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MOLECULAR AND PATHOGENIC DIVERSITY IDENTIFIED AMONG ISOLATES OF *ERWINIA CAROTOVORA* SUB-SPECIES *ATROSEPTICA* ASSOCIATED WITH POTATO BLACKLEG AND SOFT ROT

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Abstract

Twenty isolates of *Erwinia carotovora* subspecies *atroseptica* (Eca) causing blackleg of potato, were collected from tubers and diseased stems of potato plants grown in both plains and mountains of the North-Western Pakistan. The isolates were identified as Eca by biochemical tests and their identity was further confirmed by polymerase chain reaction (PCR) using subspecies-specific primers Eca 1F and Eca 2R which amplified the Eca-specific band of 690 bp. Pathogenicity test divided these 20 isolates into 4 aggressiveness groups or AGs. AG 1 (7 isolates) was the most aggressive group causing an average of 5.69 cm rot on potato stem. AG 2 (4 isolates) and AG 3 (5 isolates) were moderately aggressive producing potato stem rot of 4.93 cm and 4.49 cm, respectively. AG 4 (4 isolates) was the least aggressive, causing stem rot of 3.98 cm only. These isolates were also characterized using the technique of random amplified polymorphic DNA (RAPD). The Unweighted Pair-Group Method using Averages (UPGMA) analysis of the bi-variate data obtained as a result of RAPD, divided the isolates into four well-defined clusters showing a considerable level of genetic diversity. However, these clusters were not specific to AGs, origin or particular potato variety. Isolates with different aggressiveness levels, originated from plains or mountains and cultured from different potato varieties shared the same clusters. This means that the isolates probably derived from the same source population and got dispersed from one area to another through their hosts.

Introduction

Blackleg and soft rot are important diseases 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 the diseased tubers to the healthy ones. Multiple subsp. of *Erwinia*, including *E. carotovora* subsp. *atroseptica* (Eca) and *E. c. carotovora* (Ecc), *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). Ecb, the highly virulent enterobacteria able to cause soft rot and blackleg on potato, were reported by Duarte *et al*., (2004) from Brazil. Later on, the same subspecies (Ecb) was reported from potatoes grown in other parts of the world (Ma *et al*., 2007). *P. wasabiae* was originally found to cause disease on Japanese horseradish (Goto & Matsumoto, 1987) and was considered to be a narrow host range pathogen. However, Pitman *et al*., (2010) found this bacterium to be also associated with soft rot disease of potato in New Zealand. Interestingly, Pw isolates from New Zealand were found to be able to elicit a type III-dependent HR in tobacco but genes associated with the T3SS and the putative virulence factors HecB and DspE could not be detected.

With the exception of one strain (NZEC12), all other Pw strains failed to produce a PCR product indicative of *hrpN*, *dspE*, or *hecB*. Southern blots, probed with a DNA fragement containing *hrpN*, *dspE* or *hecB* could not locate these genes either. The researchers concluded that these virulence-associated genes were either absent or highly divergent in *P. wasabiae* strains from New Zealand. 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, which originates from the infected mother tuber (Pérombelon & Kelman, 1987).

Studies on the genetic variability of phyto-pathogenic bacteria are important to elucidate possible relationships between certain populations of the pathogen and the area from where they were originally isolated (Scortichini, 2005). Similarly, the determination of race distribution, in case of phyto-pathogenic fungi, is fundamental to guide the development of appropriate strategies for disease management according to different regions (Bayraktar and Dolar, 2012). The diversity study helps in understanding the structure of pathogen population which is a prerequisite for the control of the disease. If there are differences among the different strains of a plant pathogenic bacterium at genome level, it is of significance in understanding the ecology of the pathogen in a certain area (Seo *et al*., 2002). Moreover, it is important to have a thorough knowledge of the degree of genetic and pathogenic variation in the pathogen. This information is needed for resistance-breeding against the disease, (Arabi & Jawhar, 2007). Diversity within Ecc and *Erwinia chrysanthemi* (Ech) strains is well-established and is far greater than that within Eca. Therefore, it is possible to relate diversity within Ech and, to a lesser degree, Ecc with host range and geographical location (Maki-Valkama & Karjalinen, 1994; Nassar *et al*., 1996; Parent *et al*., 1996). However, within Eca similar correlations are not easily possible (Persson & Sletten, 1995; Parent *et al*., 1996) and this makes epidemiological studies difficult. So, to facilitate epidemiological studies, it is necessary to increase the level of discrimination within Eca strains.

Different techniques could be used to study genetic diversity present in a pathogen population. Randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), using low stringency conditions and random primers having short nucleotide sequences has been used efficiently to discriminate genetic diversity among some plant pathogenic bacteria (Mello *et al*., 2006). RAPD-PCR is popular because of its speed, simplicity, low cost and effectiveness for studying variation in pathogen populations (Jawhar *et al*., 2000; Hsiang & Wu, 2000). Although the reproducibility of RAPD fingerprints can be influenced by the reagents, thermocycler (Tyler *et al*., 1997) and intensity of amplicons used to score the fingerprints (Skroch & Nienhuis, 1995), under well-established parameters the results can be very reproducible within a laboratory (Mello *et al*., 2008). Toth *et al*., (1999) evaluated a number of phenotypic and molecular typing techniques for determining diversity in Eca and concluded that among the molecular techniques tested, RAPD analysis was the most discriminatory.

Eca is an important pathogen of potato in Pakistan, especially the North-Western part of the country. However, no research work has been done so far on the population structure of this important pathogen. Therefore, our long term goal has been to develop an effective disease management strategy and understanding the variation in the pathogen population is a must to achieve this goal. In this paper we report the pathogenic and genetic diversity found, using RAPD, among the different isolates of Eca collected from different geographical areas of the North-Western Pakistan.

Materials and Methods

Bacterial isolates: In all, 20 field isolates of Eca were analyzed (Table 1). All isolates were obtained either from potato tubers of different cultivars or stems of potato plants showing typical symptoms of blackleg. Diseased tubers and plants were collected from farmers fields (using a W-pattern at each site) during 2007-09. Using green pepper fruit as an enrichment host for the soft rot *Erwinias*, the peppers were surface-disinfested and then inserted with sterile toothpicks pre-stabbed into the margin of blackleg lesions in potato stems or soft-rotten potatoes (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. A loopful was used to streak the surface of nutrient agar (NA) plates. Single colonies were harvested, after incubating the plates at 28°C for 24 h, and purified by sub-culturing. Identity of isolates was confirmed using biochemical tests (Lelliott & Dickey, 1984; Lelliott & Stead, 1987, data not shown) supported by PCR, using the subspecies-specific primers, Eca1F (5'-CGGCATCATAAAAA CACG-3') and Eca2R (5'-GCACACTTCATCCAGC GA-3') (De Boer and Ward, 1995) (data not shown). When required, each bacterial strain was grown overnight on Luria Bertani (LB) at 27°C.

Table 1. Analysis of 20 Eca isolates and their grouping as determined by pathogencity test.

Isolate	Source	Variety	Stem rot[1]	AGs[2]
Eca 6*	Tuber	Raja	5.93 a	AG 1
Eca 8*	Tuber	Raja	5.43 ab	AG 1
Eca 9*	Stem	Raja	5.90 a	AG 1
Eca 14	Stem	Desiree	5.37 ab	AG 1
Eca 15	Tuber	Desiree	5.93 a	AG 1
Eca 16	Tuber	Desiree	5.90 a	AG 1
Eca 17	Tuber	Raja	5.37 ab	AG 1
Eca 2*	Stem	Desiree	4.93 bc	AG 2
Eca 5*	Stem	Raja	5.00 bc	AG 2
Eca 7*	Tuber	Raja	5.00 bc	AG 2
Eca 19	Tuber	Karora	4.77 bcd	AG 2
Eca 1*	Tuber	Raja	4.40 cdef	AG 3
Eca 3*	Stem	Karora	4.53 cde	AG 3
Eca 4*	Tuber	Raja	4.57 cde	AG 3
Eca 13	Tuber	Raja	4.47 cdef	AG 3
Eca 18	Tuber	Desiree	4.47 cdef	AG 3
Eca10*	Tuber	Karora	4.07 def	AG 4
Eca 11	Stem	Desiree	4.00 ef	AG 4
Eca 12	Tuber	Desiree	3.80 f	AG 4
Eca 20	Stem	Desiree	4.03 ef	AG 4

*Isolates obtained from tuber or diseased stem of potato crop grown in hilly areas of North-Western Pakistan. Isolates with no (*) were obtained from potato crop grown in plains

[1]Means of potato stem rot (cm) produced as a result of artificial inoculation of three potato varieties

[2]Aggressiveness groups AG1, AG2, AG3 & AG4

Pathogeicity tests: Pathogenicity tests were done in green-house, using the cultivars of origin (i.e., Raja, Desiree, and Karora). The tubers were spread on lab benches at room temperature in the light for 10 days for sprouting and then sown in 20 cm earthen pots (one tuber per pot) filled with heat-sterilized loamy soil. Randomized Complete Block Design (RCBD) was used with four replications, 21 treatments and 4 plants per treatment. Inoculations were done by injecting 25 µl of bacterial suspension in the base of 3-week old potato plants. Control plants were injected with sterile saline without bacteria. Bacterial suspensions were prepared by growing the isolates overnight on LB plates at 27°C, removing the bacterial growth off the plate with sterile cotton swabs, suspending in 0.85% saline and adjusting to OD_{595} = 0.3-0.5 (Toth *et al*., 1999). The plants were kept inside plastic-covered humidity chambers, incubated at 23±2°C for two weeks and checked daily for stem rot symptoms appearance. The data were recorded by measuring the stem rot for each isolate on each variety and statistically analyzed so that the isolates could be grouped according to the extent of their aggressiveness. The experiment was repeated twice with similar results.

DNA extraction: To extract DNA, bacteria were grown overnight at 27°C in 5ml LB broth in shaking incubator. Half ml of each isolate was poured in eppondorf tubes, centrifuged at 7000 rpm at 4°C for 10 minutes, supernatants were discarded and the pellets were re-suspended in 100 µl 0.5N NaOH solution (Wang *et al*., 1993). The suspension was centrifuged at 8000 rpm for 10 minutes, and supernatant was saved. For each isolate, 5µl of the supernatant were mixed with 45µl of 0.1 M TBE buffer and stored at 4°C until used. When needed, 3 µl of this mixture was directly added to the PCR tube.

RAPD analysis: To study the molecular diversity among Eca isolates, RAPD was used. Fifteen random primers including OPB-07 (5'GGTGACGCAG3'), and OPB-11 (5'GTAGACCCGT3'), (Maki-Valkama & Karjalainen, 1994) were evaluated. Only OPB-07 and OPB-11 were selected for further molecular characterization of the Eca isolates because they amplified reproducible polymorphic bands for all of the isolates. PCR master mix consisted of 2 mMol $l^{-1}MgCl_2$, 0.2 mMol l^{-1} dNTPs, and 1 µMol l^{-1} each primer. The template DNA in all PCR tubes was denatured first (95°C for 5 minutes) and then five U (1 µl) of Taq DNA polymerase (Fermentas, UK) were added to each tube. The target DNA was then amplified using MJ mini thermocycler (Bio-rad, USA). The following PCR conditions were used for each cycle (a total of 40 cycles): DNA denaturation at 94°C for 30 sec., primer annealing at 47°C for 30 sec., and primer extension at 72°C for 50 sec. A final one-time extension (at 72°C) was allowed for 8 min. However, for primer OPB 11, the annealing temperature was dropped to 26°C (because of lower Tm of the primer). The PCR amplification products (25 µl from each tube) were electrophoresed through a 2% (w/v) agarose gel (Sambrook *et al*., 1989), and stained in ethedium bromide (0.5µg/ml) solution for 15 minutes. The amplified bands were observed under UV light in UV tech machine (ESSENTIAL, D-55-20-M-Auto., UK) and the images were saved.

Data analysis: Polymorphic bands were visually inspected from the photograph of the gel containing the amplified DNA of all isolates. Presence of a band was scored as 1 and its absence as 0. Presence and absence of a band with the same molecular size was assumed to be two alleles at a locus and variation in band presence was recorded as a polymorphism (Bai *et al*., 2003). Band(s) clearly absent in at least one isolate were considered to be polymorphic and were included in the data. Using only the reproducible bands, the bi-variate (1-0) data matrix was generated for all the 20 Eca isolates. The data (bands) generated by the two random primers were used to estimate the genetic similarity among the Eca isolates using the equation: $F = 2Nxy/Nx+Ny$ (Nei & Li, 1979) where F is the value of similarity co-efficient, Nxy is the number of bands shared between isolate x and isolate y, Nx is the total number of bands present in isolate x and Ny is the total number of bands present in isolate y. Cluster analysis was performed using the un-weighted pair-group method with arithmetical average (UPGMA) of Genetyx software, version 7.0 (substituting A for 1 and T for 0) and the resulting clusters were presented as dendrogram.

Results and Discussion

Identity of the isolates: The isolates were identified bio-chemically (Lelliott & Dickey, 1984; Lelliott & Stead, 1987; data not shown) and the identification was confirmed by PCR, using the subspecies-specific primers. A 690 bp band was amplified from all isolates suggesting they were Eca (data not shown).

Pathogenicity test: Inoculation of the all 20 isolates produced stem rot symptoms on the three potato varieties used. Statistical analysis of the data divided the 20 Eca isolates into 4 aggressiveness groups (AG). AG 1 (Eca 6, 8, 9, 14, 15, 16 and 17) was the most aggressive causing an average of 5.69 cm stem rot on the base of the stem. AG 2 (Eca2, 5, 7 and 19) and AG 3 (Eca 1, 3, 4, 13 and 18) were moderately aggressive whereas AG 4 (Eca 10, 11, 12 and 20) was the least aggressive, causing only 3.98 cm stem rot (Table 1).

RAPD analysis: Both primers produced quite a large number of DNA bands. The sizes of the amplified bands ranged from 200 bp to 1 kb (Fig. 1). The dendrogram constructed from RAPD analysis of Eca isolates collected from geographically diverse zones of the North-Western Pakistan separated the 20 isolates into four major RAPD groups, (Fig. 2). The first group was formed by Eca 13, 14, 6, and 10. Eca 14 originated from the infected stems of the potato variety "Desiree" whereas the remaining three isolates were obtained from infected tubers of different potato varieties. Isolates Eca 6 and 10 were collected from potatoes sown in high mountains while Eca 13 and 14 were collected from potato crop of the low lands. Only Eca 6 and 14 belong to AG 1. The second group consisted of 10 Eca isolates, 4 of which belong to AG 1 and three to AG 2. Two isolates of this group i.e., Eca 17 and Eca 19, isolated from potato tubers grown in plains, were the most similar. The isolates Eca 20, 7, 3 and 8 formed the third group. Eca 20 originated from blacklegged potatoes grown in plains while the remaining three isolates were collected from the tubers of mountain-grown potato crop. Only Eca 8 of this group belong to AG 1. Members of the fourth group were Eca 11 and Eca 12, isolated from potato variety "Desiree" stems and tubers, respectively, grown in low land plains. Both of these isolates make AG 4. Within the different groups, the various isolates diverged little from each other. No close relationship was observed between the RAPD and AG groups or between RAPD groups and geographic origin, year of isolation, or the source (i.e. stem or tuber of a particular potato variety) of the isolate. Similar results were reported by Uddin *et al* (2013) who could not correlate the diversity found in Verticillium chlamydosporium isolates ti their host of isolation i.e., isolates obtained from tomato fell in the same RAPD cluster with isolates obtained okra.

The fact that different Eca isolates can cause different amounts of stem rot under similar experimental conditions suggests that the population of the pathogen is not homogeneous and there is pathogenic variation among the isolates tested. The less virulent isolates may have evolved from highly virulent ones simply by losing

virulence gene clusters as is the case of *P. wasabiae* isolates (Kim *et al*., 2009; Pitman *et al*., 2010). Alternatively, the weakly virulent isolates could be because of the existence of less virulent phenotypes in natural environments, along with the aggressive strains, as part of population diversity (Yap *et al*., 2004). It cannot be ruled out that these weakly virulent strains may possess a role in the survival and establishment of the population under specific environmental conditions (Yishay *et al*., 2008). Yahiaoui-Zaidi *et al*., (2010) found that Eca isolates were more aggressive on their cultivar of origin than on other potato varieties. Our data showed that there was variation in the pathogenicity of Eca. However, this variation was not linked to the aggressiveness of Eca isolates. The most aggressive, moderately aggressive or the least aggressive isolates were not clustered in any one specific region. Instead, the isolates seemed to be randomly spread among the different potato-growing areas of both, the plains and the high mountains of the North-Western Pakistan. Similar results were reported by other researchers (Arabi & Jawhar, 2007; Jawhar & Arabi, 2009) studying different plant pathogens. This can be due to genotype-isolate interactions where different virulence genes are operating in the pathosystem (Van der Plank, 1984).

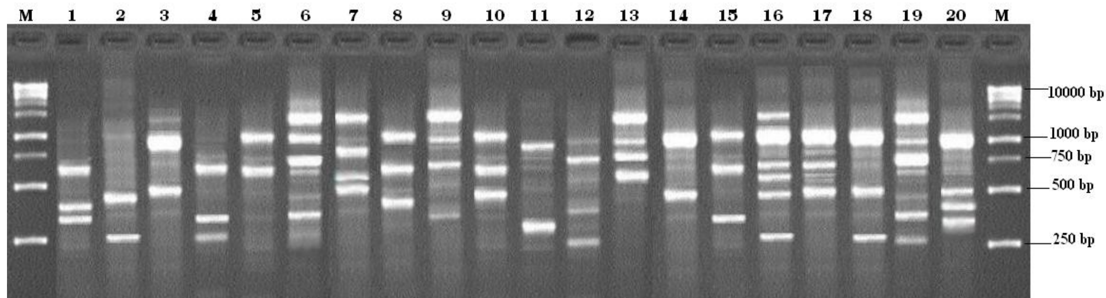

Fig. 1. Amplification profiles of 20 Eca isolates with primer OPB 11. Lane 1-20 = isolates Eca 1 to Eca 20. M = size marker

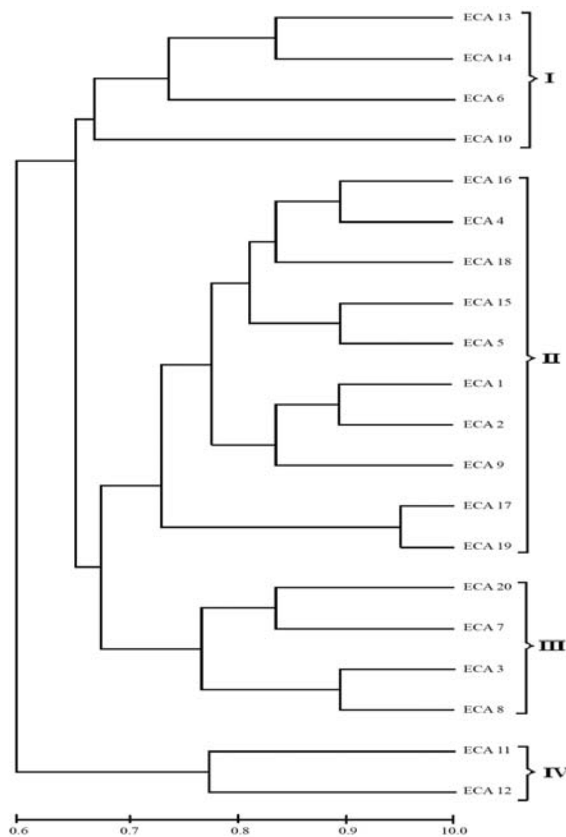

Fig. 2. Dendrogram showing genetic similarities among 20 Eca isolates using the unweighted pair-group method with arithmetical average (UPGMA) of Genetyx software, (version 7.0). RAPD clusters are indicated on the right

RAPD analysis separated our 20 Eca isolates in 4 well-defined groups. The phylogenetic structure obtained as a result of our study, however, did not correlate with the aggressiveness pattern of our Eca isolates, because the most aggressive isolates (AG 1), the moderately aggressive isolates (AG 2 and AG 3), and the least aggressive isolates (AG 4) were found within the same clusters (Fig. 2). Likewise, there was no topography-specific or cultivar-specific correlation either e.g. isolates originated from both plains and mountains, and from different potato cultivars fell in the same cluster. Other researchers, investigating different pathogens also reported similar results where there was no correlation between the genetic polymorphism of isolates and their aggressiveness or origin. Shanmugam *et al*., (2008) assessed the genetic variability of 25 different *Trichderma* isolates (having different levels of antagonism against *Fusarium oxysporum* f.sp. *dianthi* causing carnation wilt) and found that the polymorphism shown by these isolates did not correlate to their level of antagonism. Likewise, Góes *et al*., (2002) reported very high genetic variability among the 14 isolates (antagonistic against *Rhizoctonia solani*) of *Trichderma* they studied. However, they were unable to find any correlation between the polymorphism shown by these isolates and their aggressiveness or origin. The lack of correlation between the AG groups of the pathogen and the phylogenetic structure suggests that the isolates probably got derived from the same source population and dispersed from one area to another through their hosts (Arabi & Jawhar, 2007). Because the pathogen is seed-borne and seed potatoes are widely distributed, this may have contributed to the genetic diversity observed within geographic areas. The lack of correlation between PCR patterns of our Eca isolates and their origin (i.e., topography-specific or cultivar-specific) suggests the absence of any significant influence of host plant cultivar or geography.

RAPD analysis potentially provides information across the entire genome as it uses non-specific primers which bind randomly to regions over the whole genome. RAPD analysis would even detect smaller changes caused by point mutations, thus offering a higher degree of sensitivity as compared to that obtained by other methods. As compared to other related Erwinias such as Ecc and Ech, Eca is relatively homogeneous (Parent *et al*., 1996). Such relatively low levels of genetic diversity may be due to a subspecies having more recent origins, limited geographical distribution and limited host range. Avrova *et al*., (2002) however, found quite a high level of genetic diversity among 59 strains (grouped at 56.6% ± 10.4% similarity) of Eca using amplified fragment length polymorphism. Other researchers studying genetic differences among isolates of enterobacteria on potatoes also demonstrated a greater diversity of pectolytic Erwinias infecting potatoes than previously thought (Oliveira *et al*., 2003; Yahiaoui-Zaidi *et al*., 2003; Yap *et al*., 2004). Phylogentic trees of Kim *et al*., (2009) suggest that Eca is as variable as Ecc, if the branch lengths of the phylogenetic trees are considered as surrogates for diversity among strains.

Genes involved in the formation of T3SS, a molecular syringe that injects virulence (effector) proteins into host cells (Galan & Collmer 1999) as well as *dspE* (encoding the disease-specific effector) have been shown to be important for virulence of and are present in Eca (Holeva *et al*., 2004). However, some *P. wasabiae* isolates lacked genes for T3SS (Kim *et al*., 2009) and some New Zealand isolates of the same pathogen reportedly do not have *hrpN* or *dspE* genes (Pitman *et al*., 2010). The patterns generated from RAPD analysis of our studies demonstrated considerable variability among Eca isolates of the North-Western Pakistan. It would be interesting to know whether or not some of our Eca isolates are missing some of these genes.

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