Journal of Population Therapeutics & Clinical Pharmacology

Aggressiveness Analysis of *Erwinia carotovora* Isolates and Screening of Selected Commercial Potato Cultivars against Soft Rot

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Abstract

Being cash producing, potato crop has great importance in Pakistan. Soft rot caused by *Erwinia carotovora* is one of the important bacterial diseases of potato. Due to damaging of potato crop by soft rot it was essential to study the pathogen and to find out the resistance sources. On the symptomatic basis diseased tubers samples were collected from different areas of Sialkot and Rawalpindi. For the confirmation of *Ecc* staining and biochemical tests were done. The results confirmed the presence of gram negative rod shaped bacteria with white cream circular colonies. A total of the ten isolates of *Ecc* were isolated and tested for their aggressiveness on ten healthy selected potato cultivars. The isolates *Ecc* 5 group was found to be the most aggressive which caused severe and sudden rottening of tubers. The isolates *Ecc* 8 group was found to be a moderately aggressive due to intermediate level of maceration in tubers. But isolates *Ecc* 1 group was found to be low aggressive which caused slight rottening of tubers. The total weight, numbers and diseased tubers obtained from entire production were also estimated. Out of ten potato cultivars Barna and SH-651 were found highly resistant. Rodeo was found highly susceptible with 13% maceration rated 4 on 0-7 rating scale, while, SH-692 and Aladin were found (MS) moderately susceptible rated 3 on rating scale.

Key words: cultivars, maceration, susceptible

1. INTRODUCTION

Solanum tuberosum L. is a very significant and crucial crop of Pakistan. Undoubtedly it is the second most expendable and profitable food after grains. It is a worldwide crop which belongs to family *Solanaceae*. Potato is the main source of nutrients such as carbohydrates, proteins and vitamins. A normal sized potato has 20.13 gm. carbohydrates, 1.87 gm. proteins, 0.1 gm. fats, 77.0 gm. water and 2.77 gm. micronutrients such as riboflavin, vitamin C, vitamins B1, B3, B6, phosphorus, iron and potassium (Englyst 2007; Shafique et al. 2010; Statsyuk et al. 2018; Beals et al. 2019).

Being cash producing crop in Pakistan it grows favorably in cooler environment. The major potato growing areas are Lahore, Kasoor, Okara, Sahiwal, Pakpattan, Depalpur, Sialkot, Gujranwala, Mandi Bahuddin, Jhang and Faisalabad in Punjab, Swat, Mansehra, Mardan and Nowshera in KPK and Kalat, Pishin and Killa Saifullah in Balochistan. In Pakistan potatoes are cultivated in three seasons' spring, summer and autumn. Total production of potato in Pakistan was 4,578 thousand tones per 196.2 thousand hectares (Economic Survey of Pakistan 2018-2019). Punjab ranks top with cultivation of 133 thousand tons and 2782 thousand tons production, KPK ranked second with 9.02 thousand hectares and 34.6 thousand tons production, Baluchistan ranked third with 2.33 thousand hectares and yield of 3.02 thousand tons (Economic Survey of Pakistan, 2018-2019). Although potato production in Pakistan has increased many folds but it's per acre yield is far less than other parts of the worlds (Ahmad et al. 1995; Majeed et al. 2019; Rashid et al 2012 and Majeed et al. 2020).

Owing to different diseases the production of potatoes is severely affected hence this crop is considered very important. More than 18 potato diseases are reported in the country, of which 13 are of common occurrence. Their importance, however, varies considerably in different potato growing areas (Ahmad et al. 1991). Most commonly caused potato diseases in Pakistan are early blight, powdery mildew, common scab, black scurf, stem rot, soft rot, brown rot, wilts, potato cyst nematode and root knot nematode (Ahmad 1998). Bacterial pathogen has created serious problem in growing area in Pakistan especially in province Punjab. Soft rot caused by *Erwinia carotovora* is one of the important bacterial diseases of potato in Pakistan. *E. carotovora* completes its life cycle in crop in field as well as in storage tubers (Lewos 1992). *E. carotovora* subsp. *carotovora* causes the destructive soft rot disease in many vegetables such as potato,

carrot, garlic, egglplant, sweet potato and tomato (Opara and Asuquo 2016). Disease provoking organism *E. carotovora* sub species *carotovora* causes soft rot on stored potato and other vegetables such as tomato and pepper (Hades 2001). Description of the causal organism of soft rot of tomatoes and other vegetables and assessment of its most aggressive isolates (Akbar et al. 2015).

The disease symptoms appear in field typical blackleg of the basal part of the stem whereas, stolons and mother tubers shows rottening symptoms. The rot is extended to aerial leaflets and shoot with leaves becomes shriveled and stunted (De Boer 2004; Barnes 2012). The tubers from storage or farmer's markets showed two types of rot symptoms. In first tubers are showed a hard dark rot, with little odor. The rot extends to the internal portion with black brown and white cream zones. In second Internal tissues become soft, slimy and gave foul smell. In all cases the external peel is not affected (Ciampi 1997).

E. carotovora is a gram negative, rod-shaped and peritrichous flagellated bacterium (Smadja et al. 2004). This bacterium was first time isolated from carrot. *Erwinia* belongs to domain bacteria, phylum proteobacteria, class gamma proteobacteria, order entrobacteriales, genus enterobacteriaceae, species *carotovora* or *pectobacterium*. Mostly are found in tropical, warm area of the world. *E. carotovora* cannot be survived at 37^{o}C or above (Hyman et al. 1988).The optimum temperature of *E. carotovora* is 27- 30^{o}C. (Singh et al.1998). The worldwide losses of potatoes by pathogen *E.carotovora* is $ 50to $100 million annually (Perombelon 2002).

Soft rot can be controlled by two main processes using healthy seed tubers, un-infected fields and spraying tubers with chemicals before sowing (Mehrotra 1980). The second process using of chemicals are so expensive, time consuming and not accessible to the farmers. So use of resistant and environment friendly varieties is substantial solution to the problem.

Due to damaging of potato crop by soft rot it is essential to study the pathogen and to find out the resistance sources. Keeping this in view, the proposed study was carried out with following objectives of Aggressiveness analysis of potato pathogenic bacteria *Erwinia carotovora* isolated from district Sialkot and Rawalpindi and Identification of resistant varieties against soft rot disease of potato.

2. MATERIALS AND METHODS

A research was designed from collection of diseased sample to screening of diseases and healthy potatoes.

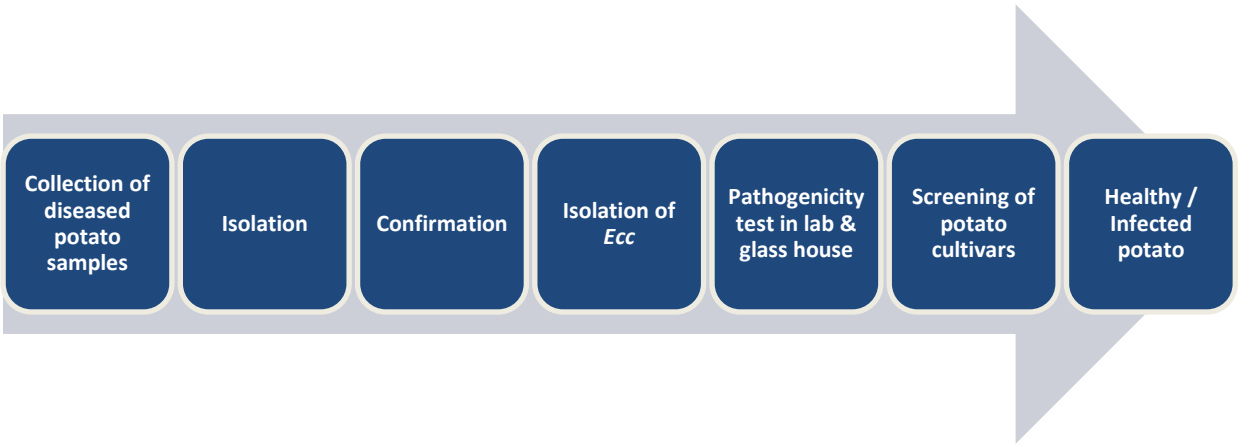

Fig 2.1 Research Design

2.1 Collection of disease samples

Potato samples were collected from different storage areas on the symptomatic basis located at different locations of Sialkot and Rawalpindi Districts. Twenty samples were collected from different locations in Daska, Jamke cheema, Akbarabad, Poorub Klair, Bheeko chor and Sahowalli in Sialkot district. Similarly, twenty samples were collected from wah cantt, Taxila, Hassanabdal and Jung Bahatar in Rawalpindi district. Each collected sample contained fifteen tubers. The samples were preserved in bags and labeled with area and the date of collection. The collected samples were brought to Plant Bacteriology Laboratory at Crop Diseases Research Institute (CDRI) in National Agricultural Research Centre (NARC) Islamabad. Samples were stored at 4^{o}C for further studies. Presence and absence of pathogen in the collected samples was confirmed through different staining and biochemical tests in the laboratory. (Farahat et al. 2011)

2.2 Disease Incidence and severity

All the fifteen tubers per location were spread over the clean open place and infected tubers with soft rot were separated from the healthy tubers (Table 3.2). The data was recorded separately on

each variety by number of diseased tubers and converted into percentage of incidence by using the following formula.

$$\text{Percentage of disease Incidence} = \frac{\text{Number of diseased tubers}}{\text{Total number of tubers}} \times 100$$

The disease severity of infected tubers was assessed by using standard visual rating scale (1-7) based on progeny tubers symptoms, (1= No or few symptoms, 2 = 1-10 % tuber area affected,3 = 11-20 % tuber area affected,4 = 21-30 % tuber area affected,5 = 31-40 % tuber area affected,6 = 41-50 % tuber area affected, 7 = 51 % or more tuber area affected, as described by (Ahmad et al.,1995).

2.3 Isolation of pathogen

For isolation of the pathogen (*E.carotovora)* Nutrient Glucose Agar (NGA) medium (Beef extract: 3gm, Peptone: 5gm, Glucose: 2.5gm, Agar: 15gm, Distilled water: 1 liter) was used (Khan *et al*., 1999, Diseased portion of the tubers of about 5 mm size were cut into small slices and disinfected by dipping in 3% Clorox for 1 minute. (Lojkowska and Kelman 2015). Three washings were given with distilled water for the removal of clorox followed by drying with filter paper to remove the excess moisture. These disinfected pieces of tubers were plated on solidified Nutrient Glucose Agar in petri plates and were incubated at 30°C under hygienic condition. The grayish white/cream circular and smooth colonies were selected after 96 hours of incubation. Selected colonies were purified by re-streaking on Nutrient Glucose Agar medium.

Fig. 2.2 Media preparation, isolation and streaking.

2.4 Identification and confirmation of *E. carotovora*

For the identification of cultures of corresponding biochemical tests Catalase test, Growth at

minimum or maximum temperature, Indole acidic acid test, Erythromycin sensitivity test, Acid production from carbohydrates test**,** Lecithina test, 3% KOH test and Growth in 5 % NaCl test were performed (Lelliot and Stead 1987; Czajkowski et al. 2015 and Salem et al. 2018).

2.4.1 Catalase test

For catalase test a fresh colony of *Ecc* was placed on a clean glass slide from NGA medium with loop. Then a drop of H_2O_2 was mixed up with colony. Some bubbles were observed from the used colony. The bubbles were obtained due to the presence of enzyme catalase which reacted with H_2O_2 to produced O_2 (bubbles). The test was repeated for 10 isolates of *Ecc* separately (Lelliot and Stead 1987; Istiqomah et al. 2013).

2.4.2 Growth at minimum /maximum temperature

A 50ml of nutrient broth was prepared and autoclaved. About 5ml medium was taken in three autoclaved test tubes. These test tubes were labeled by names H-tube (high temperature) L-tube (low temperature) and N-tube (normal temperature). All the tubes were inoculated with few colonies of fresh culture of *E. carotovora*. The H-tube kept at high temperature 40^{o}C, L-tube at minimum temperature 5^{o}C and N-tube at optimum temperature 28^{o}C for 7 days. After 7 days data on the turbidity growth of *Ecc* was recorded on all ten isolates (Lelliot and Stead, 1987); (Istiqomah et al. 2013).

2.4.3 Indole test

Kovoc's indole reagent is composed of 5g p. Dimethylaminobenzaldehyde, 75 ml Amyl alcohol and 25 ml HCl (conc.). Two tubes (experimental & control) containing tryptone / tryptophane medium were taken. A drop of culture was taken in under test while controlled tube was remained un-inoculated. These tubes were incubated at 28^{o}C for 5 days. For the confirmatory test of indole 0.5ml Kovoc's indole reagent was added into both tubes. The data was recorded on the appearance of color for all the isolates. The test was repeated for 10 isolates of *Ecc.* (Lelliot and Stead 1987; Pasco et al. 2006; Salem et al. 2018; Masum et al. 2007)

2.4.4 Erythromycin sensitivity test

Luria-Bertani (LB) medium was prepared in laboratory for the identification of bacterial growth. (Bactotrypton 10 g, Yeast extracts 5g. NaCl 10g, Agar 15g and Dist. Water 1 lit.). Out of which 75ml medium was taken into small 5ml test tubes. The rest of the medium was taken in flask. The media within tubes and flask were autoclaved. After pouring of medium into petri plates the medium in test tubes which was in the form of solution inoculated with *Ecc* bacteria. This inoculated medium was spread on basal petri plates medium. A single petri plate was kept separate and labeled as controlled tube. A disc of antibiotic erythromycin was placed on the center of all the petri plates except controlled petri plate. The petri plates were incubated at 28^{o}C. After 24 hours data was recorded on the growth of the colony for all the isolates (Lelliot and Stead 1987; Istiqomah et al. 2013).

2.4.5 Acid production from carbohydrates test

A Dye's medium C plus carbohydrate was prepared in the laboratory.(0.5g $NH_4H_2PO_4$, 0.5g K_2HPO_4, 0.2g $MgSO_4 \times 7H_2O$, 1gYeast extract, 0.7g Bromocresol purple,12g Agar and 1 liter dist. water). After autoclaving 3ml medium was poured into two tubes (experiment & controlled). The experiment tube was inoculated by streaking with *Ecc* culture and both the tubes were incubated at 28^{o}C for 20 days. The data was recorded on the appearance of yellow color (Lelliot and Stead 1987; Salem et al. 2018).

2.4.6 Lecithinase test

Lecithinase egg medium was prepared and after autoclaved poured into controlled & experiment petri dishes. (1 sterile surfaced chicken egg, yolk into sterile measuring cylinder and molten nutrient agar). The experiment plate was inoculated in center region by a single spot with *Ecc* culture. Both the plates were incubated at 28^{o}C. The data was recorded after 7 days culture on the appearance of zone growth for all the isolates (Lelliot and Stead 1987; Istiqomah et al. 2013).

2.4.7 Growth in 5% NaCl test

Nutrient broth was prepared in the laboratory with 5% NaCl. (8g Nutrient broth, 0.5ml 5% NaCl

and 1-liter dist. water). The NB medium with 5% NaCl was autoclaved and then poured into tubes. Two tubes (experiment & controlled) were selected and the experiment tube was inoculated with *Ecc* fresh culture. Both the tubes incubated at 28^{o}C for 14 days. The data was recorded on the growth of all the isolates (Lelliot and Stead 1987; Salem et al. 2018).

2.4.8 Potassium hydroxide (3% KOH) test

A fresh colony of *Ecc* was placed on a clean glass slide from NGA medium with loop. Then a drop of 3% KOH was mixed up with colony and then raised the mixed material gently upward and observed for the formation of thread like slime. This test was repeated for all 10 isolates of *Ecc* (Lelliot and Stead 1987) and (Istiqomah et al. 2013).

2.5 Gram staining

For gram staining a drop of water was placed on the center of the glass slide. A bacterial colony was mixed with distilled water for smear. After the formation of smear the slide was dried by slightly heat of spirit lamp. The slide was flooded with crystal violet for one minute and washed with tap water for 10 seconds. Gram's iodine was taken and after one minute again washed off with tap water for 10 seconds. For the appearance of coloration, 95% ethanol was used for ten seconds it was also washed with tap water for 10 seconds. The counter stain safranin was flooded and after 10 seconds it was also washed with tap water for 10 seconds. The slide was dried with little heat and covered with cover slip. A drop of mineral oil was added on the outer side of the cover slip on slide. The *Ecc* were examined with red in color under the electric microscope. It was the confirmation of *Ecc* gram negative bacteria (Lelliot and Stead 1987) and (Istiqomah et al. 2013).

2.6 Hypersensitivity test

The test was performed on non-host tobacco plant by injecting *E.carotovora* prepared suspension (10^{8}-10^{9} per ml) in sterile distilled water. The *ventral side* of *tobacco leaf* was infiltrated by *cell suspension.* The tobacco leaves were observed after 48 hours for visualizing the HR (Highly resistant) against *E.carotovora*. Observations were made after 36 hours of inoculation. The

strains which were not showing any reaction even after 72 hours of incubation were reported as non-pathogenic (Lelliot and Stead 1987; Istiqomah et al. 2013).

2.7 Maintenance of cultures

The purified cultures of were preserved by suspension in 3ml sterilized distilled water in tubes / Bijou bottles and were stored at 4^{o}C temperature (Ziaullah et al. 2011)

2.8 Pathogenicity Test/ aggressiveness analysis

The Pathogenicity of 10 different isolates collected from different locations of districts Sialkot and Rawalpindi were tested on 10 selective commercial potato cultivars to find out the aggressiveness of these isolates. All potato tubers were surface sterilized by 70 % ethyl alcohol followed by washing with sterile distilled water and were air dried. Healthy tubers were inoculated by injecting the already prepared fresh inoculums of *Ecc* (10^{8} -10^{9} per ml).While the treatment with distilled water was served as control. The inoculated tubers were bagged with wet tissue paper to provide the required moisture. The tubers were placed at 28^{o}C.The experiment was conducted in triplicate and the data was recorded on 0-7 rating scale (0 = No rottening of tubers, 1 = 50% rottening of tubers, 2 = more than 50% rottening of tubers, 3 = complete rottening of tubers) after 7-10 days by (Wright et al. 2005). The most aggressive isolate was also done on center of the healthy slice of potato. The potato's slice had rotted with soft rot disease.

3. RESULTS

3.1 Collection of disease samples

Potato diseased samples were collected from different locations of Sialkot and Rawalpindi Districts. Twenty samples were collected from different locations in Sialkot district from Daska, Jamke cheema, Akbarabad, Poorub klair, Sahowalli and Bheeko chor areas. Similarly, twenty samples were collected from Wah cantt, Taxila, Hassanabdal and Jang Bahatar in Rawalpindi district (Table 4.1)

Table 4.1 Locations, Numbers and diseased samples of potato collection

Sr. No.	Area of samples collection		No. of total samples	No. of Diseased tubers
1	Sialkot	Daska	15	7
2		Jamkecheema	15	7
3		Akbarabad	15	5
4		Poorub klair	15	0
5		Sahowali	15	7
6		Bheeko Chor	15	6
7	Rawalpindi	Wah Cantt	15	5
8		Taxila	15	8
9		Hassan Abdal	15	7
10		Jang Bahater	15	5
		Total	150	57

3.2 Disease Incidence and severity

On the basis of locations the results of different storage areas of districts Sialkot and Rawalpindi revealed that the incidence 46.6% of the tubers from Daska, Jamke Cheema and Sahowali having disease severity ranged 4 on 0-7 rating scale while it was observed 33.3% from Akbarabad district Sialkot with severity ranged 2 on scale. The incidence and severity in Poorub klair were 0.0% rated 0 on rating scales respectively. The incidence and severity in Bheeko Chor were found 40.0% and 3 on rating scale respectively.

The disease incidence and severity in Wah cantt were found 33.3.0% rated 2 on rating scale respectively. The percentage of disease incidence and severity were found in Texila district Rawalpindi 53.3% and 5 on rating scale respectively. In Hassanbdal district Rawalpindi the disease incidence was 46.6% and severity under rating scale was 4. The disease incidence and severity in Jang Bahatar were observed 33.3% rated 2 on rating scale respectively. The overall

results of disease incidence between two districts were found 37.9% and their severity with 5 on rating scale respectively. The disease incidence and severity in district Sialkot were found 35.5% with 4 on rating scale respectively. Similarly, the disease incidence and severity in district Rawalpindi were found 41.6% with 5 on rating scale. The disease incidence and severity were The disease incidence and severity were found high in district Rawalpindi 41.6%, with 5 on rating scale as compared to district Sialkot 37.5%, with 4 on rating scale (Table 4.2).

Table 4.2 Disease Incidence and severity of *E. carotovora* in different locations of Districts Sialkot & Rawalpindi

Sr. No.	Area of samples collection		No. of total samples	No. of Diseased tubers	Percentage of diseased Incidence	Severity (0-7)	District wise results
1	Sialkot	Daska	15	7	46.6 %	0-4	35.5% disease incidence And severity 0-4
2		Jamke cheema	15	7	46.6%	0-4	
3		Akbarabad	15	5	33.3%	0-2	
4		Poorub klair	15	0	0.0%	0-0	
5		Sahowali	15	7	46.6%	0-4	
6		Bheeko chor	15	6	40.0%	0-3	
7		Wah Cantt	15	5	33.3%	0-2	
8	Rawalpindi	Taxila	15	8	53.3%	0-5	indcidence 41.6 % Severity 0-5
9		Hassan Abdal	15	7	46.6%	0-4	
10		Jang Bahater	15	5	33.3%	0-2	
		Total	150	57	37.9	0-5	

3.3 Isolation of pathogen (*E. carotovora)*

E. carotovora was isolated from the samples collected from six different locations of Sialkot district and four different locations of Rawalpindi district. Each sample was designated from different location as an individual isolate on the basis of selected *E. carotovora* Bacterial colonies. The bacterial isolates were purified, multiplied and maintained on NGA medium.

3.4 Morphology of Colony

The morphological characters of the isolates exhibited circular/round, shiny, raised and creamy white in color which was the characteristic futures of the isolates of *E. carotovora* isolated from potato tubers (Table 4.4).

Table 4.4 Morphological characteristics of Selected Isolates on NGA media

Sr. No.	Isolates	Colonies	
		Color	Shape
1	*Ecc* 1	White	Round
2	*Ecc* 2	White creamy	Round
3	*Ecc* 3	White	Round
4	*Ecc* 4	White creamy	Round
5	*Ecc* 5	White	Round
6	*Ecc* 6	White	Round
7	*Ecc* 7	White creamy	Round
8	*Ecc* 8	White	Round
9	*Ecc* 9	White	Round
10	*Ecc* 10	White creamy	Round

3.5 Identification and confirmation of *E. carotovora*

For the identification and confirmation of cultures of *Ecc* corresponding biochemical tests Catalase test, Growth at minimum or maximum temperature, Indole test, Erythromycin

sensitivity test, Acid production from carbohydrates test**,** Lecithinase test, 3% KOH test and Growth in 5 % NaCl test (Table 4.5) were performed (Lelliot and Stead 1987).

Table 4.5 Confirmation of *E. carotovora* Isolates from Biochemical Tests

Tests	**Isolates**									
	Ecc 1	*Ecc* 2	*Ecc* 3	*Ecc* 4	*Ecc* 5	*Ecc* 6	*Ecc* 7	*Ecc* 8	*Ecc* 9	*Ecc* 10
Catalase	+	+	+	+	+	+	+	+	+	+
Growth at min.&max. temperature	–	–	–	–	–	–	–	–	–	–
Indole	–	–	–	–	–	–	–	–	–	–
Erythromycin sensitivity	–	–	–	–	–	–	–	–	–	–
Acid production from carbohydrates	–	–	–	–	–	–	–	–	–	–
Lecithinase	–	–	–	–	–	–	–	–	–	–
3% KOH	+	+	+	+	+	+	+	+	+	+
Growth in 5 % NaCl	+	+	+	+	+	+	+	+	+	+

All the ten isolates separately tested from biological tests in catalase test all isolates were positive. Similarly, in growth at minimum or maximum temperature test, indole test, erythromycin sensitivity test, acid production from carbohydrates test and lecithinase test all ten isolates were negative. But in 3%KOH test and growth in 5% NaCl test whole the isolates gave positively growth. In3%KOH test all isolates gave thread like structure with loop. The results of all the tests proved that the isolates from potato were *E. carotovora* .

3.6 Aggressiveness analysis

Ten freshly cultured isolates of *Ecc* were tested and compared for their aggressiveness on fresh healthy ten selected potato cultivars. The results showed different level of aggressiveness of the 10 isolates. The isolate *Ecc* 5 was found to be the most aggressive and caused severe and suddenly rottening of tubers. On the basis of aggressiveness analysis the isolates have been classified in three major groups 5, 8 and 1 based on similarity. The group 5 comprises of the isolates 2, 3, 4 and 5 on the basis of similarity and being most aggressive. This group spoiled

1.0% SH-5, 0.0% SH-651, 50.0 % SH-692, 40.0 % SH-795, 40.0 % SH-1035, 5.0 % NARC 17-19, 0.0% NARC 720-110, 51.0% Rodeo, 44.0% Aladin and 0.0% Barna. Overal 5 group rotted the tubers 23% compared to other isolates of *E. carotovora*. The group 8 comprises of the isolates 6, 7 and 8 on the basis of similarity and being moderate aggressive due to intermediate rottening of tubers. This group caused rottening in the varieties in following percentages e.g. 14.6% SH-5, 0.0% SH-651, 48.0 % SH-692, 26'0% SH-795, 25.0 % SH-1035, 20.0 % NARC 17-19, 5.0 % NARC 720-110, 41.0 % Rodeo, 51.0 % Aladin and 0.0 % Barna. The gross percentage of this group was 22.9% (Table 4.6).

Table 4.6 *E. carotovora*'s isolates Percentage of Infection in Tubers during aggressiveness analysis.

Variety	Treat	Rep	*Ecc 1*	*Ecc 2*	*Ecc 3*	*Ecc* 4	*Ecc* 5	*Ecc* 6	*Ecc* 7	*Ecc* 8
SH-5	1	1	15	5	15	27	1	5	3	15
	1	2	12	5	14	29	0	5	2	14
	1	3	14	5	16	25	2	5	4	15
SH-651	2	1	25	2	1	5	0	0	2	0
	2	2	23	2	2	5	0	1	2	0
	2	3	26	2	0	5	0	1	2	0
SH-692	3	1	50	50	0	5	51	0	0	50
	3	2	49	50	0	15	50	1	0	47
	3	3	51	49	0	15	49	1	0	49
SH-795	4	1	51	20	2	15	40	30	0	25
	4	2	50	21	2	25	40	29	0	26
	4	3	49	20	2	22	40	31	0	27
SH-1035	5	1	51	0	50	25	40	35	0	25
	5	2	50	0	50	1	41	34	0	24
	5	3	49	0	50	2	40	35	0	26
NARC 17-19	6	1	1	2	0	0	0	2	25	20
	6	2	1	2	0	0	0	2	25	20
	6	3	1	2	0	0	0	2	25	20
NARC 720-110	7	1	2	1	0	0	51	5	0	5
	7	2	2	1	0	0	51	4	0	4
	7	3	2	1	0	0	51	5	0	6
Rodeo	8	1	2	2	35	10	5	25	1	40
	8	2	1	1	36	9	5	24	1	40
	8	3	3	3	34	11	5	26	1	40
Aladin	9	1	20	20	0	0	45	0	5	51

	9	2	21	21	0	0	47	0	3	50	19	47
	9	3	19	22	0	0	40	0	7	52	21	46
Barna	10	1	1	1	20	0	1	0	2	0	10	4
	10	2	1	1	19	0	1	0	2	0	8	4
	10	3	1	1	21	0	1	0	2	0	12	4
Gross %			21.5%	10.3%	12.3%	8.3%	23.2%	10.2%	3.8%	22.9%	17.1%	13.3%

Most aggressive *Ecc 5=23.2%*, Moderate aggressive *Ecc 9=17.1%* Least aggressive *Ecc 10=13.3%* and virulent *Ecc* 7*=3.8%*

But group 1 comprises of the isolates 9, 10 and 1 on the basis of similarity and being low aggressive due to slightly rottening of tubers and placed in third rank according to aggressiveness analysis isolates. The third ranked 1 rotted the varieties according to following percentages 13.6% SH-5.0, 24.6% SH-651, 50.0 % SH-692, 50% SH-795, 50 % SH-1035, 1.0 % NARC 17-19, 2.0 % NARC 720-110, 2.0 % Rodeo, 20.0 % Aladin and 1.0 % Barna. So group 1 overall 21.3% rotted the tubers (Table 4.7). According to dendogram ranks of aggressiveness analysis of 10 isolates of *E. carotovora* group 5 was the most aggressive isolate and placed in first rank. The group 8 was the moderate isolate and placed in 2nd rank and group 1 placed in 3rd rank respectively. Mean followed by same letters are not significant (p-value 0.05) and also not significant differ at LSD value.

Table 4.7 LSD Aggressiveness of *E. carotovora*'s isolates

Varieties	***Ecc 1***	***Ecc 2***	***Ecc 3***	***Ecc* 4**	***Ecc* 5**	***Ecc* 6**	***Ecc* 7**	***Ecc* 8**	***Ecc* 9**	***Ecc* 10**
SH-5	13.66^{d}	5.00^{c}	15.00^{d}	27.00^{a}	1.000^{e}	5.00^{d}	3.00^{c}	14.66^{f}	9.66^{d}	49.66^{a}
SH-651	24.66^{b}	2.00^{d}	1.00^{e}	5.00^{bc}	0.00^{e}	0.66^{f}	2.00^{cd}	0.00^{h}	48.00^{a}	11.66^{e}
SH-692	50.00^{a}	49.66^{a}	0.00^{f}	11.66^{b}	50.00^{h}	0.66^{f}	0.00^{e}	48.66^{b}	66.00^{e}	29.66^{c}
SH-795	50.00^{a}	20.33^{b}	2.00^{e}	20.66^{a}	40.00^{c}	30.30^{d}	0.00^{e}	26.00^{d}	20.00^{c}	10.00^{e}
SH-1035	50.00^{a}	0.00^{f}	50.00^{a}	9.33^{b}	40.00^{c}	34.66^{a}	0.00^{e}	25.00^{d}	6.00^{e}	5.33^{f}
NARC 17-	1.00^{e}	2.00^{d}	0.00^{f}	0.00^{c}	0.00^{e}	2.00^{e}	25.00^{a}	20.00^{e}	1.66^{f}	1.33^{g}

19										
NARC 720-110	2.00^{e}	1.00^{e}	0.00^{f}	0.00^{c}	51.00^{a}	4.66^{d}	0.00^{e}	5.00^{g}	20.00^{c}	1.00^{g}
RODEO	2.00^{e}	2.00^{d}	35.00^{f}	10.00^{b}	5.00^{d}	25.00^{c}	1.00^{de}	40.00^{c}	30.33^{b}	14.66^{d}
ALADIN	20.00^{c}	21.00^{b}	0.00^{f}	0.00^{c}	44.00^{b}	0.00^{f}	5.00^{b}	51.00^{a}	20.00^{c}	46.00^{b}
BARNA	1.00^{e}	1.00^{e}	20.00^{c}	0.00^{c}	1.00^{e}	0.00^{f}	2.00^{cd}	0.00^{h}	10.00^{d}	4.00^{f}
LSD Alpha 0.05	1.6746	0.8795	1.0772	8.4989	2.1090	0.9833	1.2043	1.3906	2.0391	1.8657

3.7 Screening of commercial cultivars

In the pots (under plastic tunnel) some physical disease symptoms were found on the aerial parts of the potato plants. Some lesions on diseased stem were also revealed. The leaves of infected plant gave yellow color at margin region. At the lower side of the stem some irregular brownish black rottening symptoms were observed. The mother tubers of the varieties Rodeo, Aladin, NARC 17-19, SH-5, SH-692, and SH-1035 were found severely rottening due to highly susceptibility of bacterial disease. But the mother tubers of their control were not rotted. The cultivar Barna was highly resistant while SH-651 moderate resistant. It was the main source of spreading of disease. The rottening of mother tubers contaminate the progeny tubers and during favorable conditions also contaminate the storage tubers (Perombelon 2002). The disease percentage of rottening was recorded with (0-7) rating scale (Table 4.10). Disease incidence and severity level were high in cultivar Rodeo (HS) 13% rated 4 on 0-7 rating scale respectively.

Sr. No.	Variety	Incidence%	Severity (0-7)	Response
1	SH-5	9.8	0-2	S
2	SH-651	0	0-1	R
3	SH-692	11.5	0-3	S
4	SH-795	5.6	0-1	LS
5	SH1035	9.8	0-2	S
6	NARC17-19	9.8	0-2	S
7	NARC720-110	4.6	0-1	LS
8	Rodeo	13	0-4	HS

9	Aladin	11	0-3	S
10	Barna	0	0-1	R
Total		14.9%	0-4	

Table 4.9 Disease incidence and severity of selected cultivars tubers

The disease incidence and severity level of SH-692 and Aladin were moderate susceptible (MS) 11.5 %, 11 % with 3 rating scale respectively (Table 4.9). Rest of the varieties were found lowest susceptible than the above e.g. SH-5 (9.8 %), SH-651 (zero %), SH- 795 (5.6 %), SH-1035 (9.8 %), NARC 17-19 (9.8 %), NARC 720-110 (4.6 %). Barna (zero %) and the total percentage of disease incidence was 14.9 % and whole severity was 4 with rating scale. The cultivars SH- 651 and Barna were found disease resistant against soft rot. The total production of whole the ten cultivars (Table 4.10) was also estimated e.g. total number of tubers, weight of tubers and number of infected tubers etc. The higher no. of tubers (30) was found in Rodeo cultivar but their sizes were very small so had less weights. Cultivar Aladin had moderate number of tubers (27) these tubers had less weight of tubers due to smaller sizes. The numbers of tubers remaining cultivars were as (24) in SH-651, (21) in NARC 720-110, (21) in SH-5, (20) in SH-1035, (20) in NARC17-19, (26) in SH-692 and (19) tubers in SH-795. But least number of tubers (14) was found in variety Barna. The higher number of infected tubers were found in variety Rodeo (4 tubers), lowest number (zero) in Barna and SH-651 but intermediate in remaining varieties. The higher weight of tubers was found in variety Barna (0.759 kg), moderate weight in SH-651 (0.722 kg) and lower most was found in variety SH-1035 (0.639 kg). According to overall results from the screening of selected commercial potato cultivars against soft rot. The Barna and SH-651were found highly disease resistant cultivars rated 1 with ratting scale 0-7.

Table 4.10 The disease percentage of rottening.

Variety	Treatment	Production		Disease incidence			% of Disease incidence	% of Disease District wise
		No. of Tubers	Weight	No. of Healthy Tubers	No. of Infected tubers	Severity in Rating scale		
SH-5	1	20	0.599	19	2	0-2	9.8 %	**Sialkot** 7.3 %
	1	21	0.637					
	1	23	0.662					
SH-651	2	26	0.741	24	0	0-1	0.0 %	
	2	22	0.695					
	2	24	0.732					
SH-692	3	26	0.583	23	3	0-3	11.5 %	
	3	24	0.567					
	3	26	0.644					
SH-795	4	20	0.512	18	1	0-1	5.6 %	
	4	20	0.489					
	4	19	0.534					
SH-1035	5	19	0.601	18	2	0-2	9.8 %	
	5	20	0.594					
	5	22	0.712					
NARC 17-19	6	20	0.657	18	2	0-2	9.8 %	
	6	20	0.544					
	6	21	0.686					
NARC 720-110	7	20	0.581	19	1	0-1	4.6 %	
	7	23	0.607					
	7	22	0.601					
Rodeo	8	30	0.523	26	4	0-4	13.0 %	**Rawalpindi** 7.6 %
	8	30	0.426					
	8	32	0.767					
Aladin	9	26	0.611	24	3	0-3	11.0 %	
	9	27	0.522					
	9	28	0.648					
Barna	10	16	0.763	14	0	0-1	0.0 %	
	10	14	0.761					
	10	12	0.754					

4. DISCUSSION

Soft rot is the major bacterial disease of potato crop in Pakistan (Khan, et al., 1999). The potato crop a king of vegetables in Pakistan is grown with an average yield of 18.5 tons per hectare which is very less as compared to world (Anonymous 2016-2017). *E. carotovora* a causal organism of soft rot is the main reason for the low productivity of potato crop (Jones, 1901). Due to severe losses of potato crop it was necessary to identify the pathogen/ aggressiveness, incidence of disease and severity. In Pakistan the prices of potato raised high Rs. 350 / 40 kg in 2004-2005 to 690 / 40kg in 2010-11. Export of potato in these years was 16.95 million kg earned by a foreign exchange worth Rs.183.2 million. Afghanistan, Malaysia and Sri Lanka are the main markets of Pakistan potato (Anonymous 2017-18). Regarding in this view potato samples were collected from different locations of Sialkot and Rawalpindi Districts on the basis of disease symptoms on tubers.

Comparative studies on District Rawalpindi and Sialkot were conducted because of their susceptibility to *E. carotovora*. On the basis of symptoms the soft rot disease was first time reported in hilly area of Sawat valley in Pakistan in 1984 (Khan et al. 1999). The disease incidence and severity in district Sialkot were found 35.5% with (0-4) scale respectively. Similarly, the results of disease incidence and severity in district Rawalpindi were found 41.6% with (0-5) scale. The disease incidence and severity was found high in district Rawalpindi 41.6% with (0-5) scale as compared to district Sialkot 37.5% with (0-4) scale. The temperature and humidity are the main factors that affect disease incidence and severity (De Boer 1994; Malcolmson 1959; Tyner et al. 1997). Temperature of 27^{o}C was observed during the collection. There is different level of disease symptoms among hilly and plain areas in Punjab reported by Turkensteen (1986 and 1987). The disease incidence in plane areas of Punjab (districts Sialkot, Gujranwala and Faisalabad) was also found more frequent in different cultivars (Hafiz 2003). The presence of disease in these districts is fetal threat to potato production.

E. carotovora was isolated from the samples collected from six different localities of Sialkot district and four of Rawalpindi district. We designate each sample from different location as an individual isolate on the basis of selected *E. carotovora* bacterial colonies. The bacterial isolates were purified, multiplied and maintained on NGA medium (Khan et al. 1999).

The colonies of *E. carotovora* on NGA medium were circular/round, shiny, raised and creamy white in color. The morphological characteristics of colonies also correlate with the morphological results of (Ziaullah et al. 2011). The using of biochemical methods for the identification of isolates were also fast and accurate like as the previous study such as the effective and rapid methods for the isolation and identification of are necessary (Schaad 1979). Corresponding biochemical tests such as Catalase test, Growth at minimum or maximum temperature, Indole test, Erythromycin sensitivity test, Acid production from cabohydrates test**,** Lecithinase test, 3% KOH test and Growth in 5 % NaCl test all these tests gave the same results as like (Lelliot and Stead 1987; Czajkowski et al. 2015 and Nourian et al. 2002).

All the ten isolates were separately tested from biological tests in catalase test which were all positive, due to presence of enzyme catalase. At high temperature (minimum or maximum) *E. carotovora* did not show any growth because it has specific temperature of 27-30^{o}C for growth (Singh 1991; Nielson 1978 and Helias et al. 1998). A research report confirms that *Ecc* do not give any growth at 37^{o}C (Hyman et al. 1988) and in indole tests all ten isolates were also negative due to decomposition of nitrogenous compounds. The zone was formed due to the presence of enzyme lecithin's which comes from *Ecc* bacteria (Czajkowski et al. 2015). It was also found that *Ecc* unable to utilized carbohydrate source (maltose) this result also resemble with the results of (Duarte et al. 2004). But none of the ten isolates were found to be sensitive to erythromycin. The erythromycin is an antibiotic and *Ecc* retards the growth by the action antibiotic (Maniatis et al. 1989). All the isolates in acid production from carbohydrates test were negative due to minimum pH value. *Ecc* has optimum pH (7-8) so at high acidic and basic/ alkali salts bacteria do not show any growth (Elian et al. 2005; Shrestha et al. 2005).

In 3% KOH test all isolates gave thread like structure with loop, *Ecc* is a gram negative and the cell wall of gram negative bacteria have higher amount of lipid than the cell wall of gram positive In Growth in 5 % NaCl test the whole isolates gave positively growth because bacteria do not show any growth at higher acidic media during the study the incubation period was also considered (24- 96) hours this incubation period also fact on the growth of *Ecc* which were proved by day period during identification tests (Jahan et al. 2007). The results of all the tests proved that the isolates from potato were *E. carotovora*. The *Ecc* is the major causal organism of sot rot of the potato is the evidence of research.

Ten freshly cultured isolates of *Ecc* were tested and compared for their aggressiveness on fresh healthy ten selected potato cultivars. The results showed different level of aggressiveness of the 10 isolates. The isolate *Ecc* 5 was found to be the most aggressive and caused severe and suddenly rottening of tubers. On the basis of aggressiveness analysis the isolates have been classified in three major groups 5 group, 8 group and 1 group based on similarity. The group 5 comprises of the isolates 2, 3, 4 and 5 on the basis of similarity and being most aggressive. This group spoiled 1.0% SH-5, 0.0% SH-651, 50.0 % SH-692, 40.0 % SH-795, 40.0 % SH-1035, 5.0 % NARC 17-19, 0.0% NARC 720-110, 51.0% Rodeo, 44.0% Aladin and 0.0% Barna. Overal 5 group rotted the tubers 23% compared to other isolates of *E. carotovora*. The group 8 comprises of the isolates 6, 7 and 8 on the basis of similarity and being moderate aggressive due to intermediate rottening of tubers. This group caused rottening in the varieties in following percentages e.g. 14.6% SH-5, 0.0% SH-651, 48.0 % SH-692, 26'0% SH-795, 25.0 % SH-1035, 20.0 % NARC 17-19, 5.0 % NARC 720-110, 41.0 % Rodeo, 51.0 % Aladin and 0.0 % Barna. The gross percentage of this group was 22.9% (Table 4.6). But group 1 comprises of the isolates 9, 10 and 1 on the basis of similarity and being low aggressive due to slightly rottening of tubers and placed in third rank according to aggressiveness analysis isolates. The third ranked 1 rotted the varieties according to following percentages 13.6% SH-5.0, 24.6% SH-651, 50.0 % SH-692, 50% SH-795, 50 % SH-1035, 1.0 % NARC 17-19, 2.0 % NARC 720-110, 2.0 % Rodeo, 20.0 % Aladin and 1.0 % Barna. So group 1 overall 21.3% rotted the tubers (Table 4.7). According to dendogram ranks of aggressiveness analysis of 10 isolates of *E. carotovora* group 5 was the most aggressive isolate and placed in first rank. The group 8 was the moderate isolate and placed in 2nd rank and group 1 placed in 3th rank respectively.

These results also correlate with the results of aggressiveness analysis and field experiment (Molina and Harrison1989). In the field some physical disease symptoms were found on the aerial parts of the potato plants. Some lesions on diseased stem were also studied. The leaves of infected plant gave yellow color at margin region. At the lower side of the stem some irregular brownish black rottening symptoms were observed. The mother tubers of the varieties Rodeo, Aladin, NARC 17-19, SH-5, SH-692, and SH-1035 were found severely rottening due to highly susceptibility of bacterial disease. It was the main source of spreading of disease.

The rottening of tubers is the evidence of previous that the rottening of mother tubers

contaminate the progeny tubers and during favorable conditions also contaminate the storage tubers (Perombelon 2002; Bathily 2010). The percentages of rottening of tubers were estimated by rating scale were accordance to the disease percentages results of (Ahmed et al. 1995). Disease incidence and severity level of cultivars were high in Rodeo (HS) 13% rated 4 on 0-7 rating scale respectively. So disease incidence and severity level of SH-692 and Aladin were moderate (MS) 11.5%, 11% rated 3 on rating scale respectively. The results of disease incidence and severity correlate with the results of (Farran et al. 2006).

The total production of whole the ten cultivars was also estimated e.g. total number of tubers, weight of tubers and number of infected tubers etc. The higher no. of tubers 30 was found in Rodeo cultivar but their sizes were very small so had less weights. Cultivar Aladin had moderate no. of tubers 27 these tubers had also less weight of tubers due to smaller sizes. The higher no. of infected tubers were found in variety Rodeo (4 tubers), lowest (0 tubers) in Barna and SH-651 and intermediate in remaining varieties. The higher weight of tubers was found in variety Barna (0.759 kg), moderate weight in SH-651 (0.722 kg) and lower most was found in variety SH-1035 (0.639 kg) the disease incidence and severity are the specific factors which effect on potato yield rate. And this study resembles with the study which indicates that yield can decrease when incidence of disease go over 5-10% similarly 'yield' can increase when of disease and severity level fall down (Farran et al. 2006).

According to overall research work (2012) of the screening of selected commercial potato cultivars against soft rot the higher resistant cultivars are Barna and SH-651.The most susceptible cultivar is Rodeo. These results of different resistant cultivars were also equal with the eight cultivars for resistance against soft rots (Mairaj 2004; Bain and Perombelon 1988).

Conclusions and Recommendations

The most aggressive isolate was *Ecc-5* out of 10 isolates. Potato cultivars "Barna" and "SH-651" were highly resistant while "Rodeo" was the most susceptible against soft rot.

"Rodeo" is the most susceptible cultivar against soft rot of potato. Therefore, the planting of this variety should be discouraged to avoid spread of disease. "Barna" and "SH-651" were highly resistant cultivars against soft rot. The promotion of cultivation of these varieties will help to

eradicate the spread of soft rot disease in the potato growing areas especially in districts of Sialkot & Rawalpindi.

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