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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *FUSARIUM OXYSPORUM* SCH. ISOLATED FROM GUAVA WILT IN BANGLADESH

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Abstract

Wilt of guava plants (*Psidium guajava* L.) is a serious disease in Bangladesh. Sixteen isolates of *Fusarium oxysporum* Sch. were collected from the root and stem fragments of guava plants growing in six districts of Bangladesh. Species identity was based on the colony character, nature of conidiogenous cell, morphology of microconidia, macroconidia and chlamydospores. Eleven isolates were confirmed as *F. oxysporum* through polymerase chain reaction (PCR) using species specific primers designed from the conserved regions of 18S rRNA gene.

Introduction

In Bangladesh guava is one of the cheapest fruits and good source of vitamins. During 2005 - 2006, total production of guava was 145685 MT (Anon. 2008). Ten diseases of guava were reported by different workers mentioning wilt as a major pernicious disease and reported 10 - 25% loss (Talukdar 1974). Meah and Al-Mamun (1991) reported 18 - 36% guava wilt from five selected districts of Bangladesh.

Wilt disease which is a sudden, catastrophic killer, known as the "Cancer of guava production". July-October is suitable for wilt and per cent death is quicker during this time (Meah *et al*. 1995). At the seedling stage, infected plants may wilt and die soon. In older plants, vein clearing and leaf epinasty are often followed by stunting, yellowing of the lower leaves and then the tree is defoliated. When decline is rapid, leaves tend to shrivel and assume a fire scorched appearance. Browning of the vascular tissue is a strong evidence of *Fusarium* wilt. Das and Rai (1947) indicated the association of *Fusarium* sp. with the wilt disease of guava. Prasad *et al*. (1952) identified *Fusarium oxysporum* as wilt causing organism of guava and proposed the name *Fusarium oxysporum* Sch. f. sp. *psidii* Prasad, Mehta and Lal. Edward (1960a), Pandey and Dwivedi (1985), Meah and Al-Mamun (1991) and Hamiduzzaman *et al*. (1997) agreed upon the identification of wilt pathogen of guava as proposed by Prasad *et al*. (1952).

In the present investigation, an attempt was made to characterize the wilt pathogen/s of guava occurring in Bangladesh on the basis of morphological characters and PCR analysis.

Materials and Methods

The fresh root and stem fragments from guava trees showing typical wilt symptoms were collected from Barisal, Brahmanbaria, Chittagong, Faridpur, Gazipur, Khagrachari, Pirojpur and Rangpur districts of Bangladesh. Collected samples were cut into 10 - 15 cm lengths washed in running tap water followed by sterile distilled water and then splited lengthwise and cut into thin small pieces (≤ 1 cm). The cut pieces were surface sterilized with 0.1% $HgCl_2$ solution. The

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surface sterilized inocula were plated on half-strength PDA medium with three inocula per Petri plate (9 cm in dia.) (Burgess *et al*. 1975). Inoculated Petri plates were incubated at 26 ±1ºC for five days. Pure culture was obtained through culturing macroconidia in water agar (Burgess *et al*. 1994).

Purified isolates were kept at 4ºC by culturing them on PDA slants and for long time preservation cultured on water soaked wheat bran medium. Well grown cultures in wheat bran were air dried, wrapped with brown paper and kept at 2 - 4ºC for further use.

Morphological studies of the isolates were carried out by culturing them on PDA plates and wheat bran at 26 ± 1ºC for five days. Colony colour, radial growth and sporulation were recorded for all the isolates. Morphological characters were studied following Burgess *et al*. (1994) and Aneja (2003). Colony diameters of the isolates were measured after three days of incubation at 26 ±1ºC. The colony morphology was recorded on the 12th day of incubation at 25 and 20ºC during day and night, respectively.

Fusarium oxysporum specific primers were designed using Primer 3 software (Steve and Skaletsky 2000) from the conserved regions of 18S rRNA gene of the Accession No. GQ131884.1. The selected forward primer (GCCAGAGGACCCCTAAACTC) and reverse primer (CATTTTG CTGCGTTCTTCAT) were expected to produce a PCR product with a band size of 118 bp. The primers were synthesized and supplied by BIONEER Inc., USA.

Genomic DNA of 16 isolates was extracted from mycelia grown in liquid medium. A modified DNA extraction method of Ali (2002) was followed in the present investigation and purified DNA samples were kept in – 20ºC freezer for further analysis.

The PCR was initiated by a initial denaturation step at 94ºC for 5 minutes following 35 cycles of 94, 54 and 72ºC each for 30 sec, with a final extension step of 5 min at 72ºC and ended with 4ºC. The PCR was carried out in an Eppendorf Mastercycler with 25 micro tubes capacity. PCR amplified products were stored in – 20ºC freezer for analysis by resolving in 2% agarose gel. The gel was prepared with ethidium bromide (final concentration 0.5 µg/ml) and after resolving the PCR amplified DNA the gel was viewed in a gel documentation system (microDoc, Cleaver Scientific Ltd.) to take picture.

Results and Discussion

During the present investigation the wilt causing fungi has been isolated from roots and stems of recently wilted guava plants. The causal organism of guava wilt was identified as *Fusarium oxysporum*. This identification is in agreement with the findings of other earlier workers (Prasad *et al*. 1952, Edward 1960a, Akanda and Mian 1981, Pandey and Dwivedi 1985, Dwivedi and Dwivedi 1994, Dolly *et al*. 2006 and Gupta *et al*. 2010).

Root and stem inocula produced 81.11 and 62.22% colonies of *F. oxysporum* on half-strength PDA medium. Finally, a total of 16 isolates of *F. oxysporum* were purified. The colony diameter of the pathogen ranged from 3.2 - 4.5 cm on the fourth day of inoculation in PDA medium (Table 1). The mycelia of the isolates were delicate, white to creamy and tinge pink or purple tinge, sparse to abundant than floccose, margins slightly lobed or smooth on PDA (Fig.1a - d). In water soaked wheat bran isolates produced pale to dark violet or dark magenta pigment.

Numerous micro- and macroconidia were observed in case of all the 16 isolates on PDA and water soaked wheat bran (Fig. 2 a, b). Microconidia formed singly, oval to reniform and without any septation. Conidiogenous cells bearing micro- and macroconidia were monophialides type. The size of microconidia ranged from 7.50 - 16.25 and 2.50 - 4.50 µm. Macroconidia were falcate to almost straight, usually 3-septed, rarely four to 5-septed, thin walled, both ends almost pointed, notched basal cell, apical cell short and in some cases slightly curved. Macroconidia were

produced in sporodochia as well as on normal hyphae and slimy conidial masses were also observed on water soaked wheat bran. The size of the macroconidia ranged from 20.27 - 40.50 and 5.00 - 6.75 µm (Table 1).

Table 1. Macroconidial measurement and colony diameter of 11 isolates of *Fusarium oxysporum* on water soaked wheat bran and PDA medium.

Source of isolates	Isolate number	Macroconidia (Length/ breadth µm) *	Colony diameter (4th day) cm	Chlamydospores
Kazi peyara BARI, Gazipur	Fop 1	25.28 - 31.12 /5.00	3.2	+
Kazi peyara BARI, Gazipur	Fop 2	28.75 - 35.51 /5.00	3.5	+
Mukundapuri B. Baria	Fop 4	27.75 - 35.49 /6.00	3.8	+
BARI peyara-2 Khagrachari	Fop 5	21.49 - 29.23 /5.00	3.8	+
Kanchannagar Chittagong	Fop 6	29.73 - 39.43 /6.75	4.2	+
Kazi peyara Bhora, Gazipur	Fop 7	20.27 - 25.57 /5.00	4.5	+
Kazi peyara Faridpur	Fop 9	26.17 - 38.27 /6.25	3.8	+
Kazi peyara Rangpur	Fop 11	28.50 - 40.50 /6.25	4.0	+
BARI-2 Khagrachari	Fop 13	24.37 - 30.67 /6.00	3.8	+
Kazi peyara Faridpur	Fop 14	26.76 - 34.24 /6.25	3.9	+
Kazi peyara BARI, Gazipur	Fop 16	27.50 - 32.50 /5.00	4.0	+

*Dimensions obtained from standard deviation of ten readings in each case using the formula $\sqrt{(X-X)^2/n-1}$.

Out of 16 isolates 11 produced chlamydospores on culture media. Chlamydospores were thick walled, terminal or intercalary, globose, smooth or wrinkled, generally single celled (6.25 - 9.25 µm) produced in hyphae and conidia. Chlamydospores were also found in two celled or in cluster (10.0 - 30.0/7.5 µm) and in chain form (17.5 - 30.0/7.5 µm).

The above mentioned morphological characters were considered as secondary criteria for the identification of *F. oxysporum* (Burgess *et al.* 1994). Booth (1977) considered the conidiogenous cell bearing microconidia as a primary taxonomic criterion and growth rate as a secondary criterion for the identification of *F. oxysporum*. The shape of macroconidia produced in sporodochia is one of the primary defining characteristics of *Fusarium* spp. and are preferred in identification purpose. Edward (1960b) reported the wilt causing pathogen *F. oxysporum* f. sp. *psidii* exists in a variety of clonal forms which differ in pathogenicity, morphological and cultural characters.

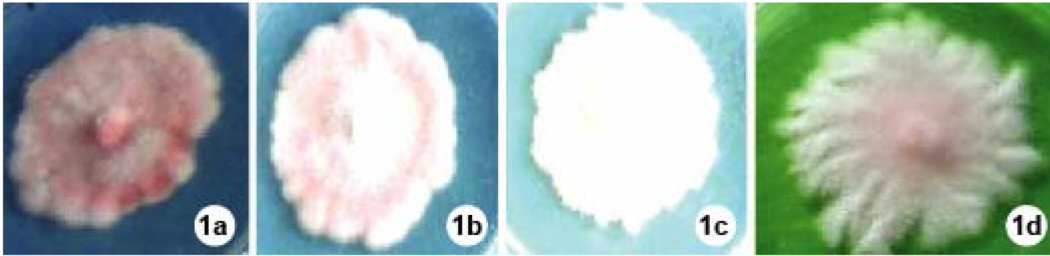

Fig. 1a - d. Colonial morphology of four virulent isolates of *Fusarium oxysporum* f. sp. *psidii*. Isolates a, b and d produced pink colony, c produced creamy white colony on PDA.

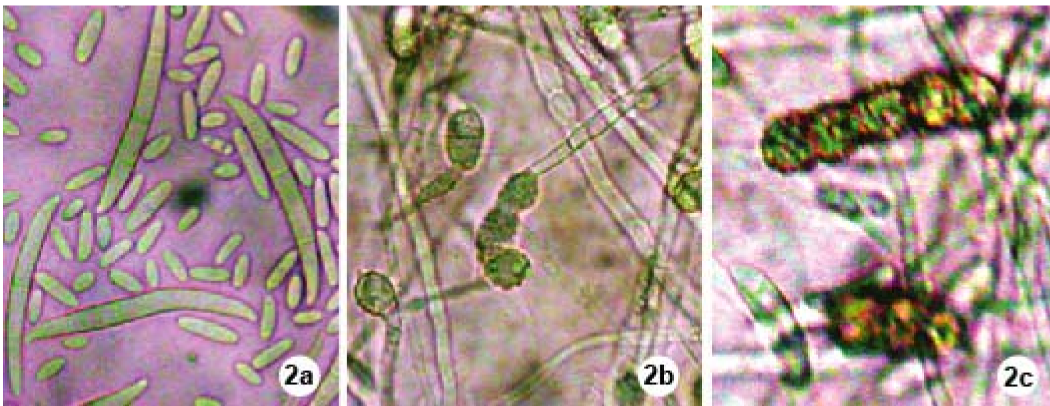

Fig. 2a-c: *Fusarium oxysporum* f. sp. *psidii*. a, macro- and microconidia; b, terminal and intercalary chlamydospores; c, chlamydospores in chain.

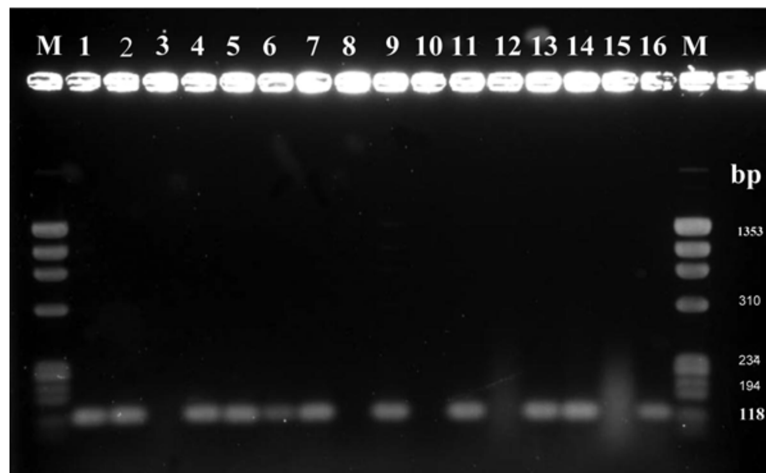

Fig. 3. PCR result of 16 isolates of *Fusarium oxysporum* by using specific primers. Lanes M are phi X 174 DNA/Hae III markers. Lanes 1 - 16 are DNA samples of *Fusarium oxysporum* isolates form wilted guava plants.

In the present investigation, sometimes it was difficult to distinguish *F. oxysporum* from other species of *Fusarium* based on the morphological features. This is in agreement with the findings of Gupta *et al*. (2010). Therefore, molecular characterization of the *F. oxysporum* was conducted for proper identification using PCR analysis. The genomic DNA isolated from morphologically identified 16 *F. oxysporum* isolates was subjected to PCR amplification. It was expected to amplify a 118 bp size fragment of the *F. oxysporum* 18S rRNA gene in the PCR amplification reaction using gene specific primers designed from the conserved regions. After agarose gel electrophoresis of the PCR amplified DNA it was observed that the selected primer pair exclusively amplified the expected 18S rDNA band of 118 bp size in 11 morphologically identified isolates of *F. oxysporum*. On the other hand, no amplification was observed in case of the isolates 3, 8, 10, 12 and 15 and was not considered as *F. oxysporum* (Fig. 3).

Development of specific primers for *F. moniliformae* was also reported by other workers (Murillo *et al*. 1998, Moller *et al*. 1999). Hence, the results described here proved that the primer pair allowed a fast, reliable and specific identification of *Fusarium oxysporum* isolates and could be suitable for early diagnosis of *Fusarium* wilt of guava by plant pathologists.

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