IDENTIFICATION IN SAUDI ARABIA OF *Pseudomonas corrugata*, THE TOMATO PITH NECROSIS PATHOGEN, AND ASSESSMENT OF CULTIVAR RESISTANCE AND SEED TREATMENT

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SUMMARY

Tomato pith necrosis disease was observed on tomato plants grown in the AL-Kharj region of Saudi Arabia. Symptoms were yellowing and wilting of lower leaves, brown areas on stems and yellowish-brown discoloration of the pith. Three bacterial strains were isolated from the stems of tomato cv. Red Gold. These strains were identified as *Pseudomonas corrugata* based on morphological, physiological, biochemical, and pathogenicity tests, as well as Biolog analysis. Seven commercial tomato cultivars were evaluated for resistance to *P. corrugata*. Of these cultivars, Alambra was the only cultivar tested that was considered resistant, Antinea was partially resistant, Agora, Farah and JV15 were susceptible whereas Newton and Red Gold were highly susceptible. Chemical seed treatments were evaluated for efficacy of disinfestation of tomato seed that had been inoculated with *P. corrugata*. Bacteria were not detected when seeds were treated with 5% hydrogen peroxide for 5 or 15 min. Treatment of seed with 0.52% sodium hypochlorite for 5 and 15 min was relatively ineffective. When sodium hypochlorite was used at a 1% concentration for 15 min, the level of bacterial infestation was reduced by 92%. Hydrogen peroxide treatments at a 5% concentration reduced seed germination up to 11.4% compared with controls. However, no significant differences in seed germination were observed between control treatments (inoculated and non-inoculated seeds and non-treated seed) and any of the other chemical seed treatments when seeds were sown in sterilized soil in the greenhouse. Effective management strategies for pith necrosis caused by *P. corrugata* should include planting of a resistant cultivar, where feasible, and seed treatment with hydrogen peroxide (5% for 15 min) or sodium hypochlorite (1% for 15 min).

Key words: pith necrosis bacteria, seed treatment, *Pseudomonas corrugata*, cultivar resistance, hydrogen peroxide, sodium hypochlorite.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is grown as a commercial crop in Saudi Arabia 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).

Tomato pith necrosis (TPN) caused by *Pseudomonas fluorescens* (biotype I) and *P. corrugata* has been reported from Saudi Arabia (Molan and Ibrahim, 2007). In addition to *P. corrugata* (Scarlett et al., 1978; Molan and Ibrahim, 2007), TPN of glasshouse tomatoes is known to be elicited by a number of other bacterial pathogens, such as *P. cicolorii* (Wilkie and Dye, 1974), *P. viridiflava* (Malathrakis and Goumas, 1987; Goumas and Chatzaki, 1998; Alippi et al., 2003; Aysan et al., 2004), *Pectobacterium carotovorum* subsp. carotovorum = *Erwinia carotovora* subsp. carotovora (Speights et al., 1967; Dhanvanthari and Dirks, 1987; Malathrakis and Goumas, 1987), *Pectobacterium atrosepticum* = *Erwinia carotovora* subsp. atroseptica (Malathrakis and Goumas, 1987), and *Pseudomonas fluorescens* (Malathrakis and Goumas, 1987; Saygili et al., 2004; Molan and Ibrahim, 2007).

*P. corrugata* is the most frequently reported causal agent of TPN world-wide (Scarlett et al., 1978; Bradbury, 1986). This pathogen is considered to be ubiquitous, has a broad host range and causes pith necrosis mainly in tomato, but also in pepper (Lopez et al., 1994) and chrysanthemum (Fiori, 1992), with the same symptomatology. The bacterium is a seed-borne pathogen and may be introduced with infested seeds (Cirvilleri et al., 2008). These authors reported that all mature tomato fruits of greenhouse-grown tomato plants that were sprayed with bioluminescent *P. corrugata* strain PVCT 4.3t lux 18 on flowers and immature fruits contained pathogen on the surface (100% in enriched samples). *P. corrugata* was recovered from the surface and from the pulp of these tomatoes after 20 days of fruit storage at 4°C. The pathogen was also recovered from the seeds of tomato fruits produced from inoculated flowers. Results suggest that *P. corrugata* can be internalized and transmitted from flowers to fruits and seeds, thus surviving in the tissues and seeds during fruit development and ripening. *P. corrugata* can cause severe losses (50% loss was es-
Identification of bacterial pith necrosis

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estimated in Lithuania (Vasinauskiene, 2002) especially in non-heated glasshouses, where a large difference in the between night and day temperature and the high levels of humidity favour the development of the disease (Scarlett et al., 1978). Little is known, however, about transmission, epidemiology and sources of inoculum. The disease can be prevented and even cured by avoiding high humidity, excessive N-fertilisation and use of balanced appropriate K and Ca fertilisation (Scarlett et al., 1978). Chemical seed treatments for the control of seed-borne pathogens have been highly successful (Humaydan et al., 1980; Leben, 1983; Lockhart et al., 1976). Common seed treatments such as those using chlorine salts, antibiotics, or hot water, are efficient in the control of several bacterial diseases (Maude, 1996). One advantage of seed treatment is that it will reduce the amount of pesticides and their environmental impact is usually quite low (Mathre et al., 1995).

The objectives of this study were: (i) identify and characterize bacteria from tomato plants exhibiting pith necrosis symptoms in Saudi-Arabia, extending earlier investigations (Molan and Ibrahim, 2007); (ii) evaluate commercial tomato cultivars grown in Saudi Arabia for resistance; (iii) evaluate surface disinfection as a means for eliminating the inoculum from seeds.

MATERIALS AND METHODS

Isolation of the pathogen from infected plant samples and pathogenicity tests. Tomato plants with typical TPN symptoms (Fig. 1) were collected from eight plastic-covered greenhouses in the AL-Kharj region (Saudi Arabia). A small piece of infected pith from the stems of tomato plants cv. Red Gold (Fig. 2) was placed in a sterile mortar and macerated in sterile distilled water with a pestle. Loopfuls of the suspension were streaked on plates of nutrient agar containing 5% D+ glucose (NGA). Plates were incubated at 28°C for 48 h. Bacterial colonies were picked from the plates and transferred to new NGA plates for purification and further tests.

Test plants. Tomato cv. Red Gold seeds were sown in a commercial potting mix in 20-cm³ plastic pots (2 kg soil), filled with a sterilized mixture of soil and sand (4:1 w/w). After emergence, each pot received 5 g of a slow-release fertilizer. Additional amounts of fertilizer were added as needed and plants were watered as required. Greenhouse experiments were carried out in an environmentally controlled unit with temperature of 26-28°C, 50-70% relative humidity (RH), and a 12 h photoperiod.

Inoculum preparation and inoculation. Twelve bacterial isolates from tomato were tested for pathogenicity by inoculation of four-week-old seedlings of cv. Red
Gold. Bacteria were grown for 72 h on NGA. Plates were flooded with sterile, phosphate-buffered saline (PBS, 3.0 g KH₂PO₄, 7.0 g Na₂HPO₄·7H₂O, 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 10⁶ CFU/ml. For each isolate, four seedlings were inoculated by injecting 50 µl of a bacterial suspension into the axils of the first true leaves. Plants inoculated with sterile water served as negative control. After inoculation, plants were covered with polyethylene bags for 24 h at 25°C, to be examined after 45 days by cutting them vertically after symptom expression. Bacteria were re-isolated from infected areas and strains were selected based on disease severity values as determined using the scale of Aysan et al. (2004): 0 = no symptoms; 1= 0.1-2.0 cm pith necrosis (PN); 2 = 2.1-4.0 cm PN; 3 = ≥ 4.1 cm PN. Bacterial strains were divided into four different virulence groups: non-virulent (no symptoms), mild virulent (scale value 1), virulent (scale value 2) and highly virulent (scale value 3).

**Identification of the bacterial isolates.** Physiological and biochemical tests were performed on the isolated bacteria using the methods described by Dye (1968, 1969), Lelliot and Stead (1987) and Klement et al. (1990). Further identification was done using the Biolog GN/GP Micro Plate systems (Biolog, USA). 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), 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.

**Evaluation of commercial tomato cultivars for resistance to pith necrosis.** This experiment was conducted in a greenhouse at 25 to 28°C with 50-70% RH, and a photoperiod of 12 h. Seven commercial tomato cultivars (Table 1) were evaluated for resistance to *P. corrugata* based on disease severity. The experiment was conducted twice as a completely randomized design with five replications of a single plant per experiment. Seeds of different commercial tomato cultivars were surface-sterilized by soaking in 70% ethanol for 3 min, washed thoroughly with sterilized distilled water and planted in sterilized mixture of soil and sand. Three bacterial isolates were used in this study. For each isolate (T1, T2 and T3) four-week-old tomato seedlings were inoculated by stem injection and disease severity was evaluated using Aysan et al. (2004) scale as mentioned above.

**Effect of seed treatment.** Preliminary tests were conducted to measure the amount of fluid absorbed by seeds. Seed suspended in 10 ml of sterile PBS, pH 7.2 for 1 h were collected, blotted to remove excess moisture, and allowed to dry on paper towels in a laminar flow cabinet for 4 h. Individual seed gained approximately 40% in weight, indicating fluid uptake into the seed, in an amount comparable to the infiltration method reported by Leben and Sleeman (1981). For inoculation of tomato seeds, bacterial isolate T3 was grown on NGA plates for 72 h at 28°C. Bacterial suspension of the tested isolate was prepared as described before. Seed aliquots (7 g) were placed in 150-ml beakers to which 75 ml of bacterial suspension were added, with shaking on an orbital platform shaker at 175 rpm for 1 h. The contents of the beakers were filtered through sterile cheesecloth and the seeds were

**Table 1. Evaluation of commercial tomato cultivars for resistance to pith necrosis bacteria.**

<table>
<thead>
<tr>
<th>Tomato cultivar</th>
<th>Average disease severity value</th>
<th>Type of reaction*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isolates</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td>Agora</td>
<td>2.7</td>
<td>2.8</td>
</tr>
<tr>
<td>Alambra</td>
<td>1.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Antinea</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Farah</td>
<td>2.6</td>
<td>2.8</td>
</tr>
<tr>
<td>JV 15</td>
<td>2.8</td>
<td>2.6</td>
</tr>
<tr>
<td>Newton</td>
<td>3.2</td>
<td>3.1</td>
</tr>
<tr>
<td>Red Gold</td>
<td>3.7</td>
<td>3.6</td>
</tr>
</tbody>
</table>

LSD (cultivar) 0.325
LSD (isolates) 0.202
LSD (interaction) 0.422

*HS= highly susceptible, S= susceptible, PR= partially resistant, R= resistant. Standard analysis of variance (ANOVA) was carried out by using the SPSS Statistical computer software program (Version 10.0). Significance was determined according to Duncan’s Multiple Range Test (P<0.05).
dried for 24 h on sterile filter paper in plastic Petri dishes in a laminar flow cabinet. Three lots of 100 seeds each were made, and each lot was placed in 10 ml of fluid in a 50-ml beaker for seed treatment. The chemicals tested were hydrogen peroxide at 1 and 5% (H) and sodium hypochlorite (So) commercial bleach at 0.52 and 1.04% concentration. Seeds were exposed to each concentration of the chemicals for 5 and 15 min. These concentrations were most effective in eliminating P. corrugata in preliminary tests where concentrations ranging from 0.5% to 3% sodium hypochlorite and 1 to 5% hydrogen peroxide were used (data not shown).

For all chemical treatments, excess diluents were drained off and seeds were placed on sterile filter paper to dry. Two control treatments were included in these experiments. Inoculated seeds treated with sterile water (In) instead of a chemical, served as a positive control. Non-inoculated seeds (None) immersed in sterile PBS instead of inoculum and treated with sterile water, served as a negative control. Seeds from all treatments were placed on NGA plates and incubated at 28°C for 3-5 days. Individual seed from which typical bacterial colonies grew were recorded as infested, and the percent of infested seed was calculated.

Three additional sets of 100 seeds were used to test the effect of each treatment on seed germination. After seed lots were treated as described above, they were dried on filter paper for approximately 2 h. Each lot was placed on a disk of sterile paper moistened with sterile distilled water, and placed on the laboratory bench (ca. 22°C). Water was added as needed to keep the paper disks moist. After 5 days, the germination percent was determined. The experiment was repeated twice.

All treatments were tested at exposure times of 5 and 15 min. For contamination studies, three lots of 100 seeds each were inoculated with a strain of P. corrugata and plated on NGA. Three additional lots of 100 seeds were placed on moistened sterile paper and germination recorded after 7 days.

RESULTS AND DISCUSSION

Isolation of the pathogen from infected plants and pathogenicity tests. TPN symptoms appeared from April to May in spring crops, consisting of yellowing and wilting of lower leaves, which progressed upwards, accompanied by brown areas on the stems and internal browning of pith tissue. Vascular discoloration also occurred, but plants generally did not wilt or collapse. Fruits did not show any symptoms. Three out of 12 bacterial isolates (T1, T2 and T3) were selected because they were highly virulent according to disease severity values (scale value = 3). No symptoms were observed on control plants inoculated with water.

Physiological, morphological and biochemical characteristics of bacterial strains. Based on morphological and physiological characteristics, all isolates were identified as P. corrugata (Buonaurio et al., 1993; Alippi et al., 2003; Quezado-Duval et al., 2007). The three isolates used in this study were Gram-negative, rod shaped and motile. All were oxidase positive and levan negative, arginine-dihydrolase positive and did not macerated potato discs. None induced a hypersensitive response on tobacco leaves. Additional tests showed that the isolates were non-fluorescent and utilised D-galactose, D-glucose, inositol, mannitol, and sucrose. All grew at 37°C and 4°C but not at 40°C. They liquefied gelatine and grew on tri-phenyl tetrazolium chloride (TTC) but did not utilize cellobiose, and sorbitol. The identity of the bacterial species was further confirmed by Biolog analysis (carbon source utilization at 37°C) with a similarity index of 0.75.

Evaluation of commercial tomato cultivars for resistance to pith necrosis. To find possible sources of resistance to P. corrugata, seven commercial tomato cultivars grown in Saudi Arabia were screened for resistance to pith necrosis. Four disease susceptibility groupings (highly susceptible, susceptible, partially resistant, and resistant) were devised based on disease severity ratings (Table 4). The highly susceptible (HS) cvs Newton and Red Gold had disease severity values (DSVs, 3.1 to 3.9) that were significantly higher than those of the other cultivars tested. In the susceptible (S) cvs Agora, Farah and JV15, the DSVs 2.6 to 2.9 were significantly lower than the DSV of the highly susceptible cultivars. The third category, partially resistant (PR), the DSVs of 1.8 was significantly lower than the DSVs of the highly susceptible cultivars. Alambra was the only cultivar tested considered resistant (R) to P. corrugata. No significant differences in virulence were observed between the three tested isolates. Only cv. Alambra, was resistant to pith necrosis whereas cv. Antinea was partially resistant and cvs Agora, Farah and JV15, were susceptible.

Molan and Ibrahim (2007), reported severe disease on cv. Red Gold, the most frequently cultivated tomato cultivar in greenhouses in Saudi Arabia.

Effect of seed treatment. Several promising seed treatments for controlling P. corrugata were identified (F value for seed chemical treatments were significant at P ≤ 0.0001). Treatment of tomato seed with hydrogen peroxide provided the best control (Fig. 3) for P. corrugata was not recovered from seed lots treated with 5% H for either 5 or 15 min. However, with treatments of 1% H for 5 and 15 min no significant differences in incidence of contaminated seeds were observed. Fifty-four percent of the seeds treated with 0.52% So for 15 min were still infested compared to 8% when treated with 1.04%. No significant difference was observed in incidence
of contaminated seed with a treatment 1.04% So for 5
and 15 min. Few seed treatments adversely affected
seed germination (Fig. 4). Germination rates were 87.6
to 100% for the seed treatments tested, as compared
with 100% of controls. Four of the five highest germi-
nation rates were observed for seeds treated with 5% H
for 5 min the highest rate being 99%. Germination of
seed treated with at 5% H for 15 min was significantly
higher (87.6%) than that of seeds treated with sodium
hypochlorite.

Kritzman (1993) reported that most of the tomato
seeds produced or imported by Israel are successfully
treated by solutions of cupric acetate, acetic acid, pen-
tachloronitrobenzene, 5-ethoxy-3(trichloromethyl)-1,2,4-
thiadiazole, and Triton x-100, for 1 h at 45±0.1
°C against _P. syringae pv. tomato_, _P. corrugata_, _Xanthomonas
campestris pv. vesicatoria_ and _Clavibacter michiganense_
subsp. _michiganense_. The same author also found that _P.
corrugata_ was controlled only after 1 h of treatment at
45°C. Sodium hypochlorite (at 1% active ingredient),
0.6 M HCl, 8 hydroxyquinoline (at 0.5-1%), copper ac-
etate (at 0.2%), streptomycin (at 50 and 100 ppm) and
bronopol (2-bromo-2-nitropropane-1,3-diol) and hot
water treatments (at 50 and 55°C) effectively eliminated
the related bacterium _P. viridiflava_ from tomato seeds.

These treatments slightly impaired the germination rate
of the seeds (range of 76% to 96%) as compared to
96% for untreated seeds (Yildiz _et al._, 2002).

We have now determined that treatment of tomato
seeds with a solution of 5% aqueous H effectively eradi-
cates _P. corrugata_ from artificially infested tomato seeds
causing only a relatively small reduction in (11.4%) in
the germination rate. The bactericidal action of oxygen
released from peroxides is well known (Black, 1999).
Dilute aqueous preparations of hydrogen peroxide have
been used for many years as a topical disinfectant. Ef-
fec tive management strategies for pith necrosis caused
by _P. corrugata_ should include planting of a resistant
cultivar, where feasible, and seed treatment with hydro-
gen peroxide (5% for 15 min) or sodium hypochlorite
(1% for 15 min).

To our knowledge, this is the first study that evaluat-
ed commercial tomato cultivars for resistance to pith
necrosis induced by _P. corrugata_.

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