AcademicPres

Available online at www.notulaebiologicae.ro

Print ISSN 2067-3205; Electronic 2067-3264

Not Sci Biol, 2012, 4(1):75-81

Notulae Scientia Biologicae

Identification and Pathogenicity of Phytopathogenic Bacteria Associated with Soft Rot Disease of Girasole Tuber

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Abstract

During 2010-2011 growing seasons six bacterial isolates were separated from naturally infected girasole plants tubers (*Helianthus tuberosus* L.) cv. 'Balady', showing soft rot, collected from experimental Farm of the Faculty of Agriculture, in El-Minia University, Egypt. Pathogenicity tests showed various virulence for the bacteria isolated from girasole tubers, found pathogenic. These organisms were characterized as rod-shaped, Gram negative, α-methyl-d-glucoside medium, reducing substances from sucrose, phos, phatase activity and deep cavities on pectate medium. Otherwise, diagnostic tests suggested that the pathogen was *Erwinia carotovora* ssp. *carotovora*. The isolated bacteria caused soft rot of wounded tubers when inoculated into tissues. The bacterial isolates were compared for their degree of pathogenicity as well as for differences in specific symptoms, induced in different hosts. The tested isolates could infect several host ranges, such as fruits of apricot, apple, olive, lemon, squash, eggplant and potato tubers, bulbs and garlic and onion cloves, roots radish, carrot, sweet potato and rape. On the other hand, no symptoms were exhibited on pods of bean and cowpea, faba bean, fruits of pepper and tomato. The extracts of experimentally diseased girasole tubers were active in pectinase and also in caboxymethyl cellulose at pH 6 compared to enzyme activities in healthy tissues. Also, the isolated bacteria increased the total and reducing sugars in infected tissues.

Keywords: *Erwinia carotovora* ssp. *carotovora*, girasole, pectinase and caboxymethyl cellulose, total and reducing sugars

Introduction

Jerusalem artichoke is grown primarily for tubers which can be eaten fresh or raw, cooked in appetizing ways similar to Irish potatoes, or pickled. Tubers are used to fatten cattle, sheep and hogs. Stems and leaves are rich in fats, protein and pectin, making good forage and silage. The alcohol fermented from the tubers is said to be of better quality than that from sugar beets. It is good weed eradicator, as it makes so dense shade that only a few other plants can compete. It is good in ridding fields of quack grass (Margaritis and Bajpai, 1982). Bacterial post-harvest diseases affect quality and availability of fruit and vegetable (Wells *et al*., 1993). Bacterial pathogens, involved in this respect, include the soft-rotting species of *Erwinia, Pseudomonas, Xanthomonas, Cytophaga* and *Bacillus* (Liao and Wells, 1987; Lund, 1983).

Plant diseases caused by plant pathogens mean a complicated process because the number of factors playing part. However, direct involvements of pectic and cellulitic enzymes, produced by the pathogen in pathogensis, were reported (Gaber *et al*., 1990; Walker *et al*., 1994). Bacteria soft-rot caused by *Erwinia carotovora* ssp. *carotovora* (Van Hall), Dye, is one of the most important and widespread bacterial disease of a wide variety plants, either in the field or during storage (Hajhamed *et al*., 2007).

The objective of this investigation is to isolate and identify the pathogenic agent, involved or associated, soft-rot disease of girasol tubers at El-Minia governorate. Furthermore, the cell wall degradation enzymes in pathogenesis were discussed.

Materials and methods

Isolation

Infected girasole tubers showing typically developed soft-rotting (Fig. 1) were subjected for isolation. During 2010 growing season samples of girasole tubers' rot (cv. 'Balady') were collected from experimental Farm of the Faculty of Agriculture, Department of Horticulture, El-Minia University, and isolation of the microorganisms, associated with these symptoms, was conducted. Diseased tubers were firstly washed with tap water then surface sterilized with 3% sodium hypochlorite solution (NaOCl) for 3 min, then washed thoroughly 3 times with sterilized water, the rotted tissues of tuber were put into sterilized mortar and homogenized, then left to stand for 20 min, being streaked into plates containing crystal violet pectate modified (CVPM) medium (Ahmed *et al*., 2000). The plates were incubated at 27±1°C for 48-72 hours. Only bacterial colonies in deep cavities (Fig. 2) were subcultured onto

Received 12 September 2011; accepted 06 February 2012

King's B medium and nutrient agar (NA) medium then stored on slants till they were used.

Identification of the causal pathogen

Six bacterial isolates, e.g. EC1, EC2, EC3, EC4, EC5 and EC6, were identified by studying their morphological, physiological and biochemical characters as recommended by Breed *et al.* (1974), Sneath *et al.* (1986), Lelliott and Stead (1987), Klement *et al.* (1990).

Pathogenicity tests on girasole tubers

The pathogenicity of the bacterial isolates was determined by inoculating intact, unblemished, healthy tubers. Each isolate was used to inoculate 5 tubers. A 1-cm wound was made in the middle of each tuber for inoculation by smearing the inside of the wound with an entomological needle, filled with 48 hour-old cultures of the bacterial isolates, individually grown on NA medium. The inoculated tubers (cv. 'Balady') were kept in sterilized boxes, containing one piece of cotton, saturated with sterilized water, to insure high humidity. These boxes were incubated at 25±1°C for seven days, rot quantity and rot severity being then assayed.

The amount of rotten tissue produced in each tuber was determined and the percentage of rotten tissue was calculated, and taken as criterion of pathogenicity for each isolate. Every tuber was weighted before and after removing the rotten portion, then calculation was done according to Kelman and Dickey's formula (1980) as follows:

Rot severity = (W1-W2)/W1 ×100

Where, W1= weight of whole tuber and W2= weight of tuber after removal of the rotten tissue.

Host range

The highly pathogenic isolate EC1, of the causal pathogen, was inoculated into 16 plant species as listed in Tab. 1. Five plants were used in each treatment. Control treatments were similarly tested with sterile water only and kept at the same conditions. Disease severity was recoded after 7 days as above.

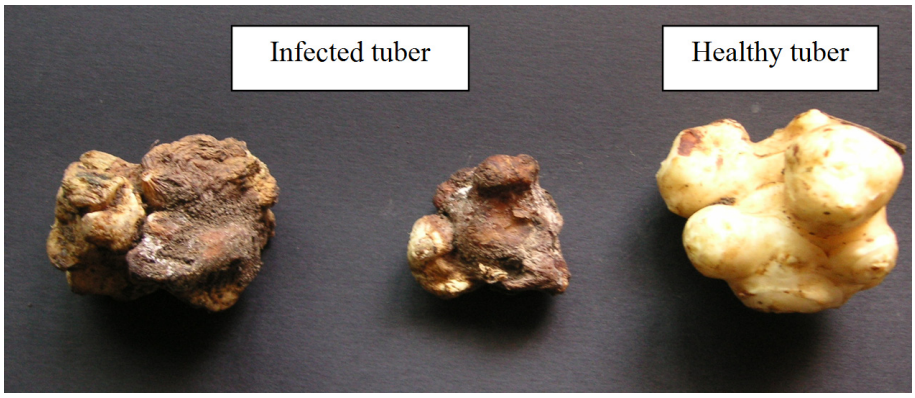

Fig. 1. Naturally infected soft-rot on girasole tubers cv. 'Balady' infected by *Erwinia carotovora* ssp. *carotovora* (left infected and right healthy tuber)

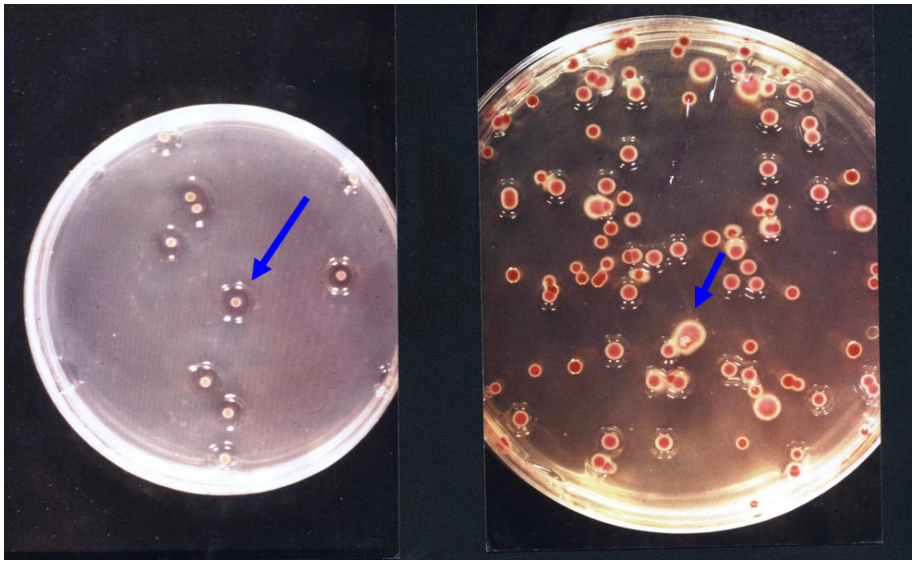

Fig. 2. Cavity formation by soft-rot bacteria after incubation at 27°C for 24 hours on the CVPM medium (left) and recovery of soft-rot bacteria from artificially inoculated tubers after 24 hours (right)

Tab. 1. List of plant species tested for their reaction to *Erwinia carotovora* ssp. *carotovora* pathogen

Hosts (Common name)	Scientific name	Family name	Variety	Part organ
Apricot	*Prunus aremeniaca*	*Rosaceae*	'Canino'	Fruits
Bean	*Phaseolus vulgaris*	*Leguminosae*	'Contender'	Pods
Carrot	*Daucus carota*	*Umbelliferae*	'Chantinay'	Storage roots
Cowpea	*Vigna unguiculata*	*Leguminosae*	'Black eye'	Pods
Cucumber	*Cucumis sativus*	*Cucurbitaceae*	'Balady'	Fruits
Eggplant	*Solanum melogena*	*Solanaceae*	'Black Beauty'	Fruits
Lemon	*Citrus limon* (L.) Burm	*Rutaceae*	'Balady'	Fruits
Tobacco	*Nicotiana tabacum*	*Solanaceae*	'Samsun'	Leaves
Pepper	*Capsicum frutesences*	*Solanaceae*	'Romy'	Fruits
Radish	*Raphanus sativus*	*Carucifera*	'Balady'	Storage roots
Onion	*Allium cepa*	*Amaryllidaceae*	'Giza 20'	Storage onions
Squash	*Cucurbita pepo*	*Cucurbitaceae*	'Eskandarani'	Fruits
Sweet potato	*Ipomea batatas*	*Convolvulaceae*	'Balady'	Storage roots
Potato	*Solanm tuberosum*	*Solanaceae*	'Diamant'	Tubers
Tomato	*Lycopersicon esculentum*	*Solanaceae*	'Super strain B'	Fruits
Turnip	*Brassica rape*	*Carucifera*	'White globe'	Storage roots

Assessment of some hydrolytic enzymes (cellulase and pectinase) in diseased and healthy girasole tubers

Assessment of pectinase and cellulase enzymes were assayed in tissue extracted from diseased and healthy tuber taken from the subjected plants during pathogenicity test.

Half gram of either healthy and/or infected rot tissues were existed and separately macerated with sterilized mortar containing 5 ml of 0.05 M phosphate buffer (pH 6). The homogenated tissue extracts were filtered through two layers of cheese-cloth, cooled to temperature near zero then centrifuged at 5000 rpm for 20 min. The clarified enzyme preparation of healthy and infected tissues was directly subjected to the viscometric assessment according to Mahadevan and Sridhar (1982).

Total carbohydrate and reducing sugars in healthy and artificially inoculated girasole tubers:

A) Total carbohydrates

The phenol-sulphuric acid method was used to determine the total sugars in clarified tissue extract as described by Hodge and Horfreir (1962).

B) Determination of reducing sugars

This was performed according to the methods of Somogyi (1952).

Statistical analysis

All experiments were performed twice. Analyses of variance were carried out using MSTAT-C program version 2.10 (1991). Least significant difference (LSD) was employed to test for significant difference between treatments at $P \leq 0.05$ (Gomez and Gomez, 1984).

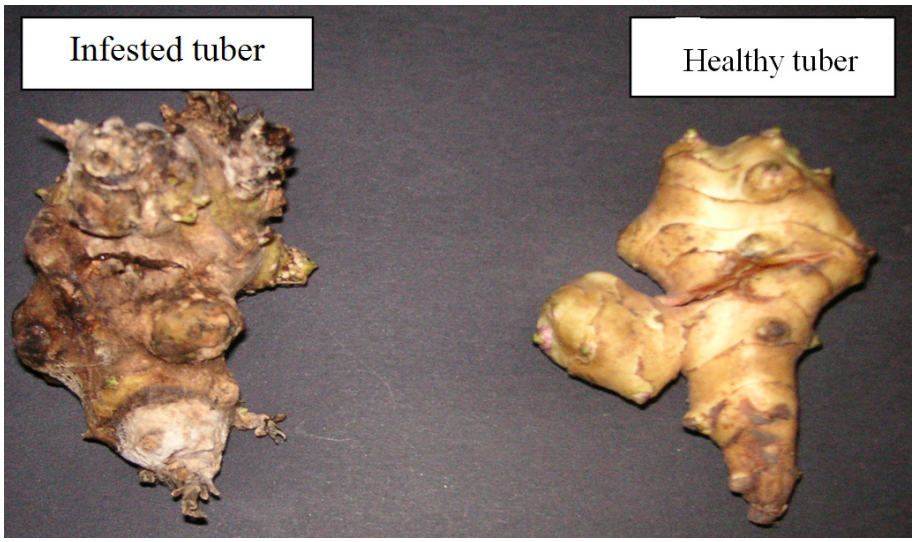

Fig. 3. Artificially infested soft-rot on girasole tubers cv. 'Balady', infected by *Erwinia carotovora* ssp. *carotovora* (left infected and right healthy tuber)

Tab. 2. Morphological, biochemical and physiological characters of bacterial isolates

Test	Bacterial isolates						Bradbury (1986)
	Ec1	Ec2	Ec3	Ec4	Ec5	Ec6	
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+	+
Gram reaction	+	+	+	+	+	+	-
Pigment on $CaCO_3$ agar	-	-	-	-	-	-	-
Sporulation	+	+	+	+	+	+	-
Potato slices	-	-	-	-	-	-	+
Aerobiosis	+	+	+	+	+	+	F
Gelatin liquefication	+	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	-
Levan production	-	-	-	-	-	-	-
Indole formation	+	+	+	+	+	+	-
Tolerance 5, and 7% NaCl	+	+	+	+	+	+	+
Maximum temperature	35	35	35	35	35	35	30
Utilization of sugars from Arabinose	-	-	-	-	-	-	?
Galactose	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	?
Lactose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	D
Insitol	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	-
Mannitol	+	+	+	+	+	+	?
Mannose	+	+	+	+	+	+	?
Sorbitol	+	+	+	+	+	+	?
Trehalose	+	+	+	+	+	+	+
Celliobiose	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	?
Raffinose	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-	-

+ = all isolates are positive, (+) = weakly reaction,-= negative reaction, D = isolates differed, F = facultative anaerobic and ? = isolates not tested

Results and discussion

Isolation and identification of the causal organisms

Six isolates of creamy-white bacteria were isolated from girasole plants showing typical tuber rot symptom (Fig. 1). Regardless of some slight differences, in certain characteristics, all bacterial isolates appeared to be representative of *Erwinia carotovora* ssp. *carotovora* (Tab. 2) according to the description of Bergeys Manual of Determinative Bacteriology (1974), also in the Bergeys Manual of Systematic Bacteriology (Sneath *et al*., 1986). However, the tests were carried out as described by Lelliott and Stead (1987) and Klement *et al*. (1990). The results in the present work revealed that all the tested bacterial isolates are rod-shaped, motile, Gram negative, non-spore forming, growing at 35°C, facultative anaerobic and negatively reacted with phosphatase production, indol formation, and H_2S production. They positively reacted toward gelatine liquefaction, rot of potato and carrot slices, grown in presence of 5% NaCl, nitrate reduction decreased. All the tested isolates produce deep cavities semi-selective (CVPM) medium. Otherwise, the bacterial isolates utilized glucose, galactose, fructose, cellubiose, lactose, mannitol, raffinose, trehalose, mannose and xylose but they did not utilize arabinose, maltose, sorbitol, sucrose and methy glucoside. Comparison of the isolated bacteria's characters with those reported by Dye *et al*. (1980), Dickey (1981) resulted their identification as being *Erwinia carotovora* ssp. *carotovora*.

Tab. 3. Pathogenicity of *Erwinia carotovora* ssp. *carotovora* on girasole tubers

Isolates	Rot weight (gm)	Rot severity (% rotted tissues)
Ec1	1.40[a]	92.0
Ec2	0.99	41.3
Ec3	1.23	74.6
Ec4	0.89	23.6
Ec5	0.68	21.3
Ec6	1.07	57.3
L.S.D. at 5%	0.12	5.88

[a] Mean of five replicates; calculated as percentage of rotted tissues

Tab. 4. Symptoms' expression with 6 bacterial isolates of *Erwinia carotovora* ssp. *carotovora* on different hosts

Hosts Bacterial isolates	Site of inoculation	*Erwinia carotovora* ssp. *carotovora* isolates					
		Ec1	Ec2	Ec3	Ec4	Ec5	Ec6
Apricot	Fruits	+ [a]	+	+	+	+	+
Bean	Pods	-	-	-	-	-	-
Carrot	Storage root	+	+	+	+	+	+
Cowpea	Pods	-	-	-	-	-	-
Cucumber	Fruit	+	+	+	+	+	+
Eggplant	Fruits	-	-	-	-	-	-
Lemon	Fruits	+	+	+	+	+	+
Tobacco	Leaves [b]	+	+	+	+	+	+
Pepper	Fruits	-	-	-	-	-	-
Radish	Storage roots	+	+	+	+	+	+
Onion	Leaves	+	+	+	+	+	+
Squash	Fruits	+	+	+	+	+	+
Sweet potato	Storage roots	-	-	-	-	-	-
Potato	Tubers	+	+	+	+	+	+
Tomato	Fruits	-	-	-	-	-	-
Turnip	Storage root	+	+	+	+	+	+

[a] Data are means of 5 replicates per treatment; [b] Hypersensitive reaction (HR)

Pathogenicity tests

Data presented in Tab. 3 indicate that all bacterial isolates, under investigation, were able to infect girasole tubers and induce soft-rot, although they varied in severity of initiated rot. Inoculation with any of these isolates showed disease symptoms appearing soft-rot at wounded sites, and eventually collapsed within two weeks. However, the control plants remained unaffected. Soft rot symptoms (Fig. 3) sites of inoculation were obvious after 5 to 10 days from the inoculation, whereas after 10 to 15 days, the tubers were collapsed. Amount of rotting, also rated from 22.2% and 42.2% after 21 days from incubation. Also, the obtained results indicate that isolate Ec1 and Ec3 could be considered as highly pathogenic, whereas other isolates were weakly virulent. Several authors reported that *Erwinia chrysanthemi* and *Erwinia carotovora* ssp. *carotovora* were isolated from different plants and caused soft-rot diseases (Hajhamed *et al*., 2007; Liu *et al*., 2002; Scortichini and Dascenzo, 2003).

Host range

Results in Tab. 4 show that all isolates produced soft-rot on most different plants tested. On the other hand, the following plants are not affected by inoculated bacteria such as pods of cowpea, bean and fruits of eggplant, pepper, sweet potato and tomato.

Production of pectolytic and cellulolytic activity by Erwinia chrysanthemi, in vivo

All tested isolates were active in secreting pectolytic and cellulolytic enzymes in tuber tissues of girasole plants after 10 days from inoculation (Tab. 5 and 6), whereas the isolate Ec1 (more virulent) was higher after 180 min than their activities with the weakly virulent (isolate Ec5). These results confirmed those reported by Galal *et al*. (2002), Ouf *et al*. (1997), Saleh (1995). They reported that the pectolytic activity of the enzymes were higher at infected tissues than at the healthy ones.

Activity of these enzymes was higher at infected tissue than at healthy ones. Data indicated that the highest activity was shown after 2 hours incubation at room temperature. These results are generally in line with those reported by previous investigators Ouf and El-Sadek (1997), Ouf *et al*. (1997). Similar results were reported for *Bacillus subtilis* and *Erwinia chrysanthemi* causing soft-rot of carrot roots

Tab. 5. Effect of extract of diseased girasole tubers on percentage of 1% citrus pectin solution's viscosity during incubation for 3 hours at room temperature

Time (min)	% Loss in viscosity of 1% citrus pectin					
	Erwinia carotovora ssp. *carotovora* isolates					
	Ec1	Ec2	Ec3	Ec4	Ec5	Ec6
0.0	6.8 [a]	5.4	5.4	2.4	2.4	2.0
30	19.7	8.2	13.2	14.5	6.5	7.1
60	33.2	11.8	20.8	17.8	15.8	17.7
120	38.0	21.4	30.4	21.3	17.3	23.6
180	38.0	21.4	30.4	21.3	17.3	23.9

[a] Values are mean of 3 replicates

Tab. 6. Effect of diseased girasole tubers' extract on percentage loss of carboxymethyl cellulose (CMC) solution's viscosity during a 3 hour incubation at room temperature

Time (min)	% Loss in viscosity of 1% carboxymethyl cellulose (CMC) solution					
	Erwinia carotovora ssp. *carotovora* isolates					
	Ec1	Ec2	Ec3	Ec4	Ec5	Ec6
0.0	22.7 [a]	15.5	16.7	11.1	0.0	10.9
30	35.4	22.1	23.7	23.0	12.2	17.8
60	43.0	28.7	39.5	27.5	20.3	22.4
120	57.4	36.1	42.6	32.6	28.9	33.4
180	57.4	36.1	43.90	32.9	28.9	33.6

[a] Values are mean of 3 replicates

(Kararah *et al*., 1985; Saleh, 1995; Saleh and Gabr, 1989; Saleh and Stead, 2003). Severin *et al*. (1985) reported that *Erwinia carotovora* pv. *carotovora*, *E. c.* ssp. *atroseptica*, *Erwinia chrysanthemi* ssp. *chrysanthemi* and *Xanthomonas campestris* pv. *pelargonii* (the causal pathogens of soft-rot of potato, dahlia and pelargonium, respectively) were able to produce pectinase (s) and cellulase (s) enzymes.

Generally data in Tab. 7 indicate that total carbohydrates were much higher at inoculated tissue extracts than at healthy ones particularly with isolate Ec1 (more virulent). Similar trends were obtained with reducing sugars. Data presented by Saleh (1995) indicate a similar effect of the pathogen (*Bacillus subtilis* and *B. pumilus*) on total carbohydrates at infected tissues.

Tab. 7. Total carbohydrate and reducing sugars at healthy and diseased tissue extracts of girasole plants inoculated with bacterial isolates

Treatment	Total carbohydrate (mg/g fresh weight)	Reducing sugars (mg/g fresh weight)
Control (healthy tissue)	36.22 ± 1.4 [a]	20.42 ± 3.3
Isolate Ec1	95.44 ± 3.4	17.82 ± 3.0
Isolate Ec2	44.13 ± 3.2	10.19 ± 2.0
Isolate Ec3	82.85 ± 2.3	13.23 ± 2.4
Isolate Ec4	41.17 ± 3.0	11.22 ± 5.0
Isolate Ec5	35.27 ± 1.0	9.12 ± 4.1
Isolate Ec6	39.12 ± 3.0	7.33 ± 3.5

[a] Data are means of 3 replicates ± SD

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