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# Occurrence of Shallow Bark Canker of Walnut (*Juglans regia*) in Southern Provinces of Iran

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**Abstract:** From April 2001 to November 2002, samples of walnut branches and trunks with symptoms of shallow bark canker were collected from Fars and Kohgiluyeh-va-Boyerahmad provinces. Symptoms of the disease were small cracks in the bark of the trunk and scaffold branches of mature trees with dark watery exudates which stained the affected trunk or limb. By removal of phelloderm, extensive necrosis of the underlying tissues was observed. In some cases, necrosis extended to cambium and outer xylem. Sixty-one strains of a bacterium were isolated from infected tissues using EMB and YDC media. On the basis of standard biochemical and physiological tests the bacterium was identified as *Brenneria nigrifluens*. The pathogen was found to be wide-spread in the provinces. Isolates were compared by physiological and biochemical characters, antibiotic sensitivity and protein electrophoretic pattern. Most of the strains were fairly similar in phenotypic features and electrophoretic profiles of whole-cell proteins were similar to each other and to reference strain (*B. nigrifluens* 5D313). Inoculation of 1-2 years-old walnut seedlings in May and June produced blackening symptoms and the bacterium survived for long period in infected tissues. This is the first report of the shallow bark canker of walnut in southern Iran.

**Key words:** *Brenneria nigrifluens*, walnut, shallow bark canker

## INTRODUCTION

Bark canker was first observed in 1957 found on a Persian walnut (*Juglans regia* L.) in California. The disease was caused by a peritrichous bacterium belonging to the genus *Erwinia*. It was described in 1957 under the name *Erwinia nigrifluens* (Wilson *et al*., 1957). Later the species was transferred to the genus *Brenneria* and the walnut pathogen was redescribed as *B. nigrifluens* (Hauben *et al*., 1998). The pathogen was also reported on Persian walnut from Italy, Spain and France (Lopez *et al*., 1994; Morone *et al*., 1998; Menard and Baudry, 2004; Loreti *et al*., 2006). Mazzaglia *et al*. (2006) reported the presence of both fungi and bacteria such as *B. nigrifluens* in walnut bark cankers. The disease is characterized by the formation of small longitudinal cracks on the trunks and scaffold branches exuding brown to black liquid during spring and summer. By removal the bark surface, brown to black necrotic area is revealed. Necrosis is usually shallow but some times progresses downward to the cambium and outer xylem (Wilson *et al*., 1957; Teviotdale, 2002; Loreti *et al*., 2006). The affected branches suffer progressive loss of vigor, foliage reduction, early senescence and death. Loreti *et al*. (2006) reported that in Italy, cankers were commonly deeper and more severe than those reported in USA for *B. nigrifluens*, resembling those caused by *B. rubrifaciens* described by Wilson *et al*. (1967). Loreti *et al*. (2006) also reported different responses of *Juglans* species to *B. nigrifluens* by artificial inoculation and an indication on the presence of resistance sources in *J. mandshurica* and *J. sieboldiana*.

In Iran, the shallow bark canker of walnut was first reported from Mazandaran province in northern Iran identified as *B. nigrifluens* (Rahimian, 1989). Further studies in Mazandaran province indicated its wide occurrence in walnut plantations causing trunk and aerial infection under humid conditions (Harighi and Rahimian, 1997).

In spite of a long history of walnut plantation in southern Iran with low rainfall, the pathogen has not been reported yet. In both Fars and Kohgiluyeh-va-Boyerahmad provinces walnut decline is wide-spread, but only *Phytophthora* sp. have been found to be associated with walnut decline (Banihashemi, 1991; Banihashemi and Ghaderi, 2006).

The objective of this research was to investigate the role of bacterial canker in walnut decline in southern provinces of Iran.

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## MATERIALS AND METHODS

**Sampling and bacterial isolation:** From April 2001 to November 2002, samples of walnut branches and trunks with symptoms of bark canker were collected from both provinces in southern Iran. Samples were cut into 5×5 cm sections and washed for 0.5 to 1 h under running tap water. Small pieces from the margin of necrotic and healthy tissues were aseptically excised, disinfested for 2 min in 0.5% sodium hypochlorite, rinsed with sterile distilled water and placed in test tubes with 10 mL Sterile Distilled Water (SDW) and left to soak for 12-18 h. After vigorous shaking test tube, one to two drops (10-20 μL) of the suspension were streaked on YDC and EMB (Difco) containing lactose and glycerol or lactose and sucrose as carbon sources. After 48 to 72 h incubation at 28°C, bright green metallic colonies from EMB and cream color colonies from YDC were selected and purified.

**Physiological and biochemical characterization:** Isolates were identified by bacteriological standard tests (Fahy and Persley, 1983; Schaad *et al*., 2001). Basal medium recommended by Ayers *et al*. (1919), was used for the utilization of carbon compounds and *B. nigrifluens* 5D313 and *B. rubrifaciens* 6D354 (supplied by Dr. Clarence Kado, Plant Pathology Department, University of California, Davis, USA) were used as reference strains.

**Pathogenecity test:** One and two-year-old walnut seedlings grown under natural conditions were used for inoculation. Fifteen strains from different orchards were randomly selected. The surface of the bark was first disinfested with 95% ethanol. The bacterial suspension in distilled water, ($10^7$ cfu $mL^{-1}$), was injected tangentially in to the bark using a sterile needle and the hole was covered for 72 h with parafilm (Loreti *et al*., 2006). Three seedlings were injected with each strain. The inoculations were performed in May and the trees were kept under observation up to August. Sterile distilled water and one strain of *Pectobacterium carotovorum* were used as negative control and the reference strain of *B. nigrifluens* 5D313 as positive control.

**Protein electrophoresis:** All of the strains were analyzed by electrophoresis of the whole cell proteins. Bacterial strains were grown for 24 h on nutrient agar. A suspension of bacterial cells in SDW was prepared and pelleted by centrifugation in an Eppendorf microcentrifuge for 15 min at 10,000 g. The pellet was diluted with SDW to optical density of 1.5 at 600 nm. To 1 mL of each sample, 0.2 mL mix B (containing Tris buffer 0.5 M, glycerol, brome phenol blue, 2-mercaptoethanol and SDS) was added and after shaking, samples were boiled for 2.5 min and electrophoresed in polyacrylamid gel using discontinuous buffer system (Laemmli, 1970; Ausubel *et al*., 1987). Concentration of acrylamid in separating gel and stacking gel was 10 and 5%, respectively.

## RESULTS

**Symptomatology:** The main symptoms of the disease consisted of exuding a dark sap from small longitudinal cracks (Fig. 1). By removal of the surface bark, brown to black necrotic area was observed (Fig. 2). In most cases,

Fig. 1: Small longitudinal cracks on the trunk outer bark (arrow) with dark exudates staining the bark of a walnut tree affected by *Brenneria nigrifluens*

Fig. 2: Outer bark removed from cankered area showing brown necrotic area on internal parts

necrotic lesions had extended to inner bark and occasionally had reached to the pith. The exudates were present mainly during spring and summer. Symptoms were often present on the trunks and rarely on scaffold branches. In some cases, the perforating bark resulted in vigorous sap exudation. Affected trees usually showed progressive lose of vigor, foliage reduction and early leaf senescence.

**Physiological and biochemical characters:** Cells were rod-shaped and slightly curved at the end, usually two celled chain or short chains. On nutrient agar containing 1% glucose, colonies were cream color and circular with entire margins. On EMB containing lactose and sucrose or lactose and glycerol, colonies were circular with entire margins. Green metallic sheen was noticed after 2 or 3 days on EMB containing lactose and sucrose and becoming dark purple but stable. On EMB that had only lactose, bacterium grew poorly and did not have green metallic sheen. On YDC, colonies were white and circular in the entire margin. On thin layer of YDC, colonies were surrounded by a clear zone, indicating acid production with the dissolution of the calcium carbonate. None of our isolates produced red pigment on YDC, in contrast to reference isolate of *B. rubrifaciens* 6D354. Physiological, biochemical and nutritional characters of the isolates are shown in Table 1. Generally the isolates did not show remarkable variation in these characters. All strains were positive for growth on 5% NaCl, production of methyl red and $H_2S$ from cysteine, esculine hydrolysis and growth at 40°C. None of the strain enabled to hydrolyse Tween 80, gelatine, casein, starch and nitrate reduction. All strains were positive for urease.

**Pathogenecity tests:** Two-weeks after inoculation, blackening symptoms appeared around the wounds. External cankers like those observed under natural conditions were not visible after two months, but small areas of necrotic tissues appeared in the bark above and below the inoculation points. When the bark was removed, brown necrotic streaks were observed. The bacterium was recovered from the lesions. Inoculated branches with diluted suspension of bacterium also showed blackening symptoms. From 15 strains inoculated, 9 produced the symptoms as described above. The reference strain of *B. nigrifluens* 5D313 caused similar

Table 1: Physiological, biochemical and nutritional characteristics of the isolates of *Brenneria nigrifluens* inciting bark canker of walnut from different parts of Fars and Kohgiluyeh-va-Boyerahmad provinces and reference strains of *B. nigrifluens* 5D313 and *B. rubrifaciens* 6D354

| | | Reference strains | |
|---|---|---|---|
| Characteristics | Strains of *B. nigrifluens* with positive reaction (%) | *B. nigrifluens* | *B. rubrifaciens* |
| Gram reaction | 0.0 | 0 | 0 |
| [Oxidase] | 0.0 | 0 | 0 |
| Fermentative growth | 100.0 | + | + |
| Tolerance of NaCl 5% | 100.0 | + | - |
| $H_2s$ production from cysteine | 100.0 | + | ND |
| Esculine hydrolysis | 100.0 | + | +(weak) |
| Tween 80 hydrolysis | 0.0 | - | - |
| Gelatin hydrolysis | 0.0 | - | - |
| Casein hydrolysis | 0.0 | - | - |
| Starch hydrolysis | 0.0 | - | - |
| Urease production | 100.0 | + | - |
| Lecithinase | 0.0 | - | - |
| Phenyl alanine deaminase | 100.0[a] | - | - |
| Argenine dihydrolysis | 0.0 | - | - |
| Phosphatase | 7.0 | + | + |
| Reducing substances from sucrose | 0.0 | - | - |
| Indole production | 0.0 | - | - |
| Nitrate reduction | 0.0 | - | - |
| Methyl red reaction | 100.0 | - | - |
| Acetoin production | 89.4[b] | +(weak) | - |
| Potato rot | 14.2[c] | - | ND |
| Levan formation | 0.0 | - | - |
| Gas production from glucose | 0.0 | - | - |
| Growth at 36°C | 100.0 | + | + |
| Growth at 40°C | 100.0 | + | - |
| **Hypersensitive reaction on:** | | | |
| Tobacco | 0.0 | - | - |
| Pelargonium | 3.5 | - | - |
| Fluorescent pigment on King's B medium | 0.0 | - | - |
| Pink pigment on YDC | 0.0 | - | + |
| **Action on litmus milk:** | | | |
| Alkaline reaction | 94.7 | + | |
| Acid reaction | 0.0 | - | |
| Reduction of litmus | 5.2 | - | |

Table 1: Continued

| Characteristics | Strains of *B. nigrifluens* with positive reaction (%) | Reference strains | |
|---|---|---|---|
| | | *B. nigrifluens* | *B. rubrifaciens* |
| **Acid production from:** | | | |
| Ethanol | 0.0 | - | - |
| Adonitol | 0.0 | - | - |
| L (+) Arabinose | 25.0 | - | - |
| Mesoerithritol | 0.0 | - | + |
| Myoinositol | 100.0 | + | - |
| Trehalose | 91.6 | + | - |
| Dulcitol | 0.0 | - | - |
| Raffinose | 93.6 | + | + |
| L (+) Rhamnose | 50.0 | ND | ND |
| D (-) Ribose | 88.8 | + | + |
| D (+) Xylose | 0.0 | - | - |
| D (+) Cellobiose | 83.6 | + | - |
| D-Sorbitol | 100.0 | + | - |
| Sucrose | 100.0 | + | + |
| D-Fructose | 100.0 | + | - |
| D(+)Galactose | 100.0 | + | + |
| D(+)Glucose | 100.0 | + | + |
| Glycerol | 100.0 | + | + |
| Lactose | 0.0 | - | - |
| Levulose | 100.0 | + | + |
| Maltose | 0.0 | - | - |
| D(+) Mannose | 100.0 | + | - |
| Mannitol | 97.6 | + | + |
| Melibiose | 46.8 | ND | ND |
| **Utilization of:** | | | |
| Asparagine | 100.0 | + | - |
| Inuline | 0.0 | - | - |
| L-Tartrate | 0.0 | - | - |
| Salicine | 100.0 | + | - |
| L-Serine | 100.0 | + | - |
| Citrate | 0.0 | - | - |
| Lactate | 20.0 | - | - |
| L-Lysine | 0.0 | - | - |
| Malonate | 0.0 | - | - |
| L-Valine | 0.0 | - | - |
| L-Hystidine | 0.0 | ND | ND |

[a]82.1% of strains with weak reaction; [b]43.8% of strains with weak reaction and 45.6% with strong reaction; [c]14.28% of strains with weak reaction; ND: Not Determined

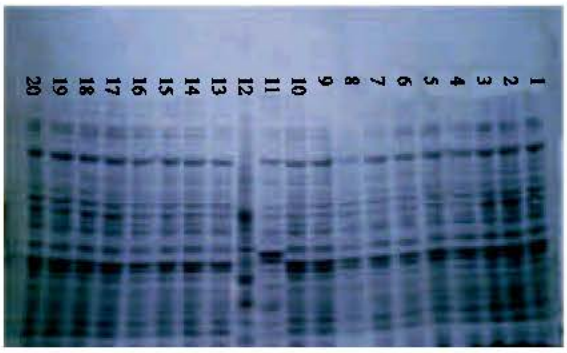


Fig. 3: Electrophoretic profile of cell proteins of the isolates of *Brenneria nigrifluens* inciting bark canker of walnut from different part of Fars and Kohgiluyeh-va-Boyerahmad provinces (1-9 and 13-20), reference strains of *Brenneria nigrifluens* 5D313 (10), *Brenneria rubrifaciens* 6D354 (11) and *Pectobactenium carotovorum* (12)

symptoms. Branches that were inoculated with sterile water and with one isolate of *P. carotovorum* did not show any visible symptoms.

**Protein electrophoresis:** Electrophoretic profiles of whole-cell proteins of 17 strains were similar to each other and to the reference strain (*B. nigrifluens* 5D313) but they were different from *B. rubrifaciens* and *P. carotovorum* profiles (Fig. 3).

## DISCUSSION

From infected tissues with symptoms of bark canker, strains of a bacterium were isolated. Based on physiological and nutritional criteria they were identified as *B. nigrifluens* (Table 1). The results of phenotypical tests of strains are similar to characters described for the type species except for positive reaction to phenylalanine deaminase test (Loreti *et al*., 2006; Hauben *et al*., 1998).

None of the strains produced red pigment, typical of *B. rubrifaciens* on YDC medium (Wilson *et al*., 1967; Schaad and Wilson, 1970).

Strains were fairly similar in phenotypic features to each other and to the reference strain but with a few variations in some characters. However, electrophoretic profiles of cell proteins were very similar to each other and to the reference strain. This similarity indicates low variability of the species. Loreti *et al.* (2006) reported that the whole cell protein patterns of *B. nigrifluens* isolates in Italy was identical to type strains.

Since the bacterium is more active during warm months and is inactive in cold months (Wilson *et al*., 1957), isolation of bacteria is more successful during warm months. EMB containing lactose and glycerol is the best medium for isolation, because colonies of *B. nigrifluens* show stable green metallic sheen. Production of green metallic sheen is dependant on pH around colonies as a result of production of acid from glycerol, in the medium (Wilson *et al*., 1967).

Although artificial inoculation of *B. nigrifluens* to walnut seedling did not lead to the formation of cankers similar to those observed in the nature, but the bacterium survived for long periods in the lesions. Difficulties in reproducing external cankers have been frequently reported by inoculating woody plants with plant pathogenic bacteria, including *B. nigrifluens*, *B. rubrifaciens* and *B. quercina* (González *et al*., 2002; Wilson *et al*., 1957). The difficulties could be due to physiological state of the tissues at the inoculation time and the environmental conditions prevail thereafter.

The results of this study showed that bark canker of walnut has a wide distribution in Fars and Kohgiluyeh-va-Boyerahmad provinces. Although it has been reported that *B. nigrifluens* does not cause much losses but it is one of the major factors of walnut decline in these provinces especially when hosts are predisposed under environmental stresses. Loerti *et al.* (2006) reported that well managed walnut trees successfully healed cankers by producing abundant callus tissue, while weak trees with numerous infected areas declined and died. Biosca *et al.* (2003) also reported that one of the major causes of dying oak trees in Spain is bark canker caused by *B. quercina.*

However, the exact estimation of loss of this disease needs more surveys and other factors including *Phytophthora* species (Banihashemi, 1991; Banihashemi and Ghaderi, 2006) and environmental stresses that predispose trees to bacterial infection should take into consideration as well.

Although Persian walnut plantation has a long history in Iran prior to its introduction to Europe and North America the presence of the bacterium in remote locations on individual local walnut trees in the area propagated from seeds might indicate its endemic occurrence for a long time.

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