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CHARACTERIZATION OF THE CAUSAL ORGANISM OF BLACKLEG AND SOFT ROT OF POTATO, AND MANAGEMENT OF THE DISEASE WITH BALANCED FERTILIZATION

HAFIZ FARHAD ALI[1*], AYESHA BIBI[1], MUSHARAF AHMAD[1], MUHAMMAD JUNAID[1], ASAD ALI[1], SHAUKAT HUSSAIN[1], SHAH ALAM[2] AND SARTAJ ALAM[1]

[1]Department of Plant Pathology, [2]Department of Plant Protection. The University of Agriculture, Peshawar.
[*]Corresponding author e-mail: hafizfarhad66@yahoo.com

Abstract

Based upon colony morphology, physio-biochemical tests and polymerase chain reaction (using species or subspecies-specific primers) studies, 20 isolates (out of a total of 42) were found to be *Erwinia carotovora* subspecies *atroseptica* (Eca), 19 were identified as *Erwinia carotovora* subspecies *carotovora* (Ecc), and 3 as *Erwinia chrysanthemi* (Ech). Results of the subspecies-differentiating biochemical tests indicated that majority of the candidate Ecc isolates did not produce acid from α-methyle glucoside (as expected) but their reaction to the production of reducing substances from sucrose was variable. Likewise, some of our Eca and Ecc strains (unexpectedly) were sensitive to erythromycin. Also, most of our Eca strains unexpectedly grew at 36°C. Our strains slightly deviate from the standard description in some of their minor characteristics but they still remain the valid members of the Eca, Ecc or Ech group as similar variations in minor characteristics have been found by other workers. The occurrence of intermediate forms of Eca and Ecc (sharing some of the characteristics of both the groups) indicates variability happening among these strains. This variability indicates the potential ability of the pathogen to break the resistance of the host. The results of the effect of balanced nutrition in controlling blackleg and soft rot of potatoes indicated that the fertilizer combination of $N_3P_1K_3$ (262/252/262 kg.ha^{-1}) which is slightly higher than the normally practiced dose (247/247/247 kg.ha^{-1}) was the best in bringing the disease to a minimum and subsequently increasing the yield.

Key words: *Erwinia carotovora* subsp. *atroseptica*, Eca, Blackleg, PCR, isolates.

Introduction

Potato (*Solanum tuberosum* L.) is one of the most important vegetable crops of the world including Pakistan. The climate of Pakistan, especially that of Khyber Pukhtunkhwa, is very suitable for the production of potatoes. In Pakistan, potato occupies an area of 133435 ha with a total production of 2581554 tons, while in Khyber Pukhtunkhwa, it occupies an area of 9600 ha with a total production of 129529 tons (Anon., 2006-2007). Among the 24 districts of Khyber Pukhtunkhwa, Nowshera, Mardan, Abbotabad, Swat, Dir Upper, Chitral, and North Waziristan are the major potato-growing districts where during 2006-2007, area under the crop was 1049, 868, 550, 917, 976, 759 and 890 ha with an average production of 17334, 7518, 5888, 16257, 20737, 11385 and 9075 tons respectively (Anonymous, 2006-2007). The average per ha production of potatoes in Khyber Pukhtunkhwa is well below its potential. One of the reasons for this low yield and quality is the occurrence of different bacterial diseases such as brown rot, ring rot, black leg and soft rot of potatoes.

Blackleg and soft rot are important diseases (caused by *Erwinia* spp., a Gram-negative, facultatively anaerobic, rod-shaped bacterium with peritrichouse flagella) of potato that cause heavy losses to potato crop not only in the field but also in the storage where the bacteria are transmitted from diseased tubers to healthy ones. Multiple subsp. of *Erwinia*, including *E. carotovora* subsp. atroseptica (Eca) and *E. c.* subsp. *carotovora* (Ecc), *E. chrysanthemi* (Ech) *E. carotovorum* subsp. *brasiliensis* (Ecb), and *Pectobacterium* (syn. *Erwinia*) *wasabiae* (Pw) attack potatoes. Ecc and Eca are the primary enterobacteria responsible for soft rotting of potato in temperate climates (Pitman *et al*., 2010). Pathogenicity of Eca is usually restricted to potatoes grown in cool and temperate climates, while Ecc (causing potato soft rot and, in some cases blackleg too) have a wider distribution in both temperate and tropical zones showing wider host ranges than those of the other subspecies (Wells & Moline, 1991). Eca is the major cause of blackleg, a blackening of the stem base of potato plants, which originates from the mother tuber (Pérombelon & Kelman, 1987). Black leg causes 10-30% whereas soft rot causes 2-10% losses in Khyber Pukhtunkhwa (Turkensteen, 1986). The pathogens are seed-borne and frequently remain undetected by the common detection methods. As much as 20% of the seed potato supplied (from open market of Punjab) on credit to small potato farmers in Khyber Pukhtunkhwa by the potato seed dealers is infected with Ecc & Eca (Musharaf Ahmad, Professor, Department of Plant Pathology, The University of Agriculture, Peshawar, Personal Communication).

Since potato seed tubers are the chief source of inoculum for many bacterial diseases, planting of pathogen-free seed tubers is a must for the control of such diseases. However, the traditional methods for the identification and detection of these bacterial pathogens in seed tubers are quite laborious and not really fool-proof. Polymerase chain reaction (PCR) that rapidly detects, identifies and characterizes microorganisms in a shorter time is a good alternate for the identification purposes. Gene-specific/ subspecies-specific/ species-specific primers could be used in a simple or multi-plex PCR to precisely identify the seed-borne phyto-pathogenic bacteria. Based upon PCR-indexing results, seed tubers could be treated to free them of the pathogens.

Controlling plant bacterial pathogens and the diseases they cause is a very serious problem. In case of human and animal bacterial diseases, antibiotics are frequently used for the control of such diseases. However, to control phytobacterial diseases, antibiotics are not recommended for two reasons; first, antibiotics are expensive and second, their long-term use might force bacteria to develop resistance against these antibiotics. This antibiotic resistance, when transferred to animal and human bacteria, can pose serious threats. Use of copper fungicides as bactericides is another option to control some plant bacterial diseases to some extent. However, copper compounds, besides being bad for the environment, can cause phytotoxicity and may exert a negative impact on the yield (Kowalska & Smolinska, 2008).

Proper fertilization, both the amount and the type of fertilizer could be manipulated to control some plant bacterial diseases. Nitrogen content of chicory plants was found to be positively correlated with the amount of nitrogen fertilization (Reerink, 1993) and with the amount of the bacterial soft rot (Wright, 1993). Likewise, Brigitte *et al.*, 1999) concluded that nitrogen treatment enhanced soft rot incidence in chicory heads. McGovern *et al*., (1985) compared three different types of N fertilizers while studying the susceptibility of *Chrysanthemum morifolium* to *Erwinia chrysanthemi.* They found that Ca $(NO_3)_2$ and NH_4NO_3, at 400 ppm level, were better than $(NH_4)_2SO_4$ in terms of reducing the disease susceptibility of *Chrysanthemum morifolium*. Some fertilizers can also increase or decrease the level of pathogeicity of a pathogen. For example, Gracia *et al*., (2004) experimentally proved that one of the reasons of the increased tuber rot was the activation of bacterial tissue macerating pectic enzymes by phosphorous of the growth medium suggesting that P fertilizers could increase plant host susceptibility to soft rot bacteria. Potassium also plays an important role in defending plants against diseases including bacterial diseases. It was found that the use of potassium decreased the incidence of 70% of fungal diseases, 69% of bacterial disease, and 41% of viral disease. Simultaneously, K increased the yield of plants infected with fungal disease by 42%, with bacterial disease by 57%, and with viruses by 78% (Perrenoud, 1990). Potassium enables plants to produce disease inhibitory compounds, such as phenols and phytoalexins. If K is low in plants, inorganic N would accumulate which results in the rapid break down of phenols thus making plants susceptible to diseases (Kiraly, 1976).

Keeping in view the importance of potato crop in Khyber Pakhtunkhwa, the losses caused by blackleg and soft rot diseases to this crop, and the lack of research work on these diseases in this province, the present research work was carried out to characterize and PCR-identify the pathogen(s), and to explore the role of proper fertilization of potato plants in controling blackleg and soft rot of potato.

Materials and Methods

Sample collection: A large number of potato plants and tubers showing typical symptoms of blackleg and soft rot were collected to isolate the causal organism. Samples were put in paper bags, kept cool and processed as soon as possible (to reduce the chances of secondary invaders) in The University of Agriculture Clinical Plant Pathology Laboratory. Samples yielding bacterial colonies having the morphology of Soft Rot Erwinias (SRES) were recorded whereas those yielding no bacteria or G+ bacteria were discarded.

Isolation of *Erwinia* spp.: Nutrient agar or NA (Bacto Agar; 10gm, NaCl; 5.0 gm, K_2HPO_4; 5 gm, KH_2PO_4; 2gm, Bactopeptone; 1.0gm and Ditilled water 1L) was used for the isolation of soft rot Erwinias. Tubers and plant samples showing disease symptoms were cleaned, surface-sterilized with 0.5% sodium hypochlorite solution (for 30 seconds), washed with sterile distilled water, and ground in sterile 0.85% saline solution using sterile mortar under aseptic conditions. The resulting bacterial suspension was left undisturbed for a few minutes. A loopful of this suspension was then streaked on the surface of plates containing nutrient agar, and the plates were incubated at 28°C for 24 h. Individual colonies (transparent, circular, raised, shiny and creamy white) growing on NA were picked up, re-suspended in 0.85% saline and streaked on NA plates, and then incubated at 28°C for another 24 h. This was done several times to obtain pure cultures.

Nutrient agar is a non-selective medium and sometimes there is a problem of over-growth of saprophytic bacteria on this medium. Therefore, in some cases, we used an indirect approach to avoid this problem. For this purpose, green pepper fruits were used as an enrichment host for the soft rot Erwinias which were subsequently isolated on NA. The peppers were surface-disinfested with 70% alcohol and 1% sodium hypochlorite (NaOCl), for 30 seconds each, and then washed with sterile distilled water. Next, sterile toothpicks were stabbed into soft-rotten tubers or the margin of blackleg lesions on potato stems and then the same toothpicks were inserted into green pepper (*Capsicum annuum* L.) fruits (Takatsu *et al*., 1981). The inoculated fruits were kept in a humid chamber at 28°C for 24-48 h. Decayed tissue was peeled off with a scalpel and crushed in 0.85% saline as described before. A loopful was used to streak the surface of NA plates. Single colonies were harvested and purified as described before.

Pure colonies were saved in 70% glycerol solution and stored at -20°C or -80°C. Cultures were also saved in 0.85% sterile saline solution and stored at 4°C. When needed, each bacterial strain was cultured on LB (Trypton 10gm, yeast extract 5gm, NaCl 10 gm, agar 15.0 gm, distilled water 1 liter) at 28°C for 2 days.

Identification of bacteria: Identification of the causal organism as Eca, Ecc or Ech was done by colony morphology, physio-biochemical tests (such as tissue maceration, yellow pigmentation on YDC, growth at 36°C, mucoid growth, reducing substances from sucrose, 5% NaCl tolerance, erythromycin sensitivity, catalase activity, and acid production from alph-methyle glucoside) and polymerase chain reaction (PCR). Tissue maceration test was carried out as reported by El-

Hendawy *et al*., (2002) and the physio-biochemical tests were performed according to the methods reported by Lelliot & Dickey (1984).

Molecular identification: The identity of the bacteria was confirmed by polymerase chain reaction (PCR) using subspecies-specific primers. Eca-specific primers, Eca1F (5'-CGGCATCAT-AAAAACACG-3') and Eca2R (5'-GCACACTTCATCCAGCGA-3') (De Boer & Ward, 1995) amplified, as expected, a 690 bp band. Ecc-specific primers, EXPCCR (5'-GCCGTAATTGCCTACCTGCTT-AAG-3') and EXPCCF (5'-GAACTTCGCA-CCGCCGACCTTCTA-3') (Kang *et al*., 2003) produced a 550 bp band whereas Ech-specific primers, ADE1 (5'-ATCAGAAAG-CCCGCAGCCAGAT-3') and ADE2 (5'-CTGTGGCCGA- TCAGGATGGTTTTGT-CGTGC-3') (Nassar *et al*., 1996) amplified a small band of 420 bp size. DNA was extracted (Wang *et al*., 1993) from the unknown (target) bacterium and 3 µl of the lysate (template) was directly used in PCR reaction. The PCR master mix included 2 mMol $l^{-1}MgCl_2$, 1 µMol l^{-1} each primer, and 0.2 mMol l^{-1} dNTPs. The concentration of the Taq buffer (Tris HCl pH 8.8) used was 67mM l^{-1}. To make the Taq DNA polymerase work for longer time, the enzyme was added to PCR tubes after the initial denaturation step. MJ mini thermocycler (Bio-rad, USA) PCR machine was used to amplify DNA. PCR steps and temperatures used were (i) one-time denaturation at 95°C for 5 minutes, (ii) repeat cycle denaturation at 94°C for 30 sec, (iii) primer annealing at 47° C for 30 sec, and (iv) primer extension at 72°C for 50 sec. The denaturation, primer annealing and primer extension steps were repeated 39 times. At the end of 40 cycles, a final extension at 72°C for 8 min was done. However, the temperatures used for primer annealing steps in case of PCR-identification of Ecc and Ech were 57°C each. After the amplification of the DNA through PCR, 25 µl from each sample tube (PCR tube) was taken and electrophoresed through a 2% (w/v) agarose gel (Sambrook *et al.,* 1989) to separte the amplified DNA bands. The separated bands were stained with ethedium bromide (0.5µg/ml) solution on agarose gel, visualized under UV light in UV tech machine (ESSENTIAL, D-55-20-M-Auto, UK) and photographed.

Disease control via host fertilization: Different fertilizers i.e., Nitrogen (Urea), Phosphorus (Diammonum phosphate) and Potash (Sulfate of potash) and their different levels (level s = 247 kg ha^{-1}, level 1 = 252 kg ha^{-1}, level 2 = 257 kg ha^{-1}, level 3 = 262 kg ha^{-1} each) were evaluated (autumn, 2008) for their effect in controlling blackleg and soft rot of potato. The experiments were conducted using RCBD (replicated three times) having 27 treatments and a control. Potato cultivar Kuroda was used in the trial. Naturally infested field (having the left-over diseased plant debris, discarded rotten tubers and possibly pathogen-harbouring solanaceous weeds as sources of inoculum) in district Mardan was chosen for these experiments. The total amount of nitrogenous fertilizer used per season was split into two parts: first half dose of nitrogen fertilizer was applied before planting and second half dose at the tuber formation/earthing up stage while the other two fertilizers were applied with sowing. Before sowing, seed tubers were kept at room temperature for a few days in the laboratory for sprouting. Data were taken on disease incidence, severity, and yield 70 days after sowing.

Disease incidence and severity: Disease incidence (%) was simply calculated by dividing the number of plants (per each random spot of 1 M^2) showing blackleg and soft rot disease symptoms by total number of plants and multiplied by one hundred. The values of all random spots (per field) were averaged together. Disease severity of each plant present in each randomly selected spot was assessed on a scale of 0-3 as reported by Wright *et al*., (2005) where 0 = no disease symptoms on plant, 1 = ≤ 50% of the plant has disease symptoms, 2 = > 50 % of the plant has disease symptoms, and 3 = plant totally dead. The disease rating values of individual plants were converted to % disease severity values using Bdliya & Dahiru, (2006) statistical equation ($S = 100\sum n/3N$; where S = black leg severity (%), $\sum n$ = sum of the ratings of all plants, N = shows the number of plants used for rating, and 3 = biggest number of the disease rating scale) to determine % disease severity. Values of all spots per field were averaged.

Results and Discussion

Identification of soft-rot *Erwinia* spp. (SRES): All the isolates either obtained from soft rotted tubers or black-legged potato plants were identified by colony morphology and physio-bicchemical tests. Regarding the morphology of the bacterial colonies on nutrient agar (NA), the colonies appeared to be transparent, circular, raised, shiny and creamy white after 48 h incubation at 28°C (Fig. 1). All the strains were catalase-positive, tolerated 5% NaCl, macerated potato (Fig. 2) and carrot tissue, and produced yellow pigment on YDC (Table 1).

The above-mentioned tests are general tests and do not differentiate Eca from Ecc or Ech. In order to know whether our isolates belong to Eca, Ecc or Ech, key diagnostic tests such as acid production from α-methyle glucoside, production of reducing substances from sucrose, sensitivity to erythromycin and growth at 36°C were performed and the results were compared with those of Lelliot & Dicky (1984) and Perombelon & Kelman (1980). Results showed (Table 1) that majority of the candidate Ecc isolates did not produce (whereas majority of candidate Eca, and Ech isolates did produce) acid from α-methyle glucoside but their reaction to the production of reducing substances from sucrose was variable. However, majority of Eca isolates produced reducing substances from sucrose. These results are in line with those of Lelliot & Dicky (1984) and Perombelon & Kelman (1980).

Sensitivity to erythromycin is also a variable character. Majority of our strains were not sensitive to this antibiotic, some were sensitive and some were weakly sensitive (Table 1). El-Hendawy *et al*., (2002) and Lelliot & Dicky (1984) found their Eca and Ecc strains not to be sensitive to erythromycin but Perombelon & Kelman (1980) reported that their Eca and Ecc strains were sensitive to this antibiotic.

Table 1. Characterization of Soft Rot Erwinia Species (SRES).

S.#	District	Location	Yellow pigment on YDC	Growth at 36co	Mucoid growth	Reducing substances from sucrose	5% NaCl tolerance	Erythromycin sensitivity	Catalase activity	Acid from α-methyl glucoside	Tissue maceration	Lab. Results
SRES 1	Abbottabad	Kakool	+	+	+	-	+	-	+	-	+	Ecc
SRES 2	Abbottabad	Hazara	+	+	+	-	+	-	+	-	+	Eca
SRES 3	Abbottabad	Kakool	+	+	+	+	+	-	+	+	+	Eca
SRES 4	Abbottabad	Kakool	+	+	+	+	+	-	+	+	+	Eca
SRES 5	Abbottabad	Logan colony	+	+	+	+	+	-	+	-	+	Ecc
SRES 6	Battagram	Pagora	+	+	+	+	+	-	+	-	+	Ecc
SRES 7	Charsadda	Umarzai	+	+	+	+	+	-	+	-	+	Ecc
SRES 8	Charsadda	Dawoodzai	+	+	+	-	+	-	+	-	+	Ecc
SRES 9	Charsadda	Mandani	+	+	+	+	+	-	+	-	+	Ecc
SRES 10	Haripur	Dobandi	+	+	+	+	+	-	+	-	+	Ecc
SRES 11	Harripur	Saraye sala	+	+	+	+	+	-	+	-	+	Ecc
SRES 12	Swat	Behrain	+	+	+	-	+	±	+	-	+	Eca
SRES 13	Swat	Behrain	+	+	+	+	+	-	+	+	+	Eca
SRES 14	Swat	Madain	+	+	+	-	+	+	+	±	+	Ech
SRES 15	Swat	Utrore (Kalam)	+	+	+	+	+	-	+	+	+	Eca
SRES 16	Swat	Kanai (Kalam)	+	+	+	+	+	-	+	+	+	Eca
SRES 17	Swat	Boyan (Kalam)	++	+	+	+	+	-	+	+	+	Eca
SRES 18	Swat	Kalam	+	+	+	+	+	-	+	+	+	Eca
SRES 19	Swat	Kas kili (Kalam)	+	+	+	+	+	-	+	-	+	Ecc
SRES 20	Mansehra	Kalgan	+	+	+	+	+	-	+	+	+	Eca
SRES 21	Mansehra	Chatti ghetti	+	+	+	+	+	-	+	-	+	Ecc
SRES 22	Mansehra	Bafa	+	+	+	-	+	+	+	±	+	Ech
SRES 23	Mardan	Sher ghar	+	+	+	+	+	-	+	+	+	Eca
SRES 24	Mardan	Torbhati	+	+	+	-	+	+	+	±	+	Ech
SRES 25	Mardan	Torbhati)	+	+	+	+	+	-	+	+	+	Eca
SRES 26	Mardan	Torbhati	++	+	+	+	+	-	+	+	+	Eca
SRES 27	Mardan	Taus(Ijara korona)	+	+	+	+	+	±	+	+	+	Eca
SRES 28	Mardan	Katlang	+	+	+	+	+	-	+	+	+	Eca
SRES 29	Nowshehra	Dag baisood	+	+	+	-	+	-	+	-	+	Ecc
SRES 30	Nowshehra	Dag baisood	+	+	+	+	+	-	+	+	+	Eca
SRES 31	Nowshehra	Aza kheil bala	+	+	+	+	+	-	+	+	+	Eca
SRES 32	Nowshehra	Wazir gari pubbi	++	+	+	+	+	-	+	-	+	Ecc
SRES 33	Peshawar	Machni	+	+	+	+	+	-	+	+	+	Eca
SRES 34	Peshawar	Kohat road	+	+	+	+	+	+	+	+	+	Ecc
SRES 35	Peshawar	Ghari sarfaraz	+	+	+	+	+	±	+	+	+	Eca
SRES 36	Peshawar	Palosay	+	+	+	+	+	-	+	-	+	Ecc
SRES 37	Shangla	Koat kili (bower)	+	+	+	+	+	-	+	-	+	Ecc
SRES 38	Shangla	Shangla top	+	+	+	+	+	-	+	-	+	Ecc
SRES 39	Shangla	Shangla	+	+	+	-	+	-	+	-	+	Ecc
SRES 40	Swabi	Manirri	+	+	+	+	+	-	+	+	+	Ecc
SRES 41	Swabi	Adena,	+	+	+	+	+	-	+	+	+	Ecc
SRES 42	Swabi	Jhangira	++	+	+	+	+	-	+	+	+	Eca

+ = Positive results, - = Negative results, and ± = Variable results

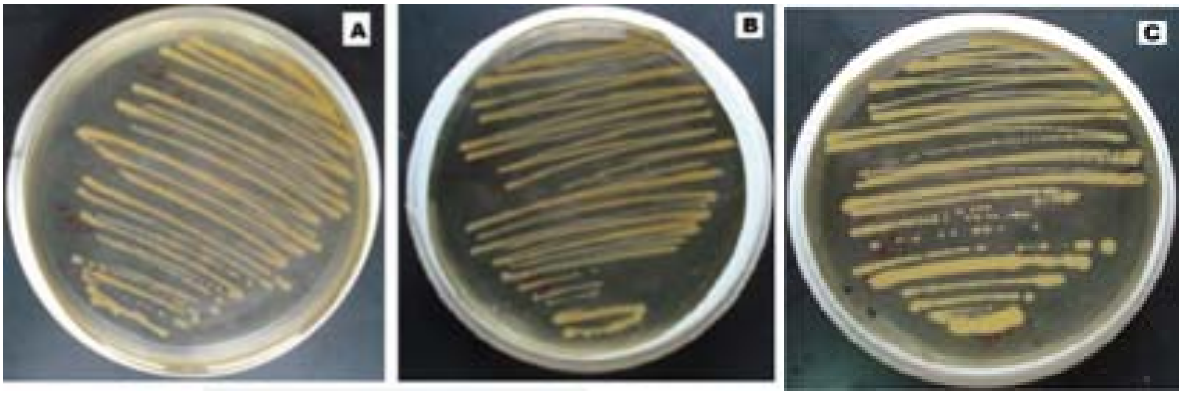

Fig. 1. Growth of *Erwinia carotovora* subspecies *carotovora* (A), *Erwinia carotovora* subspecies *atroseptica* (B), and *Erwinia chrysanthemi* (C) on nutrient agar 48 hours after incubation.

Fig. 2. Potato tissue maceration/soft rot produced by *Erwinia carotovora* subspecies *carotovora* (A), *Erwinia carotovora* subspecies *atroseptica* (B), and *Erwinia chrysanthemi* (C) 24 hours after artificial inoculation on potato halves.

The collected isolates were identified to species or sub-species level. Most isolates were found to have expected characteristics. However, some of them showed un-expected behavior. For example, all our strains were able to grow at 36°C which is the characteristic of Ecc and Ech group according to Lelliot & Dicky (1984) and Perombelon & Kelman (1980) and not that of Eca group. Based upon their growth at 36°C, our Eca strains appear to be atypical, similar to those reported by other researchers (Thomson *et al*., 1981; Helias *et al*., 1998). El-Hendawy *et al*., (2002) also reported that 21 of their Eca strains were able to grow at 36°C. Jabuonsky *et al*., (1986a) reported that unexpectedly 44 of their Ecc strains were not able to grow at 37°C. Our strains slightly deviate from the standard description in some of their minor characteristics but they still remain the valid members of the Eca group as similar variations in minor characteristics have been found by other researchers. The occurrence of intermediate forms of Eca and Ecc (sharing some of the characteristics of both the groups) indicates variability happening among these strains. This variability poses a threat towards breaking the host resistance by the pathogen.

PCR identification of soft-rot *Erwinia* spp.: To confirm the precise identity of the SRES isolates, molecular studies were done using Eca-specific, Ecc-specific and Ech-specific primers. The Eca-specific 690 bp band was amplified from a total of 20 isolates confirming that they were Eca (Fig. 3A). Nineteen isolates were found to be Ecc as the Ecc-specific primers amplified the expected band of 550 bp (Fig. 3B). A few isolates were identified to be Ech because Ech-specific primers amplified the expected 420 bp band from them (Fig. 3C).

The fact that we can successfully and accurately identify the potato-blackleg causal organisms using specific primers and our optimized PCR conditions has important implications for seed certifying agencies doing seed-potato indexing. For this purpose, random samples can be taken from seed-potato lots, crushed in 0.85% saline, plated on NA medium, DNA extracted (Wang *et al*., 1993) and PCR-tested for the amplification of the 690 bp Eca-specific, 550 bp Ecc-specific, and 420 bp Ech-specific DNA bands. If only one type of disease-causing bacterium is expected to be present in seed potatoes, single PCR reaction can be carried out. However, if two or three types of bacteria (causing blackleg and soft rot) are expected; multiplex PCR reaction could be performed.

Disease management via host fertilization

Yield (g.plant^{-1}): Significant differences ($p \leq 0.05$) were found in potato yield (g.plant^{-1}) among different combinations of nitrogen (N), phosphorus (P) and potash (K) fertilizers (Table 2). The maximum yield (579.50 g.plant^{-1}) was obtained when combination $N_3P_1K_3$ (i.e. 262/252/262 NPK kg ha^{-1}) of fertilizers was used, followed by treatments $N_3P_2K_3$ (570 g.plant^{-1}) and $N_3P_3K_3$ (537.25 g.plant^{-1}). The later two combinations showed 1.64% and 7.29% decrease in yield, respectively, over that of the best combination. The lowest yield (286.25g.plant^{-1}) was obtained when combination $N_3P_1K_1$ was used.

Table 2. Effect of different NPK combinations on the % disease incidence, severity and yield per plant of the potato crop affected with blackleg and soft rot (autumn 2008).

S. #	Treatments	Parameters					
		Disease severity (%)	Increase over the minimum (%)	Disease incidence (%)	Increase over the minimum (%)	Av. yield per plant (g)	Decrease over the maximum (%)
1.	N1 P1 K1	60.250 a	217.1053	83.000 a	253.1915	327.00 fgh	43.57204
2.	N1 P1 K2	49.250 cd	159.2105	73.000 bc	210.6383	337.25 eh	41.80328
3.	N1 P1 K3	39.750 f	109.2105	67.000 d	185.1064	350.50 dh	39.51682
4.	N1 P2 K1	56.000 b	194.7368	78.000 ab	231.9149	361.25 dh	37.66178
5.	N1 P2 K2	46.750 de	146.0526	65.000 de	176.5957	419.25 cg	27.65315
6.	N1P2 K3	37.500 fg	97.36842	61.000 ef	159.5745	366.50 dh	36.75582
7.	N1 P3 K1	48.000 de	152.6316	74.250 bc	215.9574	330.63 fgh	42.94564
8.	N1 P3 K2	30.000 jk	57.89474	61.000 ef	159.5745	431.25 cg	25.5824
9.	N1 P3 K3	25.250 mn	32.89474	61.000 ef	159.5745	436.00 cg	24.76273
10.	N2 P1 K1	52.000 c	173.6842	61.750 e	162.766	355.75 dh	38.61087
11.	N2 P1 K2	47.000 de	147.3684	51.000 ghi	117.0213	431.25 cg	25.5824
12.	N2 P1 K3	35.750 gh	88.15789	48.000 ij	104.2553	470.25 ad	18.85246
13.	N2 P2 K1	48.000 de	152.6316	64.500 de	174.4681	340.25 eh	41.28559
14.	N2 P2 K2	33.000 hi	73.68421	55.000 g	134.0426	417.50 cg	27.95513
15.	N2 P2 K3	27.000 lm	42.10526	49.250 hi	109.5745	513.75 abc	11.34599
16.	N2 P3 K1	45.500 e	139.4737	60.250 ef	156.383	336.25 eh	41.97584
17.	N2 P3 K2	31.000 ij	63.15789	47.000 ij	100	435.25 cg	24.89215
18.	N2 P3 K3	28.250 jkl	48.68421	43.250 jk	84.04255	536.75 abc	7.377049
19.	N3 P1 K1	46.750 de	146.0526	56.000 fg	138.2979	286.25 h	50.60397
20.	N3 P1 K2	28.250 jkl	48.68421	52.000 ghi	121.2766	451.00 bf	22.17429
21.	N3 P1 K3	19.000 p	0	23.500 l	0	579.50 a	0
22.	N3 P2 K1	37.750 fg	98.68421	53.500 gh	127.6596	312.50 gh	46.0742
23.	N3 P2 K2	25.250 mn	32.89474	43.250 jk	84.04255	460.25 ae	20.57808
24.	N3 P2 K3	20.500 op	7.894737	39.750 k	69.14894	570.00 ab	1.639344
25.	N3 P3 K1	36.500 g	92.10526	49.250 hi	109.5745	428.50 cg	26.05695
26.	N3 P3 K2	27.500 klm	44.73684	25.000 l	6.382979	515.25 abc	11.08714
27.	N3 P3 K3	23.000 no	21.05263	52.000 ghi	121.2766	537.25 abc	7.290768
	NsPsKs	46.750 de	146.0526	72.250 c	207.4468	330.75 fgh	42.92494
	LSD(p≤0.05)	2.981		5.0597		124.890	

N1, P1, K1 are 252; N2, P2, K2 are 257, and N3, P3, K3 are 262 Kg ha^{-1}, respectively; whereas NsPs and Ks (standard doses of NPK commonly applied by local farmers) are 247, 247, 247 Kg ha^{-1} Kg ha^{-1}, respectively. The experiment was repeated once with similar results

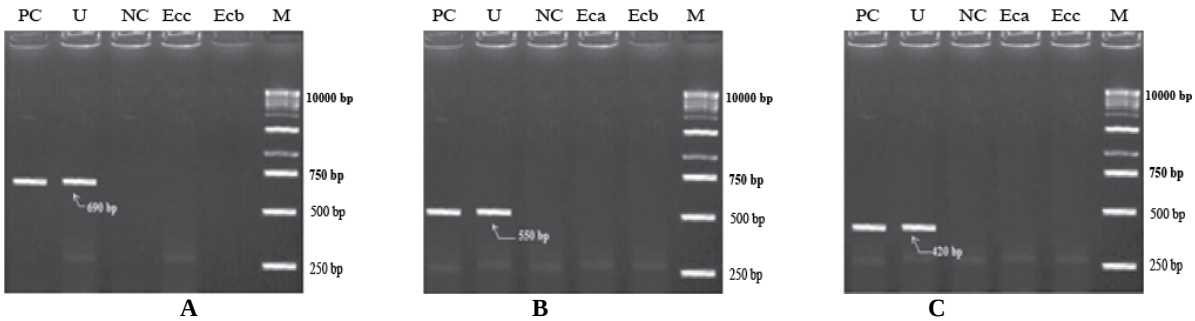

Fig. 3. Agarose gel (2%) showing expected PCR-bands of 690 bp amplified by Ecc-specific primers (A), 550 bp amplified by Eca-specific primers (B), and 420 bp amplified by Ech-specific primers (C), PC = Positive control (using DNA template of identified bacterium), U = Unidentified collected bacterial sample NC = Negative control (no template used), M = Marker. Ecc = *Erwinia carotovora* subspecies *carotovora*, Eca = *Erwinia carotovora* subspecies *atroseptica*, and Ech = *Erwinia chrysanthemi*.

Disease severity and incidence (%): The % disease severity was almost inversely proportional to the yield (Table 2). For example, the combination $N_3P_1K_3$ (which gave the best yield) allowed the minimum % diseases severity (19%). This was followed by $N_3P_2K_3$ (the second best yielder) in which case the % disease severity was 20.05%, showing an increase of 7.89 % than that of the minimum. Likewise, $N_3P_3K_3$ allowing 23% disease severity, showed 21.05 % increase over the minimum. Maximum % disease severity (60.25%) was present in plants receiving the combination $N_1P_1K_1$, showing an increase of 217.10% over that of the minimum. Likewise, in other combinations potash (K) seemed to play a role in reducing % disease severity. For example, the % disease severity in $N_3P_3K_2$ and $N_3P_3K_3$ increased by 44.74 and 21.05% over the minimum suggesting that the higher level of potash reduced % disease severity.

However, the % incidence followed somewhat different pattern as compared to the % disease severity. Plants in treatment $N_3P_1K_3$ (that gave the best yield and allowed minimum % disease severity) showed the lowest diseases incidence (23.05). It was followed by treatment $N_3P_3K_2$ which ranked 5th and 6th instead of 2nd in yield and disease severity respectively.

Means followed by the same letter(s) in the same column are not significantly different from one another at 0.05 level of probability.

Host fertilization plays an important role in disease severity and is therefore, worth investigating. The types as well as the dozes of different chemical fertilizers can either increase or decrease the severity of a disease depending upon the disease and host. Absolutely no work has been done in Khyber Pakhtunkhwa on any aspect of potato bacterial diseases including the role of fertilizers. We therefore, decided to investigate whether or not slight increase (beyond the standard dozes) in the dozes of N, P and K would have any effect on the control of potato blackleg and soft rot.

Results of the present study indicated that all the three fertilizers (N, P, K) had effect on disease severity but yield was affected only by N and K and disease incidence by P and K. With the increasing levels of N (beyond the standard level), there was initially some significant increase in yield but later it became non-significant. Increasing levels of P fertilizer did not result in any significant increase in the yield. Increasing levels of potash from K1 to K3 also did not result in any statistically significant increase in potato yield. The effect of increasing levels of nitrogen and phosphorus fertilization of potato plants on blackleg and soft rot incidence and severity followed almost the same pattern that was observed for yield. However, increasing levels of potash did show significant decrease in both severity and incidence of potato blackleg disease. Increasing levels of potash did show some significant increase in yield especially at the P2 level of phosphorus and significant decrease in blackleg severity at all levels of phosphorus.

Excessive amounts of nitrogenous fertilizers increase the succulent tissue in potato plants making them more susceptible to diseases especially bacterial diseases (Agrios, 2005). The excessive vegetative growth due to overdose of N also creates micro-climatic conditions (such as more humidity) favorable for fungal and bacterial diseases. Moreover, in case of ample N supply, there is a high demand for carbon (C) from photosynthesis via the Krebs cycle for soluble organic compounds, leaving little carbon for the synthesis of secondary compounds such as phenols and quinines. This situation weakens the plant's natural ability to defend itself against diseases. Under N limited conditions, however, much more C from the Krebs cycle is available for the synthesis of phenolic compounds. Prokkola (1994) used three different levels of nitrogenous fertilizer and found that when the disease incidence was high, the proportion of blackleg stems increased with increasing dozes of N fertilizer. However, this was not the case when disease incidence was low. Kumar *et al.,* (1991) also found that increasing dozes of nitrogenous fertilizers increased storage rots in six potato cultivars they tested. Although, our results did not show any significant increase in blackleg severity beyond P2 level of phosphorus, Gracia *et al.,* (2004) reported that P caused a significant increase in soft rot of tubers. They also found that the plant tissue-degrading enzymes (polygalacturonase and pectate lyase) of the soft rot-bacteria became more active when bacteria were grown in the presence of phosphorus.

As obvious from our studies, potash played an important role in decreasing blackleg and soft rot severity and increasing yield. Potash might have had some effect on the pathogen or on the host or both. The effect of potash on the host might have been the cross-linking and strengthening of the host cells as has been suggested for the effect Ca on host cell walls (Flego, 1997). In case of the pathogen, potash might have played role in depressing the pathogen genes responsible for the production of bacterial enzymes that degrade host plant tissue. Potassium is involved in plant enzyme activation, cation/anion balance, stomatal movement, phloem loading and photosynthate translocation and turgor regulation. Photosynthesis is increased with increasing K content of the leaves. K deficiency can result in cracks and lesions on the surface of leaves and fruits providing easy access to fungi and bacteria.

Our results indicated that disease severity increased with the increasing levels of nitrogen (Urea: $(NH_2)_2CO$) and then it dropped at the N3 level. One possibility that might explain our results may be that the variety (kuroda) we used for our experiments had some resistance against potato blackleg. This genetic resistance of the variety might have resisted the increase of disease severity with the increasing levels of nitrogen. Similar results were reported by Canaday & Wyatt (1992) who found that side dress applications of ammonium nitrate increased the incidence and severity of bacterial soft rot (caused by *Pseudomonas marginalis*) in a susceptible broccoli cultivar, Premium Crop, but had no effect on a resistant one, Shogun. McGovern *et al*., (1985) reported results similar to ours. They found that the susceptibility of *Chrysanthemum morifolium* to *Erwinia chrysanthemi* increased with the increasing rate of fertilizer and nitrogen in the form of $(NH_4)_2SO_4$. However, the susceptibility was maximum at moderate levels (100-200 ppm) of Ca $(NO_3)_2$ and NH_4NO_3 and decreased when nitrogen was increased up to 400 ppm in both forms.

The finding that nitrogen level can be increased up to a point at which disease severity starts decreasing has important implications. Increased nitrogen level will increase yield but will not increase disease severity if a proper type of nitrogenous fertilizer is used. Therefore, it is suggested that all the available types of nitrogenous fertilizers be tested to find out the one that can be used at higher doses to increase yield without increasing blackleg severity.

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