EVALUATION OF BENEFICIAL FUNGAL ROOT ENDOPHYTES AGAINST FUSARIUM ROT IN SMALL CARDAMOM

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by

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(2019-11-162)

THESIS

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KERALA, INDIA

2022

DECLARATION

I hereby declare that this thesis entitled **"Evaluation of beneficial fungal root endophytes against Fusarium rot in small cardamom**" is a *bonafide* record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

Place : Vellayani

Date : 02/04/2022

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Certified that this thesis entitled **"Evaluation of beneficial fungal root endophytes against Fusarium rot in small cardamom"** is a record of research work done independently by **Ms. Aishwarya Manoharan (2019-11-162)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
µm	Micrometer
^{0}C	Degree Celsius
CD	Critical difference
Cfu	Colony forming unit
Cm	Centimeter
CRD	Completely Randomized Design
DAC	Days after co-cultivation
et al.	And other co-workers
G	Gram
Ha	Hectares
i.e.	That is
MAT	Months after treatment application
Mg	Milligram
min.	Minute
Ml	Millilitre
OD	Optical density
PDA	Potato Dextrose Agar
PDB	Potato dextrose Broth
PDB	Potato dextrose broth
SE (m) ±	Standard error of mean
sp.	Species
spp.	Several species
Viz.	Namely

Introduction

1. INTRODUCTION

Elettaria cardamomum (L.) Maton is one of the highly priced spice crops of the world. The high price mainly reflects from the premier reputation of the pleasantly scented spice. India is one of the largest producers of cardamom covering an area of 69,190 ha with an annual production of 22,520 tonnes in 2020-21. Kerala accounts for about 56 per cent of total area and 91 per cent of total production followed by Karnataka and Tamil Nadu (Spice board India, 2021). Up until this period, India had monopolised the global export market for about three thousand years. However, since then, the global market for high-quality cardamom grown in Southern India and Kerala has dropped drastically. The main rationale for the drop is Guatemala cardamom, which is marketed at a considerably lower price despite of its inferior quality. In addition to this, high amount of pesticide residue in export cardamom has resulted in the decline in export in India by 80 per cent (Beevi *et al.*, 2014). Of late, cardamom producers have recently been in a perplexed state due to the impending risk associated with the return of consignments from foreign countries.

Another obstacle in cardamom cultivation and production is the occurrence of diseases. A number of fungi, bacteria, viruses, nematodes and pests attack this crop, leading to heavy crop loss. The most significant part of cardamom production is the generation of healthy and disease-free planting material onto the main field (Ankegowda, 2008). Propagation of cardamom is mainly accomplished through two methods *viz.*, seedlings and suckers. The appropriate establishment of planting materials in the field determines the succeeding production in the future. Cardamom is particularly susceptible to drought and other biotic or abiotic stresses, and because drought occurs frequently in the fields, it has a negative impact on seedling and sucker establishment. As a result, production and nurturing of exceptional cardamom plants with a well-established root system that can endure the challenging field circumstances during its field establishment is the need of an hour.

In India, cardamom is cultivated along the Western Ghats at altitudes between 700 m to 1500 m which receives an annual rainfall of about 1500 mm - 3000 mm. Since

cardamom farming is inextricably linked to the Western Ghats's thriving biosphere, green operations are critical for sustaining both the crop and ecology. The focus has been rapidly shifting to ecologically sound production practices with low pesticide residue that are safe for both environment and consumers while still supporting productivity and profitability. This entails the use of endophytes that can alleviate both biotic and abiotic stress conditions in cardamom production.

Endophytic fungi, which invade healthy plant tissues asymptomatically, are well known for promoting plant development, eliciting pathogen defense responses, and alleviating abiotic stressors including drought and salinity. *Piriformospora indica, an* axenically cuturable beneficial root endophyte, is well established to be effective for promoting plant development and also protecting the host from biotic and abiotic stress (Waller *et al.*, 2005; Serfling *et al.*, 2007; Ansari *et al.*, 2013; Zarea *et al.*, 2012; Johnson *et al.*, 2013; Azizi *et al.*, 2021). Similarly, mycorhizal fungus, *Glomus fasciculatum* has been proven to be useful in soil and plant health maintanence (Thomas *et al.*, 1994; Doley *et al.*, 2014; Vijayan *et al.*, 2018).

One of the most common fungal diseases in the Cardamom Hills is Fusarium rot caused by *F. oxysporum*. Under ideal climatic conditions, this disease is devastating at all stages of crop development.

In this context, the present study entitled “Evaluation of beneficial fungal root endophytes against Fusarium rot in small cardamom” was undertaken with the following objectives,

1. To evaluate the colonisation and interaction of two beneficial fungi, *Piriformospora indica* and *Glomus fasciculatum*, in cardamom.
2. To evaluate the efficacy of endophytes in the management of Fusarium rot disease of cardamom.
3. To elucidate the role of gibberellic acid in the endophytic fungi-mediated disease tolerance.

Review of Literature

2. REVIEW OF LITERATURE

Cardamom Hills Reserves (CHR) of Western Ghats is one of the important areas of production for cardamom. Cardamom cultivation in CHR is affected by many diseases among which *Fusarium* infection is the major threat (Dhanya *et al.*, 2018). The first report of *Fusarium* infection in cardamom dates back to 1979 in the form of capsule rot (Wilson *et al.*, 1979). Seed rot and seedling wilt in nurseries were also caused by this pathogen (Siddaramaiah, 1988).

Dhanapal and Thomas (1996) reported incidence of *Fusarium* in cardamom which started as stem rot and finally resulted in partial breakage and lodging of pseudostem at the point of infection. Of late, studies in Idukki districts showed wide spread "foliar yellowing and plant decline" caused by the major wilt pathogen *Fusarium oxysporum* (Vijayan *et al.*, 2009).

2.1. COLLECTION OF FUSARIUM ROT INFECTED PLANTS FROM THE HOT SPOT AREAS, ISOLATION OF PATHOGEN, PROVING KOCH'S POSTULATES AND MAINTENANCE OF THE CULTURE

In a survey conducted by Vijayan *et al.* (2009) during 2009-2011, the incidence of root rot and foliar yellowing varied from 0-48 per cent in 2009-2010 to 68 per cent in 2010-2011. The plants of age 4-6 years were more prone to panicle blight symptom which was found to be severe during April-July.

2.1.1. Symptomatology

Several plantations in Idukki districts of Kerala reported root tip rot and leaf yellowing. The disease appeared after the monsoon rains and became severe during summer months. Yellowing of the foliage later resulted in the leaf drying. The symptoms started from the older basal leaves and then reached towards the middle portion of the tillers. The basal leaves which were affected earlier became fully yellow and dried off.

Root of the affected plants also showed characteristic symptoms like decay of root which proceeds towards the plant base. Later, these roots shrivelled and resulted

in off-white to grey colour to the tips which ultimately leading to rotting when sufficient moisture was available (Vijayan *et al.*, 2009).

Another characteristic symptom by *Fusarium* in small cardamom includes pseudostem rot. It occurs in the form of dark brownish oval or round necrotic patches on pseudostem and base of the petiole. These portions elongate and later spread towards leaf sheath and in advanced stages these lesions on pseudostem splits off breaking the tillers.

Vijayan *et al.*, (2009) also reported another symptom i.e. panicle blight in plants affected by *Fusarium*. It is prevalently seen after South West monsoon. It causes drying of flower buds, young capsules and panicle tips; and in severe cases mature capsules also get dried. The drying started from panicle tip and proceeded towards base of the panicle. Pinkish to purple colour may be observed in the dried portion.

2.1.2. Isolation of pathogen

The pathogen was isolated from root and panicles and was identified as *F. oxysporum* Schlecht based on colony and conidial characters; and also revealed that the Fusarium isolates were white in colour with a purple tinge (Thomas and Vijayan, 2002). Vijayan *et al.* (2013) isolated twenty different isolates of *Fusarium* sp. from roots, rhizomes, pseudostem and panicles in PDA and conducted molecular characterization in five morphologically distinct isolates using Random Amplified Polymorphic DNA (RAPD) markers to determine genetic variability. According to the findings of the study, *F. oxysporum* consisted of a single lineage with moderate genetic diversity within that lineage.

Fusarium specific medium was used for isolation of the pathogen from root tip, pseudostem, panicle and rhizome (Vijayan *et al.*, 2014).

2.1.3. Proving Koch's postulates

Thomas and Vijayan (2002) proved the pathogenicity by pin prick method. Pin pricks were made at the base of the healthy pseudostem and 10 day old fungal culture and spore suspensions ($5x10^5$ cfu ml^{-1}) were used for inoculation. For the inoculation of panicles, detached panicles of healthy plants were used. Brownish discolouration

was observed at the base of the pseudostem in 6-10 days after inoculation with root isolates. Foliar yellowing occurred 20 days after inoculation. The reisolation proved the pathogenicity of *F. oxysporum* Schlecht.

Crude extracts of metabolites of *F. oxysporum* were used for inoculation of seedlings maintained in green house. The pathogenicity and cross infectivity of the pathogens isolated from roots, panicles, rhizomes and pseudostem of small cardamom was proved (Vijayan *et al.*, 2014).

Gopi *et al.* (2016) proved the pathogenicity of *F. oxysporum* in large cardamom by placing 5 mm mycelial plug in an opened flower and covering the spike of it by polythene bag. Symptom appeared five days after inoculation. For proving pathogenicity of stem isolates, an injury was made on the pseudostem near collar region at ground level and inoculated with mycelial plugs of pathogen. It was then covered with polythene bag as in the case of spike to maintain humidity. The blackish lesion appeared three days after inoculation.

Dhanya *et al.* (2018) worked out the Disease severity or per cent disease index (PDI) to assess the extent of damage caused by the pathogen using a 0-5 scale by visual observation of symptoms on the infected tillers and panicles.

2.1.4. Morphological, cultural and pathological characters of pathogen

According to Thomas and Vijayan (2002), the fungal isolates from infected root and panicle developed white colonies with purple tinge on Potato dextrose agar (PDA). The mycelium was delicate and the microscopic study revealed the production of macro and micro conidia by the pathogen. Macro conidia were long and slightly curved with 3-5 segments, while microconidia were small and straight. The size of the macro conidia was 23.6 x 3.48 µm, while that of micro conidia was 9.03 x 2.58 µm.

Gopi *et al.* (2016) first reported *Fusarium* infection in large cardamom. The aerial floccose mycelium of *Fusarium* was initially white which was later turned to purple. The macro conidia was multi-septate, pointed and had a curve towards the end with size ranging from 26.91 - 57.64 µm x 2.01 - 2.59 µm. The microconidia were one or two celled with size 5.62 - 8.44 µm x 1.86 - 2.71 µm.

2.2. EVALUATION OF THE COLONISATION AND INTERACTION OF *P. indica* AND *G. fasciculatum*

Plant roots provide a major ecological niche for many microorganisms. They have an important role not only in soil fertility but also in soil health improvement (Narware *et al.*, 2019).

2.2.1. Endophytes: Behaviour and colonization

The word endophyte means "in the plant" coming from two Greek words "endon" meaning within, "phyton" meaning plant. The earliest definition of endophytes was given by De Bary (1866) as organisms that can colonise internal tissues. Petrini (1991) defined endophytes as organisms that inhabit for atleast one period of their lifecycle inside plant tissues without causing any harm to the hosts. Multifaceted interaction between endophytes and plants includes reduced herbivory, priming host's defense responses, abiotic and biotic stress remediator, protection from oxidative burst, reduced expression of protein secretion systems (SSs) and genes, modulation of plant's immune system and phytoremediation (Khare *et al.*, 2018).

From the point of entry, microbes may systemically colonize the plants from roots to shoots, shoots to flowers or fruits, and/or from flowers to fruits and seeds, and they may also cause localized colonization inside plant organs (Hardoim *et al.*, 2015). For eg: *Neotyphodium and Epichloe* species systemically infect the intercellular space of leaves, reproductive stems, and seeds of their hosts (Torres *et al.*, 2012) while *Piriformospora indica* is a root endophyte (Varma *et al.*, 1998).

Successful colonization by endophytes is affected by different factors including the plant tissue type, the plant genotype, the microbial taxon and strain type, growth medium, plant age and species, inoculum density, fungal species, as well as the biotic and abiotic environmental conditions. Studies have shown the chemotactic response of endophytes to root exudates of host plants (Rosenblueth and Martínez-Romero, 2006; Compant *et al.*, 2010; Brader *et al.*, 2014). Root exudates are rich in biomolecules, which attract or are recognized by friendly microbes including endophytes. Arabinogalactan proteins (AGPs), which are highly glycosylated members of the hydroxyproline-rich glycoprotein (HRGP) superfamily of plant cell wall proteins, help

in establishing interaction of plant with microbe (including endophyte) at several stages. These proteins have a definite role in root colonization, working as repellents or attractants for microbes and in the development of infection structures (Nguema-Ona *et al.*, 2013).

2.2.2. *Piriformospora indica*

P. indica is an axenically culturable beneficial root colonising fungus belonging to the order Sebacinales. It is well known for is multi-functional activities viz., increased seed production, nutrient uptake, biomass and (a)biotic stress tolerance in plants (Johnson *et al.*, 2014 and Gill *et al.*, 2016).

According to Unnikumar *et al.* (2013) *P. indica* can colonise a wide range of plants and provide them with multifaceted amenities. *P. indica* inoculum were found to be very effective in commercial applications to various crops within the defined parameters *viz.*, inocula quantity, inoculation time point, as well as soil selection for plant cultivation. *P. indica* mediated crop improvement include increase in the biomass, seed germination, plant growth and development, and crop productivity under (un)favourable environmental conditions and it is being argued as powerful tool for crop improvement (Ansari *et al.*, 2014).

2.2.2.1. Maintanence of P. indica culture

According to Serfling (2007) endophytes require specific media for growth and development. *P. indica* culture was maintained in complete agar medium at optimum temperature for growth and spore production. Enhanced growth of *P. indica* was observed when *P. indica* was cultured in modified Kaefer medium which was maintained at 25°C with 12 h photoperiod (Sun *et al*., 2010). *P. indica* cultured on PDA medium produced hyaline, white mycelium and chlamydospores at 15 DAI (Sun *et al.*, 2010). Active stage of fungus was maintained by regular subculturing at 10 days interval in PDA (Satheesan *et al.*, 2012).

Varma *et al.* (2012) optimised the growth condition for *P. indica* in modified liquid Hill-Kaefer medium at pH 6.5, temperature 30°C at an agitation speed of 200 rpm. Maximum dry cell weight and spore yield was obtained five days after inoculation. Cheng *et al.*, (2020) obtained liquid culture of *P. indica* by placing 2-3 fungal mycelial

bits in 100 ml Potato dextrose broth taken in 250 ml Erlenmeyer flask maintained at 28°C in the dark for three days at 200 rpm agitation.

2.2.2.2. Co-cultivation of P. indica with cardamom

Two days old barley seedling's root was immersed in aqeous solution of 0.05 per cent Tween 20 containing 5 x 10^5 ml^{-1} *P. indica* chlamydospores grown in mixture of expanded clays (Deshmukh *et al.*, 2007). For establishing co-culture of *P. indica* with maize plants, the plants were grown in sterile soil inoculated with one per cent fungal mycelium that was mixed with Hoagland's solution (Kumar *et al.*, 2009). Johnson *et al.* (2013) standardised co-cultivation protocols for *P. indica* with the model plant *Arabidopsis thaliana. In vitro* co-cultivation was established by transferring nine to twelve days-old Arabidopsis seedling and four weeks-old *P. indica* plugs to modified PNM medium simultaneously. These seedlings after two weeks were transferred to sterile soil-vermiculite mix for *in-vivo* co-cultivation.

Co-cultivation of *P. indica* with tomato was carried out by Sartipnia *et al.* (2013). Two weeks old tomato seedlings were inoculated with *P. indica* chlamydospores suspension (10^6 ml^{-1}) for 12 h and was later transferred to pots containing sterilized mixture of sand and perlite substrate. The rice seedlings were dipped in chlamydospore suspension of *P. indica*, then transferred to pots filled with 1:1 (V:V) mixture of sterile sand and soil for establishing co-cultivation (Nassimi and Taheri, 2017).

Anith *et al.* (2018) established the co-culture of black pepper with *P. indica* by mixing one per cent *P. indica* mycelial mass which was grown in PDB with sterile vermiculite. Fifty gram of this inoculum was added to a small cavity made in potting medium and rooted black pepper cuttings were then placed in the cavity above the inoculum. Surface sterilized seeds of *Solanum melongena*, *Abelmoschus esculentus* and *Capsicum annuum* were transferred to medium containing MS and PDB (containing *P. indica*) in a 1: 1 ratio, for co-cultivation with *P. indica* (Jisha *et al.*, 2019). Establishment of co-culture with *P. indica* in banana was done by pouring suspension containing about 60 g mycelia mass L^{-1} and 1 x 10^5 chlamydospores

ml^{-1} directly into the soil close to the root system of one month old banana plantlets at concentration of 100 ml kg^{-1} soil (Cheng *et al.*, 2020).

2.2.2.3. P. indica colonisation

The ability of *P. indica* to colonise a wide range of plants (vascular plants to mosses) implies a highly evolved colonising strategy (Qiang *et al.*, 2012). Root colonization by *P. indica* is known to start with intracellular chlamydospore germination and formation of extracellular hyphal mats, and simultaneous penetration into rhizodermal and cortical cells (Deshmukh *et al.*, 2006; Jacobs *et al.*, 2011). The root colonisation increases with maturation and the highest mass of fungus was found near root tips (Deshmukh *et al.*, 2006). Serfling (2007) reported formation of intracellular hyphae in epidermal cells of wheat root after one week of inoculation and chlamydospores within epidermal cells and root hair after three weeks of inoculation.

According to Kumar *et al.* (2009) there was 20-30 per cent colonisation of maize roots at 10^{th} day which later increased to 70 per cent on 20^{th} day. *P. indica* colonisation in barley roots indicated inter and intracellular hyphae in rhizodermis and cortex of barley roots with fungal sporulation initiating at 14^{th} day (Schafer *et al.*, 2009). *P. indica* treated turmeric roots showed inter and intracellular root colonisation with intracellular chlamydospores with colonisation ranging from 60-70 per cent (Bajaj *et al.*, 2014). Fully developed intracellular chlamydospores having pear shape was observed after 45 days of co-culture in groundnut with root colonisation ranging from 50-60 per cent (Tarte *et al.*, 2019).

Anith *et al.* (2011) reported the colonization of *P. indica* in tissue cultured black pepper plants and its positive effect on vegetative growth. The effect of root colonisation by *P. indica* in lateral shoot branch cuttings of black pepper was studied in which the endophyte colonised seedlings had pronounced growth promotion by production of more number of leaves. There was significant difference between the inoculated and uninoculated plants with respect to the chlorophyll a and total chlorophyll content of leaf tissues. Early flowering and spike setting was observed in plants inoculated with *P. indica*. The total fresh and dry weights of berries harvested from the *P. indica* inoculated plants were significantly higher than that from the control

plants. Inoculation with the fungus also increased the total oleoresin and piperine content in the berries (Anith *et al.*, 2018).

2.2.3. Arbuscular mycorrhiza fungi (AMF)

The term mycorrhiza came from two Greek words “mykes” meaning fungus and “rhiza” meaning root implying the association of fungus with plant roots (Frank, 1885). AMF comes under the class Zygomycetes under the order Glomales (Pirozynzki and Dalphe, 1989).

AMF colonise the root cells through propagules that already exists in the soil like mature spores, mycorrhized plant fragments or mycorrhized plants in their vicinity. The hyphae of AMF are aseptate and can grow either intraradically or extraradically. Intraradical mycelium produces a characteristic branched structure in cortical cells known as “arbuscules”. AMF also produces intraradical cells known as “vesicles” which has reserve function. The spores of AMF are asexual, multinucleated and produced inside or outside the mycelium directly (Smith and Read, 2008).

Mycorrhiza has large influence on ecological and ecosystemic processes. Colonisation with arbuscular mycorhizal fungi improves crop yields as it provides efficient use of fertilizers and soil nutrients (Javaid, 2009), protection against drought stress (Porcel *et al.*, 2007, Porcel and Ruiz-Lozano, 2004) diseases (Liu *et al.*, 2007), increased N-fixation in legumes, and improved soil physical properties (Hallett *et al.*, 2009).

AMF has widest host range forming mutualistic associations with more than 90 per cent of vascular plants (Kendrick and Berch 1985). Preliminary surveys in Western Ghats suggested the occurrence of *Glomus* sp. to be dominant in root zone soils indicating their wide distribution (Muthukumar and Manian, 1993; Vasanthakrishna *et al.*, 1994).

The early report on response of cardamom seedlings to vesicular arbuscular mycorrhiza was done by Sreeramulu and Bhagyaraj (1998). The growth response of cardamom to thirteen different VAM was tested and the seedlings inoculated with mycorrhiza were taller with more number of leaves and tillers, increased biomass and uptake of nutrients compared to seedlings. The main effect of VAM in increasing

seedling growth was through increased uptake of diffusion limited nutrients especially phosphorus. Such increased P uptake has been attributed to increased· surface area of absorption and enhanced translocation. Among the different mycorrhizas tested *Gigospora margarita* and *Glomus monospora* exhibited best results. Maximum root colonisation was observed in plants inoculated with *Gigospora margarita* followed by *Glomus monospora.*

Vijayan *et al.* (2000) conducted field trial in cardamom seedlings and reported three exotic strains of VAM like *Glomus microcarpum*, *Glomus mosseae* and *Glomus fasciculatum* as efficient strains suitable for cardamom.

Microscope based method has been one of the popular method for observing the root colonisation by AMF. The roots were cleaned with KOH, neutralised with HCl, stained with Trypan blue and the presence of colonisation was assessed by the presence or absence of hyphae, vesicles and arbuscules in each segment of root (Philips and Hayman, 1970).

2.2.4. Interaction of *P. indica* and *G. fasciculatum*

Response of aerobic rice to *P. indica* and *G. fasciculatum* under greenhouse conditions was studied by Das *et al.* (2014). The biometric characters were higher in all endophyte treated plants compared to the untreated plants. After 120 days of planting, *P. indica*-treated plants had the maximum height, number of leaves, and number of tillers compared to other treatments. *P. indica*-inoculated rice plants were 1.25 times as tall as dual inoculated plants and 1.14 times as tall as *G. fasciculatum* treated plants. Endophyte treated rice plants had higher root and shoot dry weight, number of panicles per plant, and grain yield than untreated rice plants. Plants individually treated with *P. indica* or dually treated with *P. indica* and *G. fasciculatum* had higher root and shoot dry weight, number of panicles per plant, and grain yield than untreated rice plants.

According to Mansotra *et al.* (2015) significant improvement in dry weight of root was observed with combined treatment of *P. indica*, rhizobacteria and *Mesorhizobium cicer* in comparison to *M. cicer* alone at 90 days after sowing (DAS). Percentage colonization of *P. indica* improved significantly with combined treatment at 90 DAS. All the treatments significantly improved total soluble sugar content, amino

N content (1.36-1.80 fold) and stress tolerance ability (4-6 fold) over *M. cicer* treatment alone.

2.3. EFFECT OF NUTRIENT UPTAKE BY THE ENDOPHYTE COLONIZED PLANTS

Computational investigations utilising the ClustalW tool identified numerous shared motifs between the phosphate transporters from *P. indica* and 8 more *Glomus* spp. (Das *et al.*, 2014). A 3D model of Phosphate transporter protein of *P. indica* resembled a "Mayan temple", which was efficiently docked onto hydrogen phosphate, indicating the protein's affinity for inorganic phosphorus. Acid phosphatase and alkaline phosphatase activity were significantly elevated in the rhizosphere soil of rice plants infected with *P. indica*, *G. fasciculatum*, or both, contributing to higher P absorption.

According to Wu *et al.* (2018), *P. indica* promoted the availability of phosphorous to the host plants by increasing phosphatase activity and the expression of the gene ACP5 in *Brassica napus*. Phosphorous was taken up by the roots directly or by a pathway through the fungal hyphae from sources in the soil that are not accessible to the plant.

The *P. indica*-phosphate transporter (PiPT) gene which facilitated the transport of phosphates from the soil to the plant as a result of *P. indica* colonisation in maize has been reported by Yadav *et al.* (2010).

Khatun *et al.* (2020) studied the response of *G. fasciculatum* on phosphorus uptake by the plant at different developmental stages in *Coleus forskohlii*. The results showed that mycorrhizal fungus symbiotic relationship resulted in better phosphorus uptake and increased chlorophyll content in AM treated plants than in non-mycorrhizal plants, which was gradually maintained throughout their developmental stages. Plant height, root length, number of roots, number of leaves, number of branches, fresh weight of shoot, roots, tubers, and forskolin content were all considerably higher in AM treated plants than in control plants.

Kumar *et al.* (2012) reported a 1.4 fold increase in K uptake from the soil in *P. indica* colonised mung bean plants in both glasshouse and field environments, which, when combined with other nutrient uptake (N and P), may have considerably contributed to the documented growth enhancement. According to Guether *et al.* (2009), an upregulation in the expression of a plant K+ transporter in *Lotus japonicus* were observed in roots colonised with AMF.

Synergistic effect on growth of tomato on inoculation with *G. fasciculatum*, *Beijernickia mobilis* and *Aspergillus niger* has been reported by Manjunath *et al.* (1981). Combined inoculation of *Methylobacterium oryzae* strains and AM fungi resulted in significantly higher nitrogen (N) accumulation in the roots and shoots of red pepper plants compared to uninoculated controls. The combined inoculation also increased the phosphorus (P) content by 23.3 per cent compared to untreated controls. A perfect mutualism between *M. oryzae* and AMF was found which was attributed to the improved macro- and micronutrient uptake along with higher chlorophyll content in red pepper (Kim *et al.*, 2010).

Swarnalakshmi *et al.* (2017) conducted a study to evaluate the efficacy of microbial bioinoculants in chickpea (*Cicer arietinum* L.) using combinatorial approach. Phosphate-solubilizing microorganisms and plant growth-promoting rhizobacteria in presence and absence of P fertilizer was used to testify its synergistic effect with *P. indica* on symbiotic traits, growth and yield of chickpea under field conditions. Significant improvement in N and P nutrition with combined inoculation amended with 30 kg P/ha emphasized the beneficial effect of the above treatment and similar trend was reflected in productivity enhancement as well.

2.4. EFFECT OF ENDOPHYTES ON PHYTOHORMONE LEVEL

Lee *et al.* (2011) investigated the effects of *P. indica* on Chinese cabbage and Arabidopsis growth and discovered that the auxin level in infected Chinese cabbage roots was twofold higher than in uncolonized controls. A double-subtractive expressed sequence tag library was used to identify auxin-related genes (genes for cell wall acidification proteins, intercellular auxin transport carrier proteins like AUX1, and auxin signal proteins) increased by *P. indica* in Chinese cabbage roots. According to

the authors, the activation of auxin production and signalling in the roots, could be the cause of the *P. indica*-mediated growth phenotype in Chinese cabbage.

On tryptophan feeding, *P. indica* generated the phytohormones indole-3-acetic acid (IAA) and indole-3-lactate (ILA) via the intermediary indole-3-pyruvic acid, (IPA) according to biochemical investigations of the underlying biosynthetic pathways for auxin production in barley. The piTam1 gene was identified as a crucial player in time course transcriptional studies after tryptophan exposure. In green fluorescence protein (GFP) reporter study and transcriptional analysis, PiTam1 was induced during the biotrophic phase in colonised barley roots. Additionally, *P. indica* strains with the piTam1 gene silenced by RNA interference (RNAi) had reduced colonisation of barley (*Hordeum vulgare*) roots (Hilbert *et al.*, 2012).

According to Wang *et al.* (2021) AMF inoculation significantly increased the endogenous levels of auxin and cytokinin in tomato seedlings. The plant hormone signal transduction pathway was significantly enriched in root transcriptome analysis, and gene set enrichment analysis (GSEA) identified 109 genes that were positively connected with the AMF-inoculated plant phenotype, including 9 genes related to indole acetic acid (IAA). The amount of endogenous IAA in tomato seedlings increased dramatically following AMF inoculation.

2.5. *In vitro* EVALUATION OF *P. indica* AGAINST FUNGAL PATHOGENS

Kumar *et al.* (2009) conducted antibiosis assay for *P. indica* against *Fusarium verticilloides* and observed that there were no secretion of antibiotics and the resistance conferred by the endophyte against the pathogen was not due to antibiosis. Rabiey *et al.* (2015) studied the interaction of *P. indica* and *F. culmorum* causing crown rot disease of wheat in axenic culture. A five mm disc of *P. indica* was taken and kept at one of PDA plate and another five mm mycelial disc of Fusarium was taken and kept at the other end simultaneously or four days after placing *P. indica* and was incubated at 21°C ±1 °C. To see the interaction between *P. indica* and Fusarium isolates microscopically, a clean glass microscope slide was placed in the middle of Petri dishes and a thin layer of PDA poured onto it. Single five mm discs of four-day-old cultures

of *P. indica* and *Fusarium* isolates were placed at opposite ends of the slide simultaneously or three–four days apart and incubated at room temperature (21 ± 1°C). The slide was observed after three-four days after both hyphae met. Neither the *Fusarium* nor the *P. indica* was found to be affected in axenic culture. There was loose coiling of hyphae of *P. indica* around *Fusarium* but no evident mycoparasitism. It indicated no direct antagonistic activity of endophyte against *Fusarium*.

According to Dolatabadi *et al.* (2011) *P. indica* could produce limited radial growth of *F. oxysporum* f. sp. *lentis* after six days of incubation. Coiling of the endophytic fungus around the pathogen was also observed.

Johnson *et al.* (2013) conducted dual culture experiment with different pathogenic fungi like *Alternaria brassicae, Rhizoctonia solani, Phytophthora nicotianae* and observed for antibiosis and lytic enzymes (inhibition zone), coiling around the mycelium and choking, overgrowing and sporulating as parasitism, or mutualism. There was development of inhibition zone in plates of *P. indica* co-cultured with *Alternaria brassicae*. Antibiosis could be observed in plates of *P. indica* and *Rhizoctonia solani* and overgrowth and sporulation of *P. indica* could be observed in plates containing *P. indica* and *Phytophthora nicotianae*.

Sun *et al.* (2014) co-cultivated *P. indica* and *Verticillium dahliae* in agar plate for three weeks and observed strong inhibition of *Verticillium* hyphae. No inhibition zone could be detected but growth of *P. indica* was barely affected by the presence of *V. dahliae*.

2.6. *In vivo* EVALUATION OF EFFECT OF *P. indica* AND *G. fasciculatum* AGAINST *F. oxysporum* IN SMALL CARDAMOM

According to Murugan *et al.* (2011) cardamom plantations in southern India have been receiving heavy doses of chemical pesticides and the pesticide consumption of the crop has increased several times in the last 50 years. The need for safer management practices against this major disease is highly essential for an economical and eco-friendly management as well as for the benefit of the CHR and the cardamom growers.

Biological control by beneficial microorganisms is considered an important element in disease management strategies and among them the use of endophytes in disease control is gaining momentum. Endophytes suppress the pathogen by reducing the level of infection, suppressing the growth or reducing the inoculum production. Endophyte mediated biocontrol can either be direct or indirect. The direct mechanisms include antibiosis, competition, hyperparasitism or lytic enzyme production. The indirect mechanisms include induced resistance or promotion of plant growth (Gao *et al.*, 2010).

2.6.1. *Piriformospora indica* in disease management

P. indica provided tolerance to barley against root and leaf pathogens, including the necrotrophic fungus *Fusarium culmorum* (root rot) and the biotrophic fungus *Blumeria graminis*. Higher frequency of hypersensitive reaction, including host-cell death response and cell wall associated defense was observed implying an active plant response in managing the disease. Glutathione reductase activity was also higher in leaves during three weeks of *P. indica* inoculation corroborating systemic induction of antioxidant capacity of *P. indica* (Waller *et al.*, 2005). Particular genes known to be involved in plant defence reactions were shown to be systemically induced after *P. indica* inoculation (Waller *et al.*, 2008).

Harrach *et al.* (2013) found out that *P. indica* could protect the barley against the root rot causing pathogen *F. culmorum* by activating the antioxidant capacity. The roots infected with pathogen showed elevated level of lipid hydroperoxides and decreased ratios of reduced to oxidised forms of ascorbate and GSH while the *F. culmorum* inoculated roots which was pre-inoculated with *P. indica* showed ascorbate and GSH that were similar to control. The lipid peroxidation and antioxidant enzyme activities were also largely attenuated by *P. indica* inoculated roots which was later challenged by the pathogen. Deshmukh and Kogel (2007) reported that barley roots were protected from *Fusarium* infections upon inoculation with *P. indica*. The root rot symptom was evidently reduced. The *in vitro* study under axenic conditions revealed that there was no direct antagonistic activity and the retardation of *Fusarium* in roots is mediated by a plant response rather than by antibiosis.

Seed treatment of cucumber with *P. indica* before sowing in non-amended soil significantly decreased the disease severity of *Fusarium* wilt caused by *Fusarium oxysporum* in cucumber and improved plant growth (Moharam *et al.*, 2017). According to Dehghanpour-Farashah *et al.* (2019) *P. indica* could considerably reduce the disease progress of crown rot of wheat caused by *F. pseudograminearum* in seedlings and detached leaves. *P. indica* elevated the H_2O_2 levels, guaiacol peroxidase (GPX) and catalase (CAT) activity, callose deposition, relative water content (RWC) and membrane stability index (MSI) which synergistically enhanced the *P. indica* induced resistance against crown rot of wheat.

Cheng *et al.* (2020) stated that root endophytic fungus *Serendipita indica* improved the disease resistance of banana to *F. oxysporum* f. sp. *cubense* by increasing chlorophyll content, antioxidant activities and reducing symptom development in endophyte colonised "Tianbaojiao" banana plants when inoculated with *Fusarium oxysporum*. The results indicated that the colonisation of the endophyte increased superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) activities which suggests that the resistance of banana might have achieved atleast partly by the regulation of antioxidant enzymes.

Reduction in disease severity of Verticillium wilt by 30 per cent was observed in tomato plants colonised by *P. indica*. Negative effect of the pathogen on plant growth was alleviated when the plants were colonised by this root endophytic fungus. The fresh and dry weight of *P. indica* inoculated plants were higher compared to uninoculated ones when it was later challenge infected with Verticillium (Fakhro *et al.*, 2010). Ghahfarokhi and Goltapeh (2010) suggested using mycorrhizal-like fungus *P. indica*; *S. vermifera* and *Trichoderma* spp. to control root disease of wheat (take-all disease). It was found that *P. indica*, *S. vermifera* and *Trichoderma* species could coil around the mycelium of pathogen and penetrate it.

Lakshmipriya *et al.* (2016) studied the effect of *P. indica* colonisation against leaf blight in taro. The study was conducted in the varieties Sree Kiran and Muktakeshi and it was observed that *P. indica* colonised Sree Kiran had a PDI reduction of 57.6, 50.7, and 84.3 per cent during different phases of infection, while Muktakeshi had a

reduction of 39.9, 56.2, and 72.5 per cent over control. During the initial hours of infection, *P. indica* colonised plants had higher activity of defense enzymes like chitinase,-1, 3 glucanase, and total phenol than uninoculated plants.

Lin *et al.* (2019) studied the interaction of *P. indica* with *Anthurium andraeanum* and observed higher activities of stress-related enzymes of jasmonic acid levels and mRNA levels of jasmonic acid-responsive genes which suggest that the fungus prepares the plant to respond more efficiently to potentially upcoming threats, including bacterial wilt.

2.6.2. *G. fasciculatum* in disease management

Vesicular arbuscular mycorrhiza (VAM) fungus, *Glomus fasciculatum* was used in biological control of damping off of cardamom caused by *Fusarium moniliforme, Pythium vexans* and *Rhizoctonia* sp. The treatments with *G. fasciculatum* individually or in combination with pathogens showed more phosphorus in the shoots than those treated with pathogen alone. It was observed that introduction of the mycorrhizal fungus together with *F. moniliforme* reduced the severity of disease caused by the pathogen. The endophyte inoculated plants were less stunted than the control (Thomas *et al.*, 1994).

Akkopru and Demir (2005) conducted a study on *Fusarium* wilt of tomato using AMF and some rhizobacteria and it was found that AMF on single and dual inoculation of plant reduced disease severity and enhanced dry root weight effectively with better colonization.

Combination of biocontrol agents like *P. fluorescens* (2 per cent) spray with basal application of *G. fasciculatum* and *T. viride*, PGPR II 2 per cent spray with *G. fasciculatum* and *T. viride* were found effective against both tiller and panicle infection caused by *Fusarium oxysporum* in small cardamom (Dhanya *et al.*, 2018).

The efficacy of *Glomus clarum* and *G. deserticola* as biocontrol agents against *Fusarium verticillioides* strains on maize was investigated. The pathogenic effects of *F. verticillioides* on plant height and shoot weight were significantly reduced by the application of 20 g of *G. clarum* and 30 g of *G. deserticola* (Olowe *et al.*, 2018).

Kumari and Prabina, (2019) studied the influence of AMF against fungal plant pathogens. The *in vitro* interaction between *Glomus* sp. and wilt pathogen *F. oxysporum* f. sp. *lycopersici* indicated formation of clearing zone around the root which implies production of antimicrobial compounds from mycorrhizal roots that arrested the mycelial growth of fungal pathogen. The pot culture experiment also revealed that the pre-AMF and post pathogen inoculation with tomato reduced the disease incidence and increased the plant growth, dry weight, N, P, K content, chlorophyll content and yield of the plant. Yanan *et al.* (2015) reported that VAM reduced *F. oxysporum* Schlecht. f. sp. *fragariae* Winks et Williams (FO) infection in strawberry by inducing two key substance involved in disease defenses, lignin and hydroxyproline-rich glycoprotein.

Math *et al.* (2019) observed the Reactive Oxygen Species (ROS) mediated immune response in tomato. Plants inoculated with arbuscular mycorrhizal fungi (AMF) *Glomus fasciculatum* (GF) were studied in the presence of plant pathogenic fungi *F. oxysporum* f. sp. *lycopersici* (FOL). *G. fasciculatum*'s ability to alleviate the oxidative damage generated by biotic stress and buffer time taken by plants defense machinery to immunize itself at various levels were highlighted. Soil application of *T. viride* (Tv3), *P. fluorescens* (Pf2) and *G. mosseae* significantly reduced the basal rot incidence and considerably increase the biometric of onion crop (Yuvarani *et al.*, 2020).

Yaghoubian *et al.* (2014) studied the effect of *P. indica* and *G. mossae* on the growth and antioxidant defense reponses in wheat plants under drought condition. The results showed that infected wheat plants had higher vegetative growth than non-inoculated wheat plants at varied moisture levels. Dually inoculated plants had more root colonisation compared to the individually colonised plants. The plant biomass was significantly improved by all of the fungal treatments, especially in co-inoculated plants. Individually inoculated *P. indica* plants revealed higher catalase and peroxidase activity than the co-inoculated, *G. mossae* inoculated or non-inoculated plants grown under drought stress.

2.6.3. *Pseudomonas fluorescens* in disease management

Field experiments were conducted for two season to test fungicides and different combination of bioagents like *P. fluorescens* PN026, PGPR II (Consortium of *P. fluorescens* and *Bacillus subtilis*), *T. viride* T6 (Talc based formulations with cfu 10^8 /g) and *G. fasciculatum* (AMF) against *Fusarium* rot disease of cardamom under farmers' field condition. Among effective treatments, *P. fluorescens* in cowdung slurry was the best management practice against *Fusarium* rot of cardamom followed by combined application of *P. fluorescens* with AMF and *T. viride* (Dhanya *et al.*, 2018).

Vijayan *et al.* (2013) reported that fungal diseases such as Colletotrichum blight (*C. gloeosporioides*), Phoma leaf spot (*Phoma* sp.) and Leaf streak (*Pestalotiopsis royenae*) of large cardamom in Sikkim can be effectively controlled by *P. fluorescens* in the field condition. Vascular discolouration in banana associated with *Fusarium* wilt disease is reduced by the accumulation of enzymatic resistance induced by *P. fluorescens* (Saravanan *et al.*, 2004). Pot culture study using Rasthali variety of banana showed lesser discolouration index and no wilting or death. *P. fluorescens* induced defense related enzymes like peroxidase, polyphenol oxidase and phenylalanine ammonia lyase in roots.

2.6.4. Chemical check (Carbendazim)

Field trials conducted by Thomas and Vijayan (2002) revealed that *Fusarium oxysporum* disease infections in small cardamom can be brought under control with the use of chemical fungicide Carbendazim (0.2 per cent). Rajput *et al.* (2006) observed that carbendazim was an excellent fungicide against cotton wilt caused by *F. oxysporum* f. sp. *vasinfectum*. The root and shoot length recorded with carbendazim treated cotton varieties were also found to be higher than the control plants.

Yadav and Ansari (2017) revealed that *in vitro* study by poison food technique with carbendazim completely inhibited the mycelial growth of *F. oxysporum* Schlecht at concentration of 200 ppm, 500 ppm and 1000 ppm by killing the spores of the pathogen.

2.7. ELUCIDATING THE ROLE OF GIBBERELLIC ACID IN ENDOPHYTE MEDIATED TOLERANCE

A crystalline compound called as Gibberellic acid was isolated from culture filtrate of *Gibberella fujikuroi* and named it as Gibberellins (Yabuta and Sumiki, 1938). Tudzynski *et al.* (2003) reported that GA biosynthetic genes are organized in a gene cluster which consists of four cytochrome P-450 monooxygenase genes and one GA_4 desaturase gene.

According Sakamoto *et al.* (2004) *G. fujikuroi* which causes bakane disease in rice produces gibberellic acid as virulence factor. The gibberellic acid produced by the isolates of the pathogen was associated with the elongation symptom (Malonek *et al.*, 2005). Bashyal *et al.*, (2016) observed that the isolates producing more amount of Gibberellic acid produces more elongation symptom compared to those producing fusaric acid. GA_3 production by endophytic *F. oxysporum* was confirmed by high performance liquid chromatography by Ben-Rhouma *et al.,* (2020).

Tanaka *et al.*, (2006) identified a (GA)-insensitive dwarf mutant of rice, *gibberellin-insensitive dwarf1* (*gid1*) which had high concentrations of endogenous GA. The authors investigated the function of gid1 by utilising a proteomic technique to examine proteins controlled downstream of gid1. Sixteen proteins were elevated and 14 were decreased out of a total of 962 proteins identified from suspension-cultured cells in gid1 compared to its wild type. Osmotin, triosephosphate isomerase, probenazole inducible protein (PBZ1), and pathogenesis-related protein 10 were among the proteins that hyper-accumulated in gid1. Exogenous GA3 treatment enhanced the expression of only one of these four genes, PBZ1. Infection with the rice blast fungus increased expression of this gene in wild type shoots as well. As a result, the findings suggested that PBZ1 expression is regulated by GA signalling and stress, and that gid1 is involved in blast fungal resistance.

Manjili *et al.* (2012) studied the effects of phytohormones on antioxidant enzymes of various wheat cultivars under salinity stress and concluded that gibberellic acid increased antioxidant enzyme activity by lowering reactive oxygen species (ROS), leading to better stress management.

According to Buhrow *et al.* (2016), exogenous administration of GA had an antagonistic effect on the *Fusarium graminearum* that caused Fusarium Head Blight (FHB) infection in wheat plants. *F. graminearum*'s capacity to biosynthesize trichothene mycotoxin determines its pathogenicity. GA decreased actin and tri gene expression during the early stages of infection, which resulted in a decrease in disease spread. Furthermore, co-application with GA suppressed early stage gene expression of *F. graminearum* nitrogen metabolic genes such as calcium dependent aldoxime dehydratase, calcium transporting ATPase, ATP-dependent oxoprolinase, and nitroalkane oxidase, potentially reducing bioenergetic resources and redox regulation required for newly infecting fungal cells.

Wang *et al.* (2014) undertook a genome-wide investigation to discover all of the genes encoding Growth regulating factors (GRFs) in Chinese cabbage. The expression profiles of the BrGRF genes revealed that they had uneven transcript levels in different organs or tissues, and that gibberellic acid (GA3) treatment stimulated transcription of the majority of the BrGRF genes. Pan *et al.* (2017) reported that *P. indica* colonised Arabidopsis plants exhibited higher transcript levels of the GA biosynthesis genes Gibberellin 20-Oxidase 2, Gibberellin 3-Oxidase 1, and Gibberellin Requiring 1, resulting in an increase in GA4 level.

According to Shaul-Keinan *et al.* (2002), *G. intraradices* colonisation of tobacco roots significantly increased concentrations of numerous endogenous gibberellins. When compared to non-mycorrhizal controls, the majority of GAs from the earl-13-hydroxylation biosynthetic pathway – GA1, GA8, GA19, and GA20 were higher in root samples of tobacco plants after mycorrhizal colonisation.

Joo *et al.* (2005) revealed that after inoculation with the gibberellin-producing PGPR strain *Bacillus cereus* MJ-1, the amount of endogenous gibberellins in host plant shoots i.e. red pepper increased. *Burkholderia phytofirmans* PsJN, a GPR strain that does not synthesise gibberellins, stimulated the expression of the AtGA3ox1 gene in *Arabidopsis thaliana*, which encoded for an enzyme involved in one of the final stages of gibberellin synthesis (Poupin *et al.*, 2013).

Materials and methods

3. MATERIALS AND METHODS

The present study entitled "Evaluation of beneficial fungal root endophytes against Fusarium rot in small cardamom" with an objective to evaluate the colonisation and interaction of two beneficial fungi, *P. indica* and *G. fasciculatum*, in cardamom and their potential in manage Fusarium rot disease of cardamom as well as elucidation of the role of gibberellic acid in the endophytic fungi-mediated disease tolerance, was conducted at Department of Plant Pathology, College of Agriculture, Vellayani and Cardamom Research Station, Pampadumpara during the period 2019-2021. The materials and methods adopted for the study is discussed in this chapter.

3.1 COLLECTION OF FUSARIUM INFECTED PLANTS FROM HOTSPOT AREAS, ISOLATION OF PATHOGEN, PROVING KOCH'S POSTULATES AND MAINTANENCE OF CULTURE

3.1.1. Collection of diseased sample

The *F. oxysporum* infected samples of susceptible variety *Njallani* were collected from farmer's field of Pampadumpara panchayat in Idukki district during December- January. The pathogen causes eye shaped spot, root rot and panicle blight on pseudostem, root and panicles respectively. Among these, the root isolate from the infected roots were collected for isolation and proving Koch's postulates as the study included the effect of root endophytes.

3.1.2. Isolation of pathogen

Root samples were washed in running water and cut into small bits of diseased portion along with healthy portion. These small bits were dipped in one per cent sodium hypochlorite for 30 seconds to remove the saprophytic organisms present in roots. This was followed by three washings in sterile water. The excess water was blotted out using sterile tissue paper. The bits were placed in PDA plated Petri dishes and incubated.

3.1.3. Proving Koch's postulates

Pathogenicity was proved in live pseudostem of one year old suckers of *Njallani* variety raised in polybags. Small bits of five mm were cut out from seven day old fully

grown plates using a cork borer. Small pinpricks were made on pseudostem using a fine needle. The bits were placed on wounded portion of the pseudostem and covered with a thin layer of moist cotton to hold it in position. This was then covered with a perforated polythene cover to attain the favourable conditions for disease development. After the development of characteristic symptoms the pathogen was reisolated and compared with the original isolate. Concurrently, a control was placed without any pathogen inoculation.

3.1.4. Morphological and cultural characteristics

Morphological characteristics like hyphal characters and the size and shape of the conidia were studied by preparing slides which was stained with cotton blue. This was observed under 400X and 1000X magnification using Leica DM 750 (Nisha, 2018).

The cultural characteristics of the isolates were studied by growing the isolate in PDA medium and observing the characteristics.

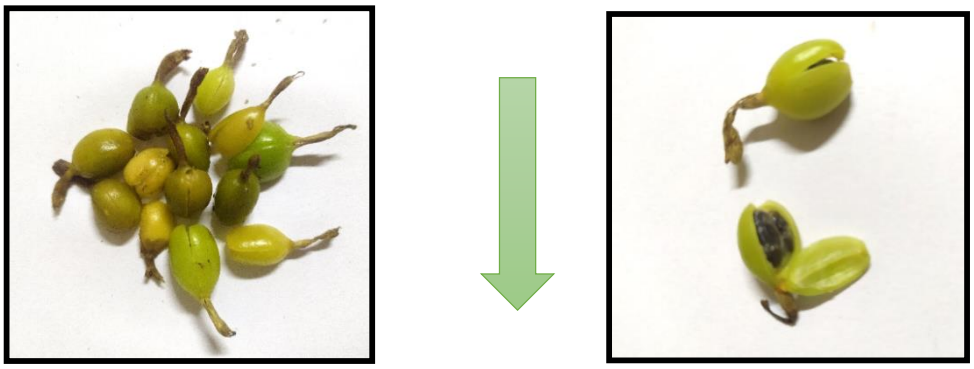
3.2. EVALUATION OF THE COLONISATION AND INTERACTION OF *P. indica* AND *G. fasciculatum*

3.2.1. Raising cardamom seedlings var. *Njallani* for the experiment

For raising seedlings for the experiment, ripened capsules of *Njallani* variety of small cardamom was taken. The seeds were extracted by pressing the capsules and were mixed with ash to remove the mucilage. The seeds were dried in shade for 2-3 days. In primary nursery, beds of steam sterilized potting mixture soil: sand: cow dung in the ratio 1:1:1 of size 2 m x 1 m x 0.3 m was laid. Seeds were sown in lines and covered with a thin layer of potting mixture. The nursery bed was mulched with dry grass to a thickness of 2 cm in order to avoid direct sunlight till germination. The bed was watered everyday in the morning and evening. The mulch was removed only on the commencement of germination.

After germination, the 2-3 leaved seedlings from the primary nursery were carefully pulled out, and colonized with the bioagents *viz.*, *P. indica* and *G. fasciculatum* individually and in combination (Plate 1).

Ripened capsules taken



Mixed with ash

Sown

Germinated seedlings colonised with bioagent in protray

Plate 1. Small cardamom seedlings var. *Njallani* raised to study the impact of endophytes in biometric characters and management of Fusarium rot

3.2.2. Co-cultivation of cardamom plants var. *Njallani* with bioagents *viz., P. indica* and *G. fasciculatum.*

3.2.2.1. Maintanence and co-cultivation of P. indica

P. indica, a beneficial fungal root endophyte from the Department of Plant Pathology was maintained in Potato Dextrose Agar (PDA) medium. Fungal discs from the hyphal tip of actively growing two weeks old culture of *P. indica* was taken and transferred to a PDA plated Petri plate and incubated at room temperature 28 ± 2°C.

P. indica colonisation was carried out as per the protocol described by Jojy *et al*., (2020). Coir pith - cow dung medium in the ratio 2:1 was used for co-inoculation and gram flour at the rate of 20 g / Kg of medium was added. The medium was moistened to field capacity and sterilized at 121°C for 20 min for 3 consecutive days. *P. indica* was cultured in 100 ml potato dextrose broth in 250 ml conical flask and incubated at room temperature (25 ± 2 º C) for 15 days. Fungal mycelial mat was harvested by filtration using muslin cloth and washed in two changes of sterile water. The mycelial mass was mixed with the sterile medium in the ratio one per cent (w/w) and filled in trays under aseptic condition, covered and kept for optimal fungal growth for one week (Plate 2).

3.2.2.2. Treatment details

For *P. indica* inoculation in seedlings, potrays were filled with the coir pith-cow dung medium and two-three leaved seedlings from the primary nursery were planted to the protrays. This was then transplanted to the polybags containing sterile potting mixture after seven days. For dual inoculation, *P. indica* inoculation was carried out as described above and after seven days vermiculite-perlite-soil based inoculum *G. fasciculatum* was incorporated at the rate of 20 g / 10 kg of soil to the polybag containing sterile potting mixture at the time of transplanting by placing the fungus in the root zone of *P. indica* treated seedlings. For co-inoculation with the endophytic *G. fasciculatum*, the bioagent in the root zone of the two-three leaved seedlings at the rate of two per cent (w/w) of soil to the polybag containing steam sterilized potting mixture at the time of transplantation to the polybags. Control plants were maintained

in the similar way without the inoculation of any bioagents. All the treatments were provided with adequate irrigation at regular intervals to maintain the field capacity (Plate 3).

For endophyte inoculation in suckers, one year old cardamom suckers were used. For individual application of *P. indica* and *G. fasciculatum,* the mass multiplied inoculum was applied to the root zone by making a depression in the solarized soil filled in the growbags at the time of planting. For dual inoculation, *P. indica* was applied as the same way as mentioned above and *G. fasciculatum* was applied seven days after by forming a ring in the polybag around the plant which is deep enough to see the roots and placed the inoculum in the ring so that it reached the root surface (Plate 4-6).

Observations on plant height, leaf length, leaf breadth, number of leaves, number of tillers, root volume, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight were recorded.

Autoclaved Coirpith- cowdung (2:1) mixture

***P. indica* fungal mat added to the autoclaved mixture and incubated**

Plate 2. Mass multiplication of *P. indica* in coirpith-cowdung medium

Plate 3. Seedlings raised to study the effect of endophytes in the vegetative characters of small cardamom var. *Njallani*

Plate 4. Suckers planted to study the effect of endophytes in the vegetative characters of small cardamom var. *Njallani*

Plate 5. *G. fasciculatum* treatment application in suckers for the experiment

Plate 6. *P. indica* treatment application in suckers for the experiment

3.2.3. Root colonization of cardamom seedlings var. *Njallani* with bioagents

Roots of cardamom seedlings var. *Njallani* treated with bio agents were collected. Roots were washed thoroughly with water to make it free from the planting medium. Small pieces of roots of length one cm was cut and transferred to test tubes containing freshly prepared 10 per cent KOH. The test tubes were kept in water bath at 65°C for five minutes. Roots were washed with water and kept in one per cent HCl for five minutes. The root bits were again washed in water and kept in lactophenol tryphan blue for staining the endophytic fungus (Philips and Hayman, 1970; Johnson *et al.*, 2013). The root bits were observed under the microscope for the presence of chlamydospores in the case of *P. indica* and arbuscules and vesicles in the case of *G. fasciculatum*.

3.3. SOIL AND PLANT NUTRIENT ANALYSIS

The major nutrients (N, P, K) from the soil and (P, K) from the plants (both seedlings and suckers) were analysed of 3.2.1 to study their role in the plant growth promotion in effect to the beneficial interaction with the bioagents. Soil samples were collected just before planting and plant samples were collected at the end of the experiment and subjected to nutrient analysis.

3.3.1. Soil nutrient analysis

3.3.1.1. Determination of nitrogen content in the soil

Twenty gram of soil sample was placed in a digestion tube with two-three beads to prevent bumping and to which one ml paraffin wax was added to prevent frothing. The distillation flask was filled with 0.32 per cent 100 ml potassium permanganate and 100 ml sodium hydroxide solution. In a conical flask holding 20 ml boric acid solution with mixed indicator, a hose was immersed. When the ammonia gas was released and gathered in the boric acid, there was a colour change from wine red to green. The distillate was collected and titrated against 0.02 N sulphuric acid until a pink colour was achieved at the end point (Subbiah and Asija, 1956).

$$\text{Nitrogen content in soil (Kg ha}^{-1}) = \frac{\text{R (Sample titer -Blank titer)} \times \text{Normality of acid} \times \text{Atomic weight of Nitrogen} \times 100}{\text{Sample weight (g)} \times 1000}$$

3.3.1.2 Determination of phosphorous content in soil (Bray's extraction and colorimetric estimation)

Five gram of soil sample and 50 ml of Bray's number 1 reagent was taken in 100 ml conical flask and shaken for five minutes. This mixture was filtered through Whatman No. 1 filter paper. 5 ml of filtrate was pipetted into the 25 ml conical flask and the volume was made up with distilled water. After 10 minutes, the colour density was read at 660 nm.

Preparation of the standard curve

Different concentrations of P solution (1, 2, 3, 4, 5 and 10 ml of 2 μg ml^{-1}) were taken in 25 ml volumetric flask and five ml extracting reagent was added to it. When the reagent was added there was a development of blue colour. Standard curve was obtained by plotting the absorbance and concentration and the calculation were done using the standard curve (Watanabe and Olsen, 1965).

$$\text{Available P (Kg ha}^{-1}) = \frac{\text{Absorbance for sample} \times 50 \times 2.24}{\text{Slope of the std. curve}}$$

3.3.1.3. Determination of potassium content in soil

Five grams of soil sample was taken in 100 ml conical flask along with 25 ml of 1 N ammonium acetate solution and shook for 5 minutes. This was filtered through Whatman No. 1 filter paper and read in flame photometer along with blank sample.

Preparation of standard curve

Different aliquots of stock solution were diluted with ammonium acetate solution to give 10 to 100 ppm of Potassium. The flame photometer was adjusted to zero for zero ppm potassium and 100 for 100 ppm potassium. A standard curve was

constructed by plotting the absorbance against different concentration of aliquots (Jackson, 1973).

$$\text{Available K (Kg ha}^{-1}) = R \times \frac{\text{Volume of the extract} \times 2.24 \times 10^6}{\text{Weight of soil taken} \times 10^6}$$

R = ppm of K in the extract (obtained from standard curve)

3.3.2. Plant nutrient analysis

Fully expanded third leaf, shoot and root of cardamom seedlings and suckers were collected and shade dried for the nutrient analysis. The shade dried samples were further dried in hot air oven at about 60 ± 2°C for three to six hours and powdered with the help of a grinder. The powdered samples were passed through 2 mm stainless steel sieves for chemical assay.

3.3.2.1. Determination of total phosphorous in plants

0.5 g ground plant sample was taken in a conical flask and 5 ml of conc. HNO_3, 2 ml mixture of equal volume of H_2SO_4 and $HClO_4$ were added to it and mixed. Heat the mixture on a hot plate until the solution turns colourless. The mixture was cooled, filtered, and the residue from the filter paper was then washed several times before being reconstituted with distilled water to a volume of 100 ml. 10 ml aliquots were transferred to a 50 ml volumetric flask to which 10 ml vanadate–molybdate solution was added, and the volume was made up to 50 ml with water. The absorbance was read at 440 nm with a blank 10 minutes after thorough mixing.

For the preparation of standard curve, 0, 1, 2, 3, 4 and 5 ml of standard P solution was separately taken in a 50 ml volumetric flask and 10 ml of vanadate-molybdate reagent was added to it. This was then made up using distilled water. The curve was prepared by plotting P concentrations on X axis and reading on Y axis (Jackson, 1973).

$$\text{Total P (\%)} = \frac{X}{10{,}00{,}000} \times 50 \times \frac{\text{Vol. of extract made}}{\text{Vol. of extract taken}} \times \frac{100}{\text{Wt. of plant sample}}$$

3.3.2.2. Determination of total potassium in plants

One gram ground sample was taken in 1000 ml digestion flask to which 20-25 ml of diacid mixture was added. The solution was heated on a heat plate and the solution turns colourless. After cooling, 20-25 ml distilled water was added to it. The solution was then passed through Whatman no. 40 filter paper and the filtrate was collected in 100 ml conical flask. The volume was made up with distilled water and the aliquots were read using flame photometer (Black, 1965).

$$K\ (\%)\ \text{in plants sample} = X \times 4 \times 10^{-3}$$

3.4. ELUCIDATING THE ROLE OF AUXIN IN PLANT GROWTH PROMOTION

IAA analysis, following the protocol of Gordon and Weber (1951) was carried out in the experimental plants of 3.2. in order to elucidate the role of auxin in plant growth promotion. One gram of root sample was dissolved in absolute alcohol and made upto 10 ml using the same. To one ml of that aliquot two ml of $FeCl_3$-$HClO_4$ (1 ml of 0.5M $FeCl_3$, 50 ml 35% $HClO_4$) reagent was added and kept for 25 minutes. The absorbance was then measured at 530 nm and the IAA content was obtained from the standard graph.

Preparation of the calibration graph

10 ppm of standard IAA was prepared. From that solution 2, 4, 6, 8 and 10 ml aliquots were taken and made upto 10 ml using absolute alcohol. To one ml of that aliquot two ml of $FeCl_3$-$HClO_4$ reagent was added and the absorbance was measured after 25 minutes. The standard curve (Fig. 1) was obtained by plotting the absorbance against concentration.

3.5. *In vitro* EVALUATION OF *P. indica* AGAINST *F. oxysporum*

Dual culture assay of *P. indica* and *F. oxysporum* was performed in PDA and the nature of antagonism, antagonism index, lysis zone, percent inhibition etc. was assessed. A five mm disc of *P. indica* was taken and kept at one end of the PDA plate and another five mm mycelial disc of *Fusarium* was taken and kept at the other end simultaneously and incubated at 21 ± 1° C (Rabiey *et al.*, 2015).

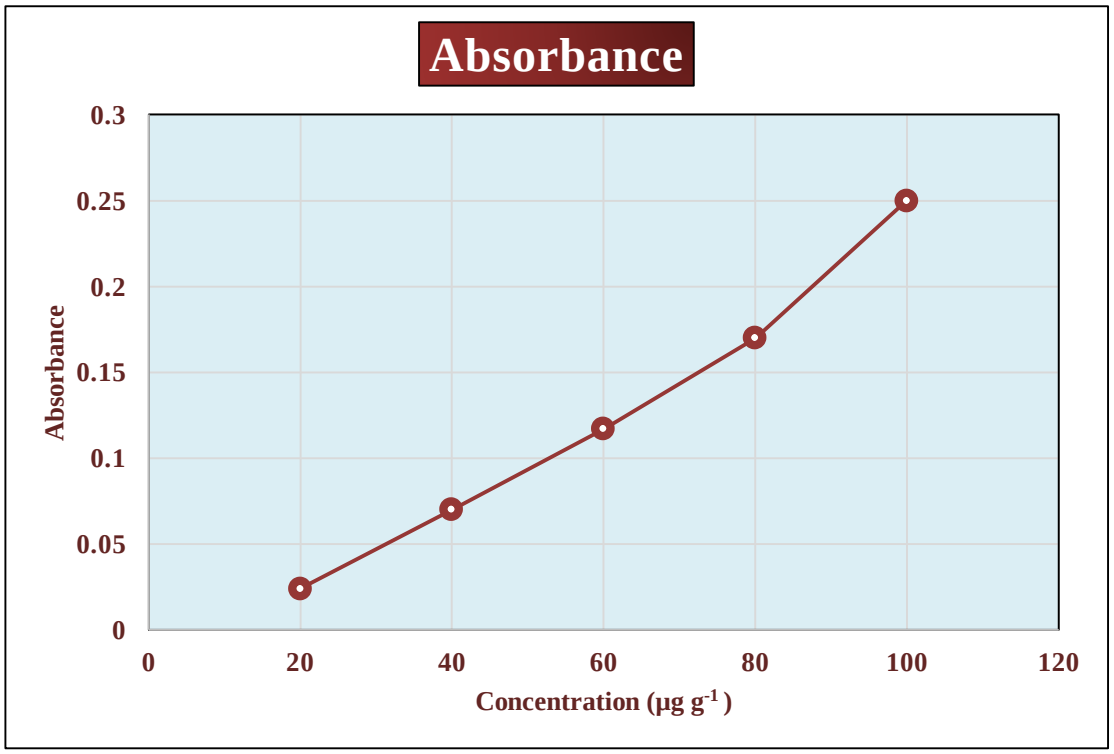


Fig. 1. Standard curve of IAA (µg g^{-1})

Percent inhibition and antagonism index was calculated using the following formulas;

$$PI = \frac{[\text{Radial growth in control (C)} - \text{Radial growth in dual culture (T)}]}{\text{Radial growth in control (C)}} \times 100$$

$$\text{Antagonism index} = \frac{RM - rm}{rm} \times 100$$

(Campanile *et al*., 2007)

RM : average of the three rays of the colony in the other directions

rm : ray of the colony towards the antagonist

3.6. *In vivo* EVALUATION OF EFFECT OF *P. indica* AND *G. fasciculatum* AGAINST *F. oxysporum* IN SMALL CARDAMOM

To study the effect of *P. indica* and *G. fasciculatum* against *F. oxysporum* under *in vivo* condition, pot culture experiments were carried out in cardamom seedlings and suckers.

3.6.1. Mass multiplication of the pathogen

Sick soil inoculation method was carried out for cardamom seedlings and suckers (Nikam *et al.*, 2011). The pure culture of Fusarium isolate was multiplied in sand-maize flour medium prepared in the ratio 9:1 (Plate 7). Firstly, sand-maize medium was mixed well with water to provide enough moisture. It was then transferred to polypropelene cover to make packets of 150 g each and sterilised in autoclave at 15 lbs for 30 minutes. Then the pure culture of Fusarium isolate was inoculated into the cover under aseptic condition and incubated at room temperature for 15 days. After 15 days, the inoculum rich medium (10^7 cfu g^{-1}) was taken out from the cover and drenched to the base of cardamom plants raised in polythene bags at the rate of 0.5 per cent w/w for seedlings and one per cent w/w for suckers (Plate 8-9).

3.6.2. Pot culture experiment using seedlings and suckers

For the pot culture experiment was carried out to assess the effect of bioagents against *F. oxysporum* in cardamom in which different management strategies including endophytes, bacterial bioagent and fungicide were employed. The endophyte colonised

seedlings and suckers were raised in the same manner as in the colonisation and interaction study (3.2) and challenge inoculation was performed. For the bacterial bioagent check (T4), two per cent *Pseudomonas fluorescens* strain PN026 was prepared by mixing 20 g talc based formulation of the bioagent in one litre cow dung slurry and drenched at the base of the plant. The fungicide carbendazim at 0.1 per cent as basal drench was used as the chemical check (T5). Treatment T6 was the untreated control without any endophyte or pathogen inoculation and T7 comprised of the absolute control with challenge inoculation alone.

After the treatments were employed, the challenge inoculation was carried out. The pathogen inoculum multiplied in sterilized sand-maize medium (9:1 ratio) with 10^7 cfu g^{-1} was inoculated as basal drench at the rate of 0.5 per cent (w/w) in seedlings and one per cent (w/w) in suckers three weeks after bioagent application.

Table 1. Bio agents and fungicide used to manage Fusarium rot of cardamom

Treatments	Basal application
T1	*P. indica* multiplied in coirpith medium @ one per cent w/w
T2	*G. fasciculatum* –KAU strain two per cent w/w
T3	*P. indica* multiplied in coirpith medium @ one per cent w/w + *G. fasciculatum* –KAU strain two per cent w/w
T4	*Pseudomonas fluorescens* (KAU strain) (2 per cent in cowdung slurry @1L pot^{-1})
T5	Carbendazim (2 g L^{-1} drench) @ 1L pot^{-1}
T6	Untreated control
T7	Absolute control

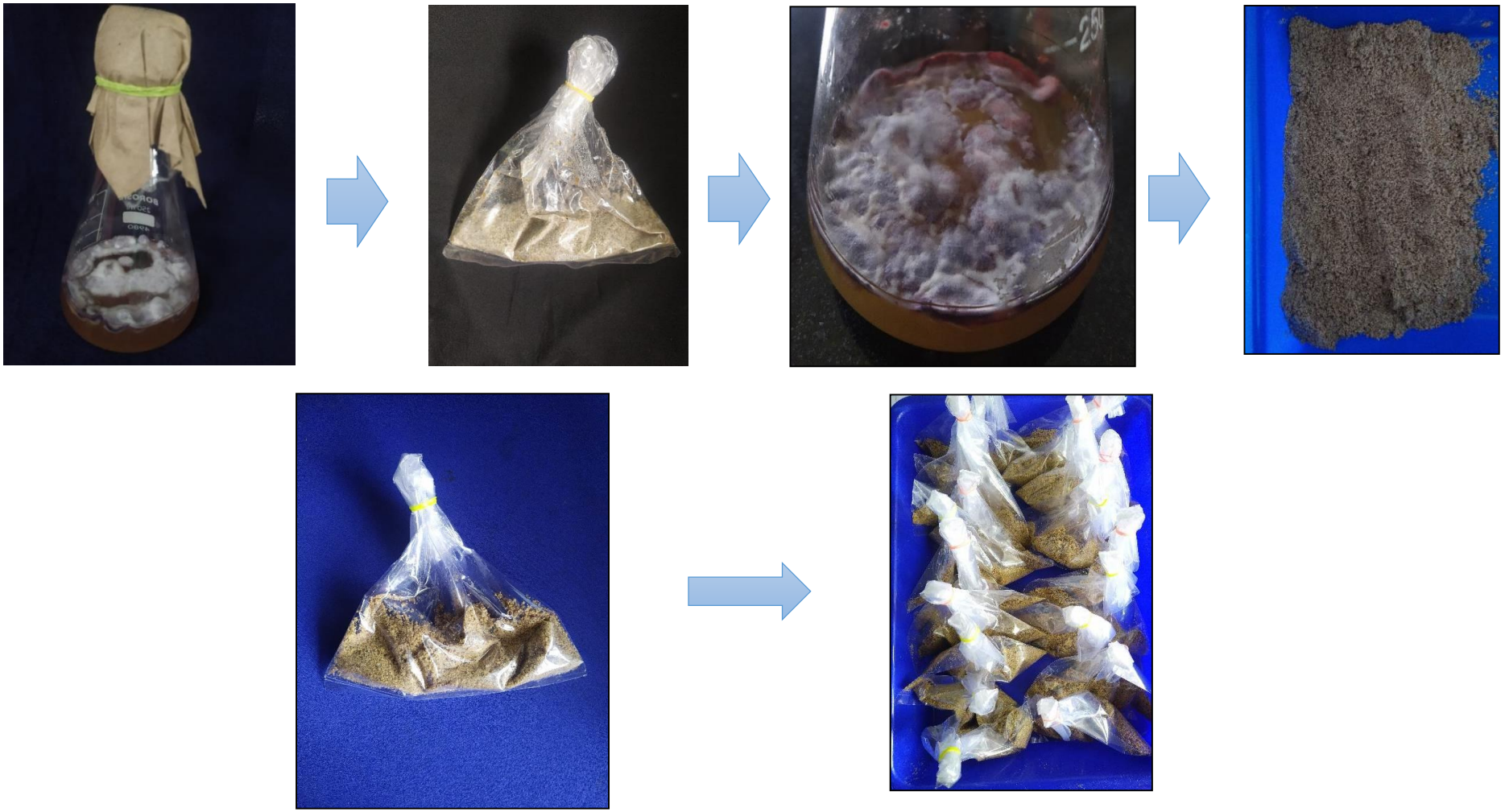


Plate 7. Mass multiplication of pathogen in sand-maize (9:1) medium

Plate 8. Seedlings challenge inoculated with the pathogen (0.5 per cent w/w)

Plate 9. Challenge inoculation in suckers by the pathogen (1 per cent w/w)

3.6.3 Lesion size and Percent disease index of cardamom plants inoculated with *F. oxysporum*

Lesion size was examined by measuring the length and width of the lesion one month after the symptom development. Disease severity / PDI of the challenge inoculated plants were evaluated using 0-4 scale score chart (Venykrishna *et al.*, 2021).

Table 2. Disease scale for scoring Fusarium rot of small cardamom

Score	Description
0	No disease
1	Lesion on 1-10% area of the tillers had fungal lesions
2	Lesion on 11-25% area of the tillers had fungal lesions
3	Root tip rotting as well as fungal lesions on 26-50% area of the of tiller
4	Root tip rotting as well as fungal lesions on 26-50% area of the of tiller

The disease severity was calculated using the following formula,

$$PDI = \frac{\text{Sum of individual scores}}{\text{Total number of tillers observed}} \times \frac{100}{\text{Maximum score given}}$$

3.7. ELUCIDATING THE ROLE OF GIBBERELLIC ACID IN ENDOPHYTE MEDIATED TOLERANCE

GA estimation was carried out from challenge inoculated plants of 3.6 using the protocol of Holbrook *et al.* (1961) in order to elucidate the role gibberellic acid in endophyte mediated tolerance. One gram of plant sample from experimental plants of 3.6 was dissolved in absolute alcohol and then diluted to 100 ml with absolute alcohol (Solution A). For the measurement of sample reading, five ml from the above prepared solution (Solution A) was added to a 100 ml volumetric flask to which 5 ml of absolute alcohol and 35 ml 30 per cent HCl was added. This was then diluted to 100 ml using

distilled water and kept in water bath at 20°± 1°C, 75 minutes. For the measurement of blank, 5 ml solution A was added to 100 ml volumetric flask to which five ml of absolute alcohol and 35 ml 35 per cent HCl was added.

The absorbance of blank and sample was read against water at 254 nm. The blank reading was subtracted from sample reading and GA content was obtained by reference to callibration graph (Fig. 2).

Preparation of standard curve

400 ppm of standard GA stock solution was prepared. 2, 4, 6, 8, and 10 ml aliquots from the stock was transferred to 100 ml conical flask and diluted to 100 ml with 30 per cent HCl commencing from smallest aliquot. This was then kept in water bath at 20°C ± 1°C for 75 minutes and read at 254 nm.

3.8. STATISTICAL ANALYSIS

The data obtained from *in vitro* and in *vivo* studies were subjected to analysis of variance (ANOVA) after appropriate transformation. When the effects were found to be significant, critical difference value was calculated for each observation using 't' values at 5 per cent level of significance. Significant treatments were compared with CD value. For biometric characters factorial analysis was carried out with different growth intervals as additional factor. All the data were analysed using GRAPES 1.0.0. Software (Gopinath *et al.*, 2020).

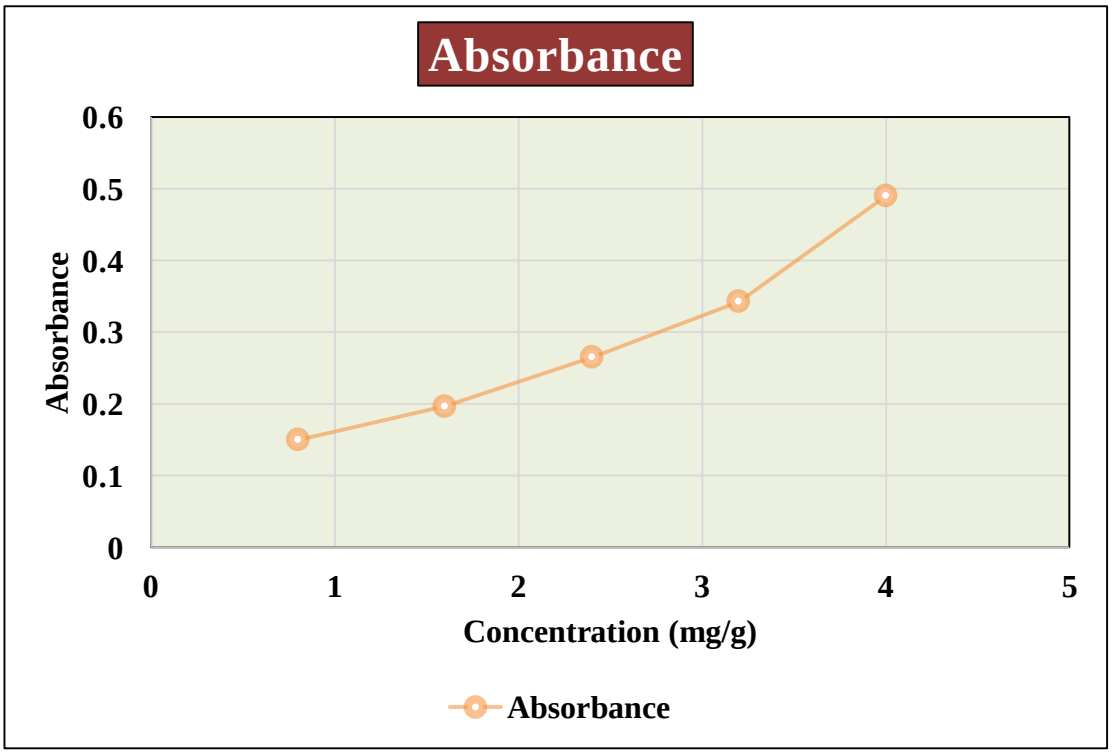


Fig. 2. Standard curve of Gibberellic acid (mg g^{-1})

Results

4. RESULTS

The study entitled "Evaluation of beneficial fungal root endophytes against Fusarium rot in small cardamom" was conducted at Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram and Cardamom Research Station, Pampadumpara, Idukki, Kerala during the year 2020-21 to evaluate the colonisation and interaction of two beneficial fungi, *P. indica* and *G. fasciculatum*, in cardamom and their potential to manage Fusarium rot disease of cardamom as well as elucidation of the role of gibberellic acid in the endophytic fungi-mediated disease tolerance.

4.1 COLLECTION OF FUSARIUM INFECTED PLANTS FROM HOTSPOT AREAS, ISOLATION OF PATHOGEN, PROVING KOCH'S POSTULATES AND MAINTANENCE OF CULTURE

4.1.1. Collection of the diseases sample and symptomatology of the disease

Characteristic symptoms of Fusarium rot of small cardamom was eye shaped lesion and dry rotting on pseudostem especially during summers. In advanced stages, the affected portion became weak at the point of infection and lodged. The root rot symptom (Plate 10) incited by the pathogen progressed from the root tip and resulted in reduced water and nutrient uptake. Symptoms in the panicle were described by drying of the tip of panicle which later resulted in burnt appearance. Fusarium infected roots were collected from the farmer's field of Pampadumpara in Idukki district (AEU 14).

4.1.2. Isolation of the pathogen, cultural and morphological characteristics

The pathogen *F. oxysporum* Scheldt.was isolated from the infected roots collected from the farmer's field in Pampadumpara of Idukki district. Standard isolation procedure was carried out as described in 3.1.2. The pathogen was characterized by pinkish- white fluffy growth in the front view and pinkish tinge at the base in the rear view (Plate 11). The rate of growth of mycelium was one cm day^{-1} covering the nine cm Petriplate in nine days. The initial growth of the fungus was slow upto five days, but after which it gained more rate of establishment.

Microscopical studies confirmed the production of macro conidia, micro conidia and chlamydospores by the pathogen. Macroconidia was hyaline in nature, two to several celled and sickle shaped having an average size of 14.8 µm. Microconidia was one to two celled ovoid in shape with an average size of 10.7 µm (Plate 12).

4.1.3. Proving Koch's postulate

The Koch's postulate was proved using artificial inoculation of the pathogen on one year old pseudostem of cardamom by pinprick method. The isolate produced typical eye shaped lesion with brown border on the pseudostem 15 days after pathogen inoculation. The lesion had an average size of 1.50 cm length and 0.25 cm width with 0.11 cm day^{-1} rate of lesion development (Plate 13).

4.2. EVALUATION OF THE COLONISATION AND INTERACTION OF *P. indica* AND *G. fasciculatum*

A pot culture experiment was carried out at Cardamom Research Station, Pampadumpara during the year 2020-21 to evaluate the colonisation and interaction of the endophytes and their role in plant growth promotion.

4.2.1. Colonisation study of the roots inoculated with endophytes

Successful root colonization was observed in *P. indica* and *G. fasciculatum* treated roots at six and seven days respectively after their inoculation in the case of seedlings and 21 days and 28 days respectively in the case of suckers (Plate 15-18). This is the first report of colonization of *P. indica* in small cardamom seedlings (Aishwarya *et al.,* 2021) and suckers (Aishwarya *et al*., 2022). Chlamydospores of *P. indica* were observed in cortical and epidermal layers under microscope upon the staining. The colonization was initiated by chlamydospores which on germination produced inter and intracellular hyphae. *G. fasciculatum* colonization was characterised by the presence of arbuscules and vesicles inside the plant cell.

Plate 10. Fusarium infection on root from farmer's field

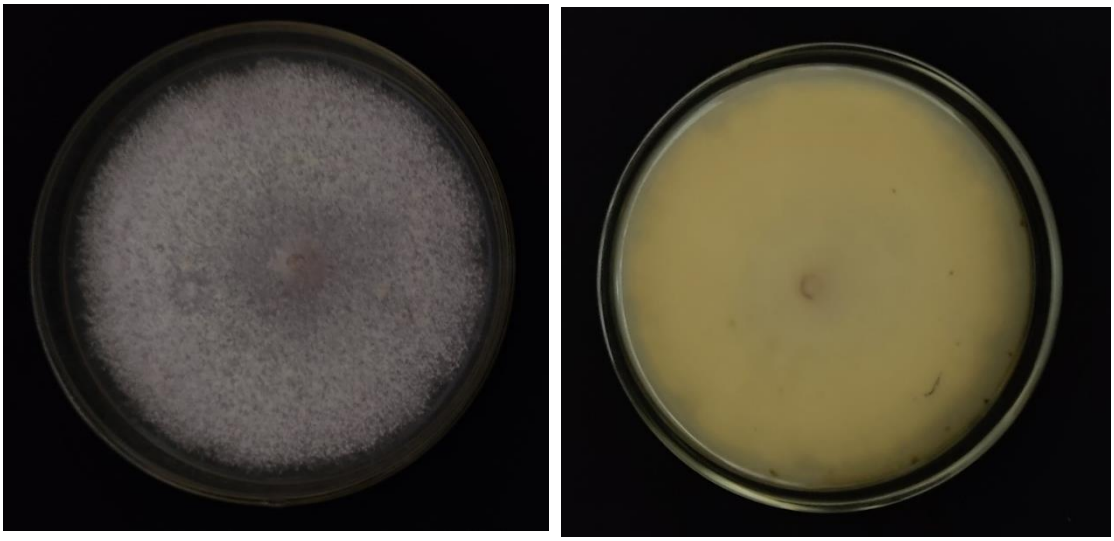
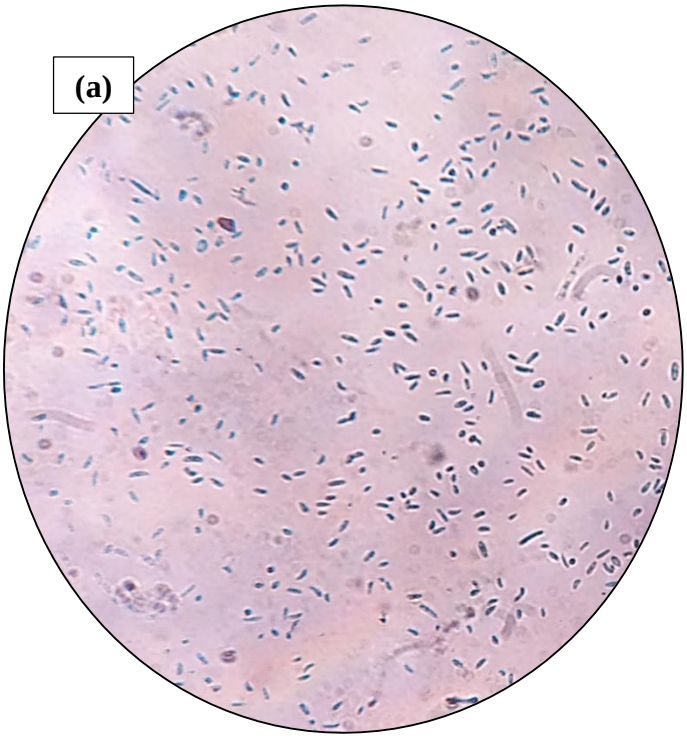
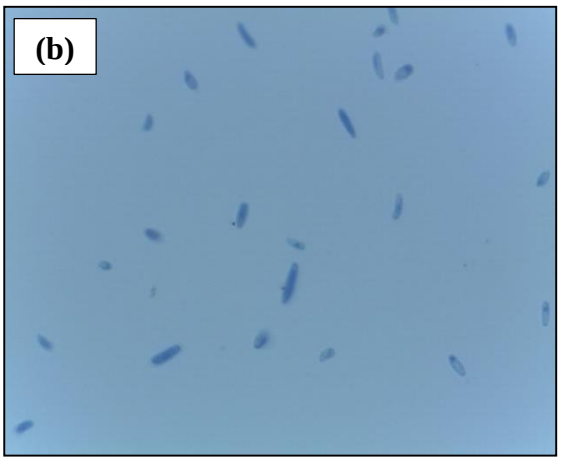


Plate 11. Nine day old *F. oxysporum* cultures isolated from roots of cardamom and grown on PDA medium





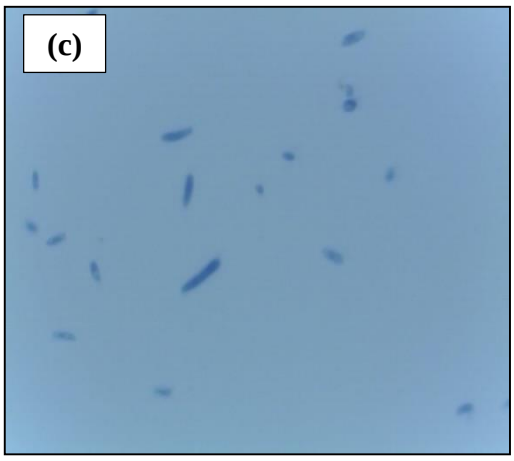


Plate 12. Conidia of *Fusarium oxysporum* isolated from roots of cardamom a) Macroconidia and microconidia under 40x b) Macroconida under 100 x c) Microconidia under 100x

Plate 13. Symptom development on pseudostem of cardamom by *F. oxysporum* on artificial inoculation by pin prick method

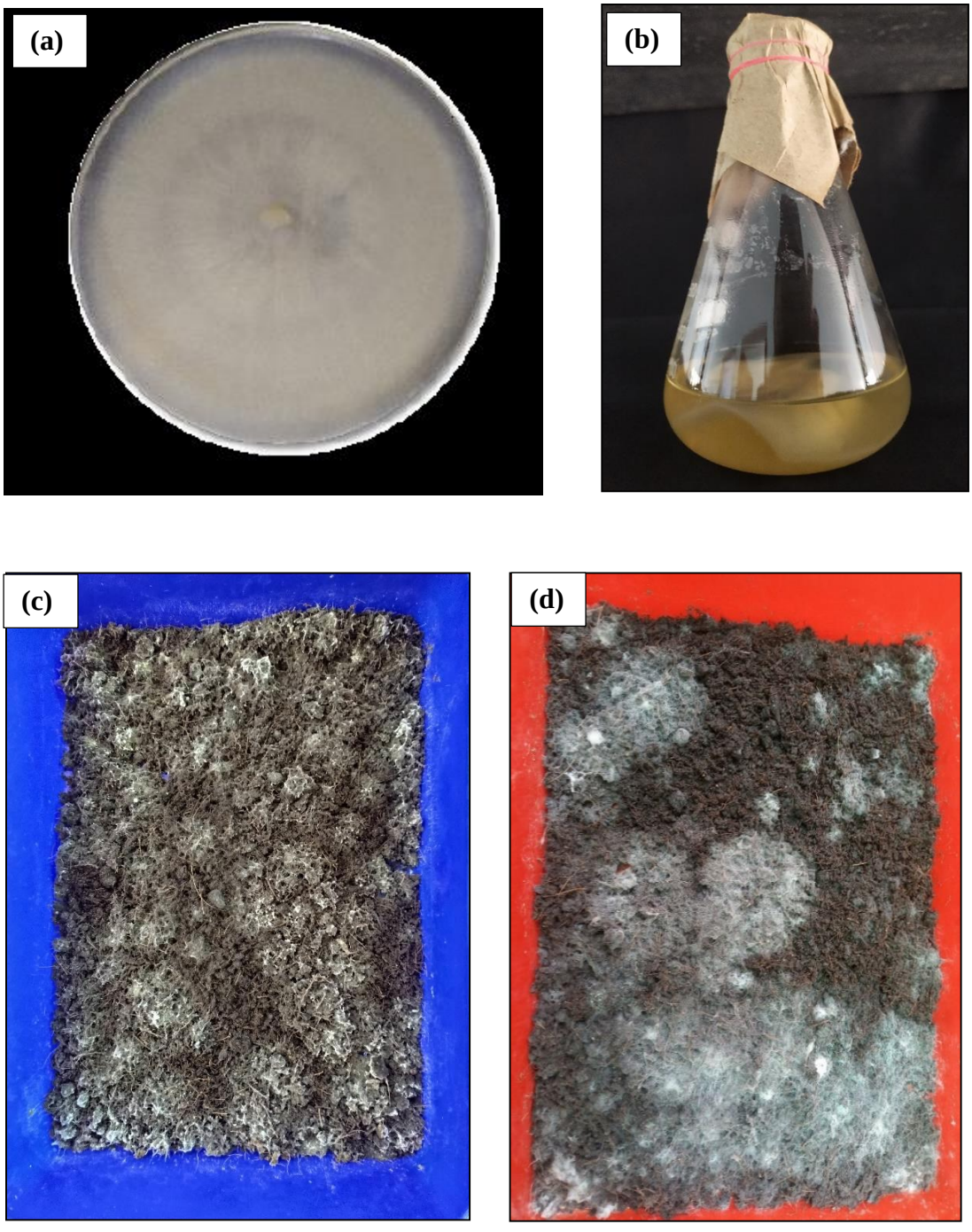


Plate 14. ***P. indica*** **in (a) PDA (b) PD broth (c) and (d) Coirpith-cowdung medium**

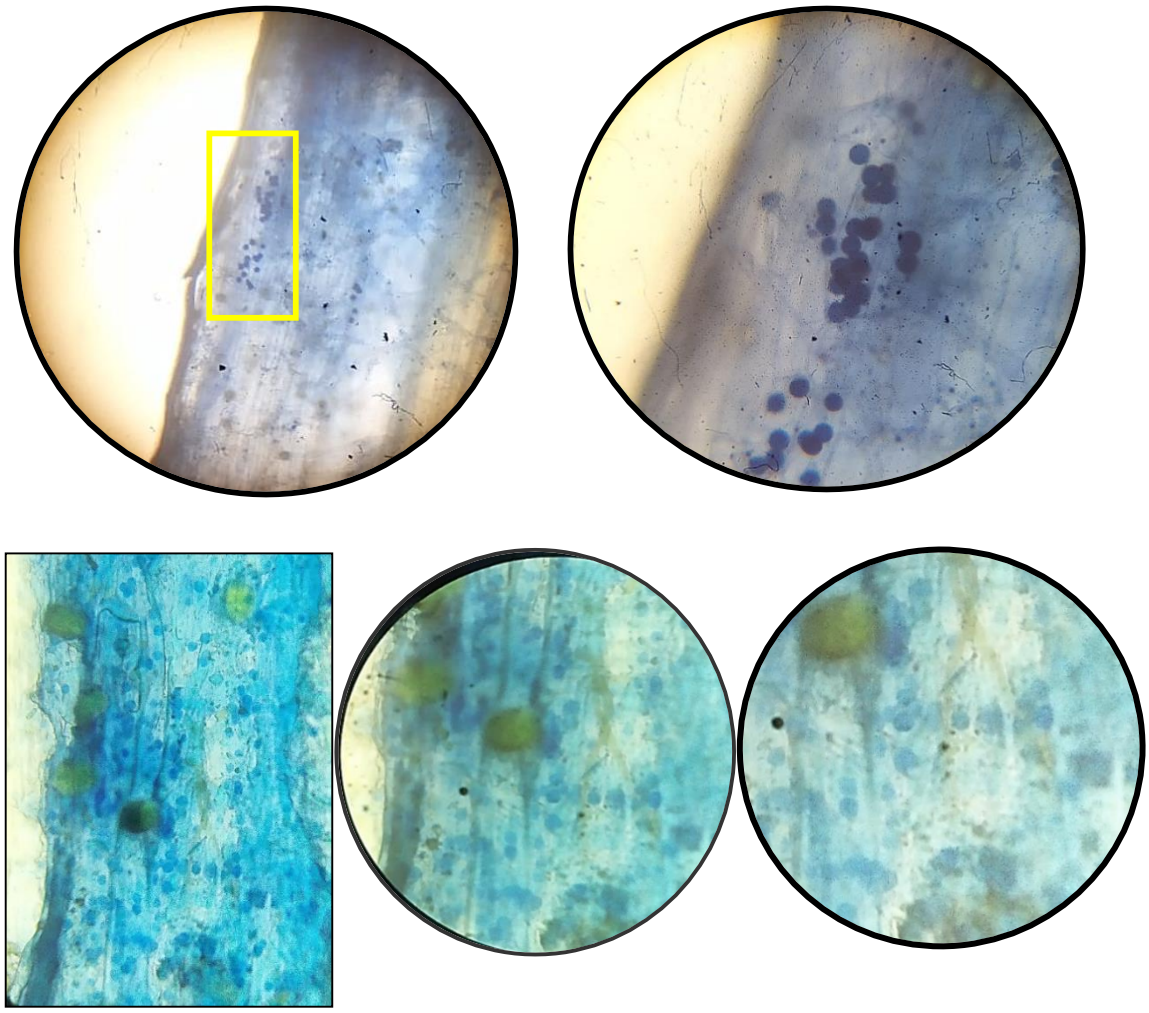


Plate 15. Root colonization of *P. indica* in cardamom seedlings var. *Njallani* under 10x and 40 x

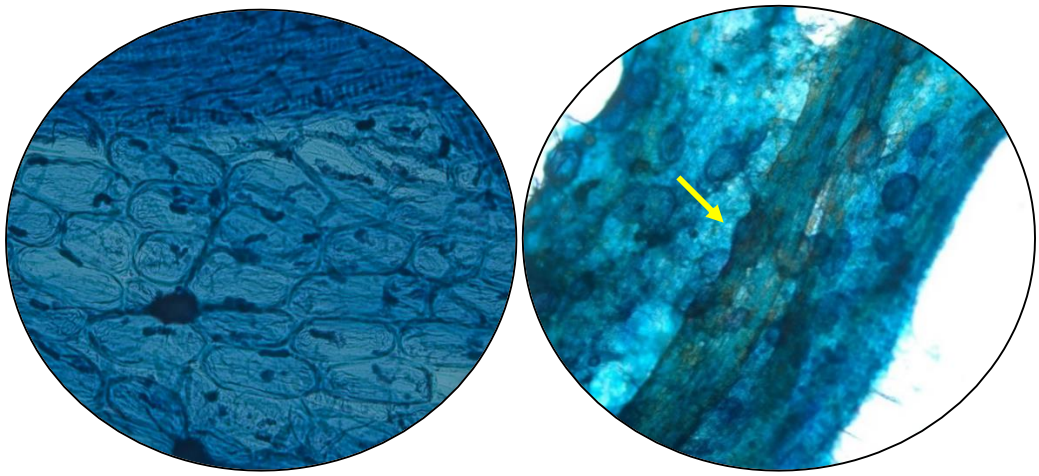


Plate 16. Root colonization of *P. indica* in cardamom suckers var. *Njallani*

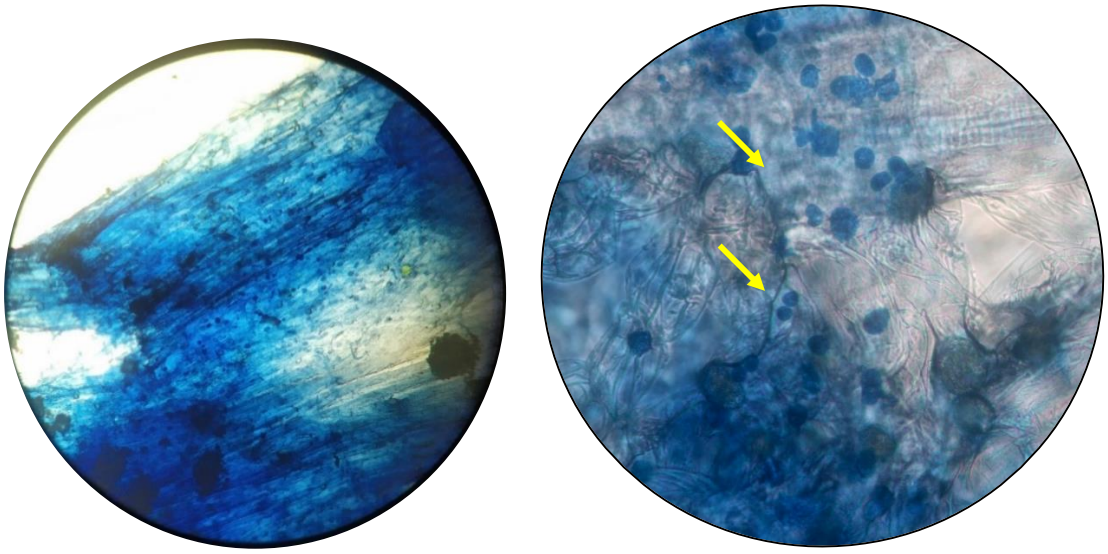


Plate 17. Root colonization of *P. indica* in cardamom suckers var. *Njallani* (10X and 40X)

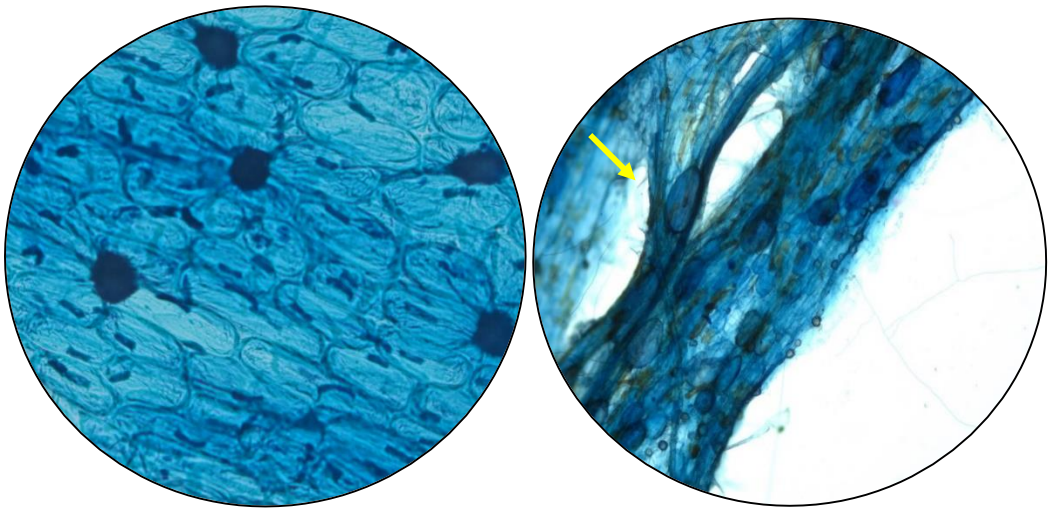


Plate 18. Root colonization of *G. fasciculatum* in cardamom suckers var. *Njallani*

4.2.2. Plant growth evaluation in bioagent colonized plants

Biometric characters such as plant height, number of leaves, leaf length, leaf breadth, number of tillers, root parameters and plant biomass were observed in the experimental plants inoculated with bioagents and uninoculated control.

4.2.2.1. Seedlings

Inoculation of the cardamom seedlings with *P. indica* and *G. fasciculatum* individually and in combination showed improved seedling vigour and vegetative growth i.e. increased plant height, number of leaves, leaf length and leaf breadth compared to the uninoculated control (Table 3) (Plate 19). Among all treatments, plants dually inoculated with *P. indica* and *G. fasciculatum* was superior at all stages of the study with maximum height, number of leaves and leaf length (70.28, 10.20 and 39.24 cm respectively) and the control plants recorded the least values i.e. 48.38, 5.40 and 30.38 cm respectively at 180 days after the fungal endophytes inoculation (just before transplanting to the field). Upto 90 days, *P. indica* and *G. fasciculatum* treated plants showed nearly same growth pattern after which the parameters like plant height and leaf length of *P. indica*-colonized plants (67.90 cm and 36.72 cm respectively) exceeded the *G. fasciculatum* inoculated plants and proceeded more towards the superior treatment i.e. the combined application of the endophytes.

Table 3. Effect of individual and combined colonization of the endophytes, *P. indica* and *G. fasciculatum* on different vegetative characters of cardamom seedlings at different intervals of growth

a) Plant height (cm)					
Treatments	**1 MAT**	**2 MAT**	**3 MAT**	**5 MAT**	**6 MAT**
P. indica	2.34±0.38^{ab}	5.52±0.08^{b}	14.00±0.80^{b}	63.06±7.27^{ab}	67.90±4.40^{ab}
G. fasciculatum	2.56±0.31^{a}	5.40±0.55^{b}	13.32±1.20^{b}	58.76±5.17^{b}	63.64±5.57^{b}
P. indica* + *G. fasciculatum	2.66±0.19^{a}	6.74±1.02^{a}	16.6±0.75^{a}	67.16±4.73^{a}	70.28±2.23^{a}
Control	2.12±0.26^{b}	3.64±0.23^{c}	7.74±0.76^{c}	41.30±6.36^{c}	48.38±4.79^{c}
SE ± (m)	0.132	0.26	0.38	2.67	2.20
CD (0.05)	0.397	0.793	1.157	8.004	5.932
CD (0.05) Interaction	4.229				

b) Number of leaves					
Treatments	**1 MAT**	**2 MAT**	**3 MAT**	**5 MAT**	**6 MAT**
P. indica	3.00±0.45	4.80±0.45^{ab}	6.60±0.44^{b}	7.00±1.58^{b}	7.60±1.34^{b}
G. fasciculatum	3.00±0.44	4.60±0.55^{b}	6.40±0.52^{b}	7.00±1.41^{b}	6.60±1.64^{b}
P. indica* + *G. fasciculatum	3.20±0.44	5.40±0.54^{a}	7.80±0.90^{a}	9.00±2.00^{a}	10.20±0.83^{a}
Control	3.00±0.35	4.40±0.64^{b}	5.40±0.21^{c}	5.00±0.10^{c}	5.40±0.55^{c}
SEm (±)	NS	0.24	0.24	0.65	0.45
CD (0.05)	NS	0.703	0.709	1.954	1.572
CD (0.05) Interaction	1.160				

c) Leaf length (cm)					
Treatments	**1 MAT**	**2 MAT**	**3 MAT**	**5 MAT**	**6 MAT**
P. indica	2.42±0.26	6.74±0.47^{ab}	13.66±1.12^{b}	27.54±4.89^{ab}	36.72±5.02^{a}
G. fasciculatum	2.36±0.36	6.06±0.58^{b}	12.64±1.23^{c}	25.96±3.41^{ab}	34.38±4.85^{ab}
P. indica* + *G. fasciculatum	2.44±0.16	7.24±0.73^{a}	14.96±0.21^{a}	30.52±3.18^{a}	39.24±1.80^{a}
Control	2.06±0.21	3.92±0.54^{c}	8.56±0.40^{d}	23.64±2.26^{b}	30.38±3.69^{b}
SEm (±)	NS	0.26	0.31	1.59	1.81
CD (0.05)	NS	0.786	0.933	4.774	5.430

d) Leaf breadth (cm)					
Treatments	**1 MAT**	**2 MAT**	**3 MAT**	**5 MAT**	**6 MAT**
P. indica	1.70±0.27[b]	2.84±0.39[b]	4.50±0.41[b]	7.9±1.30	8.96±0.15
G. fasciculatum	1.8±0.16[b]	2.62±0.29[b]	4.48±0.46[b]	7.8±1.25	8.62±1.01
P. indica* + *G. fasciculatum	1.84±0.29[a]	3.26±0.28[a]	4.96±0.49[a]	8.1±1.31	9.1±0.65
Control	1.34±0.09[c]	2.12±0.19[c]	3.32±0.33[c]	7.6±1.15	8.38±0.74
SEm (±)	0.09	0.15	0.11	NS	NS
CD (0.05)	0.274	0.443	0.577	NS	NS

e) Number of tillers	
Treatments	**1 MAT**
P. indica	1.70±0.27[b]
G. fasciculatum	1.8±0.16[b]
P. indica* + *G. fasciculatum	1.84±0.29[a]
Control	1.34±0.09[c]
SEm (±)	0.09
CD (0.05)	0.274

MAT- Months after treatment application ; Values are mean of 5 replications ± standard deviation; the values following the same letters in the superscripts are not significant at 5% level of significance

There was an exceedingly observable positive effect by the endophytes colonization, both individually and in combination on growth and development of roots compared to the control plants (Table 4) (Plate 20). The root length and number of secondary roots at six months after the endophytes application were superior and on par for all endophyte treated plants (both individual application and combination) compared to control (Table 4). The root volume was significantly highest for *P. indica*-colonized plants (27.60 cm^3) followed by the dual inoculated (23.20 cm^3) and *G. fasciculatum* inoculated (15.40 cm^3) plants whereas, the least value being observed in uninoculated plants (7.40 cm^3).

Table 4. Effect of individual and combined colonization of the endophytes, *P. indica* and *G. fasciculatum* on root parameters / architecture of cardamom seedlings at 180 days after colonization

Treatments	Root length (cm)	Root volume (cm^3)	No. of secondary roots
P. indica	56.02±2.75[a]	27.60±2.07[a]	12.40±1.82[a]
G. fasciculatum	51.58±7.53[a]	15.40±2.09[c]	10.60±1.14[a]
P. indica* + *G. fasciculatum	55.62±1.76[a]	23.20±1.76[b]	11.60±2.07[a]
Control	37.20±4.62[b]	7.40±4.63[d]	7.80±1.10[b]
SEm (±)	2.11	0.96	2.53
CD (0.05)	6.318	2.891	2.131

Values are mean of 5 replications ± standard deviation; the values following the same letters in the superscripts are not significant at 5% level of significance

In plant biomass evaluation, the fresh and dry weights of the shoot and root were taken at the end of the experiment (six months after transferring to polybag nursery). Both fresh and dry weights were more in the fungal root endophytes treated plants compared to control plants (Table 5). Among treated plants, *P. indica* inoculated plants recorded highest root weight (fresh and dry), whereas combination of endophytes contributed highest shoot weight *G. fasciculatum* were 95.62 g plant^{-1} and that for individually inoculated plants of *P. indica* and *G. fasciculatum* were 86.02 g plant^{-1} and 75.86 g plant^{-1} respectively. The root fresh weight of *P. indica* inoculated plants was 43.00 g plant^{-1} and that of combined inoculated and *G. fasciculatum* inoculated plants were 33.26 g plant^{-1} and 27.14 g plant^{-1} respectively. The fresh weights of control plants were minimal, recording 54.24 g plant^{-1} and 19.46 g plant^{-1} for shoot and root respectively. The root dry weight of combinatorial treatment was in par with *P. indica* treated plants even though there was a significant difference in their root fresh weight.

Furthermore, when *P. indica* and *G. fasciculatum* dually colonised seedlings were compared to non-colonized seedlings, the nursery duration was reduced by two months.

Table 5. Effect of individual and combined colonization of the endophytes, *P. indica* and *G. fasciculatum* on root and shoot biomass of cardamom seedlings at 180 days after colonization

Treatments	Root fresh weight (g plant^{-1})	Root dry weight (g plant^{-1})	Shoot fresh weight (g plant^{-1})	Shoot dry weight (g plant^{-1})
P. indica	43.00±1.99^{a}	4.95±0.57^{a}	86.02±10.92^{b}	12.19±1.62^{ab}
G. fasciculatum	27.14±0.51^{c}	3.98±0.52^{b}	75.86±3.34^{c}	10.76±1.79^{b}
P. indica* + *G. fasciculatum	33.26±1.42^{b}	4.62±0.93^{ab}	95.62±4.97^{a}	13.26±1.12^{a}
Control	19.46±1.15^{d}	2.93±0.16^{c}	54.24±3.60^{d}	8.61±1.59^{c}
SEm (±)	1.77	0.27	2.90	0.69
CD (0.05)	1.844	0.941	8.692	13.850

Values are mean of 5 replications ± standard deviation; the values following the same letters in the superscripts are not significant at 5% level of significance

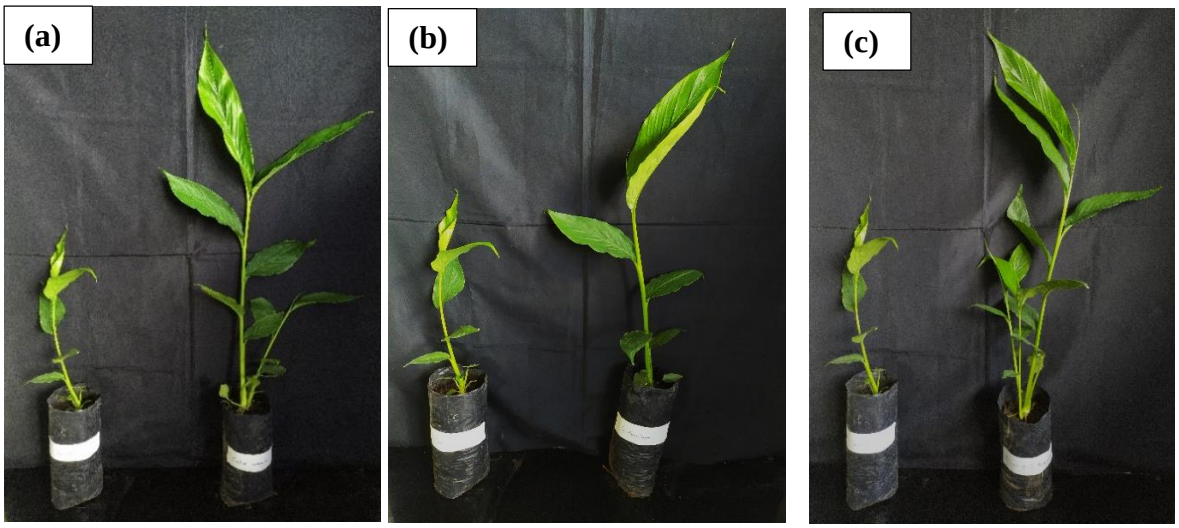


Plate 19. Comparison of plant growth (5 month) in endophyte colonised cardamom seedlings var. *Njallani* compared to the uninoculated control a) *P. indica* colonised seedling compared to control seedling b) *G. fasciculatum* colonised seedling compared to control seedling c) Dually colonised *P. indica* and *G. fasciculatum* seedling compared to control.

Plate 20. Root architecture (6 month) of endophyte colonised cardamom seedlings compared to the uninoculated control a) *P. indica* colonised seedling compared to control seedling b) *G. fasciculatum* colonised seedling compared to control seedling c) Dually colonised *P. indica* and *G. fasciculatum* seedling compared to control.

4.2.2.2. Suckers

Endophyte colonization in cardamom suckers also resulted in plant growth promotion and better root establishment. All the endophyte treated plants had better biometric parameters compared to the uninoculated control (Table 6). Eventhough there wasn't any significant difference in the plant biometric parameters upto one month after endophyte inoculation, an optimistically distinct difference in plant height, number of leaves, leaf length and number of tillers could be observed after two months in the endophyte colonized plants compared to the uninoculated control. The plant height was on par for all endophyte treated plants having 89.92 cm, 89.46 cm and 92.22 cm for *P. indica*-colonised, *G. fasciculatum*- colonized and dually-colonized plants respectively. The number of leaves were superior for dually colonized plants (17.60) followed by individually colonized which was 14.40 and 13.80 respectively for *P. indica* treated and *G. fasciculatum* treated. There wasn't any significant difference in leaf length upto two months after treatment application, thereafter the endophyte treated plants were superior to control plants with leaf lengths 43.26 cm, 42.28 cm and 48.28 cm respectively for *P. indica*-colonized, *G. fasciculatum*-colonized and dually colonized plants. The number of tillers was highest for dually-colonized plants followed by *P. indica*-colonized, which was then followed by *G. fasciculatum*.

Table 6. Effect of endophytes root colonization on vegetative characters of cardamom suckers

Treatments	a) Plant height (cm)		
	1 MAT	**2 MAT**	**3 MAT**
P. indica	51.60±2.52	89.92±1.80^{a}	136.38±4.45^{b}
G. fasciculatum	52.18±1.69	89.46±3.10^{a}	136.34±5.33^{b}
P. indica* + *G. fasciculatum	52.16±3.31	92.22±3.62^{a}	148.94±4.05^{a}
Control	51.26±2.39	81.68±6.01^{b}	121.52±3.74^{c}
SEm (±)	1.14	1.76	1.98
CD (0.05)	NS	5.28	5.942

Treatments	b) Number of leaves		
	1 MAT	2 MAT	3 MAT
P. indica	7.40±1.52	14.40±2.30[b]	15.80±1.79[ab]
G. fasciculatum	7.60±1.14	13.80±1.30[b]	15.40±1.67[b]
P. indica* + *G. fasciculatum	7.80±0.84	17.60±3.36[a]	18.80±3.70[a]
Control	7.00±1.23	11.40±1.52[b]	10.20±1.10[c]
SEm (±)	0.54	1.02	1.02
CD (0.05)	NS	3.043	3.065

Treatments	c) Leaf length (cm)		
	1 MAT	2 MAT	3 MAT
P. indica	30.46±1.89	38.16±5.83	43.26±6.08[a]
G. fasciculatum	29.12±2.70	39.84±6.48	42.28±4.59[a]
P. indica* + *G. fasciculatum	30.76±1.25	43.62±6.41	48.28±6.18[a]
Control	28.38±1.52	32.66±4.25	34.86±2.36[b]
SEm (±)	0.86	2.60	2.26
CD (0.05)	NS	NS	6.766

Treatments	d) Leaf breadth (cm)		
	1 MAT	2 MAT	3 MAT
P. indica	7.34	8.48	9.12
G. fasciculatum	7.38	8.20	9.06
P. indica* + *G. fasciculatum	7.52	8.62	9.52
Control	6.34	7.16	8.18
SEm (±)	0.36	0.38	0.34
CD (0.05)	NS	NS	NS

Treatments	e) Number of tillers		
	1 MAT	**2 MAT**	**3 MAT**
P. indica	2.0±0.71	2.8 ± 0.45^{ab}	4.4 ± 0.89^{ab}
G. fasciculatum	2.0±0.01	2.2 ± 0.84^{b}	4.2 ± 0.45^{b}
P. indica* + *G. fasciculatum	2.0±0.72	3.0 ± 0.71^{a}	5.2 ± 0.45^{a}
Control	1.8±0.45	1.6 ± 0.55^{b}	2.4 ± 0.55^{c}
SEm (±)	0.25	0.29	0.27
CD (0.05)	NS	0.874	0.821

MAT- Months after treatment application; Values are mean of 5 replications ± standard deviation; the values following the same letters in the superscripts are not significant at 5% level of significance

The root parameters were exceptionally higher for endophyte treated plants compared to the control plants (Table 7) (Plate 21-23). The root lengths were higher and were on par for *P. indica*-treated and dually-treated plants recording 96.95 cm and 100.68 cm respectively at 120 days after treatment application. The number of secondary roots and root volume were more for dually-colonized plants recording 44.75 cm^3 and 220.60 cm^3 respectively. This was then followed by individually treated plants i.e. *P. indica* and *G. fasciculatum*, where *P. indica*-colonized plants were statistically on par with the dually treated plants for both the parameters. Among the treatments least values of root parameters were observed in uninoculated plants recording 60.70 cm, 91 cm^3 and 19 for root length, root volume and number of secondary roots.

Table 7a. Effect of endophytes root colonization on root length and number of secondary roots of cardamom suckers (3 MAT)

Treatments	Root length (cm)	No. of secondary roots
P. indica	87.43±6.21[a]	39.75±4.72[a]
. fasciculatum	69.53±6.73[b]	27.75±2.50[b]
P. indica* + *G. fasciculatum	92.28±7.21[a]	43.75±6.19[a]
Control	58.75±6.59[c]	18.75±2.87[c]
SEm (±)	3.347	2.165
CD (0.05)	10.313	6.671

Table 7b. Effect of endophytes root colonization on root parameters of cardamom suckers (4 MAT)

Treatments	Root length (cm)	Root volume (cm^3)	No. of secondary roots	Root fresh weight (g $plant^{-1}$)	Root dry weight (g $plant^{-1}$)
P. indica	96.95±8.11[a]	181.00±22.42[ab]	40.50±6.46[ab]	351.05±18.29[b]	34.80±5.69[b]
G. fasciculatum	78.58±7.44[b]	157.00±40.87[b]	36.75±4.27[b]	192.96±27.13[c]	31.15±5.08[b]
P. indica* + *G. fasciculatum	100.68±8.80[a]	220.60±24.93[a]	44.75±3.86[a]	393.19±24.17[a]	48.43±6.71[a]
Control	60.70±4.33[c]	91.00±28.92[c]	19.00±4.08[c]	103.88±17.09[d]	18.49±5.10[c]
SEm (±)	3.69	13.48	2.39	11.03	2.84
CD (0.05)	11.355	40.405	7.369	33.990	8.754

Values are mean of 5 replications ± standard deviation; the values following the same letters in the superscripts are not significant at 5% level of significance

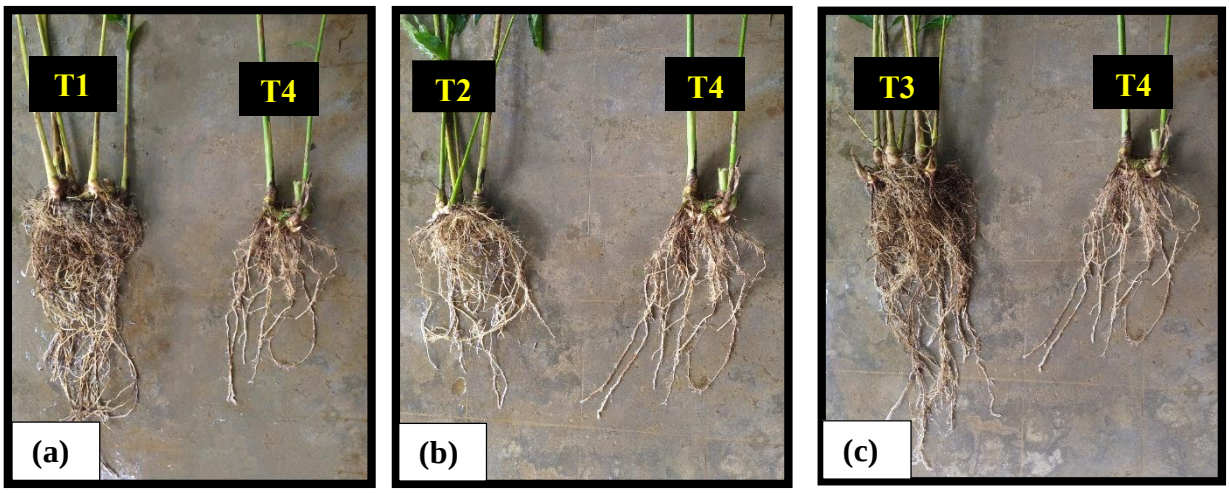


Plate 21. Root architecture (3 MAT) of endophytes colonised cardamom suckers var. *Njallani* compared to the uninoculated control a) *P. indica* colonised seedling compared to control suckers b) *G. fasciculatum* colonised suckers compared to control suckersc) Dually colonised *P. indica* and *G. fasciculatum* suckers compared to control

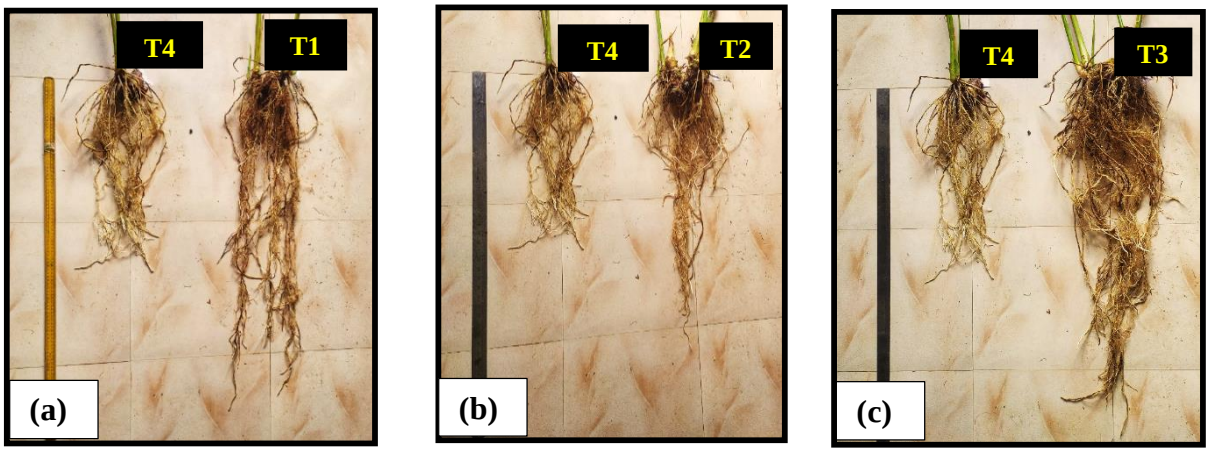


Plate 22. Root architecture (4 MAT) of endophytes colonised cardamom suckers var. *Njallani* compared to the uninoculated control a) *P. indica* colonised sucker compared to control sucker b) *G. fasciculatum* colonised sucker compared to control sucker c) Dually colonised *P. indica* and *G. fasciculatum* sucker compared to control

Plate 23. Comparison of root architecture (4 MAT) of endophytes colonised cardamom suckers to the uninoculated control

The biomass evaluation revealed that all the endophyte treatments resulted in better shoot and root biomass compared to the uninoculated control (Table 8). In contrary to the cardamom seedlings, where root biomass was higher for *P. indica*-treated plants and shoot biomass was higher for dually treated plants; here both the root and shoot's fresh and dry weights were highest in dually-treated plants followed by *P. indica*-treated which was then followed by *G. fasciculatum*-treated plants. The dually-colonized plants recorded a shoot fresh weight of 1318.68 g plant^{-1} and dry weight of 95.50 g plant^{-1} and root fresh weight of 378.71 g plant^{-1} and dry weight of 45.49 g plant^{-1}. Lowest biomass was observed in the non-colonized plants recording 93.71 g plant^{-1}, 15.13 g plant^{-1} respectively for root fresh and dry weight and 548.07 g plant^{-1}, 40.56 g plant^{-1} respectively for shoot fresh and dry weight.

Table 8. Effect of endophytes root colonization on biomass of cardamom suckers at 3 MAT

Treatments	Root fresh weight (g plant^{-1})	Root dry weight (g plant^{-1})	Shoot fresh weight (g plant^{-1})	Shoot dry weight (g plant^{-1})
P. indica	343.71±14.18^{b}	32.84±5.01^{b}	723.54±11.77^{b}	52.80±6.47^{b}
G. fasciculatum	148.71±10.86^{c}	23.05±2.34^{c}	658.34±10.57^{c}	46.82±5.82^{bc}
P. indica* + *G. fasciculatum	378.71±17.79^{a}	45.49±6.76^{a}	1318.68±13.02^{a}	95.50±7.71^{a}
Control	93.71±10.80^{d}	15.13±2.40^{d}	548.07±9.92^{d}	40.56±5.93^{c}
SEm (±)	6.86	2.27	5.69	3.26
CD (0.05)	21.125	6.978	17.533	10.049

Values are mean of 5 replications ± standard deviation; the values following the same letters in the superscripts are not significant at 5% level of significance

4.3. NUTRIENT UPTAKE BY THE BIO AGENT COLONIZED PLANTS

To investigate whether the growth promotion was associated with the better nutrient uptake by the bioagent inoculated plants, the phosphorous and potassium content in the plant and soil were estimated.

4.3.1. Soil nutrient analysis (Pre-treatment)

The pretreatment soil analysis revealed that there wasn't any statistically significant difference in the soil nutrient content of P and K between any treatments (Table 9).

Table 9. Nutrient analysis of soil before the treatment application

Treatments	Seedling			Sucker		
	OC (Kg Ha $^{-1}$)	Phosphorous (Kg Ha$^{-1)}$	Potassium (Kg Ha$^{-1)}$	OC (Kg Ha $^{-1}$)	Phosphorous (Kg Ha$^{-1)}$	Potassium (Kg Ha$^{-1)}$
P. indica	2.49	118.15	1592.28	1.99	109.89	539.55
G. fasciculatum	3.74	139.21	1220.85	1.97	122.86	498.11
P. indica* + *G. fasciculatum	2.88	140.10	1414.64	1.81	111.06	532.45
Control	2.80	145.66	1558.00	1.11	113.59	556.35
CD (0.5)	NS	NS	NS	NS	NS	NS

Values are mean of 5 replications ± standard deviation; the values following the same letters in the superscripts are not significant at 5% level of significance

4.3.2. Plant nutrient analysis (Post treatment)

The nutrient analysis of plants revealed that the beneficial fungal root endophyte's colonization resulted in a better nutrient uptake of P (Table 10) and K (Table 11) to root, shoot and leaves in both seedlings and suckers compared to the control plants.

4.3.2.1. Phosphorus uptake in experimental plants

The phosphorus uptake to root was highest in *G. fasciculatum*-colonized seedlings and suckers recording a phosphorus content of 0.110 (%) and 0.105 (%) in seedlings and suckers respectively. Nevertheless, the phosphorus uptake to the shoot was highest in the dually-colonized plants in seedlings (0.058 per cent) and *P. indica*-colonized plants in suckers (0.111 per cent). The phosphorus uptake to leaves was significantly superior in *P. indica*-colonized plants in both seedlings and suckers.

The least phosphorus content in root, shoot and leaves were observed in uninoculated plants recording 0.009 (%), 0.028 (%) and 0.026 (%) respectively in seedlings and 0.016 (%), 0.071 (%) and 0.048 (%) respectively in suckers.

Table 10. Effect of endophytes root colonization on phosphorous uptake to root, shoot and leaf in cardamom plants var. *Njallani*

Treatments	SEEDLING (%)			SUCKER (%)		
	Root	**Shoot**	**Leaf**	**Root**	**Shoot**	**Leaf**
P. indica	0.040^{b}	0.028^{c}	0.055^{a}	0.069^{b}	0.111^{a}	0.152^{a}
G. fasciculatum	0.110^{a}	0.041^{b}	0.037^{bc}	0.105^{a}	0.079^{bc}	0.065^{c}
P. indica* + *G. fasciculatum	0.050^{b}	0.058^{a}	0.041^{ab}	0.069^{b}	0.084^{b}	0.124^{b}
Control	0.009^{c}	0.028^{c}	0.026^{c}	0.016^{c}	0.071^{c}	0.048^{d}
SEm (±)	0.003	0.005	0.004	0.003	0.004	0.005
CD (0.05)	0.010	0.011	0.013	0.011	0.011	0.017

Values are mean of 5 replications ± standard deviation; the values following the same letters in the superscripts are not significant at 5% level of significance

4.3.2.2. Potassium content in experimental plants

Various bioagents positively influenced the plant's K uptake significantly. All the endophyte treated plants had better uptake / content of K in root, shoot and leaves in comparison to their uninoculated control in both seedlings and suckers (Table 11).

The K uptake in root and leaf of all the endophyte treated plants were on par with one another in seedlings whereas the K uptake to shoot was superior and on par with one another in *P. indica*-treated (4.22 per cent) and dually-treated seedlings (4.14 per cent) compared to the other endophyte treated seedlings i.e. *G. fasciculatum* (3.66 per cent). The least values for K uptake were observed in control plants recording 1.33 (%), 3.00 (%) and 2.11 (%) respectively in root, shoot and leaves respectively.

In cardamom suckers, the K uptake of all the endophyte treated plants to the leaf was superior and was on par with one another. However, the K uptake to shoot was

highest in *P. indica*-treated plants (3.07 per cent) followed by *G. fasciculatum*-treated plants (2.84 per cent) which was then followed by the dually treated plants (2.77 per cent). The *G. fasciculatum* treated plants was statistically on par with the superior treatment i.e. *P. indica*. Furthermore, the K uptake to the shoot was characterized by a higher uptake of K in dually treated plants (4.32 per cent) and *G. fasciculatum* treated plants (4.03 per cent) which were on par with each other. This was then followed by *P. indica* treated plants (3.50 per cent). The least K uptake was recorded in the control plants with K content of 2.26 (%), 3.21 (%) and 2.05 (%) K content in root, shoot and leaf respectively.

Table 11. Effect of endophyte root colonization on potassium uptake to root, shoot and leaf in cardamom plants var. *Njallani*

Treatments	Seedling (%)			Sucker (%)		
	Root	Shoot	Leaf	Root	Shoot	Leaf
P. indica	2.14^{a}	4.22^{a}	2.49^{a}	3.07^{a}	3.50^{b}	2.37^{a}
G. fasciculatum	2.21^{a}	3.66^{b}	2.46^{a}	2.84^{ab}	4.03^{a}	2.51^{a}
P. indica* + *G. fasciculatum	2.33^{a}	4.14^{a}	2.57^{a}	2.77^{b}	4.32^{a}	2.46^{a}
Control	1.33^{b}	3.00^{c}	2.11^{b}	2.26^{c}	3.21^{b}	2.05^{b}
SEm (±)	0.07	0.13	0.08	0.07	0.10	0.08
CD (0.05)	0.211	0.399	0.258	0.228	0.307	0.253

Values are mean of 5 replications ± standard deviation; the values following the same letters in the superscripts are not significant at 5% level of significance

4.4. EFFECT OF BIO AGENTS ON PHYTOHORMONE LEVEL

In order to find out the association/role of phytohormones in the evidently improved vegetative growth especially in the root architecture, the level of auxin was estimated in both endophyte colonized plants and control plants using spectrophotometric method by Gordon and Weber (1951). It was observed that there was a remarkable difference in the level of auxin in endophyte treated plants compared to the uninoculated control (Table 12). Enhanced auxin content was observed in

endophyte treated plants compared to uninoculated control. The auxin content was highest for *P. indica*-treated seedlings and suckers recording 83.80 µg g^{-1} and 94.30 µg g^{-1} respectively. This was then followed by combinatorial-treated and *G. fasciculatum*-treated plants. Moreover the *P. indica* treated seedlings had approximately twice the amount of auxin in their roots compared to all the other treatments.

Table 12. Auxin content in endophyte mediated growth promotion in small cardamom var. *Njallani*

Treatments	Cardamom seedlings (µg g^{-1})	Percentage increase over control (%)	Cardamom sucker (µg g^{-1})	Percentage increase over control (%)
P. indica	83.80±1.49[a]	106.30	94.30±6.54[a]	26.59
G.fasciculatum	43.33±0.99[b]	6.67	83.50±2.13[b]	12.10
P. indica* + *G.fasciculatum	44.80±1.62[b]	10.29	85.19±1.91[b]	14.36
Control	40.62±1.26[c]	-	74.49±8.28[c]	-
SEm (±)	0.68		2.73	
CD (0.05)	2.095		8.425	

Values are mean of 5 replications ± standard deviation; the values following the same letters in the superscripts are not significant at 5% level of significance

4.5. *In vitro* EVALUATION OF *P. indica* AGAINST *F. oxysporum*

I*n vitro* evaluation using dual culture technique was carried out to evaluate the ability of *P. indica* to suppress the pathogen. Percent inhibition of radial growth, nature of mycelial growth, inhibition zone, antagonism index etc. were recorded (Table 13). There was submissive growth of the pathogen in dual culture plates compared to the control plates (Plate 24). Percent inhibition of radial growth of pathogen by 64.4 was observed after 13 days. No inhibition zone was observed in culture plates when dual cultured with the endophyes whereas an antagonism index of 20.53 and an obscure lysis zone was also observed in dual culture plates.

Table 13. *In vitro* evaluation *P. indica* against *F. oxysporum*

Nature of mycelial growth	Submissive growth in dual culture plates
Percent inhibition*	64.4 ± 2.26
Antagonistic index*	20.53 ± 1.10

* Values are Mean of five replications ± SD

4.6. *In vivo* EVALUATION OF EFFECT OF *P. indica* AND *G. fasciculatum* INOCULATED CARDAMOM SEEDLINGS AS WELL AS SUCKERS AGAINST *F. oxysporum*

Pot culture experiments of small cardamom seedlings and suckers were conducted at CRS, Pampadumpara during 2020-21 to evaluate the *in vivo* effect of the endophytes against *F. oxysporum*. Basal application of all the treatments were carried out as described in 3.6.2. The pathogen was multiplied as mentioned in 3.6.1 and challenge inoculated to seedlings and suckers at the rate of 0.5 per cent w/w and 1 per cent w/w respectively. The lesion size and Percent Disease index/Disease severity was calculated after symptom development.

The isolate produced symptom 45 days and 70 days after challenge inoculation in seedlings and suckers respectively. All the treatments significantly reduced the lesion size and disease severity in cardamom seedlings (Table 14) (Plate 25, 26) and suckers (Table 15) (Plate 27, 28) compared to control.

4.6.1. Seedlings

4.6.1.1. Lesion size

Maximum lesion size in cardamom seedlings were observed in untreated plants inoculated with pathogen only (2.79 cm^2) and the least lesion size was observed in the chemical check i.e. carbendazim treated plants (0.30 cm^2). Among all bioagent treated plants, *P. indica*-colonised plants produced the least value for lesion size recording 0.38 cm^2 followed by combinatorial treatment (1.35 cm^2) and *P. fluorescens* (1.36 cm^2)

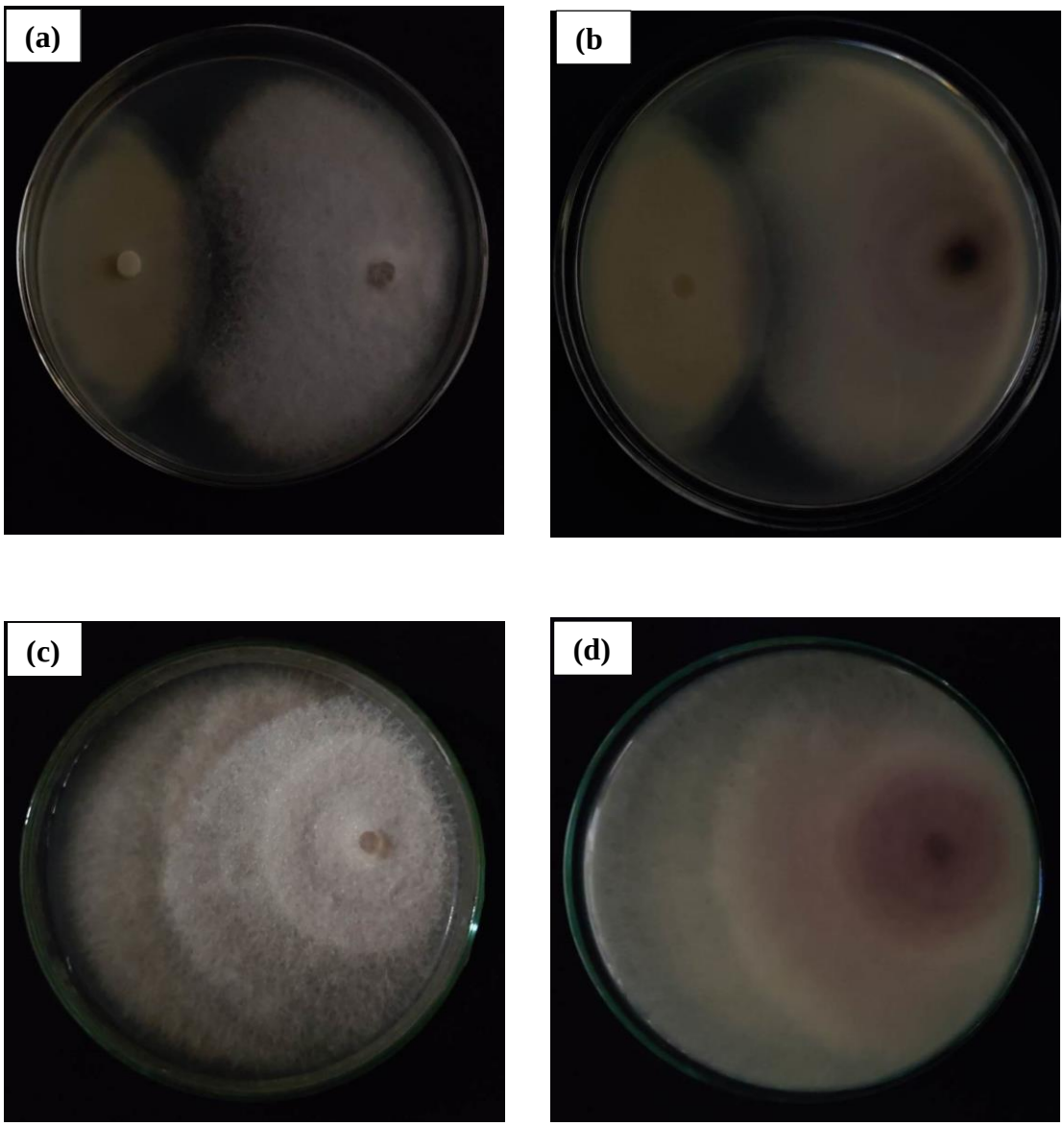


Plate 24. *In vitro* evaluation of antagonism of *P. indica* against *F. oxysporum* (a and b) Dual culture plate after 13 days (c and d) Control plates after 13 days

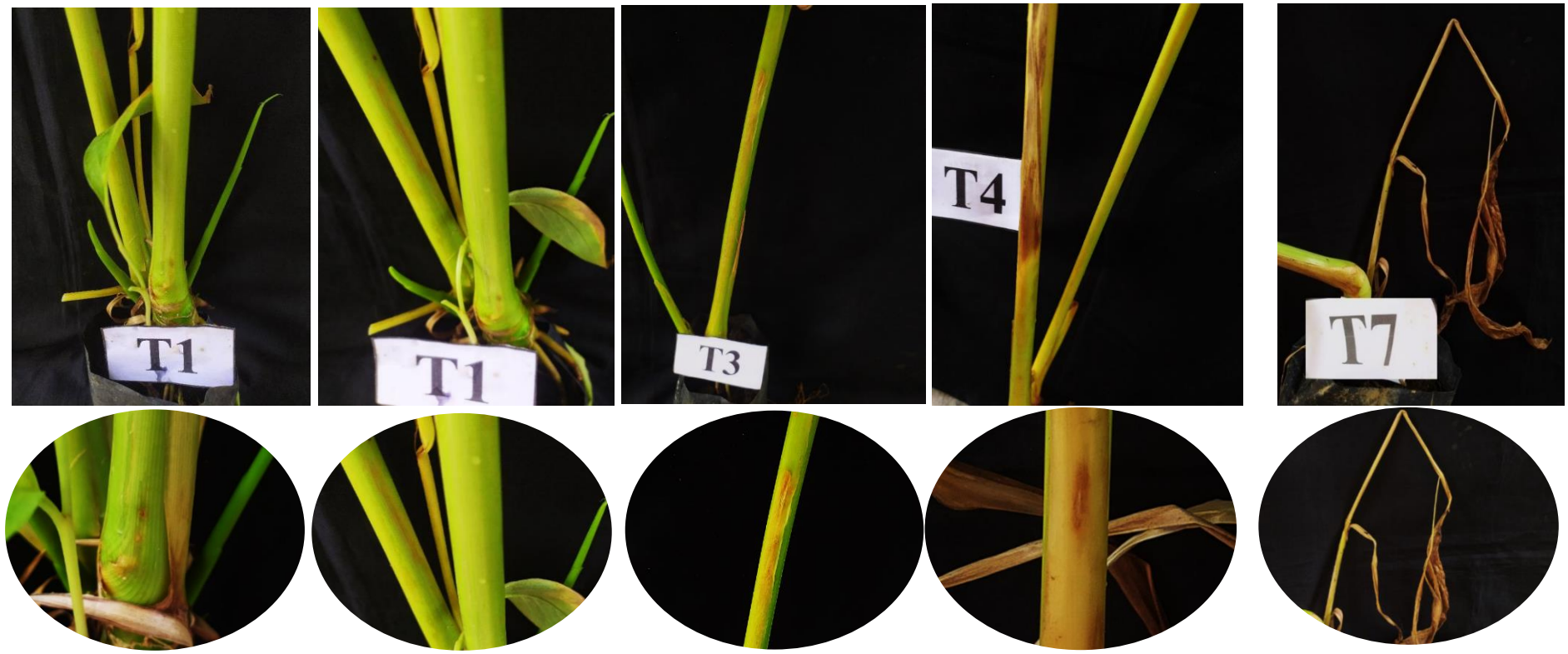


Plate 25. Reduction in symptom development of small cardamom seedlings var. *Njallani* inoculated with *Fusarium* sp. in response to the application of the best treatment (T1) second best treatments (T3 and T4) over control (T7) 45 days after challenge inoculation

P. indica treated

Absolute control

Untreated control

Plate 26. Comparison of the effect of best treatment of challenge inoculated small cardamom seedlings var. *Njallani* to the untreated and absolute control

drench which were on par. This was then followed by *G. fasciculatum* colonised plants which recorded a lesion size of 1.78 cm^2.

4.6.1.2. Percent disease index / Disease severity

The least disease severity was recorded for carbendazim treated plants (26.67 %) whereas the highest value was recorded for the untreated control (86.62 %). Out of all other bioagent treatments, the minimum PDI was recorded for *P. indica*-colonised plants (38.07 %) which was followed by combinatorial treatment (45.28 %). This was then followed by the bioagent check i.e. *P. fluorescens* drench with a PDI 49.10 (%).

Table 14. Effect of endophyte inoculation on lesion size and disease severity of Fusarium rot in small cardamom seedlings

Treatments	Lesion size (cm^2)	Disease severity / PDI (%)	Percentage reduction of PDI over control (%)
P. indica	0.38^{bc}	38.07^{d} (38.07)	56.05
G. fasciculatum	1.78^{ab}	53.08^{b} (46.76)	38.76
P. indica* + *G. fasciculatum	1.35^{abc}	45.28^{cd} (42.28)	47.73
P. fluorescens	1.36^{abc}	49.10^{bc} (42.28)	43.32
Carbendazim	0.30^{bc}	26.67^{e} (31.07)	69.21
Absolute Control	0.00^{c}	0.00^{f} (0.00)	-
Untreated control	2.79^{a}	86.62^{a} (68.95)	
SEm (±)	0.53	2.45	
CD (0.05)	1.61	7.433	

Values are mean of 3 replications ± SD; Values in the parenthesis are arc sin transformed; the values following the same letters in the superscript are not significant at 5% level of significance

4.6.2. Suckers

4.6.2.1. Lesion size

As recorded in the case of seedlings, the maximum lesion size was observed in untreated suckers having only pathogen inoculation (2.717 cm^2) and the minimum lesion size was observed in the chemical check (0.13 cm^2). On comparing the lesion size among the bioagent treated plants, *P. indica*-colonised plants recorded the least value with a lesion of size 0.43 cm^2 followed by combinatorial (1.26 cm^2) and *P. fluorescens* (1.51 cm^2) which again were on par with each other as observed in the case of seedlings. This was followed by *G. fasciculatum* which recorded a lesion size of 1.74 $cm^{2.}$

4.6.2.2. Percent disease index / Disease severity

Similar trend as that of seedlings was observed in the case of disease severity of suckers. Highest PDI was recorded in pathogen treated control suckers (76.77 %) and the least was observed in carbendazim treated suckers (26.87 %). In the case of suckers treated with bioagents, *P. indica*-colonised suckers had the least PDI recording 31.48. This was followed by *P. fluorescens* having a PDI of 37.48 (%) and combinatorial having a PDI of 37.50 (%). *G. fasciculatum* colonised plants recorded a PDI of 49.37 (%).

Table 15. Effect of endophyte colonisation on lesion size and disease severity of Fusarium rot in small cardamom suckers var. *Njallani*

Treatments	Lesion size (cm^2)	Disease severity / PDI (%)	Percentage reduction of PDI over control (%)
P. indica	0.43^{bc}	31.48^{d} (34.11)	58.99
G. fasciculatum	1.74^{ab}	49.37^{b} (34.12)	35.69
P. indica* + *G. fasciculatum	1.26^{abc}	37.50^{c} (37.74)	51.15
P. fluorescens	1.51^{abc}	37.48^{c} (37.71)	51.18
Carbendazim	0.13^{bc}	26.87^{e} (31.20)	65.00
Absolute Control	0.00^{c}	0.00^{f} (0.00)	-
Untreated control	2.72^{a}	76.77^{a} (61.17)	
SEm (±)	0.57	1.47	
CD (0.05)	1.738	4.471	

Values are mean of 3 replications ± SD; Values in the parenthesis are arc sin transformed; the values following the same letters in the superscript are not significant at 5% level of significance

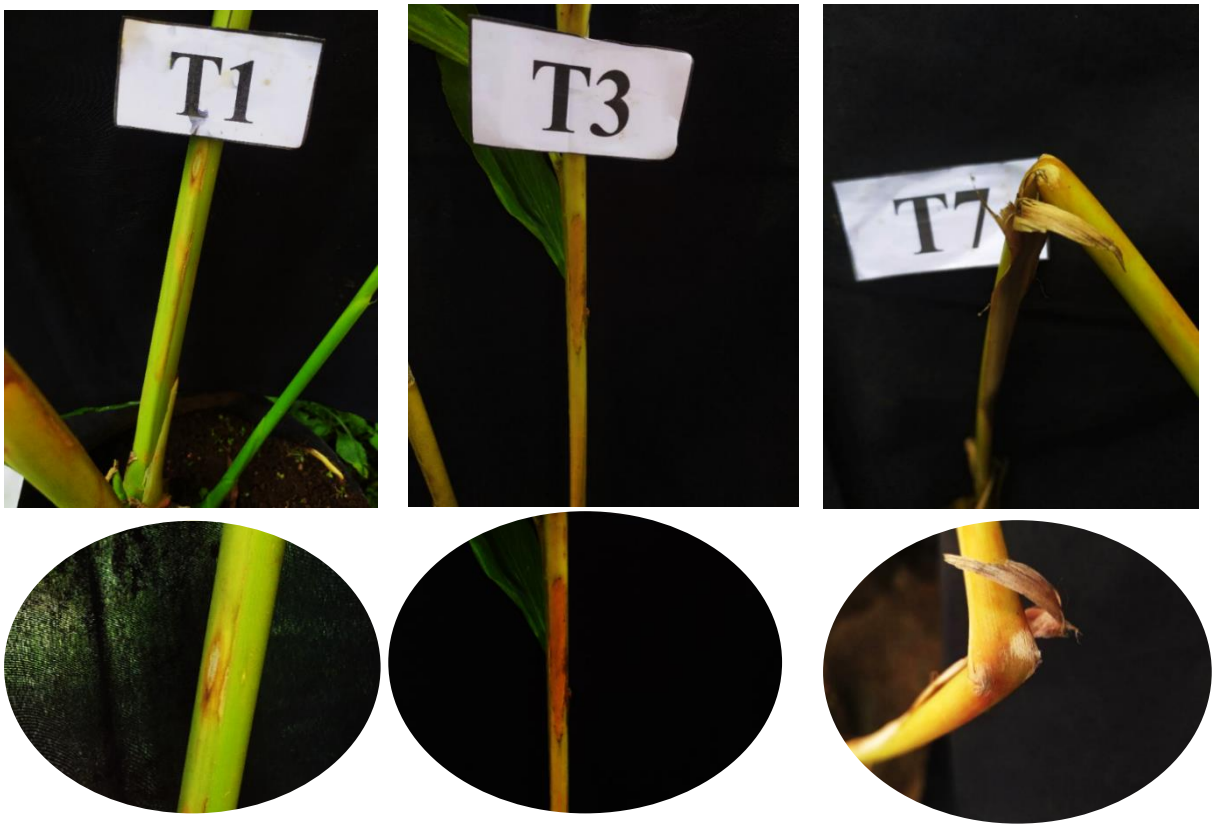


Plate 27. Reduction in symptom development of small cardamom suckers inoculated with *Fusarium* sp. in response to the application of best treatments (T1 and T3) over control (T7) 70 days after challenge inoculation

Plate 28. Reduction in symptom development of small cardamom suckers var. *Njallani* inoculated with *Fusarium* sp. in response to the application of other bio agent treatments (T4 and T2) over control (T7) 70 days after challenge inoculation

4.7. ELUCIDATING THE ROLE OF GIBBERELLIC ACID IN THE ENDOPHYTE MEDIATED TOLERANCE

Gibberellic acid estimation was carried out in the leaves of experimental plants (seedlings and suckers) of 3.6. after symptom development using the protocol of Holbrook *et. al.* (1961). The GA content was superior in all bioagent treated plants compared to the control plants (Table 16). Maximum GA content was observed in *P. indica* colonised and dually colonised plants (which were on par) in both seedlings and suckers recording 18.60 µg g^{-1} and 10.65 µg g^{-1} in *P. indica* colonised seedlings and *P. indica* colonised suckers and 18.00 µg g^{-1} and 9.71 µg g^{-1} in dually colonised seedlings and dually colonised suckers respectively. This was followed by *G. fasciculatum* treated plants which recorded 17.36 µg g^{-1} and 8.05 µg g^{-1} respectively. The least values were recorded by the absolute and untreated controls which were on par with one another. In seedlings, the absolute and untreated control recorded 2.51 µg g^{-1} and 5.36 µg g^{-1} respectively. Likewise, in suckers, the absolute and untreated control recorded 1.60 µg g^{-1} and 2.40 µg g^{-1} respectively.

Table 16. Gibberellic acid content in endophyte mediated disease tolerance in cardamom plants var. *Njallani* against Fusarium rot

Treatments	Cardamom seedlings		Cardamom suckers	
	GA (µg g^{-1})	Percentage increase over control	GA (µg g^{-1})	Percentage increase over control
P. indica	18.60 ±0.40[a]	247.01	10.65±0.95[a]	343.75
G. fasciculatum	17.36 ±7.80[ab]	223.88	8.05±0.86[b]	235.42
P. indica* + *G. fasciculatum	18.00 ±0.40[a]	235.82	9.71±0.87[a]	304.58
P. fluorescens	12.55 ±2.12[b]	134.14	5.93±0.68[c]	147.08
Carbendazim	6.21 ±1.10[c]	15.85	4.27±0.49[d]	77.92
Absolute Control	2.51 ±0.50[c]	-	1.60±0.41[e]	-
Untreated control	5.36 ±0.73[c]		2.40±0.291[e]	
SEm (±)	1.80		0.40	
CD (0.05)	5.444		1.211	

Values are mean of 3 replications ± SD; Values in the parenthesis are arc sin transformed; the values following the same letters in the superscript are not significant at 5% level of significance

Discussion

5. DISCUSSION

Indian cardamom has a long and illustrious history dating back to the dawn of civilization. This spice is thought to have originated in Southern India and Sri Lanka; and has been labelled as one of the highly priced spice crop. The crop is susceptible to different insect pests and diseases, which impairs the quality of the fruit. For instance, Fusarium rot in cardamom has been proven devastating at all phases of the crop's growth fatally affecting the yield of the crop, necessitating the administration of pesticides on a regular basis, even at 15-20 day intervals, to the capsule bearing spikes. Unfortunately, the presence of significant levels of pesticide residues has reduced cardamom exports from India by 80 per cent. Intensive cardamom production strategies currently focus on increasing yields through proper nutrient delivery; implementing more ecologically sound management techniques and judicious use of available pesticides to combat biotic stresses. Hence, the use of endophytes, which live asymptomatically inside the tissue of the host plant for the rest of host's life once colonised, was investigated.

The present study was intended in developing an ecofriendly and sustainable management approach against Fusarium rot of small cardamom using endophytes with minimal risk to environment and low pesticide residue. The work is framed around the evaluation of the colonisation and interaction of two beneficial fungi, *Piriformospora indica* and *Glomus fasciculatum* in cardamom’s biometric characters, and their potential to manage Fusarium rot disease of cardamom. Their role in plant nutrient uptake (P, K) and hormone production was also assessed in order to deduce the part of nutrients and auxin in plant growth promotion. Discussion of the results obtained is presented in this chapter.

In this study two endophytes were utilised. It is not best advised to employ biological agents for mixed inoculation on agricultural plants when there is antagonism between them. However, inoculant incompatibility could be mitigated to some extent by applying the inoculants to the root zone of the crop plants at different times. For example, despite *Trichoderma harzianum*'s antagonistic response to *P.*

indica in dual culture plates, it was demonstrated that they could be used as dual inoculants in black pepper if the root endophyte was applied at an early hardening stage of tissue cultured black pepper, followed by the application of the mycoparasitic fungus during field transplantation (Anith *et al.,* 2011). Co- inoculation of *P. indica* with *Bacillus pumilus* was reported to be more effective than single biological agent inoculation for increasing seedling growth in tomato (Anith *et al.,* 2015). Previous research on other crops (Sarma *et al.,* 2011; Kumar *et al.,* 2012) also back ups the idea of co-inoculation of *P. indica* with beneficial organisms to boost plant growth.

5.1 COLLECTION OF FUSARIUM INFECTED PLANTS FROM HOTSPOT AREAS, ISOLATION OF PATHOGEN, PROVING KOCH'S POSTULATES AND MAINTANENCE OF CULTURE

In the first part, symptomatology of the disease was studied. Fusarium rot was more prominent during the summer months (Thomas and Vijayan, 2002) and the characteristic symptoms included variable sized eye shaped lesions on pseudostem. The affected portion became weak at the site of infection and broke at advanced stages. Since the panicle initiation occurs during summer months, the Fusarium infection also occurs on panicle by producing a burnt appearance eventually affecting the yield. Similar symptoms of root rot, rhizome rot, panicle wilt and pseudostem rot were reported by Vijayan *et al.* (2013, 2014) based on a survey conducted in Fusarium infected cardamom plantations of Idukki district.

Booth's tissue isolation method (1971) was used to isolate the pathogen (Fusarium- root isolate) associated with Fusarium rot disease from diseased samples (root tip) taken from farmer's plots. Koch's postulates were proved using pin prick method on the pseudostem of one year old sucker under greenhouse conditions. The cross infectivity studies has proved that different isolates of the fungus obtained from infected tiller, panicle and root could cross-infect the other plant parts of cardamom (Vijayan *et al.,* 2013). The isolate produced typical eye shaped lesion on the pseudostem two weeks after inoculation. Previous report of *Fusarium* sp. developing root rotting and yellowing on inoculated cardamom seedlings, within 15-30 days after inoculation are available. The spore suspension of the root isolate (10^6 cfu ml^{-1})

produced root rotting symptoms when drenched to the base of the plant (Thomas and Vijayan, 2002). The root isolate produced a lesion of size 1.50 cm x 0.25 cm on the pseudostem. Kumar *et al.*, (2011) evaluated the lesion generated on the detached leaves of barley to determine resistance to Fusarium head blight. They discovered that sensitive cultivars caused larger lesions compared to the resistant ones.

The isolate on PDA were white in the front view and had pink tinge in the rear view. Colonies were pink in appearance and produced fluffy mycelium with an irregular margin. The isolate covered the nine cm Petri dish in nine days. The above results were in confirmation with the findings of Thomas and Vijayan (2002). Five different morphological types of the fungus were reported by Vijayan *et al.* (2013).

The macroconidia and microconidia were of size 14.8 µm and 10.7 µm respectively. The macroconidia and microconidia of *F. oxysporum* Schlecht in small cardamom from Idukki were first described by Thomas and Vijayan (2002). The macroconidia produced by the fungal pathogen was reported to be three-five septated with an average size of 23.6 µm ×3.48 µm, and the microconidia had a size of 9.03 µm ×2.58 µm. Similarly, the macroconidia produced by the fungus *F. oxysporum* f. sp. *cubense* which caused fusarium wilt in banana were found to have a size ranging from 27 to 55 µm × 3.3 to 5.5 µm, four- to eight-celled, and sickle-shaped, where as its micro conidia was of 5 to 16 × 2.4 to 3.5 µm size, one- or two-celled with oval- to kidney-shape. In an *in vitro* experiment Gopi *et al.* (2016) observed that *F. oxysporum* infecting large cardamom had macro and micro conidia with sizes of 26.91-57.64 to 2.01- 2.59 µm and 5.62-8.44 to 1.86- 2.71 µm, respectively.

5.2. EVALUATION OF THE COLONISATION AND INTERACTION OF *P. indica* AND *G. fasciculatum*

P. indica was maintained in PDA and PDB at 28±2°C (Cheng *et al.*, 2020). The full mycelial mat of the fungus was obtained 14-21 days after inoculating the broth. The substrate for the mass multiplication of the fungus was a sterilised combination containing coirpith and cow dung supplemented with gram flour, where it attained the maximum growth in the tray one-two week after broth inoculation. Vermiculite based

inoculum of *G. fasciculatum*, KAU strain was obtained from Department of Agricultural Microbiology, College of Agriculture, Vellayani. The utilization of sand and vermiculite, which favours the development of glomerospores with improved infectivity, was reported to be a potential system for the mass multiplication of *G. fasciculatum* (Silva *et al.*, 2007).

Co-cultivation of cardamom seedlings and suckers with beneficial endophytes revealed its colonisation in roots; and formation of chlamydospores in the case of *P. indica*, vesicles and arbuscules in the case of *G. fasciculatum*. Chippy (2020) observed chlamydospores on the bhendi root surface 15 days after co-cultivation and chlamydospores inside the roots twenty days later. Similarly, Chandran (2020) also detected fungal chlamydospores on the roots seven days after co-cultivation whereas chlamydospore production inside the root began 10 days after co-cultivation. Anith *et al.* (2015) observed the percentage of root colonisation by the endophytic fungus to be similar in tomato seedlings after application of single and co-cultured inocula of *P. indica* and *B. pumilus* after evaluating chlamydospores in the root cortex cells of tomato. In the cortical cells, however, there was a variation in the pattern of chlamydospore development. The root cortical cells occupied by chlamydospores were entirely filled with many numbers of large sized spores of the fungus after a single inoculation with *P. indica*. Only a few adjacent cells were completely devoid of spores. When dual inoculation was employed, nearly all the cells in the cortical region of the colonised roots were occupied by chlamydospores, but the distribution pattern was different. The majority of the cells were occupied by single or tiny spores (Anith *et al.*, 2015). Likewise, the synergistic effect of different bioagents on AMF colonisation has been already reported. According to Sarma *et al.* (2011), increased AMF colonisation was observed in vascular plants when co- inoculated with *Trichoderma* sp.. Boer *et al.* (2005) also revealed that co-inoculating AMF with *P. fluorescens* increased the mycorrhizal colonisation in roots by 50-60%. Kumar *et al.* (2012) corroborated the same conclusion in sorghum.

In the *in vivo* interactive study between the endophytes, plant biometric assessment of cardamom plants were carried out and a pronounced growth promotion in endophyte colonized cardamom seedlings (Fig. 3-6) and suckers (Fig. 7-10) were

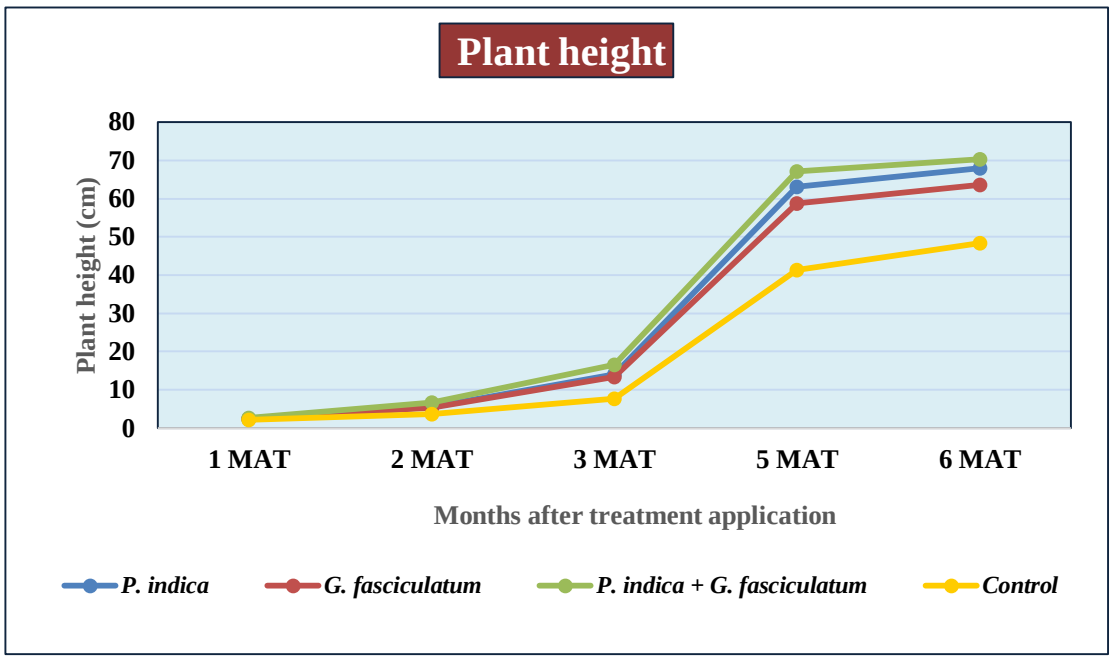


Fig. 3. Impact of endophyte colonisation on plant height of cardamom seedlings var. *Njallani*

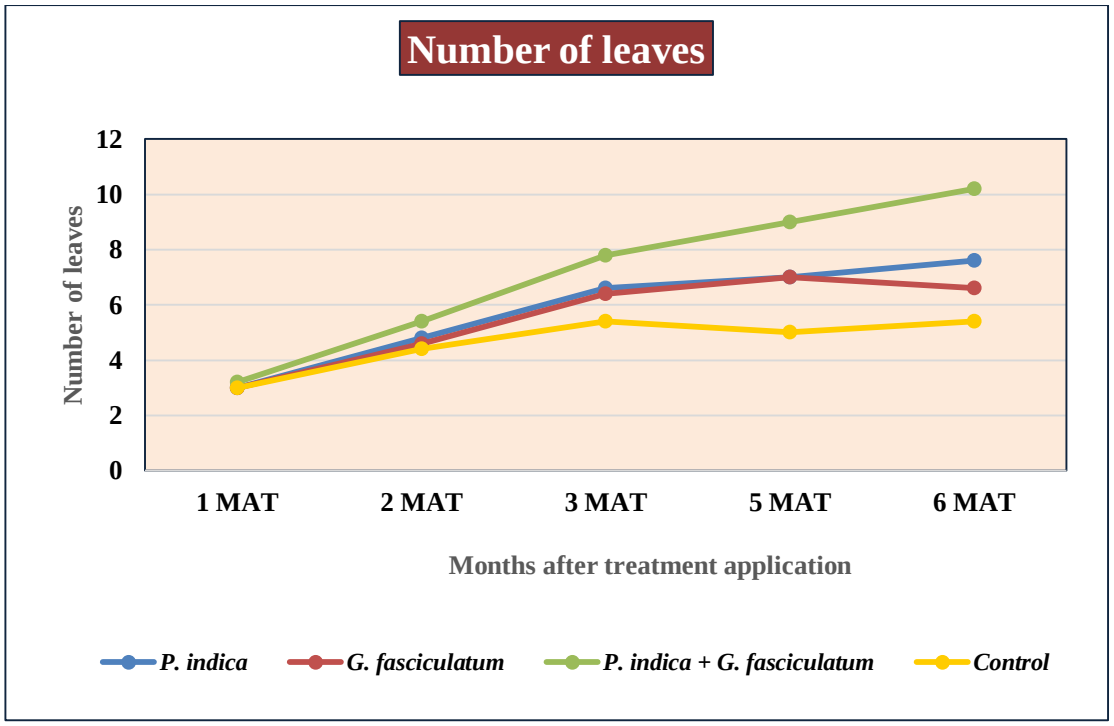


Fig. 4. Impact of endophyte colonisation on number of leaves of cardamom seedlings var. *Njallani*

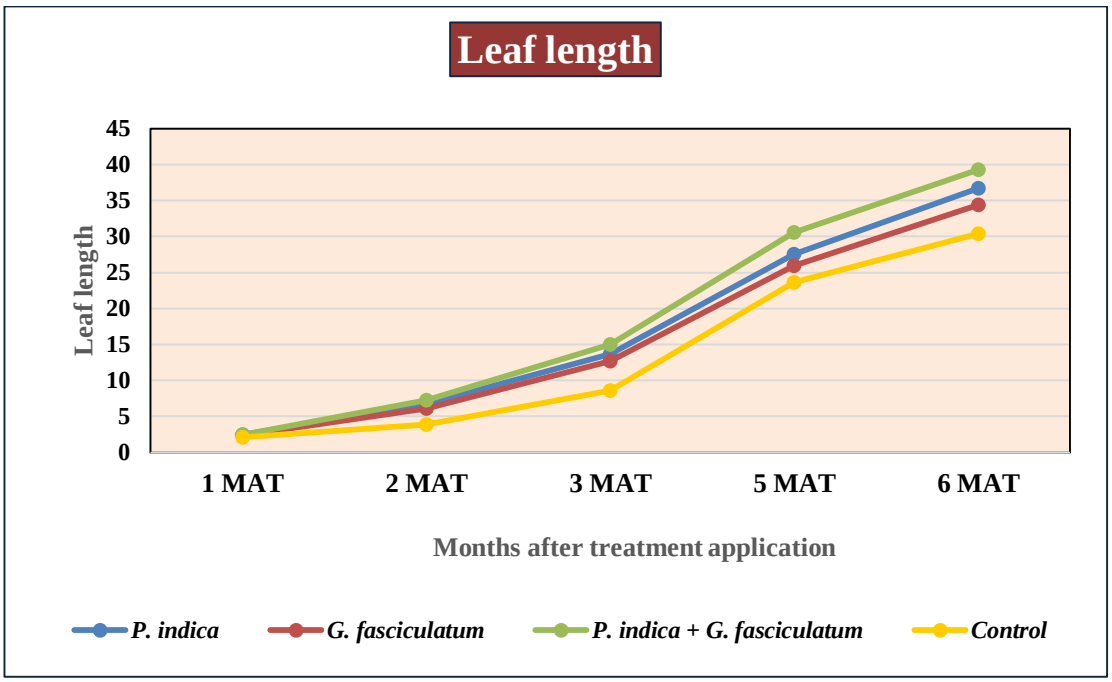


Fig. 5. Impact of endophyte colonisation on leaf length of cardamom seedlings var. *Njallani*

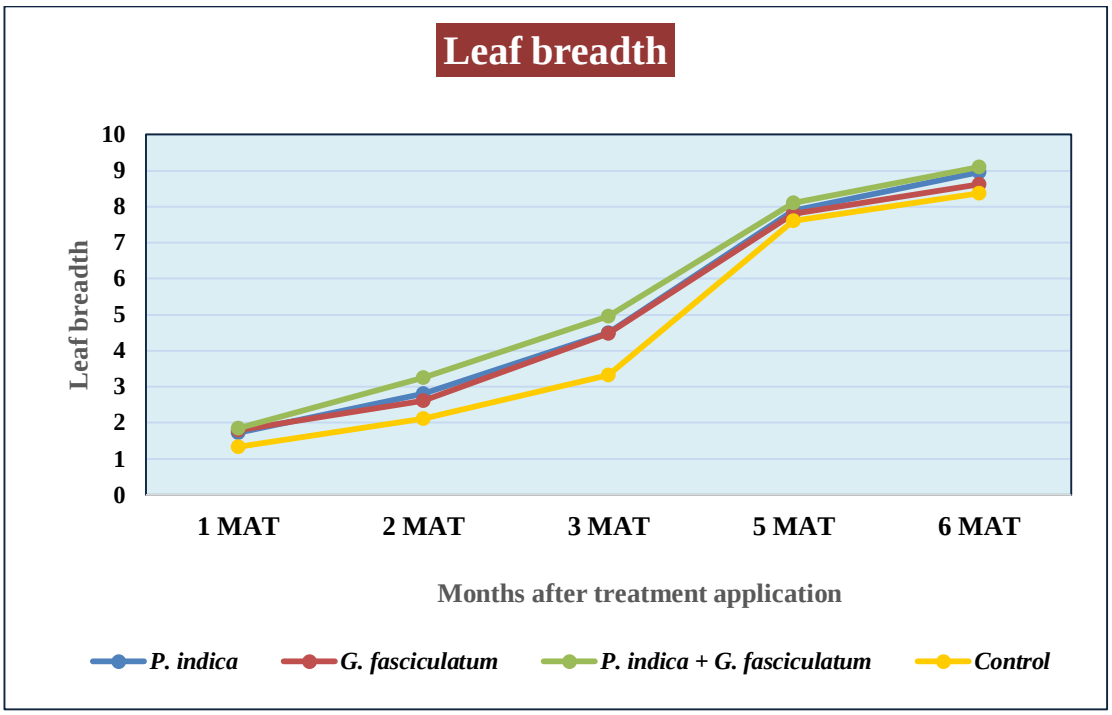


Fig. 6. Impact of endophyte colonisation on leaf breadth of cardamom seedlings var. *Njallani*

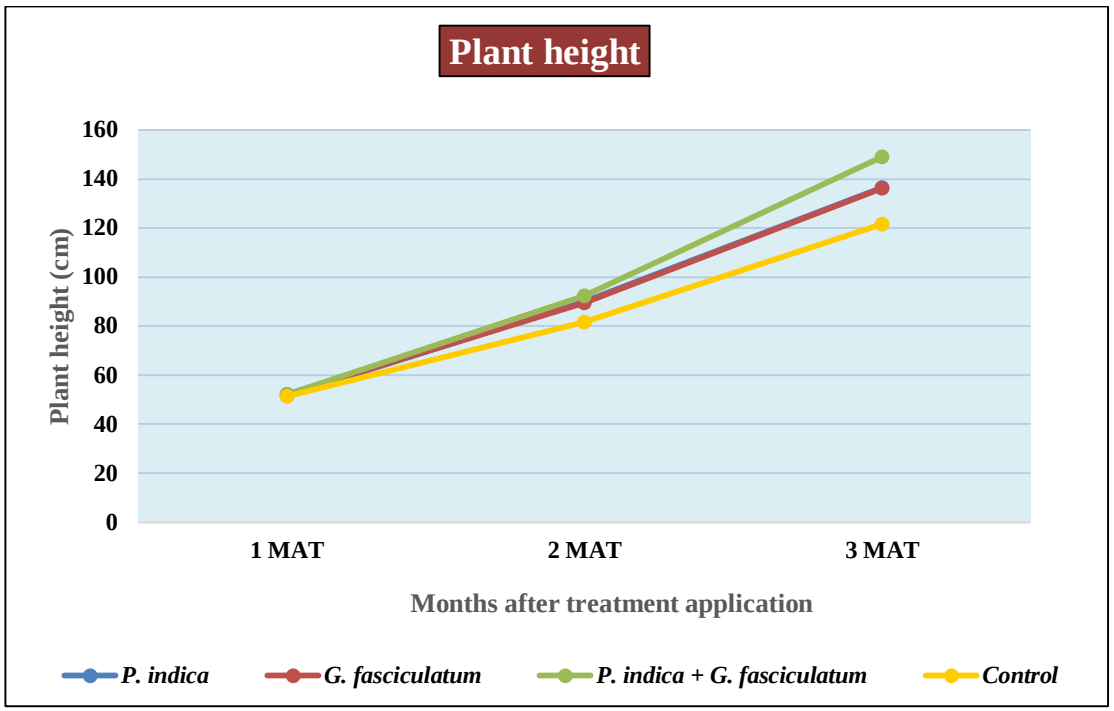


Fig. 7. Impact of endophyte colonisation on plant height of cardamom suckers var. *Njallani*

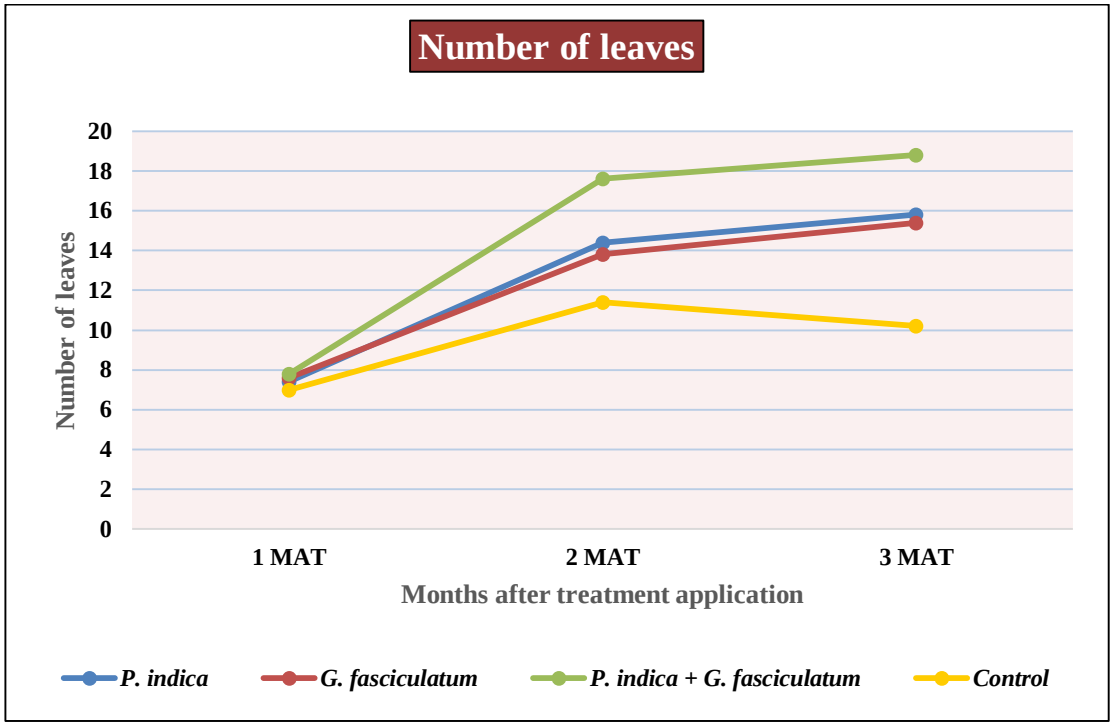


Fig. 8. Impact of endophyte colonisation on number of leaves of cardamom suckers var. *Njallani*

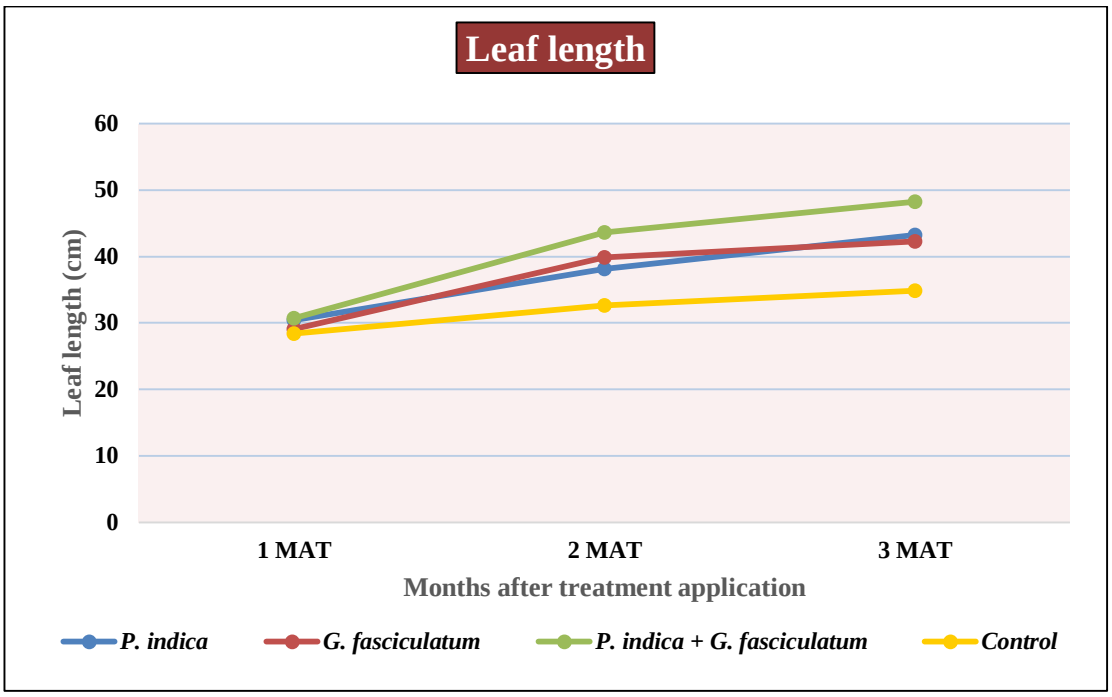


Fig. 9. Impact of endophyte colonisation on leaf length of cardamom suckers var. *Njallani*

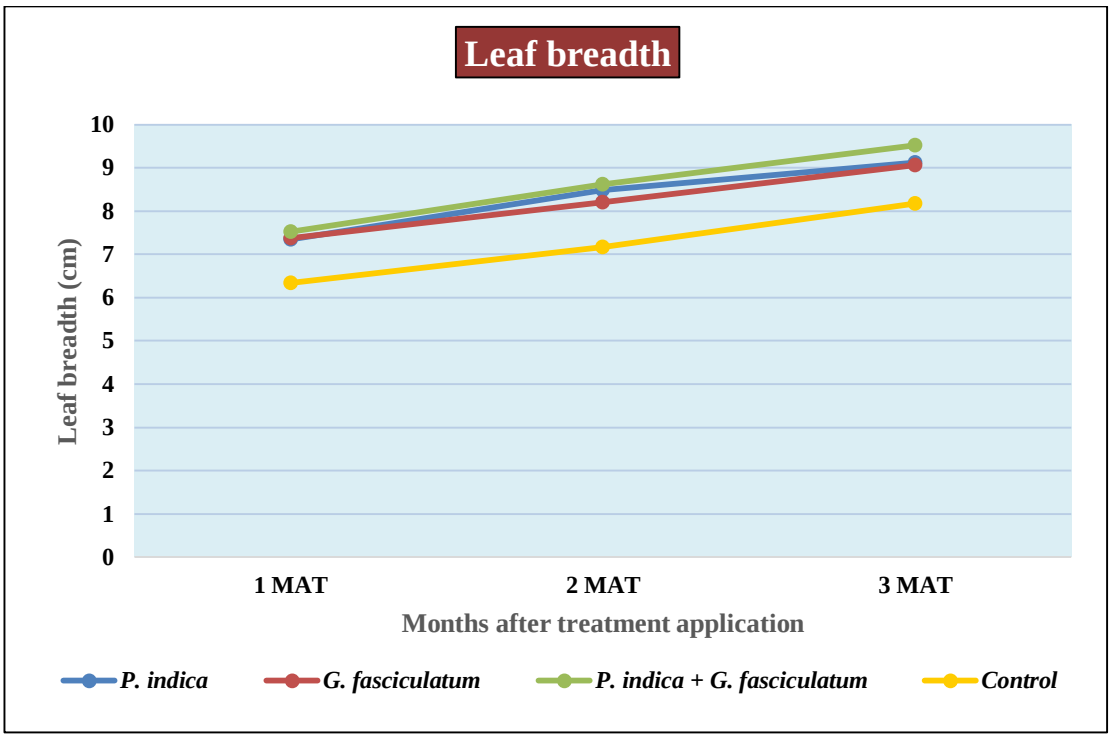


Fig. 10. Impact of endophyte colonisation on leaf breadth of cardamom suckers var. *Njallani*

observed. Regarding the seedlings, there was a per cent increase in plant height by 45.27, 40.35 and 31.54 in dually colonized, *P. indica* colonized and *G. fasciculatum* colonized plants respectively. The per cent increase in number of leaves were 88.89, 40.74 and 22.22 and that of leaf length was 29.16, 20.87 and 13.17 respectively for dually colonized, *P. indica*-colonized and *G. fasciculatum*-colonized plants. Same trend was observed in the case of suckers where there wasn't any significant difference in any of the parameter one month after treatment application. This may be due to the colonization that may be progressing in the suckers during first month as it was observed that endophyte colonization was seen in roots of cardamom suckers only 21-28 days after treatment application. There wasn't any statistically significant difference between the endophyte treated plants and control plants in the case of number of tillers in seedling whereas the number of tillers in case of suckers was significantly different and superior in endophyte treated suckers compared to the uninoculated control. There was an observable 116.67, 83.33 and 75.00 per cent increase of tillers over control in dually treated, *P. indica* treated and *G. fasciculatum* treated suckers respectively.

The plants dually inoculated with *P. indica* and *G. fasciculatum* were superior to all other treatments in all intervals of study. In seedlings, the factorial analysis of vegetative parameters at different intervals as additional factor revealed their interactive effect and it was observed that the plant height of dually-colonized seedlings at 5^{th} and 6^{th} months as well as that of *P. indica*-colonized seedlings at 6^{th} month were superior and statistically on par. The highest plant height of dually-colonized seedlings at fifth month itself helps them to transplant in the field one month before. Number of leaves was also high in the dually-colonized seedlings at 5^{th} and 6^{th} month growth stages and was on par. This is in contradictory to the results reported by Das *et al.* (2014), where the rice plants inoculated with *P. indica* was superior in plant height, number of leaves and number of tillers compared to the plants that are either dually inoculated with *P. indica* and *G. fasciculatum* or *G. fasciculatum* alone. The deviation observed in the present study maybe due to variation in the crop, region, season and weather and also on strains of the AMF. Cardamom is a robust and high input responsive crop and is mainly cultivated in organic matter rich Western Ghats at altitudes between 700 m to 1500 m having well distributed rainfall about 1500 mm - 3000 mm and temperature

ranging from 15 to 30° C. The AMF, *G. fasciculatum* used in the study was a native isolate to Kerala with a high potential in crop production and protection.

A significant positive effect on root growth of the endophyte treated plants was observed in the present study. In the case of seedlings (Fig. 11,12), there was a per cent increase of root length by 50.59, 49.52 and 39.14, number of secondary roots by 58.97, 48.71 and 35.89, root volume by 272.97,213.51 and 108.10 in *P. indica*-colonized, dually-colonized and *G. fasciculatum*-colonized seedlings respectively. The root length, number of secondary roots and root volume were superior in *P. indica* treated seedlings which was followed by dually colonized seedlings which was in turn followed by *G. fasciculatum* treated seedlings. Nonetheless, the root length and number of secondary roots of the dually colonized seedlings were in par with the superior treatment. On contrary to this, the root growth pattern in suckers (Fig. 12) was such that the dually colonized plants recorded the maximum root volume which was followed by *P. indica*-colonized plants and then *G. fasciculatum*-colonized plants. This may be due to the presence of more number of tillers in the dually-colonized suckers compared to other treatments. The other two parameters followed the same trend as that of seedlings. Similar results of plant growth promotion by *P. indica*- and *G. fasciculatum*- colonized plants have been reported earlier. Lin *et al.* (2019) examined the size of roots and shoots, and fresh weights of *P. indica*-colonized anthurium plants and the study revealed that *P. indica* promoted growth of seedlings, and enhanced number of branches, height and length of roots in the colonized plants. Better root architecture and enhanced metabolites such as GABA, oxylipin- family compounds and poly unsaturated fatty acids and auxins resulted in the growth and biomass production of *P. indica*-colonized plants in maize (Kumar *et al.*, 2009).

The present study revealed that the endophyte colonization significantly improved both aboveground and belowground biomass. With reference to seedlings, the shoot biomass was highest in the dually-colonized seedlings having 76.29 and 54.01 per cent increase over control for shoot fresh weight and shoot dry weight. The root biomass was highest in *P. indica* treated seedlings recording 120.97 and 68.94 per cent increase over control for root fresh weight and root dry weight respectively. In the case of suckers, both shoot and root biomass were higher in the dually colonized seedlings

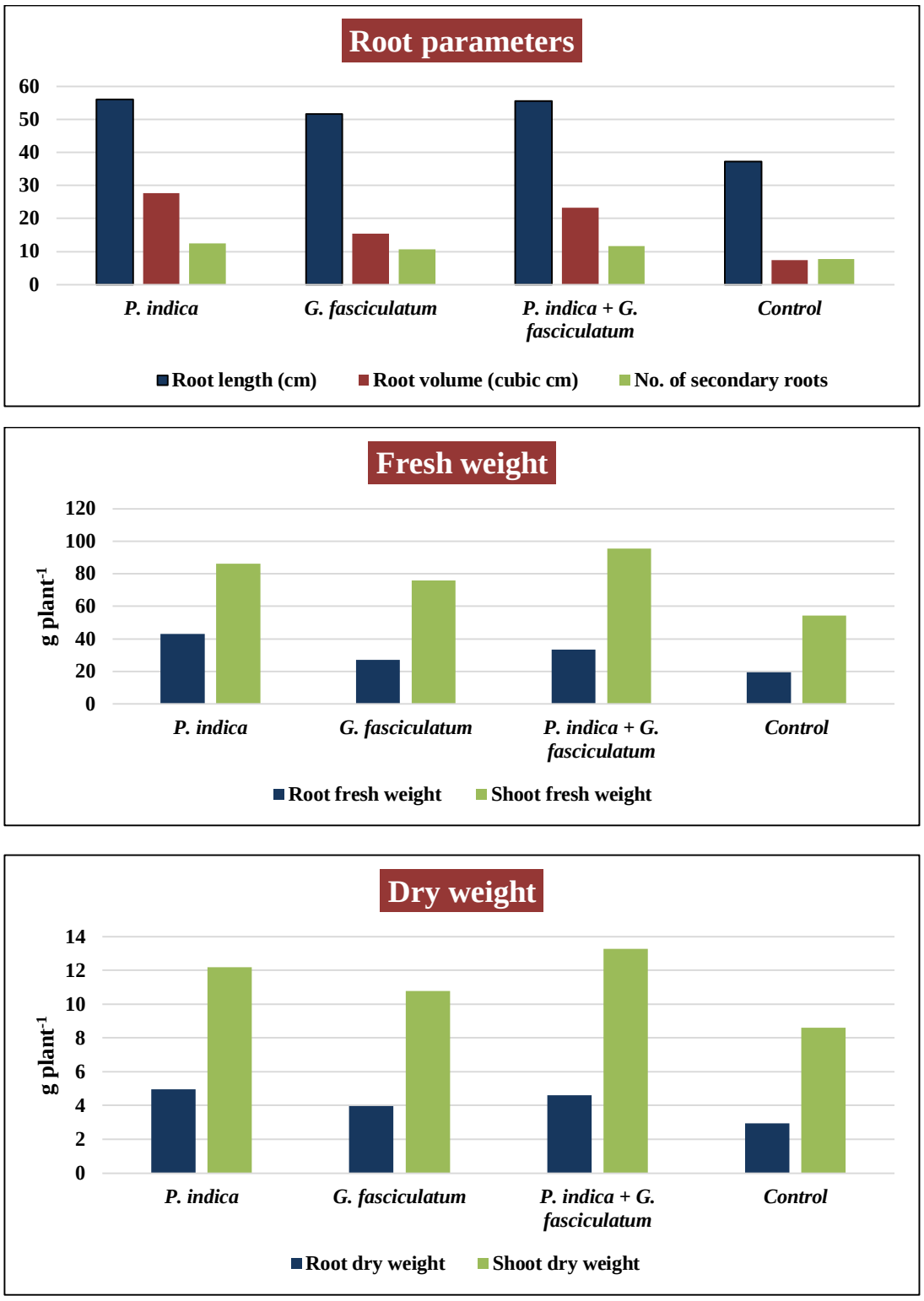


Fig. 11. Impact of endophyte colonisation on plant biomass and root characteristics of cardamom seedlings var. *Njallani* (6 MAT)

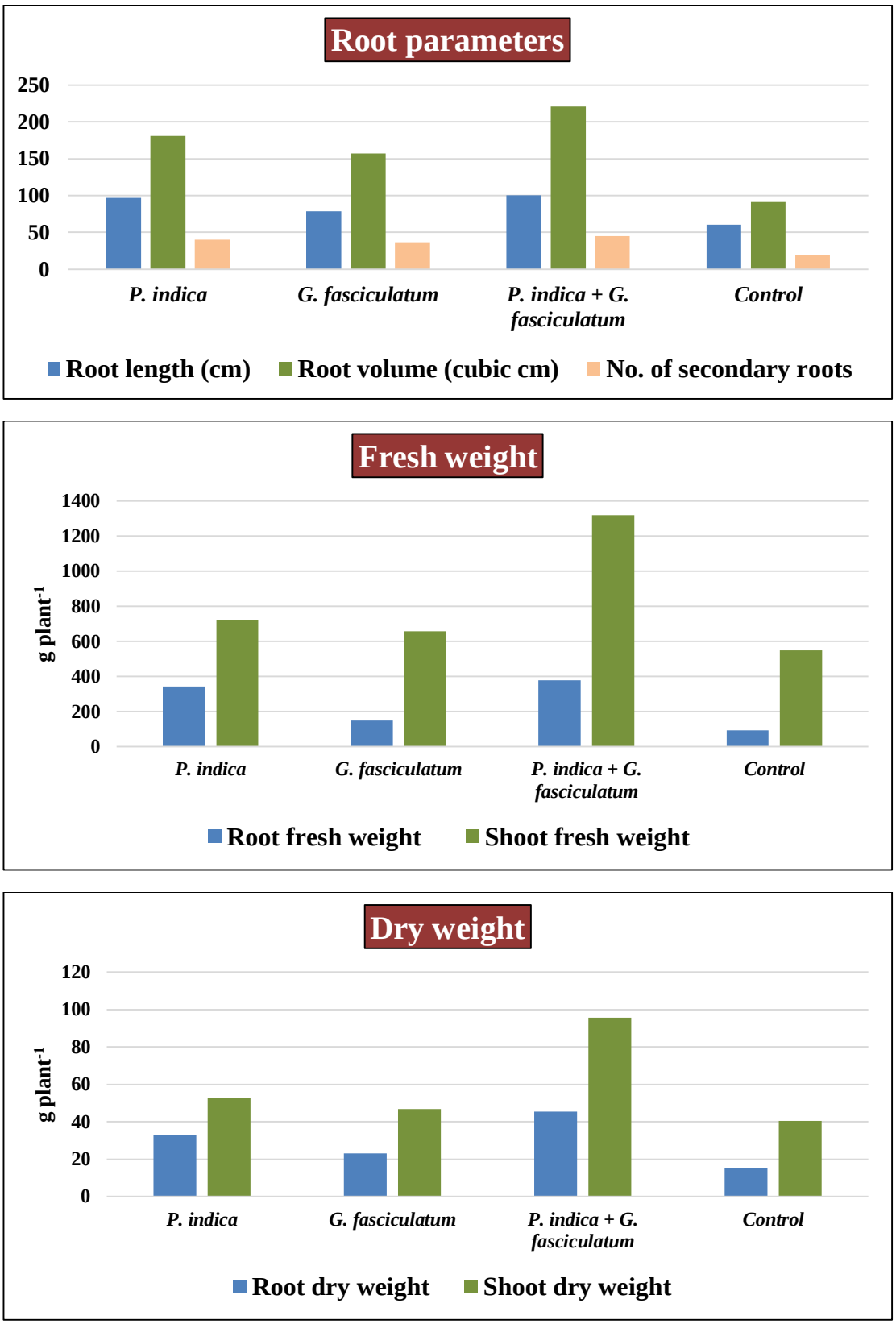


Fig. 12. Impact of endophyte colonisation on plant biomass (3 MAT) and root parameters (4 MAT) of cardamom suckers var. *Njallani*

which was followed by *P. indica*-colonized which in turn was followed by *G. fasciculatum*-colonized suckers. There was a per cent increase of 304.13, 200.66, 140.60 and 135.45 of root fresh weight, root dry weight, shoot fresh weight and shoot dry weight in the dually-colonized suckers over control. The disparity in the trend of root biomass among seedlings and suckers may be due to the statistically superior number of tiller in the case of dually colonized suckers compared to other treatments. These findings are in accordance with Yaghoubian *et al.* (2014), where they investigated the effect of *G. mossae* and *P. indica* on growth of wheat plants under drought condition and observed that the endophytes-colonized plants especially co-inoculated treatments showed a markedly improved shoot dry weight under drought condition. Das *et al.* (2014) also observed a significant increase in root and shoot biomass in *P. indica* or *G. fasciculatum* inoculated rice plants individually or in combination compared to the uninoculated control. They also revealed that the shoot biomass of *P. indica*-colonized and the dually inoculated plants did not vary significantly from each other but both of which was higher than that of *G. fasciculatum* treated plants. Ankegowda (2008) optimized the leaf stage for transplanting cardamom seedling from primary nursery to polybag nursery as four to five leaf stage. In the present study, we used three leaved plants for polybag planting and they showed a 21.2 per cent higher root length and 70 per cent more fresh weight (both shoot and root) at 180 days compared to the cardamom seedlings of the same age reported in the above study.

Moreover, the endophytes-colonized seedlings also showed better establishment in the polybag nursery compared to the control. According to Ankegowda (2008), the specification of standard healthy seedlings is four to five leaved seedlings in primary nursery, and at transplantable stage (after 180 days) to the field from polybag, a plant height of 65 cm or above and nine leaves are required for better establishment. Usually the cardamom seedlings take around 30-45 days to become four leaved stage from three leaved stage. In the present study, three leaf staged seedlings when colonized with the endophytes either individually or in combination attained the four leaves stage much before with better shoot and root characters and parameters. Additionally, when three leaved seedlings were dually inoculated with

P. indica and *G. fasciculatum*, the seedlings attained 65 cm height within five months and were ready to transplant to the fields against six months or more in the non-colonized plants. The results clearly indicate that the beneficial interaction with both the endophytes reduced the prolonged nursery period, a major constraint faced by the farmers while using cardamom seedlings for planting, by two months (30 days each in both primary nursery and polybag nursery).

5.3. NUTRIENT UPTAKE BY THE BIO AGENT COLONIZED PLANTS

The possible reason for the above growth promotion could be a significantly higher P and K uptake to root, shoot and leaf in endophyte treated plants compared to the uninoculated control (Table 3). The nutrient analysis of plant samples revealed highest uptake of phosphorus to root by *G. fasciculatum*-colonized seedlings *i.e.* approximately twelve folds higher than that of uninoculated control. Nevertheless, the phosphorus uptake to the shoot was 11 folds higher and leaves were 9 folds higher in dually colonized seedlings and *P. indica* colonized seedlings respectively compared to the control plants. In suckers, the *G. fasciculatum* colonised roots revealed a 7 fold increase in the the P content compared to the control; and shoots and leaves recorded a 1.5 fold increase and 3.1 fold increase in P content in *P. indica* colonised plants in comparison with the control plants. *P. indica* stimulates the availability of P to the host plants by higher phosphatase activity and higher expression of the gene ACP5 as reported in *Brassica napus* (Wu *et al.,* 2018). The fungus releases P from sources in the soil which are not accessible for the plant and is taken up by the roots directly or via a passage through the fungal hyphae. Apart from being a P-mobilizer, the fungus's ability to grow on a variety of P sources such as inorganic, organic, and polyphosphates reflects its role as an active P-solubilizer (Johnson *et al.,* 2014). Moreover, the *P. indica*-phosphate transporter (PiPT) gene has been identified, which aids in the movement of phosphates from the soil to the plant as a result of *P. indica* colonisation in maize (Yadav *et al.*, 2010).

Likewise, mycorrhizal association promotes phosphorus delivery to infected roots of host plants in phosphorus-limited environments (Bucher, 2007). In fact, AM fungus have active Pi transporters that take Pi from the soil and give it to the plant. Plants, on the other hand, have mycorrhizal-specific Pi transporters, which are responsible for obtaining Pi from the interfacial apoplast and delivering it to the plant cytoplasm. (Harrison and van Buuren, 1995). Khatun (2020) also studied the response of *G. fasciculatum* on phosphorus uptake by the plant at different developmental stages in *Coleus forskohlii*. The results indicated that the symbiotic association of fungus amounts to greater uptake of phosphorus and promoted different growth parameters like plant height, root length, number of roots, number of leaves and fresh weight. AMF develop fungal structures called arbuscules, which assist in the exchange of inorganic minerals and carbon and phosphorus molecules, giving host plants vigour (Li *et al.,* 2016). As a result, they can considerably increase phosphorus levels in both root and shoot systems. The increase in phosphorus uptake to root and shoot results in increased root and shoot weight, plant height, leaf sugar content etc. (Afzal and Bano, 2008). Another study conducted by Thomas *et al.* (1994) on cardamom plants inoculated with *G. fasciculatum* revealed that, the association contributed maximum plant height and leaf area index. Study of synergistic effect of *G. fasciculatum* with other microorganisms like *Pseudomonas fluorescens* on plants height has been already reported by Earanna *et al.* (2001).

Bioagent treatments positively influenced the K uptake also. All the endophyte treated plants had better uptake of K to their root, shoot and leaves in comparison to their uninoculated control. A minimum of 1.1 fold (approximately) increase of K content in root, shoot and leaves of all endophyte treated plants was observed compared to the control plants. Similar results were reported by Kumar *et al.* (2012) in *P. indica*-colonized mung bean plants where the authors observed 1.4 fold increase in K uptake from the soil in both glasshouse and field conditions which along with other nutrient uptake (N and P) may have remarkably contributed to the growth promotion observed. The contribution of AM symbiosis to plant K+ nutrition been investigated rarely. This element has been found in spores (Pallon *et al.*, 2007), hyphae (Olsson *et al.*, 2008), and vesicles (Pallon *et al.*, 2007). Additionally, the up-regulation of a plant K+

transporter has been reported in AMF colonized roots of *Lotus japonicus* (Guether *et al.*, 2009). Potassium plays a major role in metabolic processes like photosynthesis, protein synthesis and osmotic balance. It is crucial in the development of yield and the improvement of quality (Marschner, 2011; Oosterhuis *et al.*, 2014). K is also essential for cell proliferation, which is a crucial process in plant function and development (Hepler *et al.*, 2001). In terms of K's growth-promoting mechanism, it is generally agreed that K stimulates and controls ATPase in the plasma membrane to provide acid stimulation, which subsequently causes cell wall loosening and hydrolase activation, boosting cell growth (Oosterhuis *et al.*, 2014). In accordance with this, Baque *et al.* (2006) observed higher number of tillers, leaf dry weight, root dry weight, spikelet per spike and number of kernals per plant in *Phaseolus vulgaris* L. in response to higher potassium uptake.

5.4. EFFECT OF BIO AGENTS ON PHYTOHORMONE LEVEL

IAA analysis of cardamom plants revealed a higher auxin content in all endophyte treated seedlings and suckers compared to the control plants. The IAA content was highest in *P. indica* colonized plants having 106.30 per cent and 26.59 per cent increase in seedlings and suckers respectively over non colonized plants. Dually colonized seedlings and suckers had 10.29 per cent and 14.36 per cent increase over control; and *G. fasciculatum*-colonized seedlings and suckers had 6.67 per cent and 12.10 per cent increase over control. Lee *et al.* (2011) and Hilbert *et al.* (2012) stated that the promotion of growth and development of *P. indica*-colonized Chinese cabbage and barley attribute to increased level of auxin in roots. According to a double-subtractive expressed sequence tag (EST) library from Chinese cabbage roots grown in the presence or absence of the fungus, many genes involved in auxin signalling, metabolism, and functions were upregulated by *P. indica* in the colonised roots, demonstrating the positive role of auxin in *P. indica*-mediated growth promotion. This beneficial fungus is also reported to increase the level of cytokinin in colonized roots of Arabidopsis compared to the uninoculated control (Vadassery *et al.*, 2008). Likewise Yu *et al.* (2014) reported that AMF inoculation considerably increased the endogenous levels of auxin and cytokinin in tomato seedlings. Root transcriptome analysis revealed

that the plant hormone signal transduction pathway was substantially enriched, and gene set enrichment analysis (GSEA) found 109 genes that positively linked with the AMF-inoculated plant phenotype, including 9 genes related to indole acetic acid (IAA). Particularly, the level of endogenous IAA in tomato seedlings increased considerably following AMF inoculation. The above findings suggest a significant role of phytohormones *viz.* auxin and cytokinin in plant growth promotion by *P. indica* and *G. fasciculatum*. This could be a possible reason for the promotional effect observed here.

5.5. *In vitro* EVALUATION OF *P. indica* AGAINST *F. oxysporum*

The use of potentially toxic pesticides has resulted in the rejection of small cardamom during export due to higher residues in the cured products. As a result, cardamom disease management tactics are increasingly relying on consortia of biocontrol agents against plant pathogens.

The *in vitro* evaluation of the axenically culturable endophyte *P. indica* against Fusarium rot causing *F. oxysporum* revealed a per cent inhibition of 64.4 with no inhibition zone and an inconspicuous lysis zone. Production of any antimicrobial substance was also not observed. This is in uniformity with the findings of Dolatabadi *et al.* (2011) and Rabiey *et al.* (2015) where the authors carried out the dual culture assay of *P. indica* against *F. oxysporum* f. sp. *lentis* and *F. culmorum* respectively and observed that *P. indica* loose coiled around the pathogen and limited the mycelial growth. Similarly, Kumar *et al.* (2009) conducted antibiosis assay of *P. indica* against *F. culmorum* and observed the secretion of no antibiotics, which led to the conclusion that the endophyte mediated resistance to the pathogen was not attributed by antibiosis. All of these studies point to the possibility that mechanism of *P. indica* employed in combatting the pathogen here could be competition for space and nutrients, or coiling rather than antibiosis.

5.6. *In vivo* EVALUATION OF EFFECT OF *P. indica* AND *G. fasciculatum* INOCULATED CARDAMOM SEEDLINGS AS WELL AS SUCKERS AGAINST *F. oxysporum*

The pot culture experiment against Fusarium rot in small cardamom was conducted at CRS, Pampadumpara by employing various bioagents and a chemical check during the year 2020-21 revealed that above agents could successfully suppress the Fusarium rot disease by reducing the lesion size or drastically disparaging the disease severity (Fig. 13-16).

Response trend of challenge inoculated plants against various treatments in disease management were similar for both seedlings and suckers. The least disease severity and lesion size were observed in the case of the chemical check i.e. two per cent carbendazim drench recording a per cent reduction in disease severity by 69.21 and 65.00 over control in seedlings and suckers respectively. Among various bioagent treatments, the best result in the management was recorded for *P. indica*-colonized seedlings and suckers which reduced the disease severity by 56.05 per cent and 58.99 per cent respectively. This was followed by combinatorial treatment which reduced the disease severity by 47.73 per cent and 51.15 per cent; and *Pseudomonas* drench which reduced the disease severity by 43.32 per cent and 51.18 per cent respectively in seedlings and suckers respectively. Basal application of two per cent *G. fasciculatum* in seedlings and suckers reduced the disease severity by 38.76 per cent and 35.69 per cent.

Endophytes suppress the pathogen by reducing the level of infection, suppressing the growth or reducing the inoculum production. Biocontrol mediated by endophytes can be direct or indirect. Antibiosis, competition, hyperparasitism and lytic enzyme production are examples of direct mechanisms. Induced resistance and plant growth enhancement are examples of indirect processes (Gao *et al.*, 2010). Induced systemic resistance (ISR) and systemic acquired resistance (SAR) are induced indirect mechanisms by which microbes increase disease resistance. Plant cell wall-degrading enzymes damage cellular integrity in pathogenic symbioses. Damage-associated molecular pattern (DAMP) receptors detect cellular debris, ATP, and carbohydrates and

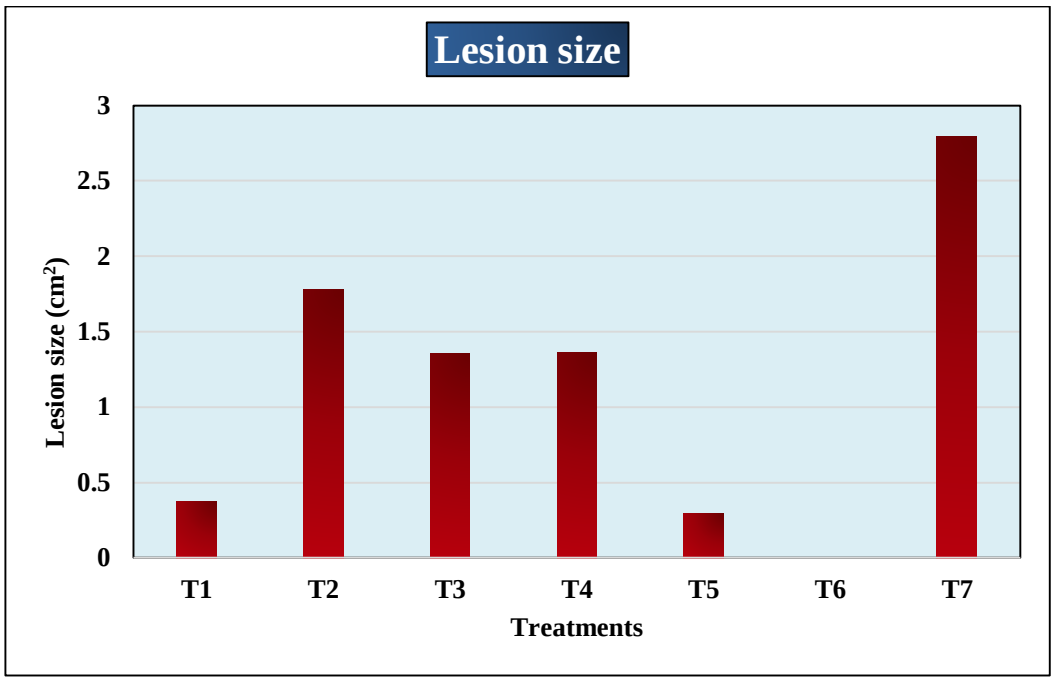


Fig. 13. Effect of bio agents on lesion size of Fusarium rot in small cardamom seedlings var. *Njallani* 45 days after challenge inoculation

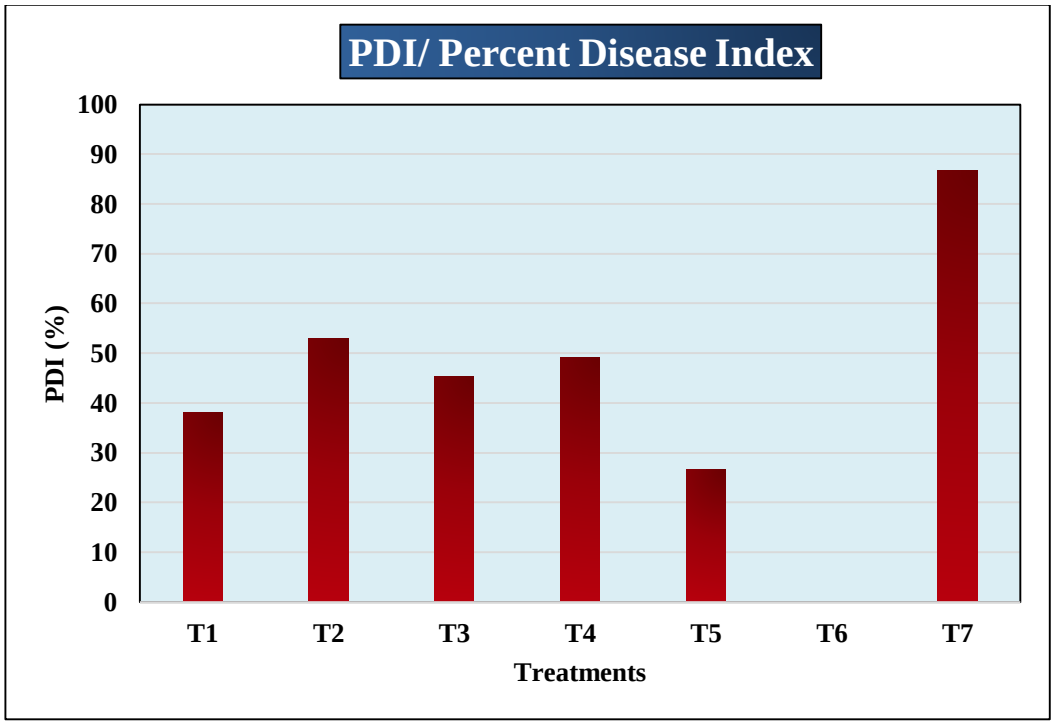


Fig. 14. Effect of bio agents on disease severity of Fusarium rot in small cardamom seedlings var. *Njallani* 45 days after challenge inoculation

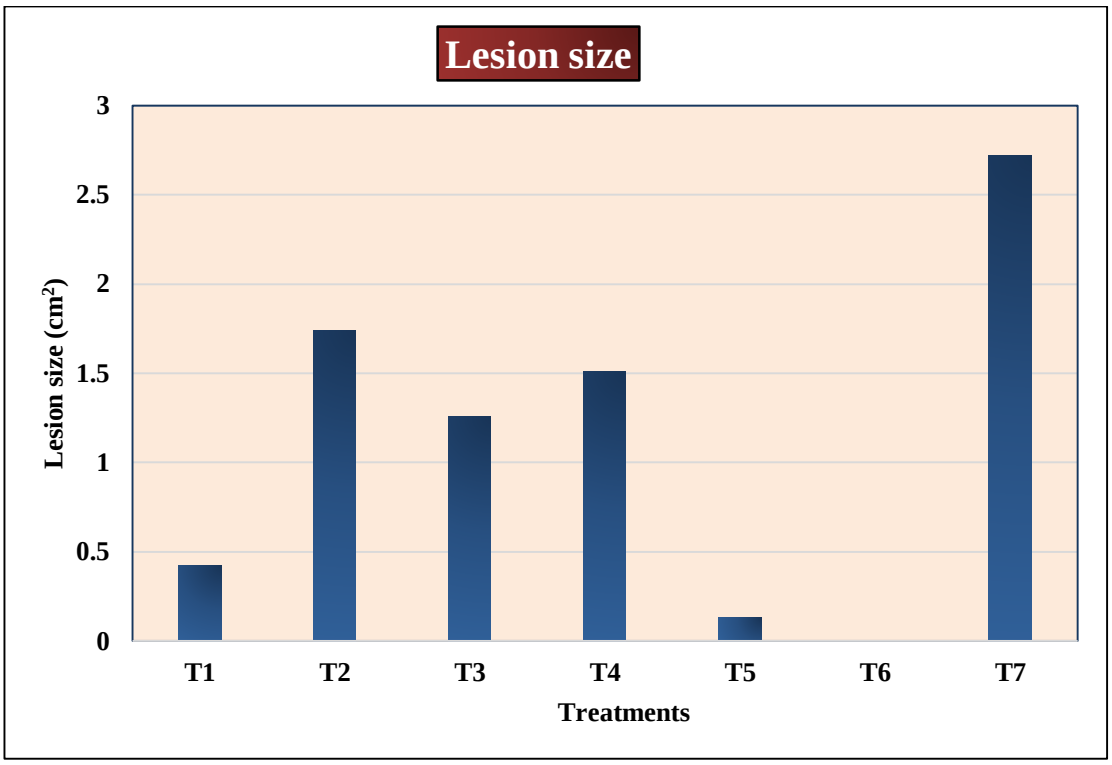


Fig. 15. Effect of bio agents on lesion size of Fusarium rot in small cardamom suckers var. *Njallani* 70 days after challenge inoculation

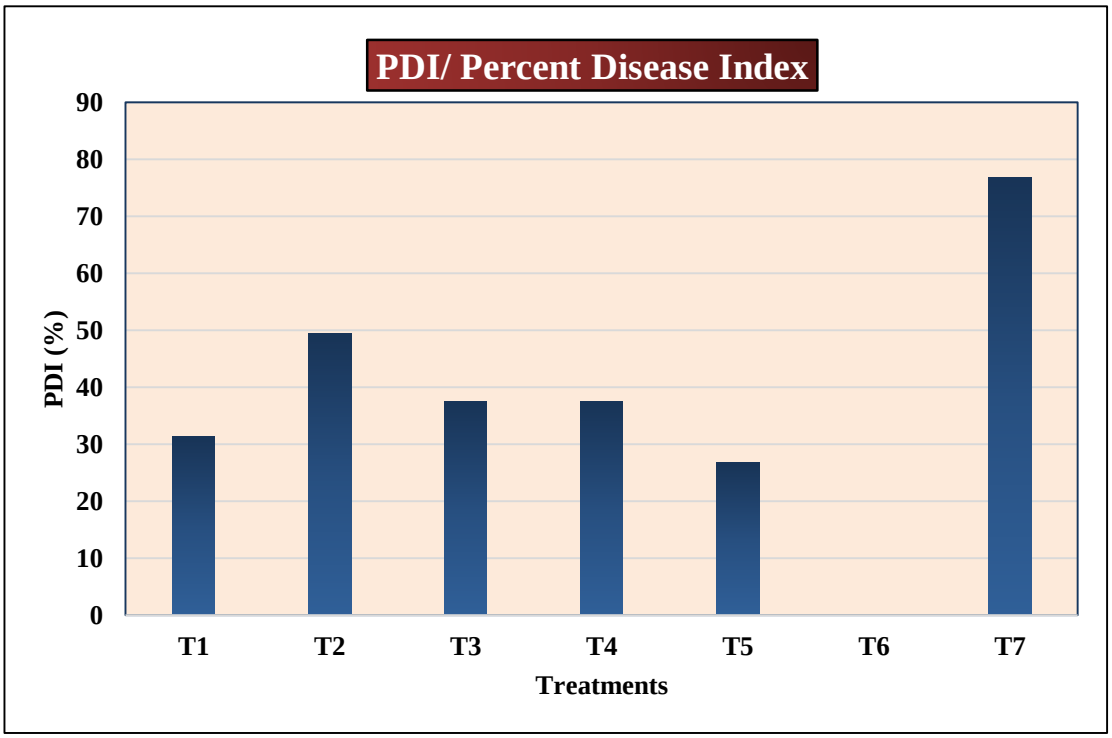


Fig. 16. Effect of bio agents on disease severity of Fusarium rot in small cardamom suckers var. *Njallani* 70 days after challenge inoculation

trigger immune response signalling in concert with signalling from microbe-associated molecular pattern (MAMP) receptors (rectangular membrane-bound structures) that detect conserved pathogenic proteins (Plett and Martin, 2018). The principal fungal cell wall components chitin and -glucans, both of which have been discovered to trigger plant responses, are MAMPs recognised by plant receptors (Lyon 2014). Endophytes secreted proteins and peptides have also been described as inducing agents and the enzymes released during the infection and colonisation process, including as xylanases, cellulases, and chitinases, can be recognised by the host either directly or via their breakdown products, and can trigger defence responses which in turn protect the host from succeeding pathogen attack (Druzhinina *et al.*, 2011).

The antimicrobial compounds produced by *P. indica* (Johnson *et al.*, 2014) is reported to deter pathogen; or act as an elicitor of defense responses mediated by PAL, peroxidase and PR proteins, thus increasing the tolerance of the host to the pathogen. The key mechanism underlying *P. indica* mediated biotic and abiotic tolerance to crops is by the strict regulation of ROS accumulation and by the modification of major components of antioxidant defense pathway (Waller *et al.*, 2005; Serfling *et al.*, 2007). *P. indica* colonization also stabilizes grana in chloroplast by scavenging ROS to protect photosystems from oxidation damage. The same specific reason could also substantiate the superior disease suppression in *P. indica* colonised plants compared to dually colonized plants as *P. indica* has been previously reported to have more accumulation of antioxidant enzymes like catalase, peroxidase and ascorbate peroxidase in individually colonized plants compared to *P. indica* and *G. mossae* dually colonized plants (Yagoubian *et al.*, 2014). Similar findings where *P. indica*-colonized tomato plants enhancing the activity of reactive oxygen scavenging enzymes such MDHAR, SOD, CAT, DHAR, APX and GR in salinity conditions in comparison with non-colonized plants were reported by Ghorbani *et al.* (2019). Waller *et al.* (2005) have also reported *P. indica* induced the resistance to disease caused by *Fusarium graminearum*, and powdery mildew caused by *Blumeria graminis* in barley and *Golovinomyces orontii* in Arabidopsis.

Likewise, when the AMF colonises the roots, the detrimental effects of soil-borne pathogens has been reported to be reduced dramatically. Colonization by AMF

has a protective effect termed mycorrhiza-induced resistance (MIR) (Nguvo and Gao, 2019). Within the mycorrhizosphere, AMF competes with soil-borne pathogens in host roots. Because of these competitive interactions, the AM fungus is pre-inoculated to improve the biocontrol agent's efficacy. Additionally, plants produce more antioxidant enzymes as a result of AMF colonisation, which can protect them from infections and other stresses. Besides the activation of plant defence mechanisms, improved nutrient status of the host plant, changes in root growth and morphology, competition for colonisation sites and host photosynthates, and microbial changes in the mycorrhizosphere have all been linked to reduced pathogen damage (Vos *et al.*, 2012). In the rhizosphere soil of *G. mosseae* treated tomato plants, Hage-Ahmed *et al.* (2009) observed that plant pathogenic fungi *F. oxysporum* sp. *lycopersici* (Fol) germination was lower than in non-mycorrhizal plants.When plants were infected with AMF and sprayed with hormone inducers (Jasmonic acid and Salicylic acid), the favourable effects were more prominent, implying a synergistic and cooperative effect between them, leading to an improved induction and regulation of disease resistance.

Kumari and Prabina (2019) reported improved disease tolerance of *Glomus* sp. colonised tomato plants against wilt caused by *F. oxysporum* f. sp. *lycopersici*. *In vitro* interactions between the AMF root and the fungal pathogen led to the formation of a clearing zone surrounding the root, suggesting that the mycorrhizal root producing antimicrobial chemicals inhibited the fungal pathogen's mycelial growth. The pre AMF – post pathogen inoculation to tomato pot culture experiment demonstrated that it reduced disease incidence and increased plant growth, dry weight, N, P, K content, chlorophyll content, and yield. Likewise, the AMF-inoculated plants were effective in controlling Fusarium wilt caused by *F. oxysporum* f. sp. *melonis* (Fom) in melon where the *G. mosseae*-inoculated plants showed the greatest capacity for reduction of disease incidence (Martínez-Medina *et al.*, 2010). Decrease in disease severity of Fusarium rot of small cardamom was observed by Venykrishna (2021) when AMF was applied basally at the rate of two per cent w/w.

Similarly, *P. fluorescens* has been demonstrated to be a promising biocontrol agent that suppresses plant diseases by preventing fungal infection of the seeds and roots. They have been shown to promote plant development and lessen the severity of

numerous fungal infections. Hass and Defago (2005) examined in depth the processes by which *P. fluorescens* regulates harmful bacteria. The formation of variety of secondary metabolites, such as antibiotics, siderophores, and hydrogen cyanide, caused this impact. Competitive exclusion of pathogens as a result of rapid colonisation of *P. fluorescens* in the rhizosphere also plays a role in disease management. Dhanya *et al.* (2018) demonstrated the efficacy of bioagents such as *P. fluorescens* and AMF against Fusarium rot of cardamom in a field trial. There was a reduction in PDI of both tiller rot and panicle wilt in 2 per cent *P. fluorescens* basal drenched plants. Venykrishna (2021) also observed a decrease in disease incidence by 37.5 per cent and disease severity by 46.17 per cent in Fusarium rot infected small cardamom plants when two per cent *P. fluorescens* were given as basal application.

All these findings were consistent with the results obtained here and suggest different mechanisms by which the bioagents conferred disease tolerance to small cardamom plants against Fusarium rot.

5.7. ELUCIDATING THE ROLE OF GIBBERELLIC ACID IN ENDOPHYTE MEDIATED TOLERANCE

Gibberellic acid estimation from the leaves of the experimental plants was carried out to explicate its role in disease development and management. The GA content in untreated control (with only pathogen inoculation) and absolute control (no pathogen or bioagent) of both seedlings and suckers were statistcally on par indicating that GA has no significant role in the disease development of Fusarium rot in small cardamom. Although there have been previous reports of endophytic *F. oxysporum* strains producing GA (Ben-Rhouma *et al.*, 2020), the GA production by the pathogenic strains of *F. oxysporum* has not yet been reported as far our knowledge. Furthermore, according to report, only through optimising cultural elements and conditions, the GA production by *F. oxysporum* was conceivable (Nwachukwu *et al.*, 2017).

In addition to this, the GA content of all bioagent treated plants was statistically superior to both absolute and untreated control. The highest GA content was observed in plants colonised with *P. indica* or dually colonised with both *P. indica* and

G. fasciculatum. *P. indica* colonised seedlings had a 3.5 fold increase and dually colonised seedlings had a 3.4 fold increase in GA content compared to the untreated control. Similarly in the case of suckers, there was an observable 4.4 fold and 4 fold increase in GA content of *P. indica* colonised and dually colonised suckers respectively. *G. fasciculatum* colonised seedlings and suckers had a 3.2 fold and 3.4 fold increase in GA content respectively; and *P. fluorescens* treated seedlings and suckers had a 2.3 fold and 2.5 fold increase in GA content respectively.

GA has been reported to be involved in stimulating pathogen resistance gene expression and enhancing resistance to ascomycetous fungus (Tanaka *et al.*, 2006). Additionally, gibberellic acid can increase the antioxidant enzyme activity by reducing reactive oxygen species (ROS), resulting in improved stress management (Manjili *et al.*, 2012). Exogenous application GA elicited opposing effect on the *F. graminearum* causing Fusarium Head Blight (FHB), infection in wheat plants (Buhrow *et al.*, 2016). The ability of *F. graminearum* to biosynthesize trichothene mycotoxin determines its pathogenicity. During the early stages of infection, GA suppressed actin and tri gene expression which correlated with a decrease in disease spread after GA co-application. Additionally, early stage gene expression of *F. graminearum*'s nitrogen metabolic genes like calcium dependent aldoxime dehydratase, calcium transporting ATPase, ATP-dependent oxoprolinase, and nitroalkane oxidase were repressed by coapplication with GA, potentially reducing bioenergetic resources and redox regulation required for newly infecting fungal cells. Similar reports of opposing metabolic pathways for GA production and nitrogen metabolism were found in *F. moniliformae* as well (Mihlan *et al.*, 2003). Together these findings strengthen the idea of a mere role of GA production by the endophytes in the disease management observed here.

Gibberellin has also emerged as a new component in the orchestration of plant-microbe symbiotic relationships (Foo *et al.*, 2019). Many phases in root development are controlled and coordinated by them, including the establishment of endosymbiotic relationships with rhizobial bacteria and mycorrhizal fungi (Yu *et al.*, 2014; Fonouni-Farde *et al.*, 2016). Gibberellic acid promotes effective uptake and ion partitioning in plants, resulting in increased growth and the maintenance of plant metabolism under normal and stress conditions. Gibberellins can also interact with other phytohormones,

eliciting critical responses and mediating tolerance mechanisms for stress tolerance including auxin, which is reported to stimulate gibberellin production (Wolbang *et al.*, 2004). To summarise, gibberellic acid affects growth, yield, mineral nutrition, and nitrogen metabolism directly.

Several studies have reported the endogenous increase in GA content when treated with *P. indica*, AMF or other PGPRs. For example, *P. indica* protect rice plants from root herbivory, and GA acted as a signal component of inducible plant tolerance to biotic stress (Cosme *et al.*, 2016). *P. indica* colonisation was reduced in barley mutants with defective GA synthesis and perception, suggesting that GA is a modulator of the root's basal defence (Schafer *et al.*, 2009). *P. indica* also recruits GA signalling to establish root cell colonisation, as indicated by a quintuple-DELLA mutant displaying constitutive GA responses and the GA biosynthetic mutant ga1-6 (for GA needing 1) showing higher and lower degrees of colonisation in Arabidopsis roots, respectively (Jacobs *et al.*, 2011). In the synthesis of phytoalexins and/or GAs, ent-kaurene synthases and ent-kaurene-like synthases were implicated and Li *et al.* (2016) demonstrated the requirement of kaurene synthase activity in successful root colonisation of *P. indica* in barley. Another report of down-regulation of the GA2ox gene associated with GA inactivation was observed in *P. indica*-colonized barley roots (Schafer *et al.*, 2009). Also, *P. indica*-induced growth promotion of Chinese cabbage and barley seedlings were linked to an increase in GA levels in colonised roots (Schafer *et al.*, 2009; Lee *et al.*, 2011). Finally, Pan *et al.* (2017) revealed that *P. indica*-colonised Arabidopsis plants had greater transcript levels of the GA biosynthesis genes Gibberellin 20-Oxidase 2, Gibberellin 3-Oxidase 1, and Gibberellin Requiring 1 consequently resulting in an increase in GA 4 level, when GA content was analysed.

Correspondingly, mycorrhizal colonisation by *G. intraradices* in tobacco roots considerably enhanced concentrations of many endogenous gibberellins in roots. Most GAs of the earl-13-hydroxylation biosynthesis pathway – GA1, GA8, GA19, and GA20 – were elevated in root samples of tobacco plants after mycorrhizal colonisation compared to nonmycorrhizal controls (Keinan *et al.*, 2002). Similarly, the roots of AM and AM + Ca^{2+}-treated peanut plants had greater levels of gibberellic acid (GA3) and flavonoids having an increase in transcripts of genes involved in the production of GA.

In AM plants, all differentially expressed genes (DEGs) encoding gibberellin 20-oxidase were up-regulated compared to the control. In Ca^{2+} + AM plants, two DEGs, gibberellins 2-oxidase and gibberellin receptor GID1, were up-regulated (Li *et al.*, 2019).

In a similar way, PGPR can regulate the amount of endogenous gibberellin in plants. Gibberellins can be synthesised by some PGPR strains (Bottini *et al.*, 2004). The level of endogenous gibberellins in the shoots of host plants rose after inoculation with gibberellin-producing PGPR strains *Bacillus cereus* MJ-1 (Joo *et al.*, 2005) and *Promicromonospora* sp. SE188 (Kang *et al.*, 2014). In mutant rice plants deficient in gibberellin synthesis, gibberellin-producing PGPR *Leifsonia soli* SE134 and *Enterococcus faecium* LKE12 boosted shoot growth. *Burkholderia phytofirmans* PsJN, a GPR strain which does not synthesise gibberellins, induced the expression of the AtGA3ox1 gene in *A. thaliana*, which coded for an enzyme involved in one of the final stages of gibberellin synthesis. (Poupin *et al.*, 2013). All these studies are in uniformity with the results obtained here revealing an increase in endogenous GA content of the plant in response to bioagent inoculation.

Taken together, these findings implies the major role of Gibberellic acid coupled with endogenous auxin production and nutrient uptake on plant growth promotion observed in the interactive study (4.2).

Summary

6. SUMMARY

Small cardamom (*Elettaria cardamomum* (L.) Maton) is one of the world's most lucrative cash crops. It is the world's third most expensive spice by weight, trailing only saffron and vanilla in terms of market value. The growing demand for spices from local to worldwide markets has piqued the interest of farmers engaged in commercial cultivation. However, several studies have identified challenges to cardamom cultivation, including production of healthy planting materials, disease management and pesticide residue. Attempting to take this into consideration, the present study entitled "Evaluation of beneficial fungal root endophytes against Fusarium rot in small cardamom" was carried out in Cardamom Research Station, Pampadumpara and College of Agriculture, Vellayani during the year 2020-21 to evaluate the colonisation and interaction of two beneficial fungi, *Piriformospora indica* and *Glomus fasciculatum*, in cardamom and their potential to manage Fusarium rot disease of cardamom and also to elucidate the role of gibberellic acid in the endophytic fungi-mediated disease tolerance.

Root rotting, eye-shaped lesion on the pseudostem, and panicle rot were the characteristic symptoms of Fusarium infection in small cardamom. Fusarium rot infected samples of roots were collected from the farmer's field of Idukki district and Koch's postulates was proved by inoculating the pseudostem of one-year plants with the isolate using pinprick method, which developed the characteristic eye-shaped lesion within two weeks, measuring 1.50 cm in length and 0.25 cm in width. The fungus had a fluffy growth pattern with pinkish white growth in front view and pinkish tinge at the base in the rear view. The macroconidia was of size 14.8 µm while microconidia had a size of 10.7 µm.

A pot culture experiment was conducted at CRS, Pampadumpara to investigate the individual and combined effect of the fungal root endophytes *P. indica* and *G. fasciculatum* on the vegetative growth of small cardamom seedlings and suckers. The effects of endophytes on biometric parameters such as plant height, number of leaves, leaf length, leaf breadth, root parameters, biomass, and so on were studied

individually and in combination. Successful root colonization was observed in *P. indica* and *G. fasciculatum* treated roots at six and seven days after their inoculation respectively in cardamom seedlings and three weeks and four weeks after their inoculation respectively in cardamom suckers. This is the first report of colonization of *P. indica* in small cardamom. The study disclosed that endophytes inoculated plants outperformed control plants in terms of shoot and root growth, with the dually colonised plants showing superior growth, followed by *P. indica*-colonized plants, and finally *G. fasciculatum* colonised plants. There was a pronounced per cent increase of 45.27, 88.89, 29.16, 49.52, 213.51 and 48.71 in plant height, number of leaves, leaf length, root length, root volume and number of secondary roots of dually colonised cardamom seedlings over control respectively. Dually colonised suckers recorded 22.56, 84.31, 38.50, 116.67, 57.07, 142.42 and 133.33 per cent increase in plant height, number of leaves, leaf length, number of tillers, root length, root volume and number of secondary roots respectively.

In the case of biomass evaluation of cardamom seedlings *P. indica*-colonised seedlings had superior belowground biomass whereas dually colonised seedlings had superior aboveground biomass. The *P. indica*-colonised seedlings had 120.97, 68.94 per cent increase in root fresh weight and root dry weight respectively while dually colonised seedlings had a per cent increase of 76.29 and 54.01 in shoot fresh weight and shoot dry weight respectively. In contrary to this, the cardamom suckers showed superiority in root and shoot biomass when dually colonised with bioagents recording a per cent increase of 304.13, 200.66, 140.60 and 135.45 in root fresh weight, root dry weight, shoot fresh weight and shoot dry weight respectively.

Furthermore, these results also suggests a reduction in nursery period by one month each in primary and polybag nursery as the endophytes treated seedlings acquired the required vegetative growth for transplantation beforehand itself.

The nutrient analysis of P and K carried out in the soil (pre-treatment) revealed no significant difference in the nutrient status of soil whereas samples (post treatment) whereas treatment application resulted in a significant difference in the nutrient uptake of plant samples. In comparison to the uninoculated control, endophyte treated plants

had exponential improvement in P and K uptake to root, shoot, and leaf. The nutrient analysis of plant samples revealed that *G. fasciculatum*-colonized seedlings had the maximum uptake of phosphorus to root, which was roughly twelve times higher than the uninoculated control. Nonetheless, when compared to control plants, phosphorus uptake to the shoot was 11 times higher and leaf uptake was 9 times higher in dually colonised seedlings and *P. indica*- colonised seedlings, respectively. In suckers, *G. fasciculatum* colonised roots had a 7-fold rise in P content compared to the control, whereas shoots and leaves in *P. indica*-colonised plants had a 1.5-fold and 3.1-fold increase in P content, respectively.

The phytohormone analysis i.e. IAA analysis in the roots of experimental plants recorded a higher auxin content in all endophyte treated plants compared to the uninoculated control. *P. indica*-colonised plants recorded the highest IAA concentration, with seedlings and suckers having 83.80 µg g^{-1} and 94.30 µg g^{-1} IAA content as against control which recorded only 40.62 µg g^{-1} and 74.49 µg g^{-1} IAA in seedlings and suckers respectively. Dually colonized seedlings and suckers documented an IAA content of 44.80 µg g^{-1} and 85.19 µg g^{-1}; and *G. fasciculatum* colonized seedlings and suckers recorded an IAA content of 43.33 µg g^{-1} and 83.50 µg g^{-1} respectively.

To investigate the effect of the axenically culturable endophyte *P. indica* in the control of *F. oxysporum* causing Fusarium rot, an *in vitro* study of *P. indica* against *F. oxysporum* was carried out. The study revealed a per cent inhibition of 64.4 per cent, antagonism index of 20.53, and an indistinct lysis zone whereas inhibition zone, antibiosis, overgrowth and sporulation etc were absent.

The *in vivo* pot culture experiment conducted at CRS, Pampadumpara utilising bioagents against Fusarium rot in small cardamom revealed that the bioagent priming successively decreased the disease severity and lesion size thus suppressing the disease. The chemical check carbendazim recorded the least disease severity/PDI (26.67 per cent) and lesion size, followed by *P. indica*, which was the superior treatment among all the bioagents. *P. indica* treated plants showed the best result having the least disease severity of 38.07 (%) and 31.48 (%) in seedlings and suckers respectively without

compromising the vegetative characters. When compared to control, colonisation with *P. indica* reduced the disease severity of Fusarium rot by 56.05 per cent and 58.99 per cent in seedlings and suckers respectively. Combinatorial and *P. fluorescens* treatments were the other treatments showing promising results in disease management. Combinatorial treatment reduced the disease severity by 47.73 (%) and 51.15 (%) in seedlings and suckers; and *P. fluorescens* treatment reduced the disease severity by 43.32 (%) and 51.18 (%) respectively in seedlings and suckers.

Total Gibberellic acid estimated from leaf samples of challenge inoculated experimental plants revealed no significant role of GA in endophyte mediated disease tolerance/ development as the GA content in both absolute control and untreated control (containing only pathogen) were on par with one another. Furthermore, the GA content of *P. indica* colonised and dually colonised plants were superior to all other treatments suggesting their critical role in the disease management by improving the vegetative characters of the plant. The GA content in *P. indica*-colonized seedlings and suckers recorded 18.60 µg g^{-1} and 10.65 µg g^{-1}; and that of dually-colonized seedlings and suckers recorded 18.00 µg g^{-1} and 9.71 µg g^{-1} with least value being observed in the absolute control recording 2.51 µg g^{-1} and 1.60 µg g^{-1} respectively.

The foregoing findings shows that beneficial endophytes promote plant growth significantly along with disease management implying the utilization of them in cardamom plantation for the better establishment and low pesticide residue.

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Appendices

APPENDIX – I

COMPOSITION OF MEDIA USED

Potato Dextrose Agar (PDA) Medium

Potato	: 200 g
Dextrose	: 20 g
Agar	: 20 g
Distilled water	: 1 L

Potato Dextrose Broth (PDB)

Potato	: 200 g
Dextrose	: 20 g
Distilled water	: 1 L

APPENDIX – II

COMPOSITION OF STAIN USED

Lactophenol Cotton Blue

Anhydrous lactophenol	: 67 ml
Distilled water	: 20 ml
Cotton blue	: 0.1 g

Anhydrous lactophenol prepared by dissolving 20 g phenol in 16 ml lactic acid and in 3 ml phenol

Abstract

EVALUATION OF BENEFICIAL FUNGAL ROOT ENDOPHYTES AGAINST FUSARIUM ROT IN SMALL CARDAMOM

by

AISHWARYA MANOHARAN

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Abstract of Thesis

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ABSTRACT

Evaluation of beneficial fungal root endophytes against Fusarium rot in small cardamom

The study entitled "Evaluation of beneficial fungal root endophytes against Fusarium rot in small cardamom" was conducted at College of Agriculture, Vellayani and Cardamom Research Station, Pampadumpara during 2020-21 to evaluate the colonisation and interaction of two beneficial fungi, *Piriformospora indica* and *Glomus fasciculatum*, in cardamom and their potential to manage Fusarium rot disease of cardamom and elucidation of the role of gibberellic acid in the endophytic fungi-mediated disease tolerance.

Fusarium infection in small cardamom is characterised by root rotting, eye shaped lesion on pseudostem and panicle rot. *Fusarium oxysporum* Schledt was isolated from the infected roots of the farmer's field of Pampadumpara in Idukki district. Koch's postulate was proved by inoculation of the pseudostem of one year plants using pinprick method where the isolate produced the typical eye shaped lesion within two weeks.

A pot culture experiment was conducted at CRS, Pampadumpara to assess the interactive effect of the fungal root endophytes *P. indica* and *G. fasciculatum* on the vegetative growth of small cardamom seedlings and suckers using four treatments and five replications. Both individual and combinatorial effects of the endophytes on the vegetative characters like plants height, number of leaves, leaf length, leaf width, root parameters, biomass etc. were evaluated. Successful root colonization was observed in *P. indica* and *G. fasciculatum* treated roots at six and seven days after their inoculation respectively. This is the first report of colonization of *P. indica* in small cardamom. The study revealed that the endophytes inoculated plants were superior to control plants in which maximum shoot growth was shown by the dually colonized plants followed by the *P. indica*-colonized which was then followed by *G. fasciculatum* colonised plants. The root parameters were higher for *P. indica*-colonized plants compared to the combined or *G. fasciculatum* colonized plants in cardamom seedlings whereas dually colonized plants had better root growth in cardamom suckers. P and K analysis of the whole plant clearly indicated the enhanced nutrient uptake to root, shoot and leaf in the endophytes-colonized plants. The IAA analysis of root samples also revealed a higher IAA content in the endophytes colonized seedlings compared to the control seedlings with *P. indica* colonized plants recording the maximum value of 83.80 µg g^{-1} in seedlings and 94.30 µg g^{-1} in suckers; and least value being observed in control plants

recording 44.80 µg g^{-1} in seedlings and 94.30 µg g^{-1} in suckers. This study stipulate a scope of better establishment of cardamom in field especially in the case of seedlings during transplantation due to well established root system. There was also a reduction in nursery period by two months in the *P. indica* and *G. fasciculatum* dually colonized seedlings compared to the non-colonized seedlings.

In vitro evaluation of *P. indica* against *F. oxysporum* and *in vivo* evaluation of both the bioagents against *F. oxysporum* in small cardamom was carried out to appraise the effect of bioagents in the management of Fusarium rot disease of cardamom. In *in vitro* evaluation, dual culture assay of *P. indica* with *F. oxysporum* was performed in PDA in which a percent inhibition of 64.4 (%), antagonism index of 20.53 and an obscure lysis zone was observed. There wasn't any presence of inhibition zone, antibiosis, coiling, overgrowth, sporulation etc. In *in vivo* evaluation, a pot culture experiment was laid out at CRS, Pampadumpara with seven treatments and three replications including a bio agent check of 2% *Pseudomonas fluorescens* and chemical check of 2 % carbendazim as basal drench. The study revealed that the beneficial interaction with bioagent successively decreased the disease severity and lesion size, among which *P. indica* treated plants showed the best result having the least disease severity of 38.07 (%) and 31.48 (%) in seedlings and suckers respectively. The disease severity of the control plants were the highest, recording 86.62 (%) and 76.77 (%) in seedlings and suckers respectively. Combinatorial and *P. fluorescens* treatments were the other treatments showing promising results in disease management.

Total Gibberellic acid was estimated from leaf samples of challenge inoculated experimental plants to elucidate the role of GA in endophyte mediated disease tolerance and the authors couldn't interpret any role of GA in disease development. Moreover, the GA content was higher in *P. indica* colonised recording 18.60 µg g^{-1} and 10.65 in µg g^{-1} in seedlings and suckers respectively; and dually colonised plants recording 18.00 µg g^{-1} and 9.71 µg g^{-1} in seedlings and suckers respectively.

Thus, the present study proclaims a very pronounced plant growth promotion in cardamom seedlings and suckers by the beneficial fungal root endophytes *viz., P. indica* and *G. fasciculatum* just before transplanting to the field and therein advocating better development and establishment in the field condition. Furthermore, the results also indicates an effective management of the destructive Fusarium rot disease in small cardamom using endophyte colonization especially in *P. indica* colonized and dually colonized plants.

സംഗ്രഹം

"വേരിൽ അന്തർവ്യാപന ശേഷിയുള്ള മിത്രകുമിളുകൾ ഉപയോഗിച്ച് ഏലത്തിൽ കണ്ടു വരുന്ന ഫ്യൂസേറിയം വാട്ടത്തിന്റെ നിയന്ത്രണം" എന്ന വിഷയത്തെ ആസ്പദമാക്കി വെള്ളായണി കാർഷിക കോളേജ് സസ്യരോഗ വിഭാഗത്തിൽ 2020-21 കാലയളവിൽ ഗവേഷണം നടത്തുകയുണ്ടായി.

ഏലത്തിൽ ഫ്യൂസേറിയം അണുബാധകൊണ്ട് വേരഴുകൽ, തണ്ടുകളിൽ കണ്ണിന്റെ ആകൃതിയിലുള്ള തവിട്ടുനിറത്തിലുള്ള പാടുകൾ, പൂങ്കുലകളിൽ ചെംചീയൽ എന്നിവ ഉണ്ടാകുന്നു. ഇടുക്കി ജില്ലയിലെ പാമ്പാടുംപാറയിലെ കർഷകന്റെ തോട്ടത്തിലെ രോഗബാധിതമായ വേരുകളിൽ നിന്നാണ് ഫ്യൂസേറിയം ഓക്സിസ്പോറം ഷ്ലെഡ് എന്ന രോഗാണു വേർതിരിച്ചെടുത്തത്. തണ്ടുകളിൽ കുമിളുകളുടെ കൃത്രിമമായ കുത്തിവയ്പ്പിലൂടെ രണ്ടാഴ്ചയ്ക്കുള്ളിൽ ഫ്യൂസേറിയതിന്റെ പ്രധാന ലക്ഷണമായ കണ്ണിന്റെ ആകൃതിയിലുള്ള പാടുകൾ കാണപ്പെട്ടു.

ഏലത്തിന്റെ വേരുകളിൽ മിത്രകുമിളുകളായ *പിരിഫോർമോസ്പോറ ഇൻഡിക്ക ഗ്ലോമസ് ഫാസിക്കുലേറ്റം* എന്നിവയുടെ അന്തർവ്യാപനശേഷിയും ചെടിയുടെ വളർച്ചയിലുള്ള സ്വാധീനവും വിലയിരുത്തി. സസ്യങ്ങളുടെ ഉയരം, ഇലകളുടെ എണ്ണം, ഇലകളുടെ നീളം, ഇലയുടെ വീതി, വേരുകളുടെ പ്രകൃതി, ജൈവോർജ്ജം തുടങ്ങിയ സസ്യ സ്വഭാവങ്ങളിൽ മിത്ര കുമിളുകളുടെ വ്യക്തിഗതവും സംയോജിതവുമായ സ്വാധീനമാണ് ഈ പഠനത്തിൽ വിലയിരുത്തിയത്. മിത്രകുമിളുകൾ കൊടുത്ത ഏലത്തിന്റെ വേരുകളിൽ അവയുടെ വിജയകരമായ കോളണൈസേഷൻ ഉണ്ടായി. ഏലത്തിലെ *പി. ഇൻഡിക്ക*യുടെ കോളണൈസേഷന്റെ ആദ്യ പഠന റിപ്പോർട്ടാണിത്. വേരുകളിലെ മിത്രകുമിളുകളുടെ കോളണൈസേഷൻ ചെടിയുടെ വളർച്ചയെ ത്വരിതപ്പെടുത്തുകയും, ശാഖ വേരുകളുടെ ഉത്പാദനം വർധിപ്പിക്കുകയും ചെയ്തു. കൂടാതെ ഇലകളുടെ എണ്ണവും വലുപ്പവും കൂടുകയും ചെയ്തു. സംയോജിതമായി മിത്രകുമിളുകൾ കൊടുത്ത ചെടികളിൽ ആയിരുന്നു ചെടിയുടെ വളർച്ച ഏറ്റവുമധികം രേഖപ്പെടുത്തിയത്, എന്നാൽ വേരുകളിലെ വളർച്ചയിൽ *പി. ഇൻഡിക്ക* കൊടുത്ത ചെടികളായിരുന്നു മുന്നിട്ടു നിന്നത്. വേരുകളിലെ ഹോർമോൺ പഠനത്തിൽ നിന്നും മിത്രകുമിളുകൾ കോളണൈസേഷൻ നടത്തിയ ചെടികളുടെ വേരുകളിൽ ഓക്സിൻ ഹോർമോൺ അധികരിച്ചതായി കണ്ടെത്തി. ഇവയിൽ *പി. ഇൻഡിക്ക* കൊടുത്ത ചെടികളിൽ ആണ് ഏറ്റവും കൂടുതൽ ഓക്സിന്റെ അളവ് കാണാനിടയായത്.

മിത്രകുമിളുകളുടെ സാനിധ്യത്തിൽ ചെടികളുടെ വേരുകളും തണ്ടുകളും ഇലകളും വളരെ വേഗം വളരുന്നതായും, മിതകുമിളുകൾ ഇടാത്ത തൈകളെ അപേക്ഷിച്ച് പി. ഇൻഡിക്ക- *ജി. ഫാസിക്കുലേറ്റം* എന്നിവ സംയോജിതമായി

കോളണൈസേഷൻ നടത്തിയ തൈകളിൽ നഴ്സറി കാലയളവ് രണ്ട് മാസത്തോളം കുറക്കാൻ സാധിക്കുന്നതായും കണ്ടെത്തി.

*എഫ്. ഓക്സിസ്പോറ*ത്തിനെതിരെ *പി. ഇൻഡിക്ക*യുടെ പ്രവർത്തനം ലബോറട്ടറിയിൽ പഠന വിധേയമാക്കി. ഏലത്തിലെ *എഫ്. ഓക്സിസ്പോറത്തി*നെതിരായി മേൽപറഞ്ഞ രണ്ട് മിത്രകുമിളുടെ *ഇൻ - വിവോ* മൂല്യനിർണ്ണയവും നടത്തുകയുണ്ടായി. ഇതിൽ ലബോറട്ടറി മൂല്യനിർണയത്തിൻറ്റെ ഭാഗമായി നടന്ന ഡ്യുവൽ കൾച്ചർ അസ്സേയിൽ രോഗാണുവിന്റെ 64.4 (%) ശതമാനം കുറവും, 20.53-ൻറ്റെ ആൻറ്റഗോണിസ്റ്റിക് ഇൻഡക്സും, അവ്യക്തമായ ലൈസിസ് സോണും കാണപ്പെട്ടു.

ഇൻ-വിവോ പഠനത്തിൽ, മിത്രകുമിളകളുടെ പ്രയോജനകരമായ ഇടപെടൽ രോഗത്തിന്റെ തീവ്രതയും പാടുകളുടെ വലുപ്പവും കുറയ്ക്കുന്നതായി കണ്ടെത്തി, ഇവയിൽ *പി. ഇൻഡിക്ക* കോളണൈസേഷൻ നടത്തിയ ചെടികളിൽ ഏറ്റവും മികച്ച ഫലം കാണപ്പെട്ടു. ഏറ്റവും കുറഞ്ഞ രോഗതീവ്രത യഥാക്രമം 38.07 (%) ഉം 31.48 (%) ഉം തൈകളിലും സക്കറുകളിലുംമായി കാണാനിടയായി. മിത്രകുമിളുകൾ കൊടുക്കാത്ത സസ്യങ്ങളുടെ രോഗതീവ്രത ഏറ്റവും ഉയർന്നതായി (തൈകളിലും തട്ടകളിലും യഥാക്രമം 86.62 (%) ഉം 76.77 (%) ഉം) കാണപ്പെട്ടു. മിത്രകുമിളുകൾ സംയോജിതമായി കൊടുത്ത ചെടികളും, *പി. ഫ്ലൂറസെൻസ്* കൊടുത്ത ചെടികളുമായിരുന്നു ഈ പഠനത്തിൽ മികച്ച രോഗ നിയന്ത്രണം കാണിച്ചത്.

മിത്രകുമിളുകൾ വഴിയുള്ള രോഗപ്രതിരോധത്തിൽ ഗിബ്ബറെല്ലിക് ആസിഡിന്റെ പങ്ക് വ്യക്തമാക്കുന്നതിനായി രോഗാണുവിനെ ചെടികളിൽ കൃത്രിമമായി കുത്തിവച്ചശേഷം സസ്യങ്ങളുടെ ഇലകളിലെ ഗിബ്ബറെല്ലിക് ആസിഡിൻറ്റെ അളവ് വിശകലനം ചെയ്തു. *പി. ഇൻഡിക്ക* കോളണൈസേഷൻ നടത്തിയ ചെടികളിലും മിത്രകുമിളുകൾ സംയോജിതമായി കോളണൈസേഷൻ നടത്തിയ ചെടികളിലും (തൈകളിലും സക്കറുകളിലും) ഗിബ്ബറെല്ലിക് ആസിഡിൻറ്റെ അളവ് കൂടുതലായി രേഖപ്പെടുത്തി. എന്നാൽ ഇതിന് രോഗനിയന്ത്രണത്തിൽ യാതൊരു പങ്കുമില്ലെന്ന് കണ്ടെത്തി.

ഗുണകരമായ മിതകുമിളുകളുടെ, അതായത്, *പി. ഇൻഡിക്ക ജി. ഫാസിക്കുലേറ്റം* എന്നിവയുടെ കോളണൈസേഷൻ ഏലത്തൈകളിലും തട്ടകളിലും സസ്യവളർച്ചയെ പ്രോത്സാഹിപ്പിക്കുമെന്ന് ഈ പഠനം നിർദ്ദേശിക്കുന്നു. തൻമൂലം തൈകളെ കൃഷിയിടത്തിലേക്ക് പറിച്ചു നടുന്നതിനുള്ള കാലതാമസം രണ്ട് മാസത്തോളം കുറക്കുമെന്നും പഠനത്തിൽ കണ്ടെത്തി. കൂടാതെ, ഇവയുടെ പ്രത്യേകിച്ചും *പി. ഇൻഡിക്ക*യുടെ കോളണൈസേഷൻ ഏലത്തിലെ വിനാശകരമായ ഫ്യൂസേറിയം ചെംചീയൽ രോഗത്തെ ഫലപ്രദമായി നിയന്ത്രിക്കുന്നതായും കണ്ടെത്തി.