogen-only control according to:

$$PI\,(\%) = \frac{A_c\text{-}A_t}{A_c} \times\ 100$$

where $A_c$is the mean colony area of the pathogen in the control treatment and $A_t$is the colony area of the pathogen in the presence of the antagonist. The procedures were based on Edgington et al. (1971) for the bacterial assays and Campanile et al. (2007) for the *Trichoderma* dual-culture assays.

In planta bioassays (dup: abstract ?)

Two independent in planta bioassays were conducted, one for dry root rot and another for black root rot. Both experiments were maintained under controlled conditions at 28°C with a 12-h photoperiod. Each experiment was arranged in a randomized complete block design with four blocks, in a 2 × 8 factorial scheme comprising two cassava cultivars, 'BRS Kiriris' and 'BRS Formosa', and eight biological treatments. Each cultivar × treatment combination comprised 20 plants, distributed as five plants per plot in each of the four blocks.

TRI81 was selected for the in planta bioassays because it showed the highest overall inhibition against the dry root rot fungi and maintained substantial activity against the black root rot pathogens. The use of a single *Trichoderma* isolate in both experiments also allowed direct comparison of treatment performance across disease groups.

The treatments were: T1, pathogen-inoculated control without a biological agent; T2, *T. asperellum* 'TRI81'; T3, '*Bacillus* mix 1', composed of isolates '399', 'APC51', 'PA05', and 'APC07'; T4, *'Bacillus mix 2'*, composed of 'BAC5' and 'BAC6'; T5, *'Bacillus mix 3'*, composed of 'BAC3' and BAC4; T6, *'Bacillus mix 1'* combined with 'TRI81'; T7, *'Bacillus mix 2'* combined with 'TRI81'; and T8, *'Bacillus mix 3'* combined with 'TRI81'. In the combined treatments, the bacterial mixtures were applied at planting, whereas TRI81 was applied 15 days after planting. TRI81 was also applied at 15 days after planting in the TRI81-only treatment. All remaining treatments received an equivalent mock application of sterile distilled water at 15 days after planting to standardize handling.

For the black root rot experiment, the pathogen mixture comprised *L. theobromae* CBPPR1002 and *N. dimidiatum* CBPPR1011. For the dry root rot experiment, the mixture comprised *F. oxysporum* CBPPR0006, *F. equiseti* CBPPR0031, *F. verticillioides* CBPPR0048, *F. solani* CBPPR0059, *F. lateritium* CBPPR0012, and *F. chlamydosporum* CBPPR0008. These isolates were obtained from the fungal culture

collection of the Plant Pathology Laboratory at Embrapa Cassava and Fruits. The pathogen isolates used in the in planta mixtures were different representatives from the same institutional collection and were not the same isolates evaluated in the in vitro screening. They were selected to represent aggressive members of the dry and black root rot complexes and to provide a broader challenge under in planta conditions.

Pathogen and *Trichoderma* isolates were grown on PDA at 25 ± 2°C under a 12-h photoperiod. After colony development, 20 mL of sterile distilled water were added to each plate, and conidia were released using a soft toothbrush. The resulting suspensions were filtered through two layers of sterile gauze. Subsequently, 40 mL of each suspension were transferred to plastic bags containing approximately 200 g of sterile rice. The bags were incubated for 15 days at 25 ± 2°C under a 12-h photoperiod.

Fungal inoculum concentrations were estimated by serial dilution and plating. Ten grams of colonized rice were suspended in 90 mL of sterile distilled water and serially diluted to 10^{-5}. Aliquots of the 10^{-4} and 10^{-5} dilutions were plated in triplicate on PDA, and colony-forming units were counted after 48 h at 25°C. The pathogen inoculum was adjusted to 10^5 CFU g^{-1} of substrate. Within each disease group, the pathogen isolates were combined in equal proportions before substrate infestation. The 'TRI81' suspension was adjusted to 10^7 CFU mL^{-1}.

The *Bacillus* isolates were initially grown on nutrient agar for 24 h and subsequently transferred to Erlenmeyer flasks containing nutrient broth. Cultures were incubated for 24 h at 25°C on a rotary shaker at 120 rpm. Bacterial suspensions were adjusted to approximately 10^8 CFU mL^{-1} based on an optical density of 0.8 at 600 nm. Isolates composing each bacterial mixture were combined in equal proportions.

Cassava stem cuttings were obtained from the Cassava Active Germplasm Bank of Embrapa Cassava and Fruits. Cuttings were standardized to 8 cm and disinfested by immersion for 3 min in a benzalkonium chloride solution at 0.5 mL L^{-1}. They were then rinsed under running water and allowed to dry for 12 h.

The experiments were conducted in 300-mL polypropylene cups containing vermiculite moistened with 30 mL of sterile distilled water. After 48 h, the substrate was infested with 3 g of pathogen-colonized rice per cup and homogenized with a sterile spatula. The cups were covered with 400-mL plastic cups to maintain a humid chamber and incubated for an additional 48 h.

Biological treatments were applied to the planting hole at a volume of 3 mL per plant. Cassava cuttings were planted vertically and partially covered with vermiculite. Plants were maintained under controlled conditions for 30 days.

Germination was recorded at 8 and 15 days after planting. At 30 days after planting, plant height, plant vigor, external disease incidence, internal colonization of the cuttings, shoot fresh mass, root fresh mass, shoot dry mass, and root dry mass were evaluated.

Plant vigor was assessed using a three-point ordinal scale: 1, low vigor; 2, intermediate vigor; and 3, high vigor. Internal colonization was evaluated visually after longitudinal sectioning of the cuttings using the following scale: 0, no internal colonization; 1, colonization of less than one-third of the internal cutting area; 2, colonization of at least one-third but less than two-thirds; and 3, colonization of at least two-thirds of the internal area. External disease incidence was recorded qualitatively as presence or absence of visible pathogen-associated symptoms. All visual assessments were performed by a single evaluator using the same scoring criteria for all treatments.

Vigor and internal colonization scores were converted into percentage indices using the McKinney index:

$$\text{Index}\,(\%) = \frac{\sum (f_i \times x_i)}{N \times x_{max}} \times 100$$

where f_i is the number of plants assigned to score x_i, N is the total number of plants evaluated in the plot, and x_{max} is the maximum score of the respective scale.

Shoots and roots were separated and weighed to determine fresh mass. The samples were subsequently dried at 50°C for 72 h until constant mass to determine shoot and root dry mass.

Statistical analysis

The in vitro bacterial and *Trichoderma* assays were analyzed separately using linear mixed effects models. Antagonist, pathogen, and their interaction were included as fixed effects, whereas experimental run was included as a random effect. When significant effects were detected, estimated marginal means were compared using Tukey adjusted pairwise comparisons at $P \leq 0.05$. Analyses were performed in R using the packages lme4, lmerTest, and emmeans.

The dry root rot and black root rot experiments were analyzed independently. Because the five plants within each block represented subsamples of the same experimental plot, plot-level means or indices were used as the experimental observations. Treatment effects were evaluated separately for each cassava cultivar using linear mixed-effects models, with biological treatment as a fixed effect and block as a random effect.

Analyses were performed in R using the packages *lme4* for model fitting, *lmerTest* for significance testing, and *emmeans* for estimated marginal means and treatment contrasts. Differences between each biological treatment and the pathogen-inoculated control were estimated using contrasts based on marginal means. Confidence intervals were calculated at the 95% level, and probability values were adjusted for multiple comparisons using the Holm method. Model assumptions were evaluated by inspection of residual and quantile–quantile plots using the performance package.

A multi-trait performance index was calculated separately within each cassava cultivar and root rot experiment. For each variable, standardized values were obtained as $z = \left(x - \overline{x}\right) / s$, where x is the plot value, $\overline{x}$ is the mean across treatments, and s is the corresponding standard deviation. The index

included germination at 15 days, plant height, vigor index, shoot fresh mass, root fresh mass, shoot dry mass, root dry mass, and internal colonization. Internal colonization was multiplied by -1 so that lower colonization contributed positively. All standardized variables received equal weight, and the index was calculated as their arithmetic mean.

Ranking robustness was assessed by recalculating the index using only dry biomass variables and by comparing treatment ranks with those obtained from the complete index. Because the index was intended for exploratory screening, small differences among treatment scores were not interpreted as definitive evidence of superiority.

Results

In vitro antagonistic activity of Bacillus spp. against cassava root rot fungi

The *Bacillus* isolates differed significantly in their ability to inhibit the mycelial growth of the evaluated cassava root rot fungi. significant antagonist × pathogen interaction was detected ($P \leq 0.05$), confirming that inhibition depended on the specific antagonist–pathogen combination. The magnitude of inhibition depended on both the bacterial isolate and the target pathogen, indicating a marked isolate-specific interaction (Fig. 1C–E).

Against *Fusarium oxysporum* 'FM09', mean mycelial growth inhibition ranged from 58.6% to 70.7%. 'BAC5' showed the highest inhibition, reaching 70.7%, followed by 'BAC6', 'APC51', and isolate 01C, with mean values of 69.7%, 67.3%, and 67.8%, respectively. 'BAC3' showed the lowest inhibition against 'FM09', with a mean of 58.6% (Fig. 1C,E).

For *Lasiodiplodia theobromae* 'COLO3', inhibition ranged from 48.1% to 67.0%. 'PA05' exhibited the highest mean inhibition, followed by 'APC51' and isolate '399', which inhibited pathogen growth by 63.1% and 61.6%, respectively. BAC4 and 'BAC3' showed the lowest inhibition values, both below 49% (Fig. 1D,E).

The response of *Neoscytalidium dimidiatum* 'SYM02' was also strongly dependent on the bacterial isolate. 'APC51' and 'APC07' produced the greatest inhibition, with mean values of 66.1% and 65.3%, respectively, followed by isolate '399' and 'BAC6'. In contrast, 'PA05' did not inhibit 'SYM02' under the conditions evaluated, resulting in a mean inhibition of 0%. This contrasting behavior illustrates that an isolate showing high antagonistic activity against one pathogen may be ineffective against another (Fig. 1D,E).

Overall, the strongest and most consistent bacterial performances were observed for 'APC51', 'APC07', isolate '399', 'BAC5', and 'BAC6', although no single isolate was superior against all pathogens. The heatmap further emphasized the specificity of the antagonist–pathogen interactions and identified combinations with comparatively high or low inhibition (Fig. 1E).

In vitro antagonistic activity of Trichoderma spp. against cassava root rot fungi

All three *Trichoderma* isolates inhibited the mycelial growth of the dry and black root rot pathogens, but their relative performance varied among pathogen isolates and disease groups (Fig. 1A,B,E). A significant antagonist × pathogen interaction was detected ($P \leq 0.05$), confirming that inhibition depended on the specific antagonist–pathogen combination.

For the dry root rot pathogens considered jointly, 'TRI81' showed the highest mean inhibition, reaching 61.2%, and differed significantly from 'T. CANA' and 'TRIBAHIA', which showed mean inhibition values of 56.3% and 54.6%, respectively. The latter two isolates did not differ significantly from each other at the disease-group level (Fig. 1A).

'TRI81' was particularly effective against *F. oxysporum* 'FM09', producing 78.1% inhibition, which was significantly higher than that obtained with 'T. CANA' and 'TRIBAHIA'. 'TRI81' also showed the highest inhibition against *F. equiseti* 'C. CITRUS FM' and *F. lateritium* 'FM12-BR', with mean values of 65.8% and 66.8%, respectively. Against *F. verticillioides* 'PR2', 'TRI81' and 'T. CANA' produced similar inhibition, approximately 60.6%, whereas 'TRIBAHIA' showed a lower value of 53.0% (Fig. 1A,E).

The three *Trichoderma* isolates showed comparatively lower activity against *F. solani* 'FM02', with mean inhibition values ranging from 46.2% to 48.9%, and no significant differences were detected among antagonists. Likewise, no differences were observed among the three antagonists against *F. chlamydosporum* 'PR4-PR' or *F. oxysporum* 'SERGIPE1' (Fig. 1A,E).

For the black root rot pathogens, 'T. CANA' and 'TRI81' showed higher overall inhibition than 'TRIBAHIA'. Mean inhibition across this pathogen group was 49.7% for 'T. CANA', 42.9% for 'TRI81', and 34.4% for 'TRIBAHIA' (Fig. 1B).

Against *L. theobromae* 'COLO3', 'TRI81' and 'T. CANA' produced mean inhibition values of 52.2% and 47.1%, respectively, whereas 'TRIBAHIA' showed substantially lower inhibition, at 22.1%. 'T. CANA' was also the most effective antagonist against *N. dimidiatum* 'SYM01' and 'SYM02', reaching 43.4% and 58.4% inhibition, respectively. For 'SYM02', 'T. CANA' differed significantly from 'TRI81', while 'TRIBAHIA' showed an intermediate response (Fig. 1B,E).

Collectively, these results show that 'TRI81' was the most effective antagonist against the dry root rot fungi, particularly 'FM09', whereas 'T. CANA' showed the most consistent activity against black root rot pathogens. 'TRIBAHIA' showed comparatively lower overall inhibition, especially against 'COLO3' and 'SYM01' (Fig. 1). Although T. CANA showed greater mean inhibition of the black root rot pathogen group, TRI81 was advanced to the in planta assays because of its broader activity across both disease groups.

In planta bioassays

Biological treatments affected cassava germination, plant growth, vigor, internal colonization, and biomass, although treatment responses varied according to cassava genotype and root rot group (Fig. 2). These bioassays used pathogen mixtures composed of different representative isolates from those included in the in vitro screen.

For ‘BRS Formosa’, most biological treatments increased germination at 15 days after planting relative to the pathogen-inoculated control. Germination in the control was 60% in both disease experiments, whereas values of 80–93% were recorded for several biological treatments. ‘Bacillus mix 1’ reached 93.3% germination under both dry and black root rot, while ‘Bacillus mix 3’ reached 90% in both experiments (Fig. 2).

‘BRS Formosa’ also showed clear treatment-associated increases in plant height and vigor. Under dry root rot, the highest mean plant height was recorded for ‘Bacillus mix 3’, at 10.0 cm, followed by ‘TRI81’ combined with ‘Bacillus mix 3’, at 9.2 cm. These same treatments showed the highest vigor indices, reaching 43.7% and 43.3%, respectively, compared with 18.6% in the control. Under black root rot, ‘Bacillus mix 3’ and ‘TRI81’ combined with ‘Bacillus mix 3’ also produced the highest vigor values, at 29.5% and 28.5%, compared with 12.8% in the control (Fig. 2).

Internal colonization in ‘BRS Formosa’ was generally reduced by the biological treatments. Under dry root rot, colonization decreased from 41.7% in the control to 20.0% with ‘Bacillus mix 3’ and to approximately 22.2–23.3% with ‘Bacillus mix 1’, ‘Bacillus mix 2’, ‘TRI81’ combined with ‘Bacillus mix 2’, and ‘TRI81’ combined with ‘Bacillus mix 3’. Under black root rot, the lowest colonization was observed with ‘TRI81’ combined with ‘Bacillus mix 3’, at 22.2%, followed by ‘Bacillus mix 3’, at 25.0%, compared with 48.3% in the control (Fig. 2).

Biomass responses in ‘BRS Formosa’ were also generally favorable. Under dry root rot, ‘TRI81’ combined with ‘Bacillus mix 2’ produced the highest shoot and root dry masses, whereas under black root rot, ‘Bacillus mix 3’ produced the greatest shoot dry mass and ‘TRI81’ combined with ‘Bacillus mix 3’ produced the greatest root dry mass (Fig. 2).

‘BRS Kiriris’ showed more variable responses. Biological treatments generally increased germination relative to the control, which showed 25% germination in both disease experiments. Germination reached 80% with ‘TRI81’ combined with ‘Bacillus mix 1’ under both dry and black root rot and 75% with ‘TRI81’ combined with ‘Bacillus mix 3’ (Fig. 2).

For ‘BRS Kiriris’ under dry root rot, ‘Bacillus mix 3’ produced the greatest plant height and vigor, reaching 9.4 cm and 38.9%, respectively. ‘TRI81’ combined with ‘Bacillus mix 3’ reduced internal colonization to 31.7%, compared with 66.7% in the control. Under black root rot, ‘Bacillus mix 3’ also produced the highest plant height and vigor, whereas ‘TRI81’ combined with ‘Bacillus mix 3’ resulted in the lowest internal colonization, at 45.0%, compared with 78.3% in the control (Fig. 2).

Adjusted comparisons with the pathogen-inoculated control confirmed that the clearest treatment effects occurred in ‘BRS Formosa’ (Fig. 3). Under dry root rot, ‘Bacillus mix 3’ increased the vigor index by 25.1 percentage points, while ‘TRI81’ combined with ‘Bacillus mix 3’ increased vigor by 24.7 percentage points. Both effects remained significant after Holm adjustment. Under black root rot, ‘TRI81’ combined with ‘Bacillus mix 3’ increased vigor by 18.3 percentage points relative to the control and was also significant after adjustment (Fig. 3).

For total dry mass in ‘BRS Formosa’ under dry root rot, ‘Bacillus mix 1’ increased biomass by 0.44 g relative to the control, whereas ‘TRI81’ combined with ‘Bacillus mix 2’ produced an increase of 0.66 g. These contrasts remained significant after Holm correction. Although several treatments showed numerical reductions in internal colonization, their confidence intervals overlapped zero after adjustment. No adjusted treatment contrast was significant for ‘BRS Kiriris’ (Fig. 3).

The multi-trait performance index integrated germination, plant growth, vigor, biomass, and internal colonization and confirmed the superior overall performance of ‘Bacillus mix 3’ and ‘TRI81’ combined with ‘Bacillus mix 3’ (Fig. 4). For ‘BRS Kiriris’, ‘Bacillus mix 3’ ranked first under both dry and black root rot, with performance indices of 0.87 and 1.19, respectively. ‘TRI81’ combined with ‘Bacillus mix 3’ ranked second, with indices of 0.62 and 0.57. When averaged across both disease groups, ‘Bacillus mix 3’ showed the highest overall index for Kiriris, followed by ‘TRI81’ combined with ‘Bacillus mix 3’ (Fig. 4A,B).

A similar pattern was observed for ‘BRS Formosa’. ‘Bacillus mix 3’ produced the highest performance index under both dry and black root rot, with values of 0.89 and 1.11, respectively. ‘TRI81’ combined with ‘Bacillus mix 3’ ranked second overall, while ‘Bacillus mix 1’ and ‘TRI81’ combined with ‘Bacillus mix 2’ also showed positive performance indices. The pathogen-inoculated control showed the lowest overall index for Formosa in both disease experiments (Fig. 4C,D).

Overall, ‘*Bacillus* mix 3’ was the most consistently effective treatment across genotypes and disease groups, whereas the combination of ‘TRI81’ with ‘Bacillus mix 3’ also showed strong performance, particularly through increased vigor, reduced internal colonization, and improved biomass. The response was more pronounced and statistically consistent in ‘BRS Formosa’ than in ‘BRS Kiriris’.

Discussion

The present study showed that the antagonistic activity of native *Bacillus* and *Trichoderma* isolates was strongly dependent on the specific antagonist–pathogen combination. This is consistent with the etiological complexity of cassava root rots, which involve several *Fusarium* species as well as *Lasiodiplodia theobromae* and *Neoscytalidium dimidiatum* (Bandyopadhyay et al. 2006; Vilas Boas et al. 2017; Brito et al. 2020). Therefore, screening against multiple pathogens is essential because no single antagonist can be assumed to control the entire root rot complex.

The inhibition produced by several *Bacillus* isolates is compatible with antibiosis, particularly because inhibition halos were observed in some pairings. *Bacillus* species are known to produce antifungal lipopeptides, hydrolytic enzymes, and other secondary metabolites involved in fungal suppression (Ongena and Jacques 2008). However, because these compounds were not characterized in the present study, their involvement should be considered a plausible explanation rather than a confirmed mechanism.

The response of the *Trichoderma* isolates was also pathogen-specific. 'TRI81' was more effective against dry root rot fungi, whereas 'T. CANA' performed more consistently against black root rot pathogens. TRI81 was selected for subsequent in planta evaluation because it combined superior activity against the dry root rot fungi with relevant inhibition of the black root rot pathogens, allowing the same fungal antagonist to be assessed consistently in both disease experiments. Such differences agree with the strain-dependent nature of *Trichoderma* biocontrol, which may involve competition, antibiosis, cell-wall degradation, and mycoparasitism. Previous studies with cassava have likewise demonstrated that different *Trichoderma* isolates can suppress root rot through distinct mechanisms (da Silva et al. 2016, 2017).

The in planta bioassays confirmed that in vitro inhibition alone did not fully predict treatment performance. Biological effects varied between 'BRS Kiriris' and 'BRS Formosa', indicating a genotype-dependent response. Differences in basal resistance, cutting establishment, physiology, and host-associated microbial communities may influence the ability of cassava cultivars to benefit from biological treatments (Teixeira et al. 2021; Li et al. 2020). The more consistent responses observed in 'BRS Formosa' therefore likely resulted from interactions among host genotype, pathogen group, and microbial treatment.

'Bacillus mix 3' showed the most consistent performance in improving early plant performance and reducing internal colonization, particularly in "BRS Formosa". Similar simultaneous effects on disease suppression and plant growth have been reported for beneficial bacterial inoculation in cassava challenged with *Fusarium* root rot (Freitas et al. 2019). Nevertheless, the present study did not distinguish whether the benefits resulted primarily from direct pathogen inhibition, plant growth promotion, induced resistance, or a combination of these effects.

The sequential combination of 'TRI81' with *'Bacillus mix 3'* also performed well, but it did not consistently surpass mix 3 alone. Thus, the results do not demonstrate true synergism. Synergy requires the combined effect to exceed an explicit additive expectation, usually tested using quantitative interaction models such as Bliss independence (Jambhulkar et al. 2018). The present findings instead indicate that the sequential application was compatible and biologically effective. The 15-day interval between bacterial and fungal applications may have reduced direct interference between the agents, which is relevant because interactions between *Bacillus* and *Trichoderma* are highly dependent on strain identity, nutrient availability, and environmental conditions (Fifani et al. 2022).

The reduction in internal discoloration suggests that some treatments restricted disease development within cassava cuttings. Because this variable was assessed visually and pathogen identity was not confirmed by re-isolation or molecular methods, it should be interpreted as a proxy for internal colonization rather than direct evidence of pathogen biomass. However, colonization was assessed visually, and several numerical reductions were not significant after adjustment for multiple comparisons. These responses should therefore be considered promising but not conclusive. Reisolation or pathogen-specific quantitative PCR would provide stronger confirmation of internal pathogen suppression.

The multi-trait index was useful for integrating disease and growth responses and consistently identified *'Bacillus mix 3'* and 'TRI81' combined with mix 3 as the best-performing treatments. However, this index should be interpreted as a screening tool because its ranking depends on the selected variables, their standardization, and equal weighting.

The main limitation of the study was the short 30-day evaluation under controlled conditions using vermiculite and standardized artificial inoculum. These conditions do not reproduce the biological and environmental complexity of natural soils. In addition, the effective number of independent experimental units was four blocks per treatment combination, which limited statistical power. Therefore, the promising treatments identified here should be validated in natural soil, over longer periods, and under greenhouse and field conditions.

Overall, the results demonstrate that biological control of cassava root rot is influenced by pathogen identity, cassava genotype, and treatment composition. 'TRI81' and 'T. CANA' showed complementary activity against dry and black root rot fungi, respectively, whereas *'Bacillus mix 3'* was the most consistent in planta treatment. Its sequential combination with 'TRI81' was also promising, but further studies are required to confirm compatibility, persistence, formulation stability, and field efficacy.

Conclusion

This study demonstrates that native *Bacillus* and *Trichoderma* isolates have potential for the biological management of cassava dry and black root rots, although their efficacy depends on the specific antagonist–pathogen combination and on the cassava genotype. *In vitro*, 'TRI81' showed greater activity against dry root rot fungi, whereas 'T. CANA' performed more consistently against black root rot pathogens. *In planta*, *'Bacillus mix 3'* was the most consistently effective treatment, particularly in 'BRS Formosa', while its sequential combination with 'TRI81' also improved early plant performance and reduced internal colonization.

The results do not demonstrate that combined applications were universally superior to single treatments or that true synergism occurred. Instead, they indicate that sequential application can be compatible and effective under controlled conditions. Treatment responses were more consistent in 'BRS Formosa' than in 'BRS Kiriris', reinforcing the importance of host genotype in the performance of biological control strategies.

Because the bioassays were conducted for 30 days under controlled conditions using artificial inoculum and vermiculite, the identified treatments should be regarded as promising candidates rather than finalized bioinputs. Further studies are required to confirm microbial compatibility, persistence, formulation stability, optimal application timing, and efficacy in natural soil and field conditions. Overall, the study supports the value of functional microbial collections as sources of locally adapted biological control agents for the development of sustainable cassava root rot management strategies.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil, Finance Code 001; the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, through Research Productivity Fellowship Grant No. 303813/2024-5 and Project Grant No. 430789/2018-1; and the Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG).

Author contributions

I.K.S.M., C.S.H., M.S.A.S.D., R.V.S.O., and D.A.S. conducted the laboratory and *in planta* experiments and contributed to data collection and analysis. C.S.H., A.B.V., M.S.A.S.D., and S.A.S.O. contributed to the experimental evaluation, statistical analysis, and interpretation of the results. I.K.S.M., C.S.H., A.B.V., M.S.A.S.D., and S.A.S.O. contributed to the original manuscript draft. S.A.S.O., C.S.H., and M.S.A.S.D. conceived and designed the study and supervised the research. All authors reviewed and edited the manuscript and approved the final version.

Acknowledgements

The authors acknowledge the Federal University of Recôncavo da Bahia (UFRB) for academic support and Embrapa Cassava and Fruits and the Plant Pathology Laboratory (LABFITO) for providing infrastructure and technical support. The authors also acknowledge the Centro de Excelência em Bioinsumos (CEBIO) and the Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG) for their support of this research.

Data availability

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

References

1. Bandyopadhyay R, Mwangi M, Aigbe SO, Leslie JF (2006) *Fusarium* species from the cassava root rot complex in West Africa. Phytopathology 96:673–676. https://doi.org/10.1094/PHYTO-96-0673
2. Brito ACQ, de Mello JF, Câmara MPS, Vieira JCB, Michereff SJ, Souza-Motta CM, Machado AR (2020) Diversity and pathogenicity of Botryosphaeriaceae species associated with black root rot and stem cutting dry rot in *Manihot esculenta* in Brazil. Eur J Plant Pathol 157:583–598. https://doi.org/10.1007/s10658-020-02024-7
3. Fifani B, Steels S, Helmus C, Delacuvellerie A, Deracinois B, Phalip V, Delvigne F, Jacques P (2022) Coculture of *Trichoderma harzianum* and *Bacillus velezensis* based on metabolic cross-feeding modulates lipopeptide production. Microorganisms 10:1059. https://doi.org/10.3390/microorganisms10051059
4. Freitas MA, Medeiros FHV, Melo IS, Pereira PF, Peñaflor MFGV, Bento JMS, Paré PW (2019) Stem inoculation with bacterial strains *Bacillus amyloliquefaciens* GB03 and *Microbacterium imperiale* MAIIF2a mitigates *Fusarium* root rot in cassava. Phytoparasitica 47:135–142. https://doi.org/10.1007/s12600-018-0706-2
5. Hohenfeld CS, Santana MP, Cairo Junior LR, de Oliveira EJ, de Oliveira SAS (2018) Modelling growth characteristics and aggressiveness of *Neoscytalidium hyalinum* and *Fusarium solani* associated with black and dry root rot diseases on cassava. Trop Plant Pathol 43:422–432. https://doi.org/10.1007/s40858-018-0228-y
6. Jambhulkar PP, Sharma P, Manokaran R, Lakshman DK, Rokadia P, Jambhulkar N (2018) Assessing synergism of combined applications of *Trichoderma harzianum* and *Pseudomonas fluorescens* to control blast and bacterial leaf blight of rice. Eur J Plant Pathol 152:747–757. https://doi.org/10.1007/s10658-018-1519-3
7. Li H, Yan C, Tang Y, Ma X, Chen Y, Chen S, Lin M, Liu Z (2020) Endophytic bacterial and fungal microbiota in different cultivars of cassava (*Manihot esculenta* Crantz). J Microbiol 58:614–623. https://doi.org/10.1007/s12275-020-9565-x
8. Ongena M, Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol 16:115–125. https://doi.org/10.1016/j.tim.2007.12.009
9. Ruano-Rosa D, Cazorla FM, Bonilla N, Martín-Pérez R, de Vicente A, López-Herrera CJ (2014) Biological control of avocado white root rot with combined applications of *Trichoderma* spp. and rhizobacteria. Eur J Plant Pathol 138:751–762. https://doi.org/10.1007/s10658-013-0347-8
10. Silva JAT, de Medeiros EV, da Silva JM, Tenório DDA, Moreira KA, Nascimento TCES, Souza-Motta CM (2016) *Trichoderma aureoviride* URM 5158 and *Trichoderma hamatum* URM 6656 are biocontrol agents that act against cassava root rot through different mechanisms. J Phytopathol 164:1003–1011. https://doi.org/10.1111/jph.12521

11. Silva JAT, de Medeiros EV, da Silva JM, Tenório DDA, Moreira KA, da Silva Nascimento TCE, Souza-Motta CM (2017) Antagonistic activity of *Trichoderma* spp. against *Scytalidium lignicola* CMM 1098 and antioxidant enzymatic activity in cassava. Phytoparasitica 45:219–225. https://doi.org/10.1007/s12600-017-0578-x
12. Teixeira JHS, Guimarães MAS, Cardoso SC, Brito AS, Peixouto YS, Ribeiro SO, de Oliveira EJ, de Oliveira SAS (2021) Distribution of resistance of cassava genotypes to dry, soft and black root diseases and correlation to yield parameters. J Phytopathol 169:350–359. https://doi.org/10.1111/jph.12987
13. Vilas Boas SA, Oliveira SAS, Bragança CAD, Ramos JB, Oliveira EJ (2017) Survey of fungi associated with cassava root rot from different producing regions in Brazil. Sci Agric 74:60–67. https://doi.org/10.1590/0103-9016-2015-0366

Figures

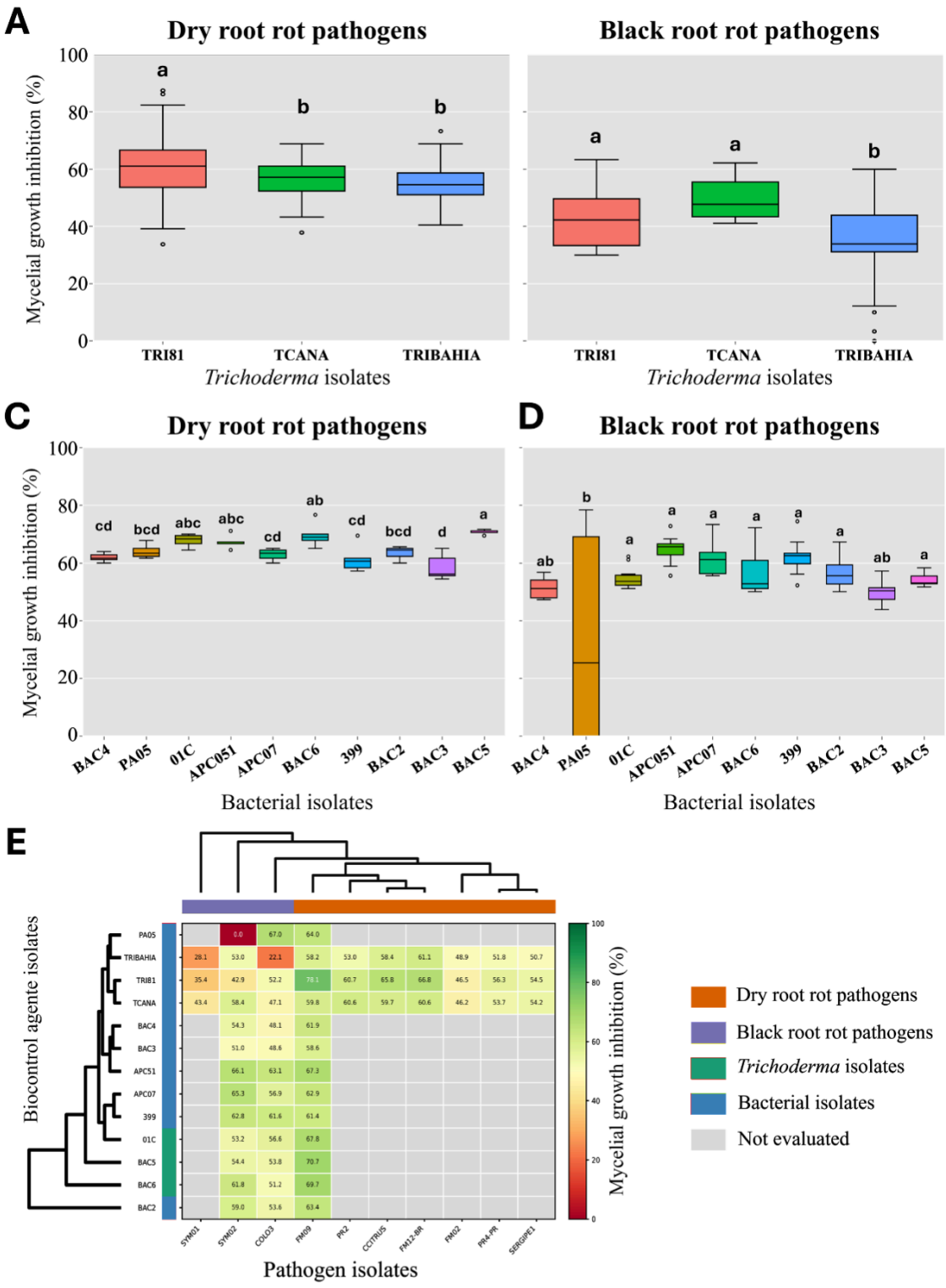


Figure 1

In vitro antagonistic activity of *Trichoderma* and bacterial isolates against cassava dry and black root rot pathogens. **(A-B)** Mycelial growth inhibition (%) by *Trichoderma*isolates against dry root rot and black root rot pathogens, respectively. **(C-D)** Mycelial growth inhibition (%) by bacterial isolates against dry root rot and black root rot pathogens, respectively. Different letters indicate significant differences among antagonists within each panel according to Tukey's HSD test ($P \leq 0.05$). **(E)** Heatmap of mean

mycelial growth inhibition (%) for each antagonist–pathogen combination. Rows represent biocontrol agents, columns represent pathogen isolates, and the color scale ranges from red (lower inhibition) to green (higher inhibition). Gray cells indicate combinations that were not evaluated.

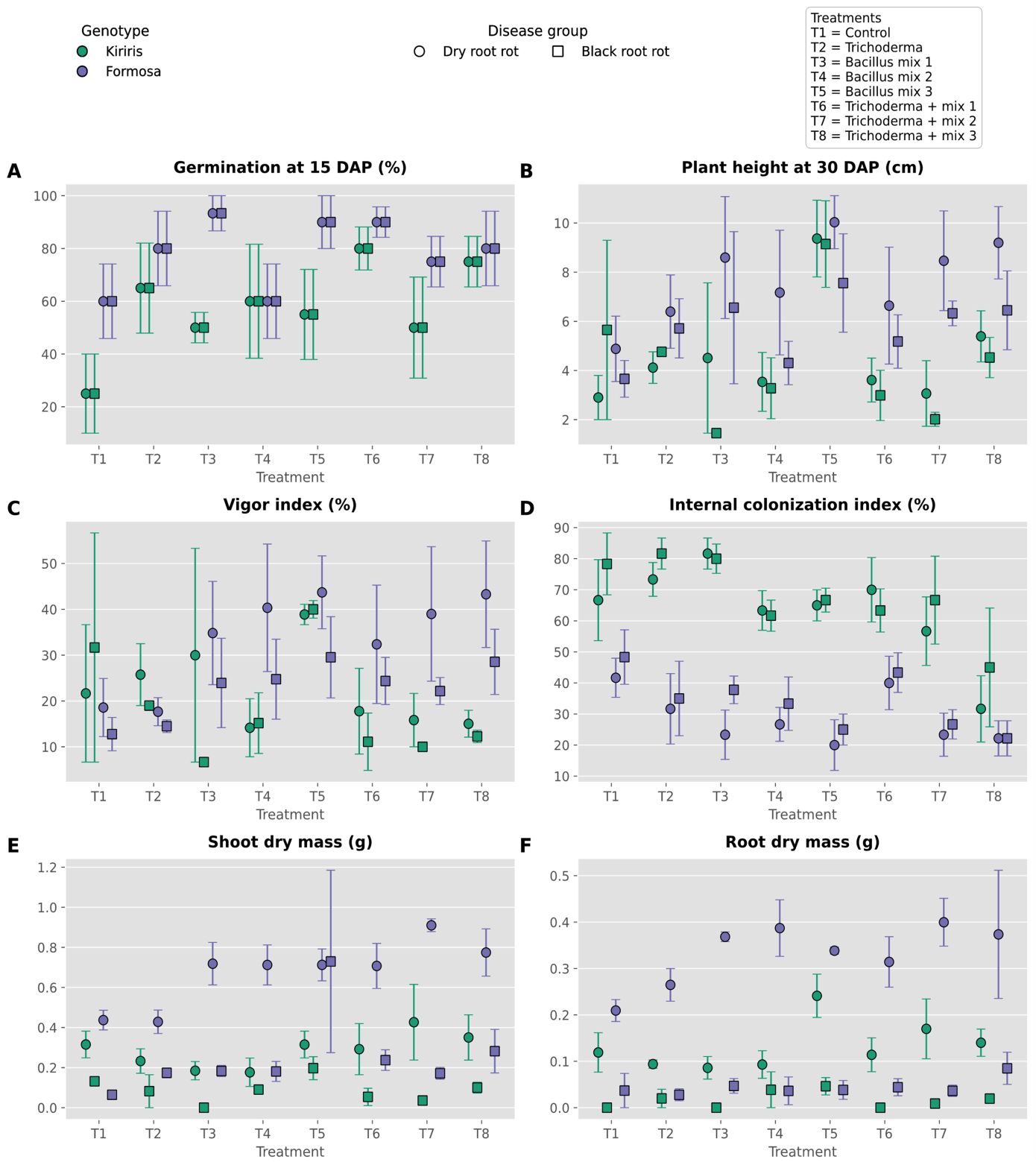


Figure 2

Effects of biological treatments on cassava germination, plant development, internal colonization, and biomass in the cup bioassay. The evaluated variables were germination at 15 days after planting (DAP), plant height at 30 DAP, vigor index, internal colonization index, and shoot and root dry mass. Points represent treatment means, and error bars represent standard errors for each cassava genotype × root rot group combination. T1, untreated control; T2, *Trichoderma*; T3–T5, *Bacillus* mixes 1–3; and T6–T8, *Trichoderma*combined with *Bacillus* mixes 1–3.

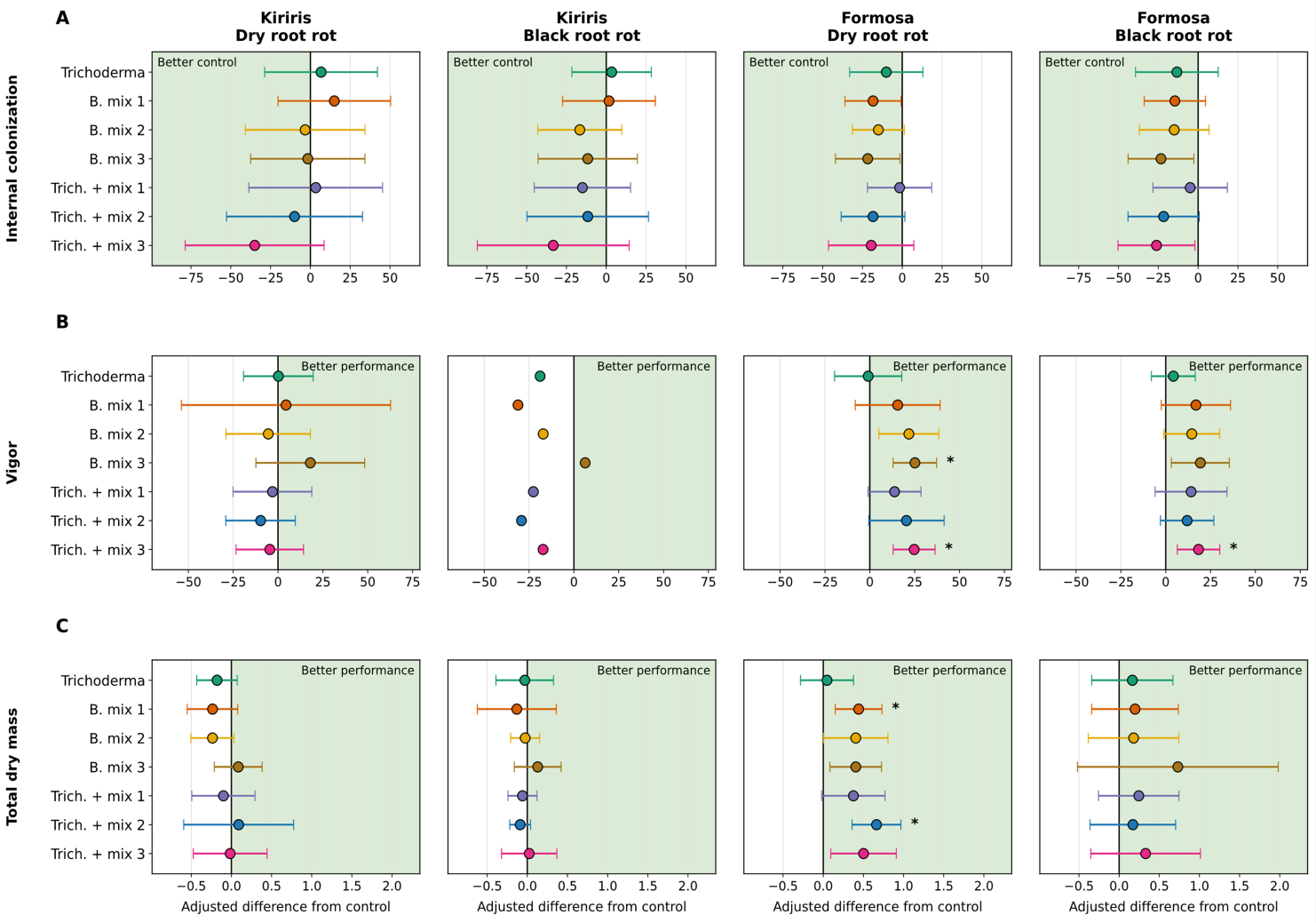


Figure 3

Genotype-specific effects of biological treatments relative to the untreated control in the cup bioassay. Treatment effects on internal colonization, vigor, and total dry mass were estimated separately for “BRS Kiriris” and “BRS Formosa” challenged with dry or black root rot pathogen mixtures. Points represent adjusted differences from the control, and horizontal bars represent 95% confidence intervals. Negative values for internal colonization indicate reduced pathogen colonization, whereas positive values for vigor and total dry mass indicate improved plant performance. Asterisks indicate significant differences after Holm adjustment at $P \leq 0.05$. T2, *Trichoderma*; T3–T5, *Bacillus*mixes 1–3; and T6–T8, *Trichoderma* combined with *Bacillus* mixes 1–3.

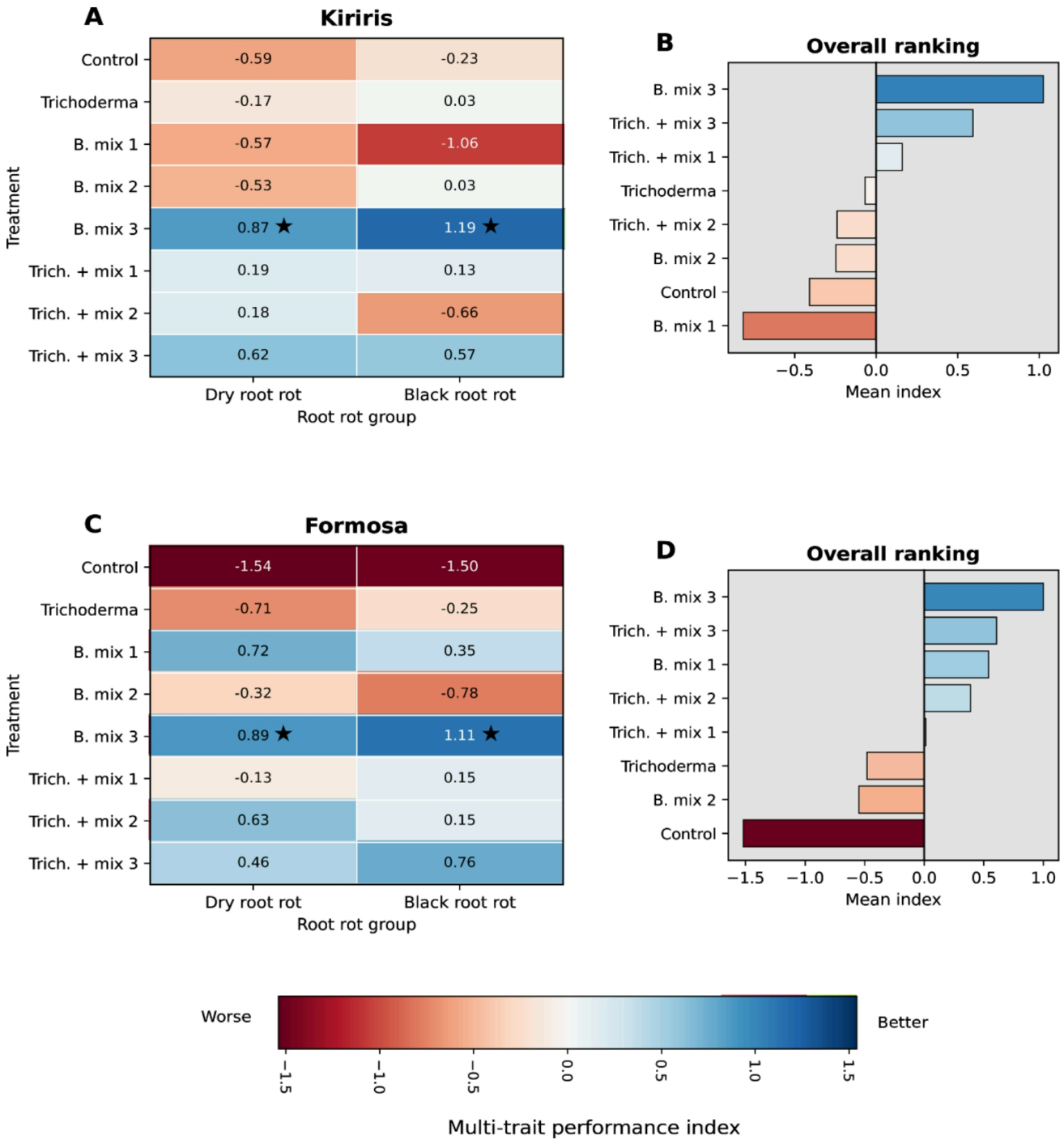


Figure 4

Genotype-specific ranking of biological treatments based on a multi-trait performance index in the cup bioassay. **(A)** Standardized treatment performance against dry and black root rot pathogen mixtures in the cassava genotype Kiriris. **(B)** Mean performance index of each treatment across both root rot groups in Kiriris. **(C)** Standardized treatment performance against dry and black root rot pathogen mixtures in the cassava genotype Formosa. **(D)** Mean performance index of each treatment across both root rot

groups in Formosa. The evaluated treatments were the untreated control, *Trichoderma*, *Bacillus* mixes 1–3, and combinations of *Trichoderma*with *Bacillus* mixes 1–3. The index represents the arithmetic mean of standardized values for germination at 15 days, plant height, vigor, shoot and root fresh mass, shoot and root dry mass, and internal colonization, calculated separately within each cultivar and root rot experiment. Internal colonization was reversed so that lower values contributed positively to treatment performance. Higher index values indicate better overall performance. The color scale ranges from red, representing the poorest performance, to blue, representing the best performance. *Stars indicate the treatment with the highest performance index within each root rot group.