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Isolation technique and morphological characteristics of *Ceratocystis fimbriata* ELL and HALST Causing wilt in pomegranate

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Abstract
Pomegranate (*Punica granatum* L.) is one of the important fruit crops belongs to the family Lythraceae. Nowadays it is highly threatened by the wilt disease caused by *Ceratocystis fimbriata* which results in complete wilting of plant and is characterized by the initial symptoms as yellowing and wilting of leaves on one to several branches. The fungus was isolated from standard tissue isolation through carrot bait technique. Ascospores mass that developed on carrot slices were transferred to Potato Dextrose Agar (PDA) in petri plates. The wilt fungus *C. fimbriata* grew well on potato dextrose agar and oat meal agar. The petri dishes were incubated at room temperature (25±1 ^{0}C) for 15 days and observed periodically for fungal growth. The morphology of mycelium was whitish grey in the beginning which later changed into brown. As the growth progressed, production of endoconidia, aleurioconidia and perithecium was observed. Perithecia were black with a globose base. Ascospores exuded from the apex of the perithecium neck in a long coil which are small, hat shaped and hyaline. Conidiophores were septate and hyaline to dark greenish brown. Thick walled endoconidia were globose to oval, olive brown in colour. Aleurioconidia were thick walled, ellipsoidal or Pyriform, golden brown in colour, borne singly or in chain.

Keywords: Pomegranate wilt, isolation, *Ceratocystis fimbriata,* Carrot bait technique, morphology

Introduction
Pomegranate (*Punica granatum* L.) is a popular highly prized fruit called as “Fruit of Paradise”. It is a long lived drought tolerant deciduous shrub belongs to the Lythraceae family, with 2n=16 number of chromosome. Both arid and semiarid zones are suitable for growing pomegranate trees. It is one of the highly remunerative horticultural fruit crops because of its nutrient dense, antioxidant rich, medicinal properties. However, in the recent years pomegranate cultivation has been highly threatened due to many diseases and pests among which disease caused by wilt, *Ceratocystis fimbriata* is the prime most important disease. Which results in complete wilting and death of plant leads to huge loss to the farmers. Somasekhara and Wali first noticed wilt of pomegranate in two areas of the Vijayapura district of Karnataka, in 1990 [8]. Prominent pomegranate growing states showed the presence of wilt of pomegranate with varying severity. Maharashtra, recorded 49.2 per cent, Karnataka recorded 61.11 per cent while Andhra Pradesh recorded 8.69 per cent [4]. Xu *et al.,* found that a new devastating disease was observed on pomegranate (*Punica granatum* L.) in Panzhihua-Xichang region of Sichuan province, southwest China that caused losses estimated to be 30 per cent as surveyed by 10 orchards [11].
The wilt disease is characterized by the initial symptoms as yellowing and wilting of leaves on one to several branches. In some orchards diseased plants were died due to wilt in patches, thereby indicating the spread of the disease from an infected to an adjacent healthy orchard. Initial symptoms appeared only on shoots; later, leaves turned yellow, then wilted. In severe cases, defoliation and dieback was accompanied by black streaks in the xylem, followed by death of the tree. The disease would be more severe in older (>15 years) than in younger plantings [1]. Splitting of root or vertical sections of diseased plant parts showed dark grayish brown streaks or distinct starburst like black discoloration in vascular and adjoining cortex tissues. Blue stains were also observed on the stem during the present investigation. Hence proper isolation technique and study of morphological characteristics of wilt causing fungus in pomegranate is the need.

Material and Methods

Isolation of *Ceratocystis fimbriata* from infected tissues through carrot bating technique

The fungus was isolated using standard tissue isolation technique. The black discolored stem bits along with some healthy portions were surface sterilized in 1% sodium hypo chloride solution for 60 seconds and washed thoroughly thrice in sterile distilled water to remove the traces of sodium hypo chloride, if any. The infected tissues from diseased roots and stem were cut into small strips of about 1 cm and carrot baited for *Ceratocystis fimbriata* by placing these between two carrot slices in desiccator for 6-7 days. Perithecia mass that developed on carrot slices were transferred to PDA in petri plates. The Petri dishes were incubated at room temperature (25±1^{0}C) and observed periodically for fungal growth. The pure colonies which developed from the bits were transferred to PDA slants and incubated at room temperature for 15 days. Pure culture of the fungus was obtained by single hyphal tip isolation method.

Hyphal tip isolation

This method was followed for maintaining of pure culture. Dilute spore suspension of the pathogen was prepared in sterilized distilled water containing eight to ten spores per ml from 15 days old culture. One ml of such suspension was spread uniformly on two per cent solidified water agar plates and observed for spores under the microscope. Single spore was marked with a marker on backside of the petri plate and it was allowed to germinate. Such plates were periodically observed for spore germination under microscope. The hyphae growing from each cell of the single spore was traced and marked with marker. The tip of the hyphae was cut carefully and transferred to PDA plates and incubated at 25±1^{0}C for 15 days. Later, mycelial bits of the fungus were transferred to the center of Petri plates containing PDA and incubated at 25±1^{0}C for 15 days. Saltation or sectoring was observed in the culture to confirm the pure culture of the fungus.

Maintenance of the culture

The hyphal tip cultures of the fungus were sub cultured on potato dextrose agar slants and kept in laboratory at 25±1^{0}C for 15 days. Such mother culture slants were preserved at 4^{0}C in refrigerator.

Results and Discussion

Pomegranate (*Punica granatum* L.) is a high value horticultural crop, suffers mainly due to wilt especially in traditional belt of Karnataka, which caused by *Ceratocystis fimbriata* Ell. and Halst. The typical symptoms of wilt complex of pomegranate results in complete wilting of plant and is characterized by the initial symptoms as yellowing and wilting of leaves on one to several branches. At times only one or two stems of the tree showed wilting and it took few weeks to some months for the entire tree to completely wilt. Wilt infected plants often revealed dried foliage and fruits being attached to the branches for many months. The xylem of the trunk turned brown to black with a star burst like pattern with blue strains on stem was noticed. Below ground symptoms like the dark black to greyish colour mycelial mat was observed on the root portion with characteristic fruity odour which attracts many of the microorganisms as well as insects leads to disease complex. In severely infected fields similar symptoms of wilt were earlier recorded by many scientists [8, 4, 5, 11, 6, 9].

Isolation of *C. fimbriata* from wilt affected samples

Standard tissue isolation through carrot baiting technique was followed to isolate *Ceratocystis fimbriata* culture from diseased sample as described in "Material and Methods". The pure culture was maintained on potato dextrose agar at 25±1 ^{0}C.

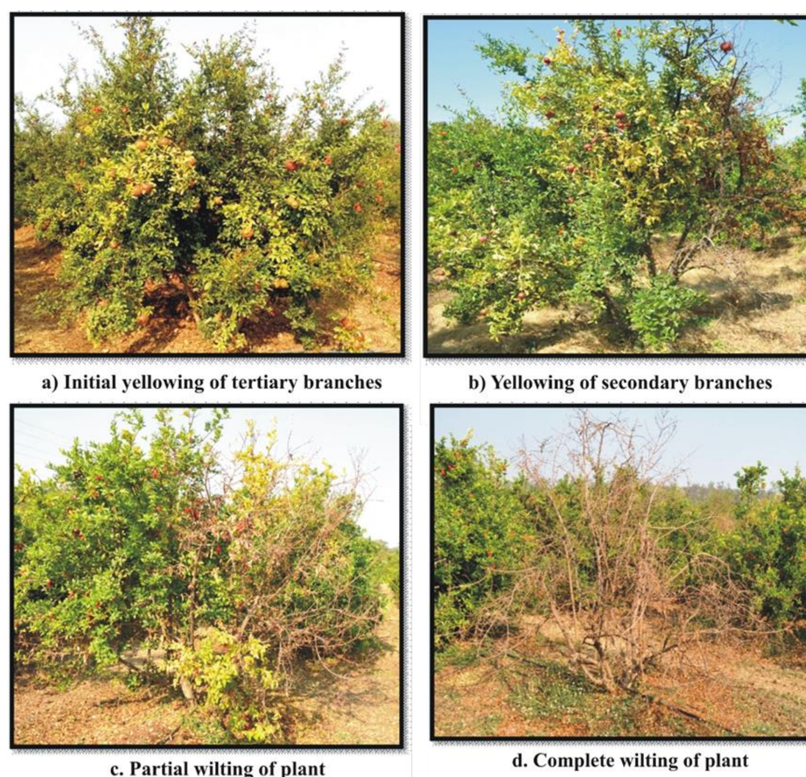

Fig 1a: Different stages of wilt symptoms in pomegranate

Sub culturing was done at every fortnight interval. The fungus isolated was confirmed as *C. fimbriata* based on their symptoms, cultural and morphological characters (Figure 1a & b).
Standard carrot bating technique was used to isolate *Ceratocystis fimbriata* culture from diseased sample. The wilt fungus *C. fimbriata* produces perithecia on carrot were transferred to media which grew well on potato dextrose agar and oat meal agar. In the beginning the mycelium was white tinged with grey colour which later turned to brown to dark grey. As mycelia follows the logistic growth, 3-4 days after incubation endoconidia and aleurioconidia were produced and 10-16 days after incubation perithecium was observed. Distinct small black perithecial bodies were observed on mycelial plate. The perithecia (72.92 x 131.2 µm) were flask shaped round base with many cells of appendages. It had characteristic long neck with opening ostiole exudes plenty of small, hat shaped hyaline ascospores which measured 18.92 x 25.84 µm. Septate and hyaline conidiophores were seen which endogenously divide and produce cylindrical endoconidia having average size of 29.26 x 6.18 µm. Septate conidiophore produced singly or in chain thick-walled pyriform to oval shaped olive brown colour aleurioconidia at the tip or in branches and were 16.97 x 26.31 µm in size. These results are in conformity with many researchers [7, 2, 10, 6, 9].

Pathogenicity test for *C. fimbriata*
The pathogenicity of the isolated fungus was proved by inoculating the pure culture of *C. fimbriata* to six month old pomegranate *cv* Bhagwa raised in sterile soil along with a control plant without adding inoculum to sterile soil. The pathogen produced wilting symptoms after 45 days of inoculation. The plants started showing initially typical wilting symptoms such as yellowing of leaves in some twigs or branches, followed by drooping and drying of leaves. Brownish discoloration of vascular tissues was observed when affected root was split opened. Blue stains on stem were also noticed. The reisolation of the fungus was made from the roots of wilted plant and was identical to original one with respect to cultural and morphological characters, thus confirmed the pathogenicity (Figure 2). The control plants which were not inoculated with the fungus did not show any symptoms of disease. The results are also in close agreement with Engelbrecht and Harrington [3, 5] and Chaudhari *et al.* [2] who isolated *C. fimbriata* from pomegranate.

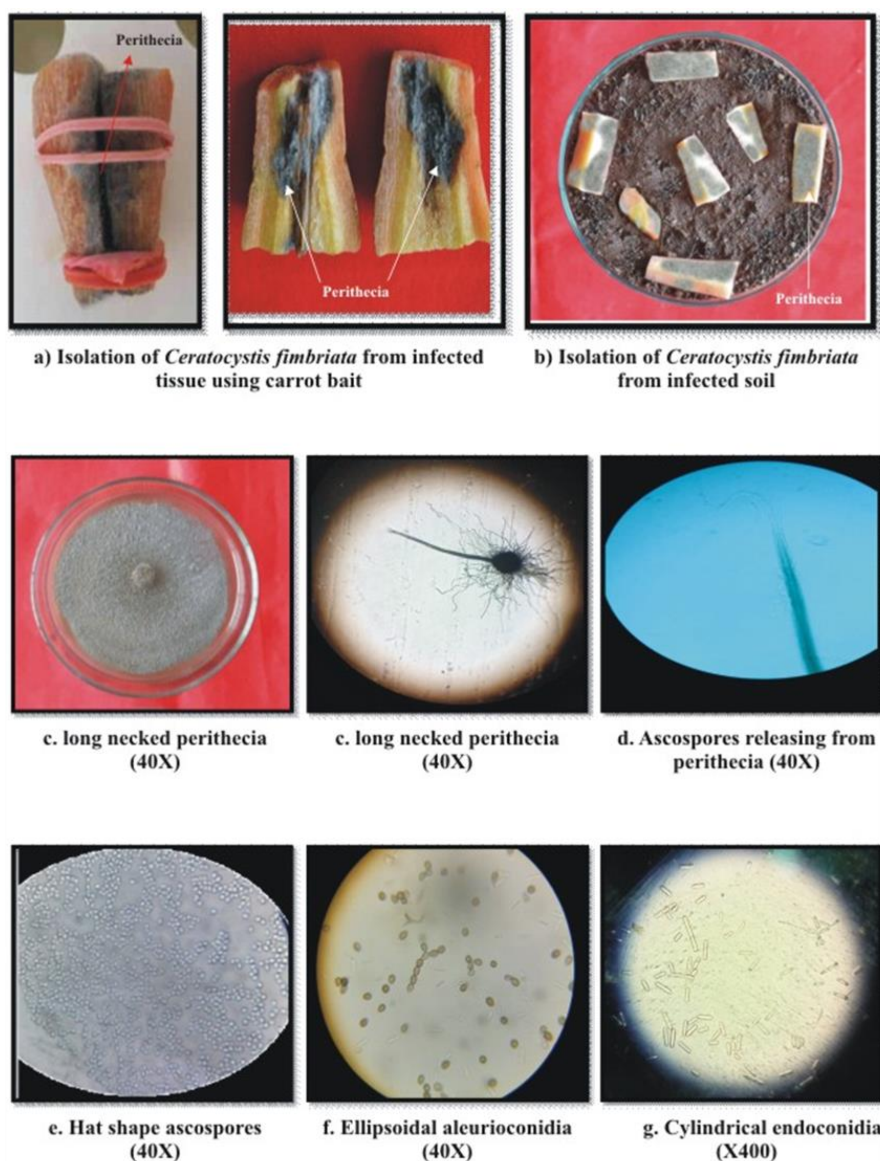

Fig 1b: Isolation and Morphology of *Ceratocystis fimbriata*

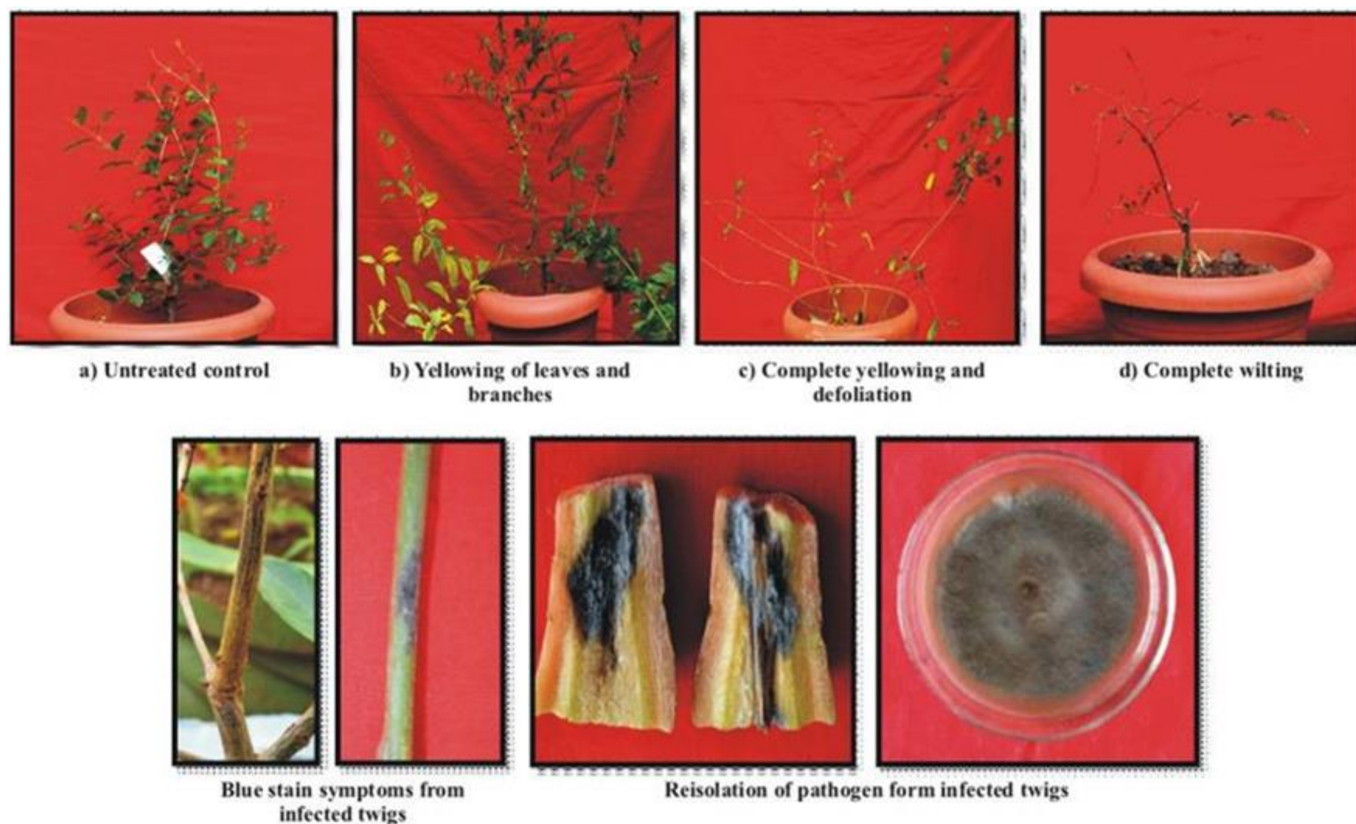

Fig 2: Pathogenicity test for *Ceratocystis fimbriata*

Conclusion

The investigation was conducted to study the isolation technique and morphological characteristics of wilt of pomegranate caused by *Ceratocystis fimbriata* Ell. and Halst. The pathogen isolated through carrot bait technique and grew well on PDA and OMA. The mycelium was whitish grey in the beginning which later on changed to brown. As the growth progressed, production of endoconidia, aleurioconidia and perithecium was observed. Ascospores were exuded from the apex of the perithecium neck which survive well in the soil and cause disease to the plant leading to death of whole plant within three months intern leads to huge losses to the farmers.

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