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Induction of Resistance against Fusarium Wilt of Banana by Application of Live RKN, Live and Dead Pathogenic Strain of *Fusarium oxysporum* f. sp. *Cubense*

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Fusarium wilt or Panama wilt disease Banana is one of the most disastrous plant diseases. In the present studies, the response of Grand Naine variety of banana plants, when interacting with dead or alive pathogen, *Fusarium oxysporum* f.sp. *cubense* (Foc), a causative agent of fusarium wilt disease of banana were investigated. The induced response of plants was evaluated in terms of induction of defense-related enzymes, *viz.*, Peroxidase (POX), Polyphenol Oxidase (PPO), ß-1,3 Glucanae, Chitinase and Phenoloics. Plants interacted with live pathogen resulted early induction of defense to check penetration as well as antimicrobial productions. However, pathogen overcome the defense of plant and caused disease. Interaction with dead pathogen resulted in acceleration of defense response in plants and so that plants inoculated with dead pathogen showed resistance to forced inoculation of live pathogens. Results obtained in the present study that the dead pathogen was able to raise defense response in plants and provide resistance to fusariam wilt disease of banana upon subsequent exposure. This study showed that dead pathogen could be a potential candidate like a plant vaccine before the onset of disease to combat fusarium wilt disease of banana.

Keywords: Banana, Fusarium, Resistance, *Fusarium oxysporum* f. sp. *Cubense,* Root Knot Nematode.

Banana (*Musa* spp.) is one of the earliest crops cultivat-ed by man which still remains to be one of the world's most important fruit crop. At present, it is grown in more than 120 countries throughout tropical and subtropical regions (Molina and Valmayor, 1999) and is the staple food for more than 400 million people. Since banana is being used as food, fiber and for medicinal, cultural and industrial purposes and also gives high returns to small holders.

In order to cater to the needs of escalating population, banana production needs to be doubled and estimated pro-duction requirement by 2020 is around 25 million tonnes (Annon, 1996). Since increase in area of cultivation is impossible, the alternative approach is to increase the pro-ductivity is the threat posed by the insect pests and diseases. Among the dis-eases *Fusarium* wilt also known as panama wilt caused by *Fusarium oxysporum* f. sp. *cubense* is the major

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constraint to banana production and the disease has major fungal disease of banana in India.

Fusarium wilt caused by *Fusarium oxysporum* f.sp. *cubense* (Foc) is the most destructive disease of banana (Moore *et al*., 2001). It has been reported in all banana-producing countries, including Asia, Central and South America, Africa, and Australia. The pathogen is soil-borne and remains viable up to several years and cause 20-80 per cent loss of banana. Several disease management strategies can be used for the control of fusarium wilt *viz* crop rotation, burning infected plants or plant parts, and application of broad spectrum systemic fungicide carbendazim, and resistant cultivars (Thangavelu *et al*., 2004). All the methods of mentioned above for the management of this disease have not fully successful. The application of synthetic fungicides may result in undesirable effects on the environment and disturbed ecosystem. Biological approach might be an alternative approach for management of fusarium wilt of banana. Bio-control agent can be alive or dead beneficial organism or its part such as cell wall, proteins and oligosaccharides (Boukaew *et al*., 2011) that can take part in management of disease. When live organisms are used as a bio-control agent, care should be taken to create appropriate conditions for its maintenance while if part of the organism such as cell wall, protein, oligosaccharides, or killed organism is used then ambient conditions are not required. Plants, humans and animals give instantaneous response to the pathogen or its part. Animals kingdom produce antibodies against pathogen or vaccine, similarly plants response to pathogen attack by producing PR-proteins, defense-related enzymes (Thangavelu *et al*., 2007), plantibodies and phytoalexins.

Studies of defense-related enzymes are key to any plant disease resistance mechanism. Farmers are being purchased tissue culture plants every year for planting in the fields with the expectations of getting high production and high profit. Foc being soil borne disease may enter the plant and cause disease anytime after planting and affects fruit production. If plants are immunized i.e. accumulation of defense-related enzymes occurs before the attack of pathogen then the pathogen can be successfully warded off and loss can be minimized. Same concept of vaccination is used here. Vaccines used for animal kingdom are derived from the same disease causing organism. But vaccines have inactive organism or attenuated organism. Elicitor used here is acting as a vaccine (derived from the dead fungus) to protect the plant. The aim of the present study was to compare the interaction of dead and live pathogen with Grand Naine banana plants. Grand Naine is a large fruit yielding dwarf Cavendish variety with height of 6.5 to7.5ft introduced to India from Israel remains choice of farmers as the bunches of banana fruits can be harvested within twelve to thirteen months from the date of planting.

The aim of the present studies was to decipher the induced response of plants by activation of defence related enzymes analysis from dead and live pathogen and also to check the response generated by using dead pathogen in the forced inoculated of live pathogen in banana.

MATERIALS AND METHODS

Maintenance of the RKN Culture

Previously isolated culture of Root Knot Nematode (RKN) from fusarium wilt infected banana plants was maintained in water broth solution at 27° C (Rajasekar *et al*., 1997). For liquid culture of RKN, 1 ml of 3-4 weak old culture was inoculated in potato dextrose broth (PDB) and incubated at 27° C for 21 day on PDB and was used as live RKN for treatment.

Maintenance of the fungal Culture

Fusarium oxysporum f.sp. *cubense* (Foc) pathogen was isolated from fusarium wilt infected banana plants and maintained on potato dextrose agar (PDA) culture medium at 27° C (Thangavelu *et al*., 2007). 6-8 mm agar plug of 3-4 weak old culture was inoculated in potato dextrose broth (PDB) and incubated at 27° C for 21 day on PDB for liquid culture. The liquid media with mycelium was autoclaved at 121° C for 20 minutes. The liquid culture was crushed in grinder and further used as dead fungi for treatment.

Plant Material

Two months old tissue culture Grand Naine banana plantlets were procured from Tissue Culture Laboratory (TCL), Bihar Agriculture University, Sabour, Bihar. Plantlets were planted and maintained in sick plot at Department of Plant Pathology, BAC, Sabour. All the cultural practices

were done for the growth and development of the plants.

Live RKN, Live and Dead Fungus Treatment

The surrounding soils of the roots of Grand Naine variety were removed carefully so that plant roots were exposed without damage. The suspensions of live RKN, live and dead fungus Foc were prepared by mixing 1 ml live RKN, 1 g of dead fungus and live fungus per liter of distilled water. 1 ml of dead and live pathogen suspension was administered per plant and control plants treated with 1 ml of distilled water in exposed root region. The changes in levels of defense related enzymes in leaves after treatment were assayed after each successive day till seventh day (Thangavelu *et al*., 2007; Thangavelu *et al*., 2011).

Forced Inoculation with live RKN and Foc

For the induction of resistance in banana, plant roots treated with dead pathogen was exposed to spore suspension of RKN and Foc (104 spores/mL) while plants treated with distilled water was used as control. Plants were kept under observation for the development of the symptoms.

Enzymatic assays

In-vitro propagated two months old disease-free plantlets were selected for this study. Live RKN, live and dead fungus Foc treatments were given as mentioned earlier to plants and distilled water treated plants were used as control plant. Up to seven days at regular interval of 24 h, leaves sample were excised from both control and treated plants for estimation of defense related enzymes, namely, POX, PPO, ß-1,3 glucanase, chitinase, and total phenolics. Fresh Banana leaves were washed in running tap water and homogenized in liquid nitrogen. The homogenized leaves were kept at 4° C until used for enzyme analyses. POX activity was measured as described by Sadashivam and Manickam (1992). PPO, ß-1, 3 glucanase, chitinase, and total phenolics assays were done as described by Meena *et al.* (2001). These experiments were repeated twice with different sets of plants under similar conditions and enzymatic analyses were performed three times.

RESULTS AND DISCUSSION

The aim of this study was to investigate interaction of live RKN, live and dead pathogen in banana plant variety Grand Naine. RKN is the carrier of *Fusarium oxysporum* f.sp. *cubense*, causative agent of panama wilt of banana plants. Interaction in terms of defense related enzymes was determined by accumulation of several defense related enzymes, namely, POX, PPO, ß-1, 3 glucanase, chitinase, and total phenolics. Plants could differentiate signals from dead and live pathogen and in response to signals from dead pathogen, it can induce defense enzymes, which could protect plants upon subsequent exposure to pathogen.

Effect of Live RKN, Live and Dead Foc on POX Activity of the Host

Peroxidases are a well known class of PR proteins and induced in host plant tissues by pathogen infection. They belong to PR-protein 9 subfamily (Van Loon, 2005) and are expressed to limit cellular spreading of infection through establishment of structural barriers by massively producing ROS and RNS (Passardi, *et al.*, 1997). In this study, POX activity induced earlier in dead and live pathogen treated plants and retained elevated levels compared to control plants. POX activity increased 2.5, 2.5 and 4 times in live RKN, live and dead fungus treated plants, respectively. The Highest POX activities in plant treated with live RKN, dead and live pathogen was observed on 4^{th} 6^{th} and 7^{th} day, respectively. POX activity remained constant throughout seven days of study in control plants. (Fig.1). The interaction of banana plants with dead and live pathogen resulted in induction of POX activity. However, POX activity induced more with dead fungus. As dead pathogen was capable to generate initial recognition signals; however, it cannot counter the plant response, which leads to induction of POX activity more than live fungus. Peroxidase activity expression in higher plants is, indeed, induced by fungi (Sasaki, *et al.*, 2004 and Thakker, 2012), bacteria (Lavania, *et al.*, 2006), nematodes (Rajasekar *et al.*, 1997), viruses (Diaz-Vivancos, *et al.*, 2006, and viroids (Vera, *et al.*, 1993). Cross-linking of the phenolic monomers in oxidative coupling of lignin subunits has been associated with peroxidase using H_2O_2 as oxidant. Acidic and basic peroxidases are capable of oxidizing p-coumaryl and coniferyl alcohol. One significant event in plant defense reactions is oxidative burst, a common early response of host plant cells to

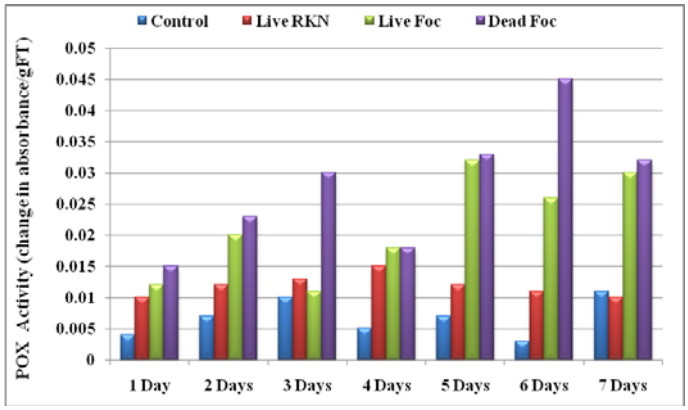

Fig.1. POX activity profile of banana for reinforcement of physical barrier as well as ROS generation in response to distilled water (Control), live RKN, live fungus and dead fungus interactions for seven days

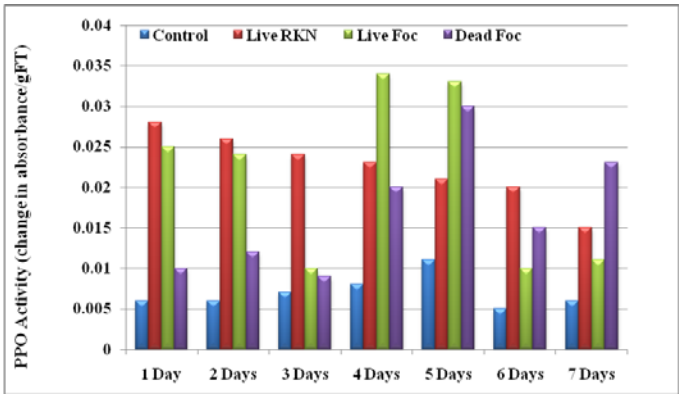

Fig. 2. PPO activity profile of banana for generation of antifungal in response to distilled water (Control), live RKN, live fungus and dead fungus interactions for seven days

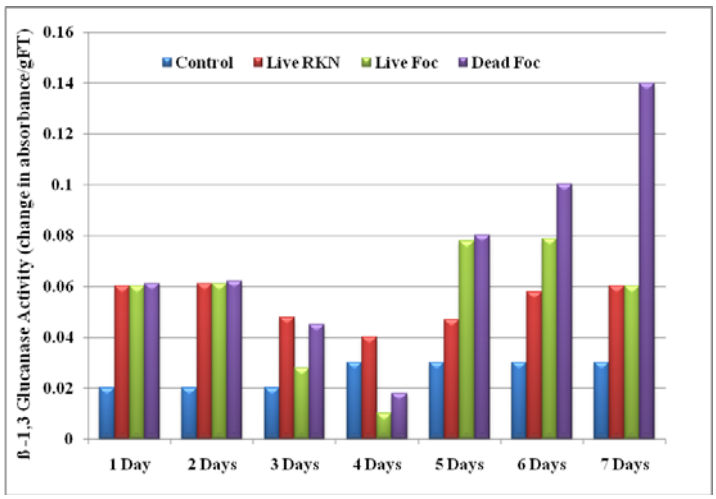

Fig. 3. ß-1, 3 Glucanase activity profile of banana for direct antifungal activity in response to distilled water (Control), live RKN, live fungus and dead fungus interactions for seven days

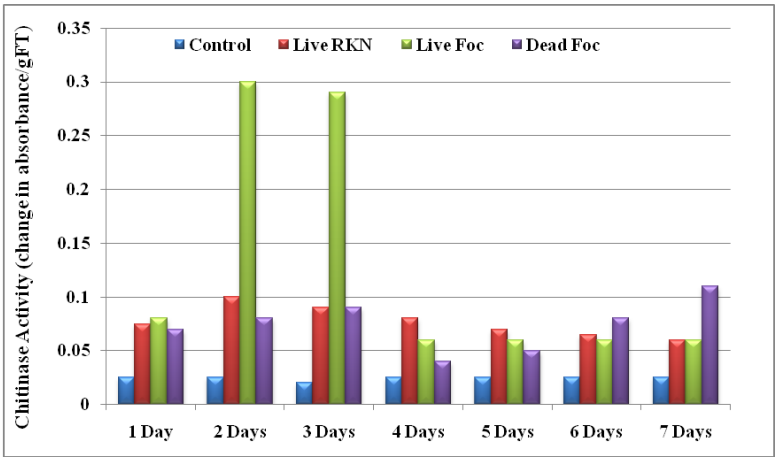

Fig. 4. Chitinase activity profile of banana for direct antifungal activity in response to distilled water (Control), live RKN, live fungus and dead fungus interactions for seven days

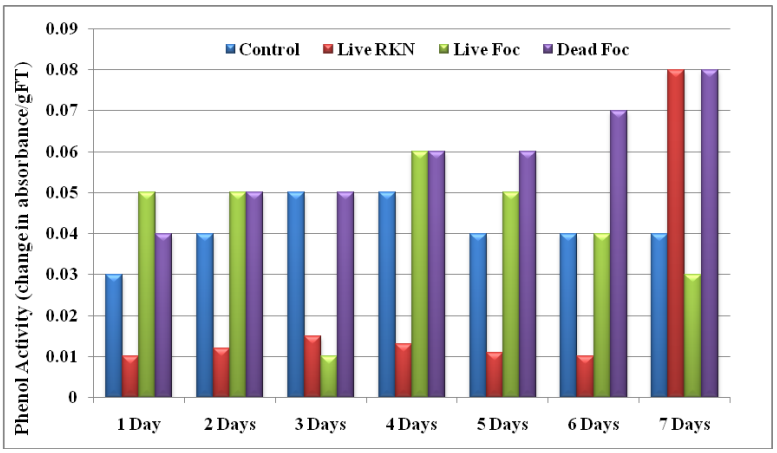

Fig. 5. Phenol activity profile of banana for direct antifungal activity in response to distilled water (Control), live RKN, live fungus and dead fungus interactions for seven days

pathogen attack and elicitor treatment. Our results showed increase in POX activity in dead fungus treated as well as fungus treated plants as compared to control. This result indicates that elicitor slowly increases level of lignin formation, suberization, and hypersensitive response. Similar results were reported in wheat heads (Mohammadi and Kazemi 2002).

Effect of Live RKN, Live and Dead Foc on PPO Activity of the Host

PPOs are a group of copper containing enzymes that catalyze oxidation of hydroxy phenols to their quinone derivatives, which have antimicrobial activity (Chunhua, *et al.*, 2001). This study showed comprehensible difference in PPO activity in banana which was treated with live RKN, dead and live pathogen. With live RKN interaction, plants showed to induce 4 times PPO activity on first day, and thereafter decreased near basal on 7th day. But, live fungus interaction, plants showed to induce 3 times PPO activity from first day onward, reached highest level on 4th day, and thereafter reached near basal. However, dead fungus treatment failed to mount significant induction in PPO activity for initial five days of interaction followed by 2 and 3 times induction in PPO activity on 6th and 7th days, respectively compared to control plants (Fig. 2). PPO plays a role in defense against plant pathogens because of its reaction products and wound inducibility (Mayer and Harel,

1979). Plant immediately responds to pathogen so there is immediate rise in PPO indicating immediate synthesis of antimicrobials to ward off pathogen. In elicitor treated plant PPO activity increases slowly day by day indicating that plant has got stimuli to increase PPO. In case of live pathogen interaction, there was immediate response by plants increasing PPO 5 times on the first day, which starts decreasing from the 6th day indicating multiplication of fungus in the plant system. However, dead pathogen completely fails to mount this response, which strongly suggests that plants are capable to differentiate signals from live and dead pathogen. Increase in PPO activity was reported in banana roots treated with Foc-derived elicitors by Thakker *et al.*, 2007 and 2012. Marked increase in PPO activity was observed in banana roots treated with *Pseudomonas fluorescens* against fusarial wilt (Sarvanan, *et al.*, 2004).

Effect of Live RKN, Live and Dead Foc on ß-1, 3 Glucanase and Chitinase Activity of the Host

ß-1,3 glucan and chitin, polymer of N-acetylglucosamin (NAG) are major cell wall components of many fungi. Since ß-1,3 glucanase and chitinases have been shown to be capable of attacking cell wall of the fungal pathogens, these enzymes have been proposed as direct defense enzymes of plants (Abeles, *et al.*, 1970 and Thakker, *et al.*, 2012). Interaction of live RKN, live and dead fungus with banana plants resulted in induction of ß-1, 3 glucanase activities from 1st day onward. Up to five days, pattern of ß-1, 3 glucanase activities was very similar in these treatments. From the 5th day onward, ß-1,3 glucanase activity stabilized at 2 to 2.5 times higher levels in live RKN and live fungus interaction as compared to control. In case of dead fungus interaction, gradual increase in ß-1,3 glucanase actively was observed from 5th day onward with highest increase 3.5-fold on 7th day as compared to control (Fig.3). Chitinase activity induced from 1st day in banana plants treated with lives RKN, live and dead pathogenic fungus. Chitinase activity increased 10 times in 2nd and 3rd day followed by rapid fall down and retention of about 1.5 times in banana treated with live fungus compared to control plants. Whereas, in both live RKN and dead fungus treated plants, chitinase activity increased two times on 1st day followed by gradual increase up to 4 times on 7th day (Fig.4). We observed an increase in chitinase and ß-1,3 glucanase activity indicating plants ready mechanism to ward of pathogen by directly degrading the pathogen cell wall and in turn protecting the plant. In our previous study, we found antifusaric activity of elicitor induced ß-1, 3 glucanase that showed swelling of mycelia after one hour incubation of pathogen with purified elicitor (Thakker, *et al.*, 2009). Endochitinase purified from barley was capable of inhibiting the growth of *Trichoderma rescei*, *Alternaria alternata* and *Neurospora crassa* (Roberts, and Selitrennikoff, 1988). In addition, (Mauch and Staehelin, 1989) reported that in combination, chitinase and ß-1,3 glucanase act synergistically to inhibit fungal growth.

Effect of Live RKN, Live and Dead Foc on Phenolics of the Host

Phenolic acids are involved in phytoalexin accumulation, biosynthesis of lignin, and formation of structural barrier, and play a main role in the resistance against pathogen. The present studies showed that total phenolics content gradually increased and reached to maximum on 7th day (2 times) In dead fungus treated plants. Whereas, in live fungus treated plants, total phenolics content increased on 3rd day followed by sharp decline to reach basal level as compared to control plants. However, live RKN treatment failed to mount significant induction in PPO activity for initial five days of interaction followed by 2 and 3 times induction in phenolics activity on 7th days, compared to control plants (Fig.5). The accumulation of phenolics was observed on the 3rd third day in fungus treated plants indicating the plants sensitivity to pathogen, and it attempt to protect itself by the formation of structural barriers. Further, from the 4th day activity decreases showing successful multiplication and establishment of pathogen by overcoming structural barriers formed by plants. In dead pathogen-treated plant, we found slow increase in phenolics activity indicating ability to recognize dead pathogen as some foreign body, but it is not multiplying; therefore, it will not break structural barrier. Increase in phenolics activity indicating ability to recognize dead pathogen as some foreign body (Thakker, *et al.*, 2009). The identified accumulation of phenols leading to suppression of fusarium wilt was observed in tomato plants (Ramanathan, *et al.*, 2000). When tomato plants

were treated with catechol, marked accumulation of phenols was observed and it resulted in suppression of fusarium wilt of tomato (Ramanathan, *et al.*, 2000). Anna and Dubey (2000) investigated that subtraction of cell-wall bound phenolics, ester-bound phenolics, glycoside bound phenolics, and free phenolics increased 6.3, 4.2, 3.0, and 2.3 times, respectively, upon induction.

Forced Inoculation

Control plants and plants treated with live RKN and dead pathogen were forced inoculated with the live fungal spores and observed for the development of symptoms. In control plants, the characteristic symptoms of fusarial wilt were observed in the first week after forced inoculation followed by aggravation of disease condition after 15 days. However, no symptoms of fusarium wilt were observed even after two months after forced inoculation in plants treated with dead pathogen. This supports the view that elicited plants are less susceptible to infection. Dead pathogen preparation was not only successful in mounting defense response but also in protecting plants upon subsequent infections. Therefore, it could be potential candidate for plant vaccine preparation to combat fusarium wilt disease of banana.

When plants are challenged by a pathogen, early local defense reactions and delayed, systemic responses get activated in order to counteract the pathogen attack. Among the early local responses, the hypersensitive response (HR) leads to a local programmed cell death in order to deprive the pathogens of their nutrition base (Greenberg and Vinatzer, 2003). Later on, the plant can develop systemic acquired resistance (SAR) leading to resistance throughout the whole plant in an unspecific manner towards a broad spectrum of pathogens. In case of SAR, signal is transmitted from infected tissue in the whole plant for induction of overall defense gene expression. This demonstrates that signal perception in initial pathogen recognition and signal transduction to initiate further defense responses is essential for plants to counteract phytopathogens (Nürnberger and Scheel, 2001). Some defenses are constitutive, such as various preformed antimicrobial compounds, whereas others activated by pathogen recognition. Recognition process includes product of a dominant resistance R gene present in the plant and the corresponding dominant avirulence (Avr) factor encoded by or derived from the pathogen. Recognition of Avr factor by host plant starts signal transduction pathways that activate several of plants defences response(Nürnberger and Scheel, 2001; Bent, 2001).

In the present study, analysis of plants response towards dead and live pathogenic strain was carried out using induction of several key marker enzymes associated with plant defense mechanism. Development of effective, durable, economic and environmentally sound strategies for the control of crop diseases could be possible through an improved understanding of the interactions between plants and pathogenic agents.

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