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COMBINATION OF DIFFERENT ANTAGONISTIC BACTERIA TO CONTROL OF POTATO BLACKLEG DISEASE CAUSED BY PECTOBACTERIUM ATROSEPTICUM UNDER GREENHOUSE AND FIELD CONDITIONS

[a]Hoda H. El-Hendawy, [b]Kamal A. M. Abo-Elyousr*
[a] Botany and Microbiology Department, Faculty of Science, Helwan University, Ain Helwan, Cairo, Egypt.
[b]Plant Pathology Dept. Faculty of Agriculture, Assiut University - 71526, Assiut, Egypt.

A B S T R A C T

Three different antagonistic bacterial isolates, *Pseudomonas fluoresces* (Pf2), *Bacillus subtilis*(Bs3) and *Rahnella acquatilis* (Ra39) restricted the growth of *Pectobacterium atrosepticum*, the causal agent of black leg disease of potato, *in vitro*. Under greenhouse and field conditions, potato plants pre-treated with the three antagonistic bacterial isolates, individually or in combination, showed reduced disease severity relative to non-treated control plants. All isolates produced siderophores in different degrees but did not produce indole acetic acid (IAA) or hydrogen cyanide (HCN).The obtained results indicated that combination of *Pseudomonas fluoresces* (Pf2), *Bacillus subtilis* (Bs3) and *Rahnella acquatilis* (Ra39) is beneficial in controlling black leg disease of potato caused by *pectobacterium atrosepticum*.

Keywords: Potato, biological control, *Pseudomonas fluorescens, Bacillus subtilis* and *Rahnella acquatilis*.

INTRODUCTION

Potato (*Solanum tuberosum*, L.) is an important food source, it is theworld's fourth main food crop after rice (*Oryza sativa*, L.), maize (*Zea mays*, L.) and wheat (*Triticum aestivum*, L.) in cultivated area and total production (Douches *et al.,* 1996). Also it harbors a high ratio of yield productivity to soil occupation as 85% of the plant is comestible whereas only 50% in cereals. Moreover, it has the ability to grow worldwide and it has a higher amount of vitamins compared with grass plant (FAO, 2008).

Unfortunately, potato is attacked by several diseases which reduce quality and market value of the produce and also spread to uncontaminated fields via latently infected tubers used as seeds. Approximately, 22% of potatoes are lost per year due to viral, bacterial, fungal, and pest attack to potato tuber and potato plant, incurringan annual loss of over 65 million tones (Ross 1986, and FAO, 2008). One of the most serious diseases is blackleg and soft rot caused by *Pectobacterium atrosepticum, Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya* species (Van der Wolf and De Boer, 2007), this disease is responsible for 30-50% of the previously mentioned huge loss (Czajkowski *et al*., 2011). Under wet conditions, the symptoms appear as a slimy, wet, black rot lesion spreading from the rotting mother tuber to the stems, whereas under dry conditions, the symptoms are yellowing, stunting, wilting and desiccation of stems and leaves (Perombelon and Kelman, 1980).

Different methods have been studied to control blackleg and tuber soft rot, of these methods: avoiding contamination, chemical methods and biological control methods. Chemical control against bacterial diseases is based on eradication of the pathogen and/or creation of unfavorable environmental conditions for disease development (Czajkowski *et al*., 2011). Several chemical compounds were tested for their ability to reduce infection on or inside potato tubers. These compounds included certain antibiotics, inorganic and organic salts or their combinations. Treatment with these compounds provided promising results, but the risk of development of resistant bacterial pathogens represents a threat to

* Corresponding Author:
Email: kaaboelyousr@yahoo.com

human and animal health (Guan *et al*., 2005). Also, although treatment with bactericides such as chlorine gas was successful, penetration in tubers could be poor and might be phytotoxic (Eckert *et al*., 1988).

Biological control of bacterial pathogens could be an alternative methods which included antagonistic bacteria. In this study, three antagonistic bacterial strains of *Bacillus subtilis, Rahnella aquatili*s and *Pseudomonas .fluorescens* that restricted the growth of *Pectobacterium atrosepticum, in vitro*, were tested for their ability to control potato blackleg disease caused by *P. atrosepticum* individually or in combination, under greenhouse and field conditions. Also, production of IAA, HCN and siderophores by these antagonistic bacteria was investigated.

MATERIALS AND METHODS

Bacterial isolates: Three antagonistic bacterial strains were used in this study. These strains are *Bacillus subtilis* (Bs3), *Rahnella aquatilis* (Ra39) and *Pseudomonas fluorescens* (Pf2), *R. aquatilis* (Ra39) *B. subtilis* and *P. fluorescens* were obtained from the stock cultures of Plant Pathology Dept. Faculty of Agriculture University of Assiut. *Pectobacterium atrosepticum* was isolated by Dr. Hoda H. El-Hendawy, from rotted potato tuber collected from a vegetable market, Alharam, Giza Governorate, Egypt and identified according to Lelliot and Dickey (1984).

Potato seedlings: Healthy tubers of potato plants (*Solanum tuberosum*L.) cv. Diamont obtained from Ministry of Agriculture, Egypt, were surface sterilized by soaking in 1% sodium hypochlorite for 5 min, washed thoroughly with sterile distilled water and planted directly in sterile plastic pots 30 cm in diameter and containing 4 kg of sterilized mixture of clay soil and sand (3:1, v/v), each pot received one tuber. Pots and soil were sterilized by 5% formalin and then left for 15 days before planting. Plants were grown in greenhouse at 24°C during the growing season. Plants were fertilized every 15 days with urea 46% (20 g/pot) and watered as required. Four weeks old potato seedlings were used in all greenhouse experiments.

Inoculations: Inoculum was prepared from 18 h old nutrient broth shaken culture. *P. atrosepticum* was grown at 25±2°C and 150 rpm. The culture was centrifuged at 6000 g and for 20 min at room temperature then the pellet was washed twice with sterile distilled water. After the final wash the number of cells in the suspension was determined from optical density measurements at OD_{600}. The suspension was diluted with sterile distilled water to give $5x10^8$cells ml^{-1}. Inoculation of seedlings was carried out by injecting 0.1 ml bacterial suspension through the stem (Klement *et al*., 1990). Control potato seedlings were injected with sterile distilled water. Inoculated seedlings were observed daily for visible blackleg symptoms.

To inoculate potato tubers, they were surface sterilized with 1% sodium hypochlorite solution then a cavity (1 cm depth and 0.5 cm width) was made in each tuber using a cork-borer the tubers were inoculated by spotting 200 µL of the bacterial suspension onto the bottom of each cavity, then covered with the removed potato plugs. Treated tubers were kept in clean sterile plastic containers each supplemented with a sterile moist cotton and incubated at 25°C for 72 h. After incubation, inoculated tubers were cut into halves to observe rotting (De Boer and Kelman, 1978). Severity of disease was recorded using the method of Yaganza *et al*. (2004) as follows:

Disease severity index was calculated by following equation:

$$DSI = \frac{A - B}{A} \times 100$$

A = Tuber weight with rotting.

B = Tuber weight without rotting.

***In vitro* antibiosis:** Antagonistic activity of *B. subtilis* (Bs3), *R. aquatilis* (Ra39) and *P. fluorescens* (Pf2) against *P. atrosepticum* was tested by the semi-solid overlay method according to Ishimaru *et al*. (1988).

Screening for indole acetic acid production: This experiment was carried out according to Shanmugam and Kanoujia (2011) as follows: Single colony from an overnight culture was streaked onto LB agar and the plates were overlaid with Whatman no. 1 filter paper. After incubation for 3 days at 30°C, the paper was removed and treated with Salkowski, s reagent (1.2% $FeCl_3$ in 37% Sulfuric acid, Bric *et al*., 1991). IAA production was identified by the formation of a red halo on the paper immediately surrounding the bacterial colony.

Screening for hydrogen cyanide production: Bacterial isolates were screened for HCN production by the method of Lorck (1948) which was adapted by Joseph *et al*. (2007). Nutrient agar plates containing glycine (4.4 g/l) were streaked with bacterial isolates. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the

plate. Plates were sealed with parafilm and incubated for 4 days at 30°C. Production of HCN was indicated by the development of orange to red colour.

Siderophore production: Siderophore production was screened on chromazurol S (CAS) agar plates according to Alexander and Zuberer (1991). A yellow halo surrounding the bacterial colony after incubation 4 – 5 days at 30°C indicated siderophore production.

Effect of antagonistic bacteria on blackleg and soft rot disease of potato under greenhouse condition: An experiment under greenhouse conditions was conducted to determine the effect of the three antagonistic bacteria, individually or in combinations, on blackleg and soft rot disease of potato tuber caused by *P. atrosepticum*. Potato seedlings were grown in sterile plastic pots 30 cm in diameter and containing 4 kg of sterilized mixture of clay soil and sand (3:1, v/v), each pot received one tuber. Nine treatments were designed as follows:1- *Pseudomonas fluoresces* (Pf2), 2- *Bacillus subtilis* (Bs3), 3- *Rahnella aquatilis* (Ra39), 4- Pf2 +Bs3, 5- Pf2+ Ra39, 6- Bs3+Ra39, 7- Pf2+Bs3+Ra39, 8- control and 9- control.

In the following experiments, all the antagonistic isolates were used at the concentration of $1x10^8$ cfu ml^{-1} and pathogen inoculation was carried out as previously mentioned, 200 ml of the bacterial suspension of each strain containing $1x10^8$ cfu ml^{-1} as mentioned above, was added as desired in each treatment. Two days later, 500 µL of the previously prepared *P. atrosepticum* suspension were inoculated by syringe into the stem of potato plants (3-4 cm) above the ground.

Pots treated with obave treatments but not inoculated with *P. atrosepticum* were used to determine the soft rot in the tuber after harvestd. Pots were arranged in randomized block design on a bench in the greenhouse at 25 ± 2 °C.

All pots were maintained in the previously mentioned greenhouse conditions and the disease incidence was recorded three weeks after inoculation. Four replicate were used and the experiment was repeated twice.

Disease severity was assessed on a scale of 0-3 as reported by Wright *et al*. (2005) where 0 = no disease symptoms on plant, 1 = less than 50 % of the plant has disease symptoms, 2 = more than 50% of the plant has disease symptoms, and 3 = plant totally dead. Disease severity (%) for each random pots was then computed according to Bdliya and Dahiru (2006) $S = 100\Sigma n/Nx3$ where S = black leg severity (%), Σn = summation of individual ratings, N = total number of plants assessed, and 3 = highest score on the severity scale.

Effect of soil treatments with antagonistic bacteria on population of *P. atrosepticum*: For the determination of bacterial multiplication in potato plant treated with the above mentioned treatments, one gram of the stem tissues of each treatment after 2 weeks from inoculation was washed with tap water, surface sterilized with 3% sodium hypochloride and washed with sterile tap water. Samples were homogenized in a sterile mortar and pestle with 10 ml of 0.1 M potassium phosphate buffer (pH 7.0). Stem homogenates then, were serial diluted from 10^{-1} to 10^{-9} with 0.1 M potassium phosphate buffer, pH 7.0. Of each dilution, 200 µL were spreadon the surface of NSA plate by using a glass rod. Plates were incubated at 25°C for 48 h and the number of bacterial colonies was counted (Roberto *et al*. 2002).

Effect of antagonistic bacteria on blackleg and soft rot disease of potato under field conditions: This experiment was carried out in the Experimental Farm of Plant Pathology Department, Faculty of Agriculture, Assiut University, Assiut, Egypt. Tested treatments were distributed in a complete randomized block design with four replicate, the experimental plot area was 25 m^2 containing five rows, each row was 3.5 meter length and distance between rows was 50 cm. Potato seed tubers were sown on the middle of the ridge at 40 cm apart, each row were seeded with 10 hells and each hell received 30 ml of bioagents. Each treatment was tested individually for blackleg disease by the above mentioned treatments before 48 hr inoculation with *P*. *atrosepticum*. Disease index was recorded 3 weeks after inoculation for blackleg disease. Tubers from all treatments were tested for soft rot disease as mentioned before. The other agricultural practices were carried out as the recommended program of the Egyptian Ministry of Agriculture for potato production. At harvest time (110 days after planting), potato tubers of six plants from each replicate were pulled to assess the total yield of each treatment (ton) per hectare.

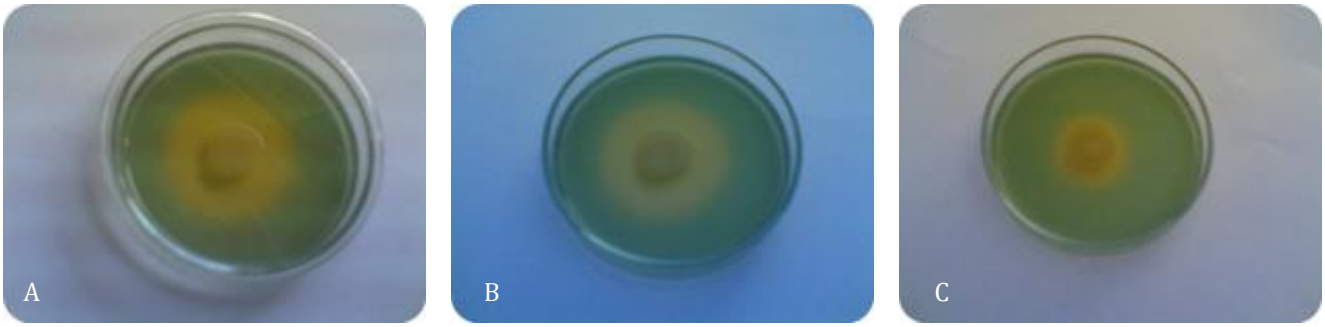
Statistical analysis: All experiments were set up in a complete randomized design. Data were subjected to analysis of variance (ANOVA), using the statistical analysis system (SAS Institute Inc. 1996). Means were compared with L.S.D. test at $P \leq 0.05$ levels.

RESULTS

Potato tuber inoculated with *P. atrosepticum* and incubated at 25°C for 72 h as described in Materials and Methods. After incubation, inoculated tuber was cut into halves to observe rotting (Figure 1). All the three strains, *Pseudomonas fluorescens, Bacillus subtilis* and *Rahnella acquatilis* restricted the growth of *P. atrosepticum* (data no show).

Siderophore production by the three antagonistic strain: The obtained results indicated that the three antagonistic strains used in this study produced siderophores with different levels as reflected by the diameter of the yellow hallo produced around the bacterial growth. *P. fluorescens* produced the highest level followed by*R. aquatilis* and then*B. subtilis* Figure 2A, B and C, respectively. None of the three isolates produced IAA or HCN.

Figure 1. Pathogenicity of *P. atrosepticum* on potato tuber.



P. fluorescens *R. aquatilis* *B. subtilis*;

Figure 2. Siderophore production by the three antagonistic strain.

Effect of antagonistic bacteria on blackleg and tuber rot severity and pathogen population in potato tuber under greenhouse condition: Before planting potato tubers were soaked in the bacterial suspension of the three antagonistic bacteria individually or in combinations. Seven treatments were used, in addition to infected control and a non-infected control. Soaking of potato tubers in the bacterial suspension of the three antagonistic bacteria, individually or in combination reduced *P. atrosepticum* population and consequently severity of blackleg disease as well as tuber rots relative to infected control. The highest level of reduction was observed in case of treatment 7 which include the three antagonists followed by treatment 6 which contains the bacterial suspension of Bs3+Ra39 then treatment 5 in comparison with the non-treated control (Table 1).

Table 1. Effect of different bioagents alone or in combination on disease severity and bacterial population of *P. atrosepticum* under greenhouse conditions.

Isolate	Blackleg severity %	Tuber rots (g)	Bacterial population 10^4
1-*P.fluoresces* (Pf2)	25 c	5.2 d	2.3 c
2- *B. subtilus*(Bs3)	17 d	7.8 c	1.2 g
3-*Rahnella acqatulis* (Ra39)	32 b	3.2 e	3.2 b
4- Pf2 +Bs3	19 d	9.2 b	3.5 bc
5- Pf2+ Ra39	16 e	6.1 c	1.50 f
6- Bs3+Ra39	12 f	2.3 e	2.00 e
7- Pf2+Bs3+Ra39	10 f	1.2 f	1.0 d
8- Health control	0.0 g	0.0 g	0.00 f
9- Infected control	42 a	10.1 a	5.43 a

Values in the column followed by different letters indicate significant differences among treatments. LSD test at 0.05.

Effect of bioagents on blackleg and tuber rot severity and yield of potato tuber under field condition: A field study was conducted over two successive seasons to determine the effect of the different treatments on disease severity and potato yield. The results presented in Table 2 indicate that all treatments significantly reduced the disease severity and increased potato yield relative to infected control plants. Application of Pf2+Bs3+Ra39 resulted in the highest reduction in disease severity as well as the highest tomato yield in comparison with the controls followed by Pf2 +Bs3, Bs3+Ra39 and thenBs3+Ra39. Data also showed that all treatment significant increases the potato yield compared to infected control and the highest increased was obtained in Pf2 and Pf2+Bs3+Ra39 and the lowest was obtained with treated Ra39 followed by Pf2 +Bs3.

Table 2. Effect of different bioagents alone or in combination on diseases severity of blackleg disease under field conditions.

Isolate	Blackleg severity %	Tuber rots	Yield (Tonn/ha)
1-*P.fluoresces* (Pf2)	15 c	7.1 c	25.3 a
2- *B. subtilis*(Bs3)	17 c	8.5 c	26.1 a
3-*Rahnella acqatilis* (Ra39)	22 b	10.1 b	20.1 b
4- Pf2 +Bs3	12 d	6.3 cd	26.1 a
5- Pf2+ Ra39	10 d	5.2 cd	27.6 a
6- Bs3+Ra39	10 d	3.3 e	27.9 a
7- Pf2+Bs3+Ra39	8 d	2.2 e	28.1 a
8- Health control	0.0 e	0.0 f	25.3 a
9- Infected control	42 a	15.1 a	10.2 c

DISCUSSION

Chemical control of plant pathogens represents a threat to animal and human health, because many of these chemicals persist and accumulate in natural ecosystems (Glick, 2015). Moreover, some pesticides support survival and growth of human bacterial pathogens (Guan *et al*., 2005). Therefore, it is desirable to use biological control agents that are more "friendly" to the environment. In an attempt to find biological control agents against *Pectobacterium atrosepticum*, three bacterial strains, *P. fluorescnes* (Pf2)*, B. subtilis* (Bs3) and *Rahnella aquatilis* (Ra39) were screened for their *in vitro* antibiosis towards *P. atrosepticum* and proved to be able to restrict the growth of the bacterial pathogen. Other studies have demonstrated the *in vitro* antagonistic activity of *P. fluorescens* (Abo-Elyousr and El-Hendawy, 2008) and *R. aquatilis* (Bell *et al*., 1995; El-hendawy *et al*., 2003) against plant pathogenic bacteria including *Pectobacterium* spp. Based on their *in vitro* antagonistic activity, these strains were further included in greenhouse and field experiments to determine their possible role in controlling potato blackleg and soft rot caused by *P. atrosepticum*. In the greenhouse experiment, treatment of potato tubers with the antagonistic bacteria resulted in reduction of *P. atrosepticum* population. Another study demonstrated the *in vivo* reduction of plant pathogenic bacteria population following treatment with the antagonistic bacteria (Abo-Elyousr and El-Hendawy, 2008). The inhibition of bacterial pathogen by the antagonistic bacteria could be exerted by several mechanisms including production of antibiotics, hydrogen cyanide (HCN), siderophores, volatile compounds, competition for nutrients (Glick, 2015). In this study the three antagonistic bacteria were screened for production of siderophores, HCN and IAA. All the strains produced siderophores but none of them produced HCN or IAA. The production of IAA, siderophores and HCN by *Bacillus* spp. and *P. fluorescence* have been reported before (Wani *et al*., 2007 and Ahmad *et al*. 2008). Also two antagonistic strains of *R. aquatilis* produced siderophores (El-Hendawy *et al*., 2003). Moreover, it is well known that many *P. fluorescens* strains produced several siderophores such as pyoverdine (Pseudobactin), pyochelin and SA (Dave and Dube, 2000). These fluorescent siderophores, which have very high affinity for ferric iron, will form ferric-siderophore complex and make it unavailable to other organisms. Due to iron starvation, the growth of pathogenic fungi and bacteria in the rhizosphere will be restricted.

HCN produced by some strains of *Pseudomonas* spp. suppressed black root rot of tobacco caused by the fungus *Thielaviopsis basicola, and* the transfer of genes encoding HCN biosynthesis increased the biocontrol activity of some bacteria. In contrast, it has been reported that many biocontrol plant growth promoting

bacteria (PGPB) strains and antibiotic-producing PGPB strains synthesize HCN in low level which may not have much biocontrol activity (Glick, 2015). However, it was suggested that HCN acts synergistically with bacterially encoded antibiotics. The synergism between antibiotic and HCN would likely prevent the development of resistance to specific antibiotics. This resistance would reduce the protective effect of antibiotic-producing PGPB strain (Glick, 2015).

The ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant. Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities (Arshad and Frankenberger, 1993; Glick, 1995). Higher level of IAA production by *Pseudomonas* was recorded by other workers (Xie *et al*., 1996).

Under greenhouse and field condition, treatment of potato tuber with the antagonistic strains individually or in combinations reduced the disease severity of blackleg and tuber soft rot in comparison with the infected control. Hossain (1987) reported that the eye plugs inoculated with *Eca* and treated with *Pf* reduced blackleg disease from 60% to 50%. However, fluorescent and non-fluorescent *Pseudomonas* spp. have shown to be potential candidates for biological control of blackleg and soft rot diseases (Czajkowski *et al*., 2011 and Kastelein *et al*., 1999). Treatment of tomato seedlings with two different strains of R. aquqtilis through leaves, seeds, soil or roots reduced the bacterial spots caused by *Xanthomonas campestris* pv. *vesicatoria* relative to non-treated seedlings (El-Hendawy *et al*., 2005). Members of Bacillus spp., *P. fluorescens* and *R. aquatilis* have been isolated from the rhizosphere of potato and are considered as plant growth promoting rhizobacteria (PGPR) (Diallo *et al*., 2011). Fluorescent *Pseudomonas* spp. applied to tubers was able to reduce populations of blackleg and soft rot bacteria on potato roots and inside progeny tubers (Kloepper, 1983). Application of individual and combinations of strains reduced the contamination of potato tuber peel by 85% and 60–70%, respectively, indicating the potential of *Pseudomonas* spp. for controlling soft rot caused by *P. atrosepticum*. They are able to survive in the potato rhizosphere and in soil (Loper & Henkels, 1999) and produce a variety of secondary antibacterial metabolites (Weller, 1988) including mainly siderophores, antibiotics and surfactants (Kloepper *et al*., 1980). In contrast, Gross (1988) demonstrated that selected strains were able to colonize plants, but were ineffective in *P. atrosepticum* disease suppression.

Reduced maceration symptoms were obtained when Sharga & Lyon (1998) tested *B. subtilis* strains for the control of potato diseases caused by *Pectobacterium* spp. The ability of members of *Bacillus* spp. to produce endospores and consequently to resist environmental stresses provided these bacteria with effective protecting activity and facilitated the development of commercial product (Jacobsen *et al*., 2004) However, *Bacillus* and *Pseudomonas* bacterial genera are included in the list of biocontrol products and some strains are registered by the United States Environmental Protection Agency (USEPA) and the European Protection Agency (EPA), (Montesinos, 2003 and Fravel, 2005), as agents against black leg and soft rot disease of potato caused by *Dickeya* spp. and *Pectobacterium* spp. by *in vitro* screening, tuber assay, soil microcosm and field trial (Kloepper, 1983; Xu and Gross, 1986;, Sharga and Lyon, 1998). *R. aquatilis* was among the cultural bacteria reported in the rhizosphere and underground organs of potato (Diallo *et al*., 2011). However, rhizobacteria are considered as the most important biocontrol agent to many plant pathogens e.g. bacteria, fungal and nematode (Obradovic *et al*., 2004; Siddiqui *et al*., 2005; Kavitha and Umesha, 2007).

In the field experiment, treatment of potato tuber with the three antagonistic strains individually or in combinations increased the crop yield. It has been reported that PGPR can directly stimulate plant growth by synthesizing hormones (phytostimulators) or by supplying the plants with nutrients (biofertilizing). Growth stimulation can also be indirectly achieved by suppressing or preventing the deleterious effects of pathogens. Thus, potato seeds bacterized with fluorescent pseudomonad strains were shown to improve the yields by about 10 % compared with noninoculated seeds. This result was obtained through trials conducted on different field sites over several years (Burr *et al*., 1978; Kloepper *et al*., 1980). Geels & Schippers (1983) also obtained a reduced loss of yield by combining a short rotation process and tuber seed bacterization with some strains of fluorescent pseudomonads. The beneficial effect was attributed to the inhibition of deleterious microbial communities.

In greenhouse and field experiments, the most effective control of *P. atrosepticum* was exhibited by combination

of all bioagents together. This combination of antagonistic bacteria resulted in significant disease reduction relative to treatment with the bacteria separately or the infected control. Other studies proved that using of bioagents in combination gave better results than that obtained when using them separately (De Boer *et al*., 1999, Abeysinghe, 2009; Hashem and Abo-Elyousr, 2011). Based on the results obtained from this study, we recommend application of bioagents in combination to control the blackleg disease and to enhance plant growth.

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