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EXPLORING THE NOVEL LEAF ENDOPHYTES FOR COMMERCIALIZATION AS BIORESOURCE TOOL TO CONTROL MANGO ANTHRACNOSE

S. THAHIR BASHA, M. NAGALAKSHMI DEVAMMA* AND N.P. ESWARA REDDY

Department of Botany, Sri Venkateswara University, Tirupati – 517502, India

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ABSTRACT

Environmental problems have raised great interest in ecofriendly sustainable agricultural practices. Mango (*Mangifera indica* L.) is one of the most important fruit crops in India and the state of Andhra Pradesh is leading in production and productivity is severely affected with the devastating anthracnose disease caused by *Colletotrichum gloeosporioides* reducing the fruit quality and responsible for 30 to 60 per cent of postharvest losses. Of all the 276 (bacteria - 247 + fungi - 29) putative leaf endophytes screened against the aggressive pathogenic isolate Cg25, the complete inhibition (100%) of the *C. gloeosporioides* pathogen was recorded by the bacterial endophytes EB07, EB35, EB39, EB99, EB57 and EB59. The potential bacterial leaf endophyte EB35 along with its compatible fungicide thiophanate methyl (25 ppm) proved to be the best combination in combating the anthracnose disease both *in vitro* and in field trials and delayed the ripening of mango fruits upto 14 days was identified as *Bacillus subtilis* (EB35) based on 16S rRNA analysis. The talc based formulations of these endophytes evaluated upto 90 days were viable at 4°C and remained potent against *C. gloeosporioides* in dual culture study. The suppressive effect of these beneficial endophytes may also be affected by environmental conditions. In this context research findings on development of cost effective and ecofriendly region specific formulations against *C. gloeosporioides* are herewith proposed.

KEYWORDS: *Colletotrichum gloeosporioides*, anthracnose, endophytes, formulations.

INTRODUCTION

Mango (*Mangifera indica* L.) is considered as one of the most popular and choicest fruit trees grown throughout the tropics and subtropics worldwide known as "King of fruits" (Shad *et al.,* 2002) is grown more than 90 countries in the world and the global production of the mango has doubled in the past thirty years. The total global area under mango is 43.69 lakh ha and the global production is to the tune of 312.51 lakh tones. India ranks first among top world's mango producing countries contributing nearly 49.62 per cent of world's area accounting for 47.80 per cent of the global production (National Horticulture Board, New Delhi). Most of the Indian mango varieties have specific eco-geographical requirements for optimum growth and yield. The major mango growing states are Andhra Pradesh, Uttar Pradesh, Karnataka, Bihar, Gujarat and Tamil Nadu. Andhra Pradesh ranks second in mango production with a share of 15.23 per cent and highest productivity. The country has exported 42,998.31 MT of fresh mangoes to the world for the worth of ₹ 302.54 crores during the year 2014-15. The major export destinations during 2014-15 are United Arab Emirates (54.98%), Nepal (22.78%), United Kingdom (4.12%), Saudi Arabia (3.85%), Qatar (2.80%), Kuwait and Bahrain (2.06%), Oman (1.17%) and Singapore (1.60%). Although a lion's share of Indian mango goes to the Gulf countries, efforts are being made to exploit European, American and Asian markets too (Agricultural and Processed Food Products Export Development Authority (APEDA) and National Horticulture Board, New Delhi). Among all the diseases, mango anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc., (teleomorph: *Glomerella cingulata*) is the most serious disease (Ploetz, 1999) and is the most important disease of mango in humid production areas (Arauz, 2000; Dodd *et al.,* 1997; Lim and Khoo, 1985; Ploetz and Freeman, 2009). Anthracnose is also the major postharvest disease of mango in all mango producing areas of the world (Dodd *et al.,* 1997) and (Swart *et al.,* 2002). The fungus prefers warm humid environment for spreading the anthracnose disease uniformly and effectively. It is the major pre and postharvest disease of mango (Arauz, 2000), and can result in serious decay of fruit during marketing and after sale. Over recent decades there has been increase in public outcry to minimize the use of synthetic fungicides in agriculture products and their presence in the

*Corresponding author, E-mail: thahirbashas@yahoo.com

environment. Synthetic fungicides are the primary means to control postharvest diseases (Eckert, 1990) either used alone, combined in mixtures, or applied separately in sequence (Ismail and Zhang, 2004). These fungicides are at risk for resistance development because a change in the pathogen at this point can render the fungicide less effective or ineffective. Several fungicides are reported to reduce disease development, but are uneconomical and also cause environmental pollution (Misra and Pandey, 1999). Endophytic bacteria are referred to as those which can be detected at a particular moment within the tissues of apparently healthy plant hosts (Hallmann *et al.,* 1997). Many endophytic bacteria possess a number of plant beneficial traits *in vitro*; few of those exhibit them *in planta* and only a small number of endophytes proved to be very effective plant growth promoting biocontrol agents (Scherwinski *et al.,* 2008). All surfaces of living plants leak nutrients that may support microbial growth and thus endophytes which colonize in internal plant tissues, thereby gain direct access to nutrients within a protected environment. The intent of this study is to provide insights into the enormous benefits of leaf endophytes, the products they make, and how some of these organisms assure to show potential use in agriculture that draws much attention as a sustainable alternative to synthetic fungicides and resistance related issues. Hence, efforts are made in order to reduce identify the potential leaf endophytes against *C.gloeosporioides* causing mango anthracnose that add value to ecofriendly agriculture and can easily adapt the specific agro climatic regions.

MATERIAL AND METHODS

Isolation and identification of pathogen

The pathogen was isolated from the mango leaves showing typical symptoms of anthracnose disease by tissue segment method (Rangaswami and Mahadevan, 1999) on potato dextrose agar medium (PDA). The fungal colonies developed were purified by single spore isolation method (Rangaswami and Mahadevan, 1999) and the pathogen was identified based on its mycelial and conidial characteristics as per standard mycological keys (Barnett and Hunter, 1972).

Pathogenicity test

One year old Baneshan mango grafts were infected by pin prick method (Bhuvaneswari and Rao, 2001) followed by spraying spore containing a load of 2.0×10^4 conidia ml^{-1}. Alcohol washed hand atomizer was used separately for spraying inoculum suspension of each isolate. The inoculated seedlings were covered with polythene bags for two days to ensure high humidity and sterile distilled water served as control. Three replications were maintained for each isolate and the disease severity was calculated based on the 0-4 disease rating scale after 8-10 days of inoculation (Agostini *et al.,* 1992).

Isolation and screening for potential endophytes against mango anthracnose

Five grams of healthy leaves were surface sterilized for five minutes with 70 per cent ethanol and homogenized in 20 ml of sterilized phosphate buffer using a mortar and pestle. Appropriate dilutions (10^{-4} for fungi and 10^{-6} for bacteria) of these suspensions are plated on PDA and NA for the isolation of fungi and bacteria, respectively. The plates were incubated at 28 ± 2°C for the development of colonies (Kishore *et al.*, 2005a). Dual culture technique was employed to identify the potential antagonistic leaf endophytes (Bhuvaneswari and Rao, 2001). Mycelial discs measuring 6 mm diameter from four days old cultures of both fungal antagonist and the test pathogen were placed at equidistant on sterile Petri plate containing PDA medium. One day old cultures of bacteria were streaked on opposite side of the pathogen on PDA medium and the Petri plates.

Compatibility of antagonistic endophytes with different fungicides

Spectrophotometric method (Kishore *et al.*, 2005b) was used to determine the compatibility of antagonistic bacterial isolates with the fungicides. Five hundred microlitres of antagonistic bacterial cultures grown in nutrient broth (NB) for 16 hours at 28 ± 2°C at 180 rpm were added to 50 ml of NB in 250 ml flasks containing different fungicides under study. Inoculated flasks were incubated at 28 ± 2°C and the bacterial growth was recorded at 600 nm after 24 hours of incubation.

Mass multiplication of potential bacterial leaf endophytes

The talc based formulations of fungicide compatible potential leaf endophytic bacteria were prepared as described by Vidhyasekharan and Muthamilan (1995). Overnight grown 10 ml of antagonistic bacterium was inoculated into one litre of NB and grown in rotary at 150 rpm for 48 h and 72 h at 28 ± 2°C respectively. One kg of talc powder (montmorillonite) was taken in a metal

tray under aseptic conditions and pH was adjusted to 7.0 by adding calcium carbonate ($CaCO_3$) @ 15 g/kg. 10 g of carboxymethyl cellulose (CMC) was added to 1 kg of talc, mixed well and the mixture was autoclaved for 30 minutes at 121°C for 2 successive days. 400 ml of the bacterial suspension containing 1×10^8 cfu/ml was mixed with carrier cellulose mixture under aseptic conditions. After drying (35% moisture content) overnight under aseptic conditions, the mixture was packed in a polypropylene bag, sealed and stored both at room temperature (28 ± 2°C) and refrigerator (4°C).

Shelf life and efficacy of bacterial formulations

The talc based formulations developed were evaluated periodically in order to determine the shelf life and efficacy of the potential endophytes for every 15 days upto 90 days.

Identification of potential bacterial leaf endophytes by16S rRNA analysis

The 16S rRNA analysis has been selected for identification of potential bacterial leaf endophytes (Marchesi *et al*., 1998. As a part of this, PCR technique has been standardized using 63F as forward primer (5^1 - CAG GCC TAA CAC ATG CAA GTC - 3^1) and 1387R (5^1 - GGG CGG (AT) GT GTA CAA GGC - 3^1) as reverse primer (Marchesi *et al.,* 1998). PCR amplifications were carried out in 0.2 ml eppendorf tubes with 25 µl reaction mixture which consists of 2.5 µl of 10x Taq buffer, 2.0 µl of 25 mM $MgCl_2$, 2.0 µl of respective primer (10 picomoles / µl), 1.0 µl of 10 mM dNTP mix, 1.25 µl of *Taq* polymerase enzyme (conc. 3 U μl^{-1}) and 11.25 µl of sterile PCR water (Genei, Bangalore) and 2 µl (40-50 ng) of DNA sample. Amplification was carried out by 5 minutes of initial denaturation at 94°C followed by 30 cycles of denaturation of 94°C for 1 minute; annealing at 56.8°C for 1 minutes; extension at 72°C for 1.5 minutes with final elongation at 72°C for 5 minutes. Amplified PCR products were subjected to 1.0 per cent agarose gel electrophoresis with 1.0 × TBE as running buffer. The banding patterns were visualized under UV trans-illuminator with ethidium bromide (10 mg ml^{-1}) staining. The DNA banding profiles were documented in the gel documentation system (Alpha Innotech) and compared with 1 kb DNA ladder (Genei, Bangalore).

RESULTS AND DISCUSSION

Twenty eight isolates of *C. gloeosporioides* were isolated from the mango leaves showing typical anthracnose symptoms appeared oval, brown to black spots with greyish centre on the leaves. The results are in agreement with the earlier findings reported by Banos *et al*., 2003 and Linh, 2007. Conidia produced on branch terminals, mummified inflorescences, flower bracts and leaves (most important) are significant sources of inoculum (Dodd *et al*., 1991; Fitzell, 1979). They are produced most abundantly when free moisture is available, but also at relative humidities as low as 95 per cent. Conidia are dispersed by rain splash and infection requires free moisture (Jeffries *et al*., 1990).

The pathogenicity of different isolates of *C.gloeosporioides* was tested by spray inoculation method on one year old baneshan mango grafts revealed that the maximum incidence of disease was recorded in the isolate Cg25 (56.14%) and the least percent disease was observed in case of Cg13 (25.48%). This highly virulent isolate Cg23 was chosen for further experiments. Based on per cent disease incidence the isolates were classified into highly virulent, moderately virulent and less virulent (data not shown). Sampath kumar *et al.,* (2007) also used the same method for studying the pathogenic variability among the isolates of *C. gloeosporioides*. Shivakumar *et al*., (2015) reported the maximum PDI was recorded in isolate Cg2 (46.89%) followed by Cg5 (46.71%) and classified as highly virulent. This experiment was able to reveal the degree of disease severity by the pathogen which depends on the fungal pathotype. Therefore, the information on pathogenic variability which was important to find the differences in disease severity and disease management was successfully carried out.

Among the 247 bacterial isolates tested under *in vitro*, complete inhibition (100%) of the *C. gloeosporioides* was recorded by the bacterial endophytes EB07, EB35, EB39, EB99, EB57 and EB59 and the least per cent of inhibition was observed in EB1 (7.28%) compared to all other antagonists. Statistically there was significant difference among the isolates. Much attention and efforts on mango anthracnose control are concentrated on the use of chemical fungicides as the disease is difficult to control in wet seasons when blossom blight is serious (Pope, 1924). Apart from guidelines of use of strategy of at-risk fungicides that may be helpful for preventing an managing resistance development, there is a need to

devise means for long term sustainable management of resistance so that adequate disease control is assured of which, Biocontrol, using antagonistic organisms offers a reliable approach either alone or in integration with other disease management practices (Patibanda and Prasad, 2004) and is in compliance with sustainable environment issues during recent years (Patel and Patel, 1998).

Several microorganisms are reported to be antagonists against plant pathogens. In such approach, fungicides need to be used with biocontrol agents without any toxic effect on antagonists (Papavizas and Lumsden, 1980). Spectrophotometric method was employed to test the compatibility of the six potential antagonistic bacterial endophytes *viz.,* EB07, EB35, EB39, EB99, EB57 and EB59 with the commonly used fungicides *viz*., carbendazim, thiophanate-methyl, propioconazole, hexaconazole, mancozeb and copper oxychloride as all the six endophytes have enormously inhibited the growth of highly virulent pathogenic isolate Cg25 under *in vitro* when compared to other antagonists. The higher OD value at 600 nm, indicates high compatibility of the antagonist with the fungicide. From the results it is evident that all the isolates were significantly differing with each other.

Among the fungicides, thiophanate-methyl was found to be highly compatible with all the six bacterial antagonists followed by propioconazole, mancozeb, hexaconazole and copper oxychloride. The potential bacterial leaf endophyte EB35 was more compatible with thiophanate-methyl (98.25%) followed by hexaconazole (81.22%), carbendazim (73.42%) and COC (69.72%) and less compatible with propioconazole (55.74%) compared to other fungicides. The other fungicides under the study were able to inhibit the growth of the biocontrol agent to some extent reveals that all the antagonists which may be efficient in controlling the pathogen individually may not integrate with the fungicide in integrated management strategies. Hence, a compatibility of biocontrol agent with the recommended fungicide plays a major role in inhibiting the growth of the pathogen. The mean compatibility of bacterial endophytes with different fungicides was highest in EB35 followed by EB39, EB57, EB99, EB59 and EB 07. In general, application of mixture of strains may results in higher biocontrol and lower variability of biocontrol, as it has been reported in other studies on different pathosystems. Application of more than one antagonist with different ecological requirements would increase the reliability and decrease the variability of biocontrol. The combination of biocontrol agents plus fungicide shall increase the effect of their biocontrol mechanism may be superior.

Talc based formulations of fungicide compatible potential bacterial endophytes *viz.,* EB07, EB35, EB39, EB99, EB57 and EB59 were developed against *C. gloeosporioides* under aseptic conditions and stored both at room temperature (28 ± 2°C) and refrigerator (4°C) respectively. The talc based formulations developed have been evaluated periodically in order to determine the efficacy and shelf life period of the potential endophytes for every 15 days upto 90 day. The bacterial population was checked on day one prior to packing in polythene bags and found to be sufficient (16.51×10^7). In the due course of storage at different temperatures, gradual decline in bacterial populations were noticed in the formulations stored at room temperature. However, the bacterial populations were almost intact in the formulations stored in refrigerator upto 120 days. Dual culture technique was employed to evaluate the efficacy of endophytes both stored at two different temperatures after the shelf life of 120 days. From the results it is evident that the potential endophytes (EB35, EB39 and EB99) stored in refrigerator were effective in inhibiting the pathogen compared to other antagonists stored at room temperature. Thus, the findings reveal that the bacterial formulations stored at 4°C has superior shelf life and retain their antagonistic potential intact in inhibiting the growth of the pathogen compared to formulations stored at room temperature.

The antagonistic bacterial isolates *viz*., EB35, EB39, EB99 and EB35 leaf endophytes having different degrees of antagonistic activity were amplified with the 63F and 1387R primers to to produce 1300 bp fragment product of 16S rRNA region, cloned into the pTZ57/RT vector using TA cloning kit. The plasmid was isolated from the transformed white colonies were analyzed for the presence of 1300 bp insert by restriction analysis using *EcoR I* and *Hind III* in order to release insert from the transformants. Alternatively, transformed white colonies were subjected to Colony PCR and finally sequenced. The fungicide compatible leaf endophyte EB35 was identified as *Bacillus subtilis.* Most broadly, biological control by novel bioagents is the suppression of damaging activities of one organism by one or more other organisms, often referred to as natural enemies. With regards to plant diseases, suppression can be accomplished in many ways.

Integrated management of postharvest mango anthracnose under tropical conditions requires knowledge of the biology of the pathosystem and the technologies available for control, their economical feasibility, and ecological acceptability. Because the plant host responds to numerous biological factors, pathogenic and non-pathogenic, induced host resistance might be considered a form of biological control. More narrowly, biological control refers to the purposeful utilization of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens.

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