https://doi.org/10.17221/77/2023-PPS

The combined application of *Bacillus velezensis* BCP6 and Jinggangmycin (JGM) to control soft rot caused by *Pectobacterium aroidearum* on *Amorphophallus konjac*

Mengjia Zhu[1#], Siyuan Ren[1#], Changlong Chen[2], Yu Tian[2], Zhijian Long[1], Zhiqiang Lin[1], Hua Xie[2], Ying Cao[1]**

[1]*School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, P. R. China*

[2]*Beijing Agro-Biotechnology Research Center, Beijing Academy of Agriculture and Forestry Sciences/ Beijing Key Laboratory of Agricultural Genetic Resources and Biotechnology, Beijing, P. R. China*

 # These authors have contributed equally to this work

**Corresponding authors: zhumengjia5954@163.com; xiehua@baafs.net.cn; caoying@swust.edu.cn*

Citation: Zhu M.J., Ren S.Y., Chen C.L., Tian Y., Long Z.J., Lin Z.Q., Xie H., Cao Y. (2023): The combined application of *Bacillus velezensis* BCP6 and Jinggangmycin (JGM) to control soft rot caused by *Pectobacterium aroidearum* on *Amorphophallus konjac*. Plant Protect. Sci., 60: 41–52.

Abstract: *Amorphophallus* spp. is an important group of crop and medicinal plants, but it is susceptible to infection by soft rot disease during both field growth and storage stages. This results in huge economic and yield losses, which must be properly addressed. Combined applications of Biological Control Agents (BCAs) and compatible chemicals have been recently considered as a more effective and reliable method to control bacterial soft rot. In the present study, we investigated the control effects against soft rot pathogenic bacteria *Pectobacterium aroidearum* MY11, using a BCA strain (i.e. *Bacillus velezensis* BCP6) and screening for three different bactericides, i.e. Jinggangmycin (JGM), Thiodiazole copper and Qingkulike. After exploring a joint application of BCP6 with chemicals, we found that JGM was the most effective and compatible bactericide to be compounded with BCP6. First, in the *in vitro* experiment, the mixture of JGM (34 mg/L) and suspension of BCP6 (1.0×10^8 cfu/mL) at 4:6 volume ratio performed with the strongest inhibitory effect on *P. aroidearum* MY11 (53.40%) and synergistic effect (1.78); this combination also significantly increased the biofilm production by BCP6, and constrained the swimming motility of *P. aroidearum* MY11 in agar plates and inhibited activities of cell wall-degrading enzymes. Second, the combined application of JGM and BPC6 reached up to 95.81% of control efficacy against *P. aroidearum* MY11 in a greenhouse experiment, and compared to JGM or BCP6 alone, combined application effectively increased konjac plant resistance to soft rot in the field, showing a synergistic action. Collectively, these results provided an alternative method for the management of soft rot disease in konjac planting.

Key words: *Bacillus velezensis*; JGM; konjac soft rot; synergistic effect

Amorphophallus spp. (Araceae) is an important economic crop and medicine plant as its tubers contain high levels of natural polysaccharide konjac glucomannan (KGM) (Chua et al. 2010). As a type of gum with high viscosity, KGM exhibits unique physical and chemical properties, which

Supported by the National Key Research and Development Projects (No. 2022YFE0199500) and the Sichuan Provincial Innovation Team Project of National Modern Agricultural Technology System (SC-CXTD-2023-12).

© The authors. This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC 4.0).

https://doi.org/10.17221/77/2023-PPS

have been widely used in food, pharmaceutical, and chemical industries (Devaraj et al. 2019). Annually, China produces 60% of the world's konjac output (Wootton et al. 1993; Douglas et al. 2005). *Amorphophallus* spp., especially *A. konjac*, is susceptible to contracting the bacterial soft rot disease (Wei et al. 2022), mainly caused by *Pectobacterium carotovorum* subsp. *carotovorum*, *P. aroidearum* and *P. chrysanthemi* (Wu et al. 2010; Wei et al. 2020). Moreover, not only does this disease damage konjac leaves and petioles in the fields during its growing season, but it also causes its tubers to rot during storage, which results in production losses of 20–30%, or more severe up to 80%, even total crop failure (Wang et al. 2019). The soft rot disease thus results in huge economic losses (Wang et al. 2019) without still any effective solutions at the moment. Therefore, developing countermeasures that control the soft rot disease is paramount to maintaining stable konjac production.

Biological control is one of the most promising strategies to tackle plant diseases. Biological Control Agents (BCAs) can, in theory, control pathogens by producing antimicrobial metabolites and/or increasing between-pathogen competition for nutrients or space (Ons et al. 2020); meanwhile, successful practices using BCAs (i.e., *Bacillus*, *Pseudomonas* and *Myxobacteria*) against soft rot pathogens has also been proven (Ghods-Alavi et al. 2012; Idowu et al. 2016; Li et al. 2018; Dong et al. 2021). Despite this progress, the recent combined applications of BCAs with bactericides or fungicides are considered a more reliable approach to managing plant diseases. This double-edged strategy presents at least two advantages to former approaches: *(i)* it can effectively reduce the reliance of BCAs on field environmental conditions while *(ii)* diminishing the dose used by chemical agents and so decreasing the risk of growing resistance (Ons et al. 2020). For instance, the combined application of *B. subtilis* plus chemicals (i.e., azoxystrobin and strobilurins) showed higher efficacy relative to their separate utilization to control zucchini powdery mildew and rice sheath blight (Gilardi et al. 2008; Liu et al. 2017). Ji et al. (2019) reported that the joint application of the fungicide Fluopimomide with *B. methylotrophicus* TA-1 also performed synergistically against gray mold in tomatoes. The application of *Trichoderma* spp. combined with thiophanate-methyl was also more effective than the independent usage of each treatment when controlling *Fusarium solani* and *F. oxysporum* in dry bean fields (Abd-El-Khair et al. 2019).

B. velezensis is a recently reclassified bacterial species producing secondary metabolites with biocontrol capacity against various plant pathogens (Devi et al. 2019; Rabbee et al. 2019). Nam et al. (2009) had earlier reported the biocontrol effect of *B. velezensis* BS87 against fusarium wilt in strawberries. To a significant extent, *B. velezensis* strains 5YN8, and DSN012 have been proven effective and significantly control the pepper gray mold disease and promote pepper growth (Jiang et al. 2018), as these strains were found to present high antagonistic and hydrolase activities.

In the previous research, a *B. velezensis* strain BCP6 isolated from the lettuce rhizosphere soil was found to have a broad antimicrobial activity *in vitro* against many phytopathogenic bacteria and fungi (Sun et al. 2020). This study explored the joint application of *B. velezensis* with bactericides to manage konjac soft rot caused by *P. aroidearum* MY11. Since the systemic bactericide Jinggangmycin (JGM) presents bacteriostatic activity against MY11 and so has biocompatibility with *B. velezensis* strain BCP6, we selected JGM to be compounded with BCP6. The present article aims to evaluate the control and synergistic effects of the combined application under three different conditions: (*i*) *in vitro* cultures, (*ii*) greenhouse, and (*iii*) field trials on konjac. Finally, the possible biocontrol mechanism against soft rot bacteria was also discussed.

MATERIAL AND METHODS

Plants, bacterial strains and bactericides. *A. konjac* cultivar (Beichuan moyu#) collected from Mianyang City (Sichuan province, China) were cultivated in greenhouse and field trials; meanwhile, soft rot pathogenic bacteria *P. aroidearum* strain MY11 (GenBank JACERL0000000000) was isolated from konjac tuber (Sichuan province, Cui et al. 2021). Collected from the rhizosphere of lettuce grown in Beijing, we isolated *B. velezensis* strain BCP6, which was identified using 16S rRNA sequencing (GenBank MN093795) and genomic analysis (VIQN00000000) (Sun et al. 2020). We stored the samples of *B. velezensis* and *P. aroidearum* strains in our lab to conduct antagonistic assays.

In addition, to carry out this experiment we used three different antipathogenic chemicals: *(i)* the sys-

https://doi.org/10.17221/77/2023-PPS

temic fungicide Jinggangmycin (JGM 20%, WP) (Huifeng Biochemistry Co. Ltd., Zhejiang Province, China); *(ii)* organic copper agent Thiodiazole copper (20%, SC) (Longwan Chemical Co. Ltd., Zhejiang Province, China); and *(iii)* botanical fungicide Qingkulike that comprises 0.5% berberine and 2.1% chlorogenic acid (Aofeng Crop Disease Control Co., Ltd, Weifang, China). They were diluted with sterile water to 10 mg/mL to prepare the stock solution.

Toxicity of three bactericides against *P. aroidearum* MY11 and their bio-compatibility with *B. velezensis* BCP6. To test the toxicity of three bactericides against *P. aroidearum* MY11, we calculated their EC_{50} (concentration for 50% of maximal effect) using the modified bacteriostatic ring method (Huang et al. 2015). In short, LB plates containing test bactericides with different concentrations (15, 30, and 60 mg/L) were prepared, and then filter paper discs (five mm diameter) soaked with a *P. aroidearum* MY11 suspension (1.0×10^8 cfu/mL) were placed at the centre each LB plate; sterile distilled water was used as control. After 48 h inside the 28 ℃ incubator, we measured the colony diameter (mm) of MY11 and calculated the inhibition rate as follows: inhibition rate (%) = (colony diameter of control – colony diameter of the chemical group) ⁄colony diameter of control. Each treatment was repeated four times.

The biocompatibility of bactericides with BCP6 was then evaluated with the method of time-dependent growth curves. BCP6 was inoculated in liquid LB medium supplemented with different concentration levels of chemicals (15, 30, and 60 mg/L) at 28 ℃ and 120 rpm; simultaneously, the bactericide-free medium was used as control. The optical density of the culture was measured at 600 nm with a spectrophotometer (Eppendorf-AG, Hamburg, Germany) after inoculation of 2, 4, 8, 16, 32, 54, and 68 hours.

Effects of *B. velezensis* BCP6 plus JGM against *P. aroidearum* MY11 *in vitro* and *in vivo*. The suspension of BCP6 (1.0×10^8 cfu/mL) and JGM solution (34 mg/L, EC_{50} value) were mixed at different volume ratios (Table 1). A dual culture bioassay evaluated the (antagonistic) activity of these mixtures against *P. aroidearum* MY11 *in vitro* (Lam et al. 2000). Filter paper discs (5 mm diameter) soaked with MY11 suspension (OD_{600} = 1.0) were placed at the centre of LB plates; subsequently, tested mixtures of BCP6 with JGM were inoculated at four cardinal points placed 25 mm from the centre of the plate using the filter paper disc, while the disc soaked with sterile water was used as control. Three replications were implemented. After three days of incubation at 28 °C, the colony diam-

Table 1. Co-toxicity of the combination *Bacillus velezensis* BCP6 and JGM to *Pectobacterium aroidearum* MY11

Treatments (V_{JGM}:V_{BCP6})[a]	Colony diameter/mm	Observed bacteriostatic rate (%)[b]	Expected bacteriostatic rate (%)	Synergistic effect[c]
Water (Control)	15.25 ± 0.11	—	—	—
0:10 (BCP6 alone)	10.08 ± 0.26	35.04^{bc}	35.04	1.00
1:9	9.68 ± 0.27	37.80^{b}	33.78	1.12
2:8	9.53 ± 0.83	38.78^{b}	32.52	1.19
3:7	9.06 ± 1.30	41.9^{ab}	31.26	1.34
4:6	7.37 ± 0.25	53.40^{a}	30.00	1.78
5:5	8.80 ± 0.85	43.73^{ab}	28.75	1.52
6:4	9.11 ± 0.61	41.63^{ab}	27.49	1.51
7:3	10.13 ± 0.03	34.71^{bc}	26.23	1.32
8:2	10.00 ± 1.06	35.57^{bc}	24.97	1.42
9:1	11.91 ± 0.31	22.63^{c}	23.71	0.95
10:0 (JGM alone)	11.94 ± 0.71	22.45^{c}	22.45	1.00

[a] Initial concentration of BCP6 suspension (1.0×10^8 cfu/mL) and JGM (34 mg/L, EC_{50} value)

[b] Observed bacteriostatic rate was evaluated relative to the water control after 24 h inoculation; 5 mm diameter of filter paper discs were used in each treatment, each value is the mean of three independent experiments; values in columns followed by the same letters are not significantly different according to Fisher's protected LSD test ($P < 0.05$)

[c] The Horsfall method was used to evaluate the synergistic inhibitory effect (Gu et al. 2017); the ratio of the observed bacteriostatic rate to the expected bacteriostatic rate > 1 indicates a synergistic effect

https://doi.org/10.17221/77/2023-PPS

eter (mm) was measured to calculate the inhibition rate: inhibition rate (%) = (colony diameter of control – colony diameter of treatment group)/(colony diameter of control – diameter of filter paper disc). Each treatment was repeated four times.

Moreover, a detached inoculated leaf assay was conducted to test the control effects of the combined application *in vivo*. For this purpose, the leaves of *A. konjac* were fully submerged in cell suspensions of BCP6 (1.0×10^8 cfu/mL) alone or in combination with the JGM solution with a 6:4 (*v*/*v*) ratio for 20 min, then the centre of leaves was infected with 15 µl *P. aroidearum* MY11 (OD_{600} = 1.0) using a syringe; water-treated leaves were used as control. All leaves were thereafter placed in Petri dishes with sterile wet filter paper on the bottom. We used two leaves in each plate and five plates in each treatment. Control effects were then estimated by leaf lesion lengths measured within 24 h post-inoculation (hpi) at room temperature.

Greenhouse pot and field bioassay. In the greenhouse pot experiment, *A. konjac* seeds were cultivated in 0.75 L plastic pots (one seedling per pot) containing sterilized peat and vermiculite (5:1, *v*/*v*); the greenhouse had 25 ℃ with 80–90% relative humidity and a photoperiod of 16 h. When *A. konjac* plants had been grown for 90 days, we pretreated the leaves of 20 cm height plants with 20 mL BCP6 suspension (1.0×10^8 cfu/mL) alone and in 6:4 (*v*/*v*) combination with JGM (34 mg/L). After five days, konjac plants with/without bacteria suspension pretreatments (i.e., BCP6 plants, BCP6 + JGM plants, JGM plants, and control plants) were infected at their base with *P. aroidearum* MY11 (OD_{600} = 1.0) using a syringe (20 µl each plant). There were six plants in each treatment, and the experiment was repeated thrice. Two days after inoculation, the severity of the disease was evaluated based on the 0–4 standard described by Wei et al. (2022). The disease index and control efficacy were then calculated using Peng et al. (2013) and Jiang et al. (2018).

As for the field bioassay, in 2021 and 2022, we selected two farmlands naturally infested with soft rot in Zitong and Jiangyou (Sichuan Province, China), respectively. The second-generation seeds of an *A. konjac* cultivar (Beichuan moyu#) were planted and divided into 12 plots; each plot had 20 m^2. We used a completely randomized design with four treatments (i.e., BCP6, BCP6 + JGM, JGM, and water control; Table 2); each treatment was replicated three times. When the konjac leaves had been fully extended (in June 2021 and 2022), these treatments were deployed with spray until water dripped from the leaves. The natural incidence rate of konjac soft

Table 2. Control efficacy of combined application of BCP6 and JGM against konjac soft rot in pot and field experiments

	Treatment *	Incidence rate (%)	Severity (%)	Disease index (%)	Control efficacy (%)	Control efficacy expected# (%)	Difference#
Pot	BCP6	52.72 ± 3.13	50.73 ± 4.51	25.37 ± 1.29	65.65 ± 4.11	–	–
	JGM	81.23 ± 7.61	49.35 ± 5.27	49.35 ± 3.35	33.18 ± 2.46	–	–
	BCP6 + JGM	1.32 ± 0.32	2.47 ± 0.15	3.10 ± 0.34	95.81 ± 7.07	77.05	+ 18.75
	water	99.45 ± 8.12	92.31 ± 8.41	73.85 ± 4.19	–	–	–
Field (2021)	BCP6	39.62 ± 2.34	–	–	52.86 ± 1.12	–	–
	JGM	69.31 ± 7.57	–	–	17.57 ± 1.32	–	–
	BCP6 + JGM	27.63 ± 1.16	–	–	67.86 ± 4.53	61.11	+ 6.75
	water	84.27 ± 9.05	–	–	–	–	–
Field (2022)	BCP6	42.75 ± 4.34	–	–	49.78 ± 6.12	–	–
	JGM	66.37 ± 5.23	–	–	19.23 ± 1.32	–	–
	BCP6 + JGM	32.58 ± 3.16	–	–	65.86 ± 5.53	59.44	+ 6.42
	water	87.82 ± 9.26	–	–	–	–	–

* BCP6 suspension (1.0×10^8 cfu/mL) and JGM solution (34 mg/L) were applicated separately or combined in the ratio of 6:4 (*v*/*v*)

The synergistic or antagonistic responses of the two treatments were calculated by the method of Colby (1967): E (control efficacy expected) = $I_J + I_N - I_J \times I_N/100$; where: I_J – the control efficacy of BCP6 suspension, I_N – the control efficacy of JGM; when the observed response is greater than expected, the combination is synergistic; otherwise, it is antagonistic

https://doi.org/10.17221/77/2023-PPS

rot was investigated in hot and humid seasons (in August 2021 and 2022). The control efficacy was calculated according to the methods of Jiang et al. (2018).

Biofilm assays of BCP6. Suspensions of BCP6 after co-cultured with JGM at different volume ratios (Table 1) were collected from 10 mL LB liquid medium. To develop the pellicle formation of BCP6, 2 µL BCP6 suspension (OD_{600} = 1.0) was added to the surface of MSgg (minimal salts glycerol glautamate) medium (200 µl) in a 96-well plate, which was sealed with parafilm. After 72 h of incubation at 28 °C static conditions, pellicles were dried and weighed. Each experiment was repeated six times.

Swimming motility assay of *P. aroidearum* MY11. Following Ahmed et al. (2022), we assessed the swimming motility of *P. aroidearum* MY11 using peptone-agar (1% peptone, 0.25% agar, and 0.5% NaCl) supplemented with the cell-free filtrate obtained from the co-culture BCP6 and JGM [6:4 ratio (*v*/*v*)]. Three other groups (control of LB liquid medium, BCP6 alone, and JGM alone) were set as a reference for comparison. Swim diameters were recorded at 48 h to check cell migration through agar. Data from three replicates were averaged.

Cell wall-degrading exoenzymes of *P. aroidearum* MY11. Cell suspension of *P. aroidearum* MY11 (OD_{600} = 1.0) mixed with LB at a 1:100 ratio (*v*/*v*) was subjected to different treatments at 180 rpm for 48 h at 28 ℃. Samples were centrifuged at 10 000 rpm for 10 min, and supernatants were collected and frozen at –80 °C until required. Pectate lyase (Pel) activity was determined by the formation of 4.5 unsaturated products, which were monitored spectrophotometrically at 235 nm (Andresen et al. 2007). Pel activity is defined as the amount of enzyme equivalent to producing 1 µm unsaturated products per minute per OD_{600} unit. Protease (Prt) activity was determined using the azocasein method following Jia et al. (2009). The enzyme unit is defined as the increase in absorbance at 436 nm per minute. Polygalacturonase (PG) activity was evaluated by estimating the increase in reducing groups (Smadja et al. 2004). One unit of PG activity is defined as the amount of enzyme that released 1 µmol of reducing groups per minute.

Data analysis. The Statistical Package for the Social Sciences (SPSS) statistics software (version 21.0) (IBM Corporation, Armonk, NY, USA) was used to analyze the results. When the ANOVA was significant ($P < 0.05$), statistical differences in means were assessed using the Fisher's protected least significant difference (PLSD) method.

RESULTS

Bactericide inhibition effects on *P. aroidearum* MY11 under culture conditions. Jinggangmycin (JGM), Thiodiazole copper, and Qingkulike are three bactericides belonging to various chemical groups, which are favoured in China because of their low cost, low toxicity, and low environmental residue (Peng et al. 2013; Feng et al. 2014). However, their efficacy against soft rot disease *P. aroidearum* is still unclear. Figure 1A shows that the three bactericides significantly suppressed the growth of *P. aroidearum* MY11 under culture conditions. Their inhibition efficiencies were correlated with the dose of bactericides. Among them, Thiediazole copper was the most effective performing with the lowest EC_{50} value (29.39 mg/L), followed by JGM and Qingkulike with EC_{50} values of 34.04 and 38.91 mg/L, respectively (Table 3).

Compatibility of bactericides with *B. velezensis* BCP6. Previous studies have indicated that *B. velezensis* strain BCP6 exhibited a stronger inhibitory activity against soft rot and sclerotia rot diseases on lettuce (Sun et al. 2020), so it might be a promising biocontrol agent. Our *in vitro* compatibility analysis showed that Thiodiazole copper suppressed the growth of BCP6; all doses were tested after 4 h culture; the inhibitory effect of Thiodiazole copper was especially significant during the BCP6 rapid-growth stage (Figure 1C). Conversely, BCP6 displayed good biocompatibility with JGM and Qingkulike for all doses tested; in addition, BCP6 was found the most insensitive to antibiotic JGM, as no differences in growth

Table 3. Toxicities of tested bactericides against *Pectobacterium aroidearum* MY11

Tested bactericides	Toxic regression equation	Correlation coefficient	EC_{50} (mg/L)
Jinggangmycin (JGM)	$y = 1.560x - 3.096$	0.9876	34.04
Thiodiazole copper	$y = 1.379x + 9.474$	0.9653	29.39
Qingkulike	$y = 1.096x + 7.356$	0.9405	38.91

EC_{50} – value id defined as the effective concentration that causes a 50 % reduction in bacterial growth rate

https://doi.org/10.17221/77/2023-PPS

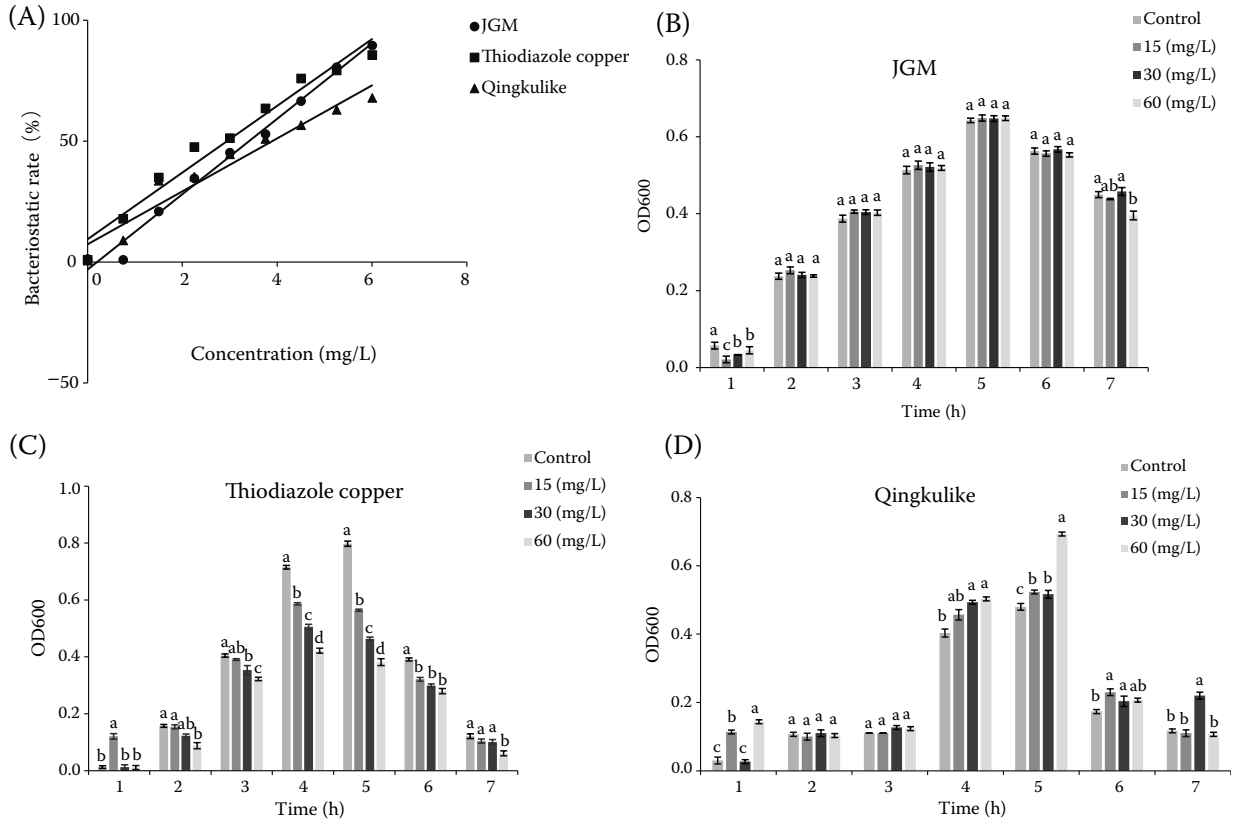


Figure 1. Effect of three bactericides on the growth of *Pectobacterium aroidearum* MY11 and antagonist BCP6 under culture conditions

A – Bacteriostatic rate of three bactericides to *Pectobacterium aroidearum* MY11; B–D – Biocompatibility of three bactericides with antagonist BCP6; values followed by the same letters are not significantly different according to Fisher's protected LSD test ($P < 0.05$)

patterns were observed between cultures with and without JGM (Figure 1B and 1D). Based on both its higher effectiveness against *P. aroidearum* MY11 and its higher compatibility with the antagonist BCP6– compared to the other tested agents, JGM was thus selected as the best compounded with BCP6.

Synergistic inhibitory effects of combined BCP6– and JGM against *P. aroidearum* MY11 under culture conditions. The inhibitory effects of combined *B. velezensis* BCP6 and JGM against *P. aroidearum* MY11 were evaluated at different volume ratios (Table 1). Our results showed that JGM and BCP6 reached a bacteriostatic rate of 34.0% and 20.6%, respectively (Table 1). Interestingly, the bacteriostatic rate substantially increased when we combined BCP6 and JGM. Their combination normally performed with synergistic effects, except for the treatment at a 9:1 volume ratio (Table 1). Furthermore, the mixture of JGM and BCP6 at a 4:6 volume ratio behaved with a noticeable inhibitory (53.40%) and synergistic effect (1.78). As a result, this combination was chosen for further analysis *in planta*.

In addition, when BCP6 was co-cultured with JGM at different (9:1, 7:3, and 4:6) volume ratios, the BCP6 biofilm formation was significantly increased. Furthermore, the pellicle weights produced in the MSgg medium increased by 5–6 times relative to weights measured in the absence of JGM, a difference that might benefit BCP6 colonization in plants (Figure 2).

Combined BCP6-JGM pretreatments enhance konjac resistance to *P. aroidearum* MY11. Firstly, inhibitory effects of BCP6 and JGM on *P. aroidearum* MY11 were investigated in detached konjac leaves. After MY11 infection, necrotic area in water-treated (control) leaves increased with prolonged infection time, reaching up to 75 mm^2 at 24 h post inoculation (hpi) (Figure 3). However, for BCP6- or JGM-treated leaves, the expansions of lesion areas

https://doi.org/10.17221/77/2023-PPS

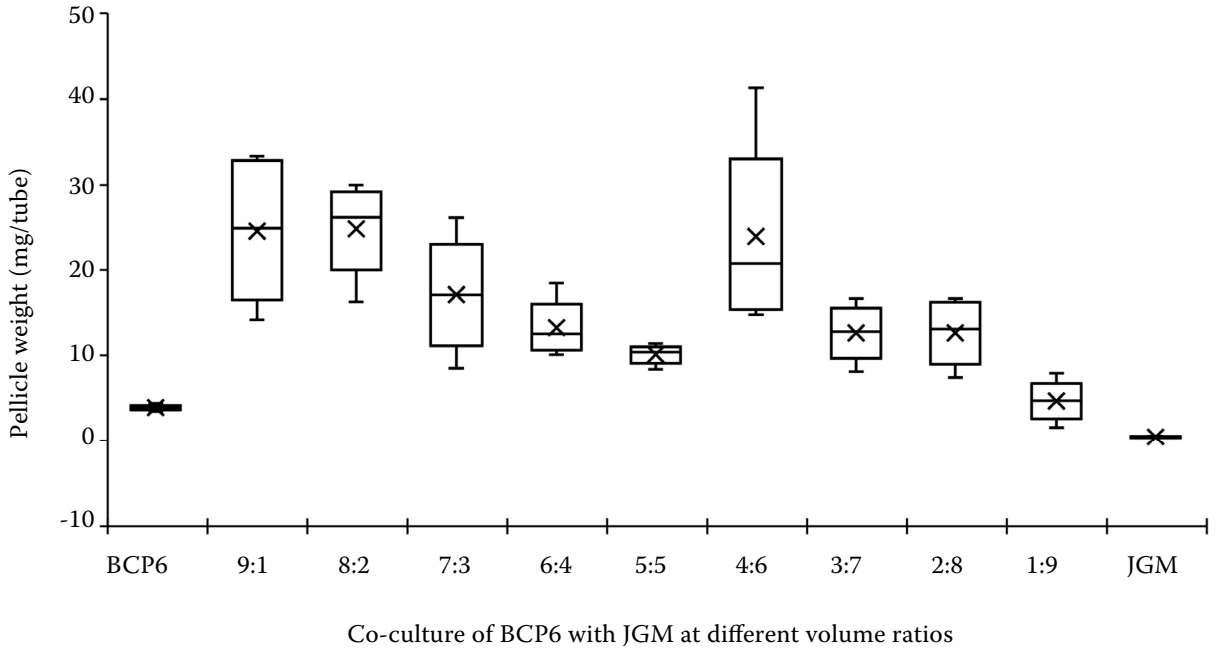
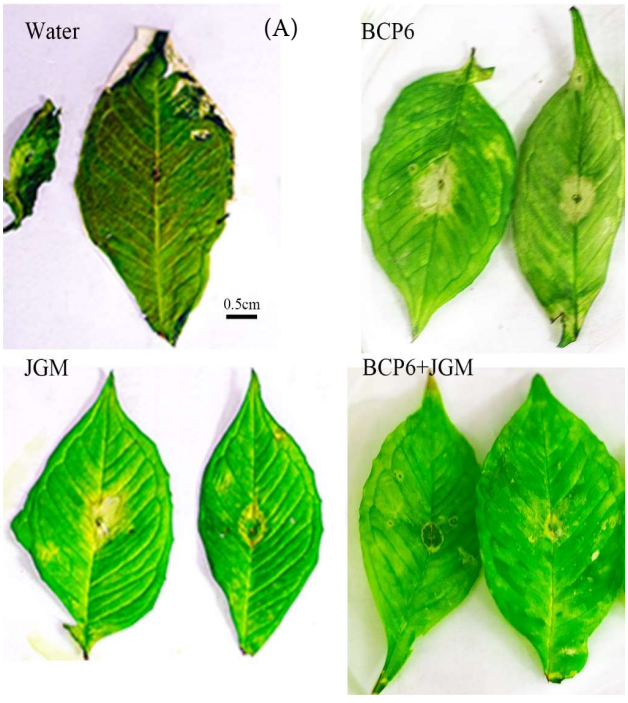


Figure 2. Biofilm formation of BCP6 when co-cultured with JGM at different volume ratios

were effectively inhibited (Figure 3). Furthermore, the combined application of BCP6 with JGM displayed a higher control effect, with less than 5 mm^2 of necrotic area within 24 hpi (Figure 3).



Secondly, after two days of *P. aroidearum* MY11 inoculation under greenhouse conditions, konjac seedlings pretreated with JGM or BCP6 displayed lower severity and disease index than non-pretreated plants. The control efficacy of BCP6 alone was 65.65%, higher than that of JGM treatment (33.18%). Meanwhile, the combined BCP6 and JGM pretreatment displayed a higher performance than in separate treatments (either of BCP6 or JGM), reaching a greater control efficacy (95.81%), which, therefore, highlights a significant synergistic effect of the combined BCP6-JGM pretreatment (Table 2).

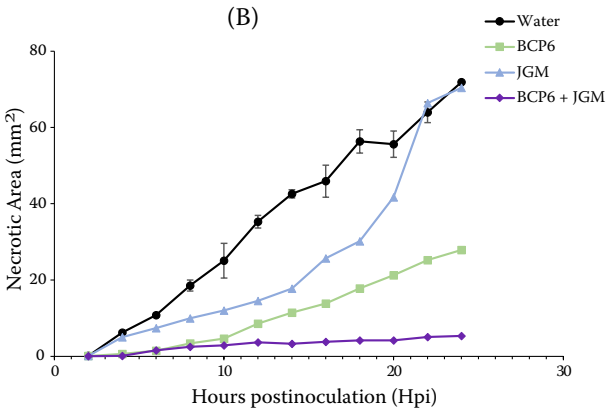


Figure 3. Control effects of BCP6 and JGM against *Pectobacterium aroidearum* MY11 on detached konjac leaves
(A) Phenotypes of konjac leaves upon MY11 infection at 18 hpi; (B) Control efficacy of BCP6 and JGM against *Pectobacterium aroidearum* MY11 in konjac leaves; the necrotic areas were measured within 24 hpi

https://doi.org/10.17221/77/2023-PPS

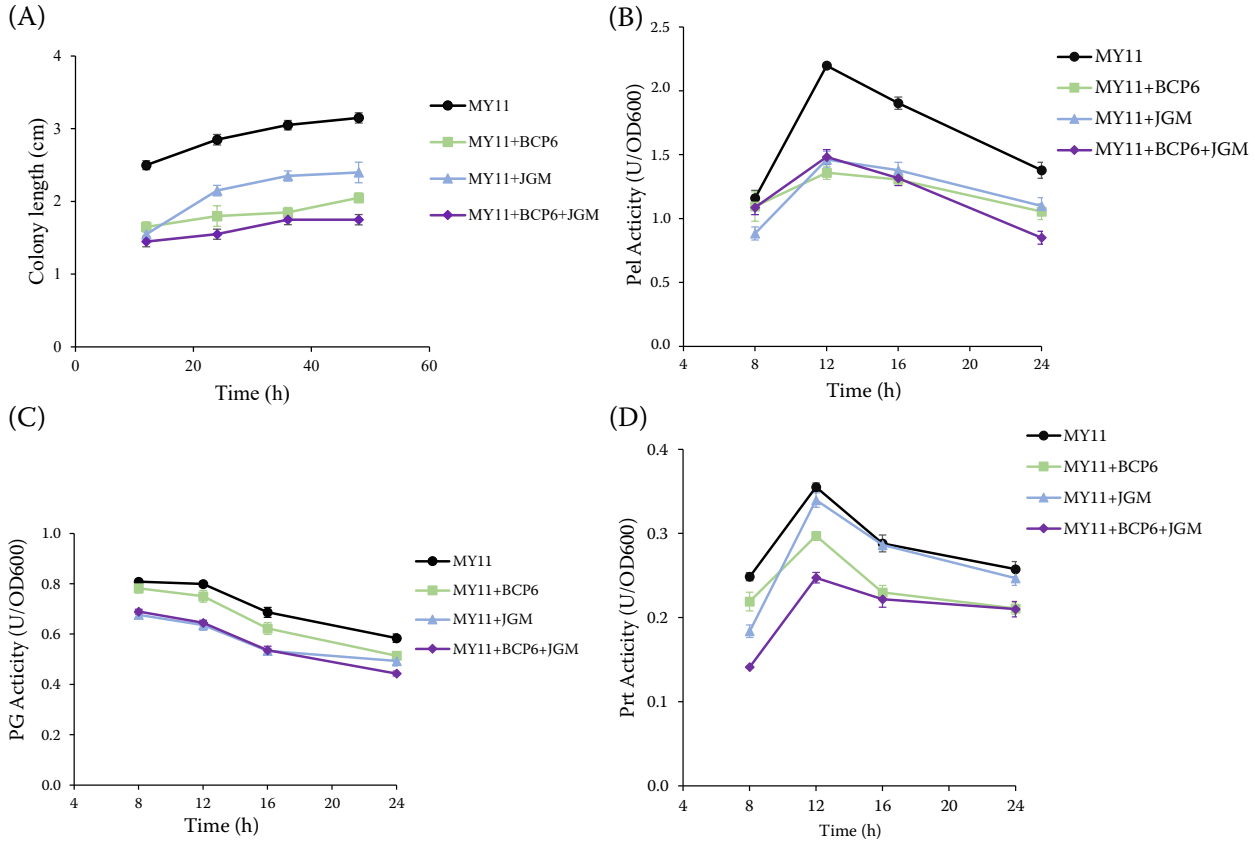


Figure 4. Effects of combined BCP6-JGM on swimming motility of soft rot bacteria MY11 (A) and activities of MY11 extracellular enzymes (B–D)

Thirdly, in the field experiments performed in 2021 and 2022, the combination of BCP6 and JGM effectively enhanced the resistance of konjac plants to soft rot disease compared to both independent pretreatments. This combined strategy rendered a net 6.42 (in 2022) or 6.75 (in 2021) synergistic effect, in terms of control efficacy of BCP6 and JGM separately (Table 2).

Effects of the combined BCP6-JGM treatment on soft rot bacteria virulence levels. Swimming motility is critical for soft rot bacteria to maintain high virulence levels (Tans-Kersten et al. 2004). As incubation prolongs, the swimming motility of *P. aroidearum* MY11 rises (Figure 4A): it increased by 3.15 ± 0.07 cm (swimming diameter) in 0.25 % agar plates after 48 h incubation. Compared with the control (MY11 alone), both BCP6 cell suspensions alone and the combined BCP6-JGM mix significantly inhibited MY11 swimming motility: the corresponding swimming diameters decreased by 34 % (2.05 ± 0.11 cm) and 44% (1.75 ± 0.14 cm), respectively, after being cultured for 48 hours (Figure 4A).

Plant-pathogenic enterobacterium *Pectobacterium* characteristically secrete extracellular plant cell wall degrading enzymes, including protease (Prt), pectate lyase (Pel), and polygalacturonase (PG), which cause tissue maceration to elicit soft rot disease (Czajkowski et al. 2011). Figure 4B–4D revealed that *P. aroidearum* MY11 extracellular enzymes were produced via a growth phase-dependent pathway, with higher activities in an early stationary phase after 12 h incubation. When grown under LB media supplemented with BCP6 cell suspensions or JGM, MY11-produced exoenzymes were significantly reduced: the activities of Prt, Pel, and PG (per OD_{600} unit) fell by 4.4 ~ 16.3 %, 33.5 ~ 38.1% and 6.1 ~ 20.4 % after 12 h incubation, respectively, relative to the control (Figure 4B–D), and for Pel and PG, the inhibitory effect was still observed after being cultured for 48 hours. The combined application of BCP6 and JGM achieved the most significant inhibitory effect for these exoenzyme productions. Still, they occurred at different time points: 12 h for Prt and 24 h for Pel and PG, respectively.

https://doi.org/10.17221/77/2023-PPS

DISCUSSION

Bacterial soft rot is a destructive disease during field growth and storage stages. It causes wilt, rot, and blackleg symptoms in potatoes, carrots, cabbage, sweet potatoes, and tomatoes, as well as on ornamental plants (Opara & Asuquo 2016). Soft rot disease causes devastating losses to crop plants worldwide, with up to 90% loss in tropical climates (Wasendorf et al. 2022). The control of bacterial soft rot is mainly dependent on agronomic techniques like crop rotation and chemical treatments. Still, these approaches have not so far achieved the desired outcomes. More recently, an integrated approach combining BCAs with chemicals has been considered more effective and reliable (Ons et al. 2020). In short, finding such an effective BCAs-chemical combined strategy is vital for controlling soft rot disease, especially under field conditions.

B. velezensis, a recently reclassified bacterial species (genus *Bacillus*), produces several secondary metabolites with biocontrol potential against various plant-pathogenic bacteria (Devi et al. 2019; Rabbee et al. 2019). For instance, Nam et al. (2009) reported the biocontrol effect of *B. velezensis* BS87 against fusarium wilt in strawberries. In addition, *B. velezensis* strains 5YN8, and DSN012 were found to have high antagonistic and hydrolase activity and significantly control pepper gray mold, promoting pepper growth (Jiang et al. 2018). In this article, *B. velezensis* strain BCP6 was found to effectively inhibit the growth of *P. aroidearum* MY11 (causing konjac soft rot disease) *in vitro* (Table 1) and *in vivo* (Figure 3 and Table 2); its cell-free filtrate significantly reduced the production of MY11 extracellular enzymes, especially for Pel and PG (Figure 4), suggesting that it might become a prospective agent to control soft rot disease.

JGM is a systemic fungicide produced from *Streptomyces* var. *jinggangen*. JGM cells are easily absorbed by bacterial cells and rapidly transported inside them, inhibiting their growth and development. JGM has been widely applied to plant-pathogen control, e.g. rice sheath blight (Peng et al. 2013). In this article, we found that JGM effectively suppressed the growth *in vitro* of *P. aroidearum* MY11 (causing soft rot disease), with an EC_{50} value of 34.04 mg/L (Table 3). This suggests its potential use to control konjac soft rot.

Previous studies have demonstrated that JGM presented higher biocompatibility with *Bacillus* spp. For instance, Chen et al. (2003) and Peng et al. (2013) found that combining JGM and *B. subtilis* Bs-916 or NJ-18 significantly increased control efficacy against rice sheath blight. Based on this experiment, we found that JGM also presented high compatibility with *B. velezensis* BCP6 (Figure 2B) as its growth in LB media was not affected by the addition of 15–60 mg/L of JGM; further, the combination of JGM and BCP6 resulted in heightened control against *P. aroidearum* MY11, both *in vitro* and under greenhouse conditions (Tables 1 and 2). Moreover, in farmlands naturally infested with soft rot, combined BCP6 and JGM also effectively increased konjac plant resistance to soft rot, relative to JGM or BCP6 alone. These findings provide a new approach to effectively preventing and controlling soft rot in konjac planting.

Various studies have revealed that biosurfactant lipopeptides iturin A, fengycin, and surfactin secreted by *Bacillus* species exhibit antibacterial and antifungal effects (Haddoudi et al. 2021). The underlying mechanism is related to the penetration capacity of *Bacillus* species through the lipid bilayer of the target cell cytoplasmic membrane. This disrupts the cell membrane, thereby increasing membrane permeability to pathogens (Carrillo et al. 2003; Guo et al. 2014). More precisely, *B. velezensis* had been found to generate similar secondary metabolites. For example, Devi et al. (2019) reported that three groups of bioactive lipopeptides (i.e., iturins, fengycins, and surfactins) were detected in the crude extract of *B. velezensis* strain DTU001, the mixture of these bioactive lipopeptides performing with higher antifungal efficacy than the single components. Jiang et al. (2018) speculated that the control capacity of *B. velezensis* against *B. cinerea* might be due to the suppression of pathogen growth by either secreting secondary metabolites or releasing the number of volatile organic compounds.

In this paper, we provide evidence of the cell-free filtrates of *B. velezensis* BCP6 significantly inhibiting the *P. aroidearum* MY11 swimming motility and constraining the activities of cell wall-degrading enzymes Prt, Pel, and PG (Figure 4) what, in turn, might lower the capacity by soft rot bacteria to infect the host plant (Table 2). More interestingly, the inhibitory effects on the swimming motility and activities of cell wall-degrading enzymes of *P. aroidearum* MY11 were enhanced after applying combined JGM and BCP6 (Figure 4). This sug-

https://doi.org/10.17221/77/2023-PPS

gests the synergistic antibacterial effect might be related to the presence of secondary metabolites of *B. velezensis* BCP6, which increases the sensitivity of *P. aroidearum* MY11 to JGM.

B. subtilis is a gram-positive bacterium that forms biofilms on inert surfaces. Biofilm matrix protects bacteria from stress and hostile environments (Kovács et al. 2012), so it is required for plant colonization (Beauregard et al. 2013; Gallegos-Monterrosa et al. 2016). *B. subtilis* cells are capable of sensing and responding to diverse extracellular cues that include internally-produced signals (e.g., surfactin) and external environmental cues like natural byproducts secreted/generated by other organisms in the soil and certain plant polysaccharides (Beauregard et al. 2013). *B. velezensis* can produce a similar biofilm to other closely related *Bacillus* species (Devi et al. 2019). When it was combined with JGM at different volume ratios, the biofilm productions of BCP6 were significantly enhanced relative to that grown in the absence of JGM, indicating one of the possible synergistic effects (Figure 3). Nevertheless, these control mechanisms of BCAs alone or combined with chemicals are often too complex and rely on different and multifaceted mechanisms. Hence, the effects of combined BCP6 and JGM on the secondary metabolites of *Bacillus*, plant disease resistance, and *in vivo* competition need to be further explored.

CONCLUSION

In this paper, we explored an integrated approach combining of *B. velezensis* with bactericides to manage konjac soft rot caused by *P. aroidearum* MY11. Our findings revealed that a systemic bactericide JGM or *B. velezensis* strain BCP6 alone could effectively suppress the growth of *P. aroidearum* MY11 *in vitro* and/or *in vivo*, and the combination of JGM and BCP6 resulted in heightened control against MY11, both *in vitro* and under greenhouse conditions. Moreover, relative to JGM or BCP6 alone, combined BCP6 and JGM also effectively increased konjac plant resistance to bacterial soft rot in the field, showing a synergistic action. These findings provide an alternative control option for soft rot disease in konjac planting.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

Abd-El-Khair H., Elshahawy I.E., Haggag H.E.K. (2019): Field application of *Trichoderma* spp. combined with thiophanate-methyl for controlling *Fusarium solani* and *Fusarium oxysporum* in dry bean. Bulletin of the National Research Centre 43: 19. doi:10.1186/s42269-019-0062-5

Ahmed B., Jailani A., Lee J.H., Lee J. (2022): Inhibition of growth, biofilm formation, virulence, and surface attachment of *Agrobacterium tumefaciens* by cinnamaldehyde derivatives. Frontiers Microbiology, 13: 4024. doi:10.3389/fmicb.2022.1001865

Andresen L., Koiv V., Alamae T., Mae A. (2007): The Rcs phosphorelay modulates the expression of plant cell wall degrading enzymes and virulence in *Pectobacterium carotovorum* ssp. *carotovorum*. FEMS Microbiology Letters, 273: 229–238.

Beauregard P.B., Chai Y., Vlamakis H., Losick R., Kolter R. (2013): *Bacillus subtilis* biofilm induction by plant polysaccharides. Proceedings of the National Academy of Sciences USA, 110. doi:10.1073/PNAS.1218984110

Carrillo C., Teruel J.A., Aranda F.J., Ortiz A. (2003): Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochimica et Biophysica Acta, 1611: 91–97.

Chen Z., Liu Y., Lu F. (2003): Synergistic effect and synergistic mechanism of Jinggangmycin and biocontrol bacterium Bs-916. Acta Plant Protection, 30: 429–434.

Chua M., Baldwin T.C., Hocking T.J., Chan K. (2010): Traditional uses and potential health benefits of *Amorphophallus konjac* K. Koch ex N.E.Br. Journal of Ethnopharmacology, 128: 268–278.

Colby S.R. (1967): Calculating synergistic and antagonistic responses of herbicide combinations. Weed Science Society of America and Allen Press, 15: 20–22.

Cui S., Chen C., Feng J. (2021): Characteristics of the soft rot pathogen *P. aroidearum* and the biocontrol effect of *B. velezensis*. China Vegetable, 385: 83–93. (in Chinese)

Czajkowski R., Pérombelon M.C.M., van Veen J.A., Vander-Wolf J.M. (2011): Control of blackleg and tuber soft rot of potato caused by *Pectobacterium* and *Dickeya* species: a review. Plant Pathology, 60: 999–1013.

Devaraj R.D., Reddy C.K., Xu B. (2019): Health-promoting effects of konjac glucomannan and its practical applications: A critical review. International Journal of biological Macromolecules, 126: 273–281.

Devi S., Kiesewalter H.T., Kovács R., Frisvad J.C., Weber T., Larsen T.O., Kovács Á.T., Ding L. (2019): Depiction of secondary metabolites and antifungal activity of *Bacillus velezensis* DTU001. Synthetic and Systems Biotechnology, 4: 142–149.

https://doi.org/10.17221/77/2023-PPS

Dong X., Fang L., Ye Z., Zhu G., Lai Q., Liu S., (2021): Screening of biocontrol bacteria against soft rot disease of *Colocasia esculenta* (L.) schott and its field application. PLoS One, 16: 7. doi:10.1371/JOURNAL.PONE.0254070

Douglas J.A., Follett J.M., Waller J.E. (2005): Research on konjac (*Amorphophallus konjac*) production in New Zealand. ISHS Acta Horticulturae, 670: 173–180.

Feng C., Zhang C.S., Kong F.Y., Wang J. (2014): Synthesis of thiodiazole copper microcapsules and release behavior of inhibiting *R. solanacearum*, RSC Advances, 4: 4478–4486.

Gallegos-Monterrosa R., Mhatre E., Kovács Á.T.(2016): Specific *Bacillus subtilis* 168 variants form biofilms on nutrient-rich medium. Microbiology (Reading, England), 162: 1922–1932.

Ghods-Alavi, Ahmadzadeh M., Behboudi K., Jamali, Behboudi M., Jamali K. (2012): Biocontrol of rhizome soft rot (*Pectobacterium carotovorum*) on valerian by *Pseudomonas* spp. under *in vitro* and greenhouse conditions. Journal of Agricultural Technology, 8: 1913–1923.

Gilardi G., Manker D.C., Garibaldi A., Gullino M.L. (2008): Efficacy of the biocontrol agents *Bacillus subtilis* and *Ampelomyces quisqualis* applied in combination with fungicides against powdery mildew of zucchini. Journal of Plant Diseases and Protection, 115: 208–213.

Gu C., Wang X., Su X. (2017): Bacillus amyloliquefaciens WH1G and fluazinam cooperated to control strawberry gray mold. Pesticide, 56: 932–936.

Guo Q.G., Dong W.X., Li S.Z., Lu X.Y., Wang P.P., Zhang X.Y., Wang Y., Ma P. (2014): Fengycin produced by *Bacillus subtilis* NCD-2 plays a major role in biocontrol of cotton seedling damping-off disease. Microbiological Research,169: 533–540.

Haddoudi I., Cabrefiga J., Mora I., Mhadhbi H., Montesinos E., Mrabet M. (2021): Biological control of *Fusarium* wilt caused by *Fusarium equiseti* in *Vicia faba* with broad spectrum antifungal plant-associated *Bacillus* spp. Biological Control 160: 104671. doi:10.1016/j.biocontrol.2021.104671

Huang B.H., Gao Z.L., Zhou B.G., Chen F.X., Xu D.F., Wang F. (2015): Toxicity test of four fungicides against Ralstonia solanacearum. Acta Tabacaria Sinica, 21: 72–75.

Idowu O., Olawole O., Idumu O., Salami A. (2016): Bio-control Effect of *Trichoderma asperellum* (Samuels) Lieckf. and *Glomus intraradices* Schenk on Okra Seedlings Infected with *Pythium aphanidermatum* (Edson) Fitzp and *Erwinia carotovora* (Jones). American Journal of Experimental Agriculture, 10: 1–12.

Ji X., Li J., Meng Z., Zhang S., Dong B., Qiao K. (2019): Synergistic effect of combined application of a new fungicide fluopimomide with a biocontrol *agent Bacillus methylotrophicus* TA-1 for management of gray mold in tomato. Plant Disease, 103: 1991–1997.

Jia Y.J., Feng B.Z., Sun W.X., Zhang X.G. (2009): Polygalacturonase, pectate lyase and pectin methylesterase activity in pathogenic strains of *Phytophthora capsici* incubated under different conditions. Journal of Phytopathology, 157: 585–591.

Jiang C.H., Liao M.J., Wang H.K., Zheng M.Z., Xu J.J., Guo J.H. (2018): *Bacillus velezensis*, a potential and efficient biocontrol agent in control of pepper gray mold caused by *Botrytis cinerea*. Biological Control, 126: 147–157.

Kovács Á.T., van Gestel J., Kuipers O.P. (2012): The protective layer of biofilm: a repellent function for a new class of amphiphilic proteins. Molecular Microbiology, 85: 8–11.

Li X., Tian Y., Zhang J., Chen C., Xie H. (2018): Identification and characterization of a *Pectobacterium aroidearum* strain causing bacterial soft rot on Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*). Acta Phytopathologica Sinica, 48: 455–465.

Liu L., Liang M., Li L., Sun L., Xu Y., Gao J., Wang L., Hou Y., et al. (2017): Synergistic effects of the combined application of *Bacillus subtilis* H158 and *Strobilurins* for rice sheath blight control. Biological Control, S1049964417302475. doi:10.1016/j.biocontrol.2017.11.011

Nam M.H., Park M.S., Kim H.G., Yoo S.J. (2009): Biological control of strawberry *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *fragariae* using *Bacillus velezensis* BS87 and RK1 formulation. Journal of Microbiology and Biotechnology, 19: 520–524.

Ons L., Bylemans D., Thevissen K., Cammue B.P.A. (2020): Combining biocontrol agents with chemical fungicides for integrated plant fungal disease control. Microorganisms, 8: 1–19.

Opara E.U., Asuquo A.A. (2016): An overview of characterization and identification of soft rot *Bacterium erwinia* in some vegetable crops. greener Journal of Biological Sciences, 6: 46–55.

Peng D., Li S., Wang J., Chen C., Zhou M. (2013): Integrated biological and chemical control of rice sheath blight by *Bacillus subtilis* NJ-18 and jinggangmycin. Pest Management Science, 70: 258–263.

Rabbee M.F., Ali M.S., Choi J., Hwang B.S., Jeong S.C., Baek K.H. (2019): *Bacillus velezensis*: a valuable member of bioactive molecules within plant microbiomes. Molecules, 24:1046.

Smadja B., Latour X., Trigui S., Burini J.F., Chevalier S., Orange N. (2004): Thermodependence of growth and enzymatic activities implicated in pathogenicity of two *Erwinia carotovora* subspecies (*Pectobacterium* spp.). Canadian Journal of Microbiology, 50: 19–27.

Sun W., Yan L., Chen C. (2020): Identification and control effect of *Bacillus Velez* BCP6, an antagonistic bacterium against lettuce soft rot and *Sclerotinia sclerotiorum*.

https://doi.org/10.17221/77/2023-PPS

China Journal of Biological Control, 36: 231–240. (in Chinese)

Tans-Kersten J., Brown D., Allen C. (2004): Swimming motility, a virulence trait of *Ralstonia solanacearum*, is regulated by FlhDC and the plant host environment. Molecular Plant-Microbe Interactions, 17: 686–695.

Wang H., Yang D., Guo B. (2019): Green and high-efficiency cultivation techniques of *Amorphophallus konjac*. China Journal of Biological Control, 35: 987–991. (in Chinese)

Wasendorf C., Schmitz-Esser S., Eischeid C.J., Leyhe M.J., Nelson E.N., Rahic-Seggerman F.M., Sullivan KE., Peters NT. (2022): Genome analysis of *Erwinia persicina* reveals implications for soft rot pathogenicity in plants. Frontiers in Microbiology, 13: 1001139. doi: 10.3389/fmicb.2022.1001139

Wei H., Yang M., Pei W.H., Wei W., Huang F.Y., Liu J.N. (2020): First report of *Pectobacterium aroidearum* causing soft rot of *Amorphophallus konjac* in China. Plant Disease, 104: 969–970.

Wei H., Yang M., Ke Y., Liu J., Chen Z., Zhao J.R., Zhao Y.T., Huang F.Y., et al. (2022): Comparative physiological and transcriptomic profiles reveal regulatory mechanisms of soft rot disease resistance in *Amorphophallus* spp. Physiological and Molecular Plant Pathology, 118: 101807. doi:10.1016/J.PMPP.2022.101807

Wootton A.N., Luker-Brown M., Westcott R.J., Cheetham P.S. (1993): The extraction of a glucomannan polysaccharide from konjac corms (elephant yam, *Amorphophallus rivierii*). Journal of the Science of Food and Agriculture, 61: 429–433.

Wu J., Diao Y., Gu Y., Hu Z. (2010). Molecular detection of *Pectobacterium* species causing soft rot of *Amorphophallus konjac*, World Journal of Microbiology and Biotechnology 27: 613–618.

Recieved: July 18, 2023
Accepted: November 2, 2023
Published online: December 19, 2023