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ASSOCIATION OF NONPATHOGENIC *FUSARIUM OXYSPORUM* SPECIES WITH CULTURED SHOOT APICES OF BANANA (*MUSA ACUMINATA*) CULTIVARS

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ABSTRACT
Fusarium wilt of banana caused by *Fusarium oxysporum* f. sp.*cubense* is the most devastating disease worldwide. Management of disease is difficult through use of chemicals and very few cultivars are resistant. Nonpathogenic strains of *Fusarium oxysporum* act as biocontrol agent for the wilt disease by antagonizing and competing with the pathogenic strains for nutrients and root colonization sites. Shoot apices cultures of four banana cultivars Alpan, Malbhog, Robusta and Kothia on MS basal medium supplemented with 4.5mgL^{-1}BAP and 0.2mgL^{-1}IAA resulted in adventitious shoot differentiations. Some cultures showed appearance of endophytic fungal mycelium from the explant surface. Macroscopic and microscopic characterization of the isolated fungus suggested it to be *Fusarium oxysporum*. The fungal isolates were tested for their pathogenicity in tissue cultured plants of Fusarium wilt susceptible banana cv. Malbhog through root dip method. The non appearance of disease symptoms established the nonpathogenic status of the isolated *Fusarium oxysporum* strain. The frequency of association of non pathogenic *Fusarium oxysporum* was more in susceptible cvs. Alpan (13.65%) and Malbhog (13.07%) compared to resistant cv. Kothia (4.59%). Thus, there were endophytic association of non pathogenic *Fusarium oxysporum* strains in banana cultivars, which might be the reason of absence of the disease in the area.

INTRODUCTION

Fusarium oxysporum is a common and cosmopolitan soil born fungi. It includes a large diversity of pathogenic and non-pathogenic strains, all saprophytic and mostly parasitic (Burgess, 1981and Garrett, 1970). Due to their ability to utilize a large variety of nutrients, these strains can colonize the rhizospheres of various plants and also enter into the endophytic stage (Garrett, 1970). The pathogenic strains are very host specific. Based on their host plant species and cultivars, there are more than 53 forms, 117 formae specials and 29 varieties of pathogenic strains (Kaur *et al.*, 2010), which produces wilt disease in many diverse plant species. In banana,formae specials,*Fusarium oxysporum* f. sp. *cubense* (FOC) produces vascular wilt disease popularly known as Panama wilt. FOC consists of four races, eight lineages and 24 Vegetative Compatibility Groups (VCGs) (Li *et al.*, 2013). Panama wilt was the most disastrous disease in banana in the agricultural history of the world and it is still the most serious disease menacing many present day cultivars.

Banana is a large perennial herb and the most widely grown tropical fruit, cultivated over 130 countries, in the tropics and subtropical regions. India is the world's biggest banana grower with an annual production of 26.509 million tonnes from an area of 0.776 million hectares with productivity of 34.2 tonnes/ hectare. Bihar ranks 6th among states in banana production in India and produces 1.702 million tonnes annually in an area of 0.033 million hectares with productivity of 51.5 tonnes/ hectare (IHD, 2013). Many prominent banana cultivars grown in Bihar are susceptible to Panama wilt. Malbhog, the most delicious banana cultivar of Bihar, is now on the verge of extinction due to severe incidence (60-82%) of Panama wilt. Alpan, another important cultivar of Bihar was recorded to have 10-25 percent wilt incidence (RAU, 2015).

Banana's roots exuded chemical compounds in response to which chlamydospores of pathogenic *Fusarium* present in soil germinate and grow towards the nearby roots and colonize the vascular tissue, which in turn disturbed water translocation and movement of nutrients to the shoots.Non availability of water and nutrients results in chlorosis, necrosis and sagging of leaves. The plants ultimately fall down and die (Forsyth *et al.*, 2006). Since, *Fusarium* is a soil born pathogen and their chlamydospores are able to survive in soil for a long time, the management of Fusarium wilt is mainly through chemical soil fumigation, use of resistant cultivars and through biological control agents. The previous two methods are not much effective. Chemicals are unable to kill the chlamydospores due to their thick wall. Thus, long living chlamydospores are resistant to the chemical fumigation (Rishbeth, 1957 and Shi *et al.*, 1991). The fungicide applied once may not persist for long duration. In this context, the biologicalcontrol is an alternative strategy for disease management (Asha *et al.*, 2011).

Use of resistant cultivars is not so effective due to emergence of new races of the pathogen over time. Moreover many reports

suggested that nonpathogenic strains of *Fusarium* were effective against fusarial wilt as biological control agent in green house conditions as well as in field trials of various crops (Fuch *et al.*, 1999; Hervas *et al.*, 1998 and Honda and Kawakub, 1998). Presence of non pathogenic *Fusarium* strains antagonized the pathogenic strains for nutrients and root colonization sites. Such nonpathogenic strains are frequently observed in soil with no occurrence of Fusarium wilt (Forsyth *et al.*, 2006). The nonpathogenic endophytic strains inter into the plant system through root and induce systemic resistance against pathogen (Kaur *et al.*, 2010). Thus, the paper deals with isolation of nonpathogenic strains of *Fusarium oxysporum* from aseptic tissue cultured shoot apices of banana and discusses their role in biological control of Fusarium wilt.

MATERIALS AND METHODS

Plant material

The experiments were carried out using *in vitro* culture of four selected cultivars of banana, Robusta(AAA), Malbhog (AAB), Alpan (AAB) and Kothia (ABB). The cultivar Kothia is resistant, Malbhog and Alpan are susceptible and Robusta is moderately susceptible to Panama wilt disease. Banana suckers were collected from the experimental field of Department of Horticulture, Rajendra Agricultural University, Pusa, and were used as a source of explants.

Media preparation

Murashige and Skoog (MS,1962) basal medium supplemented with 4.5mgL^{-1}BAP and 0.2mgL^{-1}IAA was used for the inoculation of banana explant. Half strength potato dextrose agar medium supplement with 0.1g/L streptomycin was used for the isolation of *Fusarium* and Armstrong *Fusarium* medium for increasing the inoculum of isolates of *Fusarium* (Booth, 1977).

Sterilization and preparation of banana explants

Shoot tip meristem from suckers were used as explants. They were prepared and sterilized following the methods of Suman *et al.* (2013). Banana suckers were washed in running tap water for 20 minutes. These were trimmed to the size of 2-2.5cm containing shoot-tip meristem. The explants were washed in running tap water for 20 minutes and kept for another 20 minutes in pretreatment mixture solution having ascorbic acid (0.2%), citric acid (0.4%), streptomycin (0.1%) and bavastin (0.1%). The explants were dipped for 2 minutes in 0.2% solution of $HgCl_2$, followed by washing with sterile distilled water for three times.The explants were further trimmed to the size of 1-1.5cm and inoculated onto the prepared medium (Fig. 2a). The cultures were maintained at a temperature of 25 $\pm$ 2ºC, with a photoperiod of 16 h/day.

Shoot Initiation and Association of Fungi

Shoot initiation from the shoot tip took place nearly one and half months after the inoculation. Few of the cultured explants showed a little whitish cottony growth of fungi on its external surface, which later covered the medium. These associated fungi were isolated from the explants surface on half strength potato dextrose agar medium, followed by sterile transfer in potato dextrose agar (PDA) slant. The fungal isolates were stained with cotton blue and studied under microscope for morphological characterization and identification.These observations led to the identification of the species *Fusarium oxysporum*. After one week, the mycelium from the cultures were transferred to Armstrong *Fusarium* medium to create spore suspension in 250mL Erlenmeyer flasks and kept on shaking incubator at 170 rpm and 25ºC. The spore suspension was passed through cheesecloth after five days to separate the mycelia from the spores and the filtrate was diluted to a final concentration of 10^5 to 10^6 spores mL^{-1}. The prepared spore suspension was used for testing their pathogenicity. Following the root dip method, the roots of *in vitro* developed plantlets were injured with sterilized scalpel followed by dipping in spore suspension for 30 minutes in laminar air flow and the plantlets were transferred to the rooting medium (Venkatesh *et al.*, 2013). The data were collected weekly for contamination and establishment of the explants. Nonpathogenic *Fusarium oxysporum* association with the cultured shoot apices was observed in the established sterile cultures. All the data were analyzed by executing one factor analysis of variance (ANOVA) using OP Stat.

RESULTS AND DISCUSSION

Establishment of the sterile and alive cultures was the first requirement of tissue culture. It excluded contaminated and dead cultures. Contamination of the cultures was the major problem of establishment. Establishment of cultured shoot apices of banana cultivars showed a frequency of 89.165% in cv. Malbhog to 92.498% in cv. Robusta (Fig. 1). The statistical analysis of the data suggested that there was no effect of genotype on the frequency of establishment of cultured shoot apices. The establishment depended on the surface sterilization of cultured shoot apices and not on the genotype of the donor banana plant. All the contaminations that appeared within the first week of culture were considered and were discarded in calculating the establishment frequency.The established shoot apices cultures showed initiation of adventitious shoot formation after three to four weeks of culture (Fig. 2b). The MS basal medium supplemented with 4.5mgL^{-1}BAP and 0.2mgL^{-1}IAA was suitable for adventitious shoot formation in banana genotypes. The explants before inoculation were healthy and free from any spot and externally

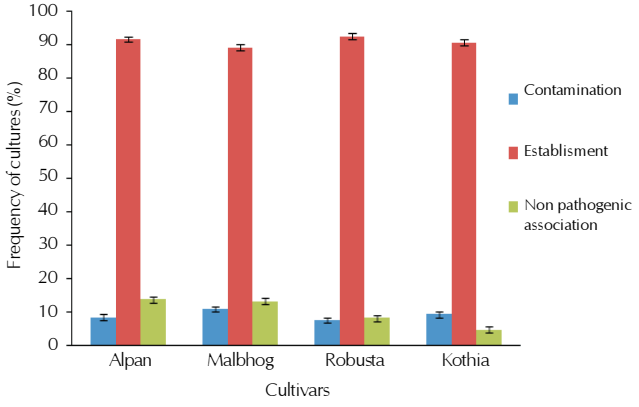

Figure 1: Effect of cultivars on frequency of cultured shoot apices of banana on establishment, nonpathogenic association and contamination

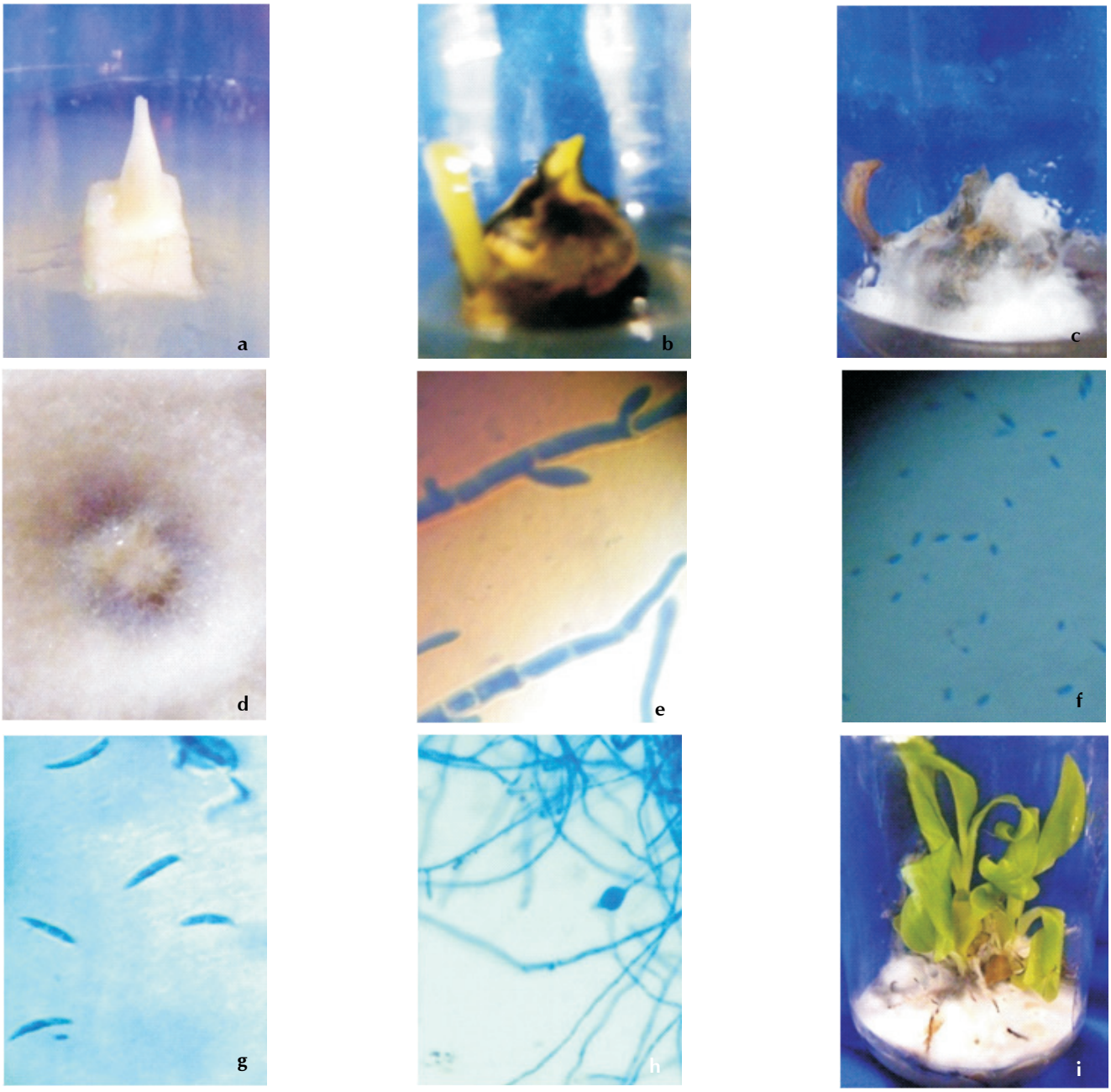

Figure 2: (a) Cultured shoot tip explant, (b) Adventitious shoot formation, (c) Appearance of enophytic non pathogenic *Fusarium oxysporum* mycelia on explants, (d) Isolated *Fusarium oxysporum* on half strength PDA, (e) Production of pale violet macroconidia in central spore mass (f) Septate hyphae and phialides of isolated fungus, (g) Abundent microconidia, (h)Macroconidia, (i) Terminal chlamydospores, (j) Tissue cultured plant of susceptible cv. Malbhog inoculated with isolate of *Fusarium oxysporum* without any symptom of wilt disease

did not show any symptom of being infected. The sterile nature of the explants and the expected tissue culture responses suggested that the explants were normal and free from any contaminations. Some of the cultured and responding shoot apices showed appearance of fungal mycelium on their surface after sixth week of culture.These fungal mycelia soon covered the entire surface of the explants and later the media (Fig. 2c). The mycelium of fungi appeared from the surface of explants in standard sterile cultural conditions, suggesting that these fungi were internally associated with the explants and thus corroborated endophytic association of the fungi (Li-Sha *et al.*, 2013). The frequency of such fungal association ranged from 4.59% in cv. Kothia to 13.65% in cv. Alpan (Fig. 1). The frequency of such fungal association was more in susceptible cultivars of banana namely Alpan (13.65%) and Malbhog (13.06%) compared to resistant one (cv. Kothia). The association was fair in moderately susceptible cultivar Robusta (8.09%). The associated fungi were isolated, purified and morphologically characterized macroscopically and microscopically.

Macroscopic features

The fungal isolates from cultured shoot apices of banana cultivars grew rapidly on half strength potato dextrose agar medium and produced white cottony, floccose, spreading colonies. Production of pale violet colour was observed in central spore mass on PDA medium (Fig. 2d). Strains of *Fusarium oxysporum* usually produced pale violet to magenta pigment on agar plates. Moreover, the ability of the isolated fungus to grow on half strength PDA medium suggested that the fungus might be *Fusarium* species (Lesile *et al.*, 2006). The isolates rapidly grew on PDA and produced colony different from that observed on half strength medium. It was more floccose, cottony and pinkish in colour. Such colonies were observed in case of *Fusarium oxysporum* cultures on PDA (Nelson *et al.*, 1983 and Sutton *et al.*, 1998).

Microscopic features

Fungal isolates had divisions within hyphae or hyphae were septate (Fig. 2e). Conidiophores or phialides were short, simple, non-septate and laterally arranged on mycelium (Fig. 2e). These monophialides were inflated structures puffed up at the middle portion. Short monophialides produced microconidia, which were abundantly present on the aerial mycelia. Microconidia were non-septate, ellipsoidal to cylindrical, slightly curved or straight, 5-14 x 2.0-4.0 μm in size and numerous in numbers (Fig. 2f). Besides microconidia, macroconidia and chlamydospores were also observed in the fungal isolates. Macroconidia were copious, boat shaped or fusiform in shape, thin-walled, with an attenuated apical cell and mostly pointed basal cell (Fig. 2g). They were three to five-septate measuring 20-50 x 3-5 μm. Chlamydospores (5-15μm diameter) were terminal in position, and occurring singly on the tip of mycelium (Fig. 2h).

The macroscopic and microscopic characters of the isolated fungus suggested it to be *Fusarium oxysporum* (Lesile *et al.*, 2006). Since this fungus has been found associated internally with the shoot apices taken from healthy rhizomes developing from a healthy banana plant without any symptom of wilt disease, it might be nonpathogenic *Fusarium oxysporum*.

Assessment of pathogenicity

Macroscopic morphological observation of growth pattern and microscopic investigation of mycelium of isolated fungal strains confirmed the species as *Fusarium oxysporum*. The isolates were tested for their pathogenicity in banana tissue culture plants. Introduction of spore suspension to potted plants were found to be an effective method for the induction and test of pathogenicity (Matsumoto *et al.*, 1995). However, Venkatesh *et al.* (2013) found that root dipped method was more efficient for the induction of Fusarium wilt disease in banana plantlets.

These fungal isolates when introduced into well established tissue culture plantlets of susceptible cv. Malbhog via root dipped method produced no symptoms of disease within 15 days of incubation. The mycelia spread over the medium after 25 days of incubation. The plantlets remained healthy in the presence of fungal mycelium and thus provided evidence for nonpathogenic nature of the isolated strain (Fig. 2i). Their nonpathogenic nature was proved from the fact that the source plants, from which the explants were obtained and consequently these *Fusarium oxysporum* strains were isolated, showed no external wilt disease symptoms. Thus, the nonpathogenic *Fusarium oxysporum* strains showed associations with all the four cultivars of banana that were susceptible, moderately susceptible and resistant to Panama wilt disease.

The nonpathogenic strain of *Fusarium oxysporum* was found to be associated with the cultured shoot apices. The occurrence of such association was proportional to the susceptibility of the genotype to the wilt pathogen. The susceptible genotypes could not distinguish between the pathogenic and non pathogenic strains of *Fusarium oxysporum* and thus allowed the entry and establishment of more associations. The resistant genotypes have mechanism to resist the entry of pathogenic *Fusarium oxysporum* and might also restrict the entry of non pathogenic strains thus limiting such associations. Many endophytes form mutuallistic relationships with their host plants, from which they obtain nutrients and in turn confer protection against biotic and abiotic stresses to the plant (Schulz and Boyle, 2005 and Anu Rajan *et al.*, 2013). Some researchers demonstrated that nonpathogenic population shows microbial antagonism with pathogenic population of *Fusarium* principally for nutrients and infection sites (Edal *et al.*, 1997). Whereas, other reports suggested that it helped the plant in developing resistance against the disease (Duijff *et al.*, 1998 and Fravel *et al.*, 2003). The prevalence of nonpathogenic strains of *Fusarium oxysporum* and the antagonistic correlation between the pathogenic and nonpathogenic strains may be one of the reasons of absence of disease incidence at the farm land of banana from where the healthy shoot apices were collected. Such nonpathogenic *Fusarium oxysporum* strains have been found in areas with no disease incidence (Alabouvette *et al.*, 1993 and Larkin *et al.*, 1996). Mechanism of action of these strains varies with their genotype(Larkin and Fravel, 1999). Two of the nonpathogenic *Fusarium oxysporum* isolates CAV 255 and CAV 241, reduced Fusarium wilt incidence by 87.4 and 75.0%, respectively (Nel *et al.*, 2006). Thus, the study showed the enophytic association of nonpathogenic strains of *Fusarium oxysporum* in the roots and rhizomes of banana cultivars at Pusa (Samastipur, Bihar, India) and suggested their exploitation as biocontrol agent against the most dreaded wilt disease of banana.

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