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1 **Plant-associated *Pseudomonas aeruginosa* harbor multiple virulence traits essential for**
2 **mammalian infection**
3
4 Sakthivel Ambreetha[1,2], Ponnusamy Marimuthu[1], Kalai Mathee[2,3,*], and Dananjeyan
5 Balachandar[1,*]
6
7 [1]Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore,
8 Tamil Nadu, India.
9 [2]Department of Human and Molecular Genetics, Herbert Wertheim College of Medicine,
10 Florida International University, Miami, FL, USA.
11 [3]Biomolecular Sciences Institute, Florida International University, Miami, FL, USA.
12
13 ***Corresponding authors:**
14 Dananjeyan Balachandar and Kalai Mathee
15 Email address: dbalu@tnau.ac.in and matheelabfor65roses@gmail.com
16
17 **Running title**
18 Virulence profile of agricultural *P. aeruginosa* strains

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19 **Summary**

20 *Pseudomonas aeruginosa* is a leading opportunistic pathogen capable of causing fatal
21 infections in immunocompromised individuals and patients with degenerative lung
22 diseases. Agricultural soil and plants are the vast reservoirs of this dreaded pathogen.
23 However, there have been limited attempts to analyze the pathogenicity of *P.*
24 *aeruginosa* strains associated with edible vegetable plants. This study aims to elucidate
25 the virulence attributes of *P. aeruginosa* strains isolated from the rhizosphere and
26 endophytic niches of cucumber, tomato, eggplant, and chili collected from
27 agricultural fields. Virulence of the agricultural strains was compared to three previously
28 characterized clinical isolates. Our results showed that 50% of the plant-associated
29 strains formed significant levels of biofilm and exhibited swarming motility. Nearly 80% of
30 these strains produced considerable levels of rhamnolipid and exhibited at least one
31 type of lytic activity (hemolysis, proteolysis, and lipolysis). Their virulence was also
32 assessed based on their ability to suppress the growth of plant pathogens
33 (*Xanthomonas oryzae, Pythium aphanidermatum, Rhizoctonia solani*, and *Fusarium*
34 *oxysporum*) and kill a select nematode (*Caenorhabditis elegans*). The plant-associated
35 strains showed significantly higher virulence against the bacterial phytopathogen
36 whereas the clinical strains had significantly higher antagonism against the fungal
37 pathogens. In *C. elegans* slow-killing assay, the clinical strains caused 50-100% death
38 while a maximum of 40% mortality was induced by the agricultural strains. This study
39 demonstrates that some of the *P. aeruginosa* strains associated with edible plants
40 harbor multiple virulence traits. Upon infection of humans or animals, these strains may
41 evolve to be more pathogenic and pose a significant health hazard.

42

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43 **Introduction**

44 *Pseudomonas aeruginosa* is a leading opportunistic pathogen that causes hospital-
45 acquired, often fatal infections in immunocompromised individuals and patients with
46 chronic pulmonary conditions (Reynolds et al., 1975; Von Graevenitz, 1977; Rosenthal et
47 al., 2020). Additionally, this pathogen can manifest as a wide variety of infections, such
48 as folliculitis, endocarditis, osteomyelitis, and sclerokeratitis, in healthy individuals
49 (Radford et al., 2000; Tate et al., 2003; Doustdar et al., 2019). *P. aeruginosa*-associated
50 mortality is a global concern in healthcare settings, which is why this bacterium is listed
51 among the 'serious threat pathogens' (CDC AR, 2019; WHO News, 2019; PHE, 2020).
52
53 *P. aeruginosa* is a well-known soil bacterium predominantly found in agricultural
54 ecosystems (Clara, 1930; Elrod and Braun, 1942; Ali Siddiqui and Ehteshamul-Haque,
55 2001; Adesemoye and Ugoji, 2009; Mondal et al., 2012; Gao et al., 2014; Yasmin et al.,
56 2014; Radhapriya et al., 2015; Arif et al., 2016; Durairaj et al., 2017; Tiwari and Singh,
57 2017; Gupta and Buch, 2019; Chandra et al., 2020). Few studies have argued that soil
58 and plants are the primary sources for transmission of *P. aeruginosa* to humans (Green
59 et al., 1974; Cho et al., 1975). Plant-associated *P. aeruginosa* first became a significant
60 concern when its presence was detected in fresh vegetables in hospital kitchens,
61 canteens, agricultural farms, retail markets, and supermarkets (Kominos et al., 1972;
62 Wright et al., 1976; Correa et al., 1991; Viswanathan and Kaur, 2001; Curran et al., 2005;
63 Allydice-Francis and Brown, 2012; Ambreetha et al., 2021).
64
65 To date, very limited studies have demonstrated the inter-kingdom pathogenicity of *P.*
66 *aeruginosa* strains. A clinical strain, *P. aeruginosa* PA14 isolated from a hospital burn

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67 ward (Mathee, 2018), was reported to elicit extensive rotting in vegetable plants, such
68 as cucumber, lettuce, potato, and tomato (Schroth et al., 1977; Schroth et al., 2018),
69 and *Arabidopsis* (Rahme et al., 2000). *P. aeruginosa* strain BP35, isolated from a black
70 pepper plant, is cytotoxic to mammalian A549 cells (Kumar et al., 2013). Clinical strains
71 of *P. aeruginosa* release a multitude of virulence factors, such as pyocyanin,
72 rhamnolipid, elastases, proteases, lipases, hemolysin, pyochelin, and pyoverdine. These
73 assist the bacterium in establishing lethal infections (Balasubramanian et al., 2012;
74 Moradali et al., 2017). However, there is a clear gap in testing the ability of plant-
75 associated strains to produce the virulence factors required for human infection.
76
77 In our previous study, we isolated the plant-associated *P. aeruginosa* strains (PPA01-
78 PPA18) from edible vegetable plants (cucumber, tomato, chili, and eggplant) directly
79 from farms in Southern India (Ambreetha et al., 2021). We reported that those PPA
80 strains were evolutionarily related to the tested clinical isolates (ATCC10145, ATCC9027,
81 and PAO1). Both the agricultural and clinical strains had comparable plant-beneficial
82 traits, such as mineral solubilization, ammonification, extracellular release of indole-3
83 acetic acid, and siderophore. These results triggered a quest to identify the virulence
84 traits shared between agricultural and clinical *P. aeruginosa* strains. In our current
85 study, we have tested the ability of the plant-associated strains to (1) release the
86 virulence factors critical for human infection and (2) cause mortality in microbial
87 systems and the animal model *Caenorhabditis elegans*.
88

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89 **Results**

90 **Biofilm formation**

91 In clinical settings, biofilm-forming *P. aeruginosa* causes chronic pulmonary infections
92 (Römling et al., 1994; Bjarnsholt et al., 2009). We hypothesized that *P. aeruginosa* strains
93 in agricultural ecosystems can form biofilms. To test this hypothesis, biofilm formation by
94 the *P. aeruginosa* PPA strains was estimated at three time points (24, 48, and 72 h) using
95 crystal violet-microtiter assay (O'Toole, 2011) and presented as the biofilm to planktonic
96 (B:P) ratio (Fig.1). The significance of the difference among the observed values was
97 assessed using the one-way Analysis of Variance (ANOVA) and Duncan's Multiple
98 Range Test (DMRT; strains that share the same letters do not differ significantly).
99 ATCC9027, a slow biofilm former, showed low levels at 24h but gradually increased after
100 48h and 72h of incubation. The well-characterized biofilm-forming strains, ATCC10145
101 and PAO1, had a high B:P ratio at all three time points. Ten (cucumber: PPA01, PPA02,
102 PPA04; tomato: PPA05, PPA07; eggplant: PPA11, PPA12; chili: PP15, PPA16, PPA18) out of
103 the 18 PPA strains were weak biofilm producers, as evidenced by their B:P ratio of less
104 than one. The cucumber and tomato endophytes, PPA03 and PPA08, produced
105 biofilms comparable to the clinical strains, ATCC9027 and ATCC10145, respectively
106 (indicated by the shared alphabets 'b' and 'c'). Among the plant isolates, the top
107 three strains (PPA03/cucumber, PPA08/tomato, and PPA10/tomato) with a high biofilm
108 population were all endophytes.

109

110 **Swarming motility**

111 Swarming motility is associated with the upregulation of multiple virulence factors in
112 many flagellated bacteria, including *P. aeruginosa* (Overhage et al., 2008; Coleman et

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113 al., 2020b; Coleman et al., 2020a). We hypothesized that the plant-associated *P.*
114 *aeruginosa* strains could exhibit swarming motility. The ability to swarm was assessed
115 using an M9 medium with 0.5% agar (Tremblay and Déziel, 2008). The swarming
116 percentage was calculated based on triple recordings of the diameter of the bacterial
117 tendrils extended on the plate surface. A non-swarming *P. chlororaphis* strain, ZSB15,
118 was used as the negative control (Fig. 2A). The three positive controls, ATCC10145,
119 ATCC9027, and PAO1, spread tendrils that covered more than 50% of the plate's area
120 (Fig. 2B). All agricultural isolates exhibited swarming patterns at varying levels. The
121 swarming phenotype of the tomato endophyte, PPA08, was significantly higher
122 (covering 80% of the plate) than the tested positive controls, indicated by the letter 'a'
123 (Fig.2A and B). Overall, four endophytes (PPA03/cucumber; PPA08, and PPA10/tomato;
124 PPA16, and PPA18/chili), and two rhizospheric strains (PPA13, PPA14/eggplant) were the
125 superior swarmers, swarming more than 50% of plate area. The rest of the strains
126 (PPA01, PPA02, PPA04, and PPA05/cucumber; PPA06, PPAO7, and PPA09/tomato;
127 PPA11, and PPA12/eggplant; PPA15, and PPA17/chili) were weak swarmers. They
128 covered less than 50% of the plate area.
129

130 **Extracellular release of rhamnolipid**

131 Rhamnolipids are a class of metabolites predominantly released by *P. aeruginosa* to
132 infiltrate mammalian lung tissues (McClure and Schiller, 1992, 1996; Zulianello et al.,
133 2006). In plants, rhamnolipids provide protection against pests and pathogens (Kim et
134 al., 2011; Yan et al., 2015; Sancheti and Ju, 2019). In this study, the agricultural strains of
135 *P. aeruginosa* were hypothesized to produce extracellular rhamnolipids. The test strains
136 were qualitatively screened for their ability to release rhamnolipids on

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137 cetyltrimethylammonium bromide (CTAB) agar plates (Fig. 3A). All *P. aeruginosa* strains
138 formed blue halo zones around the wells, thus testing positive for rhamnolipid
139 production (Fig. 3A).
140
141 Quantitative assessment of extracellular rhamnolipids was performed using the
142 gravimetric method (Zhang and Miller, 1992; Gunther et al., 2005). Rhamnolipid levels
143 were expressed as µg/ml and the statistical significance was expressed through DMRT
144 (Fig. 3B). Two of the three clinical strains, PAO1 and ATCC10145, released a high
145 quantity of rhamnolipids. The clinical isolate ATCC9027 from otitis externa (Table 1)
146 produced comparatively low rhamnolipid levels. All eighteen plant-associated strains
147 released extracellular rhamnolipids. All strains except for the eggplant isolates (PPA11-
148 PPA14) produced more rhamnolipids than ATCC 9027 (Fig. 3B).
149
150 **Lytic activity**
151 *P. aeruginosa* lytic enzymes deteriorate pulmonic health by causing vascular
152 permeability, and organ damage (Ostroff et al., 1989; Wargo et al., 2011). We
153 hypothesized that *P. aeruginosa* strains associated with agricultural plants harbor lytic
154 activity. To confirm this, the hemolytic, proteolytic, and lipolytic activities of the strains
155 were qualitatively assessed.
156
157 **Hemolysis.** We tested the ability of the *P. aeruginosa* strains to lyse blood on sheep
158 blood agar medium (Williams and Harper, 1947). Strains that partially lysed red blood
159 cells and resulted in a green discoloration on the agar were scored positive for α-
160 hemolytic activity (Table 2). Strains that did not exhibit lytic behavior were marked as γ-

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161 hemolytic. As expected, the three control strains, ATCC10145, ATCC9027, and PAO1,
162 exhibited α-hemolytic activity. More than 50% of the agricultural isolates exhibited α-
163 hemolysis, including four rhizospheric strains (PPA02 and PPA04/cucumber; PPA13, and
164 PPA14/eggplant) and six endophytes (PPA03/cucumber; PPA07, PPA08, and
165 PPA10/tomato; PPA11/eggplant; PPA16/chili). The remaining isolates did not exhibit
166 any hemolytic activity.
167
168 **Proteolysis.** *P. aeruginosa* associated lysis of the proteins casein and gelatin was tested
169 by plate assay (Atlas, 1993; Georgescu et al., 2016) and presented as positive and
170 negative scores (Table 2). Two of the three tested controls (ATCC10145 and PAO1)
171 harbored high proteolytic activity. ATCC9027 caused mild lysis of gelatin but no lysis of
172 casein. 16 out of 18 plant-associated strains showed caseinase activity whereas only 13
173 strains had gelatinase activity. The rhizospheric strains (PPA01/cucumber and
174 PPA09/tomato) were unable to hydrolyze either protein. Three other rhizospheric strains
175 (PPA02/cucumber; PPA06/tomato; PPA12/eggplant) that displayed low caesinase
176 activity did not exhibit gelatinase activity.
177
178 **Lipolysis.** The lipid hydrolytic activity of the *P. aeruginosa* strains was tested in tributyrin
179 agar medium (Atlas, 1993; Georgescu et al., 2016). Two of three control strains,
180 ATCC10145 and PAO1, showed lipolytic behavior while ATCC9027 did not lyse the
181 tested lipid (Table 2). Most plant-associated strains did not exhibit lipolysis except for
182 three rhizospheric strains (PPA06/tomato, PPA13/eggplant, and PPA15/chili) and one
183 endophyte (PPA07/tomato).
184

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185 **Antagonism against phytopathogens**

186 Agricultural *P. aeruginosa* strains have been previously shown to inhibit other
187 phytopathogens (Ali Siddiqui and Ehteshamul-Haque, 2001; Yasmin et al., 2014; Durairaj
188 et al., 2017). This study hypothesized that both agricultural and clinical *P. aeruginosa*
189 exhibit virulence against plant pathogens. To test this hypothesis, we challenged the *P.*
190 *aeruginosa* strains with common fungal (*Pythium aphanidermatum*, *Rhizoctonia solani*,
191 and *Fusarium oxysporum*) and bacterial (*Xanthomonas oryzae*) phytopathogens
192 (Sakthivel and Gnanamanickam, 1986). This is the first known attempt to test the
193 antagonism of clinical strains against phytopathogens. Inhibition of phytopathogens
194 caused by the *P. aeruginosa* strains was normalized to PAO1 (Fig. 4).

195

196 ***Pythium aphanidermatum* inhibition**. All tested strains could inhibit *Pythium*
197 *aphanidermatum* (Fig. 4A). Ten plant-associated strains (PPA03/cucumber; PPA07,
198 PAA08, and PPA10/tomato; PPA13, and PPA14/eggplant; PPA15-PPA18/chili) and two
199 clinical strains (ATCC10145 and ATCC9027) showed higher antagonism when
200 compared to PAO1. However, only four PPA strains (PPA03/cucumber; PPA07, and
201 PPA08/tomato; PPA15/chili) were significantly more antagonistic than PAO1 ($p<0.05$,
202 DMRT). The remaining strains (7 of 18) from cucumber, tomato, and eggplant inhibited
203 *Pythium aphanidermatum* significantly less as compared to PAO1.

204

205 ***R. solani* inhibition.** All tested strains could inhibit *R. solani* (Fig. 4B). Eight plant-
206 associated strains (PPA03/cucumber; PAA08, and PPA10/tomato; PPA14/eggplant;
207 PPA15-PPA18/chili) and two clinical strains (ATCC10145 and ATCC9027) showed higher
208 antagonism when compared to PAO1. Among them, six PPA strains (PPA03/cucumber;

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209 PPA08, and PPA10/tomato; PPA15, PPA16, and PPA18/chili) significantly inhibited *R.*
210 *solani* more than PAO1 ($p<0.05$, DMRT). The other *P. aeruginosa* strains (9 of 18) from
211 cucumber, tomato, and eggplant caused significantly lower inhibition of *R. solani* than
212 PAO1.
213
214 ***F. oxysporum* inhibition.** All tested strains inhibited *F. oxysporum* (Fig. 4C). Five plant-
215 associated strains (PPA03/cucumber; PAA08, and PPA10/tomato; PPA16, and
216 PPA18/chili) and one clinical strain (ATCC10145) showed higher antagonism when
217 compared to PAO1. Among them, four PPA strains (PPA03/cucumber; PPA10/tomato;
218 PPA16 and PPA18/chili) caused significantly higher inhibition than PAO1 ($p<0.05$, DMRT).
219 The rest of the *P. aeruginosa* PPA strains (13 out of 18) from cucumber, tomato,
220 eggplant, and chili were significantly less antagonistic against *F. oxysporum* when
221 compared to PAO1.
222
223 ***X. oryze* inhibition.** All tested strains could inhibit *X. oryzae* (Fig. 4C). Most of the plant-
224 associated strains (16 out of 18) showed higher antagonism when compared to PAO1.
225 Among them, 12 PPA strains (PPA01, PPA03, and PPA04/cucumber; PPA07, PPA08, and
226 PPA10/tomato; PPA13 and PPA14/eggplant; PPA15-PPA18/chili) caused significantly
227 higher inhibition than PAO1 ($p<0.05$, DMRT). Two rhizospheric strains (PPA06, and
228 PPA09/tomato) had comparatively lower antagonism of *X. oryzae* than PAO1.
229
230 **Clustering based on antagonistic potential.** Euclidean distance-based principal
231 coordinate analysis (PCoA) (NCSS, Kaysville, USA) clustered the *P. aeruginosa* strains
232 based on their combined antagonism against the phytopathogens (Fig. 5). The clinical

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233 strains (PAO1, ATCC10145, and ATCC9027) did not cluster with the PPA strains. The PPA
234 strains formed three clusters except for a chili endophyte (PPA16) and tomato
235 rhizospheric strain (PPA09). Cluster A was occupied by three tomato isolates: one from
236 the rhizosphere niche (PPA06) and two endophytes (PPA05 and PPA07). Cluster B
237 contained five endophytes and four rhizosphere strains from all four plants. Cluster C
238 contained four strains isolated from the eggplant and cucumber. Two rhizospheric
239 strains (PPA01 and PPA02) isolated from the cucumber superimposed on each other in
240 cluster C, which reflects their identical antagonism.

241

242 **Virulence in the animal model, Caenorhabditis elegans**

243 The nematode *C. elegans* has been extensively used as a model system to understand
244 the pathogenicity of *P. aeruginosa* (Mahajan-Miklos et al., 1999; Adonizio et al., 2008).
245 In this study, we hypothesized that the agricultural *P. aeruginosa* strains were capable
246 of killing the *C. elegans* worms. This hypothesis was tested through the *C. elegans* slow
247 killing assay (Tan et al., 1999). The nematodes were scored alive or dead (Fig. 6A)
248 based on their response to physical stimuli. The percentage of living nematodes after
249 feeding on the *P. aeruginosa* strains was noted every 24 h until 120 h of incubation (Fig.
250 6B). As expected, the negative control, *E. coli* OP50, did not induce mortality in the
251 worms (Fig. 6A). However, at 120 h nearly 8% of the worms died on OP50 plates due to
252 natural death (Fig. 6B). The three positive controls, ATCC10145, ATCC9027, and PAO1,
253 caused higher mortality than the plant-associated strains. All of the nematodes fed
254 with ATCC10145 and PAO1 were dead within 72 and 120 h, respectively. In contrast,
255 only 50% of the worms died after feeding with ATCC9027. Most of the plant-associated
256 strains were less virulent against *C. elegans*. Three endophytic strains from cucumber

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257 (PPA03), tomato (PPA08), and chili (PPA18) plants caused maximum mortality of 40%.
258 Only 15% of worms died after feeding on certain rhizospheric (PPA06/tomato;
259 PPA14/eggplant) and endophytic strains (PPA05, and PPA07/tomato). These four strains
260 were the least virulent among the agricultural isolates.
261
262

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263 ## Discussion

264 In the 1970s, agricultural soil and plants were recognized as reservoirs of the
265 opportunistic pathogen *P. aeruginosa* (Green et al., 1974; Cho et al., 1975). Since, *P.*
266 *aeruginosa* has been detected in fresh agricultural produce at markets, hospital
267 kitchens, and local vendors (Kominos et al., 1972; Wright et al., 1976; Correa et al., 1991;
268 Viswanathan and Kaur, 2001; Allydice-Francis and Brown, 2012; Nithya and Babu, 2017).
269 Despite these reports, there have been minimal attempts to characterize the
270 pathogenicity of the plant-associated *P. aeruginosa* strains (Lebeda et al., 1984; Kumar
271 et al., 2013). Our previous study demonstrated that *P. aeruginosa* strains (PPA01 to
272 PPA18) present in the endophytic and rhizospheric niches of cucumber, tomato,
273 eggplant, and chili produce two virulence factors, pyocyanin, and siderophores
274 (Ambreetha et al., 2021). Our current work extends our previous findings by
275 characterizing the pathogenic phenotypes of those strains. Specifically, we assessed
276 their ability to swarm, form biofilms, produce virulence factors, and kill other microbes
277 and a select nematode.

278

279 ### Vegetable-associated *P. aeruginosa* strains harbor multiple virulence traits

280 The *P. aeruginosa* strains tested in this study harbored an arsenal of virulence attributes.
281 These include biofilm formation, swarming motility, rhamnolipid production, and lytic
282 activity (hemolysis, proteolysis, and lipolysis).

283 **Biofilm.** Three endophytic (PPA03/cucumber, PPA08/tomato, PPA10/tomato),
284 and two rhizospheric strains (PPA13/eggplant, and PPA14/eggplant) produced high
285 levels of biofilm (Fig. 1). In agricultural plants, such as soybean, mung bean, sorghum,
286 and tomato, biofilm-forming *P. aeruginosa* alleviates abiotic stress and enhances plant

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287 growth (Ali et al., 2009; Tank and Saraf, 2010; Sarma and Saikia, 2014; Kumawat et al.,
288 2019). In the clinical setting, biofilm-forming *P. aeruginosa* is a dreaded pathogen and
289 accounts for significant mortality in patients with critical pulmonary conditions (Römling
290 et al., 1994; Singh et al., 2000; Nixon et al., 2001; Bjarnsholt et al., 2009). This is the first
291 report to show that the endophytic *P. aeruginosa* strains present in cucumber (PPA03)
292 and tomato (PPA08) can form biofilms comparable to clinical strains (Fig.1).

293 **Swarming motility.** There are no previous reports on the ability of plant-
294 associated *P. aeruginosa* strains to swarm. In this study, four endophytic *P. aeruginosa*
295 strains (PPA08, PPA10/tomato; PPA16, PPA18/chili) showed extensive swarming (Fig. 2).
296 The tendril tip of the swarming bacteria possesses mobile cells that can quickly spread
297 over any surface (Tremblay and Déziel, 2010). In the murine model system, it has been
298 demonstrated that pathogenic *P. aeruginosa* swarms to disseminate in the host
299 (Coleman et al., 2020a). Previous reports on clinical strains suggested that swarming
300 motility might be associated with the expression of virulence factors (Overhage et al.,
301 2008; Coleman et al., 2020b). In our study, the four superior swarmers exhibited lytic
302 activity (α-hemolysis, proteolysis, and lipolysis) and comparatively higher antagonism
303 against phytopathogens (Fig. 2, 4; Table 2).

304 **Lytic activity.** There were no previous reports on the hemolytic, proteolytic, or
305 lipolytic capability of plant-associated *P. aeruginosa*. In this study, 10 of the 18 plant-
306 associated strains exhibited α-hemolytic activity (Table 2). Hemolysin is an extracellular
307 toxin produced by pathogenic bacteria to lyse host erythrocytes thereby facilitating
308 tissue invasion (Goebel et al., 1988). Previous studies have demonstrated that in human
309 infection, *P. aeruginosa* releases hemolysins to alter host lung physiology. This in part
310 accounts for the serious morbidity and mortality associated with this bacterium (Darby

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311 et al., 1999; Wargo et al., 2011). In addition, *P. aeruginosa* extracellular lipase and
312 protease disrupt cell membrane integrity and inactivate immune components (Heck et
313 al., 1986; Parmely et al., 1990; König et al., 1996; Barker et al., 2004; Pinna et al., 2008). In
314 our current study, 13 *P. aeruginosa* PPA strains exhibited protease activity; four of which
315 also had lipase activity (Table 2).

316 **Rhamnolipid.** Both the clinical and agricultural strains of *P. aeruginosa* studied
317 released rhamnolipid (Fig. 3). Previous clinical studies have suggested that *P.*
318 *aeruginosa* rhamnolipids alter the respiratory epithelium facilitating lung infiltration
319 (McClure and Schiller, 1996; Zulianello et al., 2006). However, in the agricultural
320 ecosystem, rhamnolipids produced from *P. aeruginosa* protect the host plant against
321 fungal pathogens (Oomycetes, Ascomycota, and Zygomycetes) and green peach
322 aphid (Kim et al., 2000; Kim et al., 2011; Yan et al., 2015; Sancheti and Ju, 2019). In the
323 current study, we have observed that the clinical strains PAO1 and ATCC10145 are the
324 superior rhamnolipid producers (Fig, 3B). ATCC9027 has been previously reported as a
325 low rhamnolipid producer (Grosso-Becerra et al., 2016). In this study, 50% of the PPA
326 strains had higher levels when compared to ATCC9027.

327

328 ***P. aeruginosa* exhibits antagonism against phytopathogens**

329 *Pythium aphanidermatum, R. solani*, and *F. oxysporum* are globally distributed fungal
330 pathogens that cause rotting, blight, and wilt, respectively, in many plant species
331 (Parmeter, 1970; Martin and Loper, 1999; Michielse and Rep, 2009; Lodhi et al., 2013). *X.*
332 *oryzae* is a devastating rice pathogen that causes bacterial leaf blight (Swings et al.,
333 1990). Previous reports have described that *P. aeruginosa* in agricultural ecosystems
334 indirectly contributes to plant growth by inhibiting these harmful pathogens (Ali Siddiqui

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335 and Ehteshamul-Haque, 2001; Yasmin et al., 2014; Durairaj et al., 2017). The three
336 control isolates of human origin, PAO1, ATCC10145, and ATCC9027, have never
337 previously been tested for their ability to inhibit phytopathogens. Our current work
338 demonstrates that both clinical and agricultural *P. aeruginosa* strains antagonize the
339 tested fungal and bacterial phytopathogens (Fig. 4 and 5). This is unsurprising
340 considering the number of virulence factors harbored by these strains (Fig. 1 to 3; Table
341 2). The secondary metabolites, pyocyanin and rhamnolipid, are implicated as the
342 major determinants of *P. aeruginosa* antagonism (Kim et al., 2011; Sudhakar et al., 2015;
343 Mahmoud et al., 2016; Chen et al., 2017; DeBritto et al., 2020). The strains tested in this
344 study produced both pyocyanin and rhamnolipids (Fig. 3; Ambreetha et al., 2021)
345 which might have contributed to anti-microbial virulence (Fig.4 and 5). Compared to
346 PAO1, nearly 90% of the plant-associated strains had higher antagonism against the
347 bacterial pathogen *(*Fig. 4D). In fungal system, the clinical strains had significantly
348 higher virulence than most of the plant-associated strains (Fig. 4A to C). We suggest
349 using the phytopathogenic fungi as a simple eukaryotic model system to test *P.*
350 *aeruginosa* pathogenicity.
351

352 **Vegetable-associated *P. aeruginosa* induces mortality in *C. elegans***

353 The pathogenicity of *P. aeruginosa* in mammals is often assessed based on its lethality
354 against *C. elegans* (Mahajan-Miklos et al., 1999; Tan et al., 1999). Virulent strains of *P.*
355 *aeruginosa* accumulate in the nematode's gut and slowly cause death (Tan et al.,
356 1999; Kirienko et al., 2014). However, the non-pathogenic bacteria do not hinder the
357 growth and development of *C. elegans* (Andrew and Nicholas, 1976). In this
358 investigation, nematode mortality caused by the agricultural strains was considerably

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359 lower when compared to the clinical isolates. The most virulent agricultural strains
360 (PPA03/cucumber; PPA08, and PPA10/tomato; PPA13, and PPA14/eggplant; PPA16,
361 and PPA18/chili) induced mortality in 30-40% of the nematode population (Fig. 6).
362 Despite the multiple virulence factors observed in the plant-associated strains, the
363 mortality of *C. elegans* was higher (50-100%) when fed with clinical strains. The reduced
364 virulence of the plant-associated *P. aeruginosa* strains suggests that the clinical isolates
365 might have evolved to be more pathogenic to survive within the eukaryotic system.
366 Pathoadaptive assays would reveal if these plant-associated strains can evolve into a
367 more pathogenic form under the right conditions.

368

369 **Conclusion**

370 To date, many studies have characterized the destructive virulence factors of human-
371 associated, animal-associated, and environmental *P. aeruginosa* strains (Jaffar-Bandjee
372 et al., 1995; Alonso et al., 1999; Vives-Flórez and Garnica, 2006; Zulianello et al., 2006;
373 Balasubramanian et al., 2012; Hall et al., 2016; Moradali et al., 2017; Ruiz-Roldán et al.,
374 2020). However, limited studies have demonstrated the ability of agricultural *P.*
375 *aeruginosa* strains to infect animals and humans (Lebeda et al., 1984; Kumar et al.,
376 2013). In this investigation, we have shown the presence of extremely virulent and lowly
377 virulent *P. aeruginosa* strains in the rhizospheric and endophytic niches of four
378 vegetables (cucumber, tomato, eggplant, and chili). Virulence was not correlated
379 with the respective niche. The less virulent strains may be long-time soil dwellers and the
380 extensively virulent strains might be human- or animal-adapted ones that got recently
381 introduced into the agricultural ecosystem. These virulent strains may have entered the
382 agricultural ecosystem through animal excreta or irrigation water with run-offs from

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383 nearby sewage systems (Wheater et al., 1980; Mavrodi et al., 2012; Slekovec et al., 2012;
384 Orlofsky et al., 2016). Comparative genomic analyses will reveal the molecular
385 adaptations contributing to the variation(s) among the agricultural strains. In the future,
386 the pathoadaptive ability of the avirulent strains should be tested to find out if they
387 could evolve into pathogens under selective conditions. Overall, this study reveals that
388 agricultural plants harvested directly from soil could be a potential source for
389 transmission of *P. aeruginosa* to humans. Farmworkers and consumers face risk of *P.*
390 *aeruginosa* related infections, which are lethal in vulnerable individuals. To the best of
391 our knowledge, this study is the first comprehensive attempt to show that *P. aeruginosa*
392 strains residing within the internal tissues and rhizosphere of edible vegetables harbor
393 multiple virulence factors critical for human infection.
394

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395 **Experimental Procedures**

396 **Bacterial strains and culture conditions**

397 Plant-associated *P. aeruginosa* strains isolated and characterized in our previous study
398 were used as test strains (Ambreetha et al., 2021). Clinical strains of *P. aeruginosa*,
399 PAO1, ATCC10145, and ATCC9027 were used as controls (Table 1). All *P. aeruginosa*
400 strains were periodically sub-cultured and grown in Pseudomonas agar (for pyocyanin)
401 medium (PAP, Himedia) at 37°C. A plant pathogenic bacterium*, Xanthomonas oryzae,*
402 was cultured in a nutrient agar medium at 37°C. Plant pathogenic fungi, *Pythium*
403 *aphanidermatum, Rhizoctonia solani, and Fusarium oxysporum* were cultured in potato
404 dextrose agar medium at 37°C.

405

406 **Nematode strain and culture conditions**

407 *Caenorhabditis elegans* N2 hermaphrodite strain was used in this study (Brenner, 1974).
408 The worms were periodically cultured in nematode growth medium (NGM), overlaid
409 with *Escherichia coli* strain OP50, and maintained at 20°C (Brenner, 1974).

410

411 **Biofilm production**

412 *In vitro* biofilm production by the *P. aeruginosa* strains was quantified using microtiter
413 assay (O'Toole, 2011). Overnight cultures of the *P. aeruginosa* strains (25 µl, OD_{660}~0.5)
414 were inoculated into microtitre wells containing 225 µl of LB broth. Three sets of
415 microtitre plates were inoculated and incubated for three different time intervals (24,
416 48, and 72 hours). After the incubation period, planktonic cells were transferred to a
417 new microtitre plate and A_{660} was measured (Spectramax® i3x, USA). Biofilms stuck to the
418 plates were washed twice with sterile H_2O and flushed with 0.1 % of crystal violet. The

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419 plates were incubated for 10-15 minutes at room temperature and gently washed
420 twice with sterile H_2O. The stained plates were allowed to dry overnight at room
421 temperature. The next day, 30% acetic acid was added to the well to dissolve the
422 biofilms, and absorbance was measured @ 550 nm. The ratio between the biofilm and
423 planktonic populations was determined at three time points (24, 48, and 72 h; O'Toole,
424 2011). The experiment was repeated thrice and the results were represented as the
425 biofilm to planktonic ratio.

426

427 **Swarming motility**

428 Swarming motility of the *P. aeruginosa* strains was assessed by adding 10 µl of 24 h-old
429 test strains (OD_{660} ~ 0.5) on modified M9 plates with 0.5% agar (Tremblay and Déziel,
430 2008). The diameter of the bacterial tendrils extended on the plates due to swarming
431 was measured, and the percentage of plates swarmed within 48 h of incubation was
432 estimated (Tremblay and Déziel, 2008). The experiment was repeated thrice and three
433 different diameters were measured every time. Results were represented as the
434 percentage of the plate area swarmed in 48 h.

435

436 **Rhamnolipid**

437 **Qualitative assay.** A CTAB agar test was done to qualitatively assess the *P. aeruginosa*
438 strains for rhamnolipid production (Siegmund and Wagner, 1991). In brief, the culture
439 supernatants of the test strains were filtered using 0.45 µm filters. Ten microliters of the
440 cell-free extracts were added to 0.2 cm wells on CTAB-methylene blue agar plates and
441 incubated at 37°C for 24 h. If rhamnolipid (anionic surfactant) was present in the
442 supernatant, it reacted with the CTAB (cationic surfactant), resulting in an insoluble

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443 complex. The strains were scored positive based on the formation of a dark blue
444 precipitated zone around the culture wells. The experiment was repeated thrice to
445 confirm result consistency.
446
447 **Quantitative assay.** The strains were grown in phosphate limited protease peptone
448 ammonium salts (PPGAS) broth supplemented with 2% (v/v) sunflower oil at 37°C to
449 induce rhamnolipid production for seven days (Zhang and Miller, 1992). We used the
450 chloroform-methanol extraction method for rhamnolipid separation (Zhang and Miller,
451 1992). In brief, the cell-free culture supernatant was acidified to pH2 with 12 M
452 hydrochloric acid. The lipids were extracted using a chloroform-methanol (2:1) mixture
453 and concentrated through evaporation. Concentrated rhamnolipids were
454 gravimetrically quantified (Gunther et al., 2005). The experiment was repeated thrice
455 and results were presented as µg/ml of the culture supernatant.
456
457 **Lytic activity**
458 **Hemolysis**. The ability of the *P. aeruginosa* strains to lyse blood cells was assessed by
459 streaking the overnight cultures (OD_{660} ~ 0.5) on nutrient agar plates containing 5%
460 sheep blood (Williams and Harper, 1947). The plates were incubated for 24 h at 37°C.
461 Green discoloration of the blood with a mild halo zone was noted as α-hemolysis, and
462 the absence of lytic activity was noted as γ-hemolysis. The experiment was repeated
463 thrice for consistency.
464
465 **Lipolysis and proteolysis.** The lipolytic activity of the *P. aeruginosa* strains was assayed
466 using 1% tributyrin as a substrate (Atlas, 1993; Georgescu et al., 2016). Strains were

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467 considered positive for lipolytic activity if an opaque precipitate formed around the
468 bacterial colonies. The proteolytic behavior of the strains was assayed using 3% skim
469 milk and 3% gelatin (1;1 ratio) as the substrates (caseinase, and gelatinase activity,
470 respectively). Formation of halo zones around the colonies were indicative of casein
471 proteolysis and gelatin hydrolysis (Atlas, 1993; Georgescu et al., 2016). The strains were
472 scored based on the intensity of lysis (mild lysis, heavy lysis, or no lysis). The experiment
473 was repeated thrice for consistency.
474
475 **Antagonistic activity**
476 **Antifungal antagonism.** The antagonistic potential of the *P. aeruginosa* strains against
477 three phytopathogenic fungi (*Pythium aphanidermatum, R. solani*, and *F. oxysporum*)
478 was assessed by dual-culture assay (Sakthivel and Gnanamanickam, 1986). In brief,
479 fungal discs were placed on one corner of potato dextrose agar medium in 90 mm
480 Petri plates. *P. aeruginosa* strains were streaked 3 cm away from the fungal disc. Plates
481 were incubated @ 37°C for seven days. Inhibition in mycelial growth, as influenced by
482 the *P. aeruginosa* strains, was recorded. The percentage inhibition was estimated
483 based on the standard formula, $\frac{Dc-Dt}{Dc} \times 100$, where Dc is the diameter of the fungal
484 mycelium in the control plate and Dt is the diameter of the fungal mycelium as
485 influenced by the test strains (Riungu et al., 2008). The experiment was repeated thrice
486 for consistency.
487
488 **Antibacterial antagonism**. Antibacterial effect of the test strains against *Xanthomonas*
489 *oryzae* pv. *oryzae* was estimated by cross streak assay (Lertcanawanichakul and
490 Sawangnop, 2011). In brief, *P. aeruginosa* strains were streaked at the center of nutrient

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491 agar plates and incubated for 24 hrs. After 24 hours, *X. oryzae* was streaked
492 perpendicular to the central streak and the plates were incubated for another 24 hrs
493 @37°C. Inability of the target pathogen to grow in the confluence area was recorded
494 after incubation. The percentage inhibition of *X. oryzae,* as influenced by the *P.*
495 *aeruginosa* strains, was calculated based on the standard formula, $\frac{Lc-Lt}{Lc} \times 100$, where Lc
496 is the length of the *X. oryzae* grown in the control plate and Lt is the length of the *X.*
497 *oryzae* as influenced by the test strains (Lo Giudice et al., 2007). The experiment was
498 repeated thrice for consistency.
499

500 **C. *elegans* killing assay**

501 The ability of the *P. aeruginosa* strains to induce death in *C. elegans* was demonstrated
502 via a slow-killing assay (Tan et al., 1999). *C. elegans* gravid adults were treated with 1N
503 NaOH and 5% sodium hypochlorite (1:1) solution (Brenner, 1974). The eggs were
504 allowed to hatch in M9 buffer, and 24 h later the emerged L1-worms were released
505 over a lawn of *E. coli* OP50 (Brenner, 1974; Adonizio et al., 2008). These synchronized L1-
506 worms were grown up to the L4-stage. We prepared slow-killing plates with NGM (Tan
507 et al., 1999) seeded with overnight cultures (OD_{660} ~ 0.5) of OP5O, PPA strains, and
508 clinical strains of *P. aeruginosa.* The plates were incubated at 37°C for 24 h. The L4-
509 worms (20 per plate) were introduced into these plates and incubated at 20°C
510 (Brenner, 1974). The viability of the nematodes, as influenced by the tested bacterial
511 strains, was recorded every 24 h for five consecutive days. Worms that did not respond
512 to physical stimuli were scored as dead. The death of worms on the OP50 plate was
513 scored as natural mortality (negative control). The experiment was repeated thrice for
514 consistency.

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515 **Statistics and reproducibility**

516 All experiments were performed in triplicates. All data were subjected to a one-way
517 analysis of variance (ANOVA) with a P-value of 0.05, and Duncan's multiple range test
518 was performed between individual means to reveal any significant difference (XLSTAT,
519 version 2010.5.05 add-in with Windows Excel). Principal coordinate analysis (PCoA)
520 based on Euclidean distance was carried out using NCSS 2020 statistical software
521 (NCSS, Kaysville, USA) to cluster the *P. aeruginosa* strains based on their antagonism
522 against phytopathogens. Data analysis and scientific graphing were done in OriginPro
523 version 8.5 (OriginLab®, USA).

524

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534 providing the *C. elegans* nematode.

535

536 **Author Contributions**

537 The experiments were conceived and designed by SA and DB. The samples were
538 processed by SA and PM. The experiments were performed by SA. Critical analyses of

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539 the data were done by SA, KM, and DB. The manuscript was prepared by SA and KM.
540 Finally, all authors were involved in the critical review of this paper.
541
542 **Ethical Approval -** There were no human or animal subjects involved in this study.
543
544 **Conflict of Interest -** The authors declare no conflict of interest.
545
546 **Data Availability**
547 All sequence data generated in this study were deposited in NCBI GenBank (Accession
548 no. MT734694 to MT734711).
549

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Table 1. Microbial strains used in this study

Microorganism	Source	Infection/Niche	References
***Pseudomonas aeruginosa* (reference strains)**			
PAO1	Human	Wound infection	Holloway, (1955)
ATCC9027	Human	Otitis externa	Haynes, (1951)
ATCC10145	Human	Unknown	Picard et al. (1994)
Plant-associated *P. aeruginosa strains*			
PPA01	Cucumber	Rhizosphere	Ambreetha et al. (2021)
PPA02	Cucumber	Rhizosphere	Ambreetha et al. (2021)
PPA03	Cucumber	Endophyte	Ambreetha et al. (2021)
PPA04	Cucumber	Rhizosphere	Ambreetha et al. (2021)
PPA05	Tomato	Endophyte	Ambreetha et al. (2021)
PPA06	Tomato	Rhizosphere	Ambreetha et al. (2021)
PPA07	Tomato	Endophyte	Ambreetha et al. (2021)
PPA08	Tomato	Endophyte	Ambreetha et al. (2021)
PPA09	Tomato	Rhizosphere	Ambreetha et al. (2021)
PPA10	Tomato	Endophyte	Ambreetha et al. (2021)
PPA11	Eggplant	Endophyte	Ambreetha et al. (2021)
PPA12	Eggplant	Rhizosphere	Ambreetha et al. (2021)
PPA13	Eggplant	Rhizosphere	Ambreetha et al. (2021)
PPA14	Eggplant	Rhizosphere	Ambreetha et al. (2021)
PPA15	Chili	Rhizosphere	Ambreetha et al. (2021)
PPA16	Chili	Endophyte	Ambreetha et al. (2021)
PPA17	Chili	Endophyte	Ambreetha et al. (2021)
PPA18	Chili	Endophyte	Ambreetha et al. (2021)
Phytopathogens			
Xanthomonas oryzae	-	-	Unpublished
Pythium aphanidermatum	-	-	Unpublished
Rhizoctonia solani	-	-	Unpublished
Fusarium oxysporum	-	-	Unpublished

Table 2. Lytic behavior of *P. aeruginosa* strains

Strains	Hemolysis		Proteolysis		Lipolysis
	γ-hemolysis	α-hemolysis	Casein	Gelatin	Lipid
PPA01	+	-	-	-	-
PPA02	-	++	+	-	-
PPA03	-	++	++	++	-
PPA04	-	++	+	++	-
PPA05	-	-	+	++	-
PPA06	+	-	+	-	+
PPA07	-	++	++	++	+
PPA08	-	++	++	++	-
PPA09	+	-	-	-	-
PPA10	-	++	++	++	-
PPA11	-	++	+	++	-
PPA12	+	-	+	-	-
PPA13	-	++	++	++	+
PPA14	-	++	++	++	-
PPA15	+	-	++	++	+
PPA16	-	++	++	++	-
PPA17	+	-	++	++	-
PPA18	+	-	++	++	-
ATCC10145	-	++	++	++	+
ATCC9027	-	++	-	+	-
PAO1	-	++	++	++	++

γ-hemolysis – no lysis of blood cells; α-hemolysis - partial destruction of blood cells; '+'- mild lysis; '++' – extensive lysis; '-' – no lysis

Fig. 1

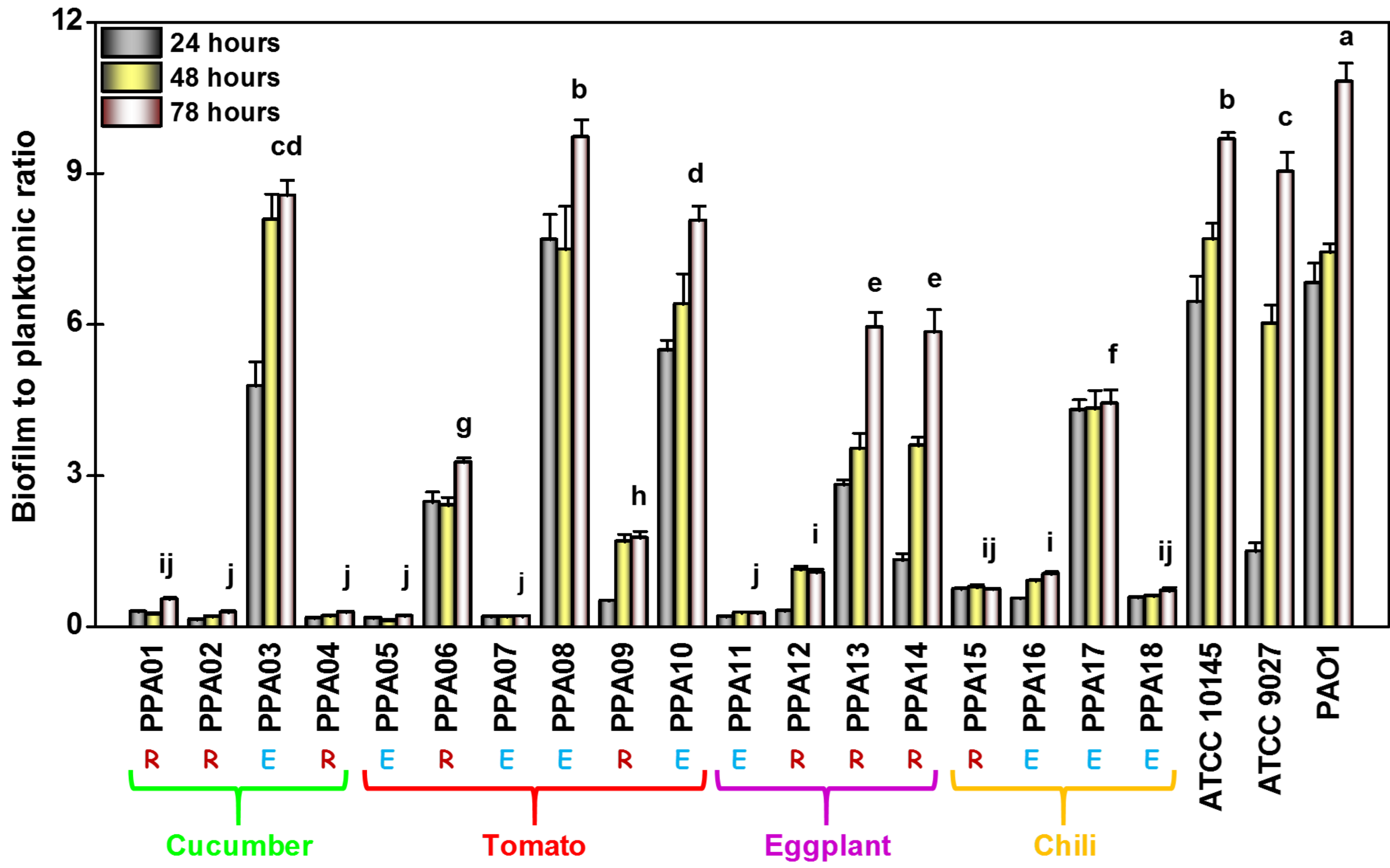

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Fig. 2

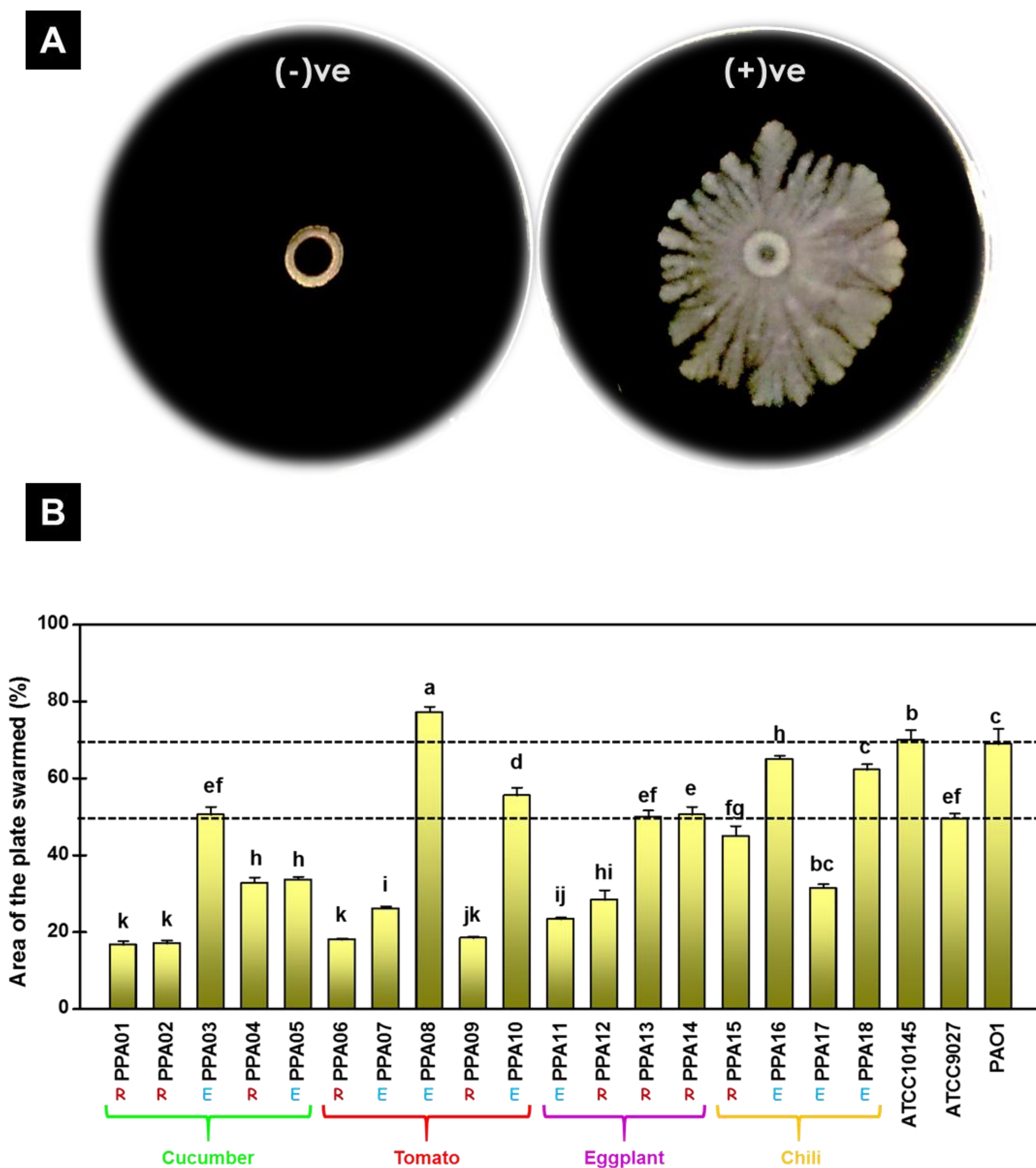

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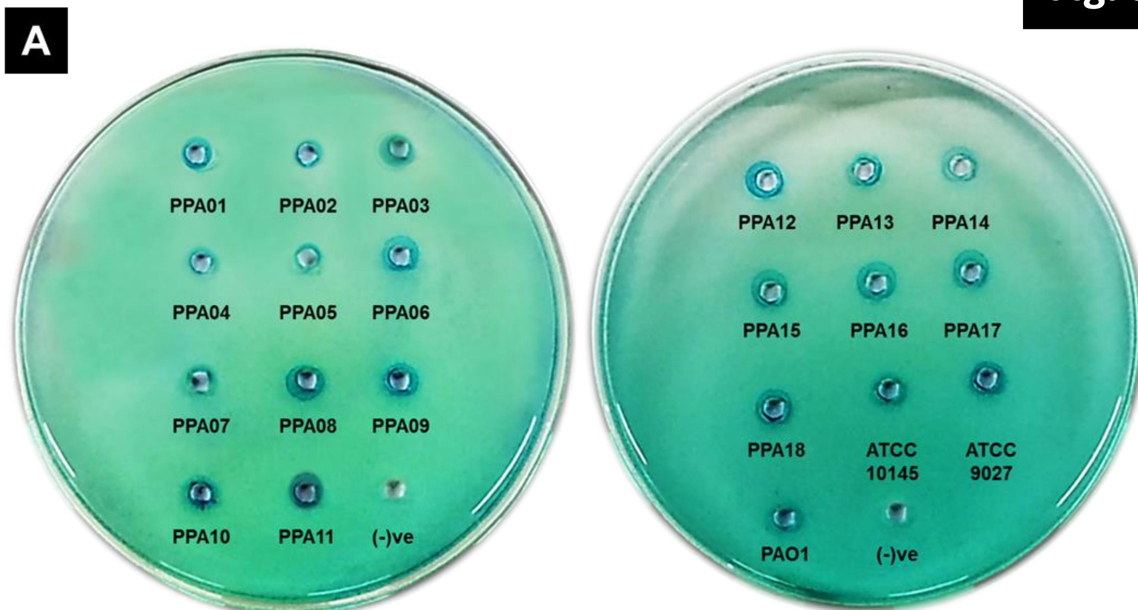
Fig. 3



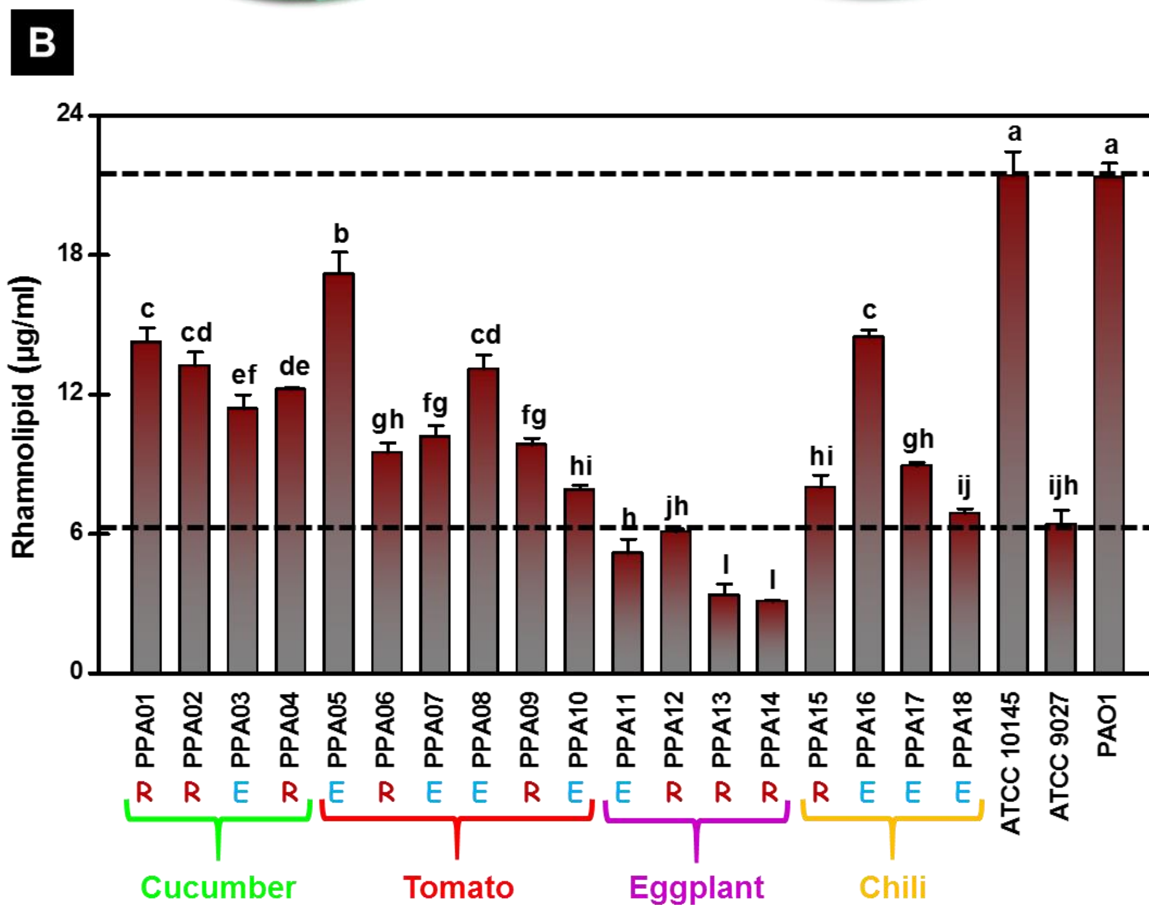


Fig. 4

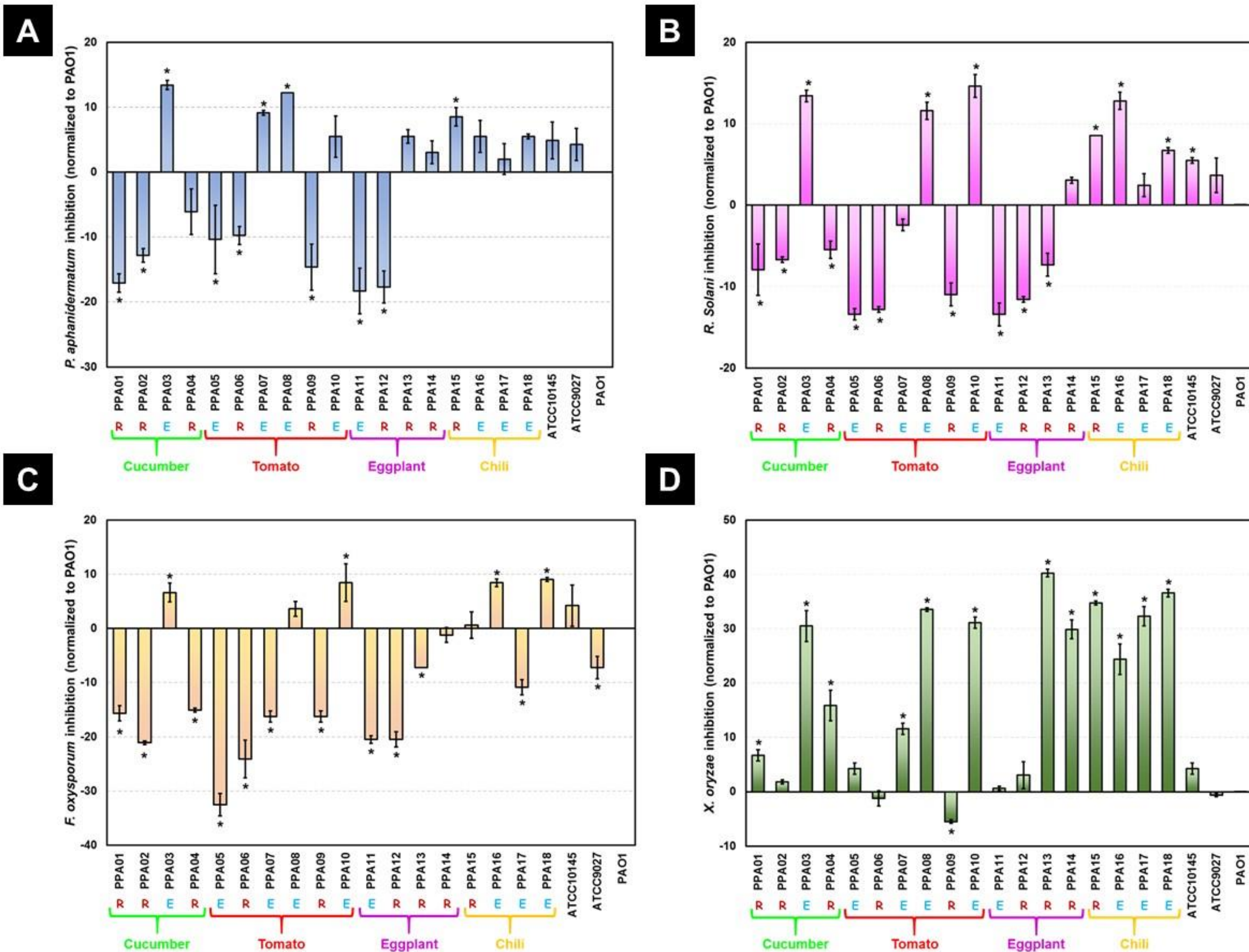


Fig. 5

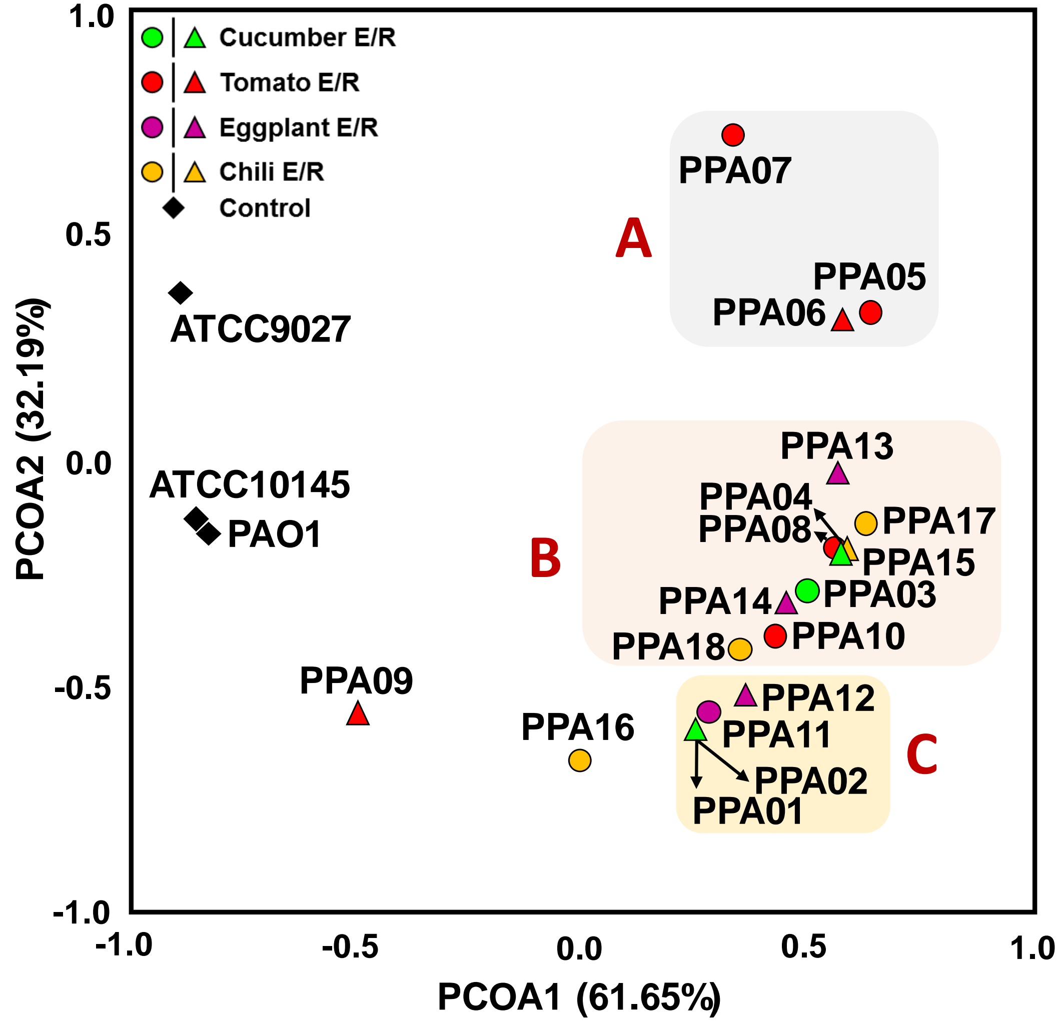


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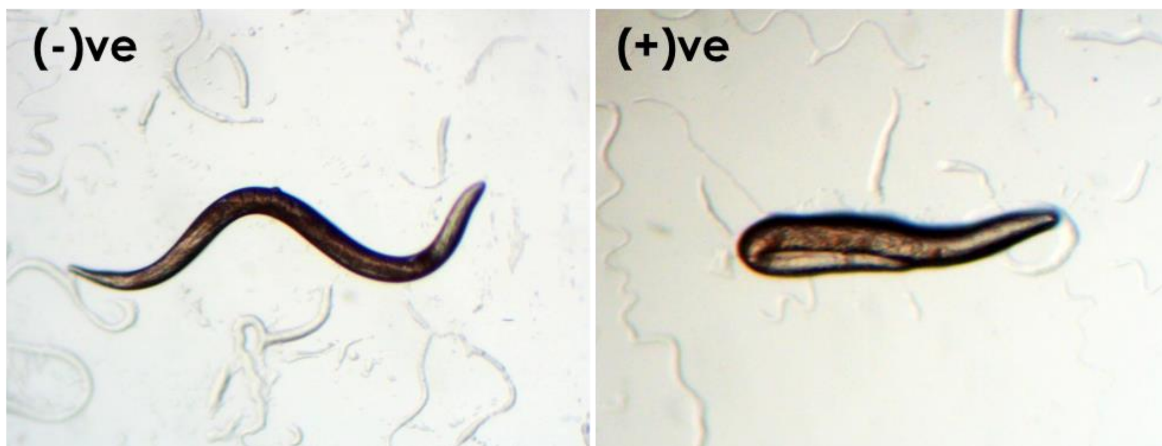
Fig. 6

A



B

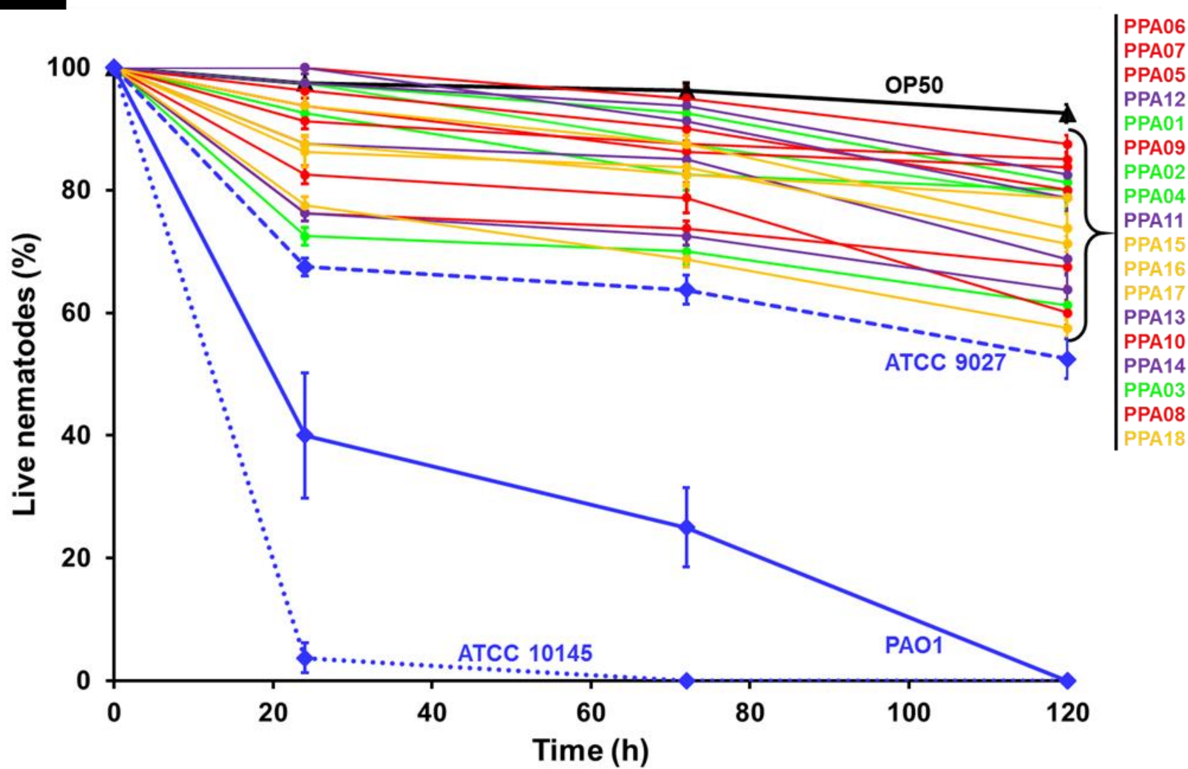


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Figure legends

Fig. 1. Biofilm production by *P. aeruginosa* strains. The graph represents the biofilm to the planktonic ratio of *P. aeruginosa* strains recorded after 24, 48, and 72 h of incubation. Values plotted are the mean of six replicates with standard errors and letters above the bars indicating the ranking of the strains (significant differences, $p < 0.05$) based on Duncan's multiple range test (DMRT). The strains are color-coded based on their plant source: cucumber (green), tomato (red), eggplant (purple), and chili (yellow). The clinical isolates, ATCC10145, ATCC9027, and PAO1 are positive controls. R, rhizosphere strain; E, endophytic strain.

Fig. 2. Swarming motility by *P. aeruginosa* strains. (A) Visualization of a non-swarming negative control, *P. chlororaphis* (left), and a superior swarmer, *P. aeruginosa* (PPA08/tomato endophyte), on M9 plates with 0.5% agar. (B) The graph represents the percentage of 90mm Petri-plates covered by the tendrils formed by the *P. aeruginosa* strains during swarming. Values plotted are the mean of six replicates with the standard errors and letters above the bars indicating the ranking of strains (significant differences ($p < 0.05$) based on Duncan's multiple range test (DMRT). The strains are color-coded based on their plant source: cucumber (green), tomato (red), eggplant (purple), and chili (yellow). The clinical isolates, ATCC10145, ATCC9027, and PAO1, are positive controls. R, rhizosphere strain; E, endophytic strain.

Fig. 3. Rhamnolipid production by *P. aeruginosa* strains. (A) Rhamnolipid production is indicated by the appearance of blue halos around the wells upon addition of cell-free supernatant of *P. aeruginosa* strains on CTAB-methylene blue agar medium. (B) Quantitative rhamnolipid levels released by *P. aeruginosa* strains. Values plotted are the mean of three replicates with the standard errors and letters above the bars indicating the ranking of the strains (significant differences ($p < 0.05$) based on Duncan's multiple range test (DMRT). The strains are color-coded based on their plant source: cucumber (green), tomato (red), eggplant (purple), and chili (yellow). The dashed lines indicate the levels of rhamnolipid made by the clinical strains, ATCC10145, ATCC9027, and PAO1 (positive controls). R, rhizosphere strain; E, endophytic strain.

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Fig 4. Biocontrol of phytopathogens by *P. aeruginosa* strains. The percentage inhibition of *Pythium aphanidermatum* (A), *Rhizoctonia solani* (B), *Fusarium oxysporum* (C), and *Xanthomonas oryzae* (D) induced by the *P. aeruginosa* strains. Values plotted are the mean of three replicates normalized to PAO1. * denotes the significant difference of PAO1 ($p < 0.05$) based on Duncan's multiple range test (DMRT). Strains are color-coded based on their plant source: cucumber (green), tomato (red), eggplant (purple), and chili (yellow). R, rhizosphere strains; E, endophytic strain.

Fig. 5. Principal coordinate analysis (PCoA) based on biocontrol ability of the *P. aeruginosa* strains. Euclidean distance-based PCoA plot for the biocontrol of bacterial and fungal phytopathogens by the *P. aeruginosa* strains. The percentage values in parentheses on the x- (PCoA1) and y-axes (PCoA2) depict the similarities and differences among the strains based on their mineral solubilizing ability. The three major clusters of PPA strains formed based on their similar biocontrol activity are named A, B, and C. The strains are color-coded based on their plant source: cucumber (green), tomato (red), eggplant (purple), and chili (yellow).

Fig. 6. *Caenorhabditis elegans* death induced by *P. aeruginosa* strains. (A) Stereomicroscopic view of L4 nematodes - live and active worm after feeding on *E. coli* OP50 (left); dead worm after feeding on the most virulent clinical isolate, ATCC 10145 (right). (B) Percentage of living nematodes after feeding on *P. aeruginosa* strains recorded over the time course of 0-120 hours. Values plotted are the means of three replicates with standard errors. The blue lines indicate the percentage of nematodes that survived after feeding on the clinical isolates, ATCC10145, ATCC9027, and PAO1 (positive controls). The black line indicates the percentage of living nematodes after feeding on *E. coli* OP50 (negative control). The PPA strains are color-coded based on their plant source: cucumber (green), tomato (red), eggplant (purple), and chili (yellow).