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Isolation, identification and pathogenicity of soil borne pathogens from tomato rhizosphere (*Solanum lycopersicum* L.)

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Abstract
The diseases that are caused by pathogens which persist (survive) in the soil matrix and in residues on the soil surface are defined as "soil-borne diseases". The soil is a reservoir of inoculum of these pathogens, the majority of which are widely distributed in agricultural soil. However, some species show localized distribution patterns. Most often damage to root and crown tissues of the plant is hidden in the soil. Thus these diseases may not be noticed until the above-ground parts of the plant are affected severely showing symptoms such as stunting, wilting, chlorosis, and death. The tomato plant is attacked by various diseases that significantly affect its growth and yield. Out of which the soil-borne diseases like fusarium wilt, bacterial wilt, stem rot, or white Mold are the most serious diseases affecting its yield. The results of the present study revealed that the three soil-borne pathogens were isolated from previously grown tomato fields and these were identified as *Fusarium oxysporium f. sp. lycopersici, Ralstonia solanacearum,* and *Sclerotium rolfsii*. The pathogenicity of the isolated pathogen was tested against the host plant and it observed that organisms were pathogenic to tomato plants.

Keywords: Soil borne, symptoms, fusarium wilt, bacterial wilt, stem rot, pathogenicity

Introduction
Tomato (*Solanum lycopersicum* L.) is one of the popular vegetables of great commercial value and is used in various forms. Among the main fruits and vegetables, tomato ranks 16th as source of vitamins. Ripe tomato fruits can be consumed either directly as a raw vegetable in salad, sand-witches, drinks or cooked foods and also utilized in the preparation of range of processed products such as powder, ketchup, soup sauce, pickle, puree and canned fruits and in many other ways. The crop is generally grown during winter months (October-April) in plains of India and in summer (April-October) in hilly regions of the country. Tomato is an important vegetable crop grown all over the world for its fleshy fruits due to its varied climatic tolerance and high nutritive value.

"The diseases that are caused by pathogens which persist (survive) in the soil matrix and in residues on the soil surface are defined as soil borne diseases". Thus the soil is a reservoir of inoculum of these pathogens, the majority of which are widely distributed in agricultural soil. However, some species show localised distribution patterns. Most often damage to root and crown tissues of the plant is hidden in the soil. Thus these diseases may not be noticed until the above-ground (foliar) parts of the plant are affected severely showing symptoms such as stunting, wilting, chlorosis and death. These diseases are often very difficult to diagnose accurately and the pathogens may be difficult to grow in culture and identify accurately.

Tomato is prone to a number of diseases like damping-off (*Pythium aphanidermatum*), early blight (*Alternaria solani*), late blight (*Phytophthora infestans*), buckeye rot (*Phytophthora nicotianae* var *parasitica*), fusarium wilt (*Fusarium oxysporum* f.sp. *lycopersici*), septoria leaf spot (*Septoria lycopersici*), powdery mildew (*Leveillula taurica*), anthracnose (*Colletotrichum phomoides*), bacterial wilt (*Ralstonia solanacearum*), Sclerotium rot or white mould (*Sclerotium rolfsii*), bacterial leaf spot (*Xanthomonas campestris* pv. *vesicatoria*), bacterial canker (*Clavibacter michiganensis* pv. *michiganensis*), tomato mosaic virus (ToMV), tomato leaf curl virus (TLCV), tomato spotted wilt virus (TSWV), tomato bunchy top virus (TBTV) and tomato big bud (TBB). Prevalence of warm and humid weather during the growing season of the crop favours the development of various diseases. Tomato plant is attacked by various diseases as mentioned above that significantly affect its growth and yield. Out of which the soil borne diseases like fusarium wilt, bacterial wilt, stem rot or white mould are the most serious diseases affecting its yield. This diseases is caused by *Fusarium oxysporum* f. sp. *lycopersic*i (Sacc.), *Ralstonia solanacearum* and *Sclerotium rolfsii*; respectively and the yield loss due to this diseases is huge.

Fusarium wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* is one of the most wide spread and destructive diseases, causing infection and losses to crop growers. It is a devastating disease causing considerable economic losses ranging from 10-80% yield loss in tomato production (Kesawan and Chaudhary 1977) [8]. Bacterial wilt is one of the major diseases of tomato and other solanaceous plants. The disease is known to occur in the wet tropics, sub-tropics and some temperate regions of the world. The disease is caused by the bacterium *Ralstonia solanacearum*, previously known as *Pseudomonas solanacearum*. It is one of the most damaging plant pathogen that affect more than 200 plant species in over 50 families throughout the world, including a wide range of crop plants, ornamentals and weeds. The major restraint to tomato production in India is bacterial wilt caused by *R. solanacearum* (Yabuuchi *et al.* 1995) [16]. The yield loss may vary between 10.8 and 90.6 percent depending on the environmental conditions and the stage at which infection occurs (Kishun, 1987) [9]. Bacterial wilt poses a constant threat to tomato in Karnataka, Madhya Pradesh, Maharashtra and West Bengal in India. The pathogen infects susceptible plants in roots, usually through wounds (Pradhanang *et al.* 2005) [10] and colonizes within the xylem preventing the water movement into upper portion of the plant tissue (Kelman, 1953) [7]. *Sclerotium rolfsii*, fungus is a soil borne facultative parasite having very wide host range which causes pre-emergence rot, damping off, collar rot, stem rot of tomato. This disease is also called by other names like southern blight, southern root rot, *Sclerotium* rot or white mould. The pathogen is polyphagous and non-target fungus.

Material and Methods

Collection of infected plant and soil sample

Infected plant and soil sample were collected from various locations *viz;* fields of Department of Plant Pathology and Agricultural Microbiology and All India Co-ordinated Vegetable Improvement Project (AICVIP), PGI experimental filed, Mahatma Phule Krushi Vidyapeeth, Rahuri. Similarly, from each location rhizospheric soil samples were also collected from diseased and healthy tomato plant. The samples were brought to the eco-friendly laboratory of department of plant pathology and agricultural Microbiology, MPKV, Rahuri for further studies.

Isolation

Isolation of pathogens were carried out by usual tissue isolation method i.e. by placing infected stem pieces on poured plates of potato dextrose agar (PDA) medium and Tripheny Tetrazolium Chloride agar (TTC) medium. The infected part were first separated, the dirt and soil partials were removed. Then cut into small pieces about 2 to 5 mm in size. They were disinfected in 1:1000 aqueous solution of mercuric chloride ($HgCl_2$) for 1 minutes and then washed with sterile water three times to remove traces of disinfectant. Four dried pieces of stem were placed on each of previously sterilized Potato Dextrose Agar (PDA) and Tripheny Tetrazolium Chloride Agar (TTC) petriplates. The plates were then incubated at room temperature at 27 ± 2^0 C for 7 days. The fugal growth was transferred on PDA slants and the bacterial growth was transferred on Nutrient Agar (NA) slants for further studies.

Identification

There were three major soil borne pathogen identified during this experimental study *viz; Fusarium oxysporium f.sp. lycopersici* causing fungal wilt, *Ralstonia solanacearum* causing bacterial wilt and *Sclerotium rolfsii* causing stem rot or white mould or southern blight. The pathogenicity for above pathogen were proved on tomato crop and microscopic morphological study was carried out for identification.

Pathogenicity

The inoculum of fungal and bacterial pathogenic organism was multiplied on PDA and NA medium at room temperature for 7 days.

Field collected soil was mixed with farm yard manure (FYM) in 3:1 proportion and sterilized in autoclave at 121 ^{0}C for 30 minutes consequently for 2 days.

The inoculum *i.e.* mycelium, spores, sclerotia and bacterial suspension of isolated pathogenic organisms were mixed with sterilized soil and then filled in pots. The pot filled in with sterilized soil without inoculum of organism served as control.

Twenty five days old seedlings of tomato variety phule kesari grown in plastic tray were transplanted in each pot and watered. Observations were recorded on the appearance of symptoms in inoculated and uninoculated pots.

Symptoms observed on artificially inoculated plants were similar to symptoms observed as infected plants under field condition.

Re-isolation of the pathogen was made from artificially inoculated infected tomato plants, showing typical symptoms and morphology *in-vitro*. The isolated culture was transferred to respective suitable media slants for further use.

Results and Discussion

Isolation and Identification of Soil Borne Pathogens

Infected plant and soil sample were collected from various locations *viz;* fields of Department of Plant Pathology and Agricultural Microbiology, All India Coordinated Vegetable Improvement Project (AICVIP) and PGI experimental filed, Mahatma Phule Krushi Vidyapeeth, Rahuri. Similarly, from each location rhizospheric soil samples were also collected from diseased and healthy tomato plant. The samples were brought to the eco-friendly laboratory of Department of Plant Pathology and Agricultural Microbiology, MPKV, Rahuri for further studies.

Isolation

Isolation of pathogen was carried out by usual tissue isolation method *i.e* by placing infected stem pieces on poured plates of Potato Dextrose Agar (PDA) medium and Tripheny Tetrazolium Chloride Agar (TTC) medium. The infected part were first separated, the dirt and soil partials were removed. Then cut into small pieces about 2 to 5 mm in size. They were disinfected in 1:1000 aqueous solution of mercuric chloride ($HgCl_2$) for 1 minute and then washed with sterile water three times to remove traces of disinfectant. Four dried pieces of stem were placed on each of previously sterilized PDA and TTC petriplates. The plates were then incubated at room temperature at 27 ± 2^0 C for 7 days. The fugal growth was transferred on PDA slants and the bacterial growth was transferred on Nutrient Agar (NA) slants for further studies.

Identification

There were three major soil borne pathogen identified during this experimental study *viz; Fusarium oxysporum f. sp. lycopersici* causing fusarium wilt, *Ralstonia solanacearum* causing bacterial wilt and *Sclerotium rolfsii* causing stem rot or white mould or southern blight. The pathogenicity for

above pathogen were proved on tomato crop and microscopic, morphological study was carried out for identification (Plate: 1).

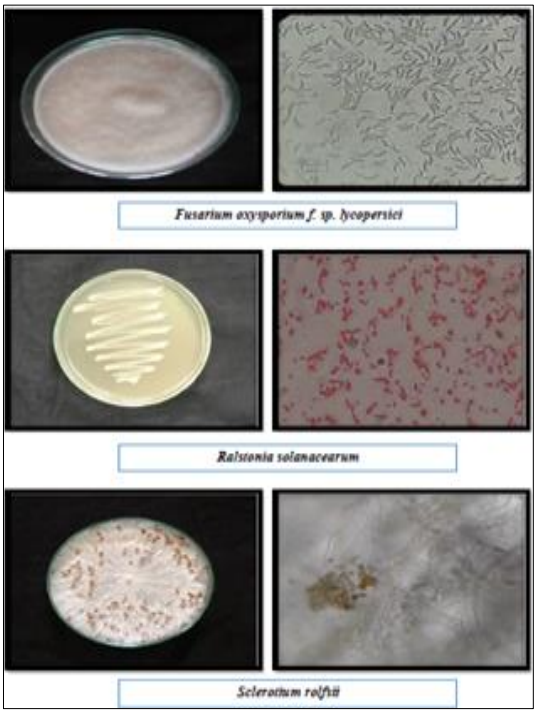

Plate 1: *In-vitro* growth and microscopic view of isolated soil borne pathogens

Pathogenicity

Symptomatology and pathogenicity for *Fusarium oxysporum f. sp. lycopersici*

Fusarium oxysporum f. sp. lycopersici was a soil borne fungal pathogen that attacks plants through roots at all stages of plant growth, cause major economic loss by inducing necrosis and wilting symptoms in plants. It was observed that *Fusarium* wilt of tomato first symptoms were yellowing of the foliage, beginning with the lower leaves and working upward. Yellowing often begins on one side of vein. Infected leaves later show downward curling, followed by browning and drying. The symptoms of *Fusarium* wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* revealed that, all leaves were completely wilted after 20 days from inoculation, showing stem drying from the apex to downward as the growing point died (Plate: 2). The pathogen was re-isolated from artificially inoculated tomato plants. The pathogen is primarily confirmed to the xylem vessels in which the mycelium branches produce micro-conidia. Based on morphological, microscopic structures such as macro and micro-conidia and after successfully proven pathogenicity, the pathogen was identified as *Fusarium oxysporum f. sp. lycopersici.*

The result findings were similar with the previous workers like Agrios (1998) [3] reported the symptoms produced due to *Fusarium oxysporum* as yellowing of the lower leaves, wilting of leaves and young stem, defoliation, marginal necrosis of remaining leaves and finally death of the entire plant. Ray (2005) [12] reported that in the *Fusarium* wilt of tomato first symptoms are yellowing of the foliage, beginning with the lower leaves and working upward. Infected leaves later show downward curling, followed by browning and drying. Singh *et al.* (2007) [15] observed that the pathogen is primarily conformed to the xylem vessels. Akrami and Yousefi (2015) [4] reported that *Fusarium oxysporum f. sp. lycopersici* is a soil borne fungal pathogen that attacks plants through roots at all stages of plant growth, cause major economic loss by inducing necrosis and wilting symptoms in plants.

Symptomatology and pathogenicity for *Ralstonia solanacearum*

The results of pathogenicity test revealed that the isolated *R. solanacearum* were able to cause wilt symptoms in tomato seedlings. It was observed that artificially inoculated plants showed typical symptoms, after 14 days from the day of transplanting of tomato seedlings in pathogen inoculated soil. The plants in the inoculated pots become infected and showed a typical bacterial wilt (*Ralstonia solanacearum*) symptoms characterized by wilting of upper leaves during the hottest part of the day, followed by recovery during the evening and early hours of morning and favourable conditions, complete wilt occurred. The vascular tissue in the lower stem of wilted plants showed a dark brown discoloration, a cross section of stem of the plant with bacterial wilt produced a white, milky strand of bacterial cell (oozing) in clear water, which indicates bacterial nature of the diseases. The pathogen was re-isolated from artificially inoculated tomato plants. The isolated bacterium grow on TZC medium produced pinkish red centred colonies, after incubation of 48 hr. at 28 ±2 ^{0}C. Based on morphological and biochemical characteristics, microscopic observation and gram staining, the pathogen was identified as *Ralstonia solanacearum* (Plate: 2).

The present study proves that *Ralstonia solanacearum* was pathogenic to tomato plant, the similar results were obtained by earlier research workers namely Ramesh (2008) demonstrated the symptomatology of bacterial wilt of brinjal. He found that initially leaf drooping, followed by wilting of entire plant within a few days. Recently wilted plants look green, a distinct symptom when compared to other vascular wilt disease which develops yellowing of the leaves. Vascular brown discoloration was found in the wilted plants. A stream of milky white bacterial ooze was be noticed when the cut ends of the stem or root were kept undisturbed for few minutes in a clear container with water. Singh *et al.* (2010) [14] proved pathogenicity test of *Ralstonia solanacearum* isolate by making slight injury to root zone and inoculating bacterial suspension (1× 10^9 cfu/ml) at root zone, using tomato cv. Pusa Ruby seedlings. They reported all the test isolates as pathogenic to tomato.

Chaudhary and Rashid (2011) [5] isolated the bacterium *Ralstonia solanacearum* from wilted tomato plant and reported that the bacterium on TZC medium produced pinkish red centred colonies, after incubation of 48 hr at 28 ±2 ^{0}C. Pathogenicity of *Ralstonia solanacearum* by root zone drenching and pin prick method on tomato seedlings and reported that the bacterium was pathogenic to tomato, which showed wilting at 14 days after inoculation. Agarwal *et al.* (2012) [1] documented the symptoms induced by *Ralstonia solanacearum* in tomato, such as brown to black vascular system, development of advantageous roots, initial drooping of leaves, yellowing of foliage, wilting of plants and finally collapse of entire plant.

Symptomatology and pathogenicity for *Sclerotium rolfsii*

It was observed that artificially inoculated plants showed typical symptoms, after one month from the day of transplanting of tomato seedlings in pathogen inoculated soil. The plants in the inoculated pots become infected and showed a typical basal stem rot symptoms such as the dense mycelial

growth that imparts a white washed appearance to colonized stem tissue. After some days the stem rot fungus got filled in stem tissues, utilized all available food and produced survival structure called sclerotia. Sclerotia were round and mustard seed in size and brown in colour. The symptoms produced were similar to as found under natural condition (Plate: 2). The pathogen was re-isolated from artificially inoculated tomato plants. Based on morphological characteristics, microscopic observation and formation of sclerotia, the pathogen was identified as *Sclerotium rolfsii*.

Similar findings were observed by previous researchers *viz;* Agarwal and Kotashane (1971) [2] reported the symptoms like a sudden yellowing, browning and wilting of the entire plant. Leaves of infected plants turn brown, dry and often cling to the dead stem. The most characteristics sign of the disease is white, fan like mat of fungal mycelium that forms on stem base, leaf debris and the soil surface around the infected plants. The mycelial mat may extend several centimeters up to the stem above the soil line. Numerous tan to brown, spherical sclerotia of about mustard seed size formed on infected plant material which was found on the soil surface. Jin and Chang (2002) [6] proved pathogenicity of *Sclerotium rolfsii*, causing stem rot in tomato plants under greenhouse conditions, by inoculating mycelial mat (form four days old culture on PDA) on the stem. The first symptom appeared 12 days after inoculation, caused severe stem rot, plant wilted and eventually died. The same fungal isolate obtained from artificially infected plant revealed similar symptoms and strong pathogenicity on tomato plants. Samsuzzaman *et al.* (2012) [13] reported the symptoms induced by *Sclerotium rolfsii* causing collar rot in tomato as, white mycelium growth on plant stem at collar region with dark brown sclerotial bodies, severe necrosis, followed by death of the plants, yellowing of leaves, brown to black lesions on roots and at collar region, which coalesced resulting in wilting and death of diseased tomato plant.

Plate 2: pathogenicity test of isolated soil borne pathogens against tomato plant

Summary and Conclusions

Isolation of pathogen was carried out by usual tissue isolation method *i.e.* by placing infected stem pieces on plates of potato dextrose agar (PDA) medium and Tripheny Tetrazolium Chloride agar (TTC) medium. Studies concluded that there were three major soil borne pathogen identified and pathogenicity of isolated pathogen were tested against the host plant. Studies revealed that organisms were pathogenic to tomato plants *viz; Fusarium oxysporum f. sp. lycopersici* causing fusarium wilt, *Ralstonia solanacearum* causing bacterial wilt and *Sclerotium rolfsii* causing stem rot or white mould or southern blight.

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