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Prevalence and Characterization of *Lasiodiplodia theobromae*–An Emerging Disease of Tuberose in Tamilnadu

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Abstract A survey was conducted to assess the occurrence of peduncle blight of tuberose at Madurai and Dindugal districts of Tamilnadu State. Tuberose was found to exhibit blossom blight followed by peduncle dieback starting from the tip, and leaf blight at the tips. Though *Lasiodiplodia theobromae* is an ubiquitous pathogen, its occurrence on tuberose is a new record, Peduncle blight in the area was observed with an incidence up to 43%. When infection occurred on blossoms it led to a total loss of flower buds. Several pycnidia were observed over the infected spike. The causal organism was identified based on spore morphology and confirmed further by Indian type culture collection. The survey resulted in a wide range of infection and severity of peduncle blight in major tuberose growing areas. Peduncle blight caused more damage to flowers which adversely affected the yield and quality.

Keywords Disease survey, Peduncle blight, *Lasiodiplodia theobromae,* Tuberose.

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Introduction

Tuberose (*Polianthes tuberosa* Linn.) is one of the most important ornamental plants which is extensively cultivated in many sub-tropical and tropical areas of the world [1]. Tuberose is commercially cultivated for cut and loose flower trade and also for the extraction of its highly valued natural flower oil. Diseases appear to be the major constraints to the production of tuberose. Peduncle blight, hitherto an unknown disease was found to be a major limiting factor to the cultivation of tuberose, Though *Lasiodiplodia theobromae* is an ubiquitous pathogen, its occurrence on tuberose is a new record [2]. The fungus induced confounding symptoms which included blossom blight, peduncle blight and leaf blight at tips as well. The present study was therefore, attempted to record the incidence of peduncle blight of tuberose and also to characterize at molecular level for the effective management of disease.

Materials and Methods

Disease survey

An intensive systemic survey was conducted in major tuberose growing areas of Tamilnadu to assess the occurrence of peduncle blight of tuberose (Table 1). The disease incidence was assessed by counting the number of affected plants out of total number of plants in each field. In each area three fields were assessed and the mean disease incidence was calculated. Diseased samples of peduncles were collected from these areas.

Table 1. Occurrence of peduncle blight in tuberose growing areas.

Sl. No.	Osolate code	Location	Variety	Age of the crop (months)	Disease Incidence (%)
1	I_1	AC and RI (Madurai)	Suvasini	24	42.60
2	I_2	Vilampatti (Dindugal dt)	Prajwal	9	19.45
3	I_3	Kodairoad (Dindugal dt)	Prajwal	14	34.33
4	I_4	Kannanur (Madurai dt)	Prajwal	8	12.00
5	I_5	Kannanur (Madurai dt	Prajwal	12	24.65
6	I_6	Sekanoorani (Madurai dt)	Prajwal	10	17.11
7	I_7	Chellampatti (Madurai dt)	Prajwal	28	25,33

Isolation of pathogen

The pathogen causing peduncle blight in tuberose was isolated from the samples by tissue segment method on potato dextrose agar (PDA) and the fungus was purified by single spore isolation and maintained on PDA. The causal organism was identified based on spore morphology and confirmed further (ID.NO. 6751/11) by Indian Type Culture Collection Centre (ITCC) of Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi.

Identification of the Pathogen

The pathogen was identified based on their cultural and morphological characters. A loop full of fungal culture were taken on a glass slide and examined under image analyzer at 40X magnifications for the presence of conidia and pycnidium. After confirming the spores, the cultures were purified by single spore isolation technique.

Pathogenicity

Detached flower bud technique

A five-mm culture disc of *L. theobromae* was placed closer to the calyx of healthy detached flower bud and kept in 150-mm-dia Petri dish over a layer of moistened filter paper. An empty five- mm disc of PDA served as control. Three replications were maintained and the plates were incubated at room temperature (28 ± 2^0C). The formation of lesion on flower bud was closely monitored and the lesion length was recorded at regular intervals.

Pathogenicity in glasshouse

The pathogenicity of the fungus was confirmed by Koch's postulates using five numbers of four-month-old healthy plants. Plants were inoculated by making a vertical cut (3 mm) in the peduncle region below the calyx using a sterilized needle and placing a fungal disc over the wound. The inoculated area was covered with moist cotton and wrapped with parafilm. The plants were covered with polythene bags to maintain humidity and monitored for symptom expression. Proper controls were maintained with PDA plugs.

Molecular characterization of *L. theobromae* isolates

Isolation of fungal DNA by cetyl trimethyl ammonium bromide (CTAB) method

Genomic DNA was extracted from the mycelial mat of *L. theobromae* isolates by Cetyl Trimethyl Ammonium Bromide (CTAB) method. Mycelium was harvested by filtration through sterile filter paper and stored at – 70 ^{0}C unit used for DNA extraction. One gram of frozen mycelium was ground to fine powder in liquid nitrogen and incubated in five ml, two per cent CTAB extraction buffer (10 mM trisbase (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, CTAB (2%), mercaptoethanol (0.1%) and PVP (0.2%) at 65 ^{0}C for one hour. The suspension was added with equal volume of phenol-chloroform-isoamylalcohol (25:24:1) mixture. It was vortexed to mix two phases, followed by centrifuged at 12,000 rpm for five minute. The supernatant was transferred to a clean tube and mixed with equal volume of ice cold isopropanol and incubated at 25 ^{0}C for DNA precipitation. The precipitate

was collected by centrifugation and the pellet was washed with 0.1 M ammonium acetate in 70% ethanol and incubated for 15 min. The pellet was resuspended in TE buffer (10 mM Tris, 1mM EDTA, pH 8.0) and the DNA concentration was estimated using spectrophotometer (Genway Genova, Bibby Scientific Ltd Dunmow, UK) at 620 nm.

PCR amplification of ITS region of L. theobromae isolates

The ITSI-5.8S-ITS2 region of ribosomal DNA from twenty six isolates of *L.theobromae* was amplified with ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers. The amplification was performed in a 50 µl reaction containing 1.5 units of *Taq* DNA polymerase (Qiagen, Germany), 1x polymerase chain reaction (PCR) buffer, 200 µM each dNTP, 0.2 µm each primer and 100 ng of template DNA. Reaction mixture were heated for 95 ^{0}C at 15 min. and then amplified for 30 cycles (0.5 min. at 94 ^{0}C, 05 min. at 56 ^{0}C and 2 min at 72 ^{0}C) with the final step at 72 ^{0}C for 7 min.

Agarose gel electrophoresis

The PCR products were separated in 1.2% (w/v) agarose gel in 1x TAE buffer (0.4 M Tris, 0.2 M acetic acid, 10mM EDTA; pH 8.4) containing 0.5 µg/ml ethidium bromide. The PCR product along with gel loading buffer (6x containing 0.25% bromophenol blue, 0.25% xylene, cyndol FF and 3% glycerol) was loaded. Electrophoresis was carried out at 100 V and the gel was documented using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA). The sizes of the PCR products were determined by comparing with standard 100 bp or 1 kb molecular marker (Bangalore Genei Pvt Ltd., Bangalore, India).

Statistical analysis

The data were statistically analyzed using the Irristat version 92 developed by the International Rice Research Institute Biometrics unit, Philippines. The percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at significant level (p <0.05) and means were compared by Duncan's Multiple Range Test (DMRT).

Results and Discussion

Occurrence of peduncle blight in tuberose growing areas

Peduncles of tuberose showing typical symptoms of blight were collected from seven locations (Table 1, Fig. 1a, 1b). The age of the crop varied from 9 to 28 months and cultivars with single flower were common than double flower types. The disease incidence ranged from 12.00 to 42.60%. The causal organism isolated was identified as *Lasiodiplodia theobromae* Pat. based on pycnidial characters and spore morphology (Fig. 2a, 2b, 2c).

Symptoms

In the current survey, blighting of flower buds of tuberose followed by dieback of peduncle from tip downward are the major symptoms observed in the field. Tests of pathogenicity by detached flower technique as well as those conducted at greenhouse yielded symptoms as observed in the field. The fungus also caused symptoms on flower buds without wound after an incubation period of seven days. However, wounding was found to enhance the symptom expression. Peduncle blight, hitherto an unknown disease was found to be a major limiting factor to the cultivation of tuberose, as the disease incidence was noticed up to 42.60% in pockets of Madurai district. Though *Lasiodiplodia theobromae* is an ubiquitous pathogen, its occurrence on tuberose is a new record. The fungus induced confounding symptoms which included blossom blight, peduncle blight and leaf blight at tips as well.

Pathogenicity in glasshouse

The pathogenecity of the isolate I_1 of *L. theobromae* was further confirmed in glasshouse. Symptoms were visible on the flower bud and the peduncle. The pathogen was reisolated and Koch's postulates were fulfilled.

Based on the symptoms and morphological characters of the fungus, it was identified as *Lasiodiplodia theobromae*. The culture was sent to the Indian Type Culture Collection, Indian Agricultural Research In-

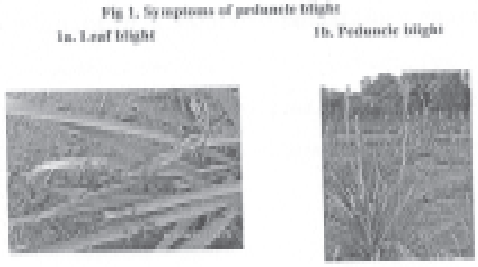

Fig 1. Symptoms of peduncle blight
1a. Leaf blight
1b. Peduncle blight

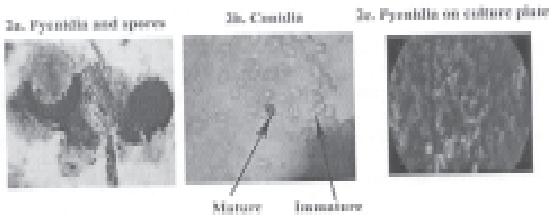

Fig 2. Spores of *L. theobromae*
2a. Pycnidia and spores
2b. Conidia
2c. Pycnidia on culture plate
Mature
Immature

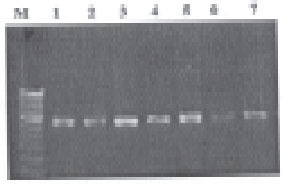

Fig 3. Confirmation of *L. theobromae* through amplification of ITS 1 & 4 region
M 1 2 3 4 5 6 7
M- 100 bp ladder 1-7 *L. theobromae* isolates

stitute, New Delhi and confirmed the pathogen as *Lasiodiplodia theobromae* (Syn: *Botryodiplodia theobromae*) (ITC No : 6751/11). Among the seven isolates of *L. theobromae,* I_1 that was fast in growth *in vitro* studies was used for pathogenicity and glasshouse studies Table 2. The results on pathogenecity of *L. theobromae* using detached flower bud technique are furnished in Table 3. Till fourth day no symptom was observed in buds inoculated with or without pin prick. Symptoms developed five days after inoculation (DAI) and the lesion length increased over time. Lesion length was more when the inoculum of the fungus was placed on the pin pricked bud than the one without pin prick. At seven DAI, the length of lesion was 570 mm in pinpricked buds while it was noly 300 mm in buds that were not pin pricked.

Molecular studies of *L. theobromae*

PCB amplified product showed the size of 560 bp in 1.8% agarose gel which confirmed the pathogen was *Lasiodiplodia theobromae* (Fig. 3). The fungus *Lasiodiplodia theobromae* has a wide host range infecting monocot and dicot plants causing an array of symptoms including shoot bight and die back. In cashew it causes drying of petals and other flower parts followed by die back of peduncles leading to inflorescence blight. The fungus causes necrosis and

Table 2. Pathogenecity of *L. theobromae* on tuberose buds *in vitro.*

Days after incubation	Lesion length (mm) Control Pin prick	Control Without Pin prick	Inoculated Pin prick	Inoculated Without Pin prick
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	50.0	30.0
6	-	-	350.0	180.0
7	-	-	570.0	300.0

Table 3. Morphological characters and growth of different isolates of *L. theobromae* on potato dextrose agar.

Sl. No.	Isolate code	Colony type	Color	Mycelial growth (mm) 48 h	Mycelial growth rate (mm/h)
1	I_1	Coarse, fluffy	Dull white	90.00	1.88
2	I_2	Sparse	Greyish white	88.30	1.84
3	I_3	Partial fluffy	Dull white	87.00	1.81
4	I_4	Coarse	Black	86.60	1.80
5	I_5	Flat	Greyish white	78.30	1.63
6	I_6	Dense, Fluffy	Black	64.30	1.34
7	I_7	Sparse	Dull white	77.00	1.60
		CD		6.44	

die back of shoots in mango [3] and grapevine [4]. The fungal colonies of *L. theobromae* on PDA were initially white turning to black or grey later. The mycelial were fast spreading, branched, septate and pycnidia were brown coloured. Conidia were initially globose to oblong, hyaline and unicellular turning brown and septate later. This is in accordance with the descriptions of *L. theobromae* reported earlier [5].

The hyaline non-septate pycnidiospores of *L. theobromae* were highly vesiculated in light microscope, while the pigmented septate ones exhibited longitudinal hyaline striations. Conidia of *L. theobromae* from mango twigs were initially hyaline, unicellular and sub-ovoid to ellipsoidal measuring 18 to 30 × 10-15 µm in size. Mature conidia were two-celled, cinnamon to dark brown, thick walled, ellipsoidal, often with longitudinal striations. In similar way, Burgess et al. [6] described three new *Lasiodiplodia* species from the tropics on the basis of their ITS and EFI-α sequence data and morphological characters. However, occurrence of peduncle blight caused by *L. theobromae* in tuberose is a new record in India. Proper identification useful for the management of disease efficiently.

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