Isolation and Characterization of Pseudomonas Species Associated with Tomato Wilt

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ABSTRACT

Bacterial wilt is an extremely serious plant disease of tomato and other crops. Infected plants wilt rapidly and often die. The disease is caused by a soil bacterium Pseudomonas and infects plants through the roots. The causal agent of tomato wilt (Pseudomonas species) was identified from the collected eleven varieties of tomato plant. A total of eleven local types of tomato were sown in different baked clay pot in a nursery and then transplanted in different lines. Diseased tomato samples were collected by simple and random collection techniques. The growth of pathogen was carried out on different specified culture media viz., triphenyl tetrazolium chloride (TTC) and yeast peptone glucose agar. The bacterial isolate was characterized by morphological, biochemical tests and its phytopathogenicity was verified by simple Rapid Streaming test.

Key words: Characterization, bacteria, tomato, disease diagnose

INTRODUCTION

In Pakistan, plant diseases caused by phytopathogenic bacteria are a major problem that has an impact on valuable agricultural crops causing decrease in yield potential annually. Tomato plant (Lycopersicon esculentum L.) is a vegetable well known for its high nutritional value rich in vitamins A and C and by its favorable influence on kidney function and digestive tract (Hernandez et al., 2008; Olaniyi et al., 2010). Tomato is one of the most important greenhouse and field grown vegetables in Pakistan. Usually, tomato plants are severely affected by pathogenic bacteria that cause total defoliation or diseases in tomato besides it also affect hardly/strongly on photosynthesis and production potential of plant (Perez et al., 1995; Bashan & De Bashan, 2002). One of these diseases is bacterial wilt, the most complex and deadly soil borne vascular bacterial disease problem found in tomato growing plants. Not only decreases yield of plants through foliar necrosis, but it also blemishes the fruits and renders them unsuitable for the fresh market (Bashan & De Bashan, 2002). This disease caused by the pathogen, Pseudomonas species; it is a highly heterogenous bacterial pathogen that causes wilting of many important plants (Rico & Preston, 2008). Bacterial diseases were mostly disseminated by water, therefore bacteria has the potential to move through plug greenhouses very quickly; thus, infested seedlings could become an important inoculums source for field epiphytotics (Schneider & Grogan, 1977). Consequently, bacteria can survive up to 20 years in the crevices and cavities of the coat of tomato seeds causing bacterial wilt, a disease characterized by rapid plant death without yellowing or spotting of the foliage (Bashan et al., 1991). Moreover, bacterial wilt infected plant become flaccid by invading and gradually blocking the vascular tissue (Cuppels & Elmhirst, 1999).

The main objectives of this study were to: (1) identify isolated bacterial species on the basis of various morphological features and (2) identify isolated bacterial species on the basis different biochemical tests.

MATERIALS AND METHODS

Collection of Tomato Seeds

Seeds of 11 Local varieties of tomato were collected from Punjab Seed Corporation Lahore (PSCL) and Ayub Agricultural Research Institute (AARI) Faisalabad (Table I).
Table I: List of Tomato varieties and their parts used for the study.

<table>
<thead>
<tr>
<th>Sowing Date</th>
<th>Seed variety</th>
<th>Parts used</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd – November - 2012</td>
<td>Rio-grande</td>
<td>The entire plant (leaf+stems+root)</td>
<td>AARI, Faisalabad</td>
</tr>
<tr>
<td></td>
<td>New-Yarker</td>
<td>The aerial parts (stem+leaf)</td>
<td>AARI, Faisalabad</td>
</tr>
<tr>
<td></td>
<td>Waya-Head</td>
<td>Leaves</td>
<td>AARI, Faisalabad</td>
</tr>
<tr>
<td></td>
<td>Siberian</td>
<td>The aerial parts (stem+leaf)</td>
<td>AARI, Faisalabad</td>
</tr>
<tr>
<td></td>
<td>Gruschowka</td>
<td>The aerial parts (stem+leaf)</td>
<td>AARI, Faisalabad</td>
</tr>
<tr>
<td></td>
<td>Pakit</td>
<td>The aerial parts (stem+leaf)</td>
<td>AARI, Faisalabad</td>
</tr>
<tr>
<td></td>
<td>Oregonspring</td>
<td>Leaves</td>
<td>AARI, Faisalabad</td>
</tr>
<tr>
<td></td>
<td>Naqeeb</td>
<td>The aerial parts (stem+leaf)</td>
<td>AARI, Faisalabad</td>
</tr>
<tr>
<td></td>
<td>Roma</td>
<td>The aerial parts (stem+leaf)</td>
<td>AARI, Faisalabad</td>
</tr>
<tr>
<td></td>
<td>Nagina</td>
<td>The aerial parts (stem+leaf)</td>
<td>AARI, Faisalabad</td>
</tr>
<tr>
<td></td>
<td>Super-special</td>
<td>Leaves</td>
<td>PSCL, Lahore</td>
</tr>
</tbody>
</table>

Sowing Nursery and Transplantation

Seeds of eleven local varieties of tomato were sowed in separate earthen pots to be grown up to nursery and then transplanted in separate rows. Transplantation of tomato plants was made 20 days after germination, in variety wise in separate rows of ridges, taken from nursery earthen pots, and tags containing their variety name sowing date etc, were fixed for each variety, under known condition of soil and water in IAGS fields in November-2012, on one side of their respective rows. Symptoms of bacterial wilt were monitored daily. No symptoms were found in any variety till the month of April.

Tomato Plants for Disease Diagnosis

Total eleven varieties of tomato plant were planted in experimental area of Institute of Agricultural Sciences, University of the Punjab Lahore. Simple random sampling technique was adopted for collection of wilted samples. On observance of symptoms in the field, wilted samples were collected in polythene bags and transported to laboratory for diagnosis the disease. The method of pathogen identification in wilted samples was performed. The presence of bacterial pathogen in stems of wilted tomato plants was indicated by the simple Rapid Streaming test. Stem was cut just above the soil level. The cut surface was suspended in a tube of clean water. Characteristic spontaneous streaming of threads of bacterial slime coming from the cut vascular bundles after few minutes was observed.

Isolation of Bacterial Pathogen from Collected Samples

The tomato plant tissues were collected at the flowering stage. Five infected plants from each variety were carefully removed, washed under tap water to remove soil. Stems and roots were cut into sections of 2-3 cm long. Infected sections were rinsed with distilled water and drained in large beaker. Afterward it was soaked in 70% ethanol for 30 seconds subsequently sterilized with 0.1% HgCl₂ for 5 minutes for stems and 3 minutes for roots and nodules. Tissues were washed ten times with sterile water (Pham & Annapurna, 2004). Later on homogenize mixture was prepared by maceration of surface-disinfected tissues aseptically. Moreover, serial dilution was made by diluting macerated tissue in 9 volumes of sterile distilled water for 10⁻¹ dilution followed by 1 ml of well-shaken suspension and adding into 9 ml water blank tubes for 10⁻⁶ dilution. After that, inoculation was done on plate by spreading 100 µl of each dilution on two different specified media, viz. triphenyl tetrazolium chloride (TTC) and yeast peptone glucose agar (YPGA) and incubated for 48 hours at 28±30°C.
Morphological and Physiological Characterization of Pathogen

Bacterial identification was carried out by observing the morphological and physiological characters viz., colony colour, colony morphology, cell shape, cell colour, motility and growth pattern. Bacterial colonies were purified followed by subjected to Gram staining and characterized using biochemical tests (catalase, oxidase, utilization of citrate, nitrate reduction, mannitol, indole and arginine tests) and consulting the pertinent literature (Benson, 1996; Holt et al., 1997; Kvitko, 2009).

Koch Postulates

For the pathogenicity tests, leaves of eleven varieties of tomato plants were inoculated by injection with a bacterial suspension (108 colony forming units (CFU mL⁻¹)). After inoculation, detached leaves were incubated on Petri plates containing 20 mL of TTC and YPGA media with rifampicin (50 μg ml⁻¹) at 28 °C. Disease development on tomato leaves was evaluated 7 days after inoculation. Re-isolations and identification were made from the diseased plants. Sterile distilled water was used as a negative control.

Identification of Bacterial Species

According to the description of Bergey’s Manual of Systematic Bacteriology (Vos, 2009), the bacterial isolate appeared to be a Pseudomonas species. Thereby, a set of specific identification tests based on morphological and biochemical characters were carried out, as described by previous literature (Bouchra et al., 2013). Thereby, a set of specific identification tests were carried out, as described by Lelliott & Stead (1987). Table 3 summarizes the results obtained. Besides, bacterial cells were Gram negative, motile, mannitol negative and oxidatively metabolized glucose. Moreover, the results revealed that the bacterial isolates showed growth on nutrient agar (N.A) medium (supplemented with 5%, w/v, glucose) and exhibit positive reaction in the presence of 3% Hydrogen peroxide (H₂O₂) solution. However, oxidase and arginine activity were absent in all bacterial isolates. All these identifying features show that the isolates correspond to Pseudomonas species. Similar strategy and microbiological tests were used by Milijasevic et al. (2009) for the identification of Pseudomonas isolates from Eastern Europe.

RESULTS AND DISCUSSION

Plant Disease Symptoms and Isolation of Phytopathogenic Bacterium

Bacterial wilt is one of the most common bacterial diseases of growing tomato plants. All plant samples used in the present study presented the symptoms of Bacterial wilt on leaves and stem, appearing as small black lesions surrounded by a yellow halo. After thorough washing, this material was used to isolate the pathogenic bacterial species. The resulting bacterial suspension was spread on TTC and YPGA media plates. After 24 h incubation at 28°C, circular bacterial colonies were observed on the media surface. Both microbiological features and plant disease symptoms are identical to those described in other studies on strain pathogenicity (Cuppels & Elmhirst, 1999; Preston, 2001; Bashan & De-Bashan, 2002).
Pathogenicity Tests

The pathogenicity of each field isolate should normally be checked before assigning it to a pathovar (Bultreys & Kaluzna, 2010). Disease development on tomato leaves was evaluated 7 days after inoculation ($10^8$ CFU mL$^{-1}$) with *Pseudomonas* sp. All infected plants showed the same first symptoms (moist spots), and after 5 days the spots became dark-brown with chlorotic halos, corresponding to the typical symptoms of bacterial wilt. No symptoms appeared on control leaves injected with distilled water. All re-isolated bacteria were identified as *Pseudomonas* sp. and eventually stored at -20°C. Similar results of pathogenicity tests on tomato leaves, pepper leaves, and immature tomato and pepper fruits were reported by Bouchra et al. (2013). Likewise, data by Milijasevic et al. (2009) indicate similar effects of other pathogenic bacteria on plant infected tissues after 4 days.

Table II: Morphological and biochemical features of the bacterial isolate.

<table>
<thead>
<tr>
<th>Identification Features</th>
<th>Morphological characters</th>
<th>Result</th>
<th>Biochemical tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell color</td>
<td>Pink</td>
<td></td>
<td>Mannitol activity</td>
<td>-ve</td>
</tr>
<tr>
<td>Cell shape</td>
<td>rod</td>
<td></td>
<td>Oxidase activity</td>
<td>-ve</td>
</tr>
<tr>
<td>Gram type</td>
<td>-ve</td>
<td></td>
<td>Catalase activity</td>
<td>+ve</td>
</tr>
<tr>
<td>Motility</td>
<td>+ve</td>
<td></td>
<td>Arginine activity</td>
<td>-ve</td>
</tr>
<tr>
<td>Growth on N.A + 5% glucose</td>
<td>+ve</td>
<td></td>
<td>Indole activity</td>
<td>-ve</td>
</tr>
<tr>
<td>Capsule</td>
<td>+ve</td>
<td></td>
<td>Citrate utilization activity</td>
<td>-ve</td>
</tr>
<tr>
<td>Grow at 28°C</td>
<td>+ve</td>
<td></td>
<td>Nitrate reduction activity</td>
<td>-ve</td>
</tr>
</tbody>
</table>

The present study revealed the widespread nature and high incidence of bacterial wilt in tested varieties of tomato plants. The bacterial wilt pathogen, *Pseudomonas* species was commonly associated to diseased plants and seeds as well as to soil samples taken in the affected fields. The high incidence of bacterial wilt indicates that bacterial wilt is a recurrent problem in Pakistan and that all popular varieties appeared to be susceptible to the disease (Burney et al., 1999). The incidence of the disease could in fact be higher as no attempts were made to isolate the pathogen from plants without symptoms. Assessment of the presence of pathogen in plants merely by scoring of disease symptoms and CFUs often gives only a superficial picture of the actual invasive properties of this organism. Latency of infection has been reported in tomato (Graham & Lloyd, 1978). The consequence of symptomless invasion of tomato plants by *Pseudomonas* is, without doubt, an important means for the survival of the pathogen resulting in the potential for infestation of soil and other plants. Bacterial wilt was easily distinguished from fungal wilt based on the brown discoloration of the vascular tissues, and in the profuse bacterial ooze observed when cut sections of the stems were placed in clear water. The morphological characteristics of the bacteria on TTC agar medium, physiological, biochemical characterization results and pathogenicity test results confirmed the identity of the pathogen as *Pseudomonas* species (Kelman 1954). In the present study, *Pseudomonas* was easily detected by the rapid streaming method. TTC agar medium proved to be useful also in the detection of pathogen from tomato plants (Kelman 1954). *Pseudomonas* on or in the seed may provide to be a potentially dangerous source of inoculum and play a role in the perpetuation of bacterial wilt in tomato crop production of small farm holders in Pakistan.

Conclusion

In this study we presented a basic method for detection and identification of the phytopathogenic bacterium *Pseudomonas* species isolated from tomato plant with their pathogenicity test. The diversity and characterization of different bacterial species that isolated from diverse tomato germplasm lines tells us about the association
between them with specific relation to the environment and crop genetics. This study may also equally help for the scientist and farmers community for the classification (on the basis of resistant, tolerant and susceptibility of tomato genotypes against various pathogens and also provides the information for further investigation in to new insight in scientific field.

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REFERENCES


