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Proteome and Quality Analysis of Potato Tubers Infected by *Pectobacterium carotovorum* sub sp. *carotovorum* (PP 4)

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***Pectobacterium carotovorum* subsp. *carotovorum* is an important soft rot pathogen that infects many vegetables in India. Healthy potato, naturally infected potato and artificially pathogen inoculated potato on third day were evaluated for differential protein expression, Polyphenoloxidase activity, total phenolic compound, titratable acidity, pH, soluble solids content, reducing sugars, colour and firmness. Totally 6 differentially expressed products were obtained in two dimensional gel electrophoresis. Among that spots numbered 1,2,5,6 were up regulated and 3, 4 down regulated indicating pathogenesis related proteins and disease resistant inhibitor proteins respectively in both naturally infected and *P. carotovorum subsp. carotovorum* (PP 4) inoculated when compared with the healthy tissue. It requires more evidence to prove their functions in future. Increase in reducing sugars, total soluble solids, polyphenoloxidase activity, total phenolic compound and decrease in firmness and colour in infected potato than the healthy exactly indicates the quality changes of potato by spoilage pathogens. To our knowledge the present investigation is the detailed report on the effects of *P. carotovorum* sub sp. *carotovorum* on the quality of post harvest potatoes.**

Key words: Differential protein expression, *Pectobacterium*, Potato, Soft rot, Two dimensional gel electrophoresis.

Potato (*Solanum tuberosum* L.) has emerged as fourth most important food crop in India after rice, wheat and maize. India is the world's third largest producer of potato and leading vegetable producing country in the world. Potato being the staple food, ranks first (26.6%) in total production of vegetables (Anonymous, 2011a). Major potato producing States in India are Uttar Pradesh, Bihar, West Bengal, Punjab, Karnataka, Assam and Madhya Pradesh. Production of potato is 423.39 Lakh tonnes in India in 2011. Only in the state of Tamil Nadu potato is harvested all around the year in Dindigul and Nilgiris (Anonymous, 2011b). Under tropical and sub-tropical conditions, the losses due to poor handling and storage of potato are reported to be 40-50 per cent in India and up to 2 per cent in Tamil Nadu (Anonymous, 2003).

Food spoilage is a metabolic process which may be brought about by microbial action and causes foods to be undesirable or unacceptable for human consumption due to deterioration in quality characteristics (Doyle, 2007). This type of spoilage is commonly associated with vegetables resulting in postharvest losses which are manifested by a loss of quantity and/or quality due to pathological, physiological

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and mechanical damages (Coursey and Booth, 1972). In most vegetable crops, these factors are interrelated since mechanical injury and physiological stress may greatly influence the susceptibility of the tubers to diseases. Spoilage microorganisms can be introduced to the crop during crop growth in the field, during harvesting and postharvest handling, or during storage and distribution (Barth *et al*., 2009).

The soft rot bacteria in the Enterobacteriaceae are economically important, because they cause serious damage worldwide on a wide variety of plants (Perombelon and Kelman, 1980). The major source for primary inoculum of these bacteria in potato crops is infected/infested seed tubers. Soft rot bacteria can survive in soil and can be transferred by irrigation water therefore, it is important to detect the pathogen in seed potatoes and other inoculum sources to eliminate the disease (Johnson, 1999). Soft rot of vegetable is caused by various species of *Bacillus*, *Pseudomonas* and *Erwinias* (Agrios, 2006). Pectobacterium carotovorum subsp. carotovorum causes soft rot disease in cabbage, potato, onion, radish and other crops during cultivation, transportation and storage, resulting in considerable economic damage. It exists on plant surfaces and in soil, and may enter the host through wound sites or natural openings. After successfully invading a plant, it resides in the intercellular space or vascular tissue, where it produces plant cell-wall-degrading enzymes (PCWDEs) in a disease-promoting environment (Perombelon, 2002; Toth *et al*., 2003).

It has been noted that the largest postharvest losses in vegetable crop tubers is mainly due to microbial infection. Investigation on the effect of soft rot pathogens on physical quality of potato by pathogens also forms part of this report. Several techniques are available to analyse differential protein expressions. Many techniques like protein microarray or gel free techniques like ICAT and iTRAQ have come up. In spite of all these new techniques, 2-DE remains the method of choice for comparative proteomics (Wu *et al*. 2006).Our study objective was to obtain data through proteome analysis that could be used to improve our understanding of the pathogenesis of *Pectobacterium*.

MATERIALS AND METHODS

Spoilage microorganisms

Spoilage pathogens used in this study were PP 1 - *Cellulosimicrobium funkei*, PP 2 - *Enterobacter asburiae*, PP 3 - *Kocuria palustris*, PP 4 - *Pectobacterium carotovorum* subsp. *carotovorum*, PE1 - *Bacillus subtilis* subsp. *inaquosorum*, PE6 - *Bacillus aryabhattai*, PE11 - *Arthrobacter nicotianae* and PE19 -*Klebsiella oxytoca* with gen bank accession numbers KC888011, KC436319, KC436321, KC425473, KC888012, KC888013, KC888015, KC888014 respectively (Ponvizhi Ramya *et al*., 2014).

Virulence assay with potato tubers

Healthy Potato tubers (H"100 g) were surface sterilized with 3% NaoCl, and isolates ($7x10^8$ cfu ml^{-1}) were inoculated in a well formed using cork borer and incubated at 28°C for 7 days. The amount of rotten zone produced in each tuber was determined and taken as criterion of pathogenicity for each isolate.

Treatments and assays

In sterile environment, healthy Potato tubers (H"100 g) were surface sterilised with 3% NaoCl, and isolates ($7x10^8$ cfu ml^{-1}) were inoculated in a well formed using cork borer and incubated at 28°C for 2 days. On third day soft rot pathogen inoculated, naturally infected and control (healthy) potatoes were evaluated for differential protein expression, PPO activity, total phenolic compound, titratable acidity, pH, soluble solids content, reducing sugars, colour and firmness.

PPO activity

Extraction procedure of PPO was carried out as described by Rocha and Morais (2001). The naturally infected, pathogen inoculated and healthy potato samples (20 g) were homogenized with 0.2 M sodium phosphate buffer with pH 6.5 (extraction buffer) plus 2% polyvinilpirrolidone (PVPP) for 3 min using a blender jar IN external ice bath, with 1 min intervals after each minute of homogenization. The homogenates were centrifuged at 4° C for 30 min at 16000 r.p.m. The supernatant was filtered through cheesecloth and its volume determined for enzymatic activity assay. Enzyme activity was assayed by measuring the rate of increase in absorbance at 420 nm in UV/VIS spectrophotometer. The reaction mixture contained

3.0 mL of catechol solution (2.2%), freshly prepared in 0.05 M sodium phosphate buffer at pH 6.5, and a fixed quantity of enzyme. The reference cuvette contained only the substrate solution. The unit for the enzymatic activity was defined as a change of 0.001 in the absorbance value under the conditions of the assay

Total phenolic compound

Each replicate of potatoes was crushed and filtered through cheesecloth. Total phenolic content was measured using the Folin-Ciocalteau reagent (Folin and Ciocalteau 1927; Singleton and Rossi, 1965). Aliquots (0.50 mL) of clear potato juice were diluted in 9.5 mL distilled water, and 4 mL of a diluted Folin-Ciocalteau reagent (1 mL plus 9 mL distilled water) was added to 1 mL of the resulting solution. In the time period between 30 s after the addition of Folin-Ciocalteau reagent, but before 8 min elapsed, 4 mL of sodium carbonate solution was added. After 1 h at 30°C plus 1 h at 0° C, the absorbance of the solution was measured at 760 nm. Dopamine was used to obtain the standard curve (0.5–5.0mg dopamine/mL), and the concentration of phenols was calculated directly from that curve, because the standard and samples were treated identically. Total phenols were expressed as mg dopamine/100 g of potato fresh weight.

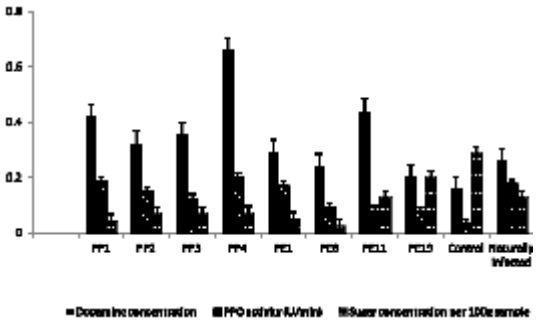

Fig. 1. Total phenolic compound, polyphenoloxidase (PPO) activity and reducing sugars of potato infected by different soft rot pathogens

Titratable acidity and pH

Aliquots (10 mL) of potato juice were diluted with 40 mL of distilled water and titrated with 0.1 N NaOH, beyond pH = 8.2. The results were calculated as a percentage of malic acid [(ml NaOH*0.1 N/mL sample titrated)*100]. The pH was measured in the juice of the crushed potato before pH determination, using a pH meter which had been previously standardized to pH 4 and pH 7 (Rocha *et al*., 2003).

Soluble solids content

The soluble solids content of non-diluted juice from crushed potatoes was determined at 20°C (room temperature) with a hand refractometer. Results were expressed in ° BRIX (1° BRIX = 1 g soluble solid/100 g solution) (Rocha *et al*., 2003).

Reducing sugars

Each replicate of potatoes was crushed and filtered through cheesecloth. Reducing sugar was measured using the dinitrosalicylic (DNS) reagent. First, aliquots (1 mL) of clear potato juice were diluted in 49 mL distilled water. About 900 m L of this solution was pipetted into a test tube, followed by addition of 100 m L of NaOH 0.1 M and 1 mL of DNS reagent. Then, the test tubes were placed in a boiling water bath (120° C) for 5 min, and 4 mL of distilled water was added. The

Table 1. Screening of soft rot isolates from Potato based on Rot zone

S.No		Spoilage pathogens	Rot zone (cm)*
1	PP-1	*Cellulosimicrobium funkei*	3.98±0.080[a]
2	PP-2	*Enterobacter asburiae*	2.89±0.058[b]
3	PP-3	*Kocuria palustris*	2.35±0.047[e]
4	PP-4	*P. carotovorum* subsp. *carotovorum*	4.01±0.080[a]
5	PE-1	*Bacillus subtilis* subsp. *inaquosorum*	2.68±0.054[d]
6	PE-6	*Bacillus aryabhattai*	2.77±0.055[c]
7	PE-11	*Arthrobacter nicotianae*	2.21±0.044[f]
8	PE-19	*Klebsiella oxytoca*	1.69±0.034[g]
		Grand mean	1.6282
		SEd	0.0143
		CD (0.05)	0.0830
		Data are means of 3 replicates ± SD.	

absorbance of the solution was measured at 540 nm. Glucose was used to obtain the standard curve (0.1–0.6 g/L), and the concentration of reducing sugar was calculated directly from that curve, because the standard and samples were treated identically. Reducing sugars were expressed as g sugars/100 g of potato fresh weight (Rocha *et al.*, 2003).

Firmness

Firmness (6 mm deformation) was measured at four different parts of five potatoes (two measurements at the midpoint and two measurements at the top and bottom of the potatoes) with texture analyser. A 100 Newton (N) load cell was used for firmness determination of the potatoes. Cross-head speed was 10 mm/min. This speed was selected considering that the chosen speed allowed relative changes of firmness of raw potatoes to be measured. A 7-mm diameter convex tip Magness-Taylor type probe was used. This test measured individual tuber firmness based on the resistance of the potato flesh to deformation by the probe (Kader, 1982).

Colour assessment

Cut potato surface colour was measured with a handheld tristimulus reflectance colorimeter. Colour was recorded using the CIE-L*a*b* uniform colour space (CIE-Lab), where L* indicates

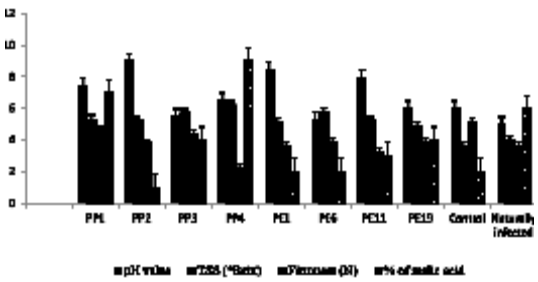

Fig. 2. pH value, soluble solids, firmness and acidity of potato infected by soft rot pathogens

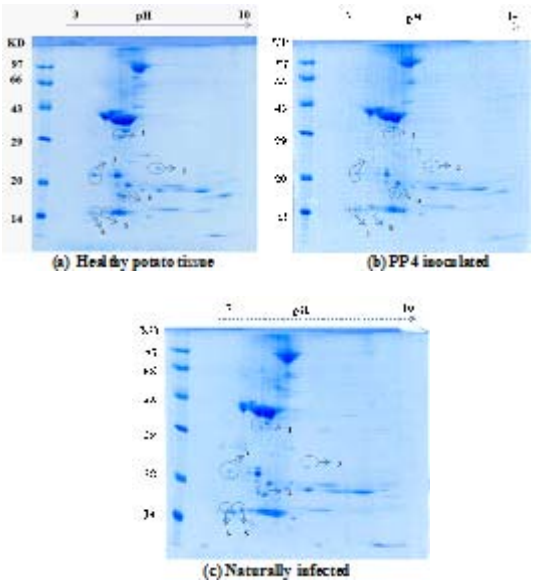

Fig. 3. Results of protein separation with 2dimensional electrophoresis

Table 2. Colour assessment of potato infected by different soft rot pathogens

S. No	Isolates	L*	a*	b*
1.	PP1	50.45±1.009^{b}	8.46±0.169^{b}	22.17±0.169^{a}
2.	PP2	50.82±1.016^{de}	8.86±0.177^{a}	22.60±0.177^{a}
3.	PP3	30.21±0.604^{c}	7.10±0.142^{e}	18.06±0.142^{d}
4.	PP4	31.53±0.631^{h}	7.26±0.145^{de}	19.04±0.145$^{b\,c}$
5.	PE1	22.54±0.451^{e}	5.03±0.101^{g}	10.37±0.101^{e}
6.	PE6	47.2±0.944^{d}	6.54±0.131^{f}	19.54±0.131$^{b\,c}$
7.	PE11	49.87±0.997^{g}	7.45±0.149^{cd}	19.69±0.149^{b}
8.	PE19	50.19±1.004^{f}	7.57±0.151^{c}	19.68±0.151^{b}
9.	Naturally infected	47.12±0.942^{a}	8.88±0.178^{a}	22.2±0.178^{a}
10.	Control	50.88±1.018^{d}	7.61±0.152^{c}	19.03±0.152^{c}
	Grand Mean	44.0867	7.4800	19.2367
	SEd	0.7404	0.1220	0.3204
	CD(.05)	1.5445	0.2545	0.6684

where L* indicates lightness
a* indicates chromaticity on a green (–) to red (+) axis
b* chromaticity on a blue (–) to yellow (+) axis.

lightness, a* indicates chromaticity on a green (–) to red (+) axis, and b* chromaticity on a blue (–) to yellow (+) axis. A decrease of L* value and an increase of a* value is a useful indicator of darkening due to rotting (Mastrocola & Lerici 1991; Monsalve-Gonzalez *et al*. 1993; Rocha & Morais 2001).

Two dimensional electrophoresis and map analysis

Protein was extracted from potato tissues using 10% Trichloro acetic acid (TCA) acetone. Protein concentration was determined by UV- VIS spectrophotometer according to Bradford method, and 1 mg/ml bovine serum albumin was used as a control. Soluble proteins from tissues of *Pectobacterium* (PP 4) inoculated, naturally infected and the control was separated by 2-dimensional electrophoresis (2-DE) method. In the first dimension the 18 cm dry strips with 3-10 of pH gradients were immobilized and 250ìg of each sample were loaded in the isoelectric focusing system (4% IEF gel -16 hours at 400V). In the second dimension 12% sodiumdeodecyl sulphate, polyacrylamide gel electrophoresis (SDS PAGE) was used in further electrophoresis (Running condition, 2 hours at 150V). Gels were stained with coomassie G-250 (Hong-mei *et al*., 2009). Differential expression spots were detected. Each sample had three replicates for 2-DE.

Statistical analysis

All experiments were performed twice. Statistical analyses of the data were done based on the methods given by (Gomez and Gomez, 1984). Least significant difference (LSD) was employed to test for significant difference between treatments at Pd”0.05.

RESULTS AND DISCUSSION

Virulent pathogen

P. carotovorum subsp. *carotovorum* (PP-4) is more virulent compared to other soft rot pathogens and exhibited a rot zone of 4.01 cm followed by *Cellulosimicrobium funkei* exhibited a rot zone of 3.98 cm (Table 1). The degradation of complex polymers of carbohydrates to simple one is brought about by the production of various pectic and pectolylic enzymes produced by *E. carotovora* var. *carotovora*. During post harvest handling and processing, pectolytic microorganisms or cell free pectic enzymes are responsible for tissue maceration (Chesson, 1980). Post harvest rots by nature, suggest maceration due to loss of tissue coherence and separation due to pectolytic activity (Dasgupta and Mandal, 1989).

Polyphenoloxidase (PPO) activity

Plants respond to bacterial invasion by activating defense responses associated with accumulation of several enzymes and inhibitors, which prevent pathogen infection. PPO levels in a plant increase when a plant is wounded or infected (Vanitha *et al*., 2009). In the present investigation PPO activity increased significantly in naturally infected and inoculated potato tubers compared to healthy potato tissue (Figure 1). This is supported by the findings of Okey *et al*., (1997), who reported significantly higher enzyme activities in inoculated and wounded cocoa clones.

Total phenols

In the current study total phenols were higher in *Pectobacterium* (PP 4) inoculated compared to healthy and naturally infected or inoculated with other pathogens (Figure 1). Phenolic compounds and PPO alone do not guarantee resistance against *Pectobacterium*, as can be seen in Montclare, which was severely macerated by the pathogen despite having a high concentration of total soluble phenols. This result supports the findings of Lojkowska and Holubowska (1992), who reported a low level of tolerance in Polish potato cultivars that had a high content of phenolic compounds and PPO. Varieties of potato that is susceptible and prone to maceration by pectolytic enzymes despite having a high concentration of total soluble phenols. High densities of pectobacteria can inhibit and reverse phenol oxidation (Lovrekovich *et al*., 1967).

Titrable acidity and pH

Mostly there is an increase in pH value of pathogen infected potatoes was observed, which was obviously correlated to the increase observed in acidity (Figures 2), nevertheless those changes were not significant. This is not desirable from the sensory point of view, because a variation in pH value would most certainly imply a negative change in flavour (Huxsoll and Bolin, 1989).

Soluble solids content

Generally, the data in Figure 3 indicate that soluble solids content were much higher in

inoculated tissue extracts than in healthy ones particularly with isolate PP 4 (more virulent). Hence, the hydrolysis of complex cell wall polysaccharides into simple sugars could be another pool responsible for increased TSS (Ben and Gaweda, 1985).

Reducing sugars

In the present study there is decrease in reducing sugars of pathogen inoculated and naturally infected tuber compared with control (Figures 1). Data presented by Ismail *et al*., (2012) indicate a similar effect of the pathogen (*Erwinia carotovora* Ec1) on reducing sugars in infected tissues.

Firmness

Many researchers have indicated that the fundamental problem in the extension of shelf life of fruits and vegetables is the loss of firmness during storage and distribution. This results from the action of endogenous enzymes on the cell wall and growth of microorganisms (Rocha and Morais 2001). It should be also noted that the retention of firmness in the potatoes during the storage period is a further indication of the absence of significant microbial spoilage of the product (Chassery and Gormley, 1994). In the current investigation, there is decrease in firmness of PP 4 inoculated compared with the healthy potato. This clearly indicates the microbial spoilage leads to the loss of firmness (Figure 2).

Colour assessment

A decrease of lightness (L* value) was observed in pathogen inoculated and naturally infected compared with the control because tubers with soft rot have brown and water-soaked areas on the surface. There is no significant change in a* and b* values (Table 2).

2D gel electrophoresis

Only the most virulent strain PP 4 was analysed for differential protein expression. The proteins were extracted in good quality and the protein spots were well distributed. In the three replicates of the inoculation treatment 37, 41 and 36 protein spots were detected with an average of 38 spots. In the control 30, 35 and 32 protein spots were detected with an average of 32 spots. In the naturally infected 48, 41 and 45 protein spots were detected with an average of 45 spots. There were more protein spots in the inoculation treatment than in the control indicating some proteins may be associated with the pathogenesis of soft rot pathogen in potato. Among that 1,2,5,6 spots were up regulated and 3, 4 spots down regulated in naturally spoiled and *Pectobacterium* inoculated compared with the healthy tissue indicating pathogenesis related proteins and disease resistant inhibitor proteins respectively (Figure 3). But it requires further studies for verification.

The upregulated expression proteins are probably phytoalexin synthetic enzymes (for instance, chalcone reductase and isoflavone synthase that catalyze the synthesis of daidzein) or pathogenesis related protein (such as PR-1a and PR-2). The metabolic enzymes involved in the transport and protein oxidoreductase were also observed to be upregulated in terms of down regulated proteins, some disease resistant inhibitor proteins were expressed in lower activities after infection of *Pectobacterium*, for example disease resistant inhibitor proteins like RIN4 of Arabidopsis (Belkhadir *et al*., 2004). Further characterization of them may provide valuable information for elucidating the molecular mechanisms of *Pectobacterium* infection.

CONCLUSION

Current research reveals the detailed effect of *P. carotovorum* sub sp. *carotovorum* on the quality of post harvest potatoes. Moreover, our results highlight the potential of proteome analyses of *Pectobacterium carotovorum* sub sp. *carotovorum* (PP 4) to assign pathogenesis related proteins and disease resistant inhibitor protein involved in infection of tubers. This approach has much to offer for future studies on the contribution of bacteria in soft rot of potato under more realistic environmental conditions.

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