Bacteriophage cocktails displayed protective effects against soft rot-causing *Pectobacterium* sp.

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Abstract:

Soft rot caused by *Pectobacterium* spp. is responsible for significant losses in vegetable production worldwide. Methods for the effective control of this disease are limited and are primarily based on good agricultural practices. The use of phages as biocontrol agents appears to be a promising alternative to combat phytopathogens. In this study, we investigated the efficacy of lytic phages against soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum*. Designated as PPc_A3, PPc_D1, and PPc_J3, three bacteriophage isolates, which were recovered from symptomatic tissues and environmental samples, were observed to effectively lyse *P. carotovorum* subsp. *carotovorum*. PPc_A3 belongs to the *Podoviridae* family, while phages PPc_D1 and PPc_J3 belong to the *Myoviridae* family based on the morphological features of the virions as observed using transmission electron microscopy. The optimal multiplicity of infection (MOI) differed greatly among the three phages. All survived incubations at 30°C, 40°C and 50°C and at pH ranging from 3.0 to 9.0, but were all inactivated at 60°C and at pH 12. Both monophage and cocktail preparations were effective in inhibiting the growth of *P. carotovorum* subsp. *carotovorum* in *in vitro* challenge tests. In the semi-*in planta* assays, monophage treatments resulted in significant reduction of tissue maceration in potato slices, while treatment with cocktail preparations completely inhibited the development of soft rot disease. Overall, these results demonstrate the efficacy of cocktail formulations of phages PPc_A3, PPc_D1, and PPc_J3 for the biocontrol of soft rot disease caused by *P. carotovorum* subsp. *carotovorum.*

Keywords: *Pectobacterium* sp., bacterial soft rot, bacteriophages, biocontrol, agricultural management

47 # 1. Introduction

48 Bacterial soft rot disease causes one of the most damage among vegetable and fruit crops
49 around the world (Bhat et al., 2010). Annual post-harvest losses due to bacterial soft rot vary
50 from 15-30% of the harvested crops and at least 30-50% of the 65 million tons of vegetables
51 in public markets (Agrios, 2005; Bhat et al., 2010; Rahman et al., 2012). Soft rot disease results
52 from the infections by several types of bacteria, but most commonly by members of the soft
53 rot *Pectobacteriaceae* (SRP) (Adeolu et al., 2016; Agyemang et al., 2020; A. O. Charkowski,
54 2018; Pérombelon, 2002; Wallace, 1948). During infection, SRPs produce enzymes like
55 pectate lyase, cellulase and polygalacturonase, which are capable of breaking down the plant
56 cell wall leading to plant tissue maceration and decay (A. Charkowski et al., 2012; Li et al.,
57 2019; Mattinen et al., 2007; Muturi et al., 2019). *Pectobacterium* species have the broadest
58 host range among soft rot bacteria, and they are considered as the leading cause of soft rot
59 disease in various vegetable crops (Davidsson et al., 2013; J.-A. Lim et al., 2017).

60 Reported practices to manageo soft rot disease include cutting-off and disposal of infected
61 tissues during or after harvest, wrapping with paper, treatment with hot water; and/or the use
62 of bactericides, such as alum powder (aluminum potassium sulfate), formaldehyde, copper
63 sulfate, and sodium or calcium hypochlorite (Bayogan et al., 2009; Bhat et al., 2010; R.
64 Czajkowski et al., 2011; Liao et al., 2003). However, these methods only slow down the
65 development of soft rot and do not effectively control the disease. Copper- and antibiotic-
66 resistant phytopathogenic bacterial strains were also reported (Behlau et al., 2011; Cooksey,
67 1990; McManus et al., 2002; Petriccione et al., 2017; Sundin & Bender, 1993). Due to the
68 limited success of the abovementioned control methods, and the currently growing global
69 threat of antimicrobial resistance, attention has been growing about the use of bacteriophages

70 or phages (viruses of bacteria) as a biocontrol agents against pathogenic bacteria (Buttimer et
71 al., 2017; Frampton et al., 2012; J.B. Jones et al., 2007; Jeffrey B. Jones et al., 2012).

72 Bacteriophages (phages) are viruses that can infect, replicate and lyse bacterial cells, When
73 environmental conditions are favorable for infection, they can effectively reduce bacterial
74 populations, especially in the case of lytic phages that undergo rapid replication (Hadas et al.,
75 1997; Sulakvelidze et al., 2001). Phages generally have high host-specificity, and they have no
76 direct negative effects on non-host cells. This allows the use of single-phage suspensions or
77 phage cocktail formulations to target only pathogenic bacteria. Such characteristic suggests the
78 use of lytic phages to be a promising alternative control method against bacterial soft-rot
79 (Buttimer et al., 2017; J.-A. Lim et al., 2017). The first *Pectobacterium* spp. phage, My1, was
80 reported in 2012 and several additional novel phage isolates were subsequently reported
81 (Buttimer et al., 2018, 2020; Kalischuk et al., 2015; Lee et al., 2012; J.-A. Lim et al., 2014,
82 2015, 2017; J. A. Lim et al., 2013; Pedersen et al., 2020; Voronina et al., 2019).

83 In this study, virion morphologies, growth characteristics and stabilities of three lytic
84 bacteriophages infecting *P. carotovorum* subsp. *carotovorum* were investigated. The lytic
85 activity and efficacy of the phages to prevent tissue maceration caused by *P. carotovorum*
86 subsp. *carotovorum* in potato slices using monophage and cocktail suspensions were also
87 determined. Based on *in vitro* and semi *in planta* assays, we found these lytic phages as
88 promising and effective candidates for the biocontrol of bacterial soft rot caused by *P.*
89 *carotovorum* subsp. *carotovorum.*

90 ## 2. Materials and Methods

91 ### *2.1. Bacterial Strains and Media*

92 *Pectobacterium carotovorum* subsp. *carotovorum* BIOTECH 1752, obtained from the Philippine
93 National Collection of Microorganisms (National Institute of Molecular Biology and Biotechnology,
94 UPLB) was used as the primary host for phage isolation. A collection of 48 *Pectobacterium* spp. isolated
95 from vegetable growing areas and market centers in the Philippines (Host-Pathogen Interaction
96 Laboratory, IWEP, UPLB) and eight other representative strains from other genera (Microbiology
97 Division, IBS, UPLB), were used for the host range assay (Supplementary Table 1). For routine tests
98 and maintenance, bacteria were grown in Tryptone Soya Agar (TSA, HiMedia®) for 16-18 hrs at 30°C.
99 For liquid preparations, bacterial cultures were grown in Tryptone Soya Broth (TSB, HiMedia®) for
100 12-18 hrs at 30°C with 125 rpm agitation.

101 ### *2.2. Bacteriophage Isolation and Characterization*

102 #### *2.2.1. Isolation and Purification of Bacteriophages*

103 Bacteriophages were isolated from rhizosphere soil, symptomatic tissues, and sewage water as
104 described by Czajkowski et al. (2014). Twenty grams (or ml) of each sample was homogenized
105 in 20 ml saline-magnesium (SM) buffer ($MgSO_4$, NaCl, $CaCl_2$, and Tris-HCl, pH 7.5), and
106 incubated at 30°C for 12-16 hrs. Five hundred µl of log phase *P. carotovorum* subsp.
107 *carotovorum* culture was added to each enrichment set-up and was incubated as above.
108 Enrichment cultures were centrifuged at 3,000 rpm for 15 minutes and the supernatants were
109 chloroform-treated in 1:20 chloroform/supernatant ratio for at least 10 min. The resulting
110 lysates were screened for the presence of bacteriophages by spot test assay (Mazzocco et al.,
111 2009). Overlay mixtures, containing 4.5 ml soft agar (TSA), 0.5 ml 20 mM $CaCl_2$, and 300 µl
112 of log phase *P. carotovorum* subsp. *carotovorum* culture were poured on Petri plates containing
113 pre-solidified TSA. Five µl of the phage lysates were spotted on the overlay and the spots were
114 absorbed into the agar for 10-15 min prior to incubation of plates at 30°C for 12-16 hrs. Zones
115 of lysis on the bacterial lawn indicated the presence of phages.

116 Phage purification was done using the double layer plaque assay (Kropinski et al., 2009). Ten-
117 fold dilutions were prepared from each positive lysate. In separate sterile 1.5-ml
118 microcentrifuge tubes, 300 µl of diluted lysate suspensions were mixed with 300 µl log phase
119 culture of *P. carotovorum* subsp. *carotovorum* in TSB. These were pre-incubated for 10 min at
120 4°C to allow adsorption of the phages. Molten overlay (4.5 ml) mixed with 0.5 ml 20 mM $CaCl_2$
121 was added to the suspensions and the resulting mixtures were poured on to Petri plates
122 containing pre-solidified TSA. The overlays were allowed to solidify for 15-20 min and the
123 plates were incubated at 30°C for 12-16 hrs. Well-isolated plaques were picked using sterile
124 pipette tips and the phages were suspended in 500 µl SM buffer. Bacteriophage isolates were
125 purified by successive single plaque isolation for at least five times or until uniform plaque
126 morphology was observed.

127 *2.2.2. Plaque Formation Using Double Layer Plaque Assay*

128 Plaque morphologies were characterized after a series of double layer plaque assays as
129 described above (Jurczak-Kurek et al., 2016). The transparency and morphology of the plaques
130 formed by each bacteriophage isolate was described and plaque diameter was measured using
131 a digital caliper.

132 *2.2.3. Bacteriophage Lysate Preparation and Titration*

133 Double layer plaque assay plates with pure phage plaques were flooded with 3-5 ml of SM
134 buffer to obtain the bacteriophage lysate. Plates were gently shaken for 15-20 min and the
135 overlay-SM buffer mixtures were collected and centrifuged at 8,000 rpm for 10 minutes. The
136 supernatants were chloroform treated in 1:20 chloroform/lysate ratio and were maintained at
137 4°C until titration. Lysates were subjected to double layer plaque assay (or spot test) for titration
138 as described above. Phage titer was determined and expressed as plaque forming units (PFU
139 ml^{-1}).

140 *2.2.4. Determination of Virion Morphology Using Transmission Electron Microscopy (TEM)*

141 High titer lysates (at least 10^{10} PFU ml^{-1}) of the three phage isolates were submitted to the
142 Electron Microscopy Laboratory of the Research Institute for Tropical Medicine (Muntinlupa
143 City, Philippines). The phage suspension was placed on carbon-coated copper grids, and 2%
144 aqueous uranyl acetate was added for three minutes to negatively stain the virions. Phages were
145 visualized by transmission electron microscopy (TEM; JEOLJEM-1220 Electron Microscope),
146 and the images were scanned with 800 ms exposure, 1.4 gain, and 1.0 bin with no sharpening,
147 normal contrast, and 1.00 gamma.

148 *2.2.5. Optimal Multiplicity of Infection (MOI)*

149 The optimal ratio of phage particles to the number of bacterial cells, also called the optimal
150 multiplicity of infection (MOI), was determined for the three phage isolates. *P. carotovorum*
151 subsp. *carotovorum* was infected with each phage isolate at four different PFU ml^{-1}/cell ml^{-1}
152 ratios (MOI): 0.01, 0.1, 1.0, and 10. Following overnight incubation at 30 °C, the suspensions
153 were chloroform-treated for 10 minutes (1:20) and were centrifuged at 8,000 rpm for 10
154 minutes. The supernatants were subjected to double layer plaque assay to determine phage titer.
155 The MOI demonstrating the highest phage titer or PFU ml^{-1} was considered optimal.

156 *2.2.6. Latent Period and Phage Burst Size*

157 One-step growth experiments were conducted to determine phage latent period and burst size
158 following previously described protocol (R. Czajkowski et al., 2014). Cells of *P. carotovorum*
159 subsp. *carotovorum* at log phase were harvested by centrifugation at 12,000 rpm for 15 min at
160 10°C, and were resuspended in TSB to an OD_{600} of 0.5 (c. 5 x 10^8 CFU mL^{-1}). Subsequently,
161 100 µl of 20mM $CaCl_2$ and 100 µl of the phage isolate were added into the bacterial culture at
162 a MOI of 0.1 (10^7 PFU mL^{-1}/ 10^8 CFU mL^{-1}). The phages were allowed to adsorb by incubation
163 at 4°C for 10 min and the suspensions were further incubated at 30°C for 10 min. The
164 suspensions were centrifuged at 12,000 rpm for 15 min at 10°C to remove unadsorbed phages.
165 The resulting pellet was resuspended in 10 ml fresh TSB. The mixtures were incubated at 30°C

166 for 60 min with shaking at 125 rpm. Aliquots were taken from the mixtures every 10 min and
167 the titer was determined using the Spot Test method. The burst size was calculated using the
168 formula: $burst\ size = \frac{PFU_{final} - PFU_{initial}}{PFU_{initial}}$ and the experiment was repeated twice with the
169 same set-up and the results were averaged.

170 *2.2.7. Adsorption Rate*

171 Phage adsorption rate was determined using previously described protocol (R. Czajkowski et
172 al., 2014). One ml of *P. carotovorum* subsp. *carotovorum* (ca. 10^8 cfu mL^{-1}) was infected with
173 each phage isolate by adding 100 µl of phage lysate (ca. 10^8 pfu mL^{-1}) to reach MOI 0.1 and
174 the resulting suspensions were incubated at 30°C for 20 min. After 0 (control), 1, 2, 4, 10 and
175 20 min, 50 µl aliquots were taken from each suspension and added to 450 µl TSB. Ten-fold
176 dilutions in SM buffer were prepared and the titer of the unadsorbed phages were determined
177 using the Spot Test method as described above. The experiment was repeated twice with the
178 same set-up and the results were averaged. Phage adsorption was calculated as follows:
179 $\%adsorption = \frac{control\ titer - residual\ titer}{residual\ titer} x100.$

180 *2.2.8. Thermal Resistance and pH Stability*

181 Thermal resistance of each of the three phages was determined at 30, 40, 50 and 60 °C. Phages
182 (10^8 PFU ml^{-1}) in SM buffer were incubated for 1 hr at each temperature condition and the
183 surviving PFU was determined using double layer plaque assay (R. Czajkowski et al., 2014).

184 The effect of pH on phage stability was determined using phages (10^8 PFU ml^{-1}) in SM buffer
185 within a pH range of 3.0 to 12.0. The experiment was carried out by incubating the phage
186 suspensions at RT for 16-24 hrs and the surviving PFU was determined using double layer
187 plaque assay.

188 *2.2.9. Host Range Determination*

189 The host range of the three phage isolates was determined using a collection of 48
190 *Pectobacterium* spp. isolates and eight other representative strains from different genera
191 (Supplementary Table 1). *Pectobacterium* spp. were isolated in the years 2017-2019 from
192 symptomatic tissues of various vegetable crops.

193 For the spot test assay, bacterial cultures were grown in TSB at 30°C for 12-16 hrs with agitation
194 at 125 rpm. From the resulting bacterial cultures, 300 µl of each was inoculated into the overlay
195 agar and was subsequently poured into a prepared TSA plate and allowed to solidify at room
196 temperature for 30 min. Five µl of the phage lysate (10^9 PFU ml^{-1}) was spotted onto the top agar
197 layer, and the plates were allowed to dry at room temperature for 10-15 min. Following
198 overnight incubation at 30ºC, plates were inspected for lysis zones.

199 ***2.3. Lytic Activity and Biocontrol Assays***

200 *2.3.1. In Vitro Challenge Test*

201 Bacterial inactivation using monophage (PPc_A3, PPc_D1, and PPc_J3) and cocktail
202 treatments (PPc_A3+D1, PPc_A3+J3, PPc_D1+J3, and PPc_A3+D1+J3) was determined
203 following the method described by Chen et al. (2018). For monophage treatments, log phase
204 culture of *P. carotovorum* subsp. *carotovorum* (OD_{600} of 0.5, c. 5 x 10^8 cfu mL^{-1}) was mixed
205 with the phage lysate at optimal MOI. For cocktail preparations, low (0.001) and high (100)
206 MOI were used for the experiment. The bacteria-phage mixtures were incubated at 30°C with
207 shaking at 125 rpm and the OD readings at 600nm were recorded (SPECTROstar^{Nano}) at 15-
208 minute intervals for eight hrs to monitor the changes in bacterial density.

209 *2.3.2 Semi-In Planta Assays*

210 *2.3.2.1 Phage Biocontrol Using Monophage and Cocktail Treatments on Tissue Slices*

211 The efficacy of monophage and cocktail treatments against soft rot caused by *P. carotovorum*
212 subsp. *carotovorum* was evaluated using a potato slice assay (Robert Czajkowski et al., 2015).

213 Potato tubers were surface sterilized by soaking in 70% ethanol for 10 minutes, washed with
214 sterile distilled water, and air dried under the laminar flow hood. The tubers were cut into 7.0
215 mm-thick transverse disks using sterile knives and three wells (5-mm diameter) were made on
216 each slice using a sterile cork borer. For monophage treatments, log phase culture of *P.*
217 *carotovorum subsp. carotovorum* was mixed with the phage lysates at optimal MOI. For
218 cocktail preparations, low (0.001) and high (100) MOIs were used for the experiment. Three
219 potato slices were used for each treatment. Wells were filled with 20 µl of the bacteria-phage
220 mixtures, 20 µl of sterile SM buffer as negative control, and 20 µl of *P. carotovorum* subsp.
221 *carotovorum* (10^8 cells ml^{-1}) as positive control. The diameter of the rotten tissues around the
222 wells inoculated with bacteria and wells co-inoculated with bacteria and phages were measured
223 after incubation at 30°C for 72 hrs. Treatment means were compared using one-way ANOVA
224 with Tukey's post-test ($\alpha = 0.05$).

225 # 3. Results

226 ## *3.1. Isolation and Characterization of Bacteriophages*

227 ### *3.1.1. Isolation of Lytic Bacteriophages*

228 Bacteriophages PPc_A3, PPc_D1 and PPc_J3 were isolated from rhizosphere soil,
229 symptomatic tissues and sewage water, respectively, using *P. carotovorum* subsp.
230 *carotovorum.* All three phages formed clear plaques typical for lytic phages, with varying sizes
231 (Table 1).

232 ### *3.1.2. Virion Morphology*

233 Examination using TEM revealed that the three phages belong to families *Podoviridae* and
234 *Myoviridae* of Order *Caudovirales*: (Table 1, Figure 1). Phage PPc_A3 belongs to the
235 *Podoviridae* family, with an icosahedral head of 80 nm and short non-contractile tail of 20 nm
236 (Table 1). Phages PPc_D1 and PPc_J3 are members of the *Myoviridae* family, with icosahedral
237 heads (75 nm) and rigid contractile tails (95-135 nm) (Table 1, Figure 1). Based on the proposed
238 unified phage naming, the phages should be formally named vB_PcaP-A3, vB_PcaM-D1 and
239 vB_PcaM-J3 (Kropinski et al., 2009) .

240 ### *3.1.3. Characterization of Bacteriophages*

241 All three phages exhibited strong adsorption to *P. carotovorum* subsp. *carotovorum,* and
242 approximately 90% of the phage particles adsorbed to the bacteria within 5 minutes (Figure 2).
243 PPc_A3 and PPc_D1 showed similar infection kinetics with a latent period of 10 min and a
244 burst size of 142-151 progeny phages/cell, whereas PPc_J3 showed a longer latent period of 20
245 min and a smaller burst size of 42 progeny phages/cell (Table 1, Figure 2A & 2B). Among the
246 three phages, PPc_A3 has the lowest optimal MOI (0.01), followed by PPc_D1 (1.0) and
247 PPc_J3 (10.0) (Table 1). All of the three phages retained infectivity at 30°C, 40°C, and 50°C,
248 and in the pH range 3-9 (Figure 2C).

249 *3.1.4. Host Range*

250 Spot test assay revealed that the three phages showed antibacterial activity specific for *P.*
251 *carotovorum* subsp. *carotovorum*. None of the three phages was able to lyse the 48
252 *Pectobacterium* spp. isolates and the other eight representative strains from other genera.

253 ***3.2. Lytic Activity and Biocontrol Assays***

254 *3.2.1. Phage Activity In Vitro*

255 Killing curves established for PPc_A3, PPc_D1 and PPc_J3 showed that all three phages were
256 highly effective against *P. carotovorum* subsp. *carotovorum* at optimal MOI (Figure 3A). The
257 bacterial cell density remained less than 0.05 (OD_{600}) throughout the 8-hr observation period.
258 The absence of any increase in OD_{600} reading also suggests that there is no observed phage
259 resistance development of the host at least in the period of observation (Muturi et al., 2019).
260 These results indicate that monophage treatments of PPc_A3, PPc_D1 and PPc_J3 based on
261 their optimal MOIs were equally effective in controlling *P. carotovorum* subsp. *carotovorum*
262 *in vitro*.

263 For the cocktail treatments, *in vitro* challenge tests were performed using low MOI (0.001) and
264 high MOI (100). Each phage was used at the same concentration and the phage cocktails
265 consisting of two or three phages were combined and evaluated. At MOI of 0.001, the killing
266 curves of the phage cocktails composed of two or three phages maintained its bacterial
267 inactivation with an $OD_{600} < 0.05$ for 8 hrs (Figure 4A). It appears that a phage cocktail
268 containing any of the three phages had the ability to control bacterial growth at low MOI during
269 the 8-hr observation period. At MOI 100, killing curves showed that all cocktail treatments
270 were highly effective against *P. carotovorum* subsp. *carotovorum* (Figure 4A). The bacterial
271 cell density remained less than 0.05 (OD_{600}) throughout the duration of the experiment.

272 *3.2.2. Phage Biocontrol Using Monophage and Cocktail Treatments on Tissue Slices*

273 The average diameter of rotting tissue was significantly lower in all monophage treatments than
274 in the set-up inoculated with *P. carotovorum* subsp. *carotovorum* only (Figure 3B-D). The set-
275 up co-inoculated with *P. carotovorum* subsp. *carotovorum* and PPc_A3 exhibited the lowest
276 diameter of rotting tissue among the three treatments (Figure 3B-D). Phage PPc_A3 was able
277 to reduce potato tuber tissue maceration to at least 60%, while phages PPc_D1 and PPc_J3 were
278 able to reduce tissue maceration to at least 50% of that observed for the control potato slices
279 inoculated with *P. carotovorum* subsp. *carotovorum* only (Figure 3B-D). All of the phage
280 cocktail treatments completely prevented tissue maceration at low and high MOI (Figure 4B-
281 D).

282 ## 4. Discussion

283 In this study, three lytic bacteriophages infecting *P. carotovorum* subsp. *carotovorum* designated as
284 PPc_A3, PPc_D1, and PPc_J3, were isolated from various environmental samples in the Philippines
285 such as rhizosphere soil, symptomatic tissues, and sewage water. We examined their morphological
286 characteristics and found that PPc_A3 belongs to *Family Podoviridae* similar to phages PP1 and PP2
287 (J.-A. Lim et al., 2017; J. A. Lim et al., 2013). PPc_D1 and PPc_J3 are members of the *Family*
288 *Myoviridae* like phages PM1 and PM2 (J.-A. Lim et al., 2014, 2015).

289 The latent period and burst size of each phage were also determined based on their one-step growth
290 curves, as well as their individual adsorption rates, optimal MOI, and stability on varying temperature
291 and pH levels. PPc_A3 has the lowest optimal MOI of 0.01. It has a latent period of 10 min and a
292 calculated burst size value of 142 PFU/cell. PPc_D1 has an optimal MOI of 0.1, latent period of 10 min
293 and a burst size of 151 PFU/cell. PPc_J3 has a high optimal MOI of 10, longest latent period of 30 min,
294 and the lowest calculated burst size of 42 PFU/cell. Since phages displaying shorter latent periods and
295 higher burst sizes are reported to provide better efficiency than phages with longer latent periods in
296 cultures where the density of the host is high, PPc_J3 showed less promising growth characteristics
297 compared to PPc_A3 and PPc_D1 (Abedon et al., 2003). All three, however, showed strong adsorption
298 to when about 90% of the phages were adsorbed within 5 min after incubation. When their stabilities
299 on varying temperature and pH levels were tested, all three survived incubations at 30°C-50°C and at
300 pH 3-9 but were inactivated at 60°C and at pH 12. These are important environmental factors in
301 biological control applications. Since temperatures under field conditions do not exceed 50°C and pH
302 levels usually range from pH 3.3-4.3, results indicate that PPc_A3, PPc_D1 and PPc_J3 can be suitable
303 for future field applications (Navarrete et al., 2017).

304 All three phage isolates also showed specificity to their isolation host, *P. carotovorum* subsp.
305 *carotovorum.* Phages are generally generally known to have narrow host ranges, typically limited to
306 strains within a certain species of their bacterial host (Ross et al., 2016). This can allow the formulation

307 of phage cocktails targeting bacterial species of interest only, which may be a certain bacterial plant
308 pathogen or a specific bacterium in a microbial community that negatively impacts plant growth or
309 health conditions. Developing a phage-based biocontrol product that can eliminate multiple genera, or
310 even different related species could be challenging,a but combining phages specific to target hosts may
311 overcome this problem (Buttimer et al., 2017; Frampton et al., 2012). The specificity of phages also
312 reduces the risk of harming the natural microbiota, especially those non-target bacteria that are
313 potentially beneficial, and limits the emergence of phage-resistant bacteria (Loc-Carrillo & Abedon,
314 2011).

315 Additionally, the efficacy of the three phage isolates, in both single-phage and cocktail suspensions
316 (combinations of two and three phages), were assessed through *in vitro* and semi-*in plant*a bacterial
317 challenge tests, and we found that both single phages and phage cocktails completely inactivate the
318 growth of *P. carotovorum* subsp. *carotovorum*. However, phages in cocktail suspensions were more
319 effective over individual application in terms of their protective effect against soft rot by *P. carotovorum*
320 subsp. *carotovorum* as indicated by the complete maceration reduction in both MOI (0.001 and 100)
321 tested in the study. Recent reports of phage cocktail formulations against *P. carotovorum* subsp.
322 *carotovorum* have also described efficient inhibition of bacterial growth both *in vitro* and *in vivo*
323 (Carstens et al., 2019; Muturi et al., 2019; Zaczek-Moczydłowska et al., 2020). Having a higher MOI
324 has also been reported to increase the chance for the bacteriophages to infect all host cells, effectively
325 killing all host cells after one round of replication (Alves et al., 2016; Thomas, 2001).

326 However, the use of higher MOI could also possibly increase the emergencebacterial variants that are
327 resistant to these phages(Chen et al., 2018). This is due to the rapid evolution of the bacterial population
328 due to the applied selection pressure that favors the propagation of phage-resistant clones over phage-
329 sensitive clones (Castillo et al., 2015; Chen et al., 2018; Hyman & Abedon, 2010; Middelboe, 2000;
330 Middelboe et al., 2001). This implies that the use of heavy phage loads is an important selection factor
331 that should be taken into account in phage therapy and that lower MOIs should also be used when
332 testing protective effect and bacterial inactivation by phages, particularly in actual plant tissues (Chen

et al., 2018). To this end, further experimental studies to optimize the phage treatment parameters and to identify the minimum effective phage number for potential field and post-harvest application should be conducted.

Data statement

All datasets presented in this paper are included in the article.

CRediT authorship contribution statement

Aeron Jade S. Parena: Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing-Original Draft, Writing-Review & Editing, Visualization. **Benji Brayan I. Silva:** Conceptualization, Methodology, Validation, Formal Analysis, Writing-Review & Editing. **Rae Mark L. Mercado:** Methodology, Validation, Investigation, Data Curation. **Adrian Adelbert A. Sendon:** Methodology, Validation, Investigation. **Freddiewebb B. Signabon:** Methodology, Validation, Investigation. **Johnny F. Balidion:** Conceptualization, Supervision, Project Administration, Funding Acquisition. **Jaymee R. Encabo:** Conceptualization, Data Curation, Writing-Review & Editing, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that the research was conducted with no known competing financial interests or personal relationships that could be construed as a potential conflict of interest.

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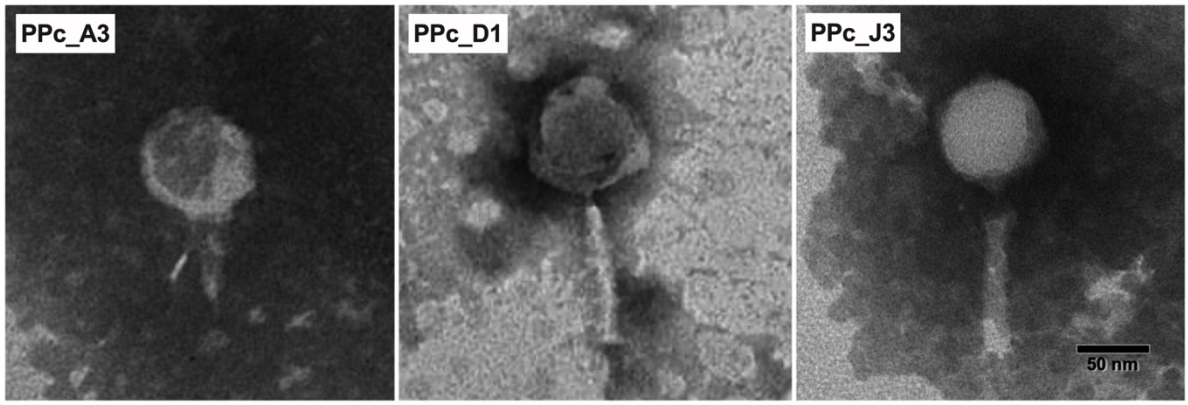
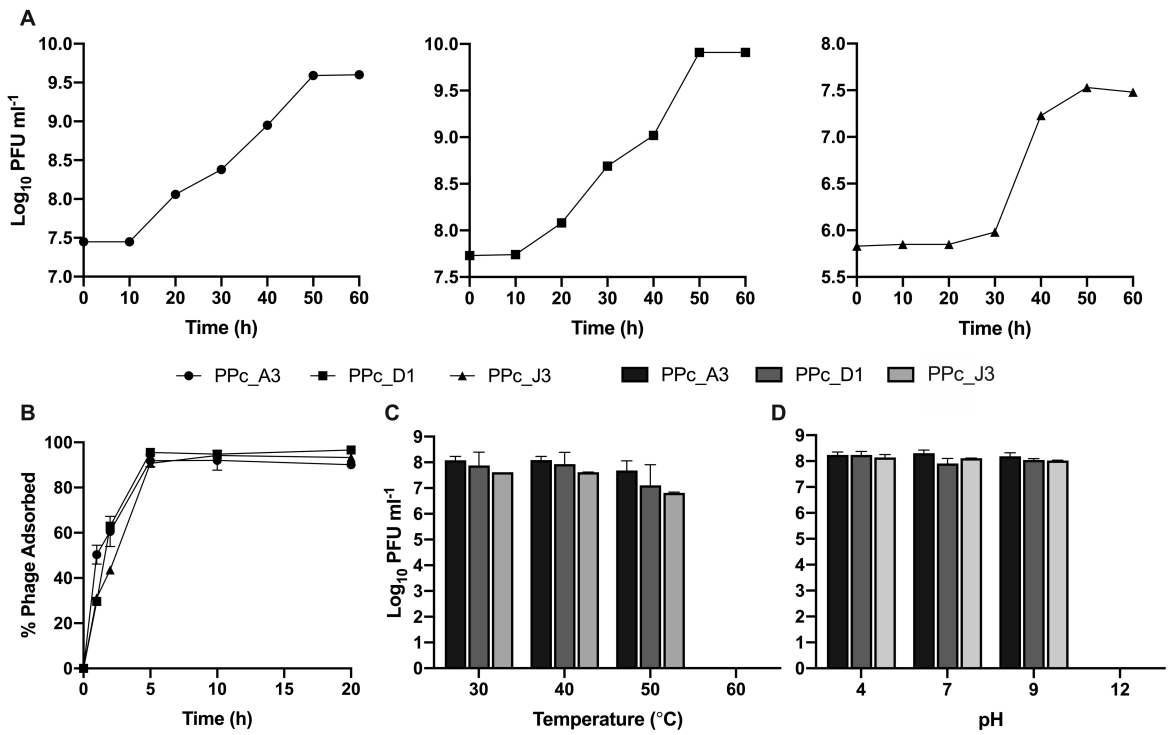
536 **Tables and Figures**

537 **Table 1.** Characteristics and biological properties of the three lytic bacteriophages isolated using *P. carotovorum* subsp. *carotovorum.*

Phage	Order	Family	Head diameter (nm)	Tail Length (nm)	Plaque Morphology		Optimal MOI	Latent Period (min)	Burst Size (PFU/cell)
					Transparency	Diameter (mm)			
PPc_A3	*Caudovirales*	*Podoviridae*	81.07 ± 7.04	19.58 ± 2.92	clear	2.0 - 3.0	0.01	10	142
PPc_D1	*Caudovirales*	*Myoviridae*	77.50 ± 4.68	98.15 ± 4.09	clear	1.0 - 1.9	0.1	10	151
PPc_J3	*Caudovirales*	*Myoviridae*	76.17 ± 2.81	137.08 ± 12.78	clear	< 1.0	10	30	42



538 **Figure 1. Transmission electron micrographs (TEM) of PPc_A3, PPc_D1, and PPc_J3 virions.**
539 High titer phage lysates were negatively stained with 2% aqueous uranyl acetate and were visualized
540 by transmission electron microscopy (TEM; JEOL JEM-1220 Electron Microscope). The images were
541 scanned with 800 ms exposure, 1.4 gain, and 1.0 bin with no sharpening, normal contrast, and 1.00
542 gamma. Bar represents 50nm.



543 **Figure 2. Growth characteristics of phages PPc_A3, PPc_D1, and PPc_J3 and their stability on**
544 **varying temperature and pH levels. (A)** One-step growth curve of each phage grown together with
545 log phase *P. carotovorum* subsp. *carotovorum* in TSB for 60 min at 30 °C. **(B)** Adsorption of each
546 phage to log phase *P. carotovorum* subsp. *carotovorum* in TSB for 60 min at 30 °C. **(C)** Stability of the
547 phages at various temperature and pH levels.

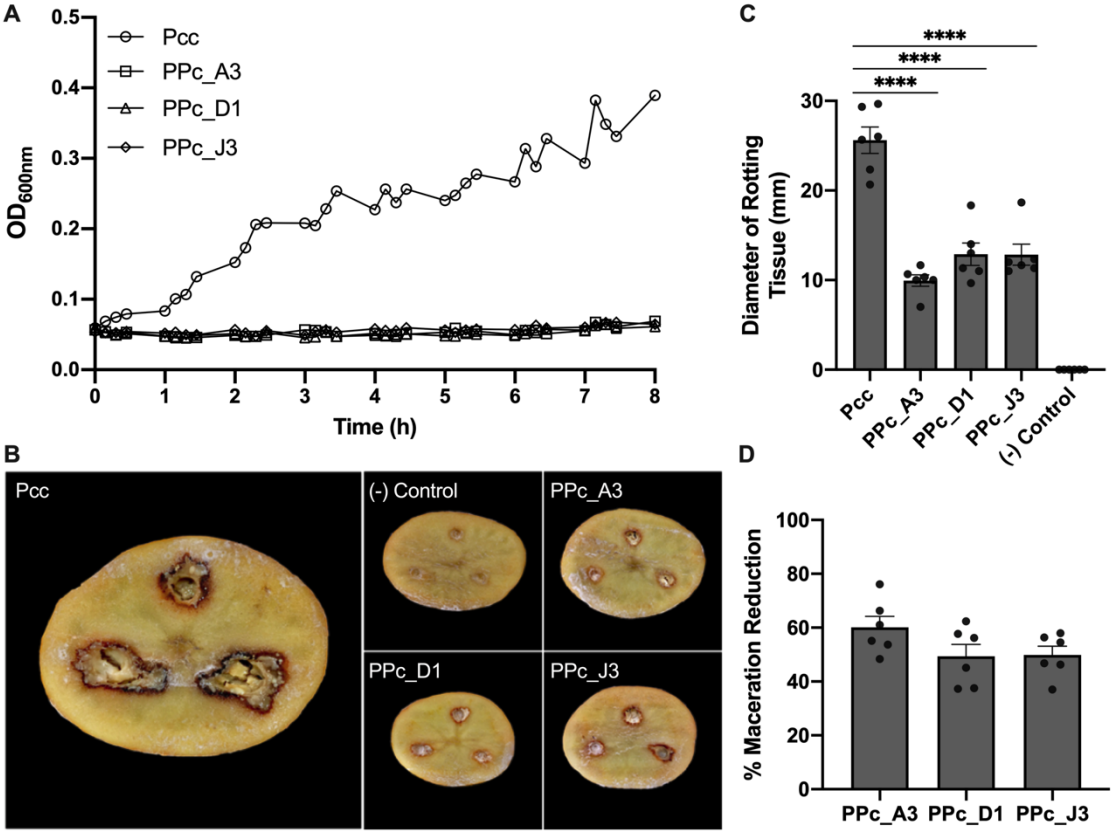


Figure 3. Bacterial killing curves of phages PPc_A3, PPc_D1, and PPc_J3 and their protective effect against *P. carotovorum* subsp. *carotovorum* on potato tuber slices based on optimal MOI. (A) Killing curves of phages PPc_A3, PPc_D1, PPc_J3 against *P. carotovorum* subsp. *carotovorum* measured as change in absorbance at OD=600 nm based on optimal MOI **(B)** Protective effect of each phage on potato tuber slices against *P. carotovorum* subsp. *carotovorum* determined by measuring the diameter of rotting tissue (in mm) after 72-hr incubation at 30°C. **(C)** Mean diameter of rotting tissue. **(D)** Percent tissue maceration reduction in tissue slices co-inoculated with *P. carotovorum* subsp. *carotovorum* and PPc_A3, PPc_D1 or PPc_J3. The data in **(A)** is mean ± SEM in duplicates (n=2). Semi-*in planta* assay **(B-D)** was representative of two independent experiments and was performed in triplicates (n=3) with three technical replicates each (n=3). Each dot in **(C-D)** is an individual potato slice (ie. replicate). Statistical significance was determined with one-way ANOVA with Tukey's post-test, ****$p < 0.0001$.

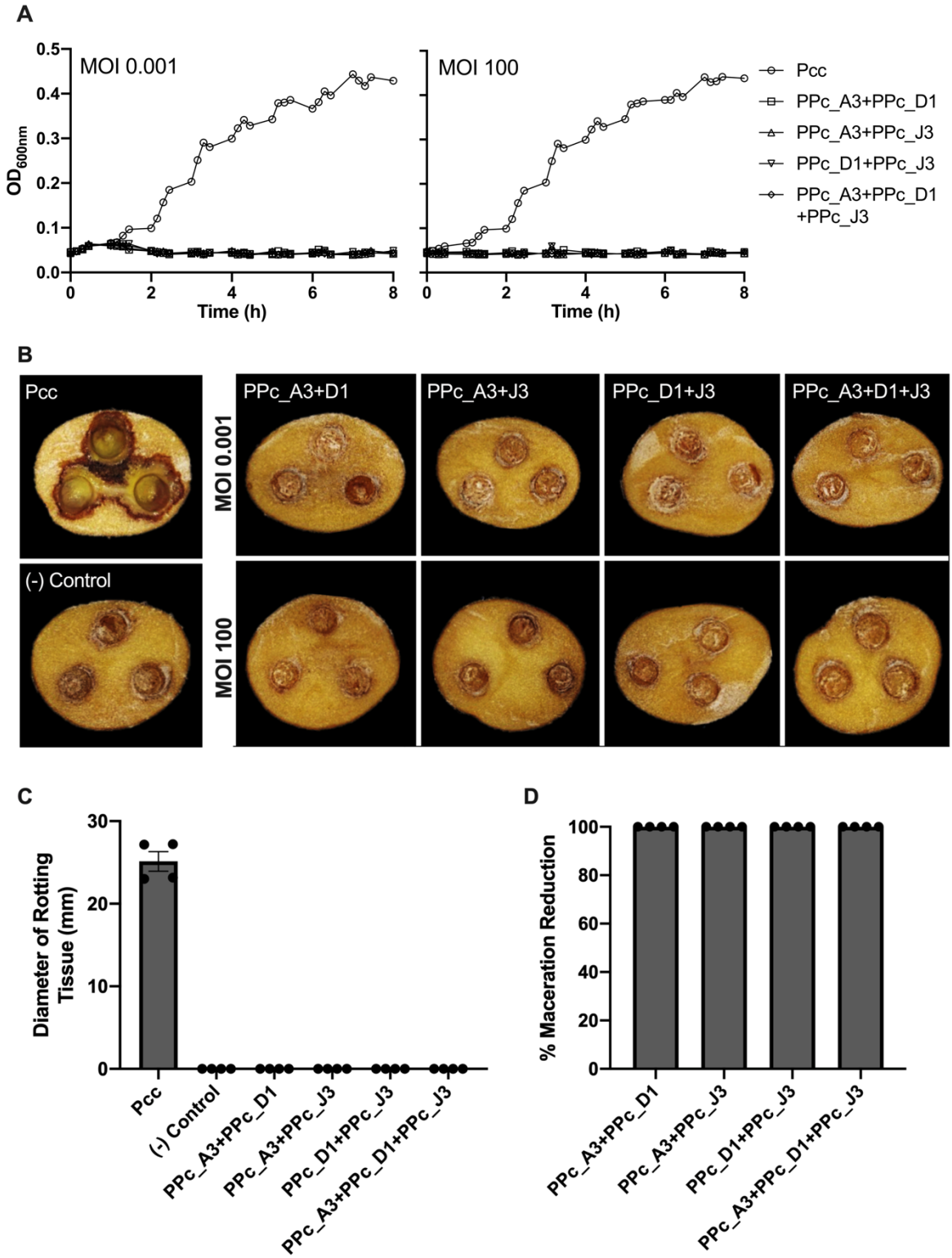


Figure 4. Suppression of *P. carotovorum* subsp. *carotovorum* by phage cocktails *in vitro* and semi-*in planta*. (A) Lytic activities of cocktails of two (PPc_A3+PPc_D1, PPc_A3+PPc_J3, and PPc_D1+PPc_J3) and three (PPc_A3+PPc_D1+PPc_J3) phages at low and high MOI against *P. carotovorum* subsp. *carotovorum* measured as change in absorbance at OD=600 nm. **(B)** Protective

564 effect of the phage cocktails on potato tuber slices against *P. carotovorum* subsp. *carotovorum*
565 determined by measuring the diameter of rotting tissue (in mm) after 72-hr incubation at 30°C. **(C)**
566 Mean diameter of rotting tissue. **(D)** Percent tissue maceration reduction in tissue slices co-inoculated
567 with *P. carotovorum* subsp. *carotovorum* and phage cocktails. The data in **(A)** is mean ± SEM in
568 triplicates (n=3). Representative images from the semi-*in planta* assay **(B-D)** were performed in two
569 independent experiments, each with duplicates (n=2) and three technical replicates each (n=3). Each
570 dot in **(C-D)** is an individual potato slice.