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In-planta Resistance to Panama Wilt of Banana is Modulated by Endophytic Bacteria: A Preliminary Study Supporting the Hypothesis

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A B S T R A C T

Fusarium wilt being caused by a major vascular pathogen of banana and it is difficult to control once it gets colonized. The present study was aimed at screening the potential endophytic bacteria against the *Fusarium* pathogen. The pathogen was first isolated from the diseased infected plant and morphologically and molecularly confirmed as *Fusarium oxysporum f.sp. cubense* (*Foc*) and its virulence confirmed. The culturable bacterial endophytes were isolated from the *Foc*-resistant banana cultivar 'Yangambi km5' and *Foc*-susceptible banana cultivar 'Ney Poovan'. Leaf, petiole, pseudostem, rhizome and root tissue samples were taken for isolation of endophytic bacteria. From the different tissues 38 culturable endophytic bacteria from cv. 'Yangambi km5' and 18 culturable endophytic bacteria from cv. 'Ney Poovan' were isolated. These isolates were screened against the *Foc* in order to elucidate their antagonistic potential. The study revealed that banana cultivar 'Yangambi km5' hosts twelve antagonist bacterial isolates, out of the thirty-eight isolates, whereas in cv 'Ney Poovan' none of the eighteen isolates was found to inhibit pathogenic fungal growth significantly. The study indicated the potential possibility of isolates from Yangambi km5 could play a key role in resisting Fusarial wilt incidence and its vascular development.

Introduction

Panama wilt of banana caused by *Fusarium oxysporum f.sp. cubense* (*Foc*) is the most devastating disease of banana, worldwide. It created havoc by destroying the Gros Michael banana in tropical America in the 1890s (Brandes, 1919). The replacement of Gros Michael with Cavendish clones such as Dwarf Cavendish, Robusta, Williams and Grand Naine over the decades has helped to sustain the banana trade due to their resistance against the Panama wilt caused by *Foc* race-1. In the recent years, occurrence of the race-4 pathogenic strain of *Foc*, more particularly from the Southeast Asia is posing severe

threat to many banana cultivars including present day Cavendish clones and it has been reported in most of the banana growing belt of the world (Ploetz, 2015) including parts of India (Damodaran *et al.,* 2018; Thangavelu *et al.,* 2019).

Even though plants have their inherent strategic cellular adaptations to mitigate most of the biotic and abiotic stresses, they also rely on their holobionts to get defended against the parasitic invaders (Moran and Sloan, 2015; Rosenberg and Rosenberg, 2011).

A holobiont is a functional entity that comprises of a multicellular host and the associate microbiota (Hassani *et al.,* 2018). It is widely known that many endophytes have the ability not only to resist invasion of pathogenic fungus but can also support the growth of the plants (Rashid *et al.,* 2012; Lodewyckx *et al.,* 2002) Probably, the genomic signature of the host plant is important in deciding the microbiota association related to the fitness of a phenotype against any particular biotic or abiotic stress (Hardoim *et al.,* 2015).

The elucidation and utilization of the *in-planta* resistance of the in a specific genotype exhibiting resistance to biotic stress can be a possible strategy to manage the vascular disease like *Fusarium*.

In the present study, attempts were made to isolate and culture the endophytes from 'Yangambi km5' (*Musa* 'AAA'), a cultivar of banana of known for its resistance to *Fusarium oxysporum fsp cubense (Foc) race 1* and as well from the Fusarial wilt susceptible 'Ney Poovan' (*Musa* 'AB') a widely grown diploid dessert cultivar in South India. The culturable isolates were tested for their antagonistic potential against *Foc*.

Materials and Methods

Isolation and characterization of the Fungus

Fusarium wilt affected plant of cv. 'Karpooravali' (Musa 'ABB') was identified by morphological symptoms in the university orchard, Horticultural College and Research Institute, TNAU, Coimbatore. The plant was transversally dissected and internal vascular discoloration was observed in the infected pseudostem and rhizome. The infected pseudostem and rhizome sample infected tissue was cut using sterile surgical blade and immediately wrapped with sterilized tissue paper to prevent the excess sap oozing. The sample was kept in the ice box and taken to the pathology laboratory. Further, surface sterilization was done by using NaOCl (1%) for 10 min, followed by 70% ethanol for 30 sec. Thorough washing was done inside the laminar air flow chamber with sterile distilled water. The samples were than sliced into fine pieces by using sterilized surgical blades and kept in half-plated PDA (Potato Dextrose Agar) media with 2% streptomycin. The plates were incubated for 3 – 5 days still fine white mycelial growth started from the plant tissue. The mycelial section was sub-cultured to the fresh PDA plate and allowed for growth. The isolated pure culture of the fungus was maintained in the lab following regular sub-culturing. The pathogen was confirmed based on the morphological characters as described by Nelson (1991) and the molecular characterization was also carried out using the ITS region as described by Mostert (2017).

Pathogenicity test (Koch's Postulates)

A recently published method (Ascensao and Dubery, 2000) was attempted for proving the pathogenicity of the isolated *Foc*. The conidial suspension was prepared by

collecting the conidia from the culture plate by using sterile distilled water. The concentration of conidia in the suspension was adjusted to 10^6 cfu ml^{-1}. One fourth diluted Murasigue and Skoog media (MS media) was taken as media for the survival of banana plants. Well hardened TC plantlets of the cultivar Ney Poovan were collected to assess the pathogenicity.

The plants roots were cleaned in the running tap water for removing the adhering cocopeat. It was taken in culture bottle and plants were placed firmly by wrapping with foam holder and cling film. The bottles were wrapped with aluminum foil in order to simulate dark condition for the root growth. The cultures were incubated in growth chamber by exposing to 16/8h NUV light/dark conditions. Finally, the observations on symptom expression were recorded.

Selection of the varieties (*Foc* resistant 'Yangambi Km 5' and *Foc* susceptible 'Ney Poovan')

Yangambi km 5 (AAA)

The banana cultivar 'Yangambi km 5', has its origin from Democratic Republic of Congo and belongs to sub group 'Ibota Bota'. It is known for its resistance reaction to Panama wilt of banana (*FOC race 1*), Sigatoka leaf spot and nematodes (Fogain and Gowen, 1998).

Ney Poovan (AB)

'Ney Poovan' is one of the traditional dessert varieties of India. Tt is also known 'Safed Velchi', 'Kadali', 'Rasabale', 'Puttubale', etc. It is highly susceptible to *Foc* race 1 and as well as *Eumusae* leaf spot pathogens, but it is extensively preferred by the farmers owing to the premium price it fetches in the market.

Isolation of endophytic bacteria from the different plant tissue

Apparently, disease free seven-month-old banana plants of both 'Yangambi km5' and 'Ney Poovan' were selected for endophytes isolation from the Experimental field of University Orchard, TNAU, Coimbatore. Different plant parts (*viz*., root, rhizome, pseudostem, petiole, leaf) were collected and washed thoroughly under tap water. Samples from each tissue was taken and surface sterilized (Thomas *et al.,* 2008). A few drops of Tween 20™ was added in separate beakers for washing each of the samples for 30 minutes in sterile water. Tissues were washed thoroughly in sterile water and transferred to laminar air flow chamber.

All the tissue samples were treated for 20 minutes with 5% NaOCl with available chlorin 5% w/v. Two sterile water washes were followed to remove the traces of NaOCl from the plant parts. A treatment with 70% ethanol was made for 30 second following a series of sterile water wash (8 times). From the 8th sterile water wash, sterile check was taken and plated on the Nutrient agar (NA) media (peptone 5%, beef extract 3%, NaCl 5% and agar 15%). Two replicates of sterile checks were maintained for each sample. The plant tissues were homogenized using sterilized pestle and mortar employing sterile peptone salt (One gram per litre each of bacteriological peptone and NaCl in sterile water) at the rate of 10ml per sample (Thomas *et al.,* 2012). The homogenate was allowed settle for 20 - 30 minutes and one ml of supernatant was pipetted from each sample for serial dilution. The samples were serially diluted up to 10^3 dilution. 100µL of dilute was pipetted from 10^1 and 10^3 dilution (Sekhar and Thomas, 2015) and plated on Nutrient Agar media with three replications with 10 petri plates per replication. Plating was done following the spread plate technique. Plates

were firmly wrapped with cling film and incubated to allow the colony growth.

In – vitro dual plate assay justifying the antagonism

In-vitro dual plate assay was carried out to confirm the efficacy of the endophytic bacterial isolates from the respective genotypes against the pathogenic *Foc* through dual culture technique. The zone of inhibition (mm) and percent inhibition of the fungal mycelia was recorded.

Results and Discussion

Fusarium wilt is one of the major devastating diseases of banana. Vascular wilt caused by the hemi-biotroph fungus *Foc* is tough to be controlled, once it gets colonized in the vascular tissue of the plant. Hence, the biological control of the disease by employing the endophytic bacteria of banana can be an ideal approach to minimize the loss caused by the pathogen. Plant system herbours numerous culturable antagonist bacteria as members of the endomicrobiota, which can be identified and employed in designing the biological control programs by artificial bacterization strategies in the disease susceptible plant genotypes.

Isolation and characterization of *Foc*

In the present study, the pathogen was isolated from the diseased infected banana plant from the field belonging to the variety 'Karpooravali' (ABB) (Fig. 1). The typical symptoms of *Fusarium* wilt, such as skirting of older leaves around the pseudostem, cracking on the basal region of the pseudostem and gradual wilting of the plant was observed. Typical vascular browning was observed on transversely dissecting the pseudostem as well as the rhizome. Such symptoms of *Fusarium* wilt have been described in many banana cultivars by Mustaffa and Thangavelu, (2011). In the present study, the pathogen was isolated from the discoloured portion and confirmed as *Foc* isolate through phenotypic and microscopic characterization. It was characterized by the presence of non-septate hyphae and numerous micro conidia as reported by Nelson (1991). The nucleotide sequence of the *Foc* isolate was submitted in NCBI data base bearing the accession number '*MK981549*'. The molecular identification by using ITS specific primer confirmed the pathogen as *Fusarium oxysporum ssp. cubense*.

Phyto-pathogenicity test

Pathogenicity of the identified *Foc* was validated hydroponically by adopting the protocol of Ascensao and Dubery, (2000) involving the conidial suspension of the fungus. Within seven days of inoculation, typical leaf yellowing symptoms of the Fusarium wilt started on the older leaves. Gradually, the symptom proceeded and complete wilting of the inoculated plants was observed on the 28th day after inoculation. The crown regions of the infected plants were cut open and typical vascular brown symptoms were observed. The characteristic external and internal symptoms of wilt confirmed the phyto-pathogenicity of the *Foc* isolate. From the infected rhizome tissue, the fungus was re-isolated and morphologically as well as microscopically validated as *Foc.*

Isolation of endophytic bacteria and *In vitro* antagonism assay

In the process of isolation of endophytic bacteria, the wash solution plated as the sterile check did not yield any bacterial CFU in none of the replicates. This in turn validated the effectiveness of the surface sterilization. Hence, the identified bacterial isolates were confirmed to be the true

culturable representatives of the endomicrobiome of banana genotypes. Thirty-eight bacterial isolates were isolated from the cv. 'Yangambi km 5' based on their colony morphology accounting ten from leaf, eight from petiole, nine from pseudostem, six from rhizome and five from the root endospheric niche. Similarly, in case of the banana cultivar 'Ney Poovan' *in-toto* 18 culturable endophytes were isolated accounting for five isolates from leaf, three each from petiole and pseudostem, two from rhizome and five isolates from the root endosphere (Fig. 2 and 3).

Fig.1

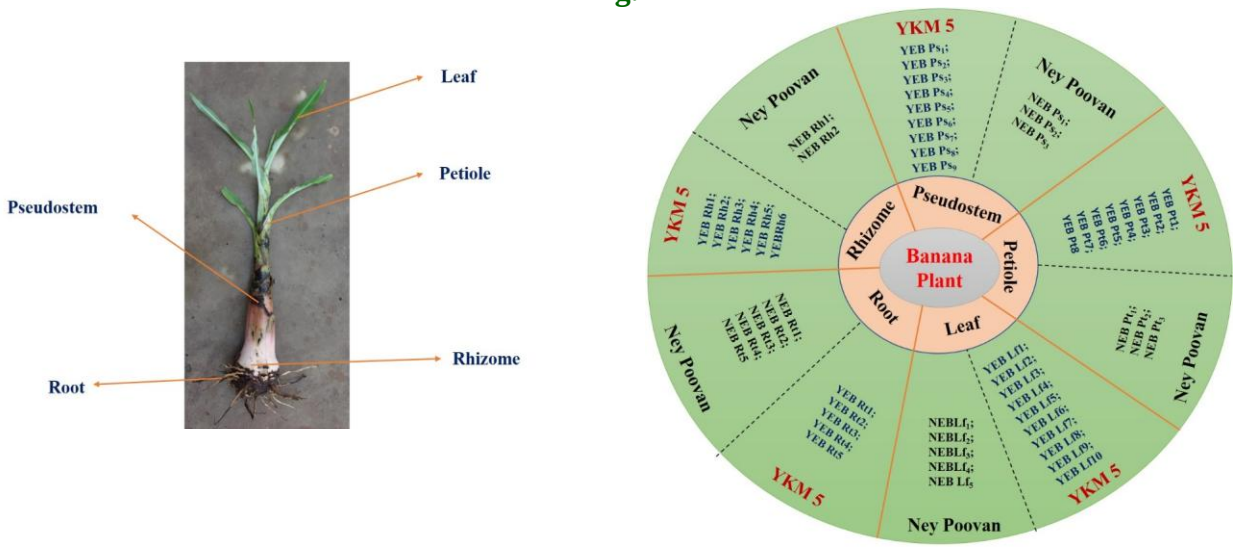


Fig.2 Antagonism of endophytes from banana cv. 'Yogambi km5' vs. *Foc*

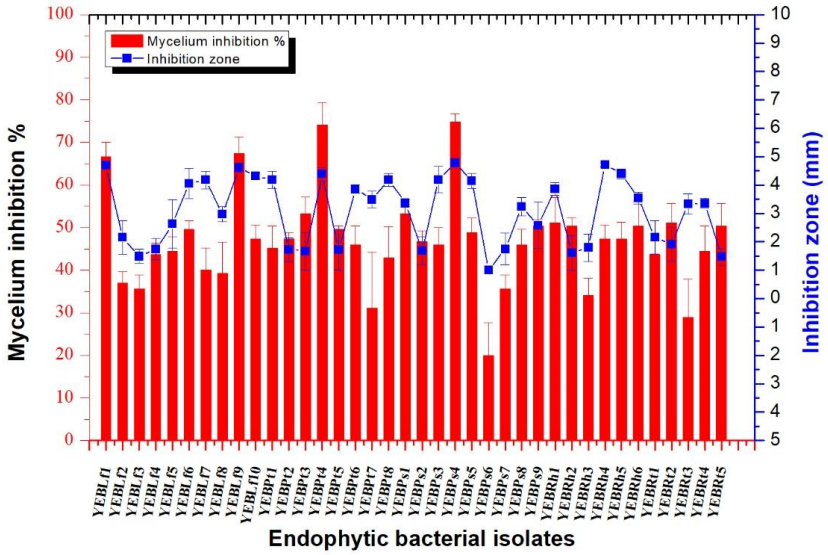
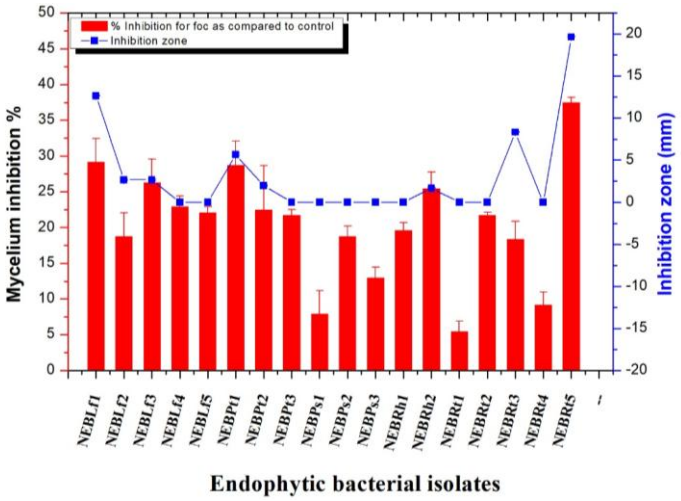


Fig.3 Antagonism of endophytes from banana cv. 'Ney Poovan' vs. *Foc*



Among the thirty-eight bacterial endophytes isolated from 'Yangambi km5', four isolates $YEBLf_1$ (66.66%), $YEBPt_4$ (74.07%), $YEBPs_4$ (74.82%) and $YEBRh_4$ (67.41%) exhibited comparatively higher per cent mycelium inhibition as compared to the control. The inhibition zone produced by the bacterial endophytes was to the extent of 21mm by $YEBLf_1$, 18.33 mm by $YEBPt_4$, 22.00mm by $YEBPs_4$ and 21.33mm by $YEBRh_4$. Most interestingly, it was observed that out of the thirty-eight endophytic bacterial isolates from the banana cultivar 'Yangambi km5', twelve isolates exhibited more than or equal to fifty percent mycelium inhibition *in vitro* against *Foc*. In case of the banana cultivar 'Ney Poovan', the endophytic bacterial isolate $NEBRt_5$ exhibited the highest percentage of mycelium inhibition accounting for 37.5% as compared to control, followed by $NEBLf_1$ (29.17%) and $NEBPt_4$ (28.75%). None of the isolates were found to inhibit even 50% inhibition. In terms of formation of the inhibition zone, $NEBRt_5$ was observed to have a clear zone of inhibition accounting for 19.67mm, followed by $NEBLf_1$ (12.67mm). Out of the eighteen isolates obtained, only two isolates (11%) *i.e.*, $NEBLf_1$ and $NEBRt_5$ produced a clear inhibition zone greater than 10mm width, indicating the antibiosis mechanism.

The population of endophytic bacteria exhibiting antagonism against *Foc* was found comparative higher in the *Foc*-resistant 'Yangambi km 5', than that of the *Foc*-susceptible 'Ney Poovan'. This indicates the probable endophytic modulation of the *in-planta* resistance in banana against the fusarium wilt disease. Earlier reports have shown that, the population of antagonistic endophytic bacteria against various diseases were found to be higher in resistant genotypes as compared to be the susceptible ones in potato (Sturz and Matheson, 1996), tomato (Feng *et al.,* 2013), tobacco (Ma *et al.,* 2004) and Citrus (Lacava *et al.,* 2004). The formation of inhibition zone as well as the inhibition of the mycelial growth in dual plate screening might be ascribed to the interaction of mycotoxins produced by the fungus and the antifungal metabolites produced by the bacteria (Muller *et al.,* 2014).

In conclusion, the present study indicates there is possibility of modulation of the *in-planta* resistance to *Fusarium* wilt in banana by the bacterial endophytes as evident from

the inhibition of mycelia growth of the pathogen by some of the endophytes especially isolated from the FOC resistant. banana cv. 'Yongambi Km 5'. The population of bacterial endophytes having antagonism against *Foc* was higher in case of the *Foc*-resistant genotype 'Yangambi km 5' as compared to the *Foc*-susceptible genotype 'Ney Poovan'. The preliminary study indicates scope for future research avenues on this aspect to target the endophytic modulated *in-plant* resistance against *Fusarium* wilt of banana. There is also a need to understand the modulation of host–pathogen interactive mechanisms governing resistance. The selection of the potential endophytes from the *Foc* resistance genotypes and involving these in triggering the *in-planta* resistance of the *Foc* susceptible banana genotypes needs to be further explored.

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