

# Influence of native endophytic bacteria on the growth and bacterial crown rot tolerance of papaya (*Carica papaya*)

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## Abstract

The native plant microbiome is composed of diverse communities that influence its overall health, with some species known to promote plant growth and pathogen resistance. Here, we show the antimicrobial and growth promoting activities of autoclaved culture metabolites (ACMs) from native endophytic bacteria (NEB) in a papaya cultivar that is tolerant to bacterial crown rot (BCR) caused by *Erwinia mallotivora*. Initially, bacterial colonization in recovering tissues of this cultivar was observed before onset of tissue regeneration or 'regrowth'. We further isolated and characterized these bacteria and were able to identify two culturable stem NEB under genera *Kosakonia* (EBW), related to *Enterobacter*, and *Sphingomonas* (EBY). We also identified root NEB (BN, BS and BT) under genus *Bacillus*. Inhibition assays indicated that ACMs from these NEB promptly (18-30h) and efficiently inhibited (60-65%) *E. mallotivora* proliferation *in vitro*. Interestingly, when ACMs from BN and EBW were inoculated in surface-sterilized papaya seeds, germination was variably retarded (20-60% reduction) depending on plant genotype, but plant biomass accumulation was significantly stimulated, at around two-fold increase. Moreover, greenhouse experiments show that ACMs from all isolates, especially EBW, significantly reduced BCR incidence and severity in susceptible genotype, at around two-fold. In general, our observations of pathogen antagonism, plant growth promotion leading to disease reduction by ACMs of native endophytic bacteria suggested its contribution to increased fitness of papaya and tolerance against the (re)emerging BCR disease.

**Keywords:** papaya bacterial crown rot, *Erwinia*, *Kosakonia*, *Sphingomonas*, *Bacillus*, endophytic bacteria, autoclaved culture metabolites

## 45 Introduction

46

47 Papaya (*Carica papaya*) is an economically important tropical fruit, mainly cultivated for food  
48 and cosmetic products. Due to its popularity, production of papaya steadily increased by 4.35%  
49 globally from 2002 to 2010 (Evans and Ballen, 2012). However, the industry still faces yield  
50 losses due to diseases, causing problems from plantation, transportation and processing until  
51 marketing (Ventura et al., 2004). Most importantly, emerging diseases greatly impair papaya  
52 production, and even cause up to 100% yield loss in some afflicted areas, especially in Asian  
53 tropical countries (Ploetz, 2004). A re-emerging disease of papaya, named bacterial crown rot  
54 (syn. soft rot, canker, decline) caused by a bacterium of genus *Erwinia* was reported in  
55 Southeast Asia (Maktar et al., 2008). Historically, the disease was reported in Java (von Rant,  
56 1931), and in other regions such as Mariana Islands (Trujillo and Schroth, 1982) and in the US  
57 Virgin Islands (Webb, 1984). In Mindoro, Philippines, a similar soft rot disease, which has  
58 water-soaked symptoms on leaves, petioles and whorl accompanied by a foul odor was  
59 reported. The pathogen was identified under the genus *Erwinia* (Pordesimo and Quimio, 1965)  
60 and remained a major problem until the 1980s (Obrero, 1980).

61

62 Re-emergence of *Erwinia* rot in papaya was reported in many regions across the globe in recent  
63 past decade. In early 2000, the bacterial ‘canker’ disease of papaya was reported in the  
64 Mediterranean region, caused by *Erwinia papayae* sp. nov. (Gardan et al., 2004). In Asia, a  
65 bacterium identified as *Erwinia papayae* was reported to cause ‘papaya dieback’ disease in  
66 Malaysia (Maktar et al., 2008). The ‘crown rot’ disease that was recently observed in the  
67 Philippines (dela Cueva et al., 2017) and ‘black rot’ disease in Japan (Hanagasaki et al., 2020)  
68 were both reported to be caused by *Erwinia mallotivora*. Interestingly, the ‘crown rot’ disease  
69 of papaya in the Republic of Tonga in Africa was reported to be caused also by *E. mallotivora*  
70 (Fullerton et al., 2011). In the Philippines, studies were reported regarding the possible  
71 resistance or tolerance of papaya against the bacterial crown rot (BCR) disease. A slow  
72 hypersensitive response exhibited by tolerant genotypes were reported. This was followed by  
73 tissue hardening and lateral stem regrowth at the junction of the diseased and healthy stem in  
74 some genotypes. The phenomenon, referred to as ‘regrowth’, is thought to be the manner by  
75 which papaya recovers from the disease, and perhaps a mechanism for tolerance (Magdalita et  
76 al., 2016). Breeding for resistance and development of management strategies against the  
77 bacterial crown rot disease are already in its early stages in the Philippines. Regrowth hybrid  
78 lines with good marketable qualities and with resistance to papaya ringspot disease are  
79 currently under preliminary field trials and back-cross F3 progenies are now in the pipeline  
80 (Magdalita et al., 2015).

81

82 However, these candidate BCR-tolerant cultivars are still undergoing field trials and there is a  
83 great need to develop augmentative solutions to combat BCR emergence and spread.  
84 Fortunately, in recent years, research in the role of endophytic bacterial community in plant  
85 defense has improved greatly (Podolich et al., 2015). In a recent study, autoclaved culture  
86 metabolites (ACMs) from *Bacillus* species were shown to significantly induce disease  
87 resistance against *Pseudomonas syringae* pv. lachrymans in cucumber and pepper (Song et al.,  
88 2017). Moreover, growing evidences have shown the important role of plant endospheric  
89 microbiome in plant defense and overall fitness (Liu et al., 2020; Trivedi et al., 2020). Here,  
90 we showed the possibility of exploiting native endophytic bacteria (NEB) of papaya stem and  
91 roots, a possibility that was recently reported in NEBs from papaya seeds (Mohd Taha et al.,  
92 2019). We prepared ACMs as previously shown (Song et al., 2017) and investigated its effect  
93 on fitness of a susceptible cultivar, contributing to BCR tolerance. Specifically, we also  
94 performed antimicrobial assays using ACMs from NEBs and investigated in effect on seed

95 germination, plant growth and response to *E. mallotivora* challenge. We hypothesize that  
96 antimicrobial activity, growth promotion and resistance-induction of ACMs from NEBs could  
97 be acquired or external mechanisms for pathogen defense in plants based. The use of these  
98 augmentative defense strategies in as early as seed or seedling stage were also suggested to  
99 help in developing more resilient genotypes or cultivars (Singh et al., 2018) that are otherwise  
100 highly susceptible to disease, but with good marketable qualities.

101

102

## 103 **Materials and Methods**

104

### 105 **Ultrastructural observation and isolation of endophytic bacteria in regrowth tissues**

106

107 Tissue samples from two-month old tolerant cultivar 5648 that were freshly recovering from  
108 BCR infection were prepared and examined using scanning electron microscope (SEM).  
109 Imaging was done using the Phenom XL™ (Phenom-World BV, Eindhoven, The Netherlands),  
110 a table top SEM. To further isolate observed bacteria, stem portions from the regrowth plants  
111 were directly obtained from the junction of the regrowth and the healthy stem part. Similarly,  
112 portions of the roots of regrowth plants tissues were prepared. The tissues underwent surface  
113 sterilization with following procedure: 95% ethanol for 1 min, 3% sodium hypochlorite  
114 solution for 6 min and 95% ethanol for 0.5 min followed by rinsing twice with sterile distilled  
115 water for 5 min (Coombs and Franco, 2003). Thereafter, sterilized tissues were submerged for  
116 10 min in 5 mL sterile water in a test tube to visibly detect bacterial ooze. A loopful of the  
117 serially diluted ( $10^{-8}$ ) bacterial suspension was streaked onto peptone sucrose agar (PSA) plates  
118 and incubated at room temperature for 24 h. Single colonies of actively growing bacteria were  
119 transferred to new PSA plates for subculture and purification. Pure colonies of the bacteria  
120 were maintained in PSA culture slants or as glycerol-skim milk stocks and stored at 4°C for  
121 future use.

122

### 123 **Cultural and biochemical characterization and pathogenicity tests of bacterial isolates**

124

125 All cultural characterization tests conducted in this part of the study were adapted from the  
126 procedures in phytobacteriology books (Goszczyńska et al., 2000; Borkar, 2017) with minor  
127 modifications. In order to prepare single colonies, a loopful of a serially diluted suspension ( $10^{-8}$ )  
128 of the isolates was streaked multiple times onto the surface of PSA plates. The color, colony  
129 diameter, morphology (*e.g.* shape, margin and elevation) and consistency were noted.  
130 Additionally, single stroke colonies were prepared in nutrient agar (NA) and described after  
131 one week. Colony color, shape, and other characteristics were likewise noted. Additionally,  
132 growth in nutrient broth was characterized in a test tube, where observations were done after  
133 one and two weeks. The morphology of the bacterial cells was characterized using gram  
134 staining methods. Biochemical tests include assay for catalase, cysteine desulfurase, lipase and  
135 gelatinase activities, while physiological test include test for oxygen requirement and  
136 utilization of starch and other sugars. All tests conducted in this part of the study were adapted  
137 from the procedures in phytobacteriology books (Goszczyńska et al., 2000; Borkar, 2017) with  
138 minor modifications. Identification based on biochemical tests were done using the *Bergey's*  
139 *Manual of Systematic Bacteriology* (Whitman et al., 2015). The tests were done in triplicates  
140 and the entire experiment was repeated twice to verify results.

141

142 On the other hand, in order to test for pathogenicity of the isolates, suspensions of the bacteria  
143 grown PSA slants were used in inoculation of two-month old seedlings at a concentration of  
144  $10^8$  cfu/mL. BCR-susceptible genotype 336 was used as the test plant. The plants were

145 wounded first at the leaf petiole and leaf midrib using a sterile needle. The inoculum was  
146 delivered using a sprayer at approximately 1.0 mL per plant. Observations were done at 7 and  
147 21 d after inoculation. Successful infection is indicated by necrosis and water-soaked lesions  
148 and progressive rotting from the point of wounding.

149

### 150 **DNA extraction and amplification of 16S rRNA**

151

152 DNA extraction was done using a simple protocol developed by Chen and Kuo, with minor  
153 modifications (Chen and Kuo, 1993). Single pure colonies were harvested from 72-h slant  
154 cultures in PSA. Both genomic DNA and diluted colony cell suspension were used as template  
155 for PCR reactions to amplify 16S rRNA of the bacterial isolates. Around 10-20 ng of DNA  
156 was used as template in PCR reactions and around 10-20 cells/mL were used as template in  
157 cell suspension PCR. For the 25  $\mu$ L PCR reaction mixture, the final concentration of PCR  
158 reagents (Invitrogen, MA, USA) was as follows: 1X PCR buffer without  $MgCl_2$ , 2.0 mM  
159  $MgCl_2$ , 0.2 mM dNTPs, 0.5  $\mu$ M of forward primer and 0.5  $\mu$ M reverse primer. *Taq* DNA  
160 polymerase was added at 0.04 U/ $\mu$ L. Universal 16S rRNA primers used were F8/rP2 and  
161 27F/1492R (Weisburg et al., 1991). The PCR program was set at 94°C for 10 min for initial  
162 denaturation, followed by 32 cycles of 94°C for 30 s, 55°C for 40 s and 72°C for 60 s, and final  
163 extension 72°C for 5 min and indefinitely at 4°C for storage. PCR amplicons were checked for  
164 correct size, at around 1300 bp, and purity in 1% agarose gel. Amplicons were then sent for  
165 Sanger sequencing at AIT Biotech, Inc. (Singapore).

166

### 167 **Bioinformatic analysis using 16S rRNA to identify bacterial isolates**

168

169 The bacterial isolates were identified up to the closest taxa using 16S rRNA sequence. For the  
170 purpose of identifying closest species-level taxon, we primarily used valid species (*i.e.* type  
171 specimens) curated in EZ-BioCloud ([www.ezbiocloud.net/identify](http://www.ezbiocloud.net/identify)), a public data and analytics  
172 for the taxonomy of bacteria and archaea (Yoon et al., 2017). The sequences of closest  
173 accessions were collected and used further in phylogenetic analysis in Molecular Evolutionary  
174 Genetics Analysis software version 7 (MEGA7) (Kumar et al., 2016). Multiple sequence  
175 alignment following the ClustalW method (Thompson et al., 1994) was performed before  
176 inferring phylogenetic tree using Neighbor-Joining method (Saitou and Nei, 1987) following  
177 the Tamura-Nei model (Tamura and Nei, 1993). Lastly, we also used BLAST (Basic Local  
178 Alignment Search Tool) in NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul et al.,  
179 1990) to check for the identity of our isolates to closest species or strains in GenBank.

180

### 181 **Antimicrobial assay using autoclaved culture metabolites (ACMs)**

182

183 A suspension of the 72-h old culture (log phase) of the endophytic bacteria and *Bacillus* isolates  
184 in PSA was diluted to obtain approximately  $10^8$  cfu/mL or  $OD_{600}$  at 0.75-0.80. A 1.0 mL aliquot  
185 consisting of approximately  $10^8$  cells was inoculated in a 100 mL 3% tryptic soy broth (TSB,  
186 BD-Difco) in Erlenmeyer flasks. The broth cultures were incubated for 72 h at room  
187 temperature with continuous shaking at 120 rpm. Thereafter, the bacterial broth cultures were  
188 incubated for 30 min at autoclaving temperature (121°C) and pressure (15 psi) in order to retain  
189 only the autoclaved culture metabolites (ACMs). The ACMs were centrifuged at 12,000 rpm  
190 for 10 min to separate cell debris. The supernatant was then filter-sterilized using a 0.2  $\mu$ m-  
191 pore size polyether sulfone (PES) membrane filter (Sterile Acrodisc, Gelman Sciences or  
192 Whatman, GE Life Sciences) to remove remaining bacterial cell debris. To use ACM filtrates  
193 for *E. mallotivora* inhibition assays, dilutions were made to obtain approximately  $OD_{600}$  of 0.25  
194 to 0.30 as standard concentrations.

195

196 Log phase colonies (72-h old) of *E. mallotivora* was prepared as suspension in sterile water at  
197 0.25 to 0.30 OD at 600 nm, in order to obtain approximately  $10^4$  cells/mL. The assay was done  
198 in a 96-well microtiter flat-bottom polycarbonate plate (Tarsons, Cat. No. 941146). ACMs and  
199 *E. mallotivora* cells were mixed in a well at 1:1 ratio (50  $\mu$ L + 50  $\mu$ L). Wells that served as  
200 controls contain *E. mallotivora* cells only and *E. mallotivora* cells in tryptic soy broth while  
201 tryptic soy broth and sterile water only served as blanks. The OD<sub>600</sub> reading and log<sub>10</sub> cfu/mL  
202 of the wells at 0, 24, 48 and 72 h were noted and compared across treatments. In order to have  
203 a good estimate of the inhibitory effect of the ACMs, percent inhibition was calculated using  
204 the formula:  $\frac{OD_{Em+TSB} - OD_{ACM}}{OD_{Em+TSB}} \times 100\%$ , where OD<sub>Em+TSB</sub> is the optical density reading of the  
205 negative check (Em+TSB) at a given time point and OD<sub>ACM</sub> is the optical density of any ACM-  
206 treated *Em* suspension at a given time point. This assay followed a randomized complete block  
207 design (RCBD), wherein the ACMs served as treatments and replicates as the blocking factor.  
208 Three replicates were prepared for each treatment containing three samples with 12  
209 subsamples. Thus, for each treatment, 96 readings were done every time the OD values were  
210 assessed.

211

## 212 **Preparation of seeds and ACM treatment**

213

214 The papaya seeds used in this study were obtained from the papaya germplasm collection at  
215 the Fruit Breeding Laboratory at Institute of Plant Breeding, College of Agriculture and Food  
216 Science, University of the Philippines Los Baños. The genotypes used were variety *Cariflora*  
217 (genotype 5648), a PRSV-tolerant and BCR-tolerant ‘regrowth’ genotype with good yield and  
218 fruit quality, and the *Solo* variety (genotype 501), which is susceptible to both PRSV and BCR.  
219 Sterilized coir dust-garden soil medium was prepared as potting medium for the seeds. Seeds  
220 were surface sterilized in an Erlenmeyer flask using 70% ethanol solution for 1 min, followed  
221 by rinsing twice in sterile water for 2 min and blot- and air-drying under the laminar flow hood  
222 for 15 min. The seeds were immersed in the ACMs for 16 h, followed by blot- and air-drying  
223 under the laminar flow hood for 30 min. Soaking in sterile tryptic soy broth only served as the  
224 mock treatment. The seeds were sowed in sterile potting medium with complete (40-40-40)  
225 fertilizer and maintained under greenhouse conditions with daily watering for six weeks. The  
226 plants were ready for inoculation if it has 4-5 leaf nodes already and about 10 cm in height.  
227 The effects of ACM treatment on seed germination was assessed based on percent of seeds  
228 germinated after four weeks. To evaluate the effect of ACM treatment on plant biomass, the 7-  
229 wk old ACM-treated plants were slowly dried in an oven at 65°C. The dry weight of the samples  
230 were assessed after 36 h. This experiment followed a randomized complete block design  
231 (RCBD), wherein the ACMs served as treatments and replicates as the blocking factor.  
232 Separate statistical analyses were done for the two genotypes.

233

## 234 **Inoculation of ACM-treated plants and disease assessment**

235

236 Six-week old ACM-treated seedlings of both papaya genotype 5648 and 501 were inoculated  
237 with *Erwinia mallotivora* as described by Supian *et al.*, with some modifications (Supian *et al.*,  
238 2017). The plants were watered first before inoculation to increase its internal turgor pressure  
239 and activate transpiration and water translocation, thus increasing inoculation efficiency. To  
240 further increase chances of infection, the apical tips, petiole and midrib of the second and third  
241 youngest leaf were pricked with a sterile syringe needle. On the other hand, the inoculum was  
242 prepared by suspending a 72-h old pure culture of *E. mallotivora* in sterile water and diluting  
243 the suspension to obtain approximately  $10^8$  cfu/mL (OD<sub>600</sub> of 0.75-0.80). The inoculum was

244 applied through spraying three times per plant, delivering approximately 1.0 mL of inoculum.  
245 After inoculation, plants were covered with clear plastic cups in order to maintain high relative  
246 humidity and favor bacterial growth for 48 h.

247  
248 Disease assessment was done at 7 and 21 days post inoculation (dpi). Percent disease incidence  
249 was calculated as  $PDI = \frac{\text{no.of diseased plants}}{\text{total no.of plants}} \times 100\%$ . Disease severity assessment followed the  
250 scoring used by Supian *et al.* (2017) with some modifications. This modified scale consisted  
251 of the following: 0 (no symptom), 1 (lesions are restricted to inoculation point only), 3 (lesions  
252 are expanding to less than half of leaf area), 5 (lesions are expanding to more than half of leaf  
253 area), 7 (lesions and rotting spread to the petiole and stem and 9 = (plant suffers severe rotting,  
254 crown falls off). Finally, percent disease severity index was calculated as  $(PDSI = \frac{\text{total of severity scores}}{\text{total no.of plants} \times \text{max.severity score}} \times 100\%$ . This assay followed a randomized complete block  
255 design (RCBD), wherein the ACMs served as treatments and replicates as the blocking factor.  
256 Three replicates were prepared for each treatment containing three samples with five  
257 subsamples each. Thus, for each treatment, 45 plants were allotted.

258

## 259 **Statistical analysis of data.**

260

261  
262 All *in vitro* experiments were done twice and greenhouse experiment were done thrice to  
263 confirm results. All data generated were subjected to statistical analysis through one-way  
264 analysis of variance (ANOVA at  $\alpha = 0.05$ ) in SAS software program version 14.3 (SAS  
265 Institute Inc., Cary, NC). Separate statistical analyses were done for the two papaya genotype  
266 used. The means were subjected to pairwise mean comparison using Tukey's honest significant  
267 difference (HSD) test. All tests are considered significant if  $P < 0.05$  (\*),  $P < 0.01$  (\*\*) or  $P <$   
268  $0.001$  (\*\*\*)).

269

## 270 **Results**

271

### 272 **Native endophytic bacteria from 'regrowth' BCR-tolerant plants**

273

274 Initially, a community of bacterial cells was observed in the tolerant genotype, 5746 (var.  
275 *Cariflora*) in early stages of regrowth (3-4 wks), but not in fully regrowth tissues (7-8 wks)  
276 (see Supplementary Figure 1). Building on the SEM observations, regrowth-associated  
277 culturable native endophytic bacteria (NEB) were isolated. Two morpho-culturally distinct,  
278 stem NEBs (EBW, EBY) and three root NEBs (BN, BS, BT) were isolated. Based on *Bergey's*  
279 *Manual of Systematic Bacteriology* (Whitman et al., 2015), EBW was identified under genus  
280 *Enterobacter* (*Gammaproteobacteria*: *Enterobacterales*: *Enterobacteriaceae*). EBY was  
281 identified under genus *Sphingomonas* (*Alphaproteobacteria*: *Sphingomonadales*:  
282 *Sphingomonadaceae*). On the other hand, the *Bacillus* isolates were not easily identified using  
283 phenotypic characteristics because their colonies are very much alike and also exhibit similar  
284 biochemical properties. Identification using 16S rRNA sequence was carried out to determine  
285 the most probable taxa of the bacterial isolates. Figure 2 shows the resulting phylogenetic trees  
286 with the percent identity to valid taxa or type specimen identified in EzBioCloud. The analyses  
287 showed that EBW is paraphyletically related to *Kosakonia* sp. clade and monophyletically  
288 related the *Enterobacter* sp. clade. It is worthwhile to note that *Kosakonia* sp. is a new genus  
289 of plant-associated, endophytic ex. *Enterobacter* species (Brady et al., 2013). Moreover, EBY  
290 is paraphyletically related to *S. endophytica* YIM 65583 (Huang et al., 2012) and  
291 *Sphingomonas phyllosphaerae* FA2 (Rivas et al., 2004), although percent identity to closest  
292 taxa is not convincingly high enough, which also implies that it could be a new species (see

293 Supplementary Table 1). On the other hand, the *Bacillus* isolates cannot be definitively  
294 identified because of inherent limitation of 16S rRNA for *Bacillus* taxonomy (Maughan and  
295 Van der Auwera, 2011). Nevertheless, it is possible that the three isolates are members of the  
296 *Bacillus subtilis* species complex (Fan et al., 2017) based on bioinformatic and phylogenetic  
297 analysis done. Lastly, all the isolates were not able to infect papaya nor cause any  
298 hypersensitive reaction, which implies no interaction with plant resistance machinery. The  
299 summary of characteristics of the isolates is shown in Table 1, while all supplementary data  
300 can be accessed in the through provided herewith.

301

### 302 ***E. mallotivora* was promptly and efficiently inhibited by ACMs.**

303

304 The microtiter plate assay for the inhibition of *Em* was proven to be useful in evaluating  
305 inhibition efficacy of the autoclaved culture metabolites (ACMs) by just monitoring the  
306 absorbance readings. Figure 3 shows the trend in absorbance of the ACM treatments, as well  
307 as percent relative inhibition. After 24 h, a drastic increase in cell density was observed in the  
308 BS, BT and EBY treatments. Nevertheless, this observed increase in cell density in the ACM  
309 treatments was still considerably lower than the negative control, at around 2-3 folds. Thus,  
310 there is still a significant inhibition of cell multiplication in these treatments. More importantly,  
311 bacterial growth was highly inhibited when *Em* was subjected to BN and EBW at 60-65% and  
312 53%, respectively.

313

### 314 **All ACMs reduced growth vigor of papaya, except ACM from *Kosakonia*.**

315

316 Treatment of surface-sterilized papaya seeds with ACMs from bacterial endophytes and  
317 *Bacillus* isolates resulted to retarded germination compared to mock-treated seeds. Figure 4a  
318 shows the average percent germination of papaya seeds of genotype 501 (var. *Solo*) and 5648  
319 (var. *Cariflora*), which were evaluated as susceptible and tolerant (*i.e.* regrowth) to the crown  
320 rot disease, respectively. Among the all treatments, BS has no significant adverse effect on the  
321 germination of papaya seeds in both genotypes. However, treatment with isolate BN and BT  
322 resulted to a significant reduction in percent germination of 501, while treatment with isolate  
323 EBW and EBY resulted to lowest percent germination in genotype 5648. This implies that  
324 papaya might respond differently to ACM treatments, depending on genotype. Plant growth  
325 was assessed by getting the dry biomass of plants at 7 wks after sowing. Figure 4b shows the  
326 accumulated biomass, measured as oven-dried weight, of the ACM-treated plants. Treatment  
327 with ACM from EBW yielded the highest dry weight, particularly in genotype 5648, which  
328 correspond to more than two-fold increase relative to mock. However, no significant increase  
329 in biomass was observed in genotype 501 in the same treatment, indicating that growth  
330 stimulation by bacterial ACMs in papaya might be genotype-dependent. Finally, all other  
331 treatments significantly reduced biomass accumulation in both genotypes as depicted,  
332 especially in BS ACM-treatment.

333

### 334 **ACMs significantly reduced the incidence and severity of BCR disease in Papaya.**

335

336 The possibility of using easy to prepare, sterile, highly-stable, and potent metabolites from  
337 beneficial bacteria as promoter of disease resistance in papaya was assessed in this study.  
338 Although plant vigor was adversely affected by the treatment of ACMs, the papaya plant would  
339 eventually recover and grow normally. Figure 5a,b shows the representative photographs of  
340 inoculated plants after two weeks of observation. Based on observations made, BN-treated  
341 plants in both genotypes seemed to have retarded growth after 14 dpi, due to infection. Necrosis  
342 within half of the inoculated leaf area was commonly observed in these plants. BS- and BT-

343 treated plants in both genotypes were not significantly affected by the pathogen challenge and  
344 continued to grow normally. At most instances, plants in this treatment only had necrosis at the  
345 inoculation point. Recorded percent disease incidence (Figure 5c) revealed that in general,  
346 ACM-treated plants have lower propensity to bacterial crown rot infection. BCR incidence  
347 continued to increase until 21 dpi, but the incidence in ACM-treated plants remained lower  
348 compared to that of the mock-treated plants, in both genotypes. On the other hand, there was a  
349 general reduction in average severity ratings in both genotypes (Figure 5d). More importantly,  
350 a two-fold reduction in disease severity was observed in EBW treatment at 21 dpi relative to  
351 control. Lowest severity was observed in EBW-treated 5648 plants relative to control.  
352 Generally, improved resistance is more pronounced in 5648 (resistant) plants than in 501  
353 (susceptible) plants.

354

## 355 Discussion

356

357 BCR tolerant genotypes such as 5648 (var. *Cariflora*) were primarily identified using the lateral  
358 stem 'regrowth' phenotype at 5-7 wks after infection (wai) as indicator of disease resistance or  
359 tolerance. In this study, it was observed through SEM that infection sites in recovering tissues  
360 from genotype 5648 were colonized by a community of bacteria at the onset of regrowth (3-4  
361 wai). Building around the initial observations, endophytic bacteria from stem and roots were  
362 isolated from regrowth genotype 5648, and further characterization and identification using  
363 16S rRNA were carried out thereafter. Root-associated *Bacillus* species were identified under  
364 the *Bacillus subtilis* species complex, while the two endophytic bacteria were identified under  
365 genus *Kosakonia* ex. *Enterobacter* (isolate EBW) and *Sphingomonas* (isolate EBY). Related  
366 species under the *B. subtilis* species complex include *Bacillus velezensis* strain CR-502 (isolate  
367 BN) that was shown to produce surfactants that confer antimicrobial activity against other  
368 microbes (Ruiz-García et al., 2005). Additionally, antibiotics from *Bacillus* successfully  
369 inhibited gram-positive bacteria that are also human-pathogenic such as *Staphylococcus aureus*,  
370 *Clostridium difficile* and *Listeria monocytogenes* (Collins et al., 2016). We report herewith the  
371 first instance that a strain of the *B. subtilis* species complex was shown to have inhibitory  
372 properties against a bacterial plant pathogen.

373

374 Species of the genus *Sphingomonas* are reported to have beneficial properties and are usually  
375 associated as endophytes in many plants. In particular, *S. paucimobilis* associated with an  
376 orchid plant (*Dendrobium officinale*), was proven to have growth promoting properties by  
377 increasing phytohormone production and aiding in atmospheric nitrogen fixation, processes  
378 which the plant cannot carry out normally (Yang et al., 2014). Likewise, *S. phyllosphaerae* and  
379 *S. endophytica* were originally isolated as endophytes in *Acacia caven* and *Artemisia annua*,  
380 respectively. On the other hand, *Kosakonia* sp. (formerly *Enterobacter* sp.) is a new taxon name  
381 assigned to endophytic, plant-associated *Enterobacteraceae* species. Two species, *K.*  
382 *oryzophilus* and *K. oryzendophytica*, were found to be key inhabitants of rice root endosphere  
383 (Hardoim et al., 2013). The two species were able to promote rice growth by supplying nitrogen  
384 and phosphorus to the plant. Similarly, *Kosakonia sacchari* (Zhu et al., 2013) (formerly  
385 *Enterobacter* sp.) is an endophyte associated with sugarcane stem, roots and rhizosphere. The  
386 bacterium was shown to help the plant in producing fixed nitrogen for its consumption. Another  
387 species of *Kosakonia*, named *K. pseudosacchari*, was discovered to be endophytically  
388 associated with field grown corn (*Zea mays*) root tissues. *K. pseudosacchari* is also a closely  
389 related species of *K. sacchari* and *K. oryzendophytica* (Kämpfer et al., 2016). However, there  
390 were no studies were reported yet regarding the antimicrobial properties of *Kosakonia* and  
391 *Sphingomonas* species other than this study. Interestingly, the aforementioned *Bacillus* species  
392 and endophytic bacteria were not able to infect papaya plants nor cause any hypersensitive

393 reaction and is resistant to latex exposure. A similar set of endophytic bacteria were also  
394 isolated from and constantly associated with tissue-cultured papaya plants based on previous  
395 studies (Thomas et al., 2007b, 2007a; Thomas and Kumari, 2010).

396

397 Endophytic bacteria and various *Bacillus* species associated with plants were reported to have  
398 growth-promoting and disease resistance-inducing properties (Hallmann et al., 1997; Ongena  
399 and Jacques, 2008; Podolich et al., 2015). Species of *Bacillus*, such as *B. paralicheniformis*  
400 MDJK30 was reported to promote growth in *Arabidopsis thaliana* (Dunlap et al., 2015;  
401 Palacio-Rodríguez et al., 2017) and suppress growth of *Fusarium solani* (Wang et al., 2017).  
402 Another strain, *B. paralicheniformis* KMS 80 was discovered to be associated with rice root  
403 endosphere and helped the plant in nitrogen fixation (Annapurna et al., 2018) (Annapurna *et*  
404 *al.*, 2018). However, assays for growth-promotion and disease resistance induction heavily rely  
405 on the availability of culturable isolates and its compatibility or ability to colonize the host  
406 plant. In this study, these challenges were surpassed by preparing culture metabolites that are  
407 easy-to-prepare, sterile and highly stable, thus the term, autoclaved culture metabolites  
408 (ACMs). Moreover, seed inoculation using ACMs of bacteria is a relatively new method used  
409 in stimulating plant growth and in turn, enhance disease resistance in plants. This method was  
410 previously demonstrated in pepper and cucumber using ACMs of *Bacillus gaemokensis* PB69  
411 (Song et al., 2017). Unlike the study by Song *et al.*, this study used unpurified preparations of  
412 ACMs, which contains mixture of metabolites that might have caused reduced germination in  
413 papaya seeds. However, it was shown that growth vigor of papaya seedlings was enhanced  
414 later on as shown in higher biomass accumulation compared to the normal or untreated ones.  
415 Comparatively, treatment of live cell suspensions of endophytic and other beneficial bacteria  
416 also led to reduced germination and seedling vigor in papaya (Thomas et al., 2007a), tomato  
417 (Worrall et al., 2012) and oil palm (Azri et al., 2018), but recovery of plant growth was  
418 observed when grown in greenhouse conditions. Nevertheless, live cell suspensions are prone  
419 to contamination and the cells are unprotected and unstable unlike when using ACMs.

420

421 On the other hand, ACMs from the NEBs contributed to the reduction of the incidence of crown  
422 rot, in both susceptible and resistant genotypes. The plants remained healthy wherein only local  
423 necrosis at the inoculation site was observed in the test plants. These observations indicated  
424 that ACM-treatment has a potential to decrease the predisposition of the papaya plant to the  
425 crown rot disease, as a collateral effect of plant growth stimulation. However, these  
426 observations still demand further molecular transcriptomic investigation to be referred to as a  
427 disease bioprimering phenomenon. Interestingly, genotype dependence of papaya responses to  
428 ACM treatments might be important to consider in breeding programs to augment disease  
429 resistance. Endophytic bacteria and many *Bacillus* species were proved to have such bioactivity  
430 in plants, may it be its native host or a non-host (Tonelli et al., 2011; Yang et al., 2014; S. et  
431 al., 2018; Wicaksono et al., 2018). However, induction of defense responses in plants comes  
432 with a penalty or cost (Cipollini et al., 2003; Dietrich et al., 2005) by delaying and lowering  
433 the plant's germination and seedling vigor. Nonetheless, we show that fully-grown seedlings  
434 eventually recuperate and even accumulate higher biomass relative to the untreated  
435 counterpart.

436

437 The use of live cell suspension remains the most popular method in exploiting beneficial  
438 microbes to promote better plant health (Hallmann et al., 1997). However, we present herewith,  
439 the use of autoclaved culture metabolites (ACMs) is relatively new but promising and only a  
440 few studies were reported to prove resistance-inducing bioactivity in plants (Noh et al., 2017;  
441 Song et al., 2017). The use of ACMs provides a convenient and fast way to screen plant-  
442 associated microbes because this method is easy to prepare, and the metabolites produced are

443 highly stable and potent. If optimally applied, disease protection and induction of systemic  
444 resistance using beneficial microbes or its metabolites will be a good alternative to chemical  
445 pesticides or resistance inducers.  
446

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448

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452

453 **Supplementary Data**

454

455 All supplementary data for this study can be downloaded through this link:

456 <https://doi.org/10.6084/m9.figshare.13499409.v1>

457

458 **Declarations**

459

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463

464 *Availability of data and material.* Sequences of the 16S rRNA gene for each bacterial isolate  
465 are deposited in NCBI GenBank. Accession numbers and sequences are also available in  
466 supplementary data [link].

467

468 *Conflict of interest.* The authors declare that they have no conflict of interest.

469

470 *Author contributions.* MPSR and PMM did the study conception and design and funding  
471 acquisition. PMM supervised all experiments. Material preparation and data collection were  
472 performed by MPSR, EPP, SFMD and EM. MPSR did the data analysis. The first draft of the  
473 manuscript was written by MPSR and all authors approved the final manuscript.

474

475 **Compliance with Ethical Standards**

476

477 *Research involving Human Participants and/or Animals.* We confirm that in this research any  
478 human and/or animals participant was not used and there is no any disagreement with informed  
479 consent.

480

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- 656

657 Table 1. Summary of morpho-cultural and biochemical properties of the bacterial isolates isolated in this study.

Properties	<i>E. mallotivora</i>	<i>Kosakonia</i> sp.	<i>Sphingomonas</i> sp.	<i>Bacillus</i> sp. BN	<i>Bacillus</i> sp. BS	<i>Bacillus</i> sp. BT
<u>Cell / Single Colony<sup>1</sup></u>						
colony color	cream white	white	deep yellow	light brown	cream white	cream white
cell shape	rods	rods	short rods	rod	rod	rod
gram stain	negative	positive	negative	positive	positive	positive
spore formation	absent	present	absent	present	present	present
diameter (mm)	0.8-1.0	6.0-6.5	5.5-6.0	6.0-6.5	7.0-7.5	5.0-6.0
shape	circular	circular	circular	irregular	irregular	irregular
margin	entire	entire	entire	undulate	undulate	undulate
elevation	convex	convex	convex	umbonate	flat	raised
consistency	fluidal	fluidal	butterous	dry	dry	dry
<u>Morpho-cultural<sup>2</sup></u>						
nutrient agar stroke	filiform, butyrous	filiform, viscid	filiform, butyrous	effuse, butyrous	effuse, rubbery	rhizoid, brittle
nutrient broth	flocculent	ring	flocculent	membranous	membranous	membranous
<u>Biochemical<sup>3</sup></u>						
oxygen requirement	facultative anaerobe	facultative anaerobe	facultative anaerobe	obligate aerobe	obligate aerobe	obligate aerobe
catalase	+	+	+	+	+	+
cysteine desulfurase	-	+	+	+	-	-
lipase	-	+	+	+	+	+
gelatinase	-	+	-	+	+	+
starch utilization	+	-	+/-	+	+	-
cellobiose utilization	-	+	+	+	+	+
glucose utilization	+	+	+	+	+	+
lactose utilization	-	+	+	+	+	+
maltose utilization	-	+	+	+	+	+
mannitol utilization	+	+	-	+	+	+
mannose utilization	+	+	+	-	-	-
sorbitol utilization	-	+	-	+	+	+

658 <sup>1</sup>Colony morphology in Peptone Sucrose Agar (PSA) after 7 d of room temperature incubation.

659 <sup>2</sup>Morpho-cultural properties after 14 d of room temperature incubation.

660 <sup>3</sup>+, positive test; -, negative test; +/-, weak positive.

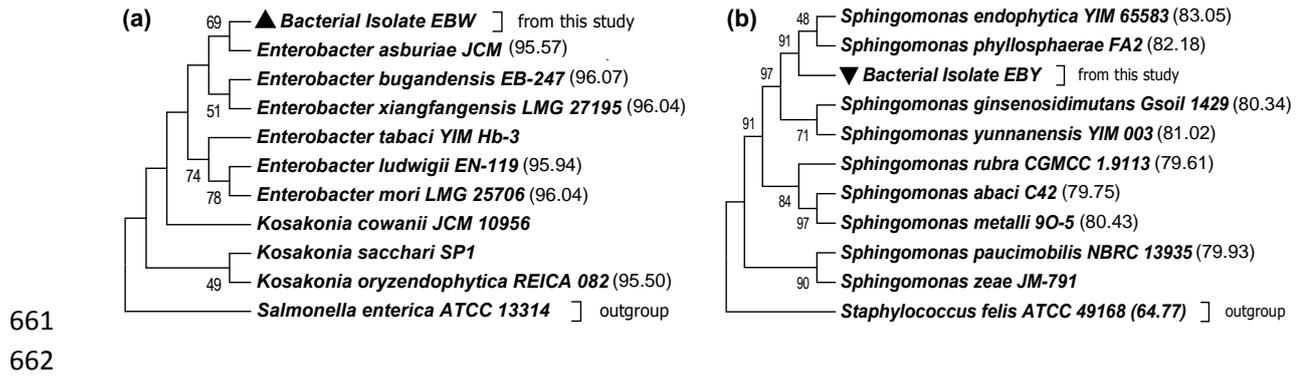
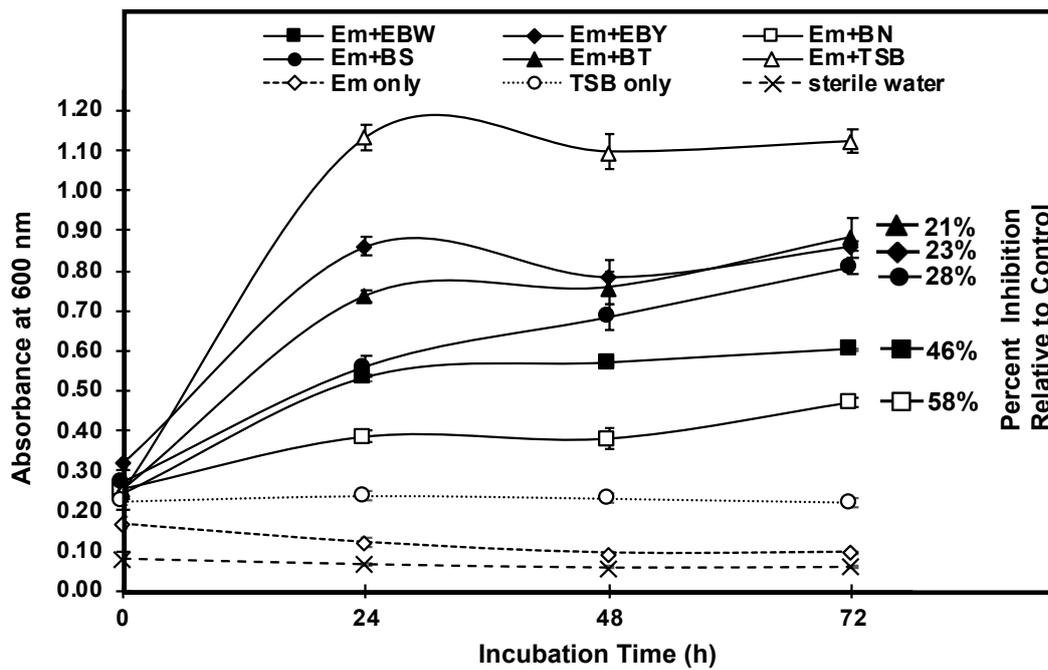


Figure 2. Phylogenetic trees showing most probable taxa and species-level identity based on 16S rRNA sequences of: (a) Isolate EBW tree showing relationship with *Enterobacter*/*Kosakonia* clade, percent identities to EBW are shown in the members of the clade; (b) Isolate EBY tree showing relationship to the *Sphingomonas* species, percent identities to EBY are shown in the members of the clade. The analysis followed Neighbor Joining following the Tamura-Nei model in MEGA7. Bootstrap values for 1000 replicates greater than 45% are shown in the nodes.

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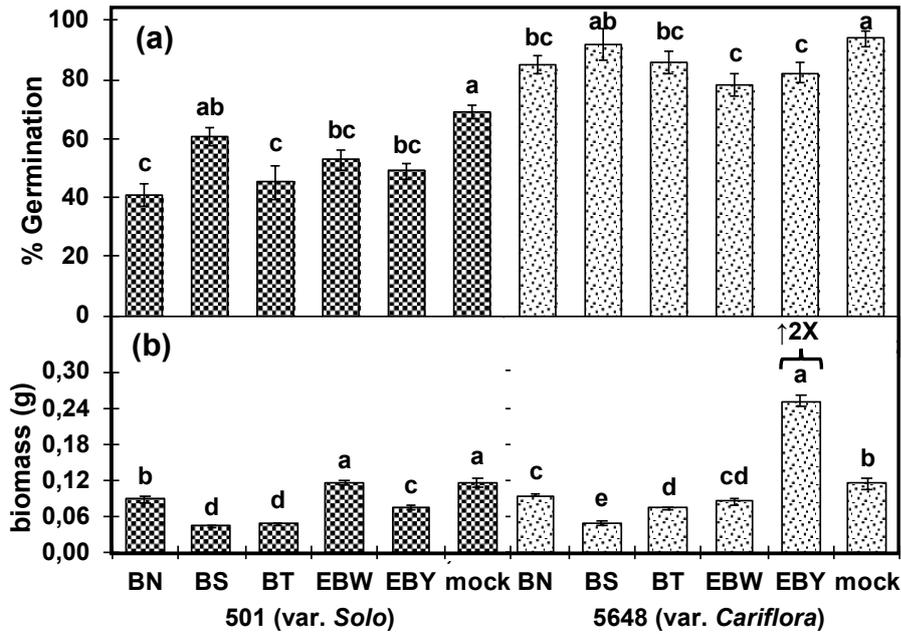


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672 Figure 3. Antimicrobial effect of autoclaved culture metabolites (ACMs) on *Erwinia mallotivora*  
673 proliferation *in vitro*. Changes in absorbance readings at 600nm (left axis) through time (horizontal axis)  
674 and percent inhibition relative to the controls (right axis) of the autoclaved culture metabolite-treated  
675 *Erwinia mallotivora* (Em) cell suspensions are shown. The treatments are as follows: Em treated with  
676 ACM from isolate EBW (*Kosakonia* sp.) (Em+EBW), EBY (*Shingomonas* sp.) (Em+EBY), isolate BN  
677 (Em+BN), isolate BS (Em+BS) and isolate BT (Em+BT). Em in tryptic soy broth (Em+TSB) and Em only  
678 served as negative controls while TSB only and sterile water only served as blanks. Standard deviation  
679 shown as error bars are depicted in each data point.

680



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682

683 Figure 4. Effect of autoclaved culture metabolites (ACMs) on the growth vigor of papaya. (a)  
 684 Germination data taken at 21 days after sowing (das) and (b) biomass taken at 49 das. Columns with  
 685 checkered shade are data from the papaya BCR-susceptible genotype and columns with dotted shade  
 686 are data from papaya BCR-tolerant genotype. The two-fold increase in dry biomass is EBY-treated  
 687 genotype 5648 is also highlighted. Treatments marked with different letters are statistically different  
 688 after HSD test at  $P < 0.05$  (\*).

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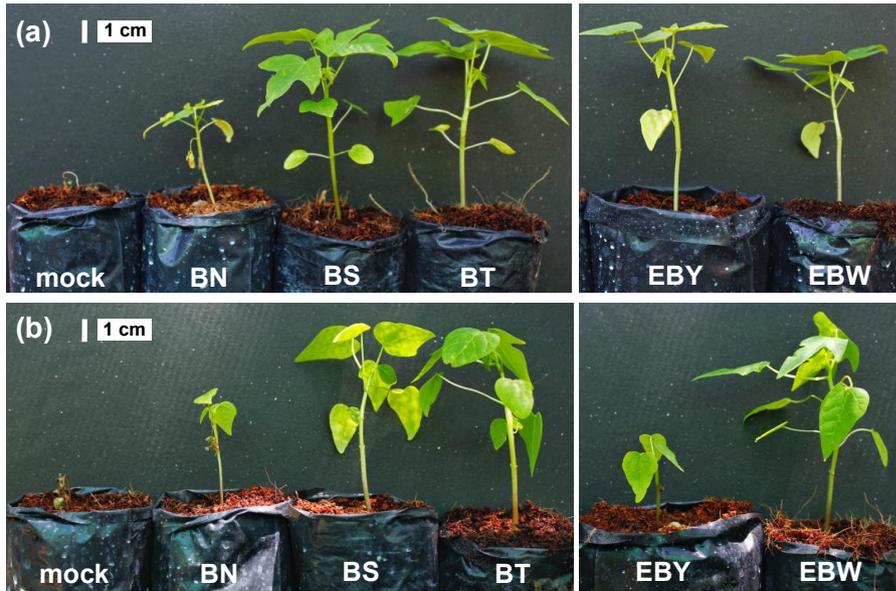
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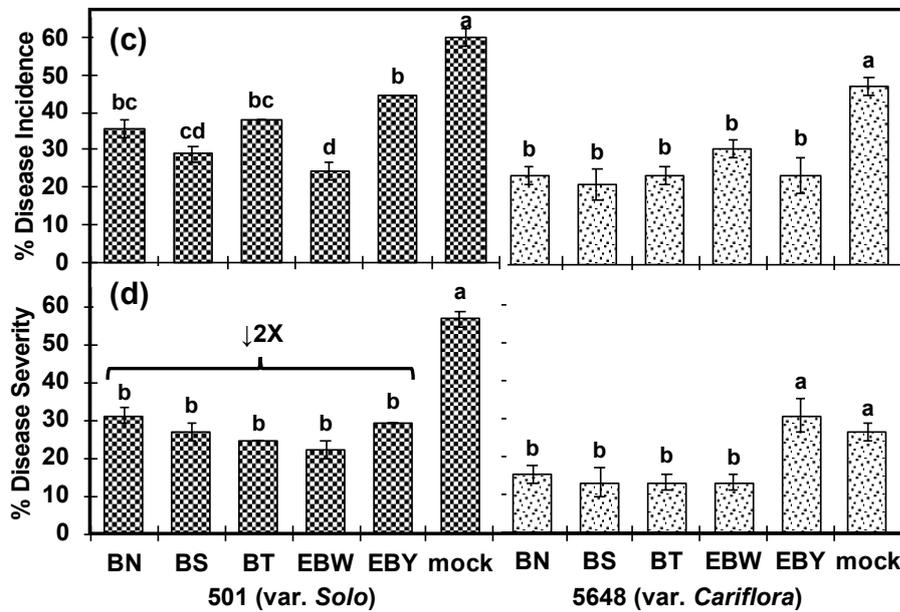
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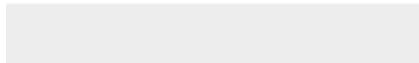
701 Figure 5. Effect of autoclaved culture metabolites (ACMs) on the incidence and severity of bacterial  
 702 crown rot disease of papaya. Representative photos of pathogen-challenged ACM-treated plants of (a)  
 703 genotype 501 and (b) genotype 5648. Scale bars are shown in the figure above. (c) Average percent  
 704 disease incidence taken at 21 days after inoculation (dai), (d) average percent disease severity at 21  
 705 dai. Columns with checkered shade are data from the papaya BCR-susceptible genotype and columns  
 706 with dotted shade are data from papaya BCR-tolerant genotype. Treatments marked with different  
 707 letters are statistically different after HSD test at  $P < 0.05$  (\*).

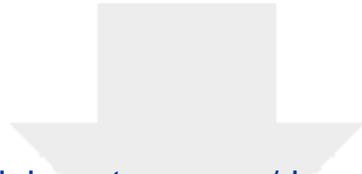


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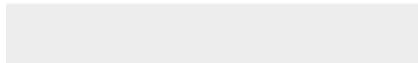
**Supplementary material**

202012\_Supplementary Figure 1 and Table 1.docx





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