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Application of Bacterial Biocontrol Agents and Different Chemicals against Potato White Mold

S. Ojaghian[1, 2*], W. Qiu[3], and L. Zhang[4]

ABSTRACT

This study was conducted to evaluate the control efficacy of *Pseudomonas chlororaphis*, *Erwinia herbicola*, *Bacillus amyloliquefaciens*, and *Bacillus subtilis*, as well as solutions of zinc sulfate, sodium malonate, and oxalic acid against potato white mold caused by *Sclerotinia sclerotiorum* under field conditions during growing seasons of 2017 and 2018 in Bahar and Lalehjin, Hamedan, Iran. The results showed that strains of *Bacillus subtilis* as well as zinc sulfate had the highest inhibitory effect against carpogenic germination of sclerotia. The myceliogenic germination of sclerotia was inhibited by solutions of zinc sulfate and sodium malonate with statistically similar results followed by oxalic acid. In addition, activities of resistance-related enzymes including *β*-N-acetyl hexosaminidase, endochitinase, chitin 1,4-*β*-chitobiosidase, *β*-1,3-glucanase, phenylalanine ammonia lyase, polyphenoloxidase, and peroxidase markedly increased in potato leaves due to application of bacteria on plants. The results showed that all treatments were able to reduce significantly (P< 0.05) the number of infected and dead plants in both years. The mixtures of five bacterial biocontrol agents and solution of zinc sulfate were found to be the most effective treatments to control white mold.

Keywords: Carpogenic germination, Myceliogenic, Polyphenoloxidase, *Sclerotinia sclerotiorum.*

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a non-host-specific fungus capable of attacking more than 400 crops and weeds worldwide (Boland and Hall, 1994). The pathogen causes an important disease on potato plants, which is called white mold. The disease causes a considerable yield loss in many potato-growing regions of Hamedan Province, Iran (Ojaghian, 2011). In addition, *S. sclerotiorum* is considered as the most important pathogen of canola in Northern provinces of Iran (Aghajani and Safaie, 2010; Karimi *et al*., 2012). Due to considerable crop damage, lack of highly resistant cultivars, and difficulty of managing sclerotinia diseases, many researches are being focused on this pathogen. *S. sclerotiorum* is an ascomycetous soil-borne fungus that overwinters as long-lasting sclerotia in soil. The sclerotia play a principal role in the disease cycle and can be viable in soil for more than eight years (Kora *et al*., 2005). Due to longevity of sclerotia and likely infection by airborne ascospores from nearby fields, nonchemical control of sclerotinia diseases is not easy. This has led

[1] Smart Agriculture Research and Application Team, Ton Duc Thang University, Ho Chi Minh City, Vietnam.
[*] Corresponding author; e-mail: seyedmohammadreza.ojaghian@tdtu.edu.vn
[2] Faculty of Applied Sciences, Ton Duc Thang University, Ho Chi Minh City, Vietnam.
[3] Ministry of Agriculture, Key Lab of Molecular Biology of Crop Pathogens and Insects, Biotechnology Institute, Zhejiang University, Hangzhou 310029, People Republic of China.
[4] Jinhua Academy of Agricultural Science, Jinhua 321000, Zhejiang Province, People Republic of China.

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to application of huge amounts of fungicides by farmers. However, the emergence of fungicide-resistant strains (Zhang *et al.*, 2003; Jo *et al.* 2006) together with environmental and human health concerns have prompted interest in looking for effective biocontrol agents against potato white mold in Hamedan Province.

Trichoderma spp., *Talaromyces flavus* and *Paraphaeosphaeria minitans* (formerly *Coniothyrium minitans*) are among the most effective fungal biocontrol agents against *Sclerotinia* diseases (Ojaghian, 2010; 2011). There are numerous studies showing that bacterial biocontrol agents are able to inhibit significantly *S. sclerotiorum* on different crops under *in vitro* and *in vivo* conditions (Savchuk and Fernando, 2004; Abdullah *et al*., 2008). Because ascospores produced by apothecia are considered as primary inoculum of the pathogen, many studies have been focused on control of carpogenic germination of the sclerotia (Huang *et al*., 2000). However, a few germinating sclerotia can cause considerable infection levels of *Sclerotinia* diseases in the field (Kora *et al*., 2005). The timely application of biocontrol agents is important in controlling *Sclerotinia* diseases because the ascospores generally infect senescing petals at the flowering stage (Ojaghian *et al*., 2016).

One of the defense mechanisms used by plants against different phytopathogens is inducing systemic resistance. The induced resistance caused by bacterial antagonists has been shown in rice against sheath blight (Nandakumar *et al*., 2001). It is well known that the occurrence of induced resistance in plants is related with various enzymes such as chitinases, β-1,3-glucanase, PolyPhenolOxidase (PPO), Peroxidase (POD) and Phenylalanine Ammonia-Lyase (PAL) (Ojaghian *et al*., 2017).

Beside biocontrol agents, non-conventional chemicals such as phytoalexin inducers and metal salts, as alternatives to fungicides, have shown inhibitory effects against different soil-borne phytopathogens (Bag and Sinha, 1997; Bhattacharya and Roy, 1998). There are numerous studies showing that Oxalic Acid (OA) is highly produced in the tissues infected with *S. sclerotiorum* (Kim *et al*., 2008). High concentrations of OA (> 10 mmol) have been reported to be toxic to plants through disrupting the integrity of the plant cell wall and acidifying the host tissues, which lead to wilting (Franceschi and Horner, 1980). However, the lower concentrations of OA have shown potential to induce resistance against phytopathogens in different crops (Toal and Jones, 1999; Sing *et al*., 2002). Sarma *et al*. (2007) showed that Zinc Sulfate (ZS), OA and Sodium Malonate (SM) solutions applied as foliar sprays decreased the incidence of chickpea (*Cicer arietinum*) stem rot caused by *S. sclerotiorum* through induction of phenolic compounds.

The main objective of this study was to determine inhibitory efficacy of different bacterial biocontrol agents including OA, ZS, and SM against carpogenic germination of *S. sclerotiorum* sclerotia. In addition, their effects were assessed against potato white mold under field conditions during two consecutive years. Effect of chemicals on myceliogenic germination of sclerotia was assessed. Another objective of this research was to determine the effects of bacterial biocontrol agents on the activity of resistance-related enzymes including β-N-acetyl hexosaminidase, endochitinase, chitin 1,4-β-chitobiosidase, β-1,3-glucanase, PAL, PPO, and POD.

MATERIALS AND METHODS

Source of Bacterial Biocontrol Agents and Chemicals

We used strains PJ41 and PJ32 of *Pseudomonas chlororaphis* (referred to as Pc-PJ41 and Pc-PJ32 in the rest of this paper), strain ER31 of *Erwinia herbicola* (referred to as Eh-ER31), strains BC4M and BCkL of *Bacillus amyloliquefaciens* (referred to as Ba-BC4M and Ba-BCKL),

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strains BD41 and BM3L of *Bacillus subtilis* (referred to as Bs-BD41 and Bs-BM3L) and strains BCF7 and BN4H of *Bacillus methylotrophicus* (referred to as Bm-BCF7 and Bm-BN4H). All strains were provided by the Institute of Biotechnology at Zhejiang University. They had already shown inhibitory effect against mycelial growth of *S. sclerotiorum* isolates under *in vitro* conditions (data not presented). The bacterial strains were stored in Nutrient Agar (Difco Laboratories, Detroit, MI, USA) with 20% glycerol at -80°C. In addition, ZS, OA and SM, which had already shown high inhibitory effect against *S. sclerotiorum* in planta (Sarma *et al*., 2007), were purchased from Sangon Company (Shanghai, China).

Effect of Biocontrol Agents and Chemicals against Carpogenic Germination of Sclerotia

The sclerotia of the pathogen were produced as described by Ojaghian *et al*. (2016). The stored bacteria were streaked onto Luria Bertani Agar plates (LBA contains 15 g agar technical, 10 g tryptone peptone, 5 g yeast extract, and 5 g NaCl L^{-1}). A single colony of each strain was inoculated and grown in LB broth with constant shaking at 250 rpm for 50 hours at 22-24°C. The cultures were centrifuged at 7,000 rpm for 10 minutes and bacterial cells were re-suspended in phosphate buffer (100 mM, pH. 7). The final concentration was spectrophotometrically adjusted to 8×10^8 CFU mL^{-1} (0.4 OD at 590 nm).

As previously described (Ojaghian *et al*., 2013, 2019a), 50 sclerotia located on a polyurethane foam block were placed in a Styrofoam cup. The above-mentioned biocontrol agents (8×10^8 CFU mL^{-1}) were sprayed on sclerotia at the rate of 10 mL/cup twice a week. In addition, solutions of ZS (10^{-3} mmol), OA (5 mmol) and SM (10^{-3} mmol) were sprayed on sclerotia at the rate of 10 mL cup^{-1} seven times in an interval of two weeks. These concentrations of biocontrol agents and chemical solutions were chosen because they had already shown the highest efficacy against *S. sclerotiorum* on chickpea and canola (Sarma *et al*., 2007; Fernando *et al*., 2007). The solutions were prepared by adding the required amount of chemicals to Sterilized Distilled Water (SDW). Pure SDW was used in the controls. The cups were kept in a dark incubator at 10°C. The cups were investigated weekly for 100 days and the sclerotia with visible stipe(s) or (pre)mature apothecia were considered as carpogenically germinated. The percent of inhibition was determined using the following formula (Ojaghian *et al*., 2019):

$I= C–T/C\times100$

Where, I= The Inhibition percentage of carpogenically germinated sclerotia, C= The number of carpogenically germinated sclerotia in the Control, and T= The number of carpogenically germinated sclerotia in the Treatment. This test was repeated three times with two repetitions for each treatment.

Effect of Non-Conventional Chemicals on Myceliogenic Germination of Sclerotia

The objective of this experiment was to determine the efficacy of three non-conventional chemicals against myceliogenic germination of sclerotia. An 8-9 mm layer of autoclaved sand was spread in 90 mm Petri plates and 20 sclerotia were spread over the sand. The solutions of ZS (10^{-3} mmol), OA (5 mmol) and SM (10^{-3} mmol) were sprayed on the sclerotia at the rate of 10 mL $plate^{-1}$ two times with an interval of seven days. The experiment was repeated twice with four replicates for each treatment. The controls received SDW. The plates were kept in a dark incubator at 22°C at 90% relative humidity. After 20, 50, 100 and 130 days, inhibition percent of sclerotia viability was measured using the method and formula described by Ojaghian *et al*. (2014a, b).

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Protein Extraction

Batches of three non-dormant medium potatoes (cultivar Agria) were sown in plastic pots filled with 4 kg field soil pasteurized at 75±5°C for 1 hour, topped with 1 cm of vermiculite and were watered as needed for 70 days. The 70-day plants were sprayed with five biocontrol agents (8×10^8 CFU mL^{-1}) including Pc-PJ41, Pc-PJ32, Eh-ER31, Bs-BD41, and Bs-BM3L at the rate of 50 mL pot^{-1}. The five biocontrol agents had already shown the highest effect against the pathogen in the above-mentioned studies. In addition, they had shown highest inhibitory efficacy in reducing the mycelial growth of the pathogen *in vitro* (unpublished data). One, three, and six days after treatment, the leaf tissues cut from three plants were sampled, weighed, and ground in liquid nitrogen to a fine powder with a pre-chilled mortar and pestle. Using 20 mM sodium acetate buffer containing polyvinylpolypyrrolidone, total proteins were extracted (Ojaghian *et al*., 2017). The extracted proteins were kept at 4°C for 24 hours to be used for assessment of chitinase and *β*-1,3-glucanase activities, as described below.

Assessment of Chitinase Activity

One, three, and six days after treatment with the biocontrol agents, the activities of *β*-N-acetyl hexosaminidase, endochitinase and chitin 1,4-*β*-chitobiosidase were evaluated with three replications (Ojaghian *et al*., 2017). The chitinase evaluation was based on colorimetric assessment of *p*-nitrophenyl, which was cleaved from chitin-analogous substrates, *p*-nitrophenyl-N-acetyl-*β*-D-glucosaminide, p-nitrophenyl-*β*-D-N-N,N″-triacetylchitotriose, and p-nitrophenyl-*β*-D-N,N′-diacetylchitobiose, respectively. In this experiment, 40 microliters of stock of each substrate solution (3 mg L^{-1}) were dissolved in 40 mM acetate buffer and added to 40 µL of the protein extracted from each sample. The absorbance was assessed at 405 nm.

Assessment of *β*-1,3-Glucanase Activity

One, three, and six days after treatment with the biocontrol agents, the activity of *β*-1,3-Glucanase was determined (Ojaghian *et al*., 2017) with three replications by calculating the production rate of reducing sugars, using laminarin as a substrate. The reaction mixture included sodium acetate buffer containing 85 µL of protein extract and 1 mg L^{-1} laminarin. After incubating for 2.5 hours at 38°C, alkaline copper reagent was added, and the combination was heated at 100°C for 2 minutes. After cooling, Nelson's chromogenic reagent was added, and absorbance was determined at 650 nm.

Effects of Biocontrol Agents on Polyphenoloxidase, Peroxidase, and Phenylalanine Ammonia-Lyase

The 70-day plants were sprayed with five biocontrol agents (8×10^8 CFU mL^{-1}) including *P. chlororaphis* (PJ41 and PJ32), *E. herbicola* (ER31) and *B. subtilis* (BD41 and BM3L) at the rate of 50 mL pot^{-1}. This test was conducted twice with three replications to determine the effect of the biocontrol agents on activity of PPO, POD, and PAL, which are known to be efficient in resistance induction. Enzymatic assessments were done two and four days after treatment as described by Ojaghian *et al*. (2013). Pure SDW was used in controls.

Field Testing of Biocontrol Agents and Chemicals

Field trials were conducted in two naturally highly infested potato fields located at Bahar and Lalehjin, two counties in Hamedan province, during the cropping seasons of 2017 and 2018. The objective of these tests was to determine protective

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effects of the biocontrol agents and the solutions of chemicals against potato white mold under field conditions. In addition, the effects of the treatments were compared with different application rates of the fungicide dichloran (Botran 5F WP, 46.7% a.i.; maximum advised application rate 450 g ha^{-1}, Gowan Ltd., Arizona, USA).

The Bahar and Lalehjin fields had been under potato cultivation for five years and contained clay loam (pH 6.3) and sandy clay loam (pH 6.4) soils, respectively. Field tests were arranged in a randomized complete block design in both years and there were three replications for each treatment. The potato tubers were sown at 4.8 ton ha^{-1} at a row spacing of 35-40 cm. Urea (400 g), superphosphate (600 g) and potassium chloride (250 g) were applied to the soil of each treatment prior to planting tubers. Individual plot sizes were 4.5×5 m in 2017 and 4×5 m in 2018, and one-meter wide borders were maintained unplanted between each replicate plot. The biocontrol agents (8×10^8 CFU mL^{-1}) as well as the solutions of ZS (10^{-3} mmol), OA (5 mmol) and SM (10^{-3} mmol) were sprayed twice on plants when approximately 20-30 and 70-80% of the potato plants were flowering. The treatments were: (**A**) Fungicide dichloran sprayed at 100% of advised rate, (**B**) Fungicide dichloran sprayed at 80%, (**C**) Fungicide dichloran sprayed at 60% , (**D**) Fungicide dichloran sprayed at 40%, (**E-I**) Pc-PJ41, Pc-PJ32, Eh-ER31, Bs-BD41, and Bs-BM3L, **(J**) Mixture of five bacterial biocontrol agents, (**K-M**) Solutions of ZS, OA, and SM, and (**N**) The control. Sterile water was used in controls. In both years, plots were irrigated with a sprinkler system as needed and weeds were removed by hand.

Potato plants were rated for incidence of white mold (Ojaghian, 2011) during harvesting (late September). Disease incidence was defined as the percentage of plants per plot infected by *S. sclerotiorum* per plot. In addition, the mean percentage of dead plants (as a proportion of infected plants) per plot was determined in each plot (Figure 1).

Data Analysis

The means of treatments recorded in percent were transformed in Sin^{-1} percentage transformation. The effects of different treatments in each test were determined by ANOVA using SAS software in completely randomized design tests.

RESULTS

Inhibitory Effect against Carpogenic Germination of Sclerotia

All tested biocontrol agents and chemical solutions were able to reduce markedly the carpogenic germination of the sclerotia (P<

Figure 1. Symptoms of potato white mold caused by *Sclerotinia sclerotiorum*. The infection of lower parts of the plants can cause just a bleached lesion (A), or this infection can develop to kill the whole plant (B).

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0.05). The highest inhibitory efficacy was observed for the treatments Bs-BD41, Bs-BM3L and ZS with statistically similar results. The next inhibitoriest treatments were those with Pc-PJ41, Pc-PJ32, OA and SM. The least inhibitory effect was observed for Bm-BCF7 and Bm-BN4H (Figure 2).

Effect of Chemical Solutions on Myceliogenic Germination

The myceliogenic germination of sclerotia was significantly reduced after 20, 50, 100, and 130 days of treatment with solutions of chemicals ($P< 0.05$) (Figure 3). In terms of ZS and SM, there was a negative relationship between the duration of treatment and myceliogenic germination of the sclerotia. The highest inhibitory effect was found for treatments with ZS and SM after 100 and 130 days at a statistically similar level. The next most inhibitory treatments were for the OA solution after 20, 50, 100, and 130 days. Finally, treatments with the ZS solution after 20 and 50 days, as well as with the SM solution after 20 days, reduced the myceliogenic germination at the least statistical level.

Effect of Bacterial Biocontrol Agents on Resistance Related Enzymes

After one day (Table 1), Eh-ER3 increased the level of β-N-acetyl hexosaminidase, endochitinase and β-1,3-glucanase, contrary to other biocontrol agents. In addition, the

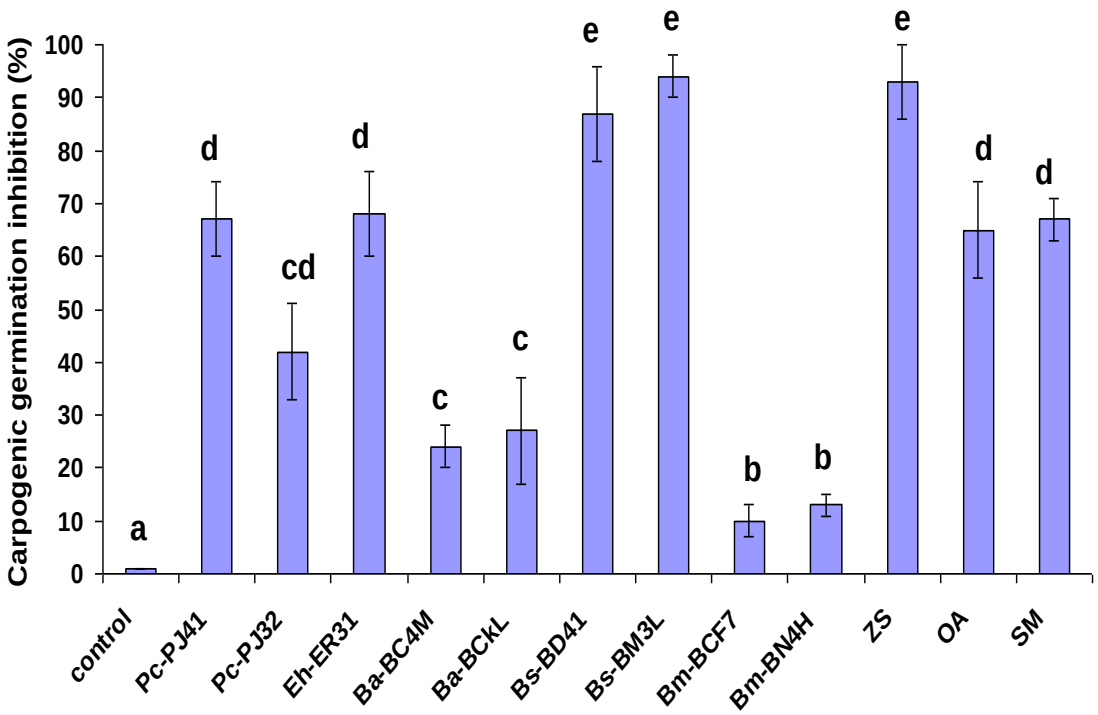

Figure 2. Inhibitory effect percentage of nine microbial strains and three chemicals against carpogenic germination of *Sclerotinia sclerotiorum* sclerotia. Pc-PJ41 and Pc-PJ32: Strains PJ41 and PJ32 of *Pseudomomas chlororaphis*; Eh-ER31: Strain ER31 of *Erwinia herbicola*; Ba-BC4M and Ba-BCkL: Strains BC4M and BCkL of *Bacillus amyloliquefaciens*; Bs-BD41 and Bs-BM3L: Strains BD41 and BM3L of *Bacillus subtilis*; Bm-BCF7 and Bm-BN4H: Strains BCF7 and BN4H of *Bacillus methylotrophicus*; ZS, OA and SM: Solutions of zinc sulfate, oxalic acid, and sodium malonate. This test was repeated three times with two repetitions for each treatment. Error bars represent the standard error. Columns having a common letter do not significantly differ ($P< 0.05$) according to Fishers' LSD test.

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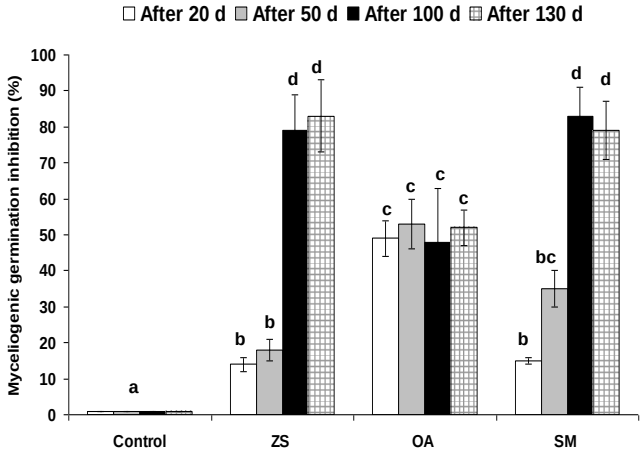

Figure 3. Inhibitory effect of solutions of Zinc Sulfate (ZS), Oxalic Acid (OA) and Sodium Malonate (SM) against myceliogenic germination of *Sclerotinia sclerotiorum* sclerotia. The experiment was repeated twice with four replicates for each treatment. Error bars represent standard error. Columns having a common letter do not significantly differ (P< 0.05) according to Fishers' LSD test.

Table 1. Effect of bacterial biocontrol agents on resistance-related enzymes in potato leaves after one, three, and six days of spraying on 70-day plants.[a]

Enzyme activity after one day (U mg^{-1} protein)					
	β-N-acetyl hexosaminidase	Endochitinase	Chitin 1,4-*β*-chitobiosidase	*β*-1,3-glucanase	Peroxidase
Control	0.00534 [a]	0.00331 [a]	0.00242 [a]	1.83 [a]	1.35 [a]
P. chlororaphis (PJ32)	0.00539 [a]	0.00335 [a]	0.00249 [a]	1.79 [a]	1.38 [a]
P. chlororaphis (PJ41)	0.00536 [a]	0.00341 [a]	0.00342 [b]	1.85 [a]	1.34 [a]
E. herbicola (ER31)	0.00655 [b]	0.00462 [b]	0.00351 [b]	2.56 [b]	2.59 [b]
B. subtilis (BD41)	0.00541 [a]	0.00339 [a]	0.00348 [b]	1.84 [a]	2.61 [b]
B. subtilis (BM3L)	0.00543 [a]	0.00342 [a]	0.00251 [a]	1.75 [a]	1.33 [a]

Enzyme activity after three days (U mg^{-1} protein)					
	β-N-acetyl hexosaminidase	Endochitinase	Chitin 1,4-*β*-chitobiosidase	*β*-1,3-glucanase	Peroxidase
Control	0.00536 [a]	0.00335 [a]	0.00245 [a]	1.85 [a]	1.36 [a]
P. chlororaphis (PJ32)	0.00679 [b]	0.00486 [b]	0.00243 [a]	1.72 [a]	1.37 [a]
P. chlororaphis (PJ41)	0.00683 [b]	0.00483 [b]	0.00352 [b]	2.63 [b]	1.38 [a]
E. herbicola (ER31)	0.00691 [b]	0.00672 [c]	0.00345 [b]	3.95 [c]	2.57 [b]
B. subtilis (BD41)	0.00602 [ab]	0.00473 [b]	0.00349 [b]	3.11 [bc]	2.61 [b]
B. subtilis (BM3L)	0.00685 [b]	0.00479 [b]	0.00248 [a]	2.66 [b]	2.67 [b]

Enzyme activity after six days (U mg^{-1} protein)					
	β-N-acetyl hexosaminidase	Endochitinase	Chitin 1,4-*β*-chitobiosidase	*β*-1,3-glucanase	Peroxidase
Control	0.00542 [a]	0.00337 [a]	0.00251 [a]	1.78 [a]	1.34 [a]
P. chlororaphis (PJ32)	0.00687 [b]	0.00678 [c]	0.00249 [a]	2.69 [b]	2.59 [b]
P. chlororaphis (PJ41)	0.00722 [c]	0.00491 [b]	0.00346 [b]	2.65 [b]	2.63 [b]
E. herbicola (ER31)	0.00729 [c]	0.00683 [c]	0.00563 [c]	4.09 [c]	3.89 [c]
B. subtilis (BD41)	0.00733 [c]	0.00595 [bc]	0.00559 [c]	2.59 [b]	3.93 [c]
B. subtilis (BM3L)	0.00688 [b]	0.00485 [b]	0.00436 [bc]	2.76 [b]	2.71 [b]

[a] Within a column and for a given enzyme, all the means followed by the same letter are not significantly different at the P> 0.05 level of confidence according to Fisher's LSD test of multiple mean comparison. U: One Unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of substrate per min.

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level of chitin and 1,4-β-chitobiosidase increased after treatment with Pc-PJ41, Eh-ER3 and Bs-BD41 with statistically similar results. A significant increase in peroxidase was observed for treatments with Eh-ER3 and Bs-BD41 (Table 1).

After three days (Table 1), all tested biocontrol agents markedly increased the level of β-N-acetyl hexosaminidase. The highest level of endochitinase was observed for the application of Eh-ER3 and other bacteria were the next treatments in the order of efficacy with statistically similar performance. Moreover, Pc-PJ41, Eh-ER31 and Bs-BD41 increased the activity of chitin 1,4-β-chitobiosidase in a statistically similar level. Eh-ER31 increased the level of β-1,3-glucanase followed by Bs-BD41, and other treatments in the order of superiority were Pc-PJ41 and Bs-BM3L. In addition, a statistically similar increase was observed in peroxidase level due to application of Eh-ER31, Bs-BD41, and Bs-BM3L (Table 1).

After six days (Table 1), the highest activity of β-N-acetyl hexosaminidase was found due to application of Pc-PJ41, Eh-ER31, and Bs-BD41; and other treatments in the order of superiority were Pc-PJ32 and Bs-BM3L with statistically at par results. The highest endochitinase level was observed due to application of Pc-PJ32 and Eh-ER31 followed by Bs-BD41, and the other two tested biocontrol agents were the next treatments in the order of efficacy with statistically similar results. In addition, Eh-ER31 and Bs-BD41 followed by Bs-BM3L showed the best efficacy in terms of increasing chitin 1,4-β-chitobiosidase. The highest activity of β-1,3-glucanase was observed due to application of Eh-ER31and other tested bacteria caused a statistically similar increase in the enzyme level. The highest level of peroxidase was observed in application of Eh-ER31and Bs-BD41 and the other three tested biocontrol agents were the next treatments in the order of efficacy with statistically similar performances (Table 1).

Effect of Biocontrol Agents on Polyphenoloxidase, Peroxidase and Phenylalanine Ammonia-Lyase

Treatments with Pc-PJ32 did not change the activity of any enzyme after two and four days (Table 2). After two days (Table 2), the highest level of PPO was observed in the plants treated with Eh-ER31 followed by Pc-PJ41, Bs-BD41, and Bs-BM3L at a statistically similar level. Moreover, the activity of POD was markedly enhanced due to treatment with Pc-PJ41, Eh-ER31, and Bs-BD41 with statistically similar performances. However, no tested bacteria changed the level of PAL after two days (Table 2).

After four days, the highest level of PPO was found to be due to treatment with Eh-ER31 followed by Bs-BD41 and the next treatment in the order of efficacy was Bs-BM3L (Table 2). Furthermore, Bs-BD41 was found to be the most effective biocontrol agent in increasing the activity of POD followed by Eh-ER31 and other treatments in the order of superiority were Pc-PJ41 and Bs-BM3L, with statistically at par results. The highest level of PAL was observed due to treatment with Bs-BM3L and other bacteria, except Pc-PJ32, were the next treatments in the order of superiority with statistically similar results (Table 2).

Field Ttrials

The results showed that all treatments were able to reduce significantly ($P< 0.05$) the number of infected plants (white mold incidence) and dead plants in both years (Table 3).

In 2017, in Bahar region, the lowest numbers of infected plants were observed in dichloran sprayed at 100% of the advised rate, mixture of five bacterial biocontrol agents, and ZS solution followed by dichloran sprayed at 60 and 80% of the advised rate, Pc-PJ41, Pc-PJ32, OA, and SM solutions with significantly similar results. Moreover, the lowest number of dead plants

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was found for dichloran sprayed at 100% of the advised rate.

In 2017, in Lalehjin, dichloran sprayed at 100% of the advised rate, mixture of five bacterial biocontrol agents and ZS solution were the most effective treatments in reducing the number of infected plants, followed by dichloran sprayed at 40, 60, and 80% of the advised rate and Pc-PJ41, which statistically showed similar performances. In addition, the least number of dead plants was observed in dichloran sprayed at 80 and 100% of the advised rate and mixture of five bacterial biocontrol agents and ZS solution.

In 2018, in Bahar, the least number of infected plants was observed in dichloran

Table 2. Effect of bacterial biocontrol agents on PolyPhenolOxidase (PPO), Peroxidase (POD) and Phenylalanine Ammonia Lyase (PAL) in potato leaves after two and four days of inoculation with *Sclerotinia sclerotiorum*.

	Enzyme activity [a]					
	PPO		POD		PAL	
	After 2 days	After 4 days	After 2 days	After 4 days	After 2 days	After 4 days
Control	68.3±9.2 [a]	67.5±12.8 [a]	5.6±1.2 [a]	5.1±2.3 [a]	9.8±2.3 [a]	10.3±1.7 [a]
P. chlororaphis (PJ32)	72.4±12.9 [a]	74.3±20.4 [a]	4.9±0.8 [a]	5.4±1.8 [a]	10.2±1.5 [a]	9.6±2.4 [a]
P. chlororaphis (PJ41)	156.5±27.4 [b]	165.2±18.7 [b]	12.8±2.7 [b]	13.9±4.5 [b]	10.5±1.6 [a]	25.9±5.8 [b]
E. herbicola (ER31)	285.9±36.2 [c]	428.4±51.5 [d]	13.2±4.5 [b]	18.8±2.1 [bc]	11.1±3.2 [a]	27.1±6.3 [b]
B. subtilis (BD41)	161.7±19.4 [b]	371.2±44.9 [cd]	11.7±3.9 [b]	25.4±5.6 [c]	9.9±2.4 [a]	28.4±3.7 [b]
B. subtilis (BM3L)	153.5±28.6 [b]	267.5±32.8 [c]	8.2±1.6 [ab]	12.7±5.4 [b]	11.4±3.1 [a]	45.9±5.2 [c]

[a] All the means within a column followed by the same letter are not significantly different at the $P<0.05$ level of confidence according to Fisher's test. The PPO activity was expressed as U398, where U398= 0.01ΔOD398 mg^{-1} protein min^{-1}. These experiments were repeated two times and there were three replicates in each treatment. The activity of POD was expressed as U460, where U460 = 0.01ΔOD460 mg^{-1} protein min^{-1}. The PAL activity was expressed as U290, where U290= 0.01ΔOD290 mg^{-1} protein min^{-1}.

Table 3. Inhibitory efficacy of bacterial biocontrol agents and chemicals solutions against incidence of *S. sclerotiorum* in potato fields of Bahar and Lalehjin during growing seasons of 2017-2018.[a]

	Disease incidence (%)							
	2017				2018			
	Bahar		Lalehjin		Bahar		Lalehjin	
	Infected plants	Dead plants	Infected plants	Dead plants	Infected plants	Dead plants	Infected plants	Dead plants
A	24.8±8.2^{d}	6.5±1.5^{d}	17.5±6.3^{d}	5.7±1.3^{d}	20.4±6.2^{d}	10.2±4.5^{d}	20.4±7.3^{d}	9.7±2.6^{d}
B	40.5±9.5^{c}	20.4±6.1^{c}	29.2±8.5^{c}	6.2±2.1^{d}	34.7±10.3^{c}	21.1±6.4^{cd}	31.9±12.6^{c}	19.2±4.1^{c}
C	44.7±11.2^{c}	19.2±3.7^{c}	31.5±10.6^{c}	19.7±5.2^{c}	32.5±6.9^{c}	24.8±7.1^{cd}	34.5±6.7^{c}	22.1±5.7^{c}
D	68.9±12.5^{b}	34.8±8.7^{bc}	33.1±6.2^{c}	20.8±3.6^{c}	50.8±13.2^{b}	40.2±12.3^{b}	44.2±17.8^{bc}	35.5±9.4^{bc}
E	44.5±11.5^{c}	35.1±1.5^{bc}	31.5±8.4^{c}	20.5±8.7^{c}	31.4±7.1^{c}	31.3±3.5^{c}	32.7±8.5^{c}	19.4±1.7^{c}
F	41.9±12.8^{c}	33.9±8.1^{bc}	33.8±7.2^{c}	23.8±6.1^{c}	34.3±5.2^{c}	32.7±6.4^{c}	30.1±6.8^{c}	22.7±4.8^{c}
G	74.4±12.3^{ab}	43.9±12.6^{b}	72.3±18.2^{ab}	38.2±8.5^{b}	63.8±12.3^{ab}	40.8±14.1^{b}	62.7±11.8^{b}	42.7±15.8^{b}
H	62.1±12.4^{b}	41.8±6.2^{b}	64.5±18.1^{b}	37.9±12.3^{b}	49.9±18.1^{b}	39.4±11.8^{b}	60.9±15.1^{b}	40.1±10.5^{b}
I	61.7±15.3^{b}	45.7±5.3^{b}	62.8±20.5^{b}	38.1±10.2^{b}	50.3±15.6^{b}	42.8±17.5^{b}	59.7±22.4^{b}	42.9±17.3^{b}
J	26.5±4.8^{d}	7.1±2.4^{d}	18.1±5.7^{d}	5.2±1.6^{d}	19.7±2.8^{d}	9.6±1.7^{d}	18.9±3.4^{d}	7.3±1.5^{d}
K	26.3±9.4^{d}	8.6±3.2^{d}	20.4±8.1^{d}	7.1±0.8^{d}	23.5±9.4^{d}	10.8±3.9^{d}	20.2±9.1^{d}	8.2±4.6^{d}
L	44.5±12.3^{c}	30.5±9.2^{bc}	42.1±14.7^{bc}	22.8±7.5^{c}	30.8±6.5^{c}	29.2±3.6^{c}	41.4±7.3^{bc}	31.1±2.8^{bc}
M	45.2±7.1^{c}	32.5±12.4^{bc}	44.5±13.8^{bc}	20.5±6.2^{c}	33.1±8.4^{c}	33.8±4.5^{c}	40.8±6.2^{bc}	35.3±0.9^{bc}
N	84.3±16.3^{a}	55.3±11.2^{a}	82.3±17.4^{a}	56.4±12.4^{a}	73.4±18.5^{a}	62.6±20.1^{a}	83.4±19.8^{a}	54.5±9.6^{a}

[a] Values in the table indicate means±standard error. There were three replications for each treatment. Columns having a common letter do not significantly differ ($P< 0.05$). (A) Fungicide dichloran sprayed at 100% of the advised rate, (B) Fungicide dichloran sprayed at 80%, (C) Fungicide dichloran sprayed at 60%, (D) Fungicide dichloran sprayed at 40%, E-I) *Pseudomomas chlororaphis* (PJ41), *Pseudomomas chlororaphis* (PJ32), *Erwinia herbicola* (ER31), *Bacillus subtilis* (BD41)and *Bacillus subtilis* (BM3L), (J) Mixture of five bacterial biocontrol agents, (K-M) Solutions of zinc sulfate, oxalic acid, and sodium malonate, (N) Control.

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sprayed at 100% the advised rate, mixture of five bacterial biocontrol agents, and ZS solution followed by dichloran sprayed at 60 and 80% of the advised rate, Pc-PJ41, Pc-PJ32, OA, and SM solutions with statistically at par results. Moreover, the most effective treatments against the number of dead plants were dichloran sprayed at 100% of the advised rate, mixture of five bacterial biocontrol agents and ZS solution with statistically similar performances.

In 2018, in Lalehjin, the number of infected plants was most reduced by dichloran sprayed at 100% of the advised rate, mixture of five bacterial biocontrol agents and ZS solution and other treatments in the order of efficacy were dichloran sprayed at 60 and 80% of the advised rate, Pc-PJ41 and Pc-PJ32. The least number of dead plants was found to be in dichloran sprayed at 100% of the advised rate, mixture of five bacterial biocontrol agents and ZS solution, which statistically showed similar results.

DISCUSSION

Although the inhibitory effect of Brassica biofumigation (Ojaghian *et al*., 2012a, b), E-cinnamaldehyde (Ojaghian *et al.*, 2015) and that of fungal biocontrol agents such as *Trichoderma* spp., *Talaromyces flavus,* and *Coniothyrium minitans* (Ojaghian, 2010; 2011) have been assessed against potato white mold, this is the first study about the effect of bacterial biocontrol agents against the disease. In addition, inhibitory effects of biocontrol agents were evaluated against the carpogenic germination of the sclerotia. The sclerotia, as overwintering structures and inoculum sources of the pathogen, play an important role in the disease cycle of *Sclerotinia* diseases. Therefore, the effect of biocontrol agents against myceliogenic and carpogenic germination of the sclerotia is suggested to be tested in biocontrol studies of *Sclerotinia* diseases. The present study revealed that all tested biocontrol agents were able to reduce significantly the carpogenic germination of the sclerotia. A previous study of Li *et al*. (2011) showed that a strain of *P. fluorescens* decreased mycelial growth and sclerogenesis of *S. sclerotiorum* by 84.4% and 100%, respectively. Essential oils of some medicinal plants have shown efficacy against *S. sclerotiorum* (Bajpai and Kang, 2012, Ojaghian *et al*., 2019b). Shehata *et al*. (2016) showed that a strain of *B. subtilis* inhibited *S. homoeocarpa in vitro* and on creeping bentgrass (*Agrostis stolonifera*) under greenhouse conditions. In a recent study, Fusarium dry rot of potato was reduced markedly through the application of probiotic bacteria (Vatankhah *et al*., 2019). Our field trials showed that all tested bacteria markedly reduced the number of infected and dead plants over two years in Bahar and Lalehjin. Interestingly, the highest protective efficacy was observed for treatments with a mixture of five bacterial biocontrol agents. This high level of disease control, which was statistically similar to application of dichloran, may be due to synergistic effects between the different bacteria because it is known that bacterial biocontrol agents show their efficacy through various modes of actions (Santoyo *et al*., 2012; Sivasakthi *et al*., 2014). Savchuk and Fernando (2004) showed that different strains of *Pseudomonas* spp. have the potential to inhibit significantly *S. sclerotiorum* on canola under field conditions. Moreover, it has been shown that foliar application of lipopeptide-producing *Bacillus amyloliquefaciens* strains could be a promising strategy for the management of sclerotinia stem rot in soybean (Alvarez *et al*., 2011). The present study showed that all tested bacterial biocontrol agents, when applied as spray on canopy, were able to increase markedly the activity of resistance-related enzymes in potato leaves. The protective effect of biocontrol agents against the pathogen may be partially due to resistance induction, because the level of several PR-proteins (chitinases, peroxidases, β-1,3-glucanases) as well as PPO, POD and

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PAL increased in the potato leaves treated with the bacteria.

It is known that chitinases play an important role in resistance induction against plant pathogens by means of degradation of fungal cell wall components (Kramer and Muthukrishnan, 1997; Ojaghian *et al*., 2018). The *β*-1,3-glucanases show their protective efficacy against plant pathogens by hydrolysing *β*-1,3-glucans available in fungal cell walls, producing compounds that can induce resistance by biological or chemical elicitors. The PODs play major roles in wall synthesizing processes. Furthermore, they have significant effects in oxidation of phenols and suberization. The PAL is an effective enzyme in the phenyl propanoid biosynthesis pathway. In this process, phenols/phytoalexins are synthesized, which can lead to various defense reactions against plant pathogens (Yu *et al*., 2014; Elsherbiny and Taher, 2018).

For the chemical control of plant diseases, the most obvious approach is to devise chemicals that inhibit directly the growth of the pathogen upon or within plants. These compounds are considered as conventional chemical agents. An alternative approach to the chemical control of plant diseases is to use unconventional chemicals that do not directly inhibit the growth of pathogens, but act against the disease itself through various mechanisms in plants. For example, Bag and Sinha (1997) reported the control of stem rot of soybean caused by *Sclerotium rolfsii* using barium sulfate, lithium sulfate, manganese sulfate, cupric chloride, and ferric chloride through an increase in the total phenol and o-dihydroxyphenol contents and of peroxidase activity in stem tissues.

The results of the present study showed that the three tested non-conventional chemicals were able to reduce markedly carpogenic and myceliogenic germination of the sclerotia. It is the first report about effects of different chemicals on sclerotia germination. In agreement with Sarma *et al*. (2007), ZS was the most effective chemical against disease incidence in our field trials in 2017 and 2018. Zinc is one of important nutrients in plants, but its application in soil as fertilizer is not a successful strategy due to organic, economic, and environmental factors (Graham and Rengel, 1993; Hacisalihoglu, 2002). Thus, foliar application of zinc may overcome the above-mentioned issues. In this study, OA significantly reduced the percentage of infected and dead plants in both years. Although it is known that *S. sclerotiorum* produces OA in diseased plants during the infection process (Marciano *et al*., 1983), the role of OA as a pathogenicity factor is still not quite clear.

Because OA, ZS, and SM have not shown considerable antifungal efficacy *in vitro* against mycelial growth of *S. sclerotiorum* (Sarma *et al*., 2007), their inhibitory effects against potato white mold may be due to induction of resistance in plants against the pathogen. Although Sarma *et al*. (2007) reported accumulation of phenolic compounds in tissues of chickpea after application of chemical solutions, the effect of chemicals on resistance-related enzymes has not been assessed.

CONCLUSIONS

This study showed inhibitory effect of bacterial biocontrol agents and chemical solutions against potato white mold. In order to commercialize the five tested bacterial biocontrol agents, more field tests are needed to prove their efficacy against different genotypes of the pathogen under different climatic situations. Therefore, numerous field trials are going to be conducted in infected fields of different crops in eastern provinces of China. Because there is no potato cultivar resistant to white mold, application of chemicals as effective, cost effective, and eco-friendly substances can be an alternative to fungicides application in potato fields. According to our previous study (Ojaghian *et al*., 2018), a number of tests are being carried out to increase resistance against *S. sclerotiorum* in

[DOR: 20.1001.1.16807073.2020.22.6.10.6]

transgenic potato plants expressing chitinase *chit42* from *Trichoderma harzianum.* Moreover, some experiments are ongoing under greenhouse and field conditions to determine the effects of chemical solutions on different resistance-related enzymes on different potato cultivars in Hamedan province. In order to make a timely decision on application of chemicals or biocontrol agents, a real-time PCR assay described by Ojaghian *et al*. (2016) is suggested to growers.

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کاربرد عوامل بیوکنترل باکتریایی و مواد شیمیایی مختلف برضد کپک سفید سیب زمینی

س. اجاقیان، و. کیو، و ل. ژانگ

چکیده

هدف از انجام این تحقیق ارزیابی توانایی کنترلی سودوموناس کلرورافیس، اروینیا هاربیکولا، باسیلوس آمیلولیکوفاشنس و باسیلوس سوبتیلیس و همچنین محلولهای سولفات روی، مالونات سدیم و اسید اگزالیک در تستهای مزرعه ای طی فصلهای زراعی ۲۰۱۷ و ۲۰۱۸ در مزارع بهار و لالجین استان همدان بر ضد کپک سفید سیب زمینی بود که عامل بیماریزای آن اسکلروتینیا اسکلروتیوروم است.

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نتایج نشان داد که استرینهای باسیلوس سوبتیلیس و همچنین محلول سولفات روی بیشترین اثر بازدارنده را بر جوانه زنی کارپوژنیک اسکلروتها داشتند. جوانه زنی رویشی اسکلروتها بر اثرمحلولهای سولفات روی و مالونات سدیم به یک میزان کاهش یافت و اسید اگزالیک نسبت به دو تیمار قبلی اثربازدارنده کمتری نشان داد. بعلاوه، فعالیت آنزیمهای مرتبط با مقاومت گیاهی شامل بتا- ان -استیل هگزوزامینیداز، اندوکیتیناز، کیتین ۴,۱- بتا- کیتوبیوزیداز، بتا - ۳,۱- گلوکاناز، فنیل آلانینین آمونیا لیاز، پلی فنول اکسیداز و پروکسیدازدر برگهای سیب زمینی درنتیجه کاربرد عوامل بیوکنترل باکتریایی روی برگها بطور معنی داری افزایش یافت. نتایج نشان داد که همه تیمارها قادربودند تا تعداد گیاهان آلوده و مرده را در هر دوسال بطور معنی داری کاهش دهند (P<۰.۰۵). ترکیب پنج عامل بیوکنترل باکتریایی ومحلول سولفات روی موثرترین تیمارها جهت کنترل کپک سفید بودند.

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