

1 **Evaluating *Bacillus* spp. As Biocontrol Agents against *Meloidogyne***
2 ***incognita* infesting Tomato**

3
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6
7 **ABSTRACT**

8 Tomato is attributed as a global host for root-knot nematode (*Meloidogyne incognita*)
9 soliciting ponderous damage. Using biocontrol agents to control plant parasitic nematodes is a
10 well-established, green approach in advance of synthetic nematicides. The role of *Bacillus* spp.
11 in inciting physiological and biochemical alterations in nematode infestation is discussed in the
12 present study. The susceptible (PKM-1) and resistant (Hisar Lalit) tomato cultivars treated with
13 *Bacillus pumilus* augmented the shoot length, root length and biomass of plants compared to
14 the standard check, *Pseudomonas fluorescens*, followed by *B. megaterium*. Accordingly, all the
15 biocontrol agent-treated susceptible plants showed reduced galling and exhibited a root gall
16 index of 3 (moderately resistant) and reduced nematode population in soil and roots. Contrarily,
17 all the resistant plants showed highly resistant reactions. *B. pumilus* showed the topmost
18 expression of all the biochemical enzymes like peroxidase (PO), polyphenol oxidase (PPO),
19 catalase (CAT), phenylalanine ammonia-lyase (PAL) and total phenols. Conclusively, *B.*
20 *pumilus* was found to be the most potential in reducing nematode infestation by embellishing
21 the plant growth and enhancing defense-related enzymes in tomatoes.

22 **KEYWORDS:** Root-knot nematode, Tomato, *Bacillus* spp., Biochemical enzymes.

23
24 **INTRODUCTION**

25 Plant parasitic nematodes (PPNs) are one of the egregious biotic stresses that are a well-
26 recognized threat to crop production and food security. PPNS are omnipresent, enabling them
27 to attack all different kinds of crop plants, field crops, and vegetable and flower crops that cause
28 annual economic losses of USD 173 billion globally (Elling *et al.*, 2013). They are also
29 responsible for multiple pathogen infection that predisposes the susceptible crops to other
30 pathogens like fungi, bacteria and thus indirectly contributing to reduced yield and crop
31 productivity (Back *et al.*, 2002).

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32 There exist more than 4000 PPNs attacking crops. Of these, the most important and prominent
33 one is the root-knot nematode (*Meloidogyne* spp.), which voraciously feeds all crops, especially
34 vegetables (Koenning *et al.*, 1999). Global vegetable production is under threat due to the
35 damage from four different species of *Meloidogyne* viz., *M. arenaria*, *M. javanica*, *M. incognita*
36 and *M. hapla*. However, *M. incognita* is one of the important pests of solanaceous crops majorly
37 in tomatoes.

38 Tomato is the second most paramount remunerable exigent solanaceous vegetable grown
39 worldwide after potato. Infestation of *M. incognita* in tomatoes impedes production and lowers
40 yield, making them vulnerable to other wilt and/or rot causing pathogens (Ogwulumba *et al.*,
41 2011). The habitual monitoring tactic employed by growers is the use of synthetic nematicides
42 which create economic and environmental constraints. Under these circumstances, substitutive
43 strategies are gaining preponderance. The current study explains one such alternative strategy
44 to combat root-knot nematode infesting tomatoes.

45 Biocontrol or biological control is an economically and environmentally well-fit tactic against
46 plant pathogens. Precisely, biocontrol of nematodes can be defined as the management of
47 nematode populations and the damage caused by them through the action of antagonists either
48 directly or indirectly by manipulating the environment favorable for nematodes (Poveda *et al.*,
49 2020) that includes many of fungi and bacterial species. The organism that shows antagonism
50 against pathogens are called biocontrol agents (BCA) where, these organisms interact with
51 pathogens through various mechanisms like competition, antibiosis and/or through inducing the
52 plant resistance against pathogens.

53 Plant resistance is one of the defensive strategies used by most of the hosts against pest and
54 pathogen attacks. Once the plant encounters the attack, the pathogen-associated molecular
55 patterns (PAMPs) will be recognized by the pattern recognition receptors (PRRs) on the cell
56 surface. Thereby it triggers a complex signaling network leading to defense responses (PAMP-
57 triggered immunity) (Ramirez-Prado *et al.*, 2018; Hou *et al.*, 2019). When it fails, these
58 biocontrol agents help the plant to trigger its innate immunity by producing microbial-
59 associated molecular patterns (MAMPs) that act as elicitors for triggering the complex defense
60 responses by the host against pathogens (Poveda *et al.*, 2020). It includes the production of
61 reactive oxygen species, hypersensitive response, production of defensive enzymes
62 (peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase, catalase, etc.), phytohormones
63 (salicylic acid, jasmonic acid and/or ethylene) and many signaling cascades.

64 In the current study, we investigated the nematicidal potential of a bacterial bioagent, *Bacillus*
65 spp. against *M. incognita* in tomato and the level of defensive enzymes across different *Bacillus*
66 spp. treated plants.

67

68 MATERIALS AND METHODS

69 The experiments were laid out in a glasshouse at AICRP (Nematodes), Zonal Agricultural
70 Research Station, GKVK, Bengaluru. Precedently, the species of *Meloidogyne* was validated as
71 *M. incognita* by using the “Perineal pattern technique” (Chitwood, 1949).

72

73 Collection of Bacterial Culture

74 Three distinct species of *Bacillus* viz., *B. subtilis* IIHR Bs-2, *B. megaterium* Bm-IIHR, *B.*
75 *pumilus* IIHR Bp-2 cultures were obtained from ICAR-Indian Institute of Horticultural
76 Research, Bengaluru whereas *Pseudomonas fluorescens* (standard check) was obtained from
77 Pathogenomics Laboratory, Department of Plant Pathology, University of Agricultural
78 Sciences, GKVK, Bengaluru.

79

80 Collection of Tomato Seeds

81 Tomato seeds of nematode susceptible variety, PKM-1 were collected from IIHR, Bengaluru
82 and tomato seeds of nematode resistant variety, Hisar Lalit (NRT 8) were collected from
83 Chaudhary Charan Singh Agricultural University, Hisar, Haryana. Tomato plants were raised
84 in trays and transplanted into pots (2 kg) filled with sterilized soil after 21 days.

85

86 Preparation of Bacterial and Nematode Suspension

87 The bacterial suspensions of respective bio-agents were prepared by growing a bacteria in 250
88 mL nutrient broth and incubating for 24 hours. The turbid broth was centrifuged at 5000 rpm
89 for 5 min and cells were resuspended with phosphate buffer (pH=7). The concentration was
90 maintained at the rate of 2.5×10^7 CFU/mL and the bacterial suspension was inoculated through
91 the seedling dip method to 25-day-old seedlings before translation for 3 hours. Here,
92 *Pseudomonas fluorescens* was used as a standard check.

93 Nematode inoculum was obtained from infected tomato roots. We extracted the nematode by
94 following Combined Cobb’s sieving and Baermann’s funnel technique (Ayoub, 1977). 50 mL
95 of nematode suspension was prepared at the rate of 20 juveniles per mL of water. After a week
96 of transplanting, the nematode suspension of 50 mL per plant with 1000 juveniles was
97 inoculated by making 4-5 holes around the seedling.

98 Nematicide-treated (carbofuran @ 1g/kg pot) plants were considered as a positive control.
99 Complete random design (CRD) was maintained in a glass house (temperature: 20°C) with
100 three replications of seven treatments. The experiment was conducted twice.

101

102 **Studies on Physiological Alterations in Tomato**

103 The efficacy of *Bacillus* spp. on the incidence and establishment of root-knot nematode in
104 tomatoes was studied through various parameters of plant growth and nematode infection under
105 pot experiments. After 45 days of inoculation, observations viz., shoot growth (cm), root
106 growth (g) and root-knot index (1-5 scale), nematode population in roots and soil were taken
107 (Narasimhamurthy *et al.*, 2017).

108 **Status of Defense-related Biochemicals in Tomato**

109 The biochemical levels in tomato-treated and untreated plants were assessed. One gram of
110 fresh root was collected from susceptible and resistant plants after 28 days of nematode
111 inoculation from each treatment for biochemical analysis. Fresh roots were washed gently in
112 running tap water and homogenized in 1 mL of 0.1 M phosphate buffer (pH 7.0) at 4 °C in a
113 pre-chilled pestle and mortar. The homogenate was centrifuged at 20,000 rpm at 4 °C for 15
114 min and the supernatant served as an enzyme source for further analysis (Anita *et al.*, 2004).

115 Here tomato plants were tested for biochemical levels of several defense-related enzymes
116 like peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL),
117 catalase (CAT) and total phenols using spectrophotometer by following standard procedures
118 (Prabhu *et al.*, 2019).

119

120 **Estimation of peroxidase (PO) activity**

121 Peroxidase activity was assayed spectrophotometrically (Chander, 1990). The reaction
122 mixture consisted of 2.5 mL of 0.05 M potassium phosphate buffer, 0.2 mL of o- phenyl diamine
123 (OPD), 0.2 mL of 0.3 per cent H₂O₂ and 0.03 mL of enzyme extract. The reaction mixture was
124 incubated at room temperature (28±1°C). The change in absorbance was recorded at 60 sec
125 intervals for 5 min. The enzyme preparation without H₂O₂ served as blank. The enzyme activity
126 was expressed as change in the absorbance at 450 nm min⁻¹ g⁻¹ on fresh weight basis.

127

128 **Estimation of polyphenol oxidase (PPO)**

129 PPO activity was determined as per the procedure given by Selvaraj and Kumar (1995). The
130 reaction mixture consisted of 2.9 mL of 0.05 M potassium phosphate buffer (pH 6.8), 0.1 mL
131 of 1.25 per cent pyrogallol and enzyme extract of 0.5 mL. The increase in absorbance was

132 measured at 450 nm up to 5 min for 1 min interval. Polyphenol oxidase activity was expressed
133 as absorbance/min/gm FW.

134

135 **Estimation of phenylalanine ammonia lyase (PAL)**

136 The PAL assay was conducted as per the method described by Whetten and Senderoff (1992).
137 0.4 mL of enzyme extract was incubated with 0.5 mL of 0.1 M borate buffer (pH 8.8) and 0.5
138 mL of 12 mM L-phenylalanine in the same buffer for 30 min at 30 °C. The reaction was arrested
139 by adding 0.5 mL of 1 M TCA and incubated at 37 °C for 5 min. The blank was prepared, that
140 contains 0.4 mL of crude enzyme extract and 2.7 mL of 0.1 M borate buffer (pH 8.8) and
141 absorbance was measured at 290 nm in spectrophotometer. Standard curve was drawn with
142 graded amounts of cinnamic acid dissolved in acetone. The enzyme activity was expressed as
143 μM of trans-cinnamic acid $\text{min}^{-1} \text{g}^{-1}$ fresh weight.

144

145 **Estimation of Catalase**

146 The catalase activity was estimated as per the procedure given by Masia (1998). The reaction
147 mixture contains 2.6 mL of 0.067 M sodium phosphate buffer (pH 7.0), 0.3 mL of 3 per cent
148 H_2O_2 and 0.1 mL of enzyme extract. The decrease in absorbance was measured at 240 nm up
149 to 5 min for 1 min time interval. Catalase activity was expressed as $\mu\text{g H}_2\text{O}_2/\text{gm FW}$.

150

151 **Estimation of Total phenol (Singleton and Rossi, 1965)**

152 Total phenol content was estimated by spectrophotometric method using Folin Ciocalteu
153 Reagent (FCR) at an absorbance of 700 nm by following procedure. Five grams of sample was
154 homogenized with 20 mL of methanol (80%) in a pestle and mortar 2-3 times and volume was
155 made to 50 mL. 0.5 mL of the extract was taken in test tubes, 0.2 mL of Folin-Ciocalteu's
156 Phenol Reagent was added followed by 3.3 mL of distilled water and mixed well. After 2 min,
157 1 mL of sodium carbonate solution was added and mixed. Allowed to stand at room temperature
158 for 30 min and blue colour was read in a spectrophotometer at 700 nm. Standard curve for
159 phenols was prepared using gallic acid (GA) as standard. The content of the total soluble phenol
160 was calculated according to a standard curve obtained from a Folin Ciocalteu reagent with a
161 phenol solution ($\text{C}_6\text{H}_5\text{OH}$) and expressed as mg gallic acid equivalent/100 g fresh weight.

162

163 **Data Analysis**

164 The data so generated were analyzed using WASP – Web Agri Stat Software Package 2.0
165 developed by ICAR. Completely Randomized Design (CRD) was used in the present study.

166 The difference between treatment means was compared with the critical difference values to
167 know a significant difference.

168

169 RESULTS

170 Physiological Transfiguration Incited by *Bacillus* spp. in Tomato

171 *Bacillus* spp. had shown its potential to embellish the shoot and root growth in relevance with
172 control treated with only nematode (Table 1). The effectuation of *Bacillus pumilus* was premier
173 among different *Bacillus* spp. and a standard check, *Pseudomonas fluorescense*.

174

175 Effects on tomato growth

176 In the case of susceptible variety, despite the fact that carbofuran-treated plants showed
177 overhead expression of shoot growth, *Bacillus pumilus*-treated plants showed the highest
178 shoot length (57.27 cm) and shoot weight (Fresh weight: 179.18 g and Dry weight: 81.72 g)
179 among different bioagents followed by *B. megaterium* (shoot length: 55.34 cm; Fresh weight:
180 175.90g and Dry weight: 77.67g). Conversely, in the case of resistant variety, regardless of
181 the bioagents, the nematode-challenged plants exhibited the highest shoot growth which was
182 comparable with healthy plants but then carbofuran-treated plants showed the least shoot
183 growth.

184 A similar trend was noticed with respect to root growth as that of shoot growth in both root-
185 knot nematode susceptible and resistant tomato varieties, respectively. In the case of susceptible
186 variety, the root growth of the bacterial bio-agents treated-*Meloidogyne incognita*-infested
187 plants showed higher values than only nematode-challenged plants, among which *B. pumilus*
188 showed maximal root length (30.73 cm) and root weight (Fresh weight: 7.67 g and Dry weight:
189 4.89 g). Even when the healthy resistant variety showed the topmost expression of root growth
190 witnessing the fact that the bioagent treatment was non-significant.

191

192 Effects on nematode infestation

193 The root-knot index was calculated by considering the number of galls produced per root
194 system (Table 2). Except for the fact that the resistant variety showed highly resistant to
195 resistant reactions with a root-knot index of 1-2, the bioagents-treated plants revealed a lesser
196 number of galls (23.33-28.33) in susceptible variety and a root-knot index of 3 imposing a
197 moderately resistant reaction. Of these, *B. pumilus* eliminated at most the production of galls
198 on the roots (Fig. 1a & 1b). Further number egg masses per root system (Table 2) and nematode
199 population was evaluated both in soil and roots. The nematode population was significantly

200 reduced in the *Bacillus* spp. treated plants compared to control showing the significance of
201 biocontrol treatment (Table 3).

202

203 **Biochemical levels after *Bacillus* spp. treatment in Tomato**

204 In this study, biochemicals like peroxidase (PO), polyphenol oxidase (PPO), catalase (CAT),
205 phenylalanine ammonia-lyase (PAL) and total phenols were analyzed in both susceptible and
206 resistant varieties. The inoculation of bio-agents to *M. incognita* infested plants showed
207 increased activity of all tested enzymes (peroxidase, polyphenol oxidase, phenylalanine lyase,
208 catalase and total phenols) over control in both susceptible and resistant varieties (Fig. 2a &
209 2b). Among bioagents, *B. pumilus* showed the topmost expression of all the enzymes. However,
210 the resistant variety showed higher enzymatic activity than the susceptible variety except for
211 the catalase enzyme.

212

213 **Studies on peroxidase (PO) activity**

214 The activity of peroxidase was assayed spectrophotometrically at 450 nm as per procedure
215 given by Chander (1990). The increased activity of peroxidase was recorded in *Meloidogyne*
216 *incognita* inoculated samples of susceptible variety along with resistant tomato variety (0.487
217 and 1.461 $\text{abs min}^{-1} \text{g}^{-1}$, respectively) compared to respective healthy plants (0.187 and 0.561
218 $\text{abs min}^{-1} \text{g}^{-1}$), but enzymatic activity was higher in resistant variety (Fig. 2aA).

219 The bacterial bio-agents treated-*Meloidogyne incognita* infested plants showed the highest
220 enzymatic activity (susceptible: 0.697-0.793 and resistant: 2.537-2.056 $\text{abs min}^{-1} \text{g}^{-1}$) compared
221 to all other treatments in susceptible as well as in resistant varieties. Among them, *Bacillus*
222 *pumilus* treated plants (susceptible: 0.793 and resistant: 2.537 $\text{abs min}^{-1} \text{g}^{-1}$) showed
223 significantly supreme activity of peroxidase, followed by *Pseudomonas fluorescens*
224 (susceptible: 0.733 and resistant: 2.482 $\text{abs min}^{-1} \text{g}^{-1}$) and *B. subtilis* (susceptible: 0.730 and
225 resistant: 2.125 $\text{abs min}^{-1} \text{g}^{-1}$).

226

227 **Studies on polyphenol oxidase estimation (PPO) activity**

228 The polyphenol oxidase activity was assayed spectrophotometrically at 450 nm by following
229 the procedure given by Selvaraj and Kumar (1995). The activity of polyphenol oxidase was
230 increased upon bacterial bio-agents treatment to *Meloidogyne incognita* inoculated susceptible
231 and resistant tomato varieties (0.076 to 0.094 and 0.171 to 0.216 $\text{abs min}^{-1} \text{g}^{-1}$, respectively)
232 compared to respective *M. incognita* infested plants (0.060 and 0.108 $\text{abs min}^{-1} \text{g}^{-1}$) however,
233 enzymatic activity was higher in resistant variety compared to susceptible variety (Fig. 2aB).

234 Among bacterial bio-agents treated-*Meloidogyne incognita* infested plants, *Bacillus pumilus*
235 treated plants showed highest activity (susceptible: 0.094 and resistant: 0.216 changes in
236 absorbance $\text{min}^{-1} \text{g}^{-1}$) and it was on par with *Pseudomonas fluorescens* (susceptible: 0.090 and
237 resistant: 0.207 changes in absorbance $\text{min}^{-1} \text{g}^{-1}$).

238

239 **Studies on phenylalanine ammonia lyase (PAL) activity**

240 The polyphenol oxidase activity was assayed according to the procedure given by Ross and
241 Senderoff (1992) spectrophotometrically at 290 nm. There was an increased enzymatic activity
242 in *Meloidogyne incognita* infested plants (susceptible: 0.337 and resistant: 0.741 μM trans-
243 cinnamic acid $\text{min}^{-1} \text{g}^{-1}$) compared to healthy plants (0.147 and 0.323 μM trans-cinnamic acid
244 $\text{min}^{-1} \text{g}^{-1}$, respectively). However, the activity was significantly higher in bacterized-*M.*
245 *incognita* infested plants.

246 The susceptible plants treated with *Bacillus pumilus* along with *Meloidogyne incognita*
247 (0.510 μM trans-cinnamic acid $\text{min}^{-1} \text{g}^{-1}$) showed higher activity of phenylalanine ammonia
248 lyase, followed by *Pseudomonas fluorescens* treated- *M. incognita* infested susceptible plants
249 (0.503 μM trans-cinnamic acid $\text{min}^{-1} \text{g}^{-1}$). Resistance plants followed a similar course (*B.*
250 *pumilus*: 1.275 and *P. fluorescens*: 1.156 μM trans-cinnamic acid $\text{min}^{-1} \text{g}^{-1}$), but they were on
251 par with each other (Fig. 2aC).

252

253 **Studies on catalase (CAT) activity**

254 The activity of catalase was assayed spectrophotometrically at 240 nm by following the
255 procedure given by Masia (1998). The increased activity of catalase was recorded in
256 *Meloidogyne incognita* inoculated samples of susceptible and resistant tomato varieties (32.01
257 and 21.340 $\mu\text{g H}_2\text{O}_2 \text{g}^{-1}$, respectively) compared to respective healthy plants (24.964 and 16.643
258 $\mu\text{g H}_2\text{O}_2 \text{g}^{-1}$), but enzymatic activity was higher in susceptible variety (Fig. 2bA). The activity
259 of catalase got significantly decreased in bacterized nematode challenged tomato varieties
260 (susceptible: 15.251- 18.614 and resistant: 9.500-11.740 $\mu\text{g H}_2\text{O}_2 \text{g}^{-1}$).

261 Among bacterial bio-agents, *Bacillus pumilus* treated plants showed least catalase activity
262 (susceptible: 15.251 and resistant: 9.500 $\mu\text{g H}_2\text{O}_2 \text{g}^{-1}$), followed by *Pseudomonas fluorescens*
263 (susceptible: 16.754 and resistant: 10.503 $\mu\text{g H}_2\text{O}_2 \text{g}^{-1}$) and both were on par and no significant
264 difference was found.

265

266

267

268

269 **Studies on total phenols**

270 Total phenols were estimated by spectrophotometric method using Folin Ciocalteu Reagent
271 (FCR) at an absorbance of 700 nm. Higher accumulation of phenol was observed in bacterial-
272 bio-agents treated plants than untreated plants. In general, the inoculation of *Meloidogyne*
273 *incognita* and bacterial-bio-agents simultaneously recorded significantly higher phenol
274 (susceptible: 65.987-68.593 and resistant: 79.184-82.311 mg gallic acid equivalent/100g fresh
275 weight) content than control with *M. incognita* inoculation (susceptible: 48.747 and resistant:
276 58.496 mg gallic acid equivalent/100g fresh weight).

277 Among the bacterial bio-agents treated-*Meloidogyne incognita* infested tomato plants, the
278 plants treated with *Bacillus pumilus* showed the highest phenol content (susceptible: 68.593
279 and resistant: 82.311 mg gallic acid equivalent/100g fresh weight) and it was on par with
280 *Pseudomonas fluorescens* (susceptible: 67.413 and resistant: 80.895 mg gallic acid
281 equivalent/100g fresh weight) (Fig. 2bB).

282

283 **DISCUSSION**

284 The influence of different *Bacillus* spp. on the physiology and biochemical status of
285 *Meloidogyne incognita*-infested tomato plants was investigated. The significant increment in
286 the shoot and root growth of tomato plants proves that the biocontrol agents do act as plant
287 growth-promoting agents. From the above observations, it is evident that bacterial bio-agents
288 have a positive effect on root and shoot growth. This may be attributed to one or more of the
289 following factors; production of phosphatases by *Bacillus* spp. facilitates the conversion of
290 insoluble phosphorus to available one for the use of plants (Abdelmoteleb and Gonzalez-
291 Mendoza, 2020), production of growth-promoting phytohormones *viz.*, indole acetic acid
292 (IAA), gibberellic acid (GA), cytokine (Calvo *et al.*, 2010), improvement of water and nutrients
293 uptake, production of antibiotic metabolites effective against soil-borne pathogens and
294 production of B-group vitamins that promote rooting capacity and affect the population of the
295 microbial community (Wu *et al.*, 2005; Rai, 2006).

296 Furthermore, the reduced galling in bacterial bio-agents treated plants might be owing to the
297 ability of the bio-agents to modify root exudates, thus hindering the feeding site recognition by
298 the nematodes in the soil (Siddiqui and Mahmood, 1999; Zhou *et al.*, 2019). Thus, affected the
299 gall formation. In addition, the bacterial bioagents produce nematicidal acids that decrease the
300 nematode population in the soil and reduce the level of infection and hence the galling of roots
301 (Iatsenko *et al.*, 2014; Lee and Kim, 2016).

302 However, when nematode-susceptible and resistant varieties are compared, the average
303 number of galls formed per root system and root-knot index were less in the case of the resistant
304 variety. These results were adjacent to the results obtained by Kumari *et al.* (2016), who made
305 a comparative study on *M. graminicola* susceptible (Pusa 1121) and resistant (Vandana)
306 cultivars of rice. It was found that after 15 dpi, all the growth parameters of nematode were
307 recorded low in the resistant variety and were significantly different from the susceptible
308 variety. However, while comparing the response of susceptible versus resistant varieties upon
309 infection with *M. incognita*, many contradictions arose may be due to nematode effects
310 (nematode biology varies with resistant and susceptible hosts), systemic hormone signaling
311 effects or tissue-specific differential expression of selected genes (Cabasan *et al.*, 2012).

312 The bio-agent activity was clearly noticed in susceptible plants infested with *M. incognita*.
313 The genetic background of the resistant variety (Hisar Lalit) contains *Mi 1.2* gene which shows
314 resistance to *M. incognita* (Reddy *et al.*, 2016) and this might be the reason for variations
315 observed in nematode infection in the resistant genotypes and it might attract nematodes
316 meagerly less than susceptible ones (Peacock, 1959). Because of the ability of a resistant variety
317 to overcome nematode infection on its own, the treatments were established to be non-
318 significant in many of the nematode growth parameters.

319 Studies on biochemical levels suggest that defense-related enzymes (peroxidase,
320 phenylalanine ammonia-lyase, polyphenol oxidase and catalase) and biomolecules (phenols)
321 get enhanced when the plants were subjected to biotic stress and abiotic stress. The above
322 observations on **biochemical studies** revealed that there was a significant accumulation of all
323 tested enzymes in bacterized tomato plants (susceptible and resistant) challenged with
324 *Meloidogyne incognita*. Among different *Bacillus* spp., *B. pumilus* treated-*M. incognita* infested
325 plants showed supreme activity of biochemicals, followed by *B. subtilis* in susceptible as well
326 as in resistant varieties compared to *Pseudomonas fluorescence* (standard check).

327 When compared to susceptible and resistant varieties, the activities of enzymes were superior
328 in the resistant variety (except catalase) probably due to avoidance of nematode invasion (Rao
329 *et al.*, 2017) which was because of its early and enhanced accumulation of enzymes in response
330 to invading pathogens. Enhanced levels of defense enzymes and phenolic content in plants
331 treated with bio-agents may contributed to resistance development, safeguarding the plants
332 without causing harm.

333 The present investigation revealed the potentiality of *Bacillus* as a bio-agent that could reduce
334 gall formation, root-knot index and nematode population, thus improving the shoot and root

335 growth under pot experiments. *Bacillus* spp. could be included in integrated disease
336 management strategies to reduce the damage caused by *M. incognita* in tomatoes. These
337 findings revealed that *B. pumilus* was the most potential *Bacillus* bio-agent in reducing *M.*
338 *incognita* infection by embellishing the plant growth and strengthening the plants by enhancing
339 defense-related enzymes, followed by *B. subtilis* and *B. megaterium*.

340

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346

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Table 1. Physiological alterations incited by Bacillus spp. in *M. incognita*-infested tomato plants.

Treatments	Susceptible variety			Resistant variety			Susceptible variety			Resistant variety		
	Shoot length (cm)	Shoot weight (g)		Shoot length (cm)	Shoot weight (g)		Root length (cm)	Root weight (g)		Root length (cm)	Root weight (g)	
		Fresh	Dry		Fresh	Dry		Fresh	Dry		Fresh	Dry
<i>M. incognita</i> + Carbofuran	60.75 (±0.08) ^b	185.25 (±0.69) ^b	86.28 (±0.29) ^b	59.71 (±0.35) ^e	163.24 (±0.28) ^f	71.86 (±0.37) ^e	33.82 (±0.36) ^b	8.90 (±0.22) ^b	5.08 (±0.08) ^{ab}	33.68 (±0.28) ^e	9.03 (±0.27) ^e	4.56 (±0.14) ^d
<i>M. incognita</i> + <i>B. pumilus</i>	57.27 (±0.51) ^c	179.18 (±0.33) ^c	81.72 (±0.33) ^c	67.65 (±0.43) ^{bc}	208.09 (±0.34) ^b	97.54 (±0.28) ^{ab}	30.73 (±0.24) ^c	7.67 (±0.27) ^c	4.89 (±0.20) ^{ab}	39.30 (±0.41) ^b	11.74 (±0.20) ^b	5.89 (±0.12) ^{abc}
<i>M. incognita</i> + <i>B. megaterium</i>	55.34 (±0.29) ^d	175.90 (±1.36) ^d	77.67 (±2.68) ^d	67.38 (±0.33) ^{cd}	206.78 (±0.31) ^c	96.37 (±0.65) ^b	29.30 (±0.33) ^d	7.29 (±0.22) ^{cd}	4.53 (±0.25) ^b	37.86 (±0.2) ^c	11.28 (±0.35) ^{bc}	5.51 (±0.33) ^{bc}
<i>M. incognita</i> + <i>B. subtilis</i>	53.63 (±0.69) ^e	171.01 (±0.89) ^f	70.89 (±0.28) ^e	66.54 (±0.3) ^d	203.19 (±0.30) ^e	92.63 (±0.53) ^d	27.39 (±0.38) ^e	6.30 (±0.24) ^{ef}	4.29 (±0.53) ^b	36.51 (±0.72) ^d	10.34 (±0.18) ^d	5.11 (±0.08) ^{cd}
<i>M. incognita</i> + <i>P. flourescens</i>	54.08 (±0.37) ^{de}	173.30 (±9.74) ^e	71.09 (±5.16) ^e	66.87 (±0.18) ^{cd}	204.51 (±0.31) ^d	94.34 (±0.70) ^c	27.82 (±0.37) ^e	6.81 (±0.26) ^{de}	4.53 (±0.32) ^b	36.03 (±0.19) ^d	10.93 (±0.32) ^{cd}	5.19 (±0.47) ^{cd}
<i>M. incognita</i> only	43.21 (±0.46) ^f	144.52 (±0.59) ^g	56.06 (±0.36) ^f	68.61 (±0.32) ^b	210.03 (±0.46) ^a	97.71 (±0.2) ^{ab}	21.57 (±0.61) ^f	5.75 (±0.02) ^f	3.36 (±0.16) ^c	40.04 (±0.22) ^{ab}	11.92 (±0.19) ^b	6.25 (±0.27) ^{ab}
Healthy control	66.48 (±0.33) ^a	200.70 (±0.52) ^a	93.75 (±0.33) ^a	69.84 (±0.22) ^a	210.34 (±0.59) ^a	98.70 (±0.32) ^a	37.36 (±0.32) ^a	10.50 (±0.31) ^a	5.50 (±0.27) ^a	40.96 (±0.199) ^a	12.83 (±0.14) ^a	6.47 (±0.29) ^a
SEm ±	0.55	0.69	0.43	0.32	0.44	0.69	0.46	0.17	0.26	0.42	0.18	0.23
CD @ 1 %	1.81	2.02	1.60	1.39	1.62	2.03	1.65	1.01	1.24	1.59	1.05	1.16

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Table 2. Effect of *Bacillus* spp. on egg masses and number of galls.

Treatments	Susceptible variety				Resistant variety			
	No. of galls per root system	Root-knot Index	No. of egg masses per root system	Per cent reduction over control	No. of galls per root system	Root-knot index	No. of egg masses per root system	Per cent reduction over control
<i>M. incognita</i> + Carbofuran	14.00 (± 0.57) ^e	3	20.67 ^e	78.16	1.67(± 0.33) ^{ab}	2	1	50
<i>M. incognita</i> + <i>B. pumilus</i>	23.33(± 0.33) ^d	3	24.33 ^d	74.30	1(± 0.57) ^b	1	1	50
<i>M. incognita</i> + <i>B. megaterium</i>	25.33(± 0.33) ^c	3	27.33 ^c	71.13	1.33(± 0.33) ^{ab}	2	1.33	33.50
<i>M. incognita</i> + <i>B. subtilis</i>	28.33(± 0.33) ^b	3	31.33 ^b	66.90	1(± 0) ^b	1	0.67	66.50
<i>M. incognita</i> + <i>P. fluorescens</i>	27.33(± 0.33) ^b	3	30.00 ^b	68.31	1(± 0) ^b	1	0.67	66.50
<i>M. incognita</i> only	103.00(± 0.57) ^a	5	94.66 ^a	-	2(± 0) ^a	2	2	-
Healthy control	0.00(± 0) ^f	1	0.00 ^f	-	0.00(± 0) ^c	1	0	-
SEm \pm	0.47		0.66		0.23		NS	
CD @ 1 %	1.67		1.98		1.18			

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Table 3. Effect of bacterial bio-agents on final nematode population.

Treatments	Susceptible variety				Resistant variety			
	Soil (200 cc)	Per cent reduction over control	Root (5g)	Per cent reduction over control	Soil (200 cc)	Per cent reduction over control	Root (5g)	Per cent reduction over control
T ₁ : <i>Meloidogyne incognita</i> + carbofuran 3G (Positive control)	253.33 ^f	35.86	50.00 ^e	64.36	15.00 ^c	16.67	5.33 ^c	33.37
T ₂ : <i>M. incognita</i> + <i>Bacillus pumilus</i>	268.33 ^e	32.06	54.33 ^d	61.28	15.67 ^a	12.94	6.67 ^{ab}	16.62
T ₃ : <i>M. incognita</i> + <i>B. megaterium</i>	269.67 ^d	31.72	57.33 ^c	59.14	16.33 ^b	9.27	7.00 ^{ab}	12.50
T ₄ : <i>M. incognita</i> + <i>B. subtilis</i>	273.33 ^b	30.80	61.33 ^b	56.29	17.67 ^{bc}	1.83	7.67 ^b	4.12
T ₅ : <i>M. incognita</i> + <i>Pseudomonas fluorescens</i>	271.67 ^c	31.22	61.67 ^b	56.05	16.00 ^{bc}	11.11	7.33 ^{ab}	8.37
T ₆ : Control with <i>M. incognita</i> inoculation	395 ^a	-	140.33 ^a	-	18.00 ^a	-	8.00 ^a	-
T ₇ : Absolute control without <i>M. incognita</i> inoculation	0 ^g	-	0 ^f	-	0 ^d	-	0 ^d	-
SEm ±	0.52		0.38		0.57		0.47	
CD @ 1 %	1.75		1.50		1.83		1.67	

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M. incognita + *Bacillus pumilus*

Control (only *M. incognita*)

(a)

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M. incognita + *Bacillus pumilus*

Control (only *M. incognita*)

(b)

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Figure 1. Effect of *Bacillus pumilus* galling of susceptible (a) and resistant (b) tomato plants infested with *Meloidogyne incognita*.

Treatments	PO (absorbance min ⁻¹ g ⁻¹ of fresh tissue)		PPO (absorbance min ⁻¹ g ⁻¹ of fresh tissue)		PAL (μM trans-cinnamic acid min ⁻¹ g ⁻¹ of fresh tissue)		CAT (μg H ₂ O ₂ g ⁻¹)		Total phenols (mg gallic acid equivalent/100g fresh weight)	
	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
T ₁ : <i>Meloidogyne incognita</i> + carbofuran 3G (Positive control)	0.377 ^d	1.074 ^d	0.05 ^c	0.092 ^{cd}	0.28 ^e	0.507 ^f	26.149 ^b	17.433 ^b	43.403 ^c	52.083 ^c
T ₂ : <i>M. incognita</i> + <i>Bacillus pumilus</i>	0.793 ^a	2.537 ^a	0.094 ^a	0.216 ^a	0.51 ^a	1.275 ^a	15.251 ^e	9.5 ^e	68.593 ^a	82.311 ^a
T ₃ : <i>M. incognita</i> + <i>B. megaterium</i>	0.697 ^b	2.056 ^b	0.076 ^{ab}	0.171 ^b	0.443 ^c	0.941 ^d	18.614 ^c	11.74 ^c	65.987 ^a	79.184 ^a
T ₄ : <i>M. incognita</i> + <i>B. subtilis</i>	0.73 ^b	2.125 ^b	0.084 ^a	0.181 ^b	0.473 ^b	1.045 ^c	17.825 ^{cd}	11.217 ^{cd}	66.71 ^a	80.052 ^a
T ₅ : <i>M. incognita</i> + <i>Pseudomonas fluorescens</i>	0.733 ^b	2.482 ^a	0.09 ^a	0.207 ^a	0.503 ^a	1.156 ^b	16.754 ^{de}	10.503 ^{de}	67.413 ^a	80.895 ^a
T ₆ : Control with <i>M. incognita</i> inoculation	0.487 ^c	1.461 ^c	0.06 ^{bc}	0.108 ^c	0.337 ^d	0.741 ^e	32.01 ^a	21.34 ^a	48.747 ^b	58.496 ^b
T ₇ : Absolute control without <i>M. incognita</i> inoculation	0.187 ^e	0.561 ^e	0.048 ^c	0.083 ^d	0.147 ^f	0.323 ^g	24.964 ^b	16.643 ^b	29.637 ^d	35.564 ^d

486 **Table 4:** Biochemical levels in bacterial bio-agents treated *Meloidogyne incognita*-infested tomato plants.

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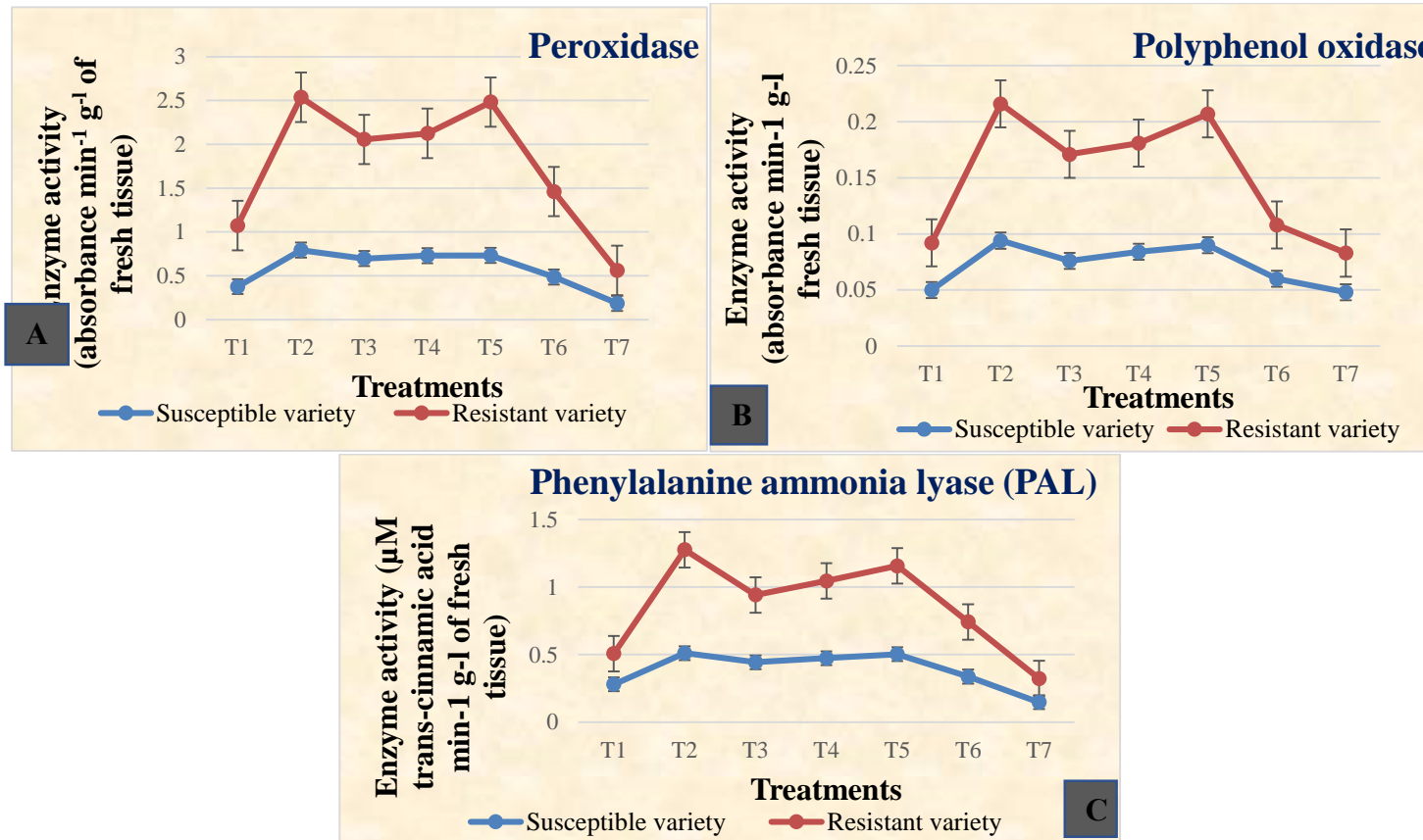
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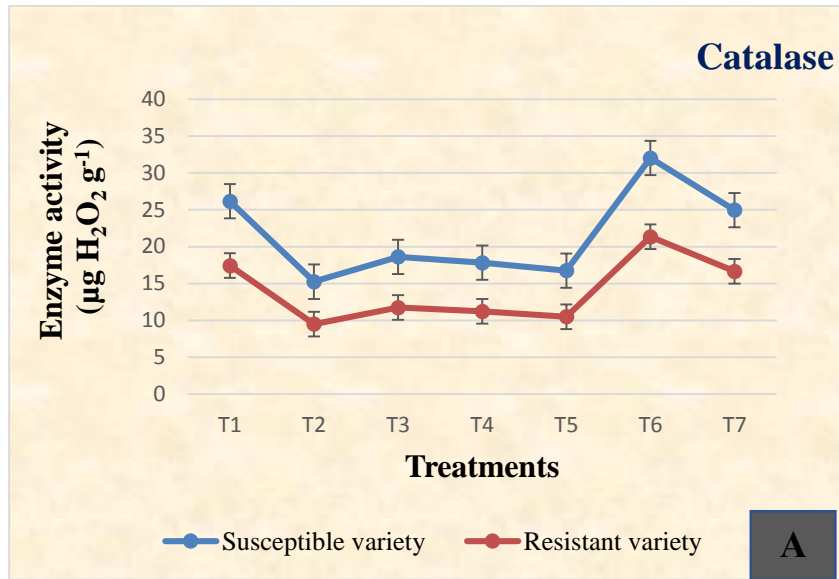
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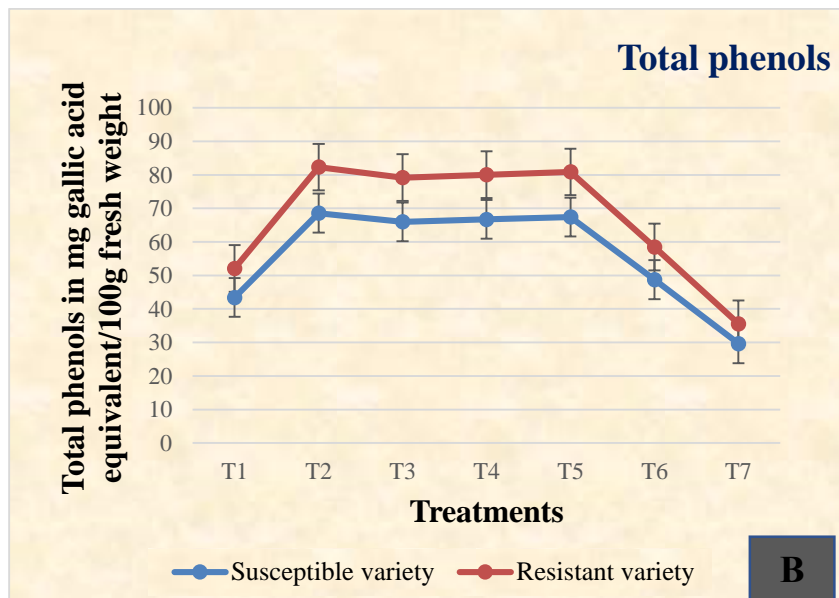
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Figure 2a. Status of biochemicals incited by bacterial bio-agents on *Meloidogyne incognita*-infested tomato plants (A) peroxidase (PO) activity, (B) polyphenol oxidase (PPO) activity and (C) phenylalanine ammonia-lyase (PAL) activity. **Note:** T₁ = *M. incognita* + carbofuran 3G, T₂ = *M. incognita* + *B. pumilus*, T₃ = *M. incognita* + *B. megaterium*, T₄ = *M. incognita* + *B. subtilis*, T₅ = *M. incognita* + *P. fluorescens*, T₆ = *M. incognita* only and T₇ = Healthy control.



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504 **Figure 2b.** Status of biochemicals incited by bacterial bio-agents on *Meloidogyne incognita*-infested tomato
 505 plants (A) catalase (CAT) activity and (B) total phenols content. **Note:** T₁ = *M. incognita* + carbofuran 3G, T₂ =
 506 *M. incognita* + *B. pumilus*, T₃ = *M. incognita* + *B. megaterium*, T₄ = *M. incognita* + *B. subtilis*, T₅ = *M. incognita*
 507 + *P. fluorescens*, T₆ = *M. incognita* only and T₇ = Healthy control.

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