Effectiveness of *Bacillus subtilis* ANT01 and *Rhizobium* sp. 11B on the control of fusarium wilt in pineapple (*Ananas comosus*) (#90983)

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Effectiveness of *Bacillus subtilis* ANT01 and *Rhizobium* sp. 11B on the control of fusarium wilt in pineapple (*Ananas comosus*)

Maria de Lourdes Adriano-Anaya [1], **Luis Fernando Pardo-Girón** [1], **Miguel Salvador-Adriano** [1], **Miguel Salvador-Figueroa** [1], **Isidro Ovando-Medina** [1], **Benjamin Moreno-Castillo** [Corresp. 1]

[1] Instituto de Biociencias, Campus IV, Universidad Autónoma de Chiapas, Tapachula, Chiapas, Mexico

Corresponding Author: Benjamin Moreno-Castillo
Email address: benjamin.moreno@unach.mx

Pineapple (*Ananas comosus*) is commonly infected by *Fusarium oxysporum*, causal agent of the fusarium wilt disease. Conventionally, growers use synthetic fungicides to control the disease, which lead to environmental pollution, hazardous effects on non-target organisms and risks on human health. The aim of this work was to assess the effectiveness of *Bacillus subtilis* ANT01 and *Rhizobium sp.* 11B to control fusarium wilt on pineapple plants. Four treatments derived from a complete factorial design were tested under field conditions. Treatments composed of *B. subtilis* ANT01 and the combination *B. subtilis* ANT01 – *Rhizobium sp*. 11B decreased disease severity by 94.4% and 86.1%, respectively. On the other hand, the treatment prepared with *Rhizobium sp.* 11B alone showed a reduction of 75.0%. Size of leaves and nutritional condition (SPAD units) of the biocontrol agents-treated plants showed no statistical differences. Moreover, *B. subtilis* ANT01 decreased by 46% the initial soil population of *F. oxysporum*, while *Rhizobium sp.* 11B, *B. subtilis* ANT01 plus *Rhizobium sp.* 11B and control, showed a population reduction of 12.5%, 24.2% and 23.0%, respectively. These results make evident the potential of *B. subtilis* ANT01 as biocontrol agent of the pathogen under field conditions.

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[1] Instituto de Biociencias, Universidad Autónoma de Chiapas, Tapachula, Chiapas, México

12

Corresponding Author:
Benjamin Moreno-Castillo
Boulevard Príncipe Akishino S/N, Tapachula, Chiapas, 30798, México
Email address: benjamin.moreno@unach.mx

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Abstract

Pineapple (*Ananas comosus*) is commonly infected by *Fusarium oxysporum*, causal agent of the fusarium wilt disease. Conventionally, growers use synthetic fungicides to control the disease, which lead to environmental pollution, hazardous effects on non-target organisms and risks on human health. The aim of this work was to assess the effectiveness of *Bacillus subtilis* ANT01 and *Rhizobium sp.* 11B to control fusarium wilt on pineapple plants. Four treatments derived from a complete factorial design were tested under field conditions. Treatments composed of *B. subtilis* ANT01 and the combination *B. subtilis* ANT01 – *Rhizobium sp*. 11B decreased disease severity by 94.4% and 86.1%, respectively. On the other hand, the treatment prepared with *Rhizobium sp.* B alone showed a reduction of 75.0%. Size of leaves and nutritional condition (SPAD units) of the biocontrol agents-treated plants showed no statistical differences. Moreover, *B. subtilis* ANT01 decreased by 46% the initial soil population of *F. oxysporum*, while *Rhizobium sp.* 11B, *B. subtilis* ANT01 plus *Rhizobium sp.* 11B and control, showed a population reduction of 12.5%, 24.2% and 23.0%, respectively. These results make evident the potential of *B. subtilis* ANT01 as biocontrol agent of the pathogen under field conditions.

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Introduction

Pineapple (*Ananas comosus*) is a bromeliad indigenous of Brazil and well-adapted to other tropical regions (Vélez et al. 2020). The plant has a short and robust stem, thick leaves and produce up to 200 inflorescences that conforms a syncarp or infruitescence commonly known as pineapple (Nassr & Abu Naser 2018). During 2021 Mexico produced 1 271 520 tons of pineapple, allowing to reach the ninth position in the world ranking of pineapple production (SIAP 2022). The state of Veracruz is the most important producer with 62.6% of the national production, followed by Oaxaca and Quintana Roo with 13.5% and 8.5%, respectively (SADER 2022a). Pineapple orchards are affected by biotic and abiotic factors (Moreira et al. 2016). Some fungal pathogens that infect pineapple plants are *Phytophthora nicotianae, Thielaviopsis paradoxa* and *Fusarium oxysporum* (Uriza-Ávila et al. 2018).

F. oxysporum is the causal agent of the fusarium wilt, a devastating disease that causes up to 80% of pineapple yield loss (SADER 2022b). The pathogen enters the plant through wounds or natural openings in the roots and colonizes the inner vascular tissues (Villa-Martínez et al. 2015), where produces a polymer that accumulates and blocks water and nutrient transport from roots to aerial parts (Hernández et al. 2019; SADER 2022b),

Conventionally, fusarium wilt is controlled by the spraying of synthetic chemical fungicides, leading to environmental pollution and hazardous effects on human health and non-target organisms (Trinidad-Cruz et al. 2017). Alternatively, the use of antifungal microorganisms as biocontrol agents has been proposed (Vinchira & Moreno 2019).

Several works have reported the effectiveness of bacterial strains of *Bacillus* and *Rhizobium* against fusarium wilt ranging from 40 to 80% in several agricultural crops, such as banana (Akila et al. 2011), in tomato (Ajilogba et al. 2013; Akram et al. 2013; Elanchezhiyan et al. 2018; Patel & Saraf 2017; Shanmugam & Kanoujia 2011), cucumber (Cao et al. 2011), chickpea (Zaim et al. 2018; Mehmood & Khan 2016), bean (Kalantari et al. 2018; Tewari & Sharma 2020) and wheat (Palazzini et al. 2016). Otherwise, some strains of *Rhizobium* are free-living and can enhance plant growth, acting as biofertilizers (Salvador-Figueroa et al. 2016). Although cell concentrations tested in the reported literature ranged from 10^4 to 10^{10} CFU/mL, there are few studies of *Bacillus* and *Rhizobium* as biocontrol agents of the fusarium wilt in pineapple orchards. Based on the former, the aim of this work was to assess the effectiveness of *Bacillus subtilis* ANT01 and *Rhizobium* sp. 11B strains against the fusarium wilt in pineapple under field conditions.

Materials & Methods

Plant material and experimental site

Pineapple shoots of about 30 cm high were collected in the municipality of Frontera Hidalgo in Chiapas state, southern Mexico (14°46'51.2"N 92°10'55.2"O). Shoots were planted at the Agroecological unit "Ayol" located in the municipality of Tapachula, Chiapas (14º49′45.3" N 92º17′48.5" O). Spacing between rows was 200 cm and 150 cm between plants, burrowed at a depth of 20 cm. Emerging weeds were manually removed and pineapple plants were irrigated when required to ensure optimum growth.

79 **Production of biofertilizers and biofertiliza**

80 Four liters of biol (organic liquid biofertilizer) were weekly applied per plant by drenching as

81 reported by Adriano et al. (2012) plus two kilograms of bocashi added bimonthly per plant.

82 Bocashi was prepared by homogenously mixing three layers composed each by 200 kg of coffee

83 pulp, 200 kg of leaves and pseudostem of banana plants, 200 kg of fresh bovine manure, 24 kg of

84 ash, 1.5 L sugarcane molasses, 2 L acid lactic bacterial broth culture and 1.5 L of yeast broth

85 culture (*Saccharomyces cerevisiae*). In order to regulate temperature and moisture, the mixture

86 was thoroughly mixed twice a day during the first seven days, then once a day during the

87 following week and finally, every 72 h during the last 14 days of the 28-day-fermentation

88 bioprocess.

89 **Treatments**

90 Treatments were set under a factorial arrangement 2^2 and a completely randomized design. The

91 two factors (*B. subtilis* ANT 01 and *Rhizobium* sp. 11B strains) were tested at two levels (Table

92 1) and each treatment consisted of 25 plants.

93 **Inoculum production of *Bacillus subtilis* ANT01 and *Rhizobium* sp. 11B**

94 Both bacterial strains *B. subtilis* ANT01 and *Rhizobium* sp. 11B were kindly provided by the

95 Microbiological collection of the BioSciences Institute of the Autonomous University of Chiapas

96 (UNACH). Strain ANT01 was cultured in potato dextrose broth (PDB) in 500 mL-flasks (24

97 g/L) during 96 h at pH 5.6 ± 0.2, 200 rpm and 28 °C. Strain 11B was cultured in nutrient broth

98 (NB) during 12 h in 500 mL-flasks (8 g/L), at pH 7, 200 rpm and 28 °C. After incubation,

99 cultures were serially-diluted and cell concentrations were estimated by the most probable

100 number microbiological protocol.

101 **Treatments application**

102 Crude cultures (cells and extracellular metabolites) were added to a mixture of vermicompost

103 leachates:water (2:10, V/V). The preparations were weekly sprayed on pineapple leaves with a

104 hand-sprayer and until drip-point.

105 **Fusarium wilt severity**

106 A scale of visible symptoms on leaves was used and consisted of five grades: grade 0= healthy

107 plants with no disease symptoms on leaves, grade 1= leaves with a yellowish decoloration less

108 than 25% of the leaf area, grade 2= leaves with yellowish decoloration covering between 25 to

109 50% of the leaf area, 3= yellowish decoloration between 50 and 75% of the leaf and grade 4=

110 decoloration above 75% of the leaf surface. With this information, severity was calculated with

111 Equation (1):

112 $$\text{Severity} = \frac{\sum(\text{number of leaves on each grade x grade of the scale})}{(\text{total leaves sampled}) \text{x (highest grade of the scale)}} \quad (1)$$

113 **Leaf size and nutritional status measured as units SPAD**

114 Leaf size was obtained measuring the length (from base to apex) and width (in the mid part) of

115 the basal leaves 2, 3 and 4 as suggested by Gordillo-Delgado & Botero-Zuluaga (2020). In these

116 same leaves, SPAD units were measured with a device SPAD-502 Plus^R (Konica Minolta).

117 **Soil population of *F. oxysporum***

118   Quantifications of *F. oxysporum* in the treated-plants soil were estimated in composite samples
119   collected in a "zigzag" sampling pattern through the experimental plots. One gram of sampled
120   soil was suspended in 9 mL of sterile Ringer´s solution and serially diluted to 10^{-2}. Then, 100 µL
121   of the dilution were spread by triplicate onto Potato Dextrose Agar (39 g/L) added with 50 ppm
122   Bengal rose in sterile petri dishes. After a nine-day-incubation at 28 °C, colonies
123   morphologically similar to *F. oxysporum* were counted and verified under a microscope. The
124   conidial and fruiting bodies morphology was also observed and compared with the reporting
125   literature (Kiffer & Morelet 2000).
126   **Data analysis**
127   The influence of the analyzed factors (bacterial strains) and its statistical significance (subjected
128   to ANOVA) during the stabilization stage of the fusarium wilt severity, the fungal soil
129   population, leaf size and SPAD units, were assessed by the statistical procedure of a complete
130   factorial design described by Gutiérrez & de la Vara (2008). In cases of statistical significances,
131   mean effects of factors and their interactions were graphically illustrated for an easier
132   interpretation.
133   **Results**
134   **Fusarium wilt severity**
135   Since the first application of the biocontrol agents and until the 56th day, an overall increasing
136   trend in fusarium wilt severity was observed (Fig. 1). In pineapple plants treated with *B. subtilis*
137   ANT01 (Treatment 2), *Rhizobium* sp. 11B (Treatment 3) and the combination of both strains
138   (Treatment 4), the maximum increments of the severity were 1.40, 1.53 and 1.75-fold,
139   respectively, as compared to their initial severity levels (mean severity=0.36). In the other hand,
140   leachates-treated plants (Treatment 1) maximum severity was 1.28-fold in comparison to its
141   initial severity level. Furthermore, after 56 days of initial treatment an overall decrement of the
142   disease severity curves was observed, and from 112th to 210th day a flattening pattern or
143   stabilization stage was observed. At the end of the field experiment, average severity in
144   treatments 1, 2, 3 and 4 represented 5.6%, 25.0%, 13.9% and 22.9%, respectively, in reference to
145   their initial severity values.
146   Data analysis of the severity at the stabilization stage revealed that *B. subtilis* ANT01 effect and
147   *B. subtilis* ANT01 - *Rhizobium* sp. 11B interaction had negative values, thus, both strains
148   decreased the fusarium wilt severity, while *Rhizobium* sp. 11B effect had a positive value and
149   increased the disease severity (Table 2). Graphs in Fig. 2 show individual effects and the
150   interaction of the bacterial strains used as biocontrol agents of fusarium wilt in this experiment.
151   The ANOVA of fusarium wilt severity revealed statistical differences in pineapple plants treated
152   with *B. subtilis* ANT01 ($F_{1,48}$=63.9; P<0.01) and *Rhizobium* sp. 11B ($F_{1,48}$=21.2; P<0.01) but not
153   in their interaction ($F_{1,48}$=1.1; P=0.31).
154   Using the effect values from Table 2 as coefficients on the linearized equation $Y = 0.1325 -$
155   0.0490 *B. subtilis* ANT01 + 0.0283 *Rhizobium* sp. 11B - 0.0063 *B. subtilis* ANT01-*Rhizobium*
156   sp. 11B, predicted values of fusarium wilt severity during the stabilization stage for each
157   treatment are given in Table 3.

158   The mean difference between experimental and predicted values was 0.1 thousandth, which is

159   very low and with a determination coefficient R^2 of 0.6425 (R^2= [(SS_{Total} - SS_{Error})/(SS_{Total})];

160   taken from ANOVA of severity, not shown).

161   **Leaf size and nutritional condition (SPAD units) of pineapple plants**

162   Throughout the experiment, the overall average leaf length of pineapple plants was 44.94 cm

163   (ranging from 43.35 cm in plants from treatment *B. subtilis* ANT01 to 46.16 cm in plants from

164   treatment *Rhizobium* sp. 11B). In addition, the overall average leaf width of pineapple plants was

165   3.5 cm (ranging from 3.15 cm in plants from treatment *B. subtilis* ANT01 to 3.87 cm in plants

166   from treatment *Rhizobium* sp. 11B). No significant differences were detected in the ANOVA

167   regarding leaf length (L) and leaf width (W) of the plants treated either with *B. subtilis* ANT01

168   (L: $F_{1,177}$=2.74; P=0.10; W: $F_{1,177}$=1.40; P=0.24) or *Rhizobium* sp. 11B (L: $F_{1,177}$=0.79; P=0.37;

169   W: $F_{1,177}$=0.39; P=0.53), neither for their interaction (L: $F_{1,177}$=0.11; P=0.74; W: $F_{1,177}$=1.47;

170   P=0.23). The determination coefficients R^2 obtained from ANOVA were 0.020 and 0.083 for L

171   and W, respectively.

172   The effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B and the interaction on leaf length and width

173   from all treatments are shown in Table 4. The highest effects were as a result of the application

174   of *B. subtilis* ANT01, but only represented 2.0% and 7.5% of the length and width total effects,

175   respectively. Prediction equations were: L = 44.94 - 0.92 *B. subtilis* ANT01 + 0.49 *Rhizobium*

176   sp. 11B +0.18 *B. subtilis* ANT01- *Rhizobium* sp. 11B, while W= 3.508 - 0.263 *B. subtilis* ANT01

177   + 0.098 *Rhizobium* sp. 11B. The proportions of both variables resulted in 2.2% and 22.9% of

178   maximum difference (Table 5).

179   The overall average of nutritional condition in pineapple leaves (measured in SPAD units from

180   leaves 2,3 and 4) was 49.0 (ranging from 47.8 in plants from treatment *Rhizobium* sp. 11B to

181   51.0 in plants from control). No statistical differences were detected in the ANOVA of SPAD

182   units from strain ANT01-treated plants ($F_{1,177}$=0.75; P=0.39), strain 11B-treated plants

183   ($F_{1,177}$=3.55; P=0.06) and in ANT01-11B interaction ($F_{1,177}$=3.54; P=0.06).

184   The highest effect was registered in treatment *Rhizobium* sp. 11B and represented 1.6% of the

185   total effect, and the highest deviation derived from prediction equation (SPAD= 48.999- 0.372 *B.*

186   *subtilis* ANT01 - 0.810 *Rhizobium* sp. 11B + 0.809 *B. subtilis* ANT01- *Rhizobium* sp. 11B) was

187   1.5% (Table 6). The determination coefficient for SPAD units was 0.0424.

188   **Colonies of *Fusarium oxysporum* from soil cultivated with pineapple plants**

189   Dynamics of *F. oxysporum* growing colonies are shown in Fig. 3. Twenty-eight days after the

190   initial treatment (DAIT) with *Rhizobium* sp. 11B - *B. subtilis* ANT01 (T4), *Rhizobium* sp. 11B

191   (T3) and control (T1) the *F. oxysporum* population decreased by 25%, 25% and 17%,

192   respectively, while treatment with *B. subtilis* ANT01 (T2) increased by 40%. At the 56th day

193   there was a peak population of 1.7, 1.6 and 2.8-fold in reference to initial values of treatments

194   T1, T2 and T4, respectively. In the case of treatment T3 the peak was at 84 DAIT, with 1.5-fold

195   of its initial value.

196   In all treatments, *F. oxysporum* soil population decreased after their maximum values and after

197   140 DAIT was less variable. The factorial design analysis of the *F. oxysporum* population at the

198   stabilization stage, showed that the effect of *B. subtilis* ANT01 has a negative value, which
199   indicates that the bacteria decreased the fungal population, while *Rhizobium* sp. 11B and the
200   interaction *B. subtilis* ANT01 - *Rhizobium* sp. 11B increased the fungal soil populations due to
201   their positive effect values (Table 7). Figure 4 shows graphically the individual and interaction
202   effects on the fungal soil populations.
203   The ANOVA of *F. oxysporum* soil populations revealed statistical differences in the case of
204   *B.subtilis* ANT01 ($F_{1,20}$=11.26; P<0.05) and *Rhizobium* sp. 11B ($F_{1,20}$=4.43; P<0.05) treatments,
205   while treatment with both bacterial strains showed no statistical significance ($F_{1,20}$=0.26;
206   P=0.61).
207   Using the effect values from Table 7 as coefficients on the linearized equation: Population of *F.*
208   *oxysporum* [10^4 CFU/g_{soil}] = 1.7042 - 0.2458 *B. subtilis* ANT01 + 0.1542 *Rhizobium* sp. 11B B +
209   0.0375 *B. subtilis* ANT01 - *Rhizobium* sp. 11B, predicted values of the fungal soil population at
210   the stabilization stage for each treatment are shown in Table 8.
211   The average difference between the experimental and predicted values was between 3.5 and
212   5.8% (which provided an accurate prediction) and with a determination coefficient R^2 of 0.4438
213   according to ANOVA of fungal soil populations.

214   **Discussion**

215   Based on the results from this work, the initial increment of fusarium wilt (increasing yellowish
216   leaf area) observed in the pineapple plants (Fig. 1), mainly in the plants treated with the
217   combination of *B. subtilis* ANT01 and *Rhizobium* sp. 11B, might be due to a plant immune
218   response or priming, as mentioned by Martínez-Medina et al. (2021) and as demonstrated by the
219   increasing activity of enzymes 1,3-glucanase and chitinase in tomato plants (*Solanum*
220   *lycopersicum* L. var Amelia) treated with the antagonistic *Glomus mosseae* and *G. cubense*
221   (Pérez et al. 2015); or the differential expression of the chalcone synthetase and phenylalanine
222   ammonia lyase genes after the infection with the pathogen fungus *Pestalotiopsis* sp., causal agent
223   of the gray blight in tea plants (*Camellia sinensis* L.) (Wang et al. 2021); or the dynamics of the
224   salicylic acid production in strawberry plants infected with *Podosphaera aphanis* (Feng et al.
225   2020). Alternatively, the increased severity of fusarium wilt observed in our experiment might
226   also has been elicited as a response of *F. oxysporum* to the presence of some antifungal
227   extracellular metabolites produced by the strains tested and/or from the naturally-living
228   microorganisms of the vermicompost leachates. This phenomena might also be interpreted as a
229   normal pattern of the antagonistic process, decreasing the disease severity once the biocontrol
230   agents reached a proper density to induce the plant defenses or to act directly on the fungus.
231   Moreover, it is not discarded that the addition of the leachates and their naturally-occurring
232   microbiota or metabolites might have influenced the rhizosphere environment, in such a way that
233   the treated plants produced more root exudates and promoted the presence of antifungal root-
234   associated microorganisms (Ren et al. 2008; Yuan et al. 2021).
235   The levels of reduction of fusarium wilt observed in the pineapple plants of 94.4%, 75.0%,
236   86.1% and 77.1%, treated with *B. subtilis* ANT01 (Treatment 2), *Rhizobium* sp. 11B (Treatment
237   3), the combination of both strains (Treatment 4) and control (Treatment 1), respectively, are in

238　the severity range reported for *F. oxysporum f.* sp. *lycopersici* in tomato plants treated with *B.*
239　*cereus* (81.2%), *B. amyloliquefaciens* (75%), *B. pumilus* (62.5%) and *B. subtilis* (62.5%)
240　(Ajilogba et al. 2013), as well as with *Penicillium* sp. EU0013_90S (ranging from 80.6% to
241　95.2% of reduction) (Hussain et al. 2016). In reference to the pathogenic *F. oxysporum f.* sp.
242　*spinaciae* of spinach plants treated with *F. equiseti* GF183, a range between 43.5% to 91.8% was
243　reported (Horinouchi et al. 2010); and in the case of *F. oxysporum f. sp. cubense* (Foc race 1) in
244　banana "Prata" the reduction levels ranged from 34% to 85% (Haddad et al. 2018). The
245　reduction levels reported in this work were higher than those reported for the control of *F.*
246　*oxysporum f.* sp. *lycopersici* in tomato plants treated with *Bacillus* sp. ERBS10 or *B. velezensis*
247　ERBS51 (34.9% and 50.2%, respectively) (Devi et al. 2022), as well as the treatment with
248　*Penicillium* sp. EU0013_90S (46.2%) (Alam et al. 2011) and in *F. oxysporum f.* sp. *cubense* (Foc
249　race 1) of banana "Grand Naine" (ranging from 27.8% to 42.2%) treated with the combinations
250　of *Pseudomonas putida* (C4r4) + *B. cereus* (Jrb1) and *Achromobacter* sp. (Gcr1) + *B. cereus*
251　(Jrb5) (Thangavelu & Gopi 2015).
252　Although the reduction in fusarium wilt was observed in the four treatments tested, data analysis
253　showed that the treatment prepared with the strain *Rhizobium* sp. 11B promoted the symptoms
254　disease, thus increasing the severity (as observed in Fig. 2A). Such increment may be due to the
255　interaction plant-microbe or any metabolite that triggers the production of reactive oxygen
256　species (ROS) provoking tissue necrosis to limit the growth of the pathogen as a defense
257　response. Otherwise, we believe that the antagonistic activities of *B. subtilis* ANT01 and the
258　combination *B. subtilis* ANT01 + *Rhizobium* sp. 11B (Table 2) on severity, was the result of the
259　direct detrimental effects of the strains or their metabolites on the fungus (Fig. 1, Fig. 2). In this
260　regard, it has been reported that the bacteria *B. subtilis* has the ability to produce several
261　antifungal metabolites such as iturin A, surfactin and bacilomicin D (Gowtham et al. 2016;
262　Theatre et al. 2021). Such metabolites help on the biofilms formation, motility, and elicit cellular
263　alterations on the fungal cell wall (Saxena et al. 2020). Moreover, the genus *Bacillus* is widely
264　known for its production of an arsenal of fungal cell wall degrading enzymes (Leelasuphakul et
265　al. 2006) as well as siderophores that limit the access of fungal pathogens as *F. oxysporum* to an
266　iron source (Goswami et al. 2016). Otherwise, since pineapple plants treated with the
267　combination of both strains showed less severity than *Rhizobium* sp. 11B-treated plants, but
268　higher severity levels than the *B. subtilis* ANT0-treated plants, a likely antibiotic activity might
269　have been occurred on *Rhizobium* strain when both biocontrol agents were combined and applied
270　on the plants. Besides, the low severity levels registered on leachates-treated plants (Treatment
271　1) might be as a function of some antifungal metabolites. The former is based on the
272　determination coefficient R^2 of treatments below 0.9 and derived from the severity ANOVA,
273　which allows to conclude not to be part of the experimental error.
274　Since leaf size (length and width) was not affected with the treatments (Table 4 and 5), a mask-
275　effect is likely to have occurred due to the biofertilizer effects of leachates. This was most
276　noticeable in *Rhizobium* sp. 11B-treated plants, since the bacteria is known for its plant growth
277　promoting traits. The lack of plant growth promoting effects observed in the pineapple plants is

278 similar to the reported by Devi et al. (2022), whose results showed a lack of promoting effects on
279 height and total leaves of tomato plants root-inoculated with *Bacillus* sp. ERBS10 or *Bacillus*
280 *velezensis* ERBS51, both with antifungal activities on *F. oxysporum f.* sp. *lycopersici*. Haddad et
281 al. (2018) also reported that height and pseudostem diameter of banana plants cv. "Prata" were
282 not increased with the inoculation of *Trichoderma harzianum*, antagonist against *F. oxysporum* f.
283 sp. *cubense* (Foc) race 1. Contrarily, Ajilogba et al. (2013) reported that inoculation of tomato
284 plants with the Foc4 antagonists *B. amyloliquefaciens*, *B. cereus*, *B. pumilus* and *B. subtilis*
285 increased plant height and root length, while Thangavelu & Gopi (2015) [40] reported that
286 banana plants cv Grand Naine inoculated with *B. flexus* (TvPr1) + *Pseudomonas putida* (Jrb2) +
287 *B. cereus* (Jrb1), produced more bunches with more quality traits and more banana hands per
288 bunch.
289 Otherwise, the lack of effect of the bacterial strains on SPAD units indicates that nitrogen
290 fertilization in all plants was similar. Additionally, a strong argument is difficult to find
291 regarding the best range of SPAD units recorded in this work, since to the best of our knowledge
292 this is the first report of SPAD assessment on pineapple plants. We only may point out that
293 SPAD units reported in this work are in the normal range reported in maize (Novoa & Villagrán
294 2002), wheat and barley (González 2009) and grape (Castañeda et al. 2018).
295 The overall time-evolution of *F. oxysporum* soil population (Fig. 3) was similar to time-
296 evolution of fusarium wilt severity (Fig. 1), including the increasing effect during the first 56
297 days of the field assay. The initial increment of the pathogen soil population might be a defense
298 response to the antifungal extracellular metabolites produced by the biocontrol agents and by the
299 naturally-occurring microorganisms from leachates. The subsequent fungal population
300 decrement (after 56th day) might be as a function of an increasing presence, or accumulation of
301 antifungal metabolites up to a detrimental or suppressing concentration in the soil. Alternatively,
302 it is likely an increment of some other fungal antagonists in the soil, due to the periodic addition
303 of leachates as biofertilizer (Ren et al. 2008; Yuan et al. 2021).
304 There are few reports on the time-evolution of fungal pathogen soil populations after the
305 treatment with biocontrol agents, and to the best of our knowledge there is no available literature
306 in reference to pineapple plants. Nevertheless, reduction values in soil population of *F.*
307 *oxysporum* reported here, were lower than the values reported by Horinouchi et al. (2010) when
308 *F. equiseti* GF183 was applied on spinach plants to control fusariosis disease, but they indirectly
309 determined the fungal population as a function of root weights.
310 Independently on the formerly mentioned, data analysis showed that *Rhizobium* sp. 11B favored
311 the presence of fusarium wilt symptoms (severity), as observed in Fig. 4A). Likely, *Rhizobium*
312 sp. 11B does not produce antifungal metabolites but fungal growth promoting metabolites. The
313 negative effect of *B. subtilis* ANT01 (Table 7) may be at cause of antifungal metabolites or cell
314 wall-degrading enzymes (Gowtham et al. 2016; Theatre et al. 2021; Saxena et al. 2020;
315 Goswami et al. 2016). In addition, the determination coefficient below 0.5 and derived from the
316 ANOVA of fungal soil population, suggests the presence of some other fungal population

317 detrimental factors, such as antifungal biomolecules or other microorganisms living on the
318 vermicompost leachates.
319 Finally, we conclude that *B. subtilis* ANT01 is effective to reduce fusarium wilt severity and has
320 the potential to be used as biocontrol agent of *F. oxysporum* in pineapple plants. Nevertheless,
321 more research is required to support and clarify the whole mode of action of these bacterial
322 biocontrol agents.

Conclusions

324 We conclude that treatments composed of *B. subtilis* ANT01 and the combination *B. subtilis*
325 ANT01 – *Rhizobium* sp. 11B decreased fusarium wilt severity in pineapple plants by 94.4% and
326 86.1%, respectively. In addition, the treatment prepared with *Rhizobium* sp. 11B alone showed a
327 reduction of 75.0%. Size (length and width) of leaves and their nutritional condition (SPAD
328 units) of the biocontrol agents-treated plants showed no statistical differences. Moreover, *B.*
329 *subtilis* ANT01 decreased by 46% the initial soil population of *F. oxysporum*, while *Rhizobium*
330 sp. 11B, *B. subtilis* ANT01 plus *Rhizobium* sp. 11B and control, showed a fungal soil population
331 reduction of 12.5%, 24.2% and 23.0%, respectively. These results make evident the potential of
332 *B. subtilis* ANT01 as biocontrol agent of the fusarium wilt of pineapple plants under field
333 conditions.

Acknowledgements

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Table 1(on next page)

Treatment design.

1 **Table 1:**

2 **Treatment design.**

Treatment	*B. subtilis* ANT01	*Rhizobium* sp.11B
1	0	0
2	10^8 CFU/mL	0
3	0	10^8 CFU/mL
4	10^8 CFU/mL	10^8 CFU/mL

3

4

Table 2(on next page)

Values of the principal effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B and their interaction on severity of fusarium wilt in pineapple plants.

1 **Table 2:**

2 **Values of the principal effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B and their interaction**

3 **on severity of fusarium wilt in pineapple plants.**

Effect	Value
Total	0.1325
B. subtilis ANT01	-0.0490
Rhizobium sp. 11B	0.0283
B. subtilis ANT01 x *Rhizobium* sp. 11B	-0.0063

4

Table 3(on next page)

Comparisons of fusarium wilt severity in pineapple plants obtained experimentally and by the principal effects linearized equation of *B. subtilis* ANT01 and *Rhizobium* sp. 11B.

1 **Table 3:**

2 **Comparisons of fusarium wilt severity in pineapple plants obtained experimentally and by**

3 **the principal effects linearized equation of *B. subtilis* ANT01 and *Rhizobium* sp. 11B.**

Treatment	*B. subtilis* ANT01	*Rhizobium* sp. 11B	Predicted	Experimental	Difference
1	-1	-1	0.1469	0.1469	0.0000
2	1	-1	0.0615	0.0615	0.0000
3	-1	1	0.2161	0.2162	-0.0001
4	1	1	0.1055	0.1054	0.0001

4

5

Table 4(on next page)

Effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B, and their interaction on leaf length and width of pineapple plants.

1 **Table 4:**

2 **Effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B, and their interaction on leaf length and**

3 **width of pineapple plants.**

Effect	Length (cm)	Width (cm)
Total	44.9372	3.5078
B. subtilis ANT01	-0.9161	-0.2633
Rhizobium sp. 11B	0.4917	0.0978
B. subtilis ANT01 x *Rhizobium* sp. 11B	0.1806	0.0000

4

Table 5(on next page)

Average values of length and width of pineapple plants obtained from the prediction equations and experimentally.

1 **Table 5:**

2 **Average values of length and width of pineapple plants obtained from the prediction**

3 **equations and experimentally.**

B. subtilis ANT01	*Rhizobium* sp. 11B	Leaf length (cm)			Leaf width (cm)		
		Predicted	Experimental	Difference	Predicted	Experimental	Difference
-1	-1	44.33	45.54	1.21	3.34	3.67	0.33
1	-1	46.35	46.16	-0.18	3.87	3.15	-0.72
-1	1	43.71	43.35	-0.36	3.15	3.87	0.72
1	1	45.18	44.69	-0.49	3.67	3.34	-0.33

4

Table 6(on next page)

Effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B, and their interaction on SPAD units of pineapple leaves, as derived from the prediction equation and experimentally.

1 **Table 6:**

2 **Effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B, and their interaction on SPAD units of**

3 **pineapple leaves, as derived from the prediction equation and experimentally.**

	Effect	Predicted	Experimental	Difference
Total	49.00	47.01	47.75	0.74
B. subtilis ANT01	-0.37	48.56	48.63	0.07
Rhizobium sp. 11B	-0.81	50.24	50.99	0.74
B. subtilis ANT01 x Rhizobium sp. 11B	0.81	49.37	48.63	-0.75

4

Table 7(on next page)

Values of the principal effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B and their interaction on *F. oxysporum* population in a soil cultivated with pineapple plants.

1 **Table 7:**

2 **Values of the principal effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B and their interaction**

3 **on *F. oxysporum* population in a soil cultivated with pineapple plants.**

Effect	Value
Total	1.7042
B. subtilis ANT01	-0.2458
Rhizobium sp. 11B	0.1542
B. subtilis ANT01 x *Rhizobium* sp. 11B	0.0375

4

Table 8(on next page)

Effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B, and their interaction on *F. oxysporum* population, as derived from the prediction equation and experimentally.

1 **Table 8:**

2 **Effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B, and their interaction on *F. oxysporum***

3 **population, as derived from the prediction equation and experimentally.**

Treatment	*B. subtilis* ANT01	*Rhizobium* sp. 11B	Predicted	Experimental	Difference
1	-1	-1	1.8333	1.8333	0.0000
2	1	-1	1.2667	1.2667	0.0000
3	-1	1	2.1045	1.0667	0.0378
4	1	1	1.5551	1.6500	-0.0949

4

5

Figure 1

Time-course evolution of fusarium wilt severity (*Fusarium oxysporum*) in pineapple treated plants (*Ananas comosus*).
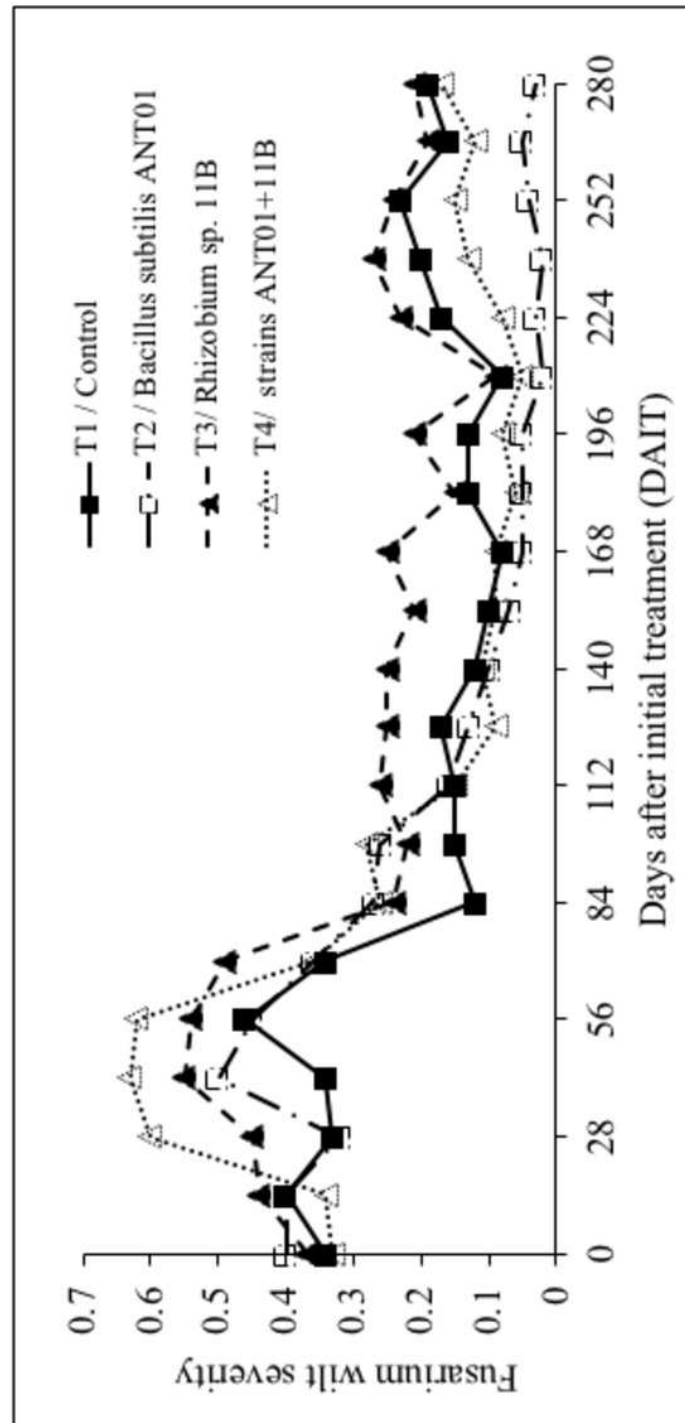
Figure 1. Time-course evolution of fusarium wilt severity (*Fusarium oxysporum*) in pineapple treated plants (*Ananas comosus*).

Figure 2

Principal effects of *B. subtilis* ANT01, *Rhizobium sp.* 11B (A) and their interaction (B) on severity of fusarium wilt during the stabilization stage of the disease.
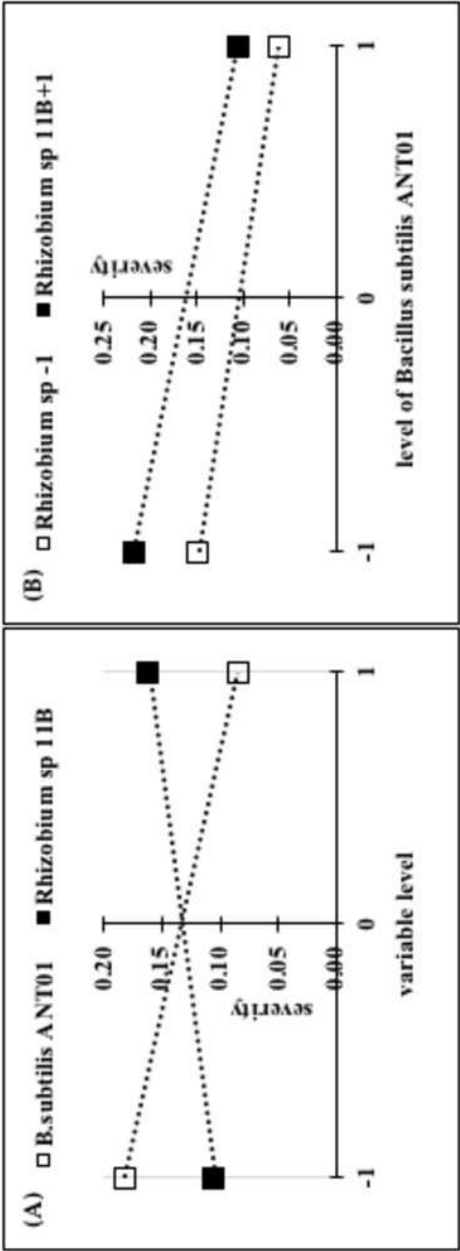
Figure 2. Principal effects of *B. subtilis* ANT01, *Rhizobium* sp. 11B (A) and their interaction (B) on severity of fusarium wilt during the stabilization stage of the disease.

Figure 3

Dynamics of estimated *F. oxysporum* populations in a soil grown with pineapple plants (*A. comosus*) treated with the bacterial strains ANT01 and 11B, alone or in combination.
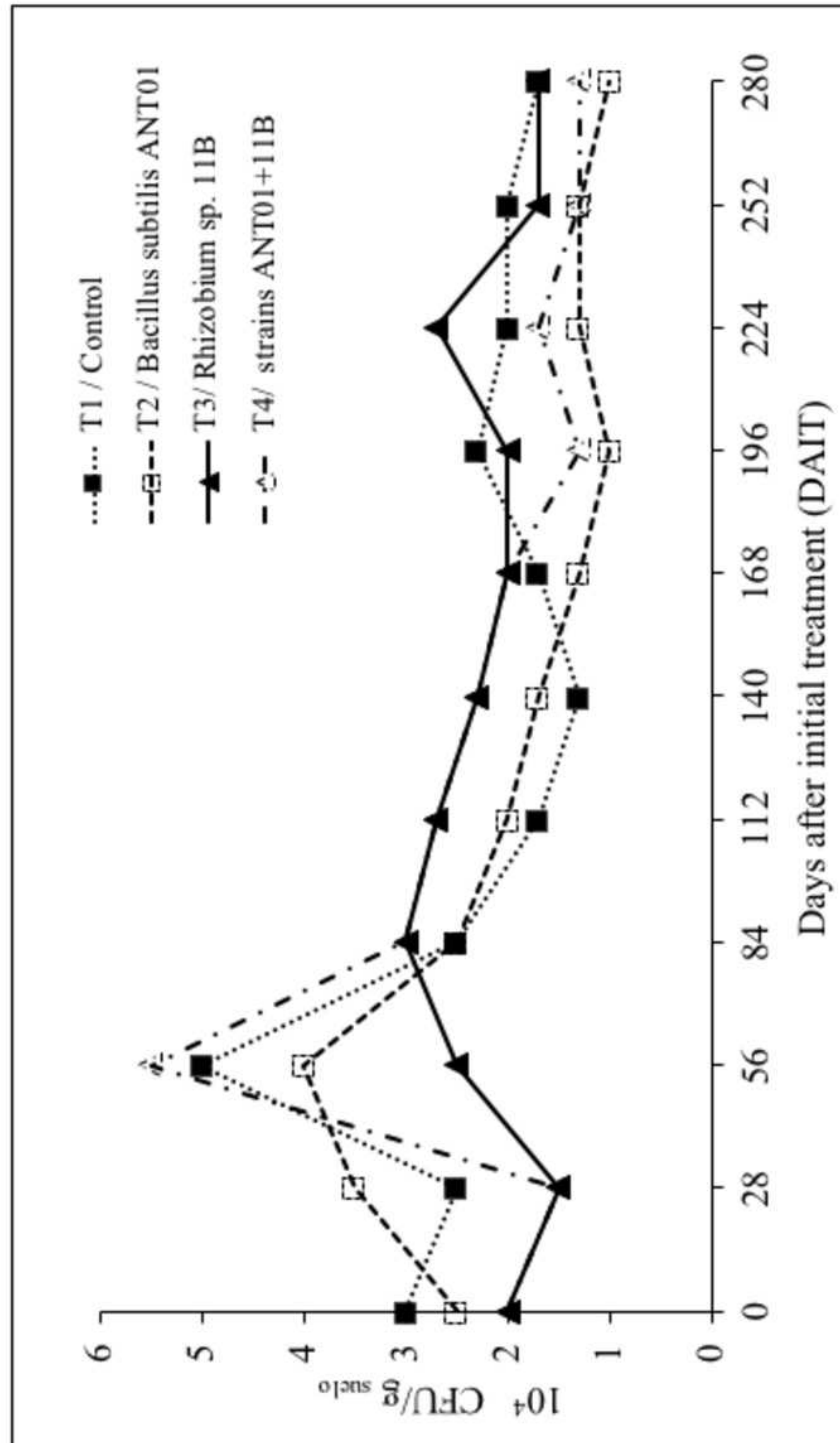
Figure 3. Dynamics of estimated *F. oxysporum* populations in a soil grown with pineapple plants (*A. comosus*) treated with the bacterial strains ANT01 and 11B, alone or in combination.

Figure 4

Principal effects of *B. subtilis* ANT01 and *Rhizobium sp* 11B (A) and their interaction (B) on *F. oxysporum* populations from a soil cultivated with pineapple plants.
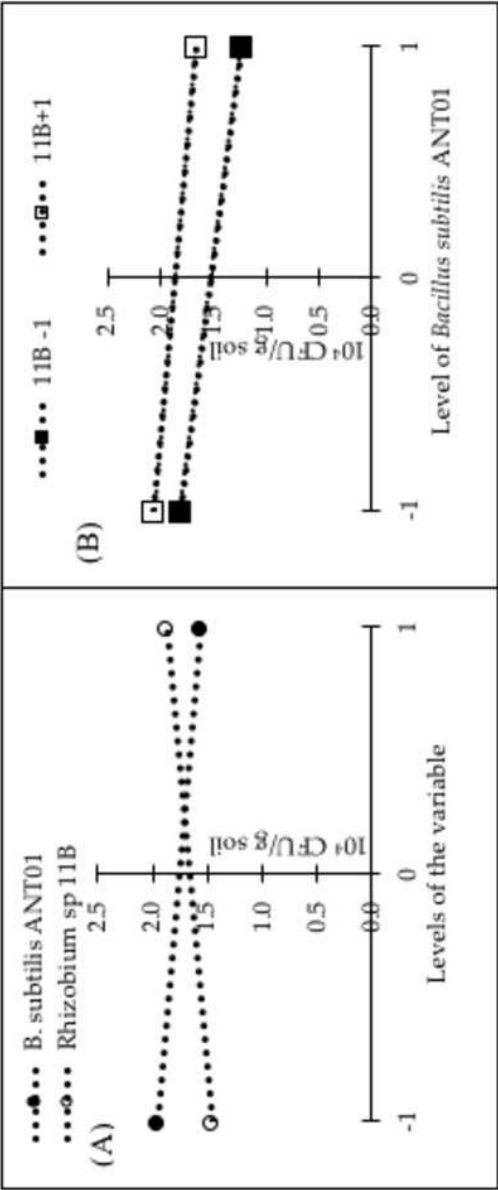
Figure 4. Principal effects of *B. subtilis* ANT01 and *Rhizobium sp.* 11B (A) and their interaction (B) on *F. oxysporum* populations from a soil cultivated with pineapple plants.