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In vitro

Response of plant growth regulators and antimalformins on conidia germination of *Fusarium mangiferae* and incidence of mango malformation

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Abbreviations: BAP, 6-benzylaminopurine; CDA, Czepeck's dox agar; CLA, carnation leaf agar; GA_3, Gibberellic acid; $HgCl_2$, mercuric chloride; NAA, naphthalene acetic acid; PDA, potato dextrose agar

Mango malformation is the most important and threatening disease of recent times, primarily because of persistent lacuna in complete understanding of its nature. Diverse *Fusarium* spp, including *F. mangiferae*, were found to be associated with the disease. Here, *F. mangiferae* from mango cv Dashehri was morphologically characterized. Typically, oval-shaped microconidia without septum and crescent-shaped macroconidia with 3-septate were more often observed, whereas not a single chlamydospore was detected. The length and width of micro- and macro-conidia were 7.5, 55, 3.2, and 3.5, respectively. The plant growth regulators such as NAA, GA_3, BAP and ethrel were found to induce in vitro germination of conidia of *F. mangiferae* after 12 h. In contrast, antimalformin silver nitrate ($AgNO_3$) inhibits conidial germination in vitro and none of conidia was germinated beyond 500 ppm, however antimalformin glutathione was highly effective in stimulating conidial germination of *F. mangiferae* in vitro at > 1000 ppm after 24 h. We observed that the response of *F. mangiferae* to germinate the conidia in vitro under influence of plant growth regulators and antimalformins is not coincided with earlier findings of reduced disease incidence by exogenous application of these compounds. The present findings do not authenticate the involvement of *F. mangiferae* in the disease, however hormonal imbalance, most probably ethylene, might be responsible for deformed functional morphology of panicle. Further, a signal transduction mechanism of stress-stimulated ethylene imbalance causing physio-morphological changes in reproductive organs of mango flower and thereby failure of fertilization and fruit set, which needs to be investigated.

Mango malformation affects mango production in several tropical and subtropical countries, which leads to 50–80% fiscal losses each year.[1] It received great interest not only because of its widespread and deleterious nature, but also because of its unresolved etiology till date.[2] Mango malformation was recognized in India at Darbhanga District of Bihar in 1981.[3] Now, it has also been recognized in several mango producing countries, such as Pakistan, the Middle East, Egypt, South Africa, Brazil, Sudan, Central America, Mexico, the USA, Cuba, Malaysia, Australia, Israel, UAE, Bangladesh, and the Sultanate of Oman.[4] The disease has been multifariously ascribed to be acarological, viral, fungal, and physiological in nature.[5] The different species of *Fusarium*, including *F. mangiferae*, were found to be associated with the disease.[6] However, the latest reports claimed that "stress ethylene" production could be involved in causing the disease.[7]

Morphological study of *Fusraium* sp revealed that colony color, hypha type, shape and size of macro- and micro-conidia, conidiogenesis, and presecnece or absence of chlamydospore are the noteworthy features employed in the characterization of *Fusraium mangiferae*.[8] The exogenous application of plant growth regulators and its inhibitors, so called antimalformins, has resulted into the control of floral malformation,[1,9] explaining the fact that hormonal imbalance and environmental stresses may act in cooperative manner to develop the disease.[7,10,11] In the present study, in vitro conidia germination technique was used to evaluate the differential response *F. mangiferae* for its tolerance or susceptibility under various treatments of plant hormones and antimalformins in relation to disease incidence.

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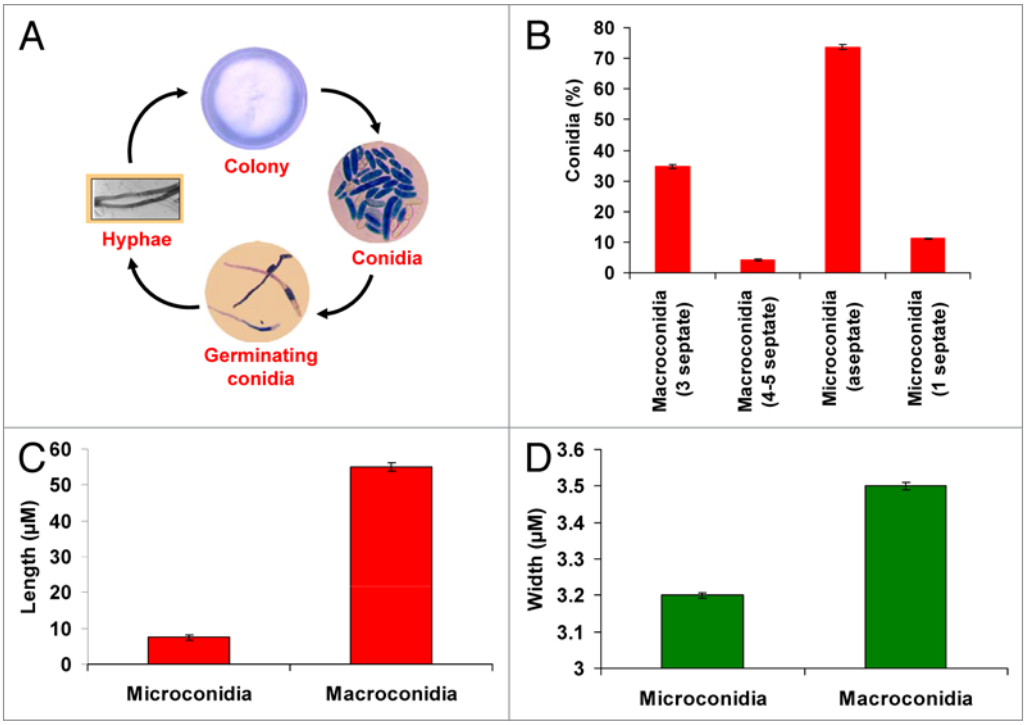

Figure 1. Morphologically diagnostic features in a life cycle of *F. mangiferae*. (**A**) The pure colony of *F. mangiferae* obtained from malformed floral tissues of mango cv Dashehri via single spore isolation technique depicting light purple along with white color. The crescent-shaped and usually 3-septate or rarely 4–5 septate macroconidia, as well as oval- to elliptical-shaped microconidia, either devoiding of septum or a few consisting one septum, were present. Both the conidia showing germination in vitro at suitable temperature (27°C) leading to the development of mycelial mat showing a long septate hyphae. (**B**) The type and number of conidia may vary in axenic suspension culture of *F. mangiferae*. (**C**) The length of micro- and macro-conidia was 7.5 and 55, respectively. (**D**) The width of micro- and macro-conidia was confined to 3.2 and 3.5, respectively.

Results

Isolation and morphological characterization of *F. mangiferae*. *F. mangiferae* isolated from malformed floral tissues of Dashehri cultivar of mango demonstrated light purple in addition white colony color. Microscopic study *F. mangiferae* revealed the presence of micro- and macro-conidia, however chlamydospores were entirely absent. Both the micro- and macro-conidia may germinate at a suitable temperature (27°C) and reproduce mycelial mat consisting of long septate hyphae (**Fig. 1A**). Macroconidia were somewhat crescent shaped. 3-septate and 4-celled macroconidia were 34.8%, whereas 4–5 septate and 5–6 celled macroconidia were 4.2%. The oval-to-elliptical shaped aseptate microconidia were present in large numbers (73.7%), however few microconidia partitioned with septa (1 septate) (11.2%) were also observed (**Fig. 1A–B**). The length and width of micro- and macro-conidia were 7.5, 55, 3.2, and 3.5, respectively (**Fig. 1C–D**).

Effect of naphthalene acetic acid, gibberellic acid, 6-benzylaminopurine, and ethrel on conidia germination of *F. mangiferae* in vitro. The plant growth regulators *viz.,* naphthalene acetic acid (NAA) and gibberellic acid (GA_3) were tested for their effect on conidia germination of *F. mangiferae*. Naphthalene acetic acid (NAA) and gibberellic acid glutathione (GA_3) were observed to have stimulatory effect on conidia germination of *F. mangiferae* after 12 h of incubation at 27°C. With increasing concentration of NAA (0–500 ppm), germination of conidia of *F. mangiferae* were found to gradually enhance. The highest conidial germination (82.47%) were recorded at 500 ppm (69.62%) with respect to control, as well as 25 ppm NAA (69.49%) (**Fig. 2A**). Likewise, the treatment of GA_3 resulted into inductive response on condial germination of *F. mangiferae*. Conidia germination was progressively increased with raising the GA_3 concentration (0–500 ppm), and the response was higher at 500 ppm (89.66%) as compared with control (70.86%) (**Fig. 2B**). Similar trends were observed in the case of 6-benzylaminopurine (BAP) and ethylene releasing agent (ethrel) applied to conidia of *F. mangiferae*, where conidia germination were steadily increased with elevated concentration of BAP and ethrel and were greatest (100% and 99.86%) at 400, 500 ppm BAP and 150 ppm ethrel with respect to untreated (control) (**Fig. 2C–D**).

Anti-malformin chemicals affect the germination of *F. mangiferae* conidia in vitro. A role of antimolformin chemicals, such as silver nitrate ($AgNO_3$) and glutathione, were evaluated in vitro for their effect on conidial germination of *F. mangiferae*. Silver nitrate ($AgNO_3$) in varying concentrations (25, 50, 75, 100, 150, 200, 250, 300, 400, and 500 ppm) showed inhibitory effect on conidia germination of *F. mangiferae*. However, inhibition was maximum with 400 ppm (1.8%) and conidia germination was nil over 500 ppm, as compared with control (70.02%) after 24 h (**Fig. 3A**). The effect of glutathione on conidia germination of *F. mangiferae* was assessed and found that it stimulates the conidial germination of *F. mangiferae* with increasing concentration of

glutathione ranging from 25 ppm to 1200 ppm. The conidial germination was highest at 1200 ppm (92.7%) after 24 h (**Fig. 3B**).

Discussion

The severity of malformation disease is the highest in mango growing areas of the world where the mean temperature preceding flowering is between 10–15°C.[1] More than one species of *Fusarium* were reported to be involved in the disease.[12] Recently, *F. mangiferae* was found to be associated with mango malformation.[13] Here, we morphologically characterized *Fusarium* sp from mango cv, Dashehri (**Fig. 1A–D**) and found that features (light purple plus white colony colors, shape and size of microconidia and macroconidia, partitionate hyphae with septa and absence of chlamydospores) were in accord with the reported standard features of species *F. mangiferae*.[8,14]

At present, several attempts have proved futile to find out the exact causal agent and the etiology of disease has not yet been determined.[15] Currently, a role of low temperature stress ethylene has been implicated in mango malformation, whereas involvement of *F. mangiferae* in causing disease either via toxic principle (TP) or malformation induction principle (MIP) is not definite at low temperature range where malformation is most sever.[1,7] In the present study, in vitro conidia germination technique was made to evaluate the degree tolerance or susceptibility of *F. mangiferae* at favorable temperature range. Plants, during their growth, development and cellular differentiation, secrete different kinds of growth regulators. It is well known that these secretions may affect the growth and development of *F. mangiferae*. Here, we observed stimulatory effect of plant growth regulators on conidia germination of *F. mangiferae* and found that conidia germination increases with increasing concentration of NAA, GA_3, BAP and ethrel (**Fig. 2A–D**). The role of hormonal imbalance in mango malformation was pointed out by Majumder et al.,[16] who achieved a significant decrease in floral malformation by exogenously applying the NAA (100–200 ppm) to the affected branches of mango cv Dashehri, Chausa, and Bobay Green. Naphthaleneacetic-acid (200 ppm) treated malformed panicles were reported to grow like healthy panicles of mango and produced fruits.[4,17] Similarly, exogenous application of GA_3 enhanced the early flowering and improved the number of healthy panicles and resulted into the reduced incidence of floral malformation.[18,19] The number, nature, and kind of cytokinins varied in healthy and malformed inflorescence during different developmental stages were studied and cytokinin content was detected to be elevated in malformed inflorescence with respect to healthy ones.[18] The higher infection of *Fusaium* and the malformation symptoms were explained to be mediated via consequence of cytokinin production and metabolism.[20,21] Ethylene is a well known "stress hormone" that may affect the extent and magnitude of spore germination. Ethrel was reported to stimulate the germination of spores and growth of fungi.[22,23] Ethylene was reported to promote the conidial germination and involved in the establishment of plant–fungus interaction.[14,24] All these reports reveal that higher population of *Fusarium* sp in diseased tissues could be due to the higher level of ethylene.

Application of antimalformin chemicals *viz.*, silver nitrate, ascorbic acid, potassium metabisulfite, and glutathione caused the disappearance of malformin from panicle, which fruited like healthy controls.[9] In the present study, the effect of silver nitrate and glutathione were evaluated on conidia germination of *F. mangiferae in* vitro (**Fig. 3A–B**). Malformed panicles sprayed with 600 ppm $AgNO_3$ were found to grow into fruit-bearing healthy panicles.[9] The effect may certainly be due to the inhibitory effect of Ag^+ in ethylene action.[25] Furthermore, inhibitory effect of silver nitrate (concentrations between 400 ppm to 500 ppm) on conidial germination of *F. mangiferae* (**Fig. 3A**) could explain the fact that

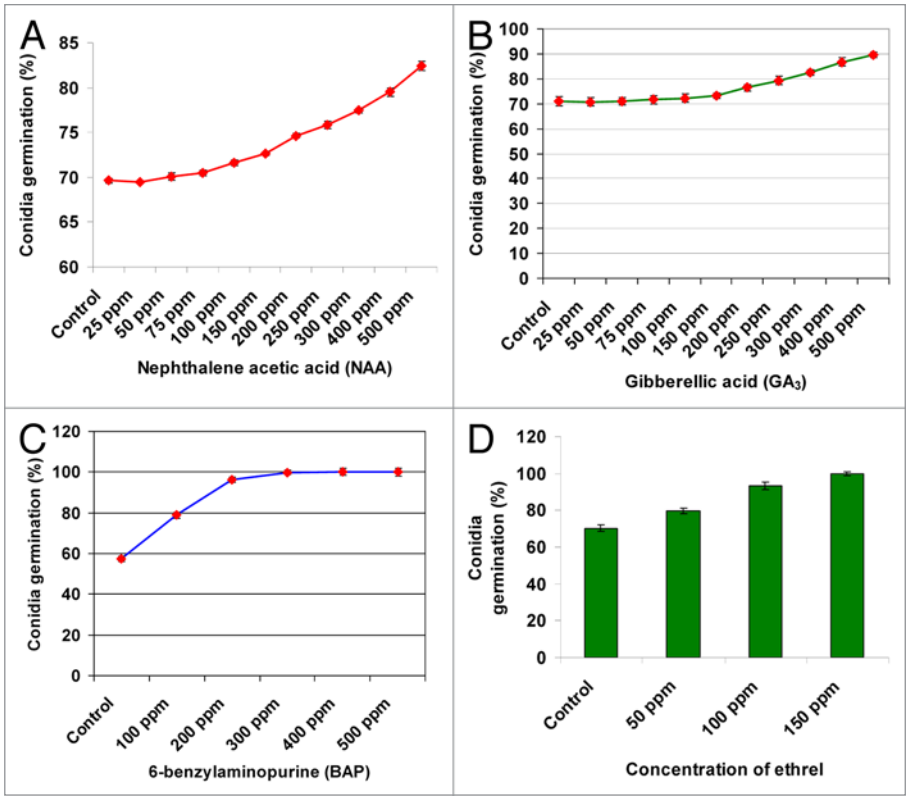

Figure 2. Evaluation of conidia germination of *F. mangiferae*in vitro under varying concentration of plant growth regulators. (**A**) Naphthalene acetic acid (NAA) (500 ppm) promotes conidia germination to 82.47% from 69.49% and 69.62% at 25 ppm and untreated control, respectively after 12 h. (**B**) The stimulatory effect of gibberellic acid (GA_3) was detected and the germination was highest (89.66%) in conidia treated with 500 ppm gibberellic acid (GA_3), whereas it was least (70.79%) at 25 ppm GA_3, after 12 h. (**C–D**) The 6-benzylaminopurine (BAP) and ethylene-releasing compounds, such as ethrel, consistently induced conidial germination of *F. mangiferae* with their increasing concentrations. Condial germination was 100% beyond 300 ppm BAP, while ethrel at 150 ppm was highly effective (99.86% conidia germination), after 12 h.

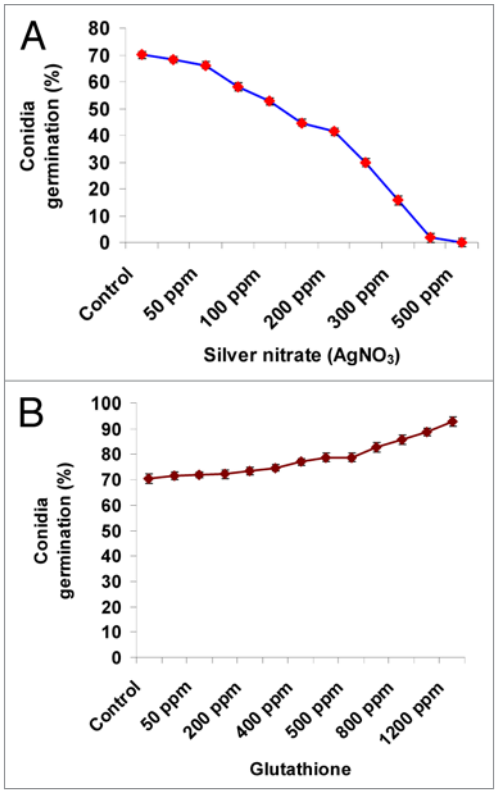

Figure 3. The response of germination of *F. mangiferaein* conidia in vitro under defined concentration of antimalformins after 24 h. (**A**) The maximum inhibition was recorded at silver nitrate ($AgNO_3$) above 400 ppm (1.8%) and conidia germination was nil over 500 ppm. (**B**) Glutathione stimulates the conidial germination from untreated control (70.4%) to 1200 ppm (92.7%).

silver nitrate may reduce the population of *F. mangiferae* contributing ethylene to stress ethylene pool in malformed panicles.[14] On the other hand, glutathione was highly effective in stimulating the conidial germination of *F. mangiferae* at concentration exceeding the 1000 ppm (**Fig. 3B**). Exogenous application of gluthione to malformed panicles resulted in normal panicles and these panicles fruited like the healthy control.[26,27] The response of glutathione to reduce the disease incidence may explain the fact that it plays a part in reducing ascorbate[28] required in the last step of ethylene biosynthesis[29] catalyzed by ACC synthase. The glutathione mediated reduced ascorbate may, therefore, affect the ethylene production. The resulted lower level of ethylene in malformed tissues may help in nullifying the malformed symptoms in mango panicles caused by over production of stress ethylene.[7]

The exogenous application of plant growth regulators *viz*. NAA and GA_3 resulted into the recovery of malformed panicles to normal panicles reported earlier[1] is not correlated with the finding of present study where such treatment cause induction of conidial germination of *F. mangiferae.* On the other hand, BAP and ethrel response to positively modulate the germination of conidia of *F. mangiferae,* explain that a higher population of *F. mangiferae* in malformed tissue might be the result of higher endogenous content of these hormones. The role of antimolformin chemicals, such as silver nitrate and glutathione in reducing floral malformation reported earlier,[30] was found to be correlated with ethylene effects and probably not *Fusarium* in causing the malformation in the present study. The exogenous application of BAP and ethrel to mango panicle to reproduce disease symptoms was not succeeded. Further, NAA and GA_3 treated mango plants expected to reduce the population of *F. mangiferae* resulting in a decline infection may lead to diminish the disease incidence,[4] but this is not the case of present in vitro study where NAA and GA_3 favored the *F. mangiferae* infection by stimulating conidia germination. The findings of present investigation do not authenticate the involvement of *F. mangiferae* in mango malformation, however hormonal imbalance might be responsible for deformed functional morphology of mango inflorescence.[7] Further study is required to trace out the mechanism involved in triggering the endogenous level of a particular hormone by the signal of various external stimuli at full bloom and prior to the full-bloom stage of flower buds and transmission of these signals via signaling cascade leading to various cellular response that in fact is responsible for loss in functional morphology of mango inflorescence.

Materials and Methods

Isolation, purification, and morphological identification of *F. mangiferae*. A single conidia isolation technique was used for producing pure cultures of the fusaria from mango cultivars. Single conidia were transferred from the axenic suspension culture to sterilized Petri plates of PDA. From these parent cultures, mycelial growth from the margin of the culture was used for sub culturing whenever required. The parent axenic cultures were kept at 5°C. The colonies of *F. mangiferae* were purified on CLA medium and the identification was done on the basis of typical macro and microconidia.[8,14]

Plant growth regulators and antimalformins affect the conidia germination of *F. mangiferae* in vitro. The harvested conidia from mycelial mat of *F. mangiferae* were placed separately into the cavity of glass slides and were treated with varying concentrations of plant growth regulators, such as naphthalene acetic acid (NAA), gibberellic acid (GA_3), 6-benzylaminopurine (BAP), ethylene releasing agent (ethrel), and anti-malformin chemicals *viz.,* silver nitrate and glutathione at the rate of 10 µL solution volume. The cavity glass slides were subsequently placed onto the moist blotting sheets of petriplates to maintain the humidity. The condia of *F. mangiferae* were then allowed to germinate at suitable temperature (27°C). The number of conidi germinating after 12 h and 24 h was counted under the compound microscope.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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