A MULTI-GENE PHYLOGENY OF *CERATOCYSTIS MANGINECANS* INFECTING MANGO IN PAKISTAN

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Abstract

Mango trees (*Mangifera indica* L.) are affected by a serious wilt disease, recognized as mango sudden death first time reported in Muzafargarh Punjab, Pakistan in 1995. Its prevalent is in almost all mango growing areas with severity ranged from 2-5% in Punjab and 5-10% in Sindh. Survey and sampling was conducted during the year 2011-12, on mango orchards in different districts of Punjab and Sindh and no location was found free from this Disease. For molecular identification, DNA was successfully extracted and was then amplified by using ITS, BT, TEF (600-800) primers through Polymerase Chain Reaction (PCR) assay and nucleotide evidence of Pakistani isolates (45 for each gene) exhibiting the maximum genetic homology with *Ceratocystis manginecans* (99-100%) followed by *C. fimbriata* (97%) and *C. omanensis* (80%) respectively. On the basics of morphological tools and comparison of nucleotide evidence of multi-genes, *C. manginecans* is different from *C. fimbriata* and *C. omanensis* which infect mango in Pakistan. The availability of disease-free planting material and management in combination with fertilization and proper irrigation system would help in improving orchard management system.

Key words: Mango, ITS, BT, TEF, Phylogeny, *Ceratocystis manginecans*.

Introduction

*Mangifera indica* L. belonging to dicotyledonous family Anacardiaceae, generally recognized as mango, is a popular, predominant seasonal fruit originated primarily in the tropical and subtropical regions of the world. Pakistan produces approximately 8.5% of the world’s total production and mostly exports to Iran, Middle East, Japan, Germany and China assembling its importance as a significant foreign exchange earning fruit crop (Anonymous, 2007). In Pakistan, soil and climatic conditions support production of mango in terms of quality and yield but the country is unable to attain the desired results. Numerous issues contribute towards the low production of mango. In Pakistan mango is susceptible to many abiotic and biotic diseases including mango malformation, mango decline, anthracnose and powdery mildew (Asad *et al*., 2007). Different species of *Ceratocystis* have been reported for canker and fruit rot, vascular discoloration, root and stem root to many trees in different parts of the world. Some of the known *Ceratocystis* species associated with fruit trees mortality were *C. sal bifundus*, *C. pirilliformis*, *C. fimbriata*, *C. manginecans*, *C. fagacearum*, *C. omanensis* and *C. paradoxa* (Engelbrecht & Harrington, 2005; Wyk *et al*., 2007). Whereas *C. fimbriata*, *C. manginecans* and *C. omanensis* were associated with mango tree (Wyk *et al*., 2007). This fungus is also known to cause sudden death or “seca” in many area of Brazil (Ploetz, 2004).

On the basis of morphological characteristics species of *ceratocystis* were redefined, like spores, ascospores and perithecia size and shape (Fateh *et al*., 2006). Distinguishing different species of *Ceratocystis* were not vague by the usefulness of morphological features and were not have treasured for phylogenetical information and it is defined by molecular analysis with the help of rDNA and internal transcribed spacer (Verkley & Starink-Willemse, 2004). The exertion of resolving relationships among *Ceratocystis* has been underlined by using single gene phylogenies (Crous *et al*., 2001). Unavailability of reliable phylogenetic information in reconstruction of trees, resulting in poorly resolved trees (Verkley *et al*., 2004; Shinwari *et al*., 1994; Shinwari, 1995).

Sequenced data was used from ITS, beta tubulin (BT), transcription elongation factor (TEF) and ribosomal gene in combination to form phylogenetic histories (Moore, 1995). Nuclear and mitochondrial genes are unlinked to each other and therefore, overruled for making phylogenetic histories (Cummings *et al*., 1989). High rate of nucleotides substitutions was found in Ribosomal DNA and it would be helpful in providing additional intuition into relationships among closely related fungus (Moncalvo *et al*., 2000).

In this study, the causal organism of MSD was confirmed through morphological characteristics and nucleotides evidence of multi-genes including ITS, BT, TEF and mtSSU.

Material and Methods

Symptomatic surveys of mango orchards was conducted in mango growing areas of Punjab and Sindh as described by Al Adawi *et al.* (2006). Morphological characteristics were compared with Domsch *et al*., 1980 and pathogenicity tests were used for further confirmation. Total nucleic acid of local isolate was extracted with modified protocol of Barnes *et al.* (2001) and used as a template (2 µl) in PCR assay. PCR reactions (50 µl) were comprised of 10X PCR reaction buffer (5 µl), 10 mM mixed dNTPS (1 µl), 25 mM MgCl2 (3 µl), 5 unit/µl Taq DNA polymerase (1 µl) and 20 pM (5 µl) of each sense and antisense primer. Amplifications of ITS and EF-1α region of local isolates was done by using primers ITS1(5’S GCTTAGGAAGCTGCG G 3’), ITS4 (5’S TCCCGCTTTATTGATATG 3’), EF1-728F(5’S
provided the accession numbers AB818966 to AB819010, sequence of 45 isolates was submitted on DDBJ and (EF1-728 and EF1-986) regions (Fig. 1a, b, c). The final (Bt 1a and Bt 1b) regions amplified approximately 600 to 648 µm in length. Whereas ascomata necks were dark brown in colour and ranged from 515-648 µm in length. Ascomata base ranged from 192-252 µm in diameter. Whereas ascomata bases were globose with brown to black and brown to black and hat like ascospores (2-3.1 µm length and 3.2-6 µm width) was also observed. Ceratocystis was homothallic, and all the isolates derived from MSD produced perithecia. The mortality of young and adult plants by MSD has become a severe thread for the mango grower in Pakistan. To identify and characterize the most reported pathogen of MSD Ceratocystis sp. the samples were collected from Multan, Shujabad, Khanewal, Rahim Yar Khan, Hyderabad, Sanghar, Tando Allah Yah, Matiyari and Muzaffar Garh showing 5 to 15% severity in orchards (Fateh et al., 2006 ). First symptoms observed in MSD were dropping of leaves which are mainly caused due to blockage of xylem vessels by the invading fungus as the nutrients and minerals cannot be transported to the aerial parts of the plants therefore the loss of turgidity occur and leaves droop. Next symptoms that appeared was bark splitting which may occur due to exposure to direct sunlight or by fungal invasion in the xylem vessels. The nutrients keep moving from soil to the collar portion of stem and accumulate their making the air pockets inside as the turgidly of cell increases internal break down or damage and varies in colours according to the severity of the disease. The fungus mostly prevails in the soil of infected orchards and when irrigation is done the spores travel from one place to another entering the wounded roots of healthy trees. Symptomology is not the reliable criteria for the confirmation of MSD because symptom develops may occur due to abiotic factors but it can play a vital role for disease diagnosis (Kazmi et al., 2007).

Results

Dead branched, turned brown leaves, brown streaks in vascular region, yellow to brown gum-like substance, bark splitting, gummositis and wilting was observed in all orchards of Punjab and Sindh. Spores were hyaline, cylindrical with truncated ends and ranged from 18-30 µm and 13-15.6 µm in length and width respectively. Hyaline, hat like ascospores (2-3.1 µm length and 3.2-6. µm width) was also observed. Ceratocystis was homothallic, and all the isolates from MSD produced perithecia. The ascomata bases were globose with brown and long neck. Ascomata base ranged from 192-252 µm in diameter. Whereas ascomata necks were dark brown in colour and ranged from 515-648 µm in length.

Internal Transcribed Spacer (ITS1 and ITS4) and BT (Bt 1a and Bt 1b) regions amplified approximately 600 to 650bp while 700 to 800bp fragments were obtained in TEF (EF1-728 and EF1-986) regions (Fig. 1a, b, c). The final sequence of 45 isolates was submitted on DDBJ and provided the accession numbers AB818966 to AB819010, AB889749 to AB889793. Bootstrap support value for this group was 98%. Whereas assembly of mtSSU sequences of all Ceratocystis isolates resulted in a data set of 685 characters, of which 13 (2.4%) were parsimony informative. The major clade 1 and 2 includes all the Ceratocystis isolates with strong bootstrap value (95%) (Fig. 4). Partition homogeneity test for the data set of ITS, BT, TEF and mtSSU gave P-values (0.02) greater than the acceptable level value (P=0.05) and they could thus be combined (Barker et al., 2000). The combined sequences data of the multi gene areas resulted in a total of 1953 characters, including gaps. The data set contained 1087 characters, 62 parsimony uninformative characters and 682 parsimony-informative characters. Tree length is about 1874 steps, with a consistency index (CI) of 0.97, homoplasy index (HI) of 0.35, a retention index (RI) of 0.85 and a rescaled consistency index (RC) of 0.68.

Discussions

The mortality of young and adult plants by MSD has become a severe threat for the mango grower in Pakistan. To identify and characterize the most reported pathogen of MSD Ceratocystis sp. the samples were collected from Multan, Shujabad, Khanewal, Rahim Yar Khan, Hyderabad, Sanghar, Tando Allah Yah, Matiyari and Muzaffar Garh showing 5 to 15% severity in orchards (Fateh et al., 2006 ). First symptoms observed in MSD were dropping of leaves which are mainly caused due to blockage of xylem vessels by the invading fungus as the nutrients and minerals cannot be transported to the aerial parts of the plants therefore the loss of turgidity occur and leaves droop. Next symptoms that appeared was bark splitting which may occur due to exposure to direct sunlight or by fungal invasion in the xylem vessels. The nutrients keep moving from soil to the collar portion of stem and accumulate their making the air pockets inside as the turgidly of cell increases internal break down or damage and varies in colours according to the severity of the disease. The fungus mostly prevails in the soil of infected orchards and when the irrigation is done the spores travel from one place to another entering the wounded roots of healthy trees. Symptomology is not the reliable criteria for the confirmation of MSD because symptom develops may occur due to abiotic factors but it can play a vital role for disease diagnosis (Kazmi et al., 2007).
The isolation of Ceratocystis isolates from infected xylem sample's confirmed the MSD disease. Investigated Ceratocystis isolates was morphologically similar to C. moniliformis, C. moniliformopsis, C. omanensis, and C. bhutanensis though some differences were also observed. The perithecial and ascomata necks were in range of C. manginecans but they are noticeably shorter than C. moniliformopsis and C. moniliformis. Hence, there are some morphological differences between investigated Ceratocystis isolates and its close relatives. It is exhibited that the sub-populations are not always expressed in teens of morphological divergence. Closely related species therefore, lack of taxonomically useful morphological differences long after the initial speciation event. In Pakistan, Ceratocystis species were identified through morphologically and no nucleotide evidence was available previously. Pathogenicity tests and nucleotide evidence are reliable tools for reporting new species in Pakistan (Abbas et al., 2014).

Multi-genes (nuclear and mitochondrial) based phylogeny for 45 local isolates of Ceratocystis exhibited unprecedented resolution between close Ceratocystis species resulting from combined use of genes. It provides sufficient evidence for confirmation of the main hypothesis and further assisting in the delimitation of closely related species in the genus Ceratocystis. With these evidences, it is confirmed that the agreement between the main results of the multigene phylogeny presented here and the previously published Ceratocystis phylogeny (Engelbrecht & Harrington, 2005; Shinwari, 1998).

By using separate data set analysis, these DNA partitions were of limited utility in supporting relationship with Ceratocystis. Only ITS did not provide the sufficient support resolution for many nodes except those nodes which are found within groups of related species. It is confirmed here that the limited utility of this gene for supporting phylogenetic resolution between and within Ceratocystis (Goodwin & Zismann, 2001. Identification from lower to intermediate taxonomic level, both nuclear and mitochondrial sequencing (ITS, BT, TEF and mtSSU-rDNA) reveals similar character variation and exhibited the identical phylogenetic (Barnes et al., 2003). These studies supported the data insufficiently resolution upto the inadequate number of informative sites found (>40%) comparatively to the large number of closely related taxa examined.
Fig. 2. Phylogenetic tree showing inter relations of *Ceratocystis* spp. with closely related species inferred from 5.8S r DNA sequences. Tree was generated using the neighbor-joining method. Bootstrap value (more than 70%), expressed as percentage of 1000 replications, are indicated at the nodes.
Fig. 3. Phylogenetic tree showing inter relations of *Ceratocystis* spp. with closely related species inferred from BT sequences. Tree was generated using the neighbor-joining method. Bootstrap value (more than 70%), expressed as percentage of 1000 replicons, are indicated at the nodes.
Fig. 4. Phylogenetic tree showing inter relations of *Ceratocystis* spp. with closely related species inferred from TEF sequences. Tree was generated using the neighbor-joining method. Bootstrap value (more than 70%), expressed as percentage of 1000 replicons, are indicated at the nodes.
Conclusion

Morpho-molecular characteristics support the first report of *Ceratocystis manginecans* infecting mango in Pakistan.

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References


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