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Antifungal potential of *Lactiplantibacillus plantarum AM2* against the banana pathogen *Fusarium oxysporum f. sp. cubense* Tropical Race 4

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ABSTRACT

Fusarium wilt of bananas is a serious disease affecting banana plantations worldwide. In an effort to sustainably manage the disease, biological control is considered a promising alternative to agrochemicals that can cause detrimental effects on humans and the ecosystem. In this study, we investigated the biological control potential of the present collection of beneficial bacteria which includes *Lactiplantibacillus plantarum* AM2, *Streptomyces morookaensis* NRRL B-12429, *Bacillus velezensis* B4158, *B. atrophaeus* B363B, and *B. amyloliquefaciens* B942 against the causal agent of Fusarium wilt disease in banana, *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc* TR4) through a dual culture assay and a greenhouse experiment. The inhibition range *in vitro* was between 31.0 to 42.1%, and the highest growth inhibition of *Foc* TR4 was observed for *L. plantarum* AM2. Infected banana plantlets that received the treatment with *L. plantarum* AM2 also showed a significant reduction in disease severity index as low as 24% compared to treatment with other beneficial bacteria. This study showed that *L. plantarum* AM2 has a good antagonistic effect on *Foc* TR4 mycelial growth and the most potential to control Fusarium wilt disease in bananas.

Keywords: Biological control, Fusarium wilt, *in Planta*, Lactic acid bacteria, Suppression.

INTRODUCTION

Musa spp. (banana) serves as an important cash crop for income generation and employment creation, especially in banana-producing countries (Caro, 2020). However, the recent discovery of Fusarium wilt disease in Latin America in 2021, together with continuing COVID-19 pandemic constraints and rising production costs had deflated the world banana export (FAO, 2022). The Fusarium wilt of bananas (FWB) is caused by a soil-borne pathogenic fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*). The fungus invades the vascular system of the host,

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blocking the transportation of nutrients and water throughout the plant. This leads to wilting symptoms and browning of the xylem (Ordóñez, 2018). *Foc* belongs to a species complex and consists of four races (1-4), each with different pathogenicity towards different banana cultivars. Race 4 mostly infects Cavendish, with Tropical Race 4 (TR4) being the most pathogenic (Pérez-Vicente et al., 2014; Ordóñez, 2018). *Foc* TR4 easily spreads through planting material, soil, and other substrates originating from infected farms. Eradication of the pathogen is difficult once it is established due to its ability to survive in several alternative hosts and produce chlamydospores that persist in the soil for a long time (Ploetz, 2015).

Due to the significant impact on the global economy, the management of FWB has been a focus of the scientific community worldwide. The management approaches include manipulation of cultural practices, chemical control, breeding for resistant cultivars, and biological control. The latter has been gaining interest recently due to increasing awareness of sustainable management of plant disease with less impact on the environment (Scortichini, 2022). The growth of the organic market, in conjunction with a reduction of pesticides, further drives the demand for more effective biocontrol agents and promotes the expansion of the biocontrol industry (Lahlali et al., 2022). The biological control agents (BCA) of FWB are dominated by endophytes such as *Trichoderma* spp. and *Bacillus* spp. (Bubici et al., 2019; Sánchez-Espinosa et al., 2020). Regardless, it does not put a hold on the quest to find new BCA candidates with different modes of action or unique secondary metabolites with better biocontrol efficacy as well as plant-growth-promoting effects. However, the majority of studies involving BCA against *Foc* TR4 were only conducted *in vitro*, with only a small number at the greenhouse level and very few that reached the field trial stage. Interestingly, based on data mining from the literature, Bubici et al. (2019) reported that biocontrol for FWB under field conditions exhibits similar disease control efficacy as observed in pot experimental conditions.

Lactic acid bacteria (LAB) are an intriguing group of microorganisms frequently present in plant-associated microbiomes (Jaffar et al., 2023). Moreover, it has been shown that LAB can generate compounds that are effective against a broad range of phytopathogens, including *F. oxysporum* (Raman et al., 2022). Compared to other common groups of BCAs, LAB possesses the upper hand since its application in food crop production presents no health risks to humans. Hence, it was given the Generally Recognized as Safe (GRAS) status by the US Food and Drug Administration (USFDA). *Lactiplantibacillus plantarum* is a type of LAB belonging to the novel *Lactiplantibacillus* genus (Zheng et al., 2020). On top of the GRAS status, *L. plantarum* was also given the Qualified Presumption of Safety (QPS) status from the European Food

Safety Authority (EFSA)(EFSA BIOHAZ Panel, 2023). Interestingly, *L. plantarum* carries more genes in its large genome compared to other LAB species, indicating its strong adaptability to different environments and high versatility (Seddik et al., 2017). Despite its huge potential, reports on the involvement of *L. plantarum* as BCA for plant pathogens have been scarce. Riolo et al. (2023) were the first to discover the potential of the fermentates of LABs from drupes of olive oil, including *L. plantarum*, as bio-fungicide against several plant pathogenic fungi and oomycetes. However, the study could not find an obvious correlation between the metabolic profile of the tested LABs and their antifungal efficacy. Not long after, Kavková et al. (2023) reported a notable inhibition of mycelial growth and conidial germination by *L. plantarum* and *L. pentosus* against *Fusarium* spp. from legumes by their cell-free supernatants.

In this study, we explored the potential of beneficial bacteria from a public culture collection and locally isolated *L. plantarum* from tempoyak, a fermented food made from durian flesh, to inhibit the growth of *Foc* TR4 *in vitro* and suppress the FWB in the greenhouse. The study aims to include more biological control candidates with proven efficacy *in planta* to the current biocontrol resources for FWB.

MATERIALS AND METHODS

Experimental Site

The study was carried out in the Plant Molecular Biology Laboratory and greenhouse of the Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, from January to October 2023.

Bacterial and Fungal Cultures

Foc TR4 isolate 9888 was obtained from the Dept. of Biology, Faculty of Science, UPM. *Bacillus velezensis* B4158, *B. atrophaeus* B363B, *B. amyloliquefaciens* B942, and *Streptomyces morookaensis* B12429 were obtained from NRRL Culture Collection, Illinois, USA. The *Bacillus* sp. was grown on Luria Bertani (LB) agar at 30°C. *S. morookaensis* B12429 was cultured on Starch-Casein agar at 30 °C. *L. plantarum* AM2 was previously isolated by a postgraduate student in the Plant Molecular Biology lab from a fermented durian (local fruit) flesh known as 'tempoyak' among the locals and deposited to the Microbial Culture Collection Unit (UNiCC), Institute of Bioscience, UPM (UNiCC Accession Number: UPMC 1489). *L. plantarum* AM2 was cultured on Man de Rosa (MRS) agar media at 37 °C.

98 ***In Vitro* Antifungal Assay**

99 The *in vitro* antifungal activity of the bacterial candidates against *Foc* TR4 was assessed in
100 a dual culture plate assay where *Foc* TR4 was co-cultured on potato dextrose agar (PDA) with
101 a single bacterial strain. Then, a mycelial plug was cut from the edge of a 5-day-old *Foc* TR4
102 culture using a 5-mm-diameter sterile cork borer. The plug was positioned in the middle of a
103 25ml solidified PDA in a 100-mm petri dish. On opposing ends of the plug, 10 µl of bacterial
104 suspension of was streaked in a straight line, maintaining a distance of 2.5 cm from the plug,
105 ensuring that the streaks of bacteria ran parallel to each other. The inhibition rate (%) was
106 measured as follows: (Colony diameter of the untreated group - colony diameter of the treated
107 group)/ colony diameter of untreated group × 100.
108
109 ***In Planta* Bioassay**

110 Three-month-old *Musa acuminata* var. Cavendish plantlets with 4 to 5 true leaves in a
111 polybag (13 cm height x 10 cm diameter) were used for the bioassay with *Foc* TR4 in this study.
112 The plantlets were purchased from Apex Phytocultures, Bangi, Malaysia and maintained in a
113 shaded greenhouse with daily watering and supplemented with inorganic fertilizer twice during
114 the bioassay period. The bioassay was conducted according to Zhu et al. (2021b) in five
115 replications for each treatment. A piece of agar with actively growing *Foc* TR4 mycelia was cut
116 from a 4-day-old PDA plate and inoculated into 100 ml Potato Dextrose Broth (PDB) in a flask.
117 The flask was cultured for 3 days on a shaker at 180 rpm at room temperature. After 3 days, the
118 suspension culture was filtered using a sterile filter paper to remove the mycelia. The filtered
119 suspension was then diluted to 1×10^6 conidia/mL using sterile double distilled water.
120 Meanwhile, individual bacterial culture was prepared by inoculating each bacterium in their
121 respective broth media of 150ml in volume as described above. The inoculated media were
122 incubated in a shaking incubator according to the specific temperature for each bacterium as
123 described above at 180 rpm for 48h. A ten-fold serial dilution was performed using 1X
124 phosphate buffer saline to obtain 1×10^8 cfu/ml spore suspension. A total of 30ml bacterial
125 culture was used to drench the roots of the banana plantlets. After 14 days of treatment, 30 mL
126 of *Foc* TR4 spore suspension was applied to the banana plantlets by pouring on the potting
127 medium. The plantlets were allowed to grow for 8 weeks, and external disease symptoms were
128 recorded by observing the yellowing and wilting symptoms. At the end of week 8, the plantlets
129 were up-rooted and cut longitudinally at the rhizome to record the internal symptoms. The leaf
130 symptom (LSI), rhizome discoloration index (RDI), and Disease Severity Index (DSI) were

calculated based on Dita et al. (2021). The disease suppressiveness of the bacterial treatment was calculated as follows: Disease suppressiveness = (control disease index − treatment disease index)/control disease index × 100.

Statistical Analysis

The data for *in vitro* antifungal assay were analyzed using analysis of variance (ANOVA), and differences between treatments were determined using Tukey's HSD test at a 5% error. The data for *in planta* bioassay were analysed using one-way ANOVA on ranks (Kruskal-Wallis's test).

RESULTS AND DISCUSSION

In Vitro Assay of Bacterial Isolates against *Foc* TR4

Five bacterial strains, including *B. velezensis* B4158, *B. atrophaeus* B363B, *B. amyloliquefaciens* B942, *S. morookaensis* B12429, and *L. plantarum* AM2, were evaluated for their antifungal efficacy against *Foc* TR4 by a dual culture assay. All the bacterial isolates tested showed the ability to inhibit the growth of *Foc* TR4 (Figure 1). The greatest reduction in mycelial growth was elicited by *L. plantarum* AM2 with the inhibition percentage 42.13%, whereas *S. morookaensis* B12429 showed the least inhibition (31%) (Table 1). On the other hand, all *Bacillus* spp. tested moderately inhibited the mycelial growth of *Foc* TR4.

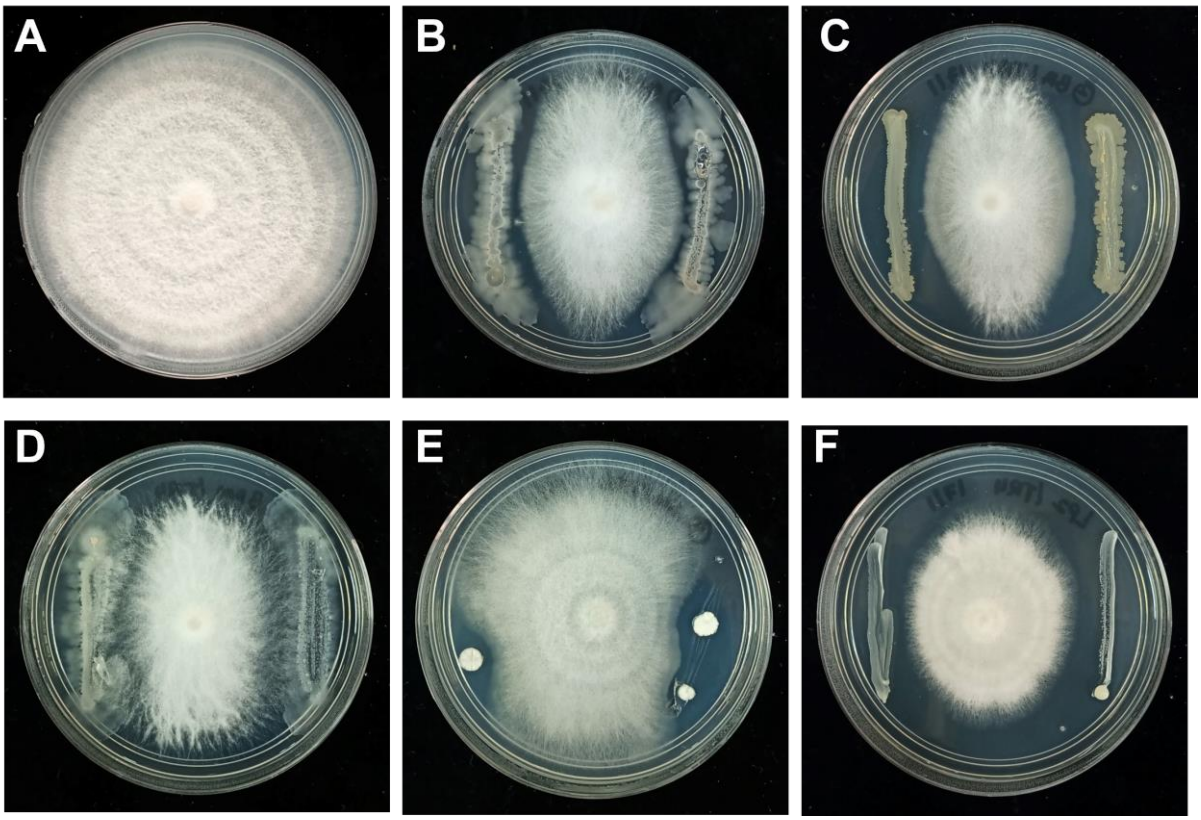

Figure 1. Dual culture assay of antagonistic bacteria against *F. oxysporum* f. sp. *cubense* (TR4) on potato dextrose agar (PDA). Control (A), culture treated with *B. velezensis* B4158 (B), *B. atrophaeus* B363B (C), *B. amyloliquefaciens* B942 (D), *S. morookaensis* B12429 (E), and *L. plantarum* AM2 (F).

Table 1. Inhibition percentage of *Fusarium oxysporum f. sp. cubense* (TR4) by antagonistic bacteria.

Antagonistic Bacteria	Inhibition Percentage (%)
Positive Control	0.00 ± 0.00^{a}
B. velezensis B4158	36.56 ± 0.46^{c}
B. atrophaeus B363B	38.55 ± 0.31^{d}
B. amyloliquefaciens B942	36.22 ± 0.46^{c}
S. morookaensis B12429	31.00 ± 0.46^{b}
L. plantarum AM2	42.13 ± 0.93^{e}

The values are the mean percentage of five replications of the inhibition diameter zone. Different letter indicates significant differences between treatments of antagonistic bacteria and control at $P < 0.05$ according to Turkey's HSD test.

L. plantarum stands out as among the most prevalent and adaptable species within the LAB family. It is well known for its role in inhibiting fungal growth and removing mycotoxins, especially in food applications (Vanitha et al., 2023; Li et al., 2023). Many *L. plantarum* strains can suppress the growth of fungi by disintegrating the cell structure under the action of its metabolites. It also has the ability to detoxify and degrade mycotoxins (Bergsma et al., 2022; Wei et al., 2020; Zhu et al., 2021a). As a hemi-biotrophic pathogen, *Foc* TR4 utilizes an array of virulent factors to infect the host plants, including phytotoxic secondary metabolites fusaric

168 acid (FSA) that could induce cell death in bananas, preparing the plants for xylem invasion (Li
169 et al., 2013). Recently, the antifungal activity of *L. plantarum* was reported against the plant
170 pathogen *F. oxysporum* (Kavková et al., 2023; Riolo et al., 2023). However, the underlying
171 mechanisms of the antifungal activity were not clearly described. It is tempting to speculate
172 that *L. plantarum* might be suppressing the growth of *Foc* TR4 mycelia via the metabolites
173 secreted into the medium that could distort hyphal structures, as observed by Deepthi et al.
174 (2016).

175 On the other hand, despite the potent antifungal effects of *Bacillus* spp. against *F. oxysporum*,
176 as previously reported (Fan et al., 2022; Saravanan et al., 2022), the *in vitro* effects of *B.*
177 *velezensis* B4158, *B. atrophaeus* B363B, and *B. amyloliquefaciens* B942 in this study were only
178 moderate and without much variation. Fan et al. (2021) reported an *in vitro* inhibition rate of
179 79.6% with endophytic *B. amyloliquefaciens* YN0904 against *Foc* TR4, while Saravanan et al.
180 (2022) observed up to 63% inhibition by *B. velezensis* YEBBR6. Conversely, *B. atrophaeus*
181 was reported to inhibit *F. oxysporum f. sp. cucumerinum* with high efficacy, but there has been
182 no report of *B. atrophaeus* against *Foc TR4* to date. The biocontrol capacity of *Bacillus* spp. is
183 mainly exhibited through direct inhibitory activity on the growth of pathogens, induction of
184 systemic resistance in host plants, and niche competition with the plant pathogens (Fira et al.,
185 2018). For instance, the application of *B. amyloliquefaciens* mixed with a compost mixture was
186 shown to induce systemic resistance of the banana plants through the upregulation of hydrolytic
187 enzyme activities such as chitinase and β-1,3-glucanase (Zhang et al., 2014). Additionally, *B.*
188 *amyloliquefaciens* and *B. velezensis* can interact with *Foc* TR4 and establish their colonies in
189 banana plants effectively to exert their inhibitory effects (He et al., 2021). Based on our findings
190 from the *in vitro* dual culture assay, the observed inhibition could be attributed to the diffusible
191 compounds released by *Bacillus spp.*, albeit less effective. In this case, *B. amyloliquefaciens*
192 and *B. atrophaeus* strains were known to produce volatile compounds for the biocontrol of plant
193 diseases (Asari et al., 2016; Rajaofera et al., 2019). The ability of the *Bacillus* spp. used in this
194 study to produce volatile compounds and their efficacy against *Foc* TR4 would be interesting
195 subjects of future studies.

196 Finally, our *Streptomyces sp.* candidate, *S. morookaensis* B12429 only managed to exert a
197 mild antagonistic activity on the growth of *Foc* TR4 *in vitro*. In contrast, another *S.*
198 *morookaensis*, strain Sm4-1986, was in the limelight recently due to its ability to promote
199 banana growth and suppress FWB (Zhu et al., 2021b). This could be attributed to the different

metabolites and genetic profile of each strain that leads to different levels of antagonistic activity.

The Suppressive Effects of Bacterial Strains on FWB *in Planta*

Despite the variation of inhibition percentage *in vitro*, all the bacterial strains were used in the greenhouse studies since the response of BCA during *in vitro* and *in planta* assay do not always correlate (Parikh et al., 2018; Besset-Manzoni et al., 2019). Interestingly, *L. plantarum* AM- inoculated plantlets showed delayed wilting symptoms compared to other treatments. The disease severity for plants co-inoculated with *L. plantarum* AM2, *B. amyloliquefaciens* B942 and *S. morookaensis* B12429 was significantly reduced (Figure 2). The lowest LSI score was recorded in *L. plantarum* AM2 at 25%, while the lowest RDI score was in *B. amyloliquefaciens* B942 at 31.76% (Figure 3). Conversely, *B. atrophaeus* B363B displayed the highest disease severity, with both LSI and RDI scores at 95 and 94%, respectively.

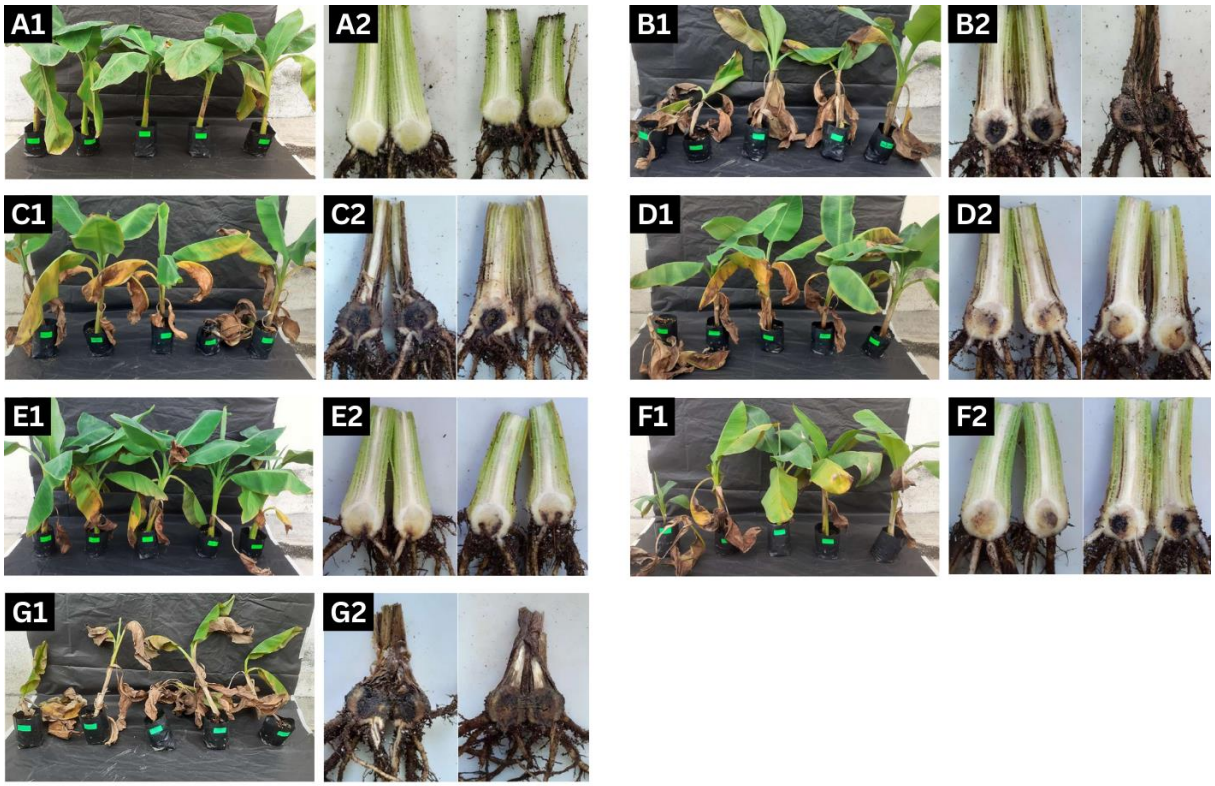

Figure 2. External and internal symptoms of different treatments on week 8. Negative control (A1-External) and (A2-Internal), *B. atrophaeus* B363B (B1- External) and (B2-Internal), *B. velezensis* B4158 (C1- External) and (C2-Internal), *B. amyloliquefaciens* B942 (D1- External) and (D2-Internal), *L. plantarum* AM2 (E1- External) and (E2-Internal), *S. morookaensis* B12429 (F1- External) and (F2-Internal), Positive control (G1-External) and (G2-Internal).

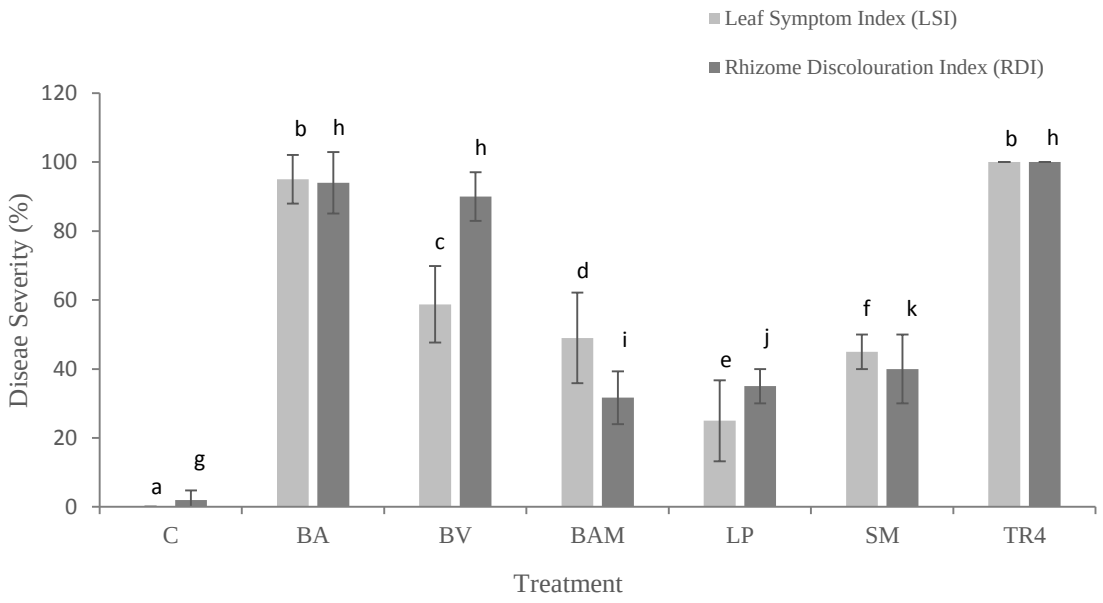

Figure 3. Leaf Symptoms Index (LSI) and Rhizome Discolouration Index (RDI) of different treatments. Negative Control - no *Foc* TR4, no biological control (C), *B. atrophaeus* B363B (BA), *B. velezensis* B4158 (BV), *B. amyloliquefaciens* B942 (BAM), *L. plantarum* AM2 (LP), *S. morookaensis* B12429 (SM), Positive Control - inoculated with *Foc* TR4, no biological control (TR4). Different letters show different significance values at P> 0.05 using one-way ANOVA on ranks (Kruskal-Wallis's test).

Banana plants inoculated with *L. plantarum* AM2 showed the lowest DSI at 24% (Table 2), which aligned with the findings from the *in vitro* assay. These findings further support the potential of *L. plantarum* AM2 as a bio-control agent against FWB. Following closely were *B. amyloliquefaciens* B942 and *S. morookaensis* B12429, with DSI at 32%. Previous research has highlighted the effectiveness of different strains of *B. velezensis* and *B. amyloliquefaciens* as biocontrol agents *in planta* (Fan et al., 2021; Fu et al., 2017; Xiang et al., 2023). However, in this study, only the *B. amyloliquefaciens B942* strain demonstrated a significant effect against FWB. Conversely, plantlets inoculated with *B. atrophaeus* B363B and *B. velezensis* B4158 exhibited severe disease symptoms, both externally and internally, despite the strong inhibition observed *in vitro*. On the other hand, *S. morookaensis* strain B12429 only demonstrated moderate antagonistic effects against *Foc* TR4 in the greenhouse experiment. To the best of our knowledge, this study is the first to report on the biocontrol potential of *S. morookaensis* strain B12429 and *B. amyloliquefaciens B942* strain against *Foc* TR4 *in planta*.

Table 2. Effects of treatment with antagonistic bacteria on the FWB development *in planta* and their biocontrol efficacy.

Antagonistic bacteria	Disease Severity Index (%)	Disease suppressiveness (%)
B. atrophaeus B363B	100	0
B. velezensis B4158	68	32
B. amyloliquefaciens B942	32	68
L. plantarum AM2	24	76
S. morookaensis B12429	32	68
Positive Control (TR4)	100	-

Nevertheless, further testing through field trials is needed to fully assess the potential of these bacteria as biocontrol agents since it offers a more reliable and realistic assessment. The interactions and competition of bio-control agents with the soil microbiome, as well as the adaptation of potential strains to the abiotic conditions, limits their efficiency against pathogens (Purkayastha et al., 2018). These underscore the necessity to validate in vivo experiments conducted in greenhouses with field trials.

CONCLUSIONS

This study explores the antagonistic potential of selected microbes as biocontrol agents against FWB in the susceptible banana cultivar, Cavendish. The results indicate that *L. plantarum* AM2, *B. amyloliquefaciens* B942 and *S. morookaensis* B12429 inhibited the mycelial growth of *Foc* TR4 *in vitro* and decreased the severity of FWB *in planta*. Inoculation with *L. plantarum* AM2 resulted in the lowest DSI at 24%. The microbes tested in this study show potential in suppressing FWB and are thus considered promising candidates for biological control. However, trials in open fields are necessary to further assess the efficacy of these antagonistic microbes in a more realistic environment.

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