Leaf Surface Fungi of Early Blight [Alternaria solani (Ellis and Martin)] Infected and Non-Infected Leaves of Tomato [Solanum lycopersicum (L.)]

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ABSTRACT

The tomato leaves infected with early blight recorded the highest leaf surface fungal species than non-infected leaves. In total twenty six fungal species (thirteen genera) were isolated from both diseased infected and non-infected leaf surface of tomato from seven districts of Meghalaya, following leaf impression, leaf washing and dilution plating and leaf washing and serial dilution plating methods. Among all the three methods used leaf impression method recovered the highest fungal population followed by serial dilution plating and leaf washing and dilution plating methods. The predominant fungal species found both in infected and non-infected leaves were Fusarium sp., Phoma sp., Penicillium sp., Aspergillus sp. Trichoderma sp. and Chaetomium sp. Whereas, Camarosporum sp., F. pallidoroseum and P. glabrum were recovered only from healthy leaf samples. The further investigation can be done to find the antagonistic potential of leaf surface fungi against the major foliar diseases of tomato.

Keywords
Leaf impression method, Leaf surface fungi, Leaf washing and dilution plating method, Leaf washing and serial dilution plating and microbial population

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Introduction

Tomato (S. lycopersicum L.) is one of the most popular vegetable crops worldwide which share a great position in India as fresh vegetable. It belongs to the Solanaceae family which originated in the Andean region of South America. It is grown in a wide range of climate and the largest production centres are in southern and central part i.e., Andhra
Pradesh, Madhya Pradesh, Karnataka, Gujarat, Odissa, West Bengal, Telangana, Chhattisgarh, Maharashtra and Bihar (Horticulture Statistics Division, Department of Agriculture, Cooperation and Farmers’ Welfare, 2018). Area under tomato cultivation in the country is about 7.3 % of the total cropped land under vegetables and the area and production during 2017-2018 was 786 hectare (ha) and 19,377 Metric Ton (MT), respectively (Department of Agriculture Cooperation and Farmers Welfare, 2017). The agro-climatic condition of Meghalaya is also favourable for the cultivation of vegetables throughout the year. Among the vegetables, tomato is one of the most popular and widely grown vegetables crop in Meghalaya. In Meghalaya, it is cultivated throughout the year during rainy, winter and summer seasons. It occupies an area of 55.081 thousand ha with production of 35.51 MT during 2017-18 (Horticulture Statistics Division, Department of Agriculture, Cooperation and Farmers’ Welfare, 2018).

Leaf surface is a habitat for a variety of microflora including pathogens and saprophytes. Leaf surface fungi are the mycota which grow on the leaf surfaces (Langvad, 1980). These group of fungi are categorized into two groups i.e., casuals and residents (Norse, 1972). Casuals land on the surface of leaves but cannot grow whereas residents can multiply on the healthy leaf surface without noticeably affecting the host (Leben, 1965). Leaf surface fungi have not been fully studied and are still misused especially compared to rhizobacteria, root and seed endophytes and pathogenic microbes. Most of the work on leaf surface fungi was concerned with the pathogens or non-parasitic fungi of economically important trees (Dickinson, 1967; Pugh and Williams, 1968; Lamb and Brown, 1970; Pugh and Mulder, 1971; Bainbridge and Dickinson, 1972; Norse, 1972; Mishra and Dickinson, 1981; Cabral, 1985). This group of fungi were studied from mangroves (Kuthubutheen, 1981, 1984; Sivakumar and Kathiresan, 1990).

Due to the deposits of nutrients on the leaf surface is a favourable environment for millions of microbes. These microbial communities can be affected by internal and external agents like temperature, humidity, nutrient availability, leaf type and age and the presence of inhibitors (chemical compounds produced by the plant) (Andrews, 1991; Kinkel, 1997; De Jager et al., 2001; Santamaria and Bayman, 2005; Evueh and Ogbebor, 2008). These microbes can either be beneficial or harmful to the host plant. Beneficial microbes cannot cause any disease symptoms unlike phytopathogens (Malfanova et al., 2013). Therefore, leaf surface microflora is essential to study the environmental microbial diversity. It will also suggest the role of such community to the health and wellbeing of the plant as well as on members of the food chain that consume them. The three methods used in this investigation include leaf washing and dilution plating, leaf washing and serial dilution plating and leaf impression for culturing culturable fungi. The present investigation was carried out from one of the important vegetable crops i.e., tomato. Three methods are employed to study leaf surface fungi (Lindsey, 1976).

**Materials and Methods**

**Sample collection**

Both early blight diseases infected and non-infected leaf samples of tomato were collected from 24 locations from seven districts i.e., Ri-Bhoi, East Khasi Hills, West Jaintia Hills, East Jaintia Hills, North Garo Hills, West Garo Hills and South West Garo Hills of Meghalaya during the tomato
growing season from 2017-2018. Randomly twenty samples (twenty leaves per sample) from each field were collected in sterile poly-bags and taken back to laboratory for isolation of leaf surface fungi. Both the healthy and disease infected leaves were handpicked by holding the petiole only and placed into a separate fresh polyethylene bag, respectively. On bringing to the laboratory, department of plant pathology, SCP, CPGS, CAU, Meghalaya samples were immediately transferred to refrigerator (4±1°C) until further processing which normally was within 24 hours (h) of collection.

Isolation method

Three isolation methods were evaluated to characterize and identify the leaf surface fungi of tomato. The leaf surface fungi were isolated by leaf impressions (Dickinson et al., 1974) and modified leaf washing method (Dickinson, 1971).

Leaf Impression method of isolation

Randomly, ten diseased infected and ten non-infected leaves were collected from the various locations. Collected samples were then rinsed for 10-15 times with tap water followed by sterilized distilled water (SDW) under a laminar air flow hood to remove externally loosely attached dusts and microbes.

The leaf impression was made by pressing both the leaf surfaces (upper and lower, separately) against potato dextrose agar (PDA) (Hi-media Ltd., Mumbai) in petri dishes to produce the leaf-imprints.

After leaving for an hour, leaves were then discarded and the plates were incubated at 28±1°C till colonies grow on the agar surface. The sample from every location was repeated for three times.

Leaf washing method of isolation

A modified leaf washing method was adopted (Dickinson, 1971) to estimate the leaf surface fungi. To estimate the leaf surface fungi, discs of 0.5 centimetres diameter was cut randomly from the washed tomato leaves with sterile cork borer aseptically. Twenty five discs for both the infected and non-infected leaves were placed separately in 250 ml conical flask containing fifty ml SDW. Then, the flask was shaken in a shaker for about 20 minutes to get a homogenous suspension of the microbial propagules. Sterilized Petri plates were poured with PDA medium and solidified. Using spread plate method one ml microbial suspension was pipetted out into the agar plates. Plates were then sealed with parafilm and incubated at 28±1°C for three days. Each sample was repeated for three times. Different colonies grown from leaf washes were subcultured, purified and fungi were preserved on PDA slants, respectively, at 4°C. The total microbial population per square cm of leaf surface of tomato was calculated by using the following formula (Vimala and Suriachandraselvan, 2006).

\[
\text{Total number of microbes in 1 ml} = \frac{\text{Total number of microbes}}{X \ 100}
\]

Total area of 25 discs x 2

(Area of 1 disc=πr², where r is the radius of disc in cm)

Leaf washing and serial dilution plating technique

From the homogenous stock suspension of the microbial propagules as prepared by the above mentioned leaf washing method, a dilution series was made upto 10⁻⁵ of each sample suspension (healthy and infected stock suspension). One ml aliquots of the 10⁻³ dilutions were surface plated in triplicate on PDA media containing petri plates (de Jager et al., 2001).
Identification and characterization of fungal isolates

Fungal colonies were subcultured after 3-4 days of incubation and pure cultures were transferred to PDA slants. Fungal isolates were studied based on cultural, morphological (Domsch et al., 1980; Kharwar et al., 2012) and microscopic characteristics viz. mycelium, conidiophore, spore structure etc. under microscope (Leica ICC50, Germany) by cover slip insertion method. And also those unidentified isolates were sent to National Centre of Fungal Taxonomy (NCFT), New Delhi for identification up to species level.

Results and Discussion

Isolation and identification

The diseased infected and non-infected leaf samples were collected from 24 locations from seven districts of Meghalaya. Altogether, twenty six fungal species of thirteen genera were isolated and identified on the basis of colony morphology, mycelia, sporangiophore and spore structure.

The predominant fungal species found both in infected and non-infected leaves were Fusarium sp., Phoma sp., Penicillium sp., Aspergillus sp. Trichoderma sp. and Chaetomium sp. Whereas, Camarosporum sp., F. pallidoroseum and P. glabrum were recovered only from the non-infected leaf samples (Table 1).

The genus Phoma recovered the total of six species followed by Trichoderma which is four species and Fusarium and Penicillium were three species.

The highest fungal isolates were recovered from the class Dothideomycetes i.e., five genus and ten species. And least fungal isolates were recovered from the class Mucoromycotina and Oomycota i.e., one genus and one species.

Among all the three methods used in this study, leaf impression infected upper leaves recovered the highest fungal population i.e., 1.9 X 10^3 microbial population/cm^2 whereas the lowest fungal population was recovered from dilution plating healthy leaves i.e., 6.2 X 10^2 microbial population/cm^2 (Table 2).

Though leaf impression method recovered higher fungal flora but it was difficult to assess the microbes quantitatively as very high density population was recovered. Also the microbial counting was difficult due to mixed nature of microbial population for their competition of nutrition and space. But, leaf impression method was a quick and simple method of isolation of leaf surface microflora (Dickinson, 1971).

Infected upper leaf surface recovered more fungal isolates than healthy leaf surface. Compared to all the three methods employed, leaf washing and dilution plating method was the most efficient method as it enabled the isolation of greater number and higher recovery of the same species. The morphological and cultural characteristics of all the fungal isolates were given in table 3.

Plant surfaces or internal tissues play as a home or niche for microorganisms. Therefore, plant surfaces are noted as a vital environment for microorganisms based on either a permanent (resident’s epiphytes, endophytes or pathogens) and transient (unspecific epiphytic saprophytes) association (Forseca and Inacio, 2006).

In aerial leaf surface many organisms are found common in several crop plants. Only some species are restricted to a particular crop plant.
Table 1 Presence (+) or absence (–) of fungi isolated from diseased infected (I) and non-infected (NI) leaves of Tomato

| Sr. No. | Name of the fungi               | Isolation Methods                                  | Leaf Impression |
|---------|---------------------------------|----------------------------------------------------|-----------------|
|         |                                 | Leaf washing and dilution plating                   | Leaf washing and Serial dilution plating | Leaf Impression |
|         |                                 | I  NI                                             | I  NI           | I  NI |
|         |                                 |                                                   | U  L            | U  L  |
| 1       | *Alternaria alternata*          | +  –                                              | –  –            | +  –  –  –  |
| 2       | *Botryodiplodia theobromae*    | –  –                                              | –  –            | +  –  +  –  |
| 3       | *Camarosporum* species novo.    | –  –                                              | –  –            | –  –  +  +  |
| 4       | *Cladosporium cladosporioides*  | –  +                                              | –  –            | +  –  +  –  |
| 5       | *Phoma glomerata*              | –  +                                              | +  –            | –  +  –  +  |
| 6       | *P. sorghina*                  | –  +                                              | –  –            | –  +  +  –  |
| 7       | *P. lingam*                    | –  –                                              | –  –            | –  –  –  +  |
| 8       | *P. exigua*                    | +  –                                              | –  –            | –  +  –  +  |
| 9       | *P. herbarum*                  | –  +                                              | –  –            | +  –  –  –  |
| 10      | *P. terrestris*                | –  –                                              | –  –            | +  –  –  +  |
| 11      | *Aspergillus niger*            | +  –                                              | +  –            | –  +  –  +  |
| 12      | *A. flavus*                    | +  +                                              | –  –            | –  –  +  +  |
| 13      | *Penicillium* sp.              | +  –                                              | –  –            | –  –  –  +  |
| 14      | *Penicillium* sp.              | +  –                                              | +  –            | –  –  –  +  |
| 15      | *P. glabrum*                   | –  –                                              | –  –            | –  –  –  +  |
| 16      | *Rhizopus* sp.                 | +  –                                              | +  –            | –  –  –  +  |
| 17      | *Pythium aphanidermatum*        | +  +                                              | +  –            | –  +  –  +  |
| 18      | *Acremonium strictum*          | –  –                                              | –  –            | –  –  +  +  |
| 19      | *Chaetomium globosum*          | –  +                                              | –  –            | –  –  –  +  |
| 20      | *Fusarium pallidoroseum*       | –  –                                              | –  –            | –  –  –  +  |
| 21      | *F. oxysporum*                 | +  –                                              | +  –            | –  +  –  +  |
| 22      | *F. solani*                    | +  –                                              | –  –            | –  –  –  +  |
| 23      | *Trichoderma* sp.              | –  –                                              | –  –            | –  –  –  +  |
| 24      | *T. harzianum*                 | +  –                                              | +  –            | –  –  –  –  |
| 25      | *T. viride*                    | –  +                                              | +  –            | –  –  –  –  |
| 26      | *T. asperellum*                | –  +                                              | –  –            | –  –  –  +  |

Table 2 Total microbial population per square cm of leaf surface of tomato [counts in colony forming units per millitre/leaf (cfu/ml)]

| Sr. No. | Methods                     | Mean fungal population in 1 ml (cm⁻²) |
|---------|------------------------------|--------------------------------------|
| 1       | Leaf impression-healthy upper leaves | 1.1 X 10³                          |
| 2       | Leaf impression-healthy lower leaves | 9.9 X 10²                           |
| 3       | Leaf impression-infected upper leaves | 1.9 X 10³                           |
| 4       | Leaf impression-infected lower leaves | 1.3 X 10³                           |
| 5       | Dilution plating-healthy leaves      | 6.2 X 10²                           |
| 6       | Dilution plating-infected leaves     | 1.3 X 10³                           |
| 7       | Serial Dilution plating-healthy leaves | 7.6 X 10²                           |
| 8       | Serial Dilution plating-infected leaves | 1.7 X 10³                           |

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Table 3: Morphological and cultural characteristics of leaf surface fungal isolates of tomato

| Sr. No. | Fungal isolates     | Class                  | Colour of isolate | Culture characteristics | Microscopic observation | Conidia (Shape and colour) | Nutrition |
|---------|---------------------|------------------------|-------------------|-------------------------|-------------------------|---------------------------|-----------|
|         |                     |                        | Front             | Reverse                 | Growth rate             | Text          | Margin   | Conidium and Conidiophore |                                                                 | Nutrition  |
| 1       | A. alternata        | Dothideomycetes        | Dark black        | Dark black              | S                       | P            | Cr       | Septate and branched brown colour mycelium and conidiophores containing long chain conidia | Large, dark brown obclavate conidium in chain with short conical beak at the tip | Saprophyte |
| 2       | B. theobromae       |                        | Greyish black     | Dark black              | F                       | C            | I        | Conidiophores are hyaline, simple, sometimes septate, rarely branched cylindrical and arising from the inner layers of cells lining the pycnidial cavity. Pycnidia dark brown colour | Immature conidia whitish with thin walls and mature conidia dark brown with septa and thick walls | do         |
| 3       | Camarosporum species novo. |                        | Greyish black     | Greasy black            | F                       | C            | Cr       | Septate and dark coloured mycelium. Conidiomata pycnidal | Conidia hyaline, aseptate and obclavate | do         |
| 4       | C. cladosporioides  |                        | Dark grey         | Dark black              | M                       | V            | Cr       | Branched hyphae. Cylindrical branched conidiophore | Dark, single celled lemon shaped conidia | do         |
| 5       | P. glomerata        |                        | Pale pink         | Pale pink               | F                       | WC           | Cr       | Dark brown septate hyphae. Chlamydomycoses in branched or unbranched chains. Chlamydomycoses showed both longitudinal and transverse septations as in commonly seen in the genus Alternaria. | Single celled hyaline, ovoid to ellipsoidal conidia. Conidia are bi-guttulate (containing 2 oil droplets) | do         |
| 6       | P. sorghina         |                        | White later greyish| Greyish                 | F                       | C            | Cr       | Hyphae septate and hyaline. Chlamydospores are unicellular, dark brown and botryoid-alternarioid shape. Pycnidia grey, globose and ostiolate | Conidia ellipsoidal to cylindrical, smooth, hyaline and aseptate | do         |
| 7       | P. lingam           |                        | Whitish           | Whitish                 | F                       | WC           | Cr       | Hyphae septate and hyaline. Pycnidia dark, globose and ostiolate | Conidia ellipsoidal, smooth, hyaline and single celled | do         |
| 8       | P. exigus           |                        | Pale pink         | Pale pink               | F                       | C            | Cr       | No chlamydospores, dark walled pycnidia | Oblong to elliptic or often irregular hyaline conidia | do         |
| 9       | P. herbarum         |                        | Whitish grey      | Greyish                 | F                       | C            | I        | No chlamydospores, dark walled pycnidia, ostioles often with short beaks | Oblong to cylindrical with rounded ends hyaline conidia | do         |
| 10      | P. terrestris       |                        | Pale pink         | Reddish                 | F                       | C            | Cr       | Mycelium hyaline and septate. Pycnidia dark brown, subglobose, ostiole and occur singly. | Conidia oblong to ovoid, hyaline and aseptate | do         |
| 11      | A. niger            | Eurotiomycetes         | Dark black        | Pale white              | F                       | P            | I        | Mycelium septate, branched and hyaline. Erect, unbranched, single and club shaped conidiophore | Round shaped black coloured conidia, arranged in a long chain and single-celled | do         |
| 12      | A. flavus           |                        | Greenish          | Pale white              | F                       | P            | I        | do | Round shaped green coloured conidia, arranged in a long chain and single-celled | do         |
| 13      | Penicillium sp.     |                        | Olive green with concentric circle | Creamish             | S                       | FV          | I       | Hyphae septate, branched and hyaline. Erect, unbranched and septate conidiophore | One-celled hyaline and globose conidia | do         |
| 14      | Penicillium sp.     |                        | Light blue        | Creamish h with reddish pigments | S                       | FV          | I       | do | do | do | do |
| 15      | P. glabrum          |                        | Brownish          | Brownish               | F                       | FV           | I        | do | do | do | do |
| 16      | Rhizopus sp.        | Mucoromycota           | Greyish           | Greyish                 | F                       | CC           | Cr      | Coecyctic with branched hyphae. Sporangia are supported by a large apophysate columella atop a long stalk, the sporangiophore. Sporangiohphores arise from root-like rhizoids. | Sporangiospores globose, brown coloured and one-celled. | do         |
| 17      | P. aphantidermatum  | Oomycota               | White             | White                   | F                       | CF           | Cr      | Coecyctic and hyaline hyphae | Oogonia terminal, globose and smooth, antheridia intercalary, thick walled oospores and lobed sporangia | Facultative parasite |
| 18      | A. strictum         | Sordariomycetes        | White             | Pale white              | M                       | C            | I       | Hyphae are hyaline and produce mostly simple awl-shaped erect phialides with inconspicuous collarettes | Conidia hyaline, cylindrical and single celled in chains or in conidioid masses arising from short unbranched single phialides | Saprophyte |
| 19      | C. globosum         |                        | Olive green but later | Olive green | F                       | P            | Cr      | Mycelium often grows in conglomerate masses that resemble ropes. Ostiodar dark perithecia with flat lemon-shaped and olive brown ascospores within clavate ascospores | Flat lemon-shaped and olive brown ascospores within clavate ascospores | do         |
In this present investigation total twenty six fungal species from thirteen genera were recovered from three different isolation methods. It was found that the composition of tomato leaf surface fungi showed some similarities to other plant species like Egyptian wheat (Mazen et al., 1985), Spinacia oleracea (Singh et al., 1986), Capsicum annuum (Basha et al., 2010), Persea bombycina (Bhuyan et al., 2013) and Abelmoschus esculentus (Ogwu and Osawaru, 2014). The leaf impression method was the quickest and simplest method for the isolation of leaf surface microflora. But counting of colonies was also very difficult because of mixed nature of microbial population; may be slow growing colonies were hidden by fast growing microbes (Gunasekera, 1994).

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