

Full Length Research Paper

Identification and pathogenicity of phytopathogenic bacteria associated with soft rot disease of girasole tuber in Egypt

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Six bacterial isolates were isolated from naturally infected tubers of girasole plants (*Helianthus tuberosus* L. cv. Balady) showing soft rot, which were collected from the experimental farm of the Faculty of Agriculture, Department of Horticulture, El-Minia University, during 2010 and 2011 growing seasons. Pathogenicity tests showed the virulence of the isolated bacteria in girasole tubers which harbored the pathogen; the bacteria were characterized as rod-shaped, Gram negative, α -methyl-d-glucoside medium, reducing substances from sucrose, phosphatase 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 the specific symptoms induced in the different hosts. The tested isolates could infect several host range such as fruits of apricot, apple, olive, lemon, squash, eggplant, potato tubers, cloves of bulbs, garlic and onion; root of radish, carrot, sweet potato and rape, were infected by the tested isolates. 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 at pH 6 and also caboxymethyl cellulose on pH 6 compared to enzyme activities in healthy tissues. The bacterial isolates also increased the total and reducing sugars in infected than healthy tissues.

Key words: Girasole, *Erwinia carotovora* ssp. *carotovora*, 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, and make 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 a shade that few other plants can compete.

It is good in ridding fields of quack grass (Margaritis and Bajpai, 1982).

Post harvest diseases caused by bacteria affect quality and availability of fruit and vegetable (Wells et al., 1993). Bacterial pathogens involved in this respect include the species of soft-rotting *Erwinia*, *Pseudomonas*, *Xanthomonas*, *Cytophaga* and *Bacillus* (Liao and Wells, 1987; Lund, 1983).

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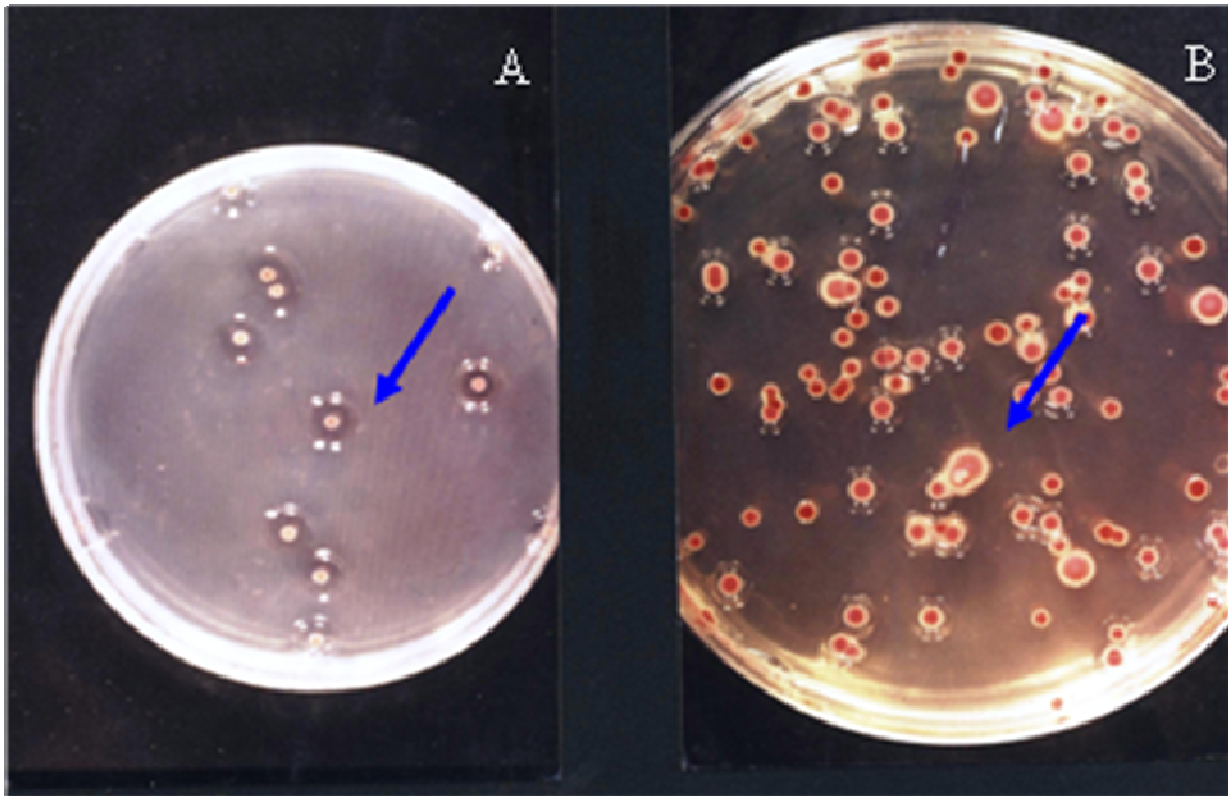


Figure 1. a) Cavity formation by soft-rot bacteria after incubation at 27°C for 24 h on the CVPM medium; b) recovery of soft-rot bacteria from artificially inoculated tubers after 24 h.

Plant diseases caused by plant pathogens are a complicated process because a number of factors play a part. However, direct involvements of pectic and cellulitic enzymes produced by the pathogen in pathogenesis 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 of 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 with 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 (Figure 1) were subjected for isolation. Samples of girasole tubers rot (cv. Balady) were collected from experimental farm of Faculty of Agriculture, Department of Horticulture, El-Minia University, during

2010 growing season; the isolation of the microorganism(s) 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 distilled water; the rotted tissues of tuber was put into sterilized mortar and homogenized then left to stand for 20 min then streaked into plates containing crystal violet pectate modified (CVPM) medium (Ahmed et al., 2000). The plates were incubated at 27±1 °C for 48 to 72 h. Only bacterial colonies in deep cavities (Figure 2) were subcultured onto King's B medium and nutrient agar (NA) medium and stored on slants till they were used.

Identification of the causal pathogen

Six bacterial isolates - 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), and 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. This was done by making a 1 cm wound



Figure 2. Naturally infected soft rot on girasole tubers cv. Balady infected by *E. carotovora* ssp. *carotovora*.

Table 1. List of plant species tested for their reaction to *Erwinia carotovora* sub sp. *carotovora* pathogen.

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

in the middle of the tuber, and then inoculating by smearing the inside of the wound with an entomological needle filled with 48 h old cultures of the bacterial isolates grown on NA medium individually. Inoculated tubers (cv. Balady) were kept in sterilized boxes containing piece of sterilized distilled water-saturated cotton to insure high humidity and then incubated at $25\pm 1^\circ\text{C}$. Seven days later, rot quantity and rot severity were assayed. The amount of rotten tissue produced in each tuber was determined and the percentage of rotten tissue was calculated and taken as a criterion of the pathogenicity to each isolate. Every tuber was weighed before and after removing the rotten portion, and calculation was done via the following formula put forward by Kelman and Dickey (1980):

$$\text{Rot severity} = (W1 - W2) / W1 \times 100$$

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 Table 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 recorded after 7 days.

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 tubers taken from the subjected plants during pathogenicity test. Half gram of either healthy and/or infected rot tissues were extracted and macerated separately with sterilized mortar containing 5 ml of 0.05 M phosphate buffer (pH 6). The homogenated tissue extracts were

Table 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 liquefaction	+	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	-
Levan production	-	-	-	-	-	-	-
Indole formation	+	+	+	+	+	+	-
Tolerance 5, and 7% NaCl	+	+	+	+	+	+	+
Maximum temperature (°C)	35	35	35	35	35	35	30
Utilization of sugars from Arabinose	-	-	-	-	-	-	?
Galactose	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	?
Lactose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	D
Inositol	+	+	+	+	+	+	+
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.

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 viscometrical assessment according to Mahadevan and Sridhar (1982).

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

Total carbohydrates

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

Determination of reducing sugars

Determination of reducing sugars 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).

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 (Figure 1). Regardless of some slight differences in certain characteristics, all bacterial isolates appeared to be representative of *E. carotovora* ssp. *carotovora* (Table 2) according to the description of Bergey's Manual of

Table 3. Pathogenicity of *E. carotovora* sub sp. *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.

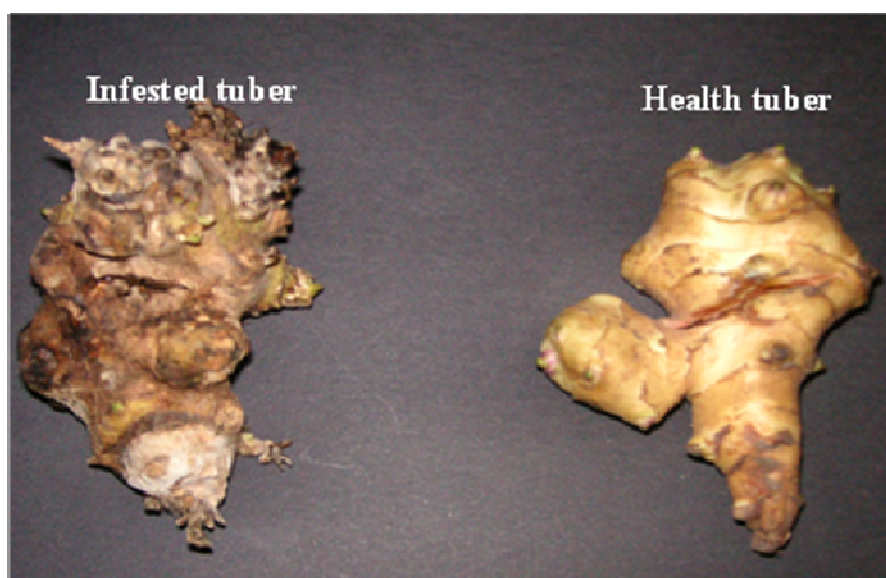


Figure 3. Artificially infested soft rot on girasole tubers cv. Balady infected by *E. carotovora* sub sp. *carotovora*.

Determinative Bacteriology (1974) and Bergey's 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 of the present work revealed that all the tested bacterial isolates are rod-shaped, motile, gram negative and non-spore forming, and they grow at 35°C. They are facultative anaerobic, negatively react with phosphatase production, indol formation and H₂S production, while they positively react with gelatine liquefaction, rot of potato and carrot slices; however, they grow in the presence of 5% NaCl, and reduce nitrate reduction. 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 characters of the isolated bacteria with those reported by Dye et al. (1980) and Dickey (1981) could be identified as *E. carotovora* ssp. *carotovora*.

Pathogenicity tests

Data presented in Table 3 indicate that all bacterial isolates under investigation were able to infect girasole tubers and induce soft rot, although they varied in severity of rot they initiated. Inoculation with any of these isolates showed disease symptoms appearing as soft rot at wounded sites, and eventually collapsed within two weeks. However, the control plants remained unaffected. Soft rot symptoms (Figure 3) sites of inoculation were obvious 5 to 10 days after inoculation, whereas from 10

Table 4. Symptoms expression with 6 bacterial isolates of *E. carotovora* ssp. *carotovora* on different hosts.

Hosts bacterial isolates	Site of inoculation	<i>Erwinia carotovora</i> ssp. <i>carotovora</i> 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).

Table 5. Effect of extract of diseased girasole tubers on percentage of viscosity of 1% citrus pectin solution during incubation for 3 h at room temperature.

Time(min)	% Loss in viscosity of 1% citrus pectin					
	<i>Erwinia carotovora</i> sub sp. <i>carotovora</i> isolates					
	Ec1	Ec 2	Ec 3	Ec 4	Ec 5	Ec 6
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.

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 weak virulent. Several authors reported that *Erwinia chrysanthemi* and *E. carotovora* ssp. *carotovora* were isolated from different plants and caused soft rot diseases (Liu et al., 2002; Scortichni and Ascenzo, 2003; Hajhamed et al., 2007).

Host range

Results in Table 4 show that all isolates produced soft

rot on most different plant tested. On the other hand, plants such as pods of cowpea, bean and fruits of eggplant, pepper, sweet potato and tomato are not affected by inoculated bacteria.

Production of pectolytic and cellulolytic activity by *E. chrysanthemi* in vivo

All tested isolates were active in secreting pectolytic and cellulolytic enzymes in tuber tissues of girasole plants after 10 days of inoculation (Tables 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 Saleh (1995),

Table 6. Effect of extract of diseased girasole tubers on percentage loss of viscosity of 1% carboxymethyl cellulose (CMC) solution during incubation for 3 h at room temperature.

Time(min)	% Loss in viscosity of 1% carboxymethyl cellulose (CMC) solution					
	<i>Erwinia carotovora</i> sub sp. <i>carotovora</i> isolates					
	Ec1	Ec 2	Ec 3	Ec 4	Ec 5	Ec 6
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.

Table 7. Total carbohydrate and reducing sugars in 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.

Ouf et al. (1997), and Galal et al. (2002). They reported that the pectolytic activity of the enzymes were higher in infected tissues than in the healthy ones.

Activity of these enzymes was higher in infected tissue than in healthy ones. Data indicated that the highest activity was shown after 2 h 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 *E. chrysanthemi* causing soft rot of carrot roots (Kararah et al., 1985; Saleh and Gabr, 1989; Saleh, 1995; Saleh and Stead, 2003). Severin et al. (1985) reported that *E. carotovora* pv. *carotovora*, *E. carotovora* ssp. *atroseptica*, *E. 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, the data in Table 7 indicate that total carbohydrates were much higher in inoculated tissue extracts than in 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 (*B. subtilis* and *B. pumilus*) on total carbohydrates in infected tissues.

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