

Enhanced growth of cabbage seedlings by a *Paenibacillus* isolate in the presence of *Xanthomonas campestris* pv. *campestris*

H. Ghazalibiglar, J.G. Hampton, E. van Zijll de Jong and A. Holyoake

Bio-Protection Research Centre, PO Box 85084, Lincoln University, Lincoln 7647, New Zealand

Corresponding author: hoda.ghazali@lincoln.ac.nz

Abstract *Paenibacillus* spp. are rhizobacteria that can promote plant growth through a range of mechanisms. A New Zealand isolate of *Paenibacillus*, P16, has reduced the incidence of black rot, caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*), in brassicas. To investigate if this response was provided through plant growth promotion, isolate P16 was co-applied with *Xcc* as a seed treatment. In the presence of *Xcc*, P16-treated seedlings had significantly greater root length, leaf area, and root and shoot dry weight compared to the positive control (*Xcc* alone). There were no significant differences in plant growth parameters between P16-treated seedlings in the absence of the pathogen and the negative control (seeds without *Xcc* or P16). Isolate P16 enabled plants to survive and grow normally by preventing disease development; the mechanism of disease suppression requires further investigation.

Keywords biological control agent, black rot, brassica, *Paenibacillus* spp., plant growth parameters.

INTRODUCTION

Plant growth promotion by biological control agents (BCAs) has been reported in both the presence and absence of a pathogen. Under pathogen attack, BCAs can improve plant growth indirectly by reducing the impact of the pathogen. This may occur through antibiosis, competition for space and nutrients or induced systemic resistance (Gamalero & Glick 2011). Some BCAs also improve plant growth directly by means of phytostimulation, biofertilization and/or modulation of the plant abiotic stress response (Lugtenberg & Kamilova 2009). These direct effects may occur in the presence and absence of any pathogen threat.

Plant growth promotion can be one of the mechanisms involved in the biocontrol activity of *Paenibacillus*. Protection may be provided

by enabling plants to rapidly outgrow the stage when they are sensitive to the pathogen attack (Timmusk 2003). However, some negative effects of BCAs on growth and development of the host have also been reported (Timmusk et al. 2005). The protection provided by the BCA appears to come at a “fitness cost” (Heil 2002). The BCA may, through activation of the plant defense response, cause disproportionate re-allocation of plant resources away from plant growth towards protection against pathogen attack. The phytohormones produced by BCAs, in addition to those produced by the plant, may also negatively affect plant growth (Gamalero & Glick 2011).

Black rot, caused by the seed borne bacterium *Xanthomonas campestris* pv. *campestris* (*Xcc*), occurs in all cultivated brassicas and is regarded

as the most serious disease of brassicas worldwide (Williams 2007). From infected seedlings, the pathogen can spread readily within a crop, particularly in warm and wet conditions, and in severe outbreaks crop losses can reach 100% (Williams 2007). In New Zealand, *Xcc* is a problem for hybrid cabbage seed producers for whom production contracts require *Xcc*-free seed for export seed lots. Failure to do so can result in losses ranging from \$1500 to \$8000/ha (van Zijl de Jong et al. 2010). A New Zealand isolate of *Paenibacillus*, P16, has been shown to consistently reduce cabbage black rot caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*), when applied to the seed (Ghazalibiglar 2014). This paper reports on the effect of isolate P16 on cabbage plant growth promotion, both in the presence and absence of *Xcc*, to examine if the black rot reduction on cabbage was provided through this mechanism.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Isolate *Xcc*32 (isolated from cabbage seed and stored in the Smart Seeds Collection, Lincoln University, New Zealand) was streaked on Petri dishes containing yeast dextrose chalk agar (YDCA: 15 g/litre bacto agar, yeast extract 10 g/litre, CaCO₃ 20 g/litre, D-Glucose 20 g/litre; Asma et al. 2014) and incubated at 25°C in the dark for 72 h. Colonies of *Xcc* were then suspended in sterile 0.1% (w/v) bacteriological peptone (BP; Oxoid) water.

Paenibacillus isolate P16 (isolated from forage rape seed and stored in the Smart Seeds Collection) was cultured aerobically in 5 ml sterile Luria-Bertani broth (LB; Sigma-Aldrich) on an orbital shaker (MaxQ 5000, Barnstead) at 180 rpm, 30°C in the dark. After 24 h, 2 ml of the bacterial culture was transferred to a 250 ml conical flask containing 98 ml sterile LB and incubated on an orbital shaker at 180 rpm, 30°C in the dark for 18 h. *Paenibacillus* cells were then harvested by centrifugation (3220 × *g* for 20 min).

The concentrations of *Xcc* and *Paenibacillus* isolates were adjusted to 1×10⁹ and 5×10⁹ CFU/ml, respectively, in BP water using a spectrophotometer (Jenway, Model 6305). The quantity and purity of inocula were confirmed by the microdot method whereby an aliquot of

the inoculum was serially diluted in BP water and three 10 µl aliquots of selected dilutions were run across the surface of nutrient agar (Difco). Petri dishes were incubated at 28°C for 24 h in the dark. The actual inoculum concentration was calculated from the mean number of colonies.

Pot experiment setup

Seeds of cabbage cv. Kameron were immersed (3 ml per 1 g seed) in *Xcc* suspension of 1×10⁹ CFU/ml or sterile 0.1% BP water (negative control and P16 only treatment) and gently mixed under vacuum (ca 6.7 KPa) for 5 min. Seeds were collected by filtration through sterile Mira Cloth, and air-dried in open Petri dishes in a laminar flow cabinet overnight in the dark. The next day, 0.6 ml *Paenibacillus* suspension of 5×10⁹ CFU/ml or sterile 0.1% BP (positive and negative controls) was added per 1 g of seeds. Inoculated seeds were incubated in closed but unsealed Petri dishes in a laminar flow cabinet overnight in the dark.

In order to investigate the effect of the BCA on plant growth, a low nutrient potting mix was used containing Southland peat (600 litres/m³, New Zealand Growing Media), pumice (400 litres/m³; Egmont Commercial) and the following fertilizer additions per cubic metre: 1 kg Osmocote Exact Mini (Everris International; containing 16% N, 3.5% P, 9.1% K), 4 kg dolomite lime (Golden Bay Dolomite), and 1 kg Hydroflo (Everris International). The seeds were sown to a depth of 1 cm. One seed was sown in each cell of a 4-cell tray (25 cm³) for the early assessment times, and four seeds in the larger pots (1 litre) for the late assessment times. Cell trays and pots were placed in a growth room adjusted to a 13 h photoperiod at 400 µmol/m²/s light and 25°C, 11 h dark at 15°C, and 79% relative humidity, and were watered daily.

Assessment of pot experiment

Seven days after sowing seeds (DAS), seedling emergence was recorded. The effect of P16 on plant growth parameters and black rot incidence was assessed 14-35 DAS (Ghazalibiglar 2014). To avoid confounding effects due to the plants becoming root-bound, plants sown in the cell trays were only used for the early assessments (7, 14 and 21 DAS) and those in the large pots for

the late assessments (28 and 35 DAS). Plants were carefully uprooted at each sampling time, and separated into roots and shoots. The roots were washed in running tap water before scanning using a WinRhizo (Regent Instruments, Inc) to determine their total length (Himmelbauer 2004). The leaf area was measured by scanning all fully extended cotyledons and true leaves. Samples were then dried in an oven at 65°C for 2 days after which the dry weights were recorded.

The average of cabbage growth and black rot disease incidence were measured using the following equation for the area under the growth and disease progress curve (AUC) (Campbell & Madden 1990);

$$AUC = \sum_{i=1}^{n-1} \{[(y_i + y_{i+1})/2] \times (t_{i+1} - t_i)\}$$

where “n” is the number of evaluations, “y” is the percentage of disease incidence or growth parameter measurement, and “t” is the time in days after sowing seeds. The AUC values of growth and disease incidence, rather than individual values, were used in order to reflect the average growth and disease progress throughout the period of assessment.

Experimental design and statistical analyses

The experiment was run in two mini-trials for the two different pot sizes. Each mini-trial was set up as a randomized complete block design with 15 and 12 replicates for the cell trays and pots, respectively. Each replicate (block) contained 12 cell trays and 8 pots for the three early and two late assessment times, respectively. This allowed for destructive sampling at each assessment time. For each assessment time, four cell trays or pots were assessed from each block; one negative control (non-inoculated with *Xcc* or P16), one positive control (treated with *Xcc* only), one treated with P16 only, and one inoculated with both *Xcc* and P16 (a total of 180 cell trays and 96 pots from each mini-trial).

Statistical analyses of the data were performed using GenStat software (14th edition) and analysis of variance (ANOVA) was used to test the significance of the effect of seed treatment with P16 on black rot incidence. As there were no black rot symptoms on the seedlings that had

not been inoculated with *Xcc* (Negative control and P16 treatment), they were not included in the analyses of data. The influence of P16 and *Xcc* seed treatments on plant growth parameters was analysed for each assessment time separately in a 2 × 2 factorial design with P16 and *Xcc* as the treatment factors. The least significant difference (LSD P=0.05) was used to compare different treatments with each other. The interaction between P16 and *Xcc* was calculated as follows: [(*Xcc* + P16) - *Xcc*] - [P16 - Negative control], and the least significant interaction (LSInteraction (P=0.05) = LSD × √2) was used to test the significance of the interaction.

RESULTS

Paenibacillus and *Xcc* seed applications did not reduce cabbage seedling emergence in either the cell trays or pots compared to the negative control (Table 1). Isolate P16 significantly reduced black rot incidence compared to the positive control at all assessment times from the onset of symptoms (14 DAS) until the end of the experiment (35 DAS). The average black rot incidence from 14 to 21 DAS was reduced by 44% in the P16 treatment (P<0.001). Similarly from 28 to 35 DAS, P16-treated seedlings caused a 49% decrease in black rot incidence in comparison to the positive control (P<0.001). All cabbage seedlings that had not been inoculated with *Xcc* (negative control and P16 only treatment) were free of black rot symptoms.

Application of *Paenibacillus* isolate P16 to the seed in the presence or absence of *Xcc* altered plant growth in cabbage. Differences in root length, leaf area, root and shoot dry weights of cabbage were detected (Table 2). From 7-21 DAS *Xcc* had a negative effect on root length (P=0.027), root and shoot dry weights (P=0.033 and P=0.049, respectively) and no effect on leaf area (P=0.327), whereas P16 had no effect (P>0.05). In the first week, however, P16 significantly decreased root length (P=0.022), leaf area (P=0.016), root dry weight (P=0.011) and shoot dry weight (P=0.004). From 28-35 DAS all of the plant growth parameters were reduced by *Xcc* (P<0.001) but increased by P16 (P<0.05).

Up to 21 DAS there was a significant negative interaction between P16 and *Xcc* for root length

($P=0.034$). P16 caused a greater reduction in root length in the presence of *Xcc*. This was not detected at 28-35 DAS. A highly significant positive interaction occurred between *Xcc* and P16 at 28-35 DAS for both root and shoot dry weight ($P=0.003$ and $P=0.005$, respectively). In the presence of *Xcc*, P16 had a large positive effect on both root and shoot dry weight as compared to a small negative effect in the absence of the

pathogen. There was no statistically significant interaction between P16 and *Xcc* on the average leaf area during the early and late assessment periods ($P=0.124$ and $P=0.068$, respectively).

DISCUSSION

Some strains of *Paenibacillus* spp. have been reported to be plant growth promoting bacteria that enhance the growth of plants directly and/or

Table 1 Cabbage seedling emergence (%) and black rot incidence (%) at 7 and 35 days after sowing, respectively, for seeds treated with P16 and/or *Xcc* and untreated seeds.

Treatment	Seedling emergence		Disease incidence	
	Cell tray	Pot	Cell tray	Pot
Negative control	97	94	0	0
P16	92	92	0	0
<i>Xcc</i>	92	93	50	51
<i>Xcc</i> +P16	96	90	28	26
LSD ($P=0.05$)	6	8	10	12
Significance of difference ¹	NS	NS	***	***

¹***: $P < 0.001$; NS: not significantly different.

Table 2 Cabbage seedling root length (cm/plant) and root dry weight (mg/plant), leaf area (cm²/plant) and shoot dry weight (mg/plant) at 7-12 and 28-35 days after sowing seeds treated with P16 and *Xcc*.

	Root length		Leaf area		Root dry weight		Shoot dry weight	
	7-21	28-35	7-21	28-35	7-21	28-35	7-21	28-35
Xcc								
No <i>Xcc</i>	196	2836	9.6	185	8.4	209	44	1275
<i>Xcc</i>	184	2304	9.3	166	7.7	161	41	1038
LSD ($P=0.05$)	11	169	0.6	9	0.6	17	2.8	95
Significance ¹	*	***	NS	***	*	***	*	***
P16								
No P16	192	2375	9.5	168	8	174	42	1105
P16	188	2765	9.4	183	8	196	42	1207
LSD ($P=0.05$)	11	169	0.6	9	0.6	17	2.8	95
Significance	NS	***	NS	***	NS	*	NS	*
Treatment means								
Negative control	192	2723	9.39	181	8.08	212	42.6	1294
P16	201	2950	9.77	189	8.68	207	44.1	1256
<i>Xcc</i>	192	2028	9.55	154	7.97	137	41.6	917
<i>Xcc</i> +P16	176	2579	9.07	178	7.38	185	39.5	1158
LSD (5%)	16	239	0.78	12	0.91	24	4.0	134
Interaction ²	-24	324	-0.86	16	-1.20	53	-3.6	279
LSInteraction ³ ($P=0.05$)	22	337	1.11	17	1.30	34	5.6	190
Significance of interaction	*	NS	NS	NS	NS	**	NS	**

¹*: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ns: not significantly different.

²Interaction = [(*Xcc* + P16) - *Xcc*] - [P16 - Negative control].

³LSInteraction = $LSD \times \sqrt{2}$.

indirectly (Timmusk et al. 1999; Cakmakci et al. 2006). Isolate P16 applied to cabbage seeds alone (in the absence of *Xcc*) did not enhance any of the growth parameters in comparison to the negative control. *Paenibacillus* spp. have been shown to improve root development and consequently promote plant growth through the production of phytohormones such as auxins and cytokinins (Timmusk et al. 1999; da Mota et al. 2008). P16 may either not produce phytohormones or produce them at levels insufficient to stimulate plant growth, but this has yet to be assessed. Other factors may also have contributed because the effect of phytohormones on plant growth depends on soil type and plant variety (Bent et al. 2001; Egamberdiyeva 2007). The outcome of this study was similar to that reported by Lindberg et al. (1985) who found no significant growth promotion in wheat in response to *P. polymyxa* in the absence of a pathogen.

The effect of isolate P16 on plant growth promotion was indirect and appeared to be due to its biocontrol activity. It was observed that *Xcc* reduced plant growth parameters, but in the presence of P16 these negative effects were diminished. This was also associated with a reduction in black rot incidence. *Xcc* disrupts the growth of the plant by blocking the xylem vessels, causing leaf senescence and reducing photosynthesis (Williams 2007). By preventing the development of these symptoms, P16 decreased the negative effects of *Xcc* on cabbage growth. As already noted, *Paenibacillus* spp. indirectly promote plant growth through various mechanisms (Timmusk 2003; Senthilkumar et al. 2007; Lee et al. 2012) and a combination of these mechanisms may be employed. Ryu et al. (2006) reported that *P. polymyxa* applied to sesame seed indirectly promoted plant growth in the presence of a pathogen through root colonization and antibiotic production.

The beneficial effects of P16 appeared to be associated with an initial cost on plant fitness. Plant growth was reduced in P16 treatments in the first week. P16 may induce systemic resistance in the plant, a process that places heavy demands on plant resources and restricts the energy allocated for plant growth (Heil 2002; Phi et al. 2010). Alternatively P16 may compete with the emerging

seedling for available nutrients in the soil. *Brassica* seeds lack a true endosperm that in other seeds is used as a food supply to support the emerging seedling, so the young radicles may compete with microorganisms in the soil for nutrients during the early stages of plant growth before the cotyledons have developed. Moreover, the production of phytotoxins or phytohormones may lead to imbalances and contribute to a decrease in plant growth (Heil 2002). The application rate of the BCA could be reduced to decrease the cost of fitness, but this may impact on biocontrol performance (Ghazalibiglar 2014). In this study, the cost of fitness of P16 was transient and was not associated with any negative effect on seedling emergence.

In conclusion, in comparison to the control, isolate P16 enhanced cabbage growth in the presence of *Xcc*, but in the absence of the pathogen it did not. The results support the hypothesis that P16 can only indirectly promote the growth of the plant. The indirect effect of biocontrol agents on plant growth promotion has been reported to be due to preventing the deleterious effects of pathogens and a reduction in the level of the disease through different mechanisms including production of antibiotics, colonizing the root system and inducing plant systemic resistance. The mechanism(s) by which isolate P16 improves plant growth in the presence of *Xcc* requires further investigation.

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