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Research Paper

COLONIZATION AND PLANT GROWTH PROMOTION IN SOMATIC EMBRYO DERIVED BLACK PEPPER PLANTS BY FUNGAL ENDOPHYTES

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Abstract

Black pepper (*Piper nigrum* L.) plants (variety Sreekara) were produced through somatic embryogenesis and studied for endophytic fungal association and the role endophytic fungi in hardening tissues and its growth promotion. Interestingly, somatic embryo derived black pepper plants are found to be free from fungal association. No Fungus could be obtained from the tissues of somatic derived black pepper plants. The colonization studies on somatic plants by endophytic fungi showed that stem and root tissues were successfully colonized by the inoculated endophytic fungi such as *Diaporthe* sp., *Annulohypoxylon nitens*, *Daldinia eschscholtzii*, *Fusarium oxysporum and, Phomopsis* sp. The colonization of black pepper tissues by the endophytic fungi were confirmed by re-isolation from the treated plants. Endophyte enriched sterile sand is a promising medium for the hardening of somatic derived black pepper plants with enhanced survival rate of 56% -69% when compared to control (50%). Application of endophytic fungi in black pepper showed a significant increase in growth parameters like plant height, number of leaves, number of nodes, fresh and dry weight of plant in under green house. This is the first report of using endophytic fungi for hardening tissue cultured plants of black pepper.

Key words: *Black pepper; Endophytic fungi; Growth promotion; Somatic embryogenesis.*

INTRODUCTION

Black pepper (*Piper nigrum* L.) is an important spices crop and is believed to be originated in the Western Ghats of India. Black pepper is propagated vegetatively as well as through seeds. As the crop is heterozygous in nature, seedlings raised from seeds will not breed true to type. Hence vegetative propagation through stem cuttings is preferred for commercial cultivation, which is taken mainly from runner shoots,

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although terminal shoots can also be used (1). Black pepper cuttings are raised in nurseries using rapid multiplication techniques by bamboo splits method, the serpentine method, the column method, and even soil-less culture (2). The internal tissue of plants represents an ecological niche where number species of fungal endophytes live and may have an important role in the adaptation of plants to some particular environment (3). Fungal endophytes have profound impacts on plant health as the endophytes can increase plant fitness by making plants resistant to stress from both abiotic and biotic sources; increase biomass, plant growth, and yield either through increasing nutrient uptake or through suppressing pathogens (4; 5).

Somatic embryogenesis is the development of any somatic cell into embryos that finally forms to whole plant. In black pepper somatic embryogenesis is a suitable method for large scale production of planting materials (6; 7). Micropropagation has many advantages over conventional methods of vegetative propagation but is limited by the acclimatization stage, one of the most critical stages of this process. A high percentage of micropropagated plantlets are lost or damaged during transfer from *in vitro* to *in vivo* (8). The presence of fungi in internal tissue (endophytic fungi) of black pepper has been known (9; 10; 11). But no information was available for the presence of endophytic fungi in somatic embryo derived black pepper plants and their growth promotion. Hence this study was undertaken to check, endophytic fungal association in somatic embryo derived black pepper plants, and its role in the hardening of tissue cultured black pepper plants and also their effect on growth promotion.

MATERIALS AND METHODS

The study was conducted in a series of experiments like production of *invitro* somatic embryogenic plants, screening somatic plants for endophytic fungal association, screening the colonization of endophytes in *invitro* derived plants, inoculation of endophytic fungi in the hardening medium etc.

Endophytic fungal isolates used for the experiments

The following fungi isolated from black pepper were used for the study; BPEF11- *Diaporthe sp.* (KF219919), BPEF25- *Annulohypoxylon nitens* (KF151846), BPEF38- *Annulohypoxylon niten* (KF254768), BPEF41- *Daldinia eschscholtzii* (KF151848), BPEF72- *Fusarium oxysporum* (KF151847), BPEF73- *Daldinia eschscholtzii* (KF151849), BPEF75- *Fusarium oxysporum* (KF151850), BPEF81- *Ceriporia lacerata* (KF151851) and

BPEF83- *Phomopsis* sp.(KF219920). The isolates BPEF72 and BPEF75 were identified by comparing both molecular and morphological features as *F. oxysporum*.

Production of *in vitro* black pepper plants through somatic embryogenesis (Direct somatic embryogenesis from black pepper seeds)

Mature seeds of black pepper variety Sreekara were collected from field grown plants at ICAR-IISR, Experimental Farm, Peruvannamuzhy, Kozhikode, India. Fully ripened seeds were selected and used as source material for initiating primary somatic embryogenic cultures. The seeds were soaked overnight in water in order to remove the outer pericarp. The seeds were washed several times in sterile water before surface sterilization in order to completely remove the outer pericarp. Surface-sterilization was done by dipping the seeds in 0.1% mercuric chloride for 5 min and rinsed 2-3 changes in sterile distilled water followed by drying on sterile filter paper under aseptic conditions in laminar air flow chamber. The surface sterilized seeds were cultured on agar gelled full-strength, plant growth regulator free SH medium (12) with a pH of 5.8 containing 3.0% (W/V) sucrose under darkness for 90-100 days. The plates were periodically observed for the production of somatic embryos (7).

Induction of secondary somatic embryogenesis

Primary somatic embryos (PEs) derived from germinating seeds after 90 days on SH-30 were used for inducing secondary somatic embryogenic cultures. The developing primary embryos on the germinating seeds were carefully detached and transferred on half strength, SH medium (pH-5.8) containing 1.5% sucrose (SH-15) and gelled with 0.8% agar. Cultures were incubated in dark at a temperature of 25 ± 1 °C. After mass production, the PEs were uniformly spread over the surface of SH - medium with 1.5% sucrose and incubated in dark for 15 days.

Secondary embryo proliferation and regeneration of black pepper plants

Embryogenic clumps (25 mg each) from secondary embryogenic cultures were inoculated into 500 mL flasks containing 250 mL of liquid SH-15 medium and incubated under dark in a shaker at 100 rpm for 20 days. After 20 days of culturing, embryos of similar developmental stages were allowed to develop further in SH-30 medium with 12 h light and dark cycles for 10 more days. Every 5 days, 20 mL of medium was withdrawn and replaced with the same quantity of fresh medium. After 30 days of growth in SH-30 medium, cotyledon stage plants were developed and plants with one tap root were transferred to Woody Plant Medium (13). Embryogenic clumps (25 mg

each) from secondary embryogenic cultures were transferred into 500 mL flasks containing 250 mL of liquid SH-15 medium and incubated under darkness in shaker at 100 rpm for 20 days. After 20 days of culturing, embryos of similar developmental stages were allowed to develop further in SH-30 medium with 12 h light and dark cycles for 10 more days. Every 5 days, 20 mL of medium was withdrawn and replaced with the same quantity of fresh medium. After 30 days of growth in SH-30 medium, cotyledon stage plants were developed and plants with one tap root were transferred to WPM for further growth and maturation. The plantlets were maintained in the above condition until they grew and produced more than two leaves.

Screening of somatic black pepper plants for endophytic fungal association

For testing endophytic association in somatic embryo derived plants, 50 contamination free healthy plants were randomly selected and removed from the WPM using sterile forceps. Among these 50 plants, 25 plants were surface sterilized by immersing the whole plant in a test tube containing sodium hypochlorite (3-5% available chlorine) for 2 min. followed by treatment with 70% ethanol for 2 min. Then the plants were washed thoroughly in sterile distilled water thrice. The surface sterilized plants were kept in a sterile filter paper placed on laminar air flow for surface drying. Stem, root and leaf tissues of each plant was cut into small pieces of approximately 5 mm size and each tissue was transferred to Petri dishes containing Malt Extract Agar (MEA) amended with 0.1% stock antibiotic solution. (Antibiotic stock was prepared by adding 0.02 g each tetracycline, streptomycin and penicillin in separate test tube containing 10 mL sterile distilled water followed by filter-sterilization through 0.2-µm filter and added to MEA at bearable temperature of 45- 50°C). The inoculated plates were sealed with cling film and incubated for 30 days at room temperature under dark. The plates were examined periodically for the appearance of fungal colony.

Screening the colonization of endophytes in *in vitro* grown plants

Rooted somatic embryo derived black pepper plants with two leaves grown on WPM were used for endophytic colonization experiment. There were ten treatments (T_1- T_{10}) with thirty replications in each treatment; The treatments include T1= *Diaporthe sp.* (KF219919), T2= *Annulohypoxylon nitens* (KF151846), T3= *Annulohypoxylon niten* (KF254768),T4= *Daldinia eschscholtzii* (KF151848), T5= *Fusarium* sp. (KF151847), T6= *Daldinia eschscholtzii* (KF151849), T7= *Fusarium* sp. (KF151850), T8= *Ceriporia lacerata* (KF151851) T9= *Phomopsis* sp.(KF219920) and T10= control.

Inoculum preparation for sporulating and non sporulating endophytic fungi

The sporulating endophytic fungi mentioned above were mass cultured in Potato dextrose broth (PDB) and after 10 days of growth, spore suspension was prepared in sterile distilled water and estimated the spore count using haemocytometer and adjusted the spore to10^6 spore/mL with sterile water. In the case of non sporulating fungi, each isolate was grown on PDB for 14 days at room temperature. From this, 50g of mycelial mat was taken and homogenized with sterile motor and pestle and the final volume was made up to 100 mL.

Endophyte inoculation and planting

For each treatment, the plants were immersed completely in respective endophytic suspension for 2 h and planted singly in tissue culture tubes with sterile sand (moisture was maintained by addition of sterile distilled water under aseptic conditions). All these procedures were carried out aseptically in laminar air flow chamber. The plants were allowed to grow in the growth chamber set at 24°C with 12 h light and dark cycles for one month. After one month, ten healthy plants were uprooted from each treatment as well as from control, washed in sterile distilled water and kept for surface drying. From each plant, stem, leaf and root were separated and each tissue was cut into approximately 5mm size aseptically. From these, 100 tissues were screened for endophytic association as described elsewhere. The plates were observed periodically for fungal growth. Once growth was observed, each isolate was sub cultured on to purified PDA medium and compared with the pure cultures of each isolate.

Hardening of somatic embryo derived black pepper plants using selected endophytic fungi

To study the role of endophytic fungi on the survival of somatic derived plants during hardening, the following experiment was conducted. Somatic embryo derived black pepper plants with uniform growth on WPM were carefully uprooted and planted in sterile sand enriched with endophytic fungi (population of 10^6 CFU/g of sand) in plastic cups. There were ten treatments with six replications (25plants/ replication)/ treatment. The treatment details are T1= *Diaporthe sp.* (KF219919), T2= *Annulohypoxylon nitens* (KF151846), T3= *Annulohypoxylon niten* (KF254768), T4= *Daldinia eschscholtzii* (KF151848), T5= *Fusarium* sp. (KF151847), T6= *Daldinia eschscholtzii* (KF151849), T7= *Fusarium* sp. (KF151850), T8- *Ceriporia lacerata* (KF151851) T9- *Phomopsis* sp.(KF219920) and T10= control. All the plants were

maintained in the Greenhouse with regular watering and observation were recorded on survival percentage in each treatment after two months of hardening. The survival percentage was calculated by the number of plants survived in each treatment x 100/ total number of plants kept in each treatment.

In planta evaluation of endophytic fungi for growth promotion in black pepper

In order to study the efficacy of endophytic fungi on growth promotion in black pepper, a series of experiments were conducted with nine endophytic fungi mentioned above, *T. harzianum* and *P. chlamydosporia*.

Preparation of endophytic fungal inoculum

Sand culture method was used for the inoculum production. Endophytic fungal inoculum was prepared by mass culturing the isolates in 500 mL conical flask containing 250 mL PDB. After ten days of growth, mycelia of each isolate was removed from conical flask, and blended using sterile mortar and pestle with small volume of sterile distilled water and made up the final volume to 100 mL. From this suspension, 10 mL was transferred to a 100 mL conical flask containing sterile sand with 30% moisture. The inoculated flasks were incubated for 30 days at 30°C with regular mixing at 2 days interval. The final population of inoculum was adjusted to 10^6 CFU/g of sand.

Effect of endophytic fungi on establishment of black pepper cuttings

Single node cuttings of the variety Sreekara were planted in protrays (25 cuttings / protray) filled with sterile sand. At the time of planting the following treatments were imposed with three replications (25 plants/ replication); T1= *Diaporthe sp.* (KF219919), T2= *Annulohypoxylon nitens* (KF151846), T3= *Annulohypoxylon niten* (KF254768), T4= *Daldinia eschscholtzii* (KF151848), T5= *Fusarium* sp. (KF151847), T6= *Daldinia eschscholtzii* (KF151849), T7= *Fusarium* sp. (KF151850), T8= *Ceriporia lacerata* (KF151851) T9= *Phomopsis* sp.(KF219920), T10= *T. harzianum*, T11= *P. chlamydosporia* and T_{12}= absolute control. The fungal inoculum was prepared and the final population was adjusted to a 10^6 CFU/g as described above and inoculated to the sand. After one month of planting, data recorded on number of sprouted cuttings and number of leaves. Spouting percentage in each treatment was calculated using the formula, Number of sprouted cuttings x 100/total number of cuttings planted.

Effect of endophytic fungi on growth promotion in black pepper

The uniform sized rooted cuttings selected from each treatment above were planted in grow bags containing potting mixture of soil: sand: farmyard manure (1:1:1) and

imposed the same treatments described above. The experiment was carried out in Green house with an average temperature of 28°C. After one month of growth, booster dose of inoculum was given for each treatment; Watering was done regularly at 24 h interval. After three months of growth, plants were uprooted and observations were recorded on growth parameters like plant height, number of nodes, fresh and dry weight of plants and data were statistically analyzed.

Statistical analysis

Statistical package SAS (SAS Institute, Cary, North Carolina) software (Version 9.3) was used for data analysis. The significant differences among the treatments were calculated by analysis of variance (Two-way ANOVA).The data were subjected to mean separation by DMRT *(Duncan's Multiple Range Test)* with Least Significant Difference (LSD) at, $P < 0.05$.

RESULTS

Production of *in vitro* plants through somatic embryogenesis

Primary somatic embryo was observed in germinating seeds after 90 days of incubation on SH-30 agar. About 10% of the germinating seeds showed primary somatic embryo induction with an average 2-4 primary embryos/ seeds. Primary somatic embryos (PEs) derived from germinating seeds were utilized for inducing high frequency secondary somatic embryogenic cultures. The primary embryos from the germinating seeds were inoculated on SH -15 agar and produced numerous embryos with in 20 days.

Secondary embryo proliferation and regeneration of black pepper plants

After 20 days of incubation of PEs, secondary embryo masses with different developmental stages were observed under microscope like globular, heart and torpedo shapes The embryogenic mass was maintained by repeated sub culturing on SH medium containing 1.5% sucrose and 0.8% agar (pH was maintained at 5.8). The prolonged incubation of secondary embryos on SH- 15 medium resulted in the development of plantlets with different growth stages. High germination percentage of secondary embryo was observed during incubation on single flask broth containing SH 30. Different developmental stages of embryos to plants were also observed in the single flask broth containing SH 30. After 30 days of incubation under shaking, cotyledon stage plantlets were developed in broth containing SH 30, further growth and maturation was carried out in WPM (Fig. 1).

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Screening of somatic embryo derived black pepper plants for endophytic fungal association

No fungal growth was observed in any of the tissue plated even after 45 days of incubation. All the tissues (stem, root and leaves) were found to be free from culturable endophytic fungi. The tissues kept for incubation without surface sterilization also did not yield any fungal growth.

Screening of endophytic nature of selected endophytes on *in vitro* grown plants

Fungal growth with similar culture morphology of the inoculated fungi only recorded. The growth was compared with the culture plates of the inoculated isolates and confirmed the identity. Out of the three tissues (stem, root and leaf) analyzed fungi could not be isolated from leaf tissues. Incubation time (growth of fungi on tissue) ranged from 10- 25 days, mycelium was observed after 12th day of incubation in plants treated BPEF11, BPEF 72 (both in root and stem). The growth of BPEF75 from root tissues were observed on 14th day where as growth observed on stem tissues on 15th day of incubation. The stem and root tissues of BPEF 83 were observed on 17th day of incubation. Growths of other isolates were observed after 20 days of incubation. Fungi were re isolated from all the treated plants except BPEF81. No fungal growth was observed in the tissues of control plants (plants without exposure to fungal inoculum).

Hardening of somatic embryo derived black pepper plants using selected endophytic fungi

Application of endophytic fungi the time of hardening significantly enhanced the percentage survival of somatic embryo derived black pepper plants. After two months of hardening. Among the treatments highest percentage of survival (Table 1) was observed in treatment with BPEF 72 (68.95%) followed by BPEF 38 (68.44%), BPEF 75 (67.43%), BPEF 25 (67.37%), compared to control (50.63).

Effect of endophytic fungi on establishment of black pepper cuttings

In this study, all treatments resulted in significant increase in sprouting percentage compared to the control plants (Table 2). Among the treatments, highest sprouting percentage was recorded (Fig. 2) in treatment with *D. escholtzii* -BPEF 41 (57.60%) followed by *C. lacerate*- BPEF 81 (56.59%), *T. harzianum*-MTCC 5179 (56.38%), *P. chlamydosporia* (56.10%) and *F. oxysporum*- BPEF75 (56.10%) compared to control (36.49%).

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Effect of endophytic fungi on growth promotion in black pepper

Black pepper plants (Fig.4) showed a significant increase in various growth parameters like plant height, number of leaves, number of nodes, fresh and dry weight of plant (Table 3). In case of plant height , all the treatments resulted in significantly higher plant height compared to control plants with highest plant growth in treatment with BPEF11 (96.33cm) followed by treatment with *T. harzianum* (91.00 cm) compared to control plants (66.00cm). Considering the number of leaves with highest number of leaves was observed in *T. harzianum* (18.33) treated plants followed by treatment with BPEF 25 (17.00) compared to control plants (13.00). Considering the number of nodes produced by the treatments, all treatments except treatment with *P. chlamydosporia*, BPEF 83, BPEF 41 and BPEF 38 resulted in significant increase in number of nodes compared to control plants with highest number of nodes in

T. harzianum treated plants (19.00) followed by treatment with BPEF 25 (17.67) compared to control plants (13.33).

Table 1. Effect of endophytic fungi on primary hardening of somatic embryo derived black pepper plants.

Treatment	Organism	Survival (%)
T1	*Diaporthe* sp. (BPEF11)	61.24^{c}
T2	*A. nitens* (BPEF25)	67.37^{ab}
T3	*A. nitens* (BPEF38)	68.44^{a}
T4	*D. eschscholtzii* (BPEF41)	61.09^{c}
T5	*F. oxysporum* (BPEF72)	68.95^{a}
T6	*D. eschscholtzii* (BPEF73)	60.32^{c}
T7	*F. oxysporum* (BPEF75)	67.43^{ab}
T8	*C. lacerata* (BPEF81)	64.03^{bc}
T9	*Phomopsis* sp. (BPEF83)	56.22^{d}
T10	Control	50.63^{e}

Data with same letter designation are not different according to DMRT at p= 0.05 (LSD value = 3.72).

Table 2. Effect of endophytic fungi on establishment of black pepper cuttings

Treatments	Sprouting (%)*	No. of leaves*
Diaporthe sp. (BPEF11)	53.10^{c}	1.0 ^{b}
A. nitens (BPEF25)	53.80^{c}	2.3^{a}
A. nitens (BPEF38)	50.70^{d}	2.3^{a}
D. eschscholtzii (BPEF41)	57.60^{a}	2.0^{ab}
F. oxysporum (BPEF72)	56.10^{b}	2.3^{a}
D. eschscholtzii (BPEF73)	53.90^{c}	2.3^{a}
F. oxysporum (BPEF75)	53.80^{c}	2.3^{a}
C. lacerata (BPEF81)	56.59^{ab}	2.0^{ab}
Phomopsis sp. (BPEF83)	50.70^{d}	2.3^{a}
T. harzianum (5179)	56.38^{b}	2.3^{a}
P. chlamydosporia	56.10^{b}	2.3^{a}
Control	36.49^{e}	1.0^{b}
LSD at P ≤ 0.05	1.17	0.99

* Mean data of three replications. Data with same letter designation are not different according to DMRT at p= 0.05.

Table 3. Effect of endophytic fungi on growth parameters

Treatments	Height of plant (cm)*	No. of leaves*	No. of nodes*	Fresh weight (g)*	Dry weight (g)*
BPEF II	96.33	15.67	16.00	63.33	23.67
BPEF 25	83.67	17.00	17.67	72.33	29.67
BPEF 38	79.00	13.67	14.33	50.33	21.00
BPEF 41	75.00	10.33	11.33	63.00	21.00
BPEF 72	85.00	15.00	15.00	69.67	24.00
BPEF 73	81.00	15.00	15.33	60.67	26.33
BPEF 75	78.67	16.67	17.33	62.00	22.00
BPEF 81	87.67	15.67	16.33	56.00	23.00
BPEF 83	83.00	14.00	14.33	52.67	22.33
T. harzianum (5179)	91.00	18.33	19.00	47.33	29.00
P. chlamydosporia	73.67	14.33	14.33	45.33	21.00
Control	66.00	13.00	13.33	39.33	15.67
LSD at P ≤ 0.05	4.65	1.52	1.63	3.94	2.29

*Pooled data of three experiments

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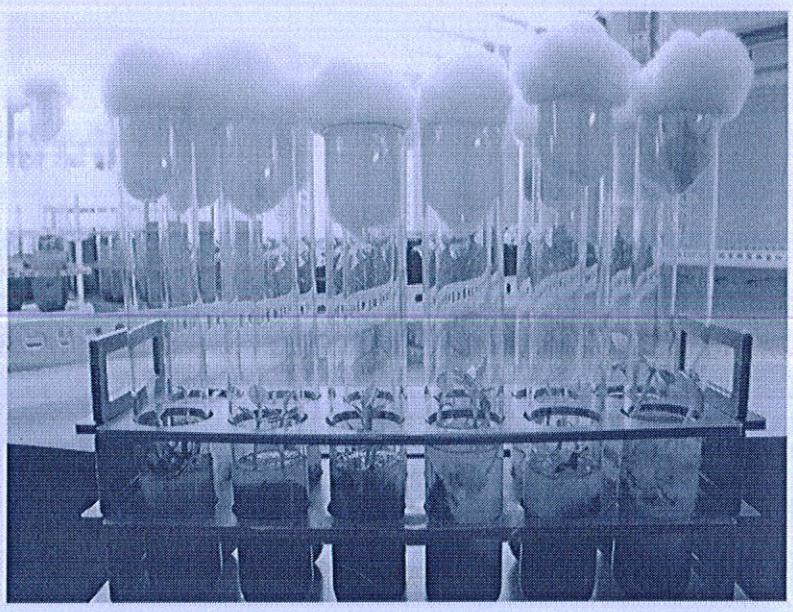

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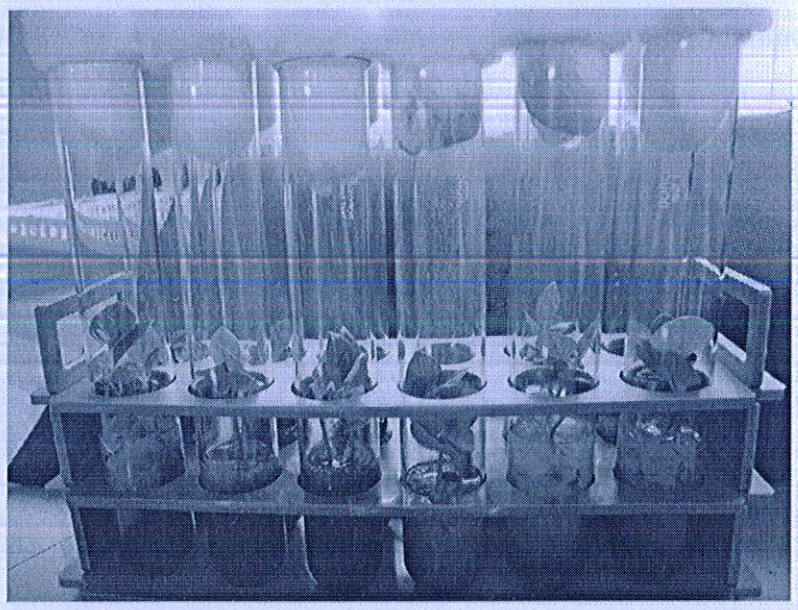

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Figure 1. Growth and maturation of somatic embryo derived plants in WPM. a) Growth after 15 days, b) Growth after 30 days.

Figure 2. Growth of somatic embryo derived plants in primary hardening stage

Figure 3. Growth of black pepper plants in growth promotion experiment

DISCUSSION

The somatic plants were found to be free of endophytic fungi as no fungi were obtained in isolation from the tissues were as the tissues of somatic plants grown in normal conditions were found to be colonized by the fungi. Axenic plants are the plants which are free from any other living forms such systems are limited to cell, tissue, or callus cultures grown on semisolid media or in liquid suspension (14). Production of axenic *Arabidopsis* plants through sucrose supplemented liquid culture was reported (15). This study confirmed that somatic tissue derived black pepper plants are free of endophytic fungi and there were also reports that *in vitro* grown black pepper seedlings are free of endophytic bacteria (16).

Our study reported the successful colonization of endophytic fungi in tissue cultured plants, and the inoculated fungi were re-isolated from the treated plants. Similar colonization studies were reported (17; 18). Hardening of black pepper plants using selected endophytic fungi resulted in better survival (56% -69%) of the plants whereas, only 50% plants survived in control. The hardening of somatic plants in endophyte enriched sterile sand showed better survival this result was in agreement of earlier report that the bio-hardening with fungi (19;20) thus endophytic fungi might be included in the hardening medium. The germination percentage of black pepper stem cuttings in endophytic fungi enriched sand was found to be better than control. The growth promoting ability of endophytic fungi was known (21).

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