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IMMUNOGOLD LOCALIZATION OF FUNGUS *Fusarium mangiferae* IN MALFORMED BUDS OF MANGO

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ABSTRACT

The studies to ascertain the etiological agent of mango malformation through immunogold labeling were pioneered in the world to our knowledge. On examination of ultra thin bud sections under Transmission Electron Microscope (TEM), inter and intracellular presence of fungal hyphae indicating fungal infection in malformed bud tissues of cultivar Malda was found. No fungus was detected in healthy tissues, cells or intercellular spaces. Immunogold labeling revealed the gold labeled localization of FM-2 and FM-17 isolates of causal fungus *Fusarium mangiferae* using anti rabbit immunoglobulin G (IgG). A characteristic gathering of gold granules on the isolate specific hyphal cross sections was observed. The results derived from a novel electron microscopical technique constitute an additional support to unveil the myth and mystery of mango malformation. The present studies clued to explore the potential infection site and provided an excellent and precise confirmation that a fungus, identified as, *F. mangiferae*, is responsible for this malady. These findings will be helpful to end the conflict regarding the causative relationship of malformation and devise the target specific management strategies to curb mango malformation in Pakistan.

Key words: *Mangifera indica*, malformation, infection site, immunoglobulin, gold labeling

INTRODUCTION

Mango (*Mangifera indica* L.) ranks second in area and production in Pakistan. In 2003, 26.28 million tonnes fruits was produced globally. In Pakistan the area under mango cultivation is 0.1 million ha with an annual production of 1.072 million tonnes (FAO, 2004). Mango malformation has become a crux amongst animate diseases of mango. It's the major obstacle in the establishment of profitable orchards. Since its first record, it is intriguing the scientists as to its cause and control. The disease affects vegetative shoots of juvenile and adult plants leading to extensive losses in both nurseries and orchards. The disease also affects floral panicles resulting in phyllody and hypertrophy without necrosis. The etiological relationships viz. nutritional, acarological and physiological have been refuted (Kanwar and Kahlon, 1987; Das et al., 1989;

Singh, 1997). Results from electron microscopy, transmission, cultural and serological studies have disapproved the involvement of a virus or mycoplasmal agent (Kishtah et al., 1985). Recent literature supports the evidence that a fungus *Fusarium mangiferae* (Britz.) (synonym: *F. subglutinans* P.E. Nelson, T.A. Toussoun & Marasas Comb.nov) is the causal agent.

Mango buds are considered to be the primary infection site. Freeman et al. (1999) unequivocally proved the causal relationship between *F. mangiferae* and mango malformation. They transformed a wild type isolate with a GUS reporter gene and the hygromycin resistant gene. After inoculation to healthy plants, the presence of mycelia in floral and vegetative buds of symptomatic plants was viewed microscopically that stained positive for GUS, thus fulfilling Koch's postulates.

The disease is more severe in grafted or budded mango trees and the transmission of disease through buds taken from branches of trees bearing malformed inflorescences and budded on the branches of a healthy mango tree is possible (Kausar, 1959). Barkouki et al. (1978) observed the histological changes in dormant terminal buds of Taimoor trees to observe the symptoms of floral malformation. A modification in the anatomical features of some buds was observed at the 4th stage in January. The buds might bear the malformed tissues and produce the malformed organs. Scanning electron microscopic studies exhibited fungal mycelial infection at the base of the malformed buds. The fungus was later identified as *F. subglutinans* [*mangiferae*] (Usha et al., 1997).

A variety of detection systems are employed to identify the pathogenic organisms. Biologically based detection methods provide potentially the most effective systems as they involve the direct measurement of fungal activity and/or presence. One such system is the immunoassay, which uses the specific interaction of antigen with antibody to tell about the concentration of antigen or antibody in samples (Edwards, 1985). The potential of *Bacillus pumilus* (PGPR strain SE 34) for introducing defense reaction in tomato plants inoculated with the vascular fungus, *F. oxysporum* f.sp. *radicis lycopersici* was studied by Transmission electron microscopy. When gold complex was applied to tissue sections, labeling was found strictly associated with the walls of invading *Fusarium* hyphae (Benhamou et al., 1998).

The main objectives of the present study were to examine the healthy and malformed mango buds at ultrastructural level to explore the potential infection site and confirm the etiological agent. This information will be useful in assessing future efforts to eradicate the inoculum and manage the major floral disorder in Pakistan.

MATERIALS AND METHODS

Ultrastructural Assay

1. Sample's Collection

For ultrastructural assay, the studies were carried out in a local variety Malda. The trees of uniform age (10 years old), growing under similar soil and climatic conditions were selected for the study. Young emerging and fully swollen diseased and healthy buds were obtained from malformed and healthy branches, respectively. The respective branches were tagged in the preceding flowering cycle of the year, 2002. Healthy buds excised from non malformed branches served as control.

2. Culture Proliferation

The sterilized 5 mm long bud pieces excised from healthy and malformed buds were immersed in liquid potato dextrose medium in 1.5 ml eppendorf tubes. The tubes were incubated at 25°C with 12 hr alternating cycles of light and darkness for 96 hours. The proliferating fungal growth picked from malformed bud pieces under aseptic conditions was subjected to microscopic examination. The culture was pelleted by centrifugation at 10,000 rpm, for ultrastructure examination. Healthy bud pieces yielded no growth of the fungus.

3. Culture and Tissue Processing

Tissue pieces and fungal culture were placed in individual sterilized 1.5 ml eppendorf tubes and processed for TEM. Glutaraldehyde (5%) was added and left for vacuum infiltration/fixation for 16-18 hr. Fixative was replaced with 0.2 M PIPES buffer, pH 6.8 and washed 2 x 1 hr in fresh buffer. Post fixation for ultrastructure was done with 0.2% osmium tetroxide made in 0.2 M PIPES buffer, pH 6.8, for 16-18 hrs and washed 2 x 30 min with PIPES and then 1x15 min with distilled water. The samples were treated with 5% aqueous uranyl acetate for 16-18 hrs and washed with sterilized distilled water for 2 x 30 min. Dehydration was done through a graded alcohol series for 30 min each in 30, 50 and 70% ethanol. Absolute alcohol was added twice, keeping for 2 x 30 minutes each time, followed by addition of fresh alcohol and kept over night (16-18 hr). Alcohol was exchanged with 100% acetone (2 x 15 min). Fresh acetone was added and kept overnight (16-18 hr). Infiltration was done for 16-18 hr in 1:1 acetone/spur resin, then for 16-18 hr in spur resin alone. The samples were transferred to flat embedding moulds and polymerized for 72 hr at 62°C. Polymerized resin blocks were removed from oven and left at room temperature for at least 24 hours before cutting ultrathin sections (Roberts, 2002). Ultrathin sections 120-200 nm thick were cut on Ultramicrotome RMC-7000 and ribbons of sections were lifted on copper grids. Sections were double stained with uranyl acetate and lead citrate and examined under JEOL JEM-1010, TEM (Japan) operating at 80 kv.

Immunogold Labeling

1. Sample's Collection

Infected mango shoots bearing malformed panicles were collected randomly from different cultivars after surveying different districts of Pakistan during the year 2002. The samples were kept in ice box immediately after clipping to avoid heating during transit and ensure maximum recovery of the fungus.

2. In vitro Culture

Ten tissue pieces 5 mm long, excised from healthy and malformed buds of each sample were surface disinfested for 10 sec in 70% ethanol and for 2 min in 1% NaOCl solution. The tissues were rinsed twice in sterilized deionized water, dried on sterile blotting papers and placed into 9 cm wide glass petri plates containing potato dextrose agar (PDA) medium (Ploetz and Gregory, 1993). The plates were incubated at 25°C temperature under cool white light with a photoperiod of 12 h light and 12 h darkness. Identification to the species was verified by typical microconidia, macroconidia, morphology and growth pattern (Nelson et al., 1983; Britz et al., 2002). The isolates were designated with different prefixion keeping in view their origins. The cultures were lyophilized and additional copies were stored at 4°C for future use.

Three isolates viz. FM-2, FM-14 and FM-17 identified from Katkan Allahabadi, Chaunsa and Langra cultivars, respectively were subjected to immunolocalization (Roberts, 2002). The tagged buds of cultivars infected with respective isolates were processed as described under ultrastructural assay except that treatment with osmium tetraoxide and uranyl acetate was omitted and ultra-thin sections were finally lifted on plastic coated nickel grids.

3. Preparation of Antigen

The isolates were revived in static liquid potato dextrose medium at 25°C for ten days until mycelia covered the top of culture in 250 ml glass flask and exhibited typical pigmentation. Fungal mats were harvested by filtration through Whatman No.1 filter paper, washed with distilled water until the filtrate ran clear and freeze dried. Each individual antigen suspension was prepared by grinding lyophilized mycelium in a mortar and pestle and mixing it with phosphate-buffered saline (PBS 0.01 M, pH 7.4) to form slurry.

4. Production of Antisera

Antisera were raised in New Zealand six month old white albino female rabbits against a whole cell antigen preparation of *F. mangiferae*. For immunization, mycelial preparations were used at a concentration of 5mg ml⁻¹ PBS. One and a half ml of mycelial slurry was mixed with an

equal volume of Freund's complete adjuvant to give an emulsion which was then inoculated subcutaneously at six dorsal sites. Booster injections of the same antigen preparation mixed with Freund's incomplete adjuvant were given two weeks later. Rabbits were bled from the marginal ear vein 10 days after the booster injection. Subsequently, rabbits were repeatedly given booster injections followed by three bleeds at fortnightly intervals (Glancy et al., 1989). Trial bleed was done from marginal ear vein for titer determination through agglutination (serial dilution).

Blood (30-50 ml) was obtained by cardiac puncture from rabbit and allowed to clot overnight. The serum was separated and centrifuged at 15000 rpm for 15 min at 4°C to remove red blood cells. Merthiolate (final concentration 1:10,000) was added and stored in sterilized tubes at -20°C.

5. Fractionation of Serum Globulins

Serum collected from rabbits contained many kinds of proteins such as albumin, L, B, M, G globulin etc. Protein that acts as antibody is a globulin called as immunoglobulin (Ig). For purification of immunoglobulin G (IgG's), equal volume of cold 4M (NH₄)₂ SO₄ was added drop wise by constant stirring to each serum placed in crushed ice to precipitate the serum globulins. The mixture was left overnight at 4°C. Separation of globulins was done by centrifugation at 10,000 rpm for 20 min, discarding the supernatant and dissolving the precipitates in saline to assign the original volume. Precipitation and centrifugation procedure was repeated thrice to render the globulins completely white and free of hemoglobin. Globulins were desalted by dialysis against 0.85% saline (pH 8) in a cold cabinet with constant stirring overnight and stored in sterilized test tubes at -20°C.

Immunogold labeling (IGL) of sections

Sample Processing

Ultrathin sections of buds processed for IGL were mounted on plastic coated nickel grids. Petridishes were prepared with wet filter papers as incubation chambers and waxed slides were used for the treatment drops. Grids were floated on 30-40 µl drop of immunogold labeling buffer (IGL) for 1 hr. Grids were removed, drained, transferred to drops of antisera diluted in IGL buffer (1:100) and incubated at room temperature (25-26°C) for 16-18 hr. Grids were picked, drained and washed twice for 10 min on IGL buffer. Sections were thereafter transferred to a drop of colloidal gold probe diluted 1:50 with IGL buffer for 4-6 hr. Grids were rinsed with IGL buffer and subsequently with distilled water for 10 min each and allowed to dry before staining. Finally grids were double stained with uranyl acetate and lead citrate and observed and photographed on JEOL 1010 T E M.

RESULTS

Ultrastructural Studies

1. Culture Proliferation

The in vitro grown fungal culture yielded from malformed buds of Malda cultivar revealed longitudinal and crosswise arranged sections of hyphae when observed under TEM (Figure 1A). The longitudinal sections of hyphae were septate. Oval to fusiform, single celled, microconidium, characteristic feature of *F. mangiferae* was also observed with similar morphology as under light microscope but varying in magnification and degree of resolution.

2. Tissue Infection

On examination of ultra thin bud sections, no fungus was detected in healthy tissues, cells or intercellular spaces. Well organized and clear cell morphology was observed in case of healthy tissues of Malda (Figure 1B). No such distinction could be made in tissues of malformed buds wherein cell walls were mostly thick and closely packed. Invasion of the fungus was inter and intracellular (Figure 2).

Immunogold Labeling

The technique was used to study the serological relationship of *F. mangiferae* to develop a diagnostic test for the organism. The immunolocalization of isolate FM-2 showed highly specific labeling with proteins of fungal fragments only. Osmicated and double stained cross section of the hypha displayed intermediate labeling at higher magnification. An intense gold labeling was observed on hyphal cross section of FM-17. The ultrathin sections trimmed from buds infected with isolate FM-2 showed labeling specificity when sections coated with respective IgG were examined under TEM. Gold particles, 15 nm in size were seen dusted with peculiarity over the surface of osmicated cross sections lying within tissue of malformed bud only.

FM-2 and FM-17 were precisely localized against which antisera were raised. However no gold labeling was observed on samples of FM-14. Specificity of gold labeling was assessed by comparison with healthy parts. Immunogold labeling was absent in healthy tissues.

DISCUSSION

Ultrastructural Assay

The etiology of mango malformation has remained controversial since its first manifestation. Although latest literature supports the evidence that a fungus *F. mangiferae* is responsible for disease causation, doubts still exist about the etiology as the problem defies complete solution. Dearth of reliable information has led to misconceptions. Complementary informations at the ultrastructural level were essential to provide further insight to unfold the cause of the disease.

The results of the present study demonstrate that a fungus *F. mangiferae* is the cause of mango malformation. Examination of ultra thin bud sections revealed that successful penetration of the fungus was both inter and intracellular. This is in conformity with the findings of Kumar and Beniwal (1992) who reported inter and intracellular invasion by the fungus. When hyphae of the pathogen multiplied extensively, they started filling the space originally occupied by the host cytoplasm. This massive cell colonization coincided with the expression of external symptoms as swelling and hypertrophy of bud cells.

The mango buds are reported to be the primary site of infection. A single macroconidium which lands the bud can cause infection. Ramification ensues and the cells of the buds are conditioned to produce malformed growth. Spin lattice relaxation time (T_1) measured using nuclear magnetic resonance (NMR) technique in different tissues showed that the malformed parts had a higher T_1 when compared with healthier parts and seemed to be related to a lower solute concentration in the malformed parts. NMR technique also showed that primary site of infection is probably the meristematic region (Usha et al., 1996).

Usha et al. (1997) using scanning electron microscope found fungal infection at the base of fully swollen malformed buds of variety Amrapali. No such mycelial growth was observed in healthy buds. As the fungus could not be identified with scanning electron micrographs, healthy and malformed tissues were cultured in vitro. Mycelial growth of *F. subglutinans* [*mangiferae*] formed on the 4th day from malformed tissues only. Among other possibilities this may suggest that the fungus, which is closer to vascular channels of the mother plant, competes for the nutrients by acting as a more powerful sink than the buds of the malformed inflorescence and could be a reason for the low uptake of assimilates by the malformed buds.

The present ultrastructural studies supported the preceding findings wherein *F. mangiferae* proved to be the dominant fungus. The current data provide convincing evidence for novel subcellular localization of the same fungus.

Immunogold Labeling

This is the first report in the world on immunogold labeling of *F. mangiferae* within the malformed tissues of mango buds. The results indicate that identification by immunological methods is feasible through antisera using mycelial extracts as immunogens. The polyclonal antisera produced were found specific towards the isolates of *F. mangiferae*. The immunolocalization proved to be a complement to ultrastructural studies. Polyclonal antisera have already reported to be raised against the mycelial/spore suspension of *F. oxysporum* and *Tilletia indica* (Chakraborty et al., 1997; Gupta et al., 2001).

Antisera conjugated gold labeling on the fungal sections confirmed the specificity for the isolate against which antisera were actually raised. Osmicated, electron dense cross sections identified from culture, matched precisely with similarly treated sections carrying gold particles, observed within tissues. Incubation of ultrathin sections with the fungal IgG's resulted in discernible gold labeling. The specific labeling was found strictly associated with the sections of invading hyphae and the pattern obtained with the gold complex confirmed that isolates successfully penetrated tissues of the bud. The same uniform distribution of gold particles was observed in immunogold application against *Pneumocystis carinii* too (Kwon et al., 1998). The labeling density in non specific parts was absent. Non-osmicated cross sections did not exhibit well defined organelles but almost equally good labeling was observed. This finding suggested the binding of antibody to specific fungal cell components. Meagre immunogold labeling was evident on plant cell wall due to nonspecific binding or some common proteins of prokaryotic system. Although some antibodies or antibody fragments might be fused with plant components as confirmed by immunogold electron microscopy as being secreted through the cell wall, despite, cell wall porosity limitations retained other antibodies in the cytoplasm (Yuan et al., 2000).

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FIGURES

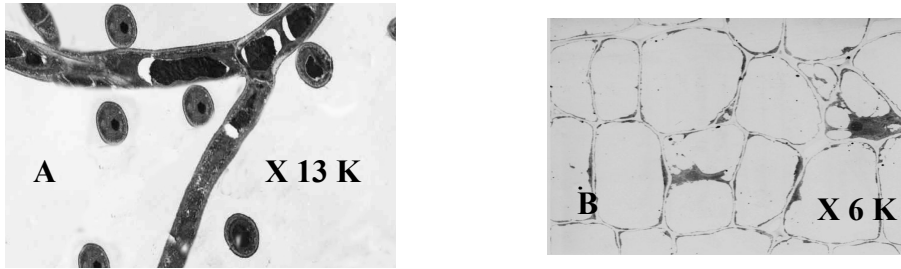


Figure 1:

A= Cross and longitudinal sections of fugal hyphae yielded from culture of *F. mangiferae*.

B= Healthy bud cells of Malda cultivar free of fungal infection.

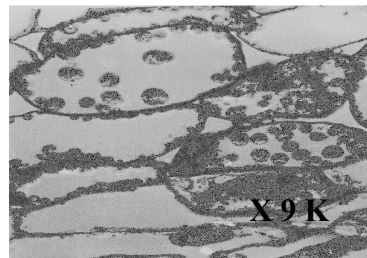


Figure 2: Intracellular cross sections of hyphae.