

Isolation and characterization of *Erwinia herbicola* associated with internal discoloration of tomato fruits (*Lycopersicon esculentum* Mill) in Saudi Arabia

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Abstract: Tomato fruits grown in greenhouses were collected from Al-Karj region during summer 2008. Seven bacterial isolates that characterized as Gram negative, short rod-shaped, yellow-pigmented bacterium were isolated and recovered from the internal discoloration tissue of there collected fruits. Symptoms of infected fruits were blackening of the vascular vessels of tissue reached to seeds and lack any external symptoms. Identification and pathogenicity tests of the isolated bacterium were revealed that it belongs to the species *Erwinia herbicola*. Artificial inoculated showed that red ripe fruits were more affected than other fruit stages. Also, injection method of various tomato fruits were more effective method when compared to other methods tested. Screening the tomato fruits obtained from various cultivars showed significant differences when were inoculated. To our knowledge this is the first report of *E. herbicola* recovered from tomato fruits in Saudi Arabia.

Key words: *Erwinia herbicola*, tomato fruit, screening, Saudi Arabia

عزل ووصف البكتيريا ايروينيا هيربيكولا المصاحبة للتلون الداخلي لثمار الطماطم بالمملكة العربية السعودية

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الملخص: تم جمع عينات من ثمار الطماطم من منطقة الخرج خلال صيف 2008. أظهرت نتائج العزل من النسيج الداخلي لثمار الطماطم التي تم جمعها وجود سبع عزلات بكتيرية سالبة لصبغ جرام وعصوية قصيرة وتفرز صبغة صفراء و كانت أعراض الثمار المصابة عبارة عن اسوداد النسيج الوعائي والبذور دون ظهور أعراض خارجية على الثمار. أظهرت نتائج التعرف والقدرة الامراضية أن العزلات تتبع البكتيريا ايروينيا هيربيكولا. أظهرت نتائج العدوى الصناعية أن الثمار مكتملة النضج أكثر تأثرا من الثمار الأخرى. وكانت طريقة الحقن لثمار الطماطم أكثر تغيرا عند مقارنتها بالطرق الأخرى. ثمار الطماطم المتحصل عليها من أصناف مختلفة أظهرت معنوية مختلفة عند عداها. يعتبر هذا أول تسجيل للبكتيريا ايروينيا هيربيكولا المعزولة من ثمار الطماطم في المملكة العربية السعودية.

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Introduction

Erwinia herbicola is widespread in nature as an epiphyte on many plants (Starr, 1981), bio-control agent (Thomson et al., 1976) and also reported to have ice nucleation activity (Lindow et al., 1978 and 1983). However, some strains of this bacteria have evolved into plant pathogens that induce yellow brown lesions on honeydew melons (*Cucumis melo* L.) fruits (Wells et al., 1987), blight on *Aglaonema* spp (Contrerass et al., 1994), wilting of cut rose (*Rosa* spp) flowers (Van Doorn and De Witte, 1997), rot of alfalfa (*Medicago sativa* L.) sprouts (Moline and Kulik, 1997), internal necrosis of immature cotton (*Gossypium* L.) boll (Galal, 2001 and Ashworth et al. 1970), internal rot to peach (*Prunus persica* L.), apricot (*Prunus armeniaca*, plum (*Prunus cerasifera*) and apple (*Malus domestica*) fruits (Abdel-Gowad et al., 2001) and internal discoloration to tomato (*Lycopersicon esculentum* Mill) (Galal et al., 2003). In addition, O'Brien (1993) reported that, the bacterium was able to colonize the site of inoculation with the pathogen *E. amylovora*.

A previous study of Galal et al. (2003) indicated that strains of *E. herbicola* induced typical symptoms of internal discoloration characterized by blackening of the vascular vessels of tomato fruits and they reported that the bacteria can infect flowers, leaves and seeds.

Tomato is grown as a commercial crop with approximately 14,699 ha planted annually and an estimated yield of 522,152 tonnes (Ministry of Agriculture, Central Administration of Economic Studies and Statistics, 2008) in Saudi Arabia. Existence of this bacterium on the plants could cause severe damage for the tomato crop especially when the night temperature drops to 0°C. Moreover, frost damage may render the crop to be sensitive to infection with other microorganisms (Abdel-Gowad et al., 2001). Fruits have healthy appearance but when they were cut they

showed vascular and seed discoloration with no signs of insect infestation was collected from the Riyadh city (The first observation was showed in tomato fruits which collected from Supermarket). Repeated isolations from infected tissue revealed consistently the presence of bacterial growth. The aims of this work were to: (i) identify the associated bacterium on tomato fruits by physiological, biochemical tests and Biolg, (ii) determine an efficient artificial inoculation technique for tomato fruits using this bacterium, and (iii) test the response of different tomato cultivars to artificial inoculation with this bacterium.

Materials and Methods

Collection of tomato fruits samples

Tomato fruit samples which did not shown any external symptoms were collected during summer 2008 from five greenhouses in Al-Karj region. Fruits were placed in paper bags and brought to the laboratory for the isolation of the target pathogen and further studies.

Bacterial isolation

Collected tomato fruits were cut with a sterilized scalpel under aseptic conditions and small portions from the fruits with internal discoloration that characterized as blackening vascular vessels of tissues reached to seeds (Figure 1) was crushed in small quantity of sterile water in sterilized mortars (Galal, 2003). A loopful of the resulting suspension was streaked onto plates of nutrient glucose agar medium (NGA), incubated for 72 h at 25-28 oC and observed for colony growth. Presumptive colonies of the pathogen (pale to strongly yellow, mucoid colonies) were purified by sub-culturing single colonies. One colony of the purified presumptive pathogen from each sample was selected and retained on NGA slants for further tests.

Identification of bacterial strains

In order to identify the microorganisms, physiological and biochemical tests were performed on the isolated bacteria using the methods described by Dye (1968, 1969), Lelliott and Stead (1987) and Klement et al. (1990). Further identification was done using the Biolog GN/GP Micro Plate

systems (Biolog, USA). Bacterial isolates for Biolog analysis were grown on tryptone soya agar (TSA) for 24 h at 28°C. Biolog microplates were inoculated with a bacterial suspension in sterile saline (0.85% NaCl), then adjusted in density to the Biolog system turbidity standard, and incubated for 24 h at 30°C. Plates were read on an automated microplate reader.



Figure 1. Typical symptoms of natural infection of tomato fruits which *E. herbicola* was consistently isolated.

Pathogenicity test

Seven bacterial isolates from tomato fruits were tested for their pathogenicity. For inoculation of tomato fruits, isolates were grown for 72 h on NGA. Plates were flooded with sterile, phosphate-buffered saline (PBS, 3.0 g KH_2PO_4 , 7.0 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 4.0 g NaCl per litre of distilled water, pH 7.2) (Leben et al., 1968) and the resulting suspension was adjusted turbidimetrically to approximately 1×10^6 CFU/ml. Intermediate stage of tomato fruits cv. Farah were surface sterilized by dipping in 0.5 % sodium hypochlorite solution for 2 min, rinsed in a sterile water and left to dry. For each isolate, six fruits were injected with bacterial suspension (200 μ l). Control fruits were inoculated with buffer only. Fruits were kept at 25°C in a sterile

plastic container, and evaluated after 7 days for the development of symptoms.

Disease assessment

Vascular discoloration severity was assayed using an arbitrary 0-5 scale, where 0= no symptoms, 1= 1-25%, 2= 26-50%, 3= 51-75% and 4= 76% to completely discolored tomato fruits. Disease severity index was calculated according to Liu et al. (1995).

Response of tomato fruits to different inoculation methods with *E. herbicola*

Bacterial suspension of three isolates was prepared as described before (10^6 CFU/ml). Fruits were surface sterilized and three methods of inoculation were applied as follow:

1) Fruits were atomized with the bacterial suspension to run off, 2) fruits

were dusted with carborundum and then rubbed with cotton tips which had been soaked in the bacterial suspension and 3) fruits were injected as described above and the bacterial suspension was deposited by a sterile syringe. Control fruits were treated similarly but inoculated with sterile buffer only. Fruits were incubated in sterile plastic containers supplied with water-wetted piece of cotton to maintain high level of relative humidity at room temperature for 7 days and the symptoms developed were assessed. Six fruits were inoculated for each isolate. Disease severity was calculated as described above.

Effect of developmental stages of tomato fruits on infection with *E. herbicola*

Tomato fruits were collected from commercial farms near Riyadh at three different stages of development which are small green, intermediate green and full ripened. Ten fruits of each stage were inoculated by the injected method as described above. Control fruits were treated similarly but inoculated with sterile buffer. Fruits were incubated as described above and symptoms developed were recorded after 7 days. Three bacterial isolates were used in this study.

Reaction of tomato cultivars to infection with *E. herbicola*

Five commercial tomato cultivars (Table 3) were evaluated to infection with bacterial suspension of *E. herbicola*. Six fruits at the full ripened stage were surface sterilized and inoculated with the three tested bacterial isolates by the injected method for each isolate in this study.

Statistical analyses: all of the experiments mentioned were conducted twice. The data were pooled for statistical analyses. Disease severity data were analyzed according to the Kruskal-Wallis nonparametric test statistics. Analysis of variance was performed and means were separated according to the Student-Newman-Keuls test.

Results

Isolation and identification of the bacterium

Yellow pigmented colonies on NGA were consistently isolated from tomato fruits, which showed internal discoloration, and characterized as blacking vascular vessels of tissues reached to seeds. The seven isolates used in this study were Gram-negative, rod shaped, motile, nonspore forming and grew at 37°C. All isolates were able to hydrolyze starch, facultative aerobic, produced a diffusible yellow pigment, positively reacted with Voges-Proskauer. Moreover, all isolates reduce nitrate to nitrite and macerated potato tissues. In addition, the tested bacterial isolates utilized glucose, glycerol, lactose, mannitol, sucrose and xylose. All tested isolates have ice nucleation activity at concentration of 10^5 and 10^7 CFU/ml. Based on these tests; all isolates were identified as *E. herbicola* (Lohnis) Dye. The identity of the bacterial species was further confirmed by Biology analysis (carbon source utilization at 37°C) with a similarity index of 0.85.

Pathogenicity test

Significant variances among isolates were showed in Table 1. Isolates EH2, EH4 and EH5 were the most pathogenic isolates expressed by disease severity index. The highest severity index (3.8) was provided by isolate EH5, followed by both two isolates EH4 (3.5) and EH2 (2.8). While the two isolates EH3 and EH7 reacted as the weakest pathogenic ones. Three of seven bacterial isolates (EH2, EH4 and EH5) were selected for further experiments because they were highly virulent according to disease severity index. No symptoms were observed on control fruits inoculated with buffer. Re-isolation from the artificially inoculated fruits revealed similar isolates to the original ones.

Table 1. Pathogenicity of seven isolates of *E. herbicola* on tomato fruits cv. Farah.

Bacterial isolates	Disease severity index	Pathogen re-isolated
EH1	2.2 c	+
EH2	2.8bc	+
EH3	2.0 c	+
EH4	3.5a	+
EH5	3.8a	+
EH6	2.4 bc	+
EH7	2.0c	+
Control	0.0	-

Response of tomato fruits to different inoculation methods with *E. herbicola*

Results of the inoculation methods experiment (Table 2) showed that spraying the cell suspension (1×10^6 CFU/ml) of *E. herbicola* in tomato fruits did not produce any disease symptoms during the two experiments. While fruits developed restricted brown spot but did not extend

further using carborundum-rub method. All tomato fruits resulted in the appearance of disease symptoms with injection technique one week after inoculation. No significant differences were observed among the two isolates EH4 and EH5 while both of them were significant than EH2.

Table 2. Response of tomato fruits cv. Farah to different inoculation methods with *E. herbicola*.

Bacterial isolates	Inoculation Methods		
	Atomized	Carborandum – rubbed*	Injection
	Disease severity index		
EH2	0.0	0.0	2.8b
EH4	0.0	0.0	3.7a
EH5	0.0	0.0	4.2a
Control	0.0	0.0	0.0

* Symptoms appeared as restricted lesion.

Reaction of tomato cultivars to infection with *E. herbicola*

Infected fruits from five different local tomato cultivars were showed variable internal discoloration symptoms (Table 3). Significant differences in virulence were observed between the three tested isolates. Isolate EH5 was the most virulent followed by EH4. Significant differences were observed between all tested cultivars. Tomato cultivar JV15 was the most susceptible to infection followed by cvs. Newton and Alambra.

Effect of developmental stages of tomato fruits on infection with *E. herbicola*

Data in Table 4 indicated that all the three stages of fruits showed internal discoloration. Full ripened red fruits were the most affected compared to the other stages. Significant differences were observed between the intermediate green fruits, full ripened and small green stages while no significant results was observed between EH2 and EH4 isolates. Isolate EH5 reacted as the most pathogenic isolate.

Table 3. Reaction of tomato cultivars to infection with *E. herbicola*.

Tomato cultivar	Bacterial isolates		
	EH2	EH4	EH5
Disease severity index			
JV15	2.3ab	4.2de	4.8f
Newton	2.0a	4.0cde	4.8f
Alambra	2.6b	4.0cde	4.4ef
Red Gold	2.1ab	3.8cd	4.2de
Sultana	2.5ab	3.5c	3.8cd

Table 4. Effect of development stages of tomato fruits cv. Farah on infection with *E. herbicola*.

Bacterial isolates	Disease severity index		
	Small green	Intermediate green	Full ripened
EH2	2.2a	3.2c	3.5c
EH4	2.4ab	3.5cd	3.8de
EH5	2.8bc	3.8de	4.2e
Control	0.0	0.0	0.0

Discussion

The Gram negative, yellow pigmented bacteria isolated from tomato fruits which exhibit internal discoloration were identified as an isolates of *Erwinia herbicola* (Lohnis) Dye (Lelliott, 1974) (synonymy *Pantoea agglomerans* (Gavini et al., 1989). It was selected by biochemical and physiological properties, as well as with biologic analysis. Also, strains of this bacterium are reported to have ice nucleation activity (Lindow et al., 1978, 1983). Pathogenicity test showed that the bacterial isolates were able to induce internal discoloration with different severity index. The highest severity index (3.8) was provided by isolate EH5, followed by both two isolates EH4 (3.5) and EH2 (2.8). While the two isolates EH3 and EH7 reacted as the weakest pathogenic ones. Three of seven bacterial isolates (EH2, EH4 and EH5) were selected for further experiments because they were highly virulent according to disease severity index. These data were similar to

findings of Galal (2003). They reported that tomato fruits cv. Super Strain B showed internal discoloration characterized by blackening of the vascular vessels of tomato tissue reached to seeds. Also, Ashworth et al. (1970) indicated that, strains of *E. herbicola* induced typical symptoms of internal necrosis of immature cotton bolls when flowers and bolls were inoculated. The inoculation experiments performed in this study and the failure of the inoculum spray method or wounding by carborundum to cause disease symptoms may suggest that the bacterium is seed borne pathogen. Superficial wounding using carborundum develops only a restricted brown spot that did not extend downward. This result may due to inhibition of bacterial growth under aerobic conditions.

Our results corroborate with previous research that showed wounding by carborundum or spray methods of inoculation did not cause infection with *E. herbicola* (Abdel Gwad et al., 2001; Galal,

2003). Our data showed that the tested tomato fruits of different cultivars which are most of cultivars grown in the greenhouses in Saudi Arabia exhibited various levels of infection to *E. herbicola*. Different response in tomato cultivars to infection with *E. herbicola* were reported previously (Galal et al., 2003). This may increase the risk of an epidemic if all environmental conditions are optimal for the development of this disease.

The response of different stages of tomato fruits to infection with *E. herbicola* showed variable differences. Ripened fruits were highly susceptible to infection when compared to the other stages (intermediate and small green). This could be the toxic substances present in immature fruits which inhibit the growth of the pathogens such as tannins (Prusky et al., 1983; Larsen et al., 1980). These results are in contrast to results obtained by Galal (2003) they reported that, green immature fruits were more affected than red ripe fruits.

The results reported in this study indicate that *E. herbicola* was able to infect different cultivars and stages of tomato fruits. Galal et al. (2003) reported that *E. herbicola* infect the leaves, flowers and seed. In another study, Moline and Kulik (1997) indicated that, *E. herbicola* was recovered from beneath the seed coats of three seed lots used for the commercial production of alfalfa sprouts. These observations suggested that *E. herbicola* contaminated seeds may be a significant primary inoculum source in disease outbreaks in tomato grown areas in Al-Karj region.

Further investigations are needed to study the consideration of contaminated seeds as the primary inoculum source of this serious bacterial disease in order to develop effective management programs.

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