Synthetic Seed Production in *Chirita zeylanica* as a Conservation Method

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Abstract
Chirita zeylanica is an endemic flowering herb in Sri Lanka that possesses a greater potential of being an ornamental pot plant. Currently, the plant is subjected to extinction due to collecting plants from its natural habitats. Therefore, there should be a reliable conservation method and also, there should be an efficient way to produce large number of plants in a short period of time. In the present study, production of synthetic seeds was attempted from this species. The structure of the beads and the percentage of germination from encapsulated shoot tips were influenced by the concentration of sodium alginate and the growth regulators used. It was found that among the concentrations tested, 4% sodium alginate produced optimal beads with firm, clear, round and uniform size, and were convenient for handling. It was also observed that the beads produced with 4% sodium alginate and, growth regulator combination of 2.0 mg/L BAP and 0.2 mg/L NAA showed the highest percentage of germination (80%) after 4 weeks from the establishment. The shoot tips of the beads remained green after storage at 4 ^{0}C for a period of 8 weeks. However, all the beads turned to brown color after 4 weeks from the establishment. The findings suggested that the encapsulation method for micro-shoots could be used as a conservation method for *Chirita zeylanica* after developing a procedure to acclimatize the beads for growth under room temperature before establishment.

Keywords: BAP, *Chirita zeylanica*, NAA, Sodium alginate, Synthetic seeds
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Introduction

Chirita zeylanica is an endemic herb with 10–35 cm height which belongs to family Gesneriaceae. It grows in undisturbed montane forests of the moist and intermediate zones at elevations above c. 750 (Dassanayake, 1987). It possesses a greater potential of being an ornamental pot plant due to its beautiful flowers. Currently the plant is subjected to extinction due to collecting it from its natural habitats.

Therefore, *Chirita zeylanica* is named as a threatened plant in the IUCN Red Data Book published in 2012.To commercialize *Chirita zeylanica* as a pot plant, there should be reliable conservation methods. Also, there should be an efficient way to produce large number of plants in short period of time for commercial level production. Micropropagation is an ideal option for obtaining large number of plants. However, when using in-vitro cultures, sub culturing should be done within short intervals, thus making it less-efficient as a conservation method.

Artificial seed production technology is currently considered as an effective and efficient alternative method of propagation of several commercially important agronomic and horticultural crops. Synthetic seeds are small in size and can store for a certain period of time. Therefore, synthetic seeds can be considered as one of the most efficient ways for conservation and commercialization of *Chirita zeylanica*.

The present work was conducted with the main objective of developing a methodology for synthetic seed production in *Chirita zeylanica* and the specific objectives were; to find the best sodium alginate concentration that gives best structure and germination percentage for the beads, to find the best growth regulator combination and to find the storage and germination ability of the synthetic seeds produced.

Materials and Methods

Micro shoots were obtained from *In vitro* grown *Chirita zeylanica* plants maintained under optimum physical conditions (temperature of 26 ± 2 ^{0}C, 75% relative humidity and 16 hour photo period) in a culture room. MS medium was used with 3.0mg/L BAP as culture maintenance medium. Encapsulation matrix consisted of sodium alginate solution at two concentrations (2and 4%) was prepared in MS basal medium solution (pH 5.7) added with 100mg/L Myo-inositol and 30g/Lsucrose.

Two concentrations were selected based on the results of previous studies on different plant species. For hardening process 100mM(14.7g/L) $CaCl_2.2H_2O$ 5 Solution was prepared by dissolving $CaCl_2.2H_2O$ in distilled water.

Separated shoot tips were individually dipped for a few seconds in sodium alginate solution. Then a single shoot tip and alginate mixture was picked up by sterile 2 mm stainless steel spoon. Single coated shoot tip was then dropped into $CaCl_2.2H_2O$ solution. After 30 minutes, the beads were washed with sterilized distilled water.

The best sodium alginate concentration which gives the best structure for the beads was selected by giving marks out of 100 for each bead by observing the characters such as size, shape, strength, whether the shoot was fully covered or not and on easiness for preparation and handling. Collected data were statistically analyzed using Mann-Whitney Test. Thirty replicates were used in each concentration.

Best results were obtained with 4% sodium alginate solution and for the next experiment beads were prepared with 4% sodium alginate solution containing four different growth regulator combinations (Table1). Concentrations for the growth regulators were decided based on the previous studies done on micropropagation of *Chirita zeylanica* and also synthetic seed production of other plant species.

Table 1: Growth regulator combinations used with sodium alginate solutions for the production of synthetic seeds

Treatment	BAP (mg/L)	NAA (mg/L)
T1	2	0.2
T2	2	-
T3	-	0.2
T4	-	-

The encapsulated beads of two sodium alginate solutions and four different growth regulator combinations were stored in a refrigerator at low temperature (4 ± 1°C). The survival rates for germination after 0, 2, 4, 6 and 8 weeks were recorded. For germination purpose, the beads were maintained under the culture room conditions of temperature at 26±2°C, 75% relative humidity and 16hour photo period under fluorescent illumination.

MS medium containing 3mg/L BAP was used as the substrate. Five replicates were tested at each time. The experiments were conducted using completely randomized design. Data collected were statistically analyzed using ANOVA and the means were separated by Duncan's Multiple Range Test (DMRT).

Results and Discussion

According to the Mann-Whitney Test, the beads Prepared with 4% sodium alginate solution showed a significantly higher median than the beads prepared with 2% sodium alginate solution. The beads prepared with 2% sodium alginate solution were hard to prepare, hard to handle, very fragile when handling with the forceps, formed without a proper shape and their size was also not equal. Plant material was also not covered properly. The beads prepared with 4% sodium alginate solution were easy to prepare compared to that with 2% sodium alginate solution. Damages did not happen to the beads when handling with the forceps and the beads were considerably hard. Round shaped beads were obtained and the beads were equal in size. Plant material was properly coated.

It has been reported that the concentration of sodium alginate needed for encapsulation of somatic embryos or micro shoots varies depending on species (Redenbaugh *et al.*, 1986). The hardness of the beads or capsules mainly depends on the number of sodium ions exchanged with calcium ions. 4% sodium alginate solution contains hither number of sodium ions than 2% sodium alginate solution. Hence, the beads prepared with 4% sodium alginate solution were much harder than beads prepared with 2% sodium alginate solution. The beads prepared with 2% and 4% sodium alginate solutions were established in an MS medium containing 3 mg/L BAP for germination just after preparation and after the storage periods of 2, 4, 6 and 8 weeks.

Beads established just after preparation were started to germinate after one week from the establishment. At the 4th week from the establishment, 52% of beads (prepared with 4% sodium alginate solution) were germinated whereas only 26% was germinated from the beads prepared with 2% sodium alginate solution. There was a significant difference in germination percentages ($p < 0.05$).

In stored beads, the green color of the shoot tip was still remained when taken out of the storage even after eight weeks of storage period. After establishment, the shoot tips of the stored beads turned to brownish green color within few days (2–4 days) and gradually turned to brown color. After 4 weeks, all the shoot tips (100%) turned to brown color. The beads prepared with 4% sodium alginate solution containing four different growth regulator combinations were established in an MS media containing 3mg/L

BAP for germination just after preparation and after the storage periods of 2, 4, 6 and 8 weeks. Beads established just after preparation started to germinate one week after the establishment. At the 4th week of the establishment, 80% of beads were germinated from T1 whereas 56%, 66% and 52% beads were germinated from T2, T3 and T4, respectively. The germination percentage of the T1 was significantly different from the other treatments at the 4th week of the establishment ($p < 0.05$).

In previous studies, sodium alginate (encapsulation matrix) has been prepared with different solutions, i. e., either distilled water only, distilled water with hormones or MS solution with hormones (Daud *et al.*, 2008). The beads can potentially serve as a reservoir for nutrients that may aid the survival and speed up growth. In plant tissue culture, auxins promote, mainly in combination with cytokinins, the growth of calli, cell suspensions and organs, and also regulate the direction of morphogenesis (George *et al.*, 2008). In this study, the best results were observed in the treatment containing both auxin and cytokinin. The higher performance may be due to the same reason.

In the experiment, the stored beads were gradually turned in to brown color after establishment. But the shoot tips were still green when they were taken out from the storage. Therefore, it can be assumed that the beads were still viable when taking out from the storage and the viability has been lost after the establishment. The beads established without storage (just after preparation) were germinated without any problem. Therefore the medium had no effect on the viability of the beads. The problem may be in the acclimatization of the beads to the growth room temperature when taking out from the storage. The sudden temperature change may be a stress condition for the shoot tips. For this reason, the artificial seed matrix should be supplemented with nutrients successive in storage. Application of stress preventing chemicals to the encapsulation matrix may be a better option to prevent browning after the establishment of the stored beads.

Synthetic seeds of *Chirita zeylanica* prepared with 4% sodium alginate concentration and growth regulator combination of BAP 2 mg/L and NAA 0.2 mg/L give the best structure and best germination percentage without storage. When storing, further studies are needed for the acclimatization of the beads for the growth at room temperature.

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Screening and Evaluation of the Potential Use of *Fusarium* spp. for Effective Stimulation of Resin Production in *Gyrinops walla*

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Abstract
A study was conducted to explore the efficacy of different *Fusarium* strains in inducing resin production in *Gyrinops walla* and to optimize conditions for *in vitro* mass cultivation of the selected fungal strains. Four *Fusarium* strains isolated from infected plant tissues were used for the screening. Mycelial suspensions (separately or mixed) prepared in 20% and 30% coconut and toddy palm treacle were injected through the drilled holes of two year old *G. walla* stems (girth 32 cm^2). Two months after inoculation, characteristic stem lesions were observed with all the tested strains that indicated successful induction of the defense response leading to agarwood production. All the treatments produced the necrotic streaks along the stem, starting from the inoculation point whereas; the control treatment did not produce such streaks. To identify the optimum conditions for *in-vitro* fungal growth, the fungal cultures on potato dextrose agar (PDA) were subjected to three light levels (total dark, 12 h light/dark, and total light) and three incubation temperatures (25 ^{0}C, 28^{0}C, 32^{0}C).Results revealed that the fungal growth was optimum at 25^{0}C and at 12 dark/light incubation ($P<0.05$). Among three different liquid media (potato broth, chickpea broth and soybean broth) tested for the mass culture of *Fusarium* strains, chickpea broth resulted the highest fresh weight and dry weight of mycelia ($P<0.05$). The study concludes that *Fusarium* spp. can successfully used to artificially induce resin production in *G. walla* and the inoculums can be conveniently produced in large scale under the given laboratory conditions.

Keywords: Agarwood, *Fusarium* spp., *Gyrinops walla,* Mass culture
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Introduction
Agarwood is a highly valued perfumery product obtained from resins of certain tropical tree species such as *Gyrinops walla* and *Aquilaria* spp These resins are produced as induced defense responses to fungal infections. One of the well known fungi reported to induce agarwood formation is an Ascomycetous mold *Phaeoacremonium parasitica* (Crous *et al.*, 1996) However, different fungal strains such as Aspergillus spp., *Botryodiplodia* spp., *Fusarium oxysporum, F. solani, Penicillium* spp., and *Pythium* spp. have been found to be associated with agarwood formation (Soehartono and Mardiastuti, 1997). Since, the availability of this product is very rare, there is always a limited supply compared to the demand. As a solution research has found that agarwood plants could be artificially inoculated with certain fungi to induce the agarwood production. Since the quality of agarwood mostly depends on the plant species and the fungal species involved (Akter *et al.*, 2013), identification of highly efficient fungal strains and optimization of their production conditions are of importance.

Therefore, this study was conducted with the aim of exploring the efficacy of different *Fusarium* strains in inducing resin production in *G. walla* and optimizing the conditions for *in vitro* mass cultivation of the selected fungal strains for inoculum production.

Materials and Methods
This research was carried out in the Kamburupitiya area of Sri Lanka (Agro ecological Zone WL_2) in 2014.

Fungal strains: Five *Fusarium* strains were isolated from infected chilli plants and papaya fruits. Pure cultures were maintained on potato dextrose agar (PDA) and their identity WAS confirmed based on the spore morphology, using a reference manual.

Preparation of inocula: Ten milliliter mycelial and spore suspensions of each of the four *Fusarium* isolates were prepared using sterilized distilled water. Seven days old *Fusarium* cultures were flooded with 10 ml of sterile water, and the surface of the culture was gently scarped using a sterile scalpel. Each 10 ml of the prepared fungal suspension was mixed with coconut and toddy palm treacle at two concentrations each at 20% and 30%.

Inoculation: Two year old *G. walla* plants were selected in a plantation at Kamburupitiya area.

Three replicates were maintained. Holes were made in the plants using a drill up to 5 mm depth at 32 cm^2 girth. The gaps between the holes on the stem were 10 cm (horizontal) x 20 cm (vertical). The prepared inocula were inserted into the holes using a sterile syringe as summarized in the table 1.

Optimum temperature for the fungal growth:Four *Fusarium* strains cultured on PDA, were incubated at three temperatures (25°, 28° and 32° C), in triplicate in completely randomized design (CRD) and the diameters were recorded (average of two perpendicular axes) at 3 and 7 days after inoculation (DAI).

Table 1: Treatments used for the inoculation of *G. walla* plants

Treatment No	Treatment	Carrier substance and its concentration (%)	
1	*Fusarium* strain 1	Coconut treacle	20
			30
		Palm treacle	20
			30
2	*Fusarium* strain 2	Coconut treacle	20
			30
		Palm treacle	20
			30
3	*Fusarium* strain 3	Coconut treacle	20
			30
		Palm treacle	20
			30
4	*Fusarium* strain 4	Coconut treacle	20
			30
		Palm treacle	20
			30
5	Control	Coconut treacle	20
			30
		Palm treacle	20
			30
6	Combined inoculum	Coconut treacle	20
			30
		Palm treacle	20
			30

Optimum light level for the fungal growth:The *Fusarium* cultures were incubated at three light levels (total darkness, 12 light/dark cycles, 24h light) at 25°C, in triplicate in completelyrandomized design (CRD) and the diameters were recorded as explained above.

Best mass cultivation media for fungi:Soya bean dextrose broth (SDB), chickpea dextrose broth (CDB) and potato dextrose broth (PDB) were checked for a suitable mass production media using two of the *Fusarium* isolates (isolates 1 and 3), which showed the highest growth rates. To prepare the broth, 200g of soya bean, 200g of potato and 40 g of chick pea were used after sorting to remove stones and dirt. The material were weighed, washed and boiled in 200 ml of distilled water until soften. The suspensions were filtered using three layers of cheesecloth. Then, 20g of dextrose was added to each filtrate and the volumes were adjusted to 1L, using distilled water. The media were dispensed into 250ml flasks and sterilized using an autoclave at 121°C and 15 psi for 20 minutes. Once cooled, 100ml of each broth were transferred to sterile 100ml conical flasks in triplicate and 5x5 mm^2 agar blocks containing the fungal cultures were introduced to the flasks. The broth cultures were maintained at 27 °C on a mechanical shaker (50 rpm) for 14 days. After the incubation period, mycelial mats in each of the flask were filtered out, washed in distilled water once, blot dried between two paper towels and the fresh weights were obtained. To obtain the dry weights, the mycelia mats on filter papers were kept in a warm oven (40 °C) for 72 hours (Singh *et al.*, 2012) and the weights were recorded.SAS software package (version 5.1.2600) was used to analyze experimental data. ANOVA was performed and the treatment means were separated using DMRT.

Results and Discussion

Assessment of resin production

All the four *Fusarium* strains and their combinedinoculum were able to induce resin formation in the treated plants.The average lesion length produced by theisolates and their combined inoculum rangedfrom 2.5 to 3.5 cm (Figure 1).

Effect of temperature on in vitro proliferation of Fusarium spp.

In all the tested isolates, the highest mycelia growth was recorded at 25 °C and the lowest at 28°C (Figure 2A).

Effect of light on in vitro proliferation of Fusarium

Out of the four strains, the strain four showed maximum growth at 12 hours of light period (Figure 1B). All the strains showed approximately similar growth rate in alternative 12hours of light and dark cycles. The lowest growth was observed at 24 hours light period. Only the strain 1 showed maximum growth at complete darkness.

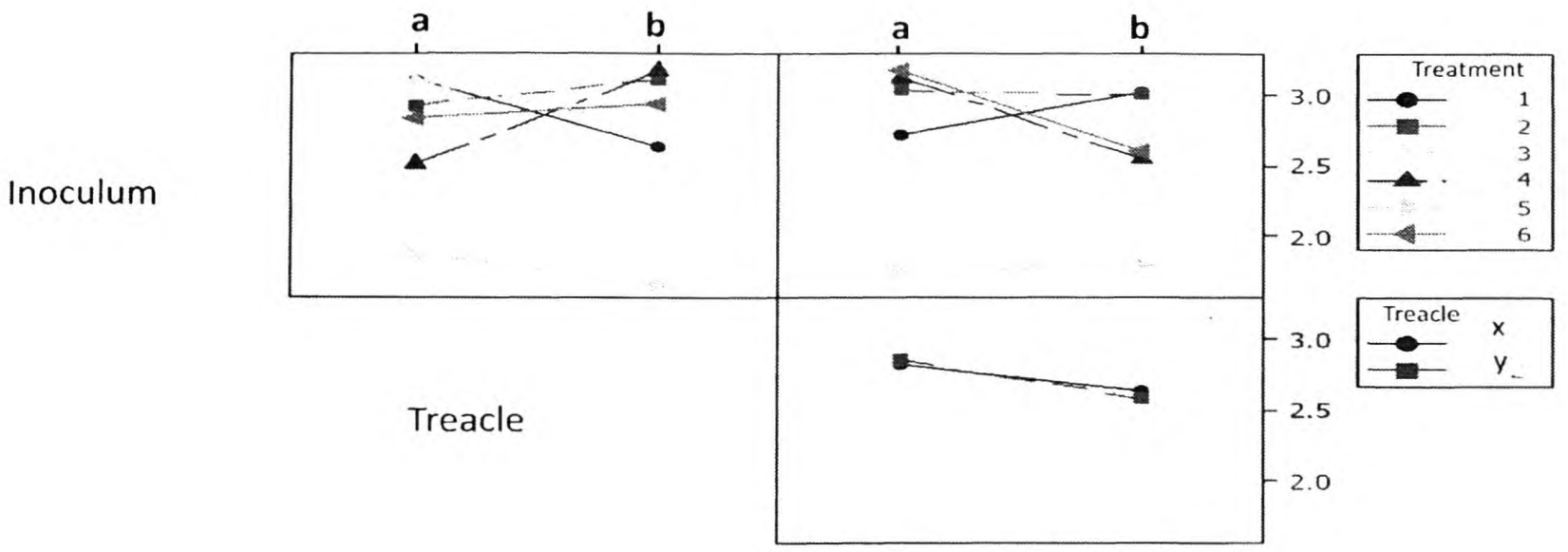

Figure 1: Interaction plot for mean length of lesions on treated *G. walla* stems resulted from four *Fusarium* strains (1-4) and combined inoculum (6) in comparison with the control (5) applied at two concentrations (a-20% and b-30%) of coconut treacle (x) and palm treacle (y)

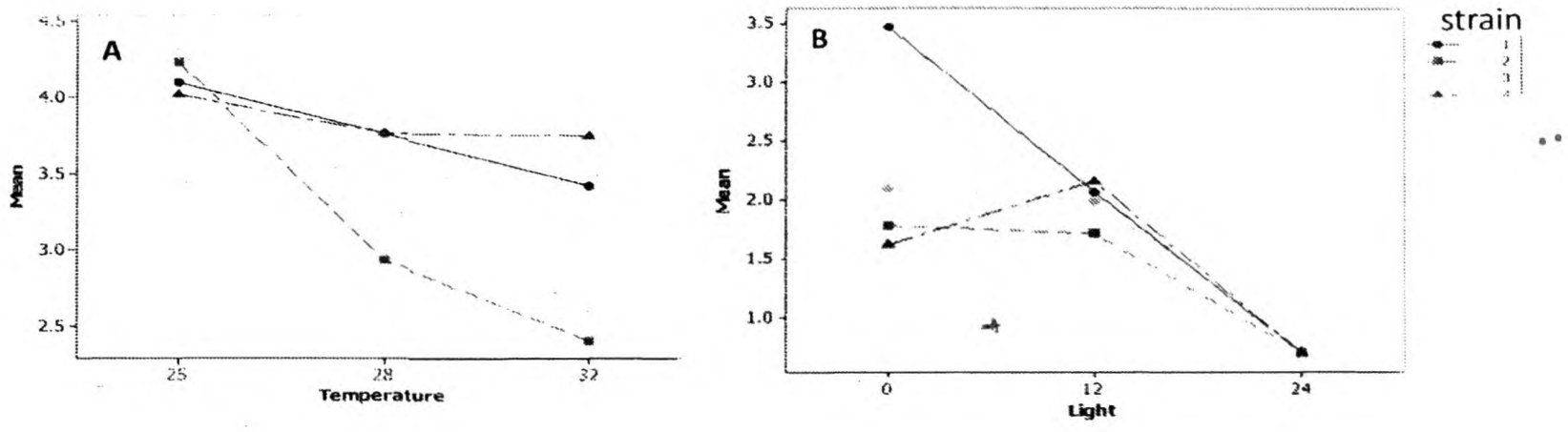

Figure 2: Effect of temperature (A) and light (B) on radial growth of *Fusarium* isolates

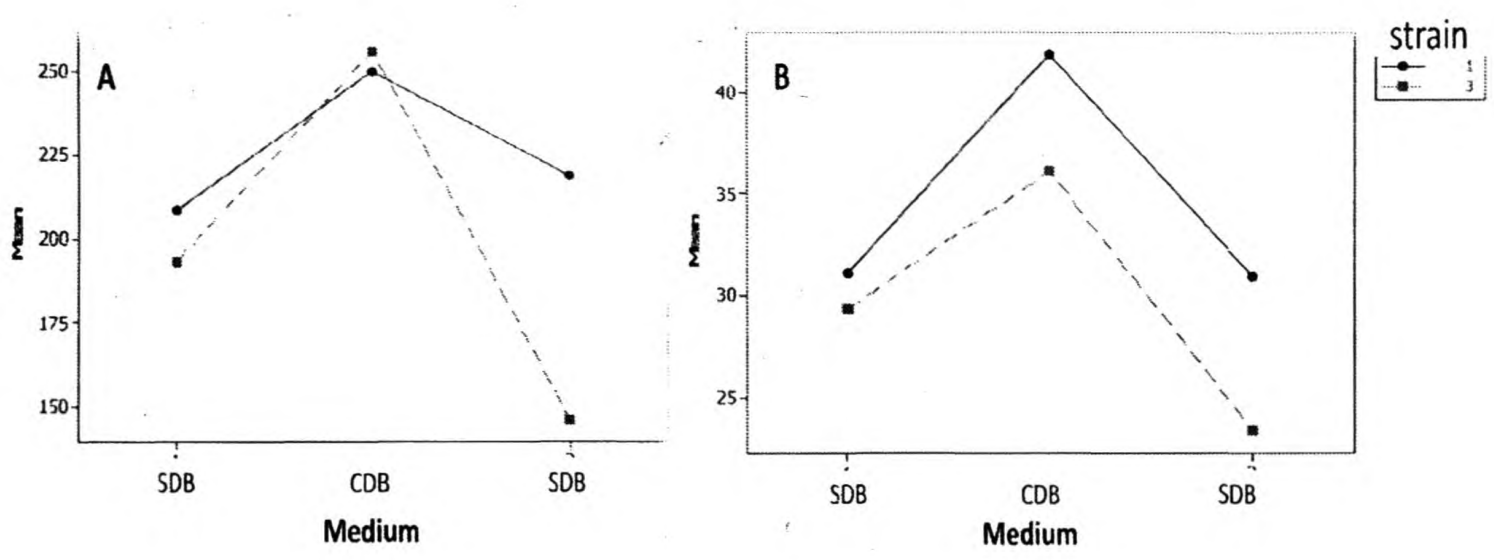

Figure 3: Fresh (A) and dry (B) weights of 14 day old mycelial mats produced by *Fusarium* isolates on different liquid media

Effect of light on in vitro proliferation of Fusarium

Out of the four strains, the strain four showed maximum growth at 12 hours of light period (Figure 1B). All the strains showed approximately similar growth rate in alternative 12 hours of light and dark cycles. The lowest growth was observed at 24 hours light period. Only the strain 1 showed maximum growth at complete darkness.

Evaluation of a liquid media for mass cultivation

The two tested *Fusarium* isolates produced highest fresh and dry weights in chickpea

Dextrose broth (Figure 3). The isolate 1 produced higher fresh/dry weights in SDB and PDB media compared to the isolate 3.

Conclusions

All the four *Fusarium* isolates used in this study effectively induced resin production in *G. walla.* The selected *Fusarium* isolates showed their optimum *in vitro* growth at 25^{0}C and 12 h light/dark conditions. Out of the tested mass cultivation media, chick pea dextrose broth was more suitable in the establishment of liquid cultures for inoculation.

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