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Endophytic *Acremonium kilense* as a potential biocontrol agent against Leaf blotch disease of Clove

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Endophytic microorganism (fungi & bacteria) isolated from leaf samples collected from different agro climatic zones of Kerala state, in order to investigate their biocontrol potential against major foliar pathogens of tree spices (nutmeg, clove & cinnamon). In vitro study was conducted to check its antagonistic potential against leaf blotch disease of clove. Majority of endophytic fungi belonging to Hyphomycetes was observed in the present study. Quantification of mode of action of endophytes was also tested by diffusible, non-volatile metabolitic activity and a significant reduction (65.5 per cent) was observed.

Key words: *Syzygium aromaticum e*ndophytic fungi, *Acremonium kilense*, *Cylindrocladium quinqueseptatum*

INTRODUCTION

The term endophyte is an all- encompassing topographical term which includes all organisms that, during a variable period of their life symptomlessly colonies the living internal tissues of their hosts. This definition is broad enough to include virtually any organism residing inside a plant host. The word endophyte came from two Greek words, 'endon' means within and 'phyton' means plant. Literatures reveal that microorganism including fungi having symbiotic relationship with plants, virulent foliar pathogenic and mycorrhizal root symbionts were included initially in the category of endophytes (de Bary, 1866; Petrini, 1986). Later on Carrol (1986) excludes pathogenic and mycorrhizal fungi from endophytic category.

The plants studied so far harboured at least one species of endophytic fungi in each while others exhibit literally scores of species (Sieber, 2007). Volume of fungi may vary with genotype diversity, type of tissue, chemical and nutrient composition and has ability to enhance the physiology of host plant to enable them to counter abiotic stress and climatic changes besides influencing the plant growth and building resistance to pest and diseases.

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Sopalan *et al.* (2003) have been investigated endophytic fungi *Muscudor albus* residing in cambium tissue of nutmeg (*Myristica frgrans*) in Thailand. Hatem *et al.* (2013) have also reported the antagonistic nature of *Acremonium kilense* against Fusarium wilt of Date palm. However in depth studies have not been made from India for exploiting the potential of endophytic microbes in case of nutmeg and the biocontrol potential against clove diseases. So an attempt is made to investigate the antagonistic potential of endophytes against major foliar disease of clove.

MATERIALS AND METHODS

Isolation of pathogen

The pathogen causing leaf blotch disease of clove was isolated from the infected leaves collected from different locations of Kerala. The diseased leaf samples were brought to the laboratory, washed under tap water and the infected area along with healthy portion were cut in to small bits. The bits were surface sterilized with one per cent sodium hypochlorite solution for one minute followed by washing in three changes of sterile distilled water. The surface sterilized bits were placed on Potato dextrose agar (PDA) medium in Petri dishes and incubated at room temperature

(26 ± 2 °C). When the fungal grew was visible, small bits of mycelia were transferred to PDA mediated Petri dishes and the isolates were purified by hyphal tip method. These purified cultures were maintained in PDA slants for further studies. The cultural and morphological characters of the isolates were also studied.

Collection of plant materials

Nutmeg (*Myristica fragrans*) grew in different districts of Kerala state (10.8505°N, 76.2711° E). Samplings were done for a period of eight months representing two seasons summer season (May to July) and monsoon (August to October) during 2014 to 2015**.** The leaf samples were collected from individual healthy and symptomless plants from each site. The collection of leaves, were made from each selected plant above the ground with a help of ethanol-disinfected knife and placed in sterile polyethylene bags separately. The samples were brought to the laboratory, washed thoroughly in running water and shade dried under fan before isolation procedure was undertaken.

Sterility check

The surface of leaves harbour a lot of epiphytes, thorough surface sterilization was needed to eliminate them. The concentration of sterilant and time of exposure were standardized so as to get the maximum number of endophytes with no growth on sterility check. Three different concentrations of sodium hypochlorite *viz.,* one, two and three per cent were tried for three different exposure times *viz.* two, five and ten minutes. Further, three different weights of leaf samples viz.0.5g, 1.0g and 2.0g were also tried. Since the isolation from the 2g of sample after surface sterilization with two per cent sodium hypochlorite for 10 min yielded good number of colonies with no growth in sterility check, it was selected for further studies.

Isolation of endophytes

For the isolation of endophytes from healthy leaves of nutmeg healthy samples were brought to laboratory and weighed out to 2g each. It was followed by washing in three changes of sterile water and blot dried. The leaf bits were then transferred to sterilized mortar containing 8 ml sterile Potassium phosphate buffer (PB 0.1M, pH) washed thoroughly in the buffer. From the final buffer wash, one ml was pipetted out and poured into sterile Petri plate, to this molten and cooled medium was added and this served as a sterility check. If microbial growth was observed in sterility check within four days, the isolates obtained from particular samples were discarded. The surface sterilized leaves of nutmeg were triturated (McInroy and Kloepper, 1995) using sterile mortar and pestle with 8 ml of sterile buffer. The triturate was serially diluted in sterile PB up to 10^{7}. One ml of diluted triturate was pipetted in to sterile Petri plate poured with Potato Dextrose Agar medium (PDA), supplemented with penicillin-G (60mg L^{-1}) and streptomycin sulphate (80 mg L^{-1}) to inhibit the bacterial contamination. Each sample, plated with media were incubated at room temperature (30° C approx.) for 4-6 weeks in dark. The plated segments were observed once a day for the growth of endophytic fungi. Hyphal tips growing out on the plates were immediately transferred into PDA slants, purified and maintained at 4°C. The fungal isolates were identified based on their morphological and reproductive characters using the standard identification manuals (Barnett and Hunter, 1998). The fungal cultures that failed to sporulate were categorized as sterile mycelia. All the isolates were maintained in Potato Dextrose Agar slants.

Determination of antifungal activity of the endophytic fungi against Cylindrocladium quinqueseptatum

Fifteen fungal isolates were evaluated for their antagonistic potential against the pathogens of nutmeg and leaf blotch disease of clove, by dual culture method (Skidmore and Dickson, 1976) in comparison with standard culture of *Trichoderma viride*. The organisms were inoculated on dual cultures after giving due consideration of their growth rate. Mycelial disc of the pathogen from seven day old culture grown on PDA was placed on one side of the plate and incubated at room temperature (26 ± 2 ° C) for two days. The mycelial disc, (10 mm) of antagonistic fungi were placed on other side of the plate, four cm away from the pathogen and incubated. Three replications were maintained for each isolate. The pathogen grown as monoculture served as control. The plates were observed daily after 24 h of inoculation of antagonists till the pathogen grew and covered the plate kept as control. The per cent inhibition of the pathogen was calculated using the formula suggested by Vincent (1927).

$$PI = \frac{C - T}{C} \times 100$$

PI = Per cent inhibition, C = Growth of the pathogen in control (mm), T = Growth of the pathogen in dual culture (mm)

Based on the per cent inhibition of mycelial growth of the pathogen, the efficient antagonists were selected for further studies. The nature of antagonistic action of endophytic fungi against the foliar pathogens of these tree spices was assessed by the method of Purkayastha and Bhattacharya (1982) and assigned to four categories.

A. Overgrowth : Pathogen overgrown by test organism
B. Homogeneous : Free intermingling of hyphae
C. Cessation of growth : Cessation of the growth at line of contact
D. Aversion : Development of clear inhibition zone

Determination of diffusible, non-volatile metabolites activity

Antibiosis test for production of diffusible, nonvolatile inhibitory metabolite was carried out using cellophane paper method described by Dennis and Webster (1971). Cellophane paper of 9 cm diameter was taken and sterilized in autoclave at 121°C for 15 min and then each sterilized disc was aseptically placed over the PDA inoculated plates. Ten mm discs was taken from the growth of each isolate of endophytes was placed at the centre of the cellophane paper and incubated for 72 h. After this, the cellophane paper along with adhering antagonists was removed carefully and 8 mm disc of pathogens of nutmeg, clove and cinnamon was immediately placed on the medium at the central position previously occupied by antagonist. The growth of the pathogen was incubated for 48 h up to seven days and the growth was compared with that in control. Three replications were maintained and the per cent inhibition of the pathogen was calculated.

RESULTS AND DISCUSSION

Identification of pathogen

The fungal growth was slow and it took 13-14 days to attain full growth in PDA mediated Petri dishes. Hyphae septate, highly branched, and light reddish brown in colour. Conidiophores produced in masses with primary, secondary and tertiary sterigmata having broom shaped appearance. The culture sporulate and produced darker reddish brown pigmentation in to the medium. Conidia hyaline, straight, cylindrical with rounded ends and 1 to 3 septate and 32.20 to 35.80 x 3.58 to 6.58 µm in size, and sterile filament consisted of vesicle at the tip. (Fig. 1) Based on these characters and further confirmation from N.C.F.T New Delhi with ID.NO (6766.15) the organism was identified as *Cylindrocladium quinqueseptatum.*

Table 1 : Occurrence of endophytic fungi in leaves of *Myristica fragrans* in different districts of Kerala

District	Endophytic fungi	Summer	Monsoon	Total
Thrissur	*Asperigillus*	-	2	2
Wayanad	*Penicillium*	-	2	2
Calicut	*Colletotrichum gloeosporioides*	2	1	3
Palakkad	*Acremonium kilense*	-	1	1
Pathanamtitta	*Mycelia sterilia* sp. 1	1	2	3
Thiruvananth-apuram	*Mycelia sterilia* sp. 2	1	1	2

A total of 15 isolates belonging to ten taxa including three isolates of sterile mycelia and two unidentified species were obtained from the leaves of *Myristica fragarans*. Of the total species isolated majority of endophytes constituted was Hyphomycetes followed by Zygomycetes. Among the endophytes, species of *Aspergillus, Penicillium, Fusarium, Colletotrichum* and sterile mycelia were dominant. Maximum endophytes were recovered from the leaves during the monsoon season, and it was also noticed that maximum endophytes were obtained from the samples collected from natural eco system than in the farmers fields. However less number of endophytes was isolated from the samples collected during the summer season. Among the isolates *Acremonium* was isolated during the summer season and isolates like *Asperigillus* and *Penicilium* were isolated during the late monsoon season. In general, more endophytes were isolated during the monsoon than in the summer season. The different species of endophytic fungi isolated from leaf samples collected from different locations of Kerala state are presented in Table 1.

Total 15 endophytic fungal isolates were screened against the leaf blotch disease of clove. Only one isolate *Acremonium kilense* exhibited considerable level (60 per cent) of inhibition over all the test pathogens.

Determination of diffusible non volatile metabolites

Similarly when *Acremonium kilense* is tested for its ability to produce diffusible non volatile metabolities it recorded 64.55 per cent activity against *Cylindrocladium quinqueseptatum*. against the leaf blotch pathogen of clove, and it showed

overgrowth type of mechanism of action. The effective bioagent was tried to identify in Department of Plant Pathology, College of Horticulture, Vellanikkara. The cultures grew rapidly in 12 days, hyphae thin walled, hyaline, conidiogenous cells, phialidic, mostly solitary, conidia produced singly at the tip of phialides and aggregating into slimy heads, ellipsoid to cylindrical with rounded ends. Straight or sometimes slightly curved single celled and hyaline (Fig. 2) Based on these characters and coupled with confirmation

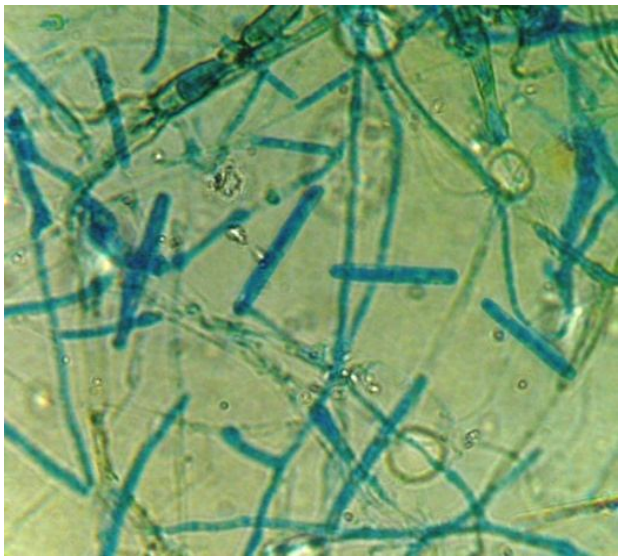

Fig.1: *Cylindrocladium quinqueseptatum* conidiophores (400 X)

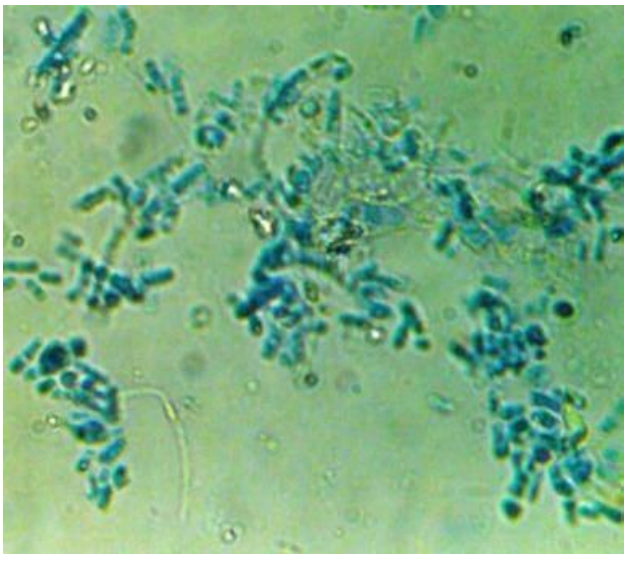

Fig. 2: *Acremonium kilense* conidia (400X)

of identification from National Centre for Fungal Taxonomy (N.C.F.T), New Delhi (ID No. 6762.15) the antagonist was identified as *Acremonium kilense.*

Endophytic fungi had been found associated with every plant species investigated so far from tropical and temperate hosts, yet they are poorly investigated because of their cryptic and ephemeral nature (Rodrigues and Petrini, 1997; Strobel *et al.* 2001). There were few reports on the endophytic fungi of invasive plant species, despite their abundance in certain invasive hosts (Shipunov *et al*. 2008). In the present study, rich endophytic fungal comprising Zygomycetes, Coelomycetes, Hypomycetes, sterile mycelia and unidentified genera from the leaf tissues of *Myristica fragrans*. Among the endophytes, class Hyphomycetes was dominant. Such dominance of Hyphomycetes as endophytes had also been reported from several plants such as *Azadirachta indica* and *Terminalia indica* (Mahesh *et al*. 2005; Manoharachary and Nagaraju, 2016). indicating their ubiquity among the plant kingdom. It was generally believed that plants growing in lush tropical rainforests, where competition for light and nutrients might be severe, could be most likely to host the greatest number of endophytes than the temperate parts of the world. The dominant endophytic isolates were species of *Aspergillus*, *Acremonium* and *Colletotrichum*. These fungi are generally found as plant pathogens and they might have evolved to endophytic life style due to loss of virulence (Freeman and Rodrigues ,1993). But at present we had isolated some endophytes and some of them displaying biological activity. (Kour *et al*. 2008; Deng *et al.* 2009 ; Hatem *et al.* 2013). It also showed higher species richness than the summer isolates. This could be due to higher precipitation rates which might have favoured spore germination in fungi indicating horizontal transfer of the endophytes in the host tissues. A strong correlation had been observed between the endophyte infection levels and cumulative precipitation (Wilson, 2000). In many instances, leaves sampled during the wet season harboured more endophytes than those screened during the dry season (Rodrigues 1994; Suryanarayanan *et al*. 1998). It was known that the endophytic fungi existing in the plant are potential sources of antimicrobial substances (Strobel and Daisy, 2003) and this had also been demonstrated in earlier studies (Samanta and Dutta, 2004; Mohanta *et al.* 2008 ; Deepa and Mathew, 2015). Production of different levels of diffusible non volatile metabolites by *Acremonium Kilense* suggested the presence of active substances. Similarly, Kurian (2011) noticed the production of volatile metabolites.

However the results presented a base for the role of secondary metabolites in the mechanism of antagonism, and it emphasized the importance of quantification of the secondary metabolites that are involved in the mechanism of action of endophytes. Considering their unusual biology and adaptability in adverse environmental conditions, tree spices that grow in warm humid conditions might harbour interesting endophytic microbes with multiple applications. The present study on endophytes of *Myristica fragrans* with antimicrobial activity against the leaf blotch of clove caused by *Cylindrocladium quinqueseptatum* had widened the biocontol spectrum of endophytes. This research could throw a lime light on broad spectrum activity of endophytes.

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