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In-vitro Screening of some Chemicals and Biocontrol Agents against *Erwinia carotovora* subsp. *carotovora,* the Causal Agent of Soft Rot of Potato (*Solanum tuberosum*)

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Abstract

Erwinia carotovora subsp. *carotovora* was isolated from the infected potato tuber and was identified by pathological, morphological and biochemical studies. Five chemicals viz. Cupravit 50 WP (Copper oxychloride), Sulcox 50 WP (Copper oxychloride), Champion 77 WP (Copper hydroxide), Indofil M-45 (Mancozeb) and Bavistin 50 WP (Carbendazim) and five biocontrol agents viz. *Bacillus subtilis, Pseudomonas fluorescens, Rhizobium leguminosarum, Trichoderma harzianum* and *Aspergillus flavus* were screened out *in vitro* against the growth of *Erwinia carotovora* subsp. *carotovora* by well diffusion method measuring the inhibition zone. Among the chemicals, Sulcox 50 WP (Copper oxychloride) was highly effective against it with 31.00 mm inhibition zone after 48 hours of incubation at 0.2% concentration when 100 µl/well was used. In case of biocontrol agents, *Bacillus subtilis* was the best against the bacterium with 16.67 mm inhibition zone after 48 hours of incubation.

Keywords: Potato, soft rot, *in-vitro* screening, chemicals, biocontrol agents

1. Introduction

Potatoes (*Solanum tuberosum*) are grown worldwide and the crop is usually considered to be the fourth most important staple food source after rice (*Oryza sativa*), maize (*Zea mays*) and wheat (*Triticum aestivum*) (Czajkowski *et al*., 2011). It is a popular and important vegetable in Bangladesh. Annual potato production in Bangladesh is 6648 thousand metric tons (WFP, 2013). *Erwinia carotovora* subsp. *carotovora*, the causal agent of soft rot of potato is a very economically important pathogen in terms of postharvest losses and is a common cause of decay in stored fruits and vegetables (Pérombelon, 2002). Approximately 22% of potatoes are lost per year due to viral, bacterial, fungal and pest attack in potato tuber and potato plant, incurring an annual loss of over 65 million tones and bacterial soft rot alone accounts for 30-50% of this huge loss (Czajkowski *et al.*, 2011). Copper-based compounds provided better control of bacterial soft rot (Blom and Brown, 1999). But the indiscriminate use of agrochemicals leads to degradation of the ecosystem, which may induce pathogen resistance to the pesticide, and may cause human and animal health problems (Huang, 1997). Therefore, public concern is focused on alternative methods of pest control, which can play a role in integrated pest management systems to reduce our dependence on chemical pesticides (Sutton, 1996). *Bacillus subtilis, Pseudomonas fluorescens, Rhizobium leguminosarum, Trichoderma harzianum* and *Aspergillus flavus* are most commonly used

antagonistic organisms against plant pathogen. *Bacillus subtilis* shows biological activity against phytopatogenic bacteria by producing peptide antibiotics (Backman *et al*., 1997). Soil fluorescent and non-fluorescent *Pseudomonas* spp. have shown biological control of soft rot disease of potato by producing a variety of secondary antibacterial metabolites including siderophores, antibiotics and surfactants (Compant *et al.*, 2005). Despite of economic importance, sufficient information has not been generated so far in respect of isolation, identification and management of this disease. Therefore, the current study was conducted to identify the causal agent and evaluate the effect of potential antibacterial chemicals and antagonists against it.

2. Materials and Methods

The study was conducted at Disease diagnostic laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka during April, 2012 to March, 2013. The study included the isolation and identification of the causal organism of soft rot of potato and *in vitro* screening of some chemicals and biocontrol agents against it.

2.1. Isolation and purification of the causal organism

Erwinia carotovora subsp. *carotovora* was isolated from the infected potato tuber collected from a local market of Dhaka. Potato tuber was surface sterilized with 5% sodium hypochlorite solution for 5 minutes, washed thrice with sterilized distilled water. Sections of lesion margins were placed for 30 minutes in sterile water in a test tube. Tenfold dilution was made from this solution, which was repeated four times to make final dilution up to 10^{-4}. One hundred micro litre of each dilution was spreaded over NA plates at three replications as described by Goszczynska and Serfontein (1998) and was incubated at 30±1ºC for 48 hr. Colonies of bacteria were purified on NA plate.

2.2. Identification of the pathogen

Identification of the pathogen was done by the following tests:

2.2.1. Gram's staining reaction

Part of a young colony was smeared on a glass slide and the bacterium was heat fixed on it. The slide was flooded with Crystal violet solution for 1 minute, rinsed under running tap water for a few seconds and air dried. Then it was flooded with Lugol's iodine solution for 1 minute, decolorized with 95% Ethanol for 30 seconds and again rinsed with running tap water and air dried. It was counterstained with 0.5% Safranine for 10 seconds, rinsed under running tap water for a few seconds and air dried. Then the glass slide was examined at 40x and 100x magnification using oil immersion (Gerhardt, 1981).

2.2.2. Biochemical characters

Gram's staining result was confirmed with potassium hydroxide (3% KOH) test (Suslow *et al.*, 1982). During this test, a loopful of bacteria was stirred in 3% KOH on a glass slide and any change in the viscosity was recorded. For starch hydrolysis test, pure colony of bacterium was spot inoculated on nutrient agar plate containing 0.2% soluble starch, incubated at 30±1ºC for 48 hr and flooded with Lugol's iodine solution (Cowan, 1974), the absence or presence of clear zones in stained media was recorded. For catalase test, a few drops of freshly prepared 3% H_2O_2 (Hydrogen peroxide) was added with 48 hr old pure culture of bacteria grown on NA plate (Schaad, 1988) and formation of bubbles were recorded. For oxidase test, part of a colony was smeared onto the moistened oxidase disk containing 1 ml 1% aquous w/v solution of NNN'N- tetramethyl- p- phenylene- diamine-dihydrochloride solution. Colour changed to dark purple was recorded (Kovacs, 1956). Citrate utilization test was carried out by streak inoculating the bacterium on simmon's citrate agar slant (Schaad, 1988). After incubation green colour of simmon's citrate agar slant changed into a bright blue colour indicated positive test. For motility indole urease agar (MIU) test, pure colony of bacterium was stub inoculated into the

motility indole urease agar (Schaad, 1988). Bacterium migrated away from the original line of inoculation after 48 hr of incubation indicated positive test. Gelatine liquefaction test was performed by stub inoculating the bacterium into the tube containing 12% (w/v) gelatin. After 24 hours of incubation gelatin liquefied microorganisms were determined by the formation of liquid culture after keeping it at 5°C in refrigerator for 15 minutes (Salle, 1961). Pectiolytic test was performed by spot inoculating the bacterium in sterile distilled water on a nick of potato slice placed on a petri dish (Goszczynska *et al*., 2000).

2.3. Pathogenicity test

A bacterial suspension (5 µl) containing 10^8 colony forming units per ml (CFU ml^{-1}) was inoculated into potato tuber with a sterile syringe. The control tuber was inoculated with same amount of sterile water. Tubers were then placed in polyethylene bags, which were tightly sealed and incubated in the dark at 25°C for 7–14 days. After incubation, the tubers were cut in half longitudinally through the inoculation site with a sterile knife and examined for the extent of soft rot. To confirm Koch's postulates, bacteria reisolated from diseased tubers were streaked on NA plate and reidentified using the methods outlined by Lelliott and Stead (1987).

2.4. Isolation and purification of biocontrol agents

Trichoderma harzianum from Bangladesh Agricultural Research Institute (BARI), Gazipur and *Aspergillus flavus* from Plant Pathology Laboratory of Sher-e-Bangla Agricultural Unversity (SAU), Dhaka were isolated and purified on PDA plates following the method described by Ashrafuzzaman (1976). Antagonistic bacteria were isolated from rhizosphere soil of dhaincha and tomato plant by dilution plate technique as described by Goszczynska and Serfontein (1998). One gram of soil was placed in the test tube containing 9 ml of sterile water and stirred thoroughly for few minutes. Tenfold dilution was made from this solution, which was repeated four times to make final dilution up to 10^{-4}. One hundred micro litre of each dilution was spreaded over NA plate, King's B (KB) plate and YMA plate at three replications and incubated at 30±1ºC for 48 hr. Colonies of bacteria were purified on NA plates. For isolation of spore forming *Bacillus subtilis,* stock solution was heated at 85°C for 10 minutes (Nemeckova *et al*., 2011).

2.5. Identification of antagonistic bacteria

Identification of antagonistic bacteria was done by gram's staining reaction (Gerhardt, 1981), potassium hydroxide (3% KOH) test (Suslow *et al.*, 1982), starch hydrolysis test (Cowan, 1974), catalase test (Schaad, 1988), oxidase test (Kovacs, 1956), citrate utilization test (Schaad, 1988), motility indole urease agar (MIU) test (Schaad, 1988) and gelatine liquefaction test (Salle, 1961) as described above.

2.6. In-vitro screening of chemicals against Erwinia carotovora subsp. carotovora

A supply of cotton swabs on wooden applicator sticks was prepared following the method described by Vandipitte *et al*. (1991). Five selected chemicals viz. Cupravit 50 WP (Copper oxychloride), Sulcox 50 WP (Copper oxychloride), Champion 77 WP (Copper hydroxide), Indofil M-45 (Mancozeb) and Bavistin 50 WP (Carbendazim) were screened against the bacterium by well diffusion method measuring the inhibition zone (Anon, 1996). Four wells of 5 mm in diameter were made in the same NA plate maintaining equal distance and the broth culture of *Erwinia carotovora* subsp. *carotovora* was spreaded uniformly on it with sterile cotton swabs. Chemical suspension at definite concentration with different volume was added into the well each at three replications. In case of control, only sterile water was used instead of chemical. The plates were incubated at 30±1ºC. Zone of inhibition around the wells were measured and recorded after every 24 hours for 5 days.

2.7. In vitro screening of biocontrol agents against Erwinia carotovora subsp. carotovora

Five biocontrol agents viz. *Bacillus subtilis, Pseudomonas fluorescens, Rhizobium leguminosarum, Trichoderma harzianum* and *Aspergillus flavus* were screened against the growth of *Erwinia carotovora* subsp. *carotovora* by well diffusion method measuring the inhibition zone (Yenjerappa, 2009). Supernatant of biocontrol agents were dropped into the well of previously swabbed plate with the pathogenic bacteria each at three replications. In case of control, only sterile water was used instead of supernatant. The plates were then incubated at 30±1ºC. Zone of inhibition around the wells were measured and recorded after every 24 hours for 5 days.

Data on different parameters were analyzed by using computer software MSTAT-C (Anon., 1989) and level of significant differences under different treatments and parameters were determined.

3. Results and Discussion

3.1. Isolation and purification of the causal organism

The infected potato tuber yielded well separated, round, convex, creamy white colonies of *Erwinia carotovora* subsp. *carotovora* on NA plates after 48 hours of incubation at 30±1ºC (Fig. 1). Similar types of colonies were found by Abd El-Khair and Karima (2007).

3.2. Identification of the pathogen

The isolated bacteria identified as *Erwinia carotovora* subsp. *carotovora* according to its morphological and biochemical characters (Table 1).

The bacterium was rod shaped with rounded ends, cells appeared singly and also in pairs, gram negative (red colour) and capsulated under the compound microscope at 100x magnification with oil immersion. It produced a mucoid thread when lifted with the loop, showed amylase activity, formed bubbles after adding 3% H_2O_2, formed dark purple colour on oxidase disk, used citrate as a carbon source for their energy, migrated away from the original line of inoculation, gelatin was liquefied and decayed the potato slice (Fig. 2). These results agreed with those recorded by Pérombelon (2002), Schaad *et al.* (2001) and Cuppels and Kelman (1974).

3.3. Pathogenicity test

The characteristic symptoms were observed on potato tuber after ten days of inoculation as small, water-soaked spots on the surface. These spots were rapidly enlarged and the tissue was decomposed in a soft, blister-like area on the surface of the tuber. Re isolation was carried out from these lesions and comparison was made with the original culture to confirm the identity of the pathogen. Artificially inoculated plants yielded the bacterial colonies similar to the original ones.

Table 1. Reaction of isolated *Erwinia carotovora* subsp. *carotovora* to different tests

Name of tests	Reaction
Gram's staining	+
KOH solubility test	+
Starch hydrolysis test	+
Catalase test	+
Oxidase test	+
Citrate utilization test	+
Motility indole urease agar (MIU) test	+
Gelatine liquefaction test	+
Pectiolytic test	+

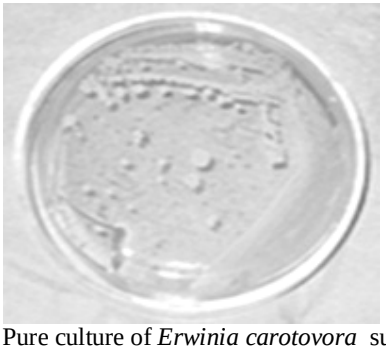

Fig. 1. Pure culture of *Erwinia carotovora* subsp. *carotovora*

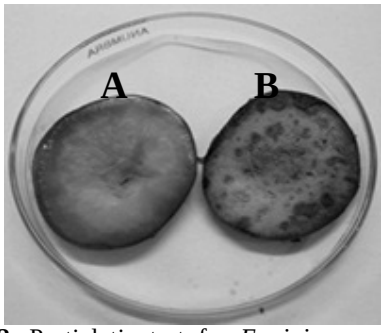

Fig. 2. Pectiolytic test for *Erwinia carotovora* subsp. *carotovora* (A. control and B. inoculated potato slice)

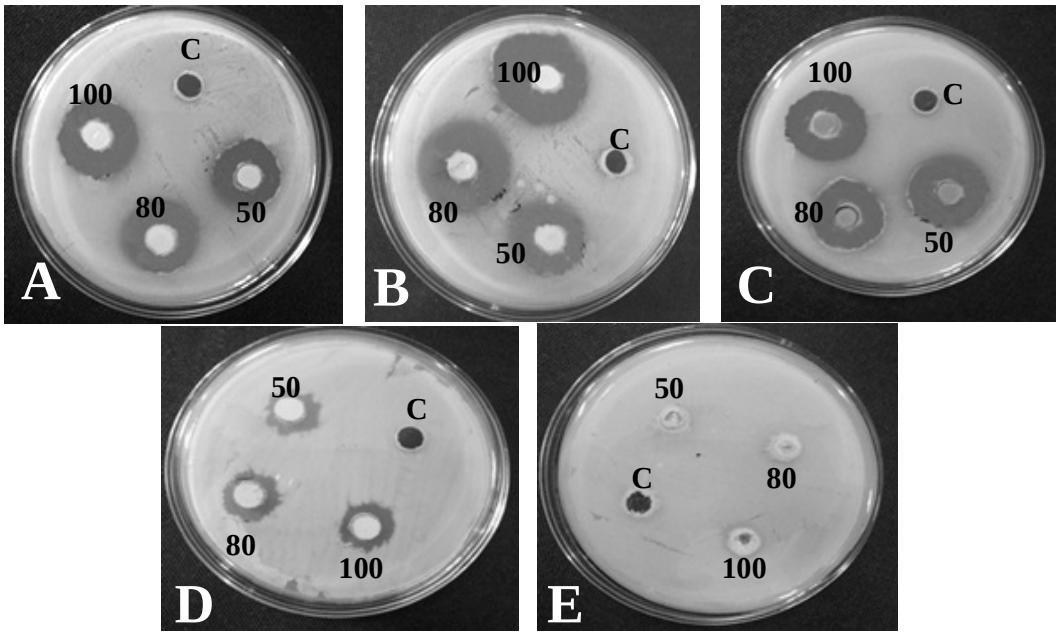

Fig. 3. Screening of different chemicals against *Erwinia carotovora* subsp. *carotovora* (A) Cupravit 50 WP (B) Sulcox 50 WP (C) Champion 77 WP (D) Indofil M-45 and (E) Bavistin 50 WP after 48 hours of incubation

3.4. In-vitro screening of chemicals against Erwinia carotovora subsp. carotovora

Among the five chemicals, Sulcox 50 WP (Copper oxychloride) showed the highest inhibition zone after 24 (28.00 mm), 48 (31.00 mm), 72 (29.33 mm), 96 (26.67 mm) and 120 hours (23.67 mm) of incubation at 0.2% concentration when 100 µl/well was used where Bavistin 50 WP (Carbendazim) showed the lowest inhibition zone (Table 2 and Fig. 3). Garza *et al.* (2002) reported that copper-based compounds suppressed soft rot bacterial growth at various cupric ion concentrations *in vitro*.

Table 2. Comparative efficacy of some chemicals against *Erwinia carotovora* subsp. *carotovora*

Antibacterial chemicals	Conc. (%)	Volume (µl)	Inhibition zone (mm)				
			24 h	48 h	72 h	96 h	120 h
Cupravit 50 WP	0.2	100	26.67 b	29.00 b	26.33 b	24.33 b	20.67 b
Sulcox 50 WP	0.2	100	28.00 a	31.00 a	29.33 a	26.67 a	23.67 a
Champion 77 WP	0.2	100	25.67 b	28.33 b	26.00 b	23.33 c	21.00 b
Indofil M-45	0.3	100	19.33 c	18.67 c	16.33 c	13.67 d	11.67 c
Bavistin 50 WP	0.3	100	10.33 d	0.00 d	0.00 d	0.00 d	0.00 d
CV %			2.99	4.59	2.79	2.74	3.56

Each data represents the mean of three replications.
Values followed by the same letter within a column are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test.

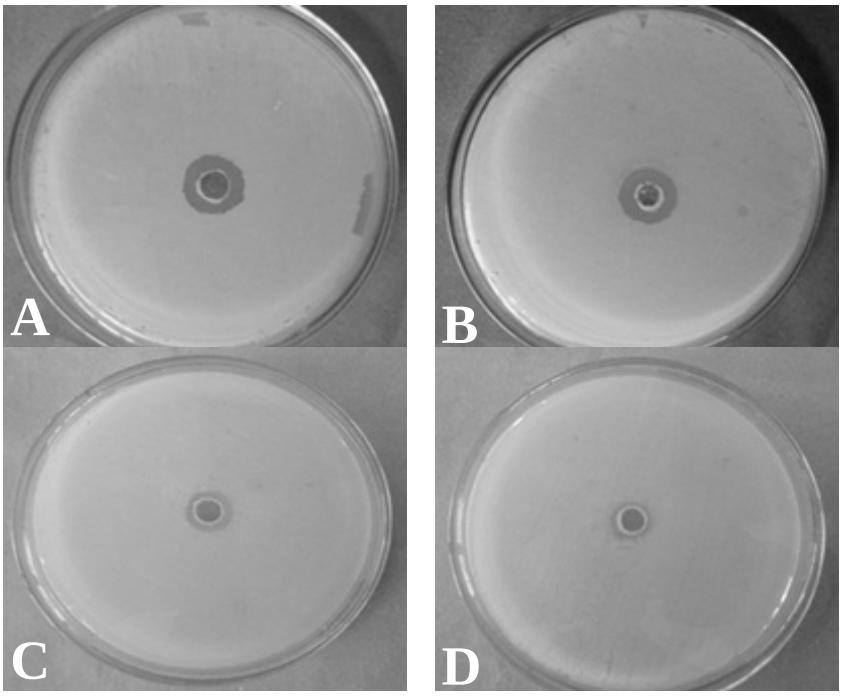

Fig. 4. Screening of different biocontrol agents against *Erwinia carotovora* subsp. *carotovora* (A) *Bacillus subtilis* (B) *Trichoderma harzianum* (C) *Pseudomonas fluorescens* and (D) *Rhizobium leguminosarum* after 48 hours of incubation

Table 3. Efficacy of some biocontrol agents against the growth of *Erwinia carotovora* subsp. *carotovora*

Biocontrol agent	Inhibition zone (mm)				
	24 h	48 h	72 h	96 h	120 h
Bacillus subtilis	14.33 b	16.67 a	15.67 a	13.33 a	9.33 a
Pseudomonas fluorescens	11.67 c	14.33 b	13.33 b	12.00 b	8.00 b
Rhizobium leguminosarum	11.33 c	13.67 b	13.33 b	11.33 b	7.67 b
Trichoderma harzianum	16.33 a	13.67 b	0.00 c	0.00 c	0.00 c
Aspergillus flavus	0.00 d	0.00 c	0.00 c	0.00 c	0.00 c
Control	0.00 d	0.00 c	0.00 c	0.00 c	0.00 c
CV %	5.65	4.47	5.59	7.11	8.76

Each data represents the mean of three replications.
Values followed by the same letter within a column are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test.

3.5. In-vitro screening of biocontrol agents against Erwinia carotovora subsp. carotovora

Among the five biocontrol agents, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Rhizobium leguminosarum* and *Trichoderma harzianum* were significantly superior in inhibiting the growth of *Erwinia carotovora* subsp. *carotovora* (Table 3 and Fig. 4). *Bacillus subtilis* showed highest inhibition zone (16.67 mm) after 48 hours of incubation followed by *Trichoderma harzianum* with inhibition zone (16.33 mm) after 24 hours of incubation (Table 3). *Aspergillus flavus* was ineffective as it failed to inhibit the growth of *Erwinia carotovora*. Long *et al*. (2003) reported that the genus *Bacillus* and *Pseudomonas* have antagonistic activity against various plant pathogenic bacteria including soft rot bacterium *E. carotovora* subsp. *carotovora in vitro*. Similar results were also observed by Abd El-Khair and Karima (2007); Raju *et al.* (2006). Abd El-Khair and Karima (2007) reported that *T. harizanum* and *B. subtilis* were strangely effective in reducing the soft rot disease.

4. Conclusions

Erwinia carotovora subsp. *carotovora*, the causal agent of soft rot of potato was isolated from the infected tuber and was identified. The present findings revealed that among the five selected chemicals Sulcox 50 WP (Copper oxychloride) was highly effective and among the five biocontrol agents, *Bacillus subtilis* was highly effective against this pathogen *in vitro*.

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