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.

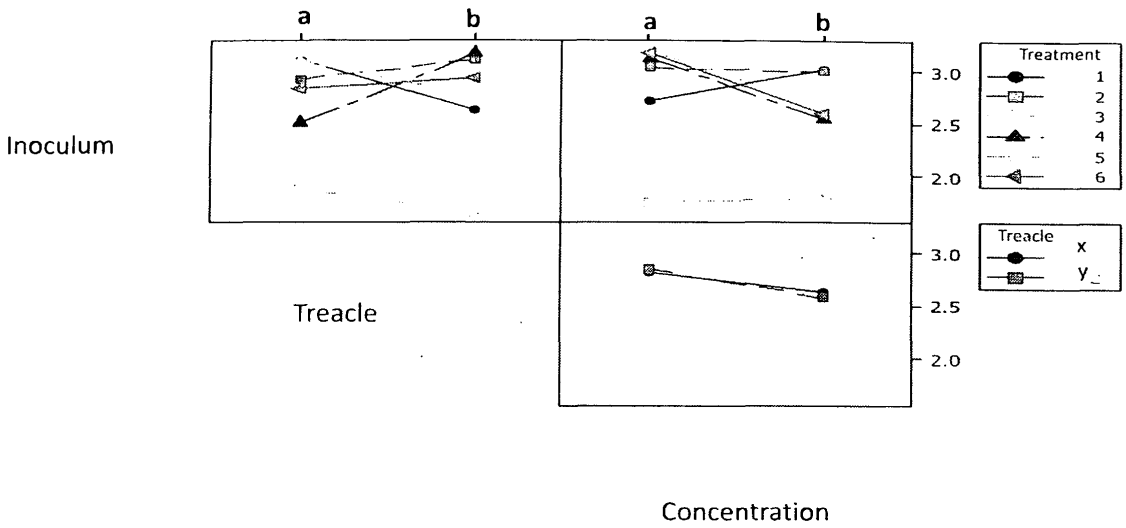

Figure 1: Interaction plot for mean length of lesions on treated *G. walla* stems resulted from four *Fusarium* strains (1-4) and combined inoculum (6) in comparison with the control (5) applied at two concentrations (a-20% and b-30%) of coconut treacle (x) and palm treacle (y)

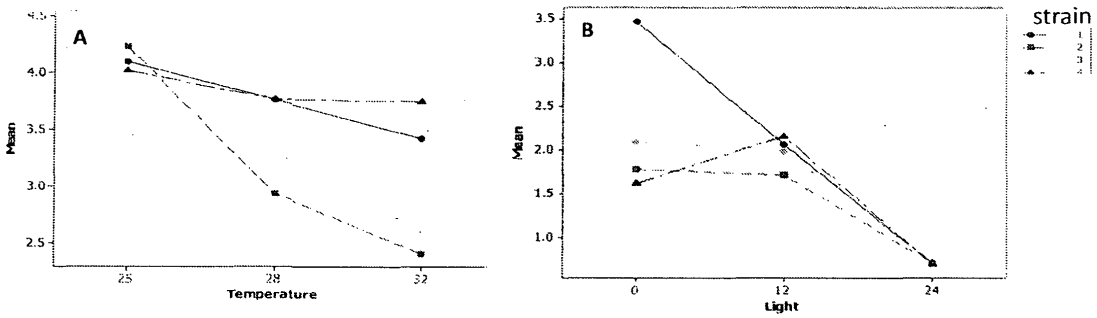

Figure 2: Effect of temperature (A) and light (B) on radial growth of *Fusarium* isolates

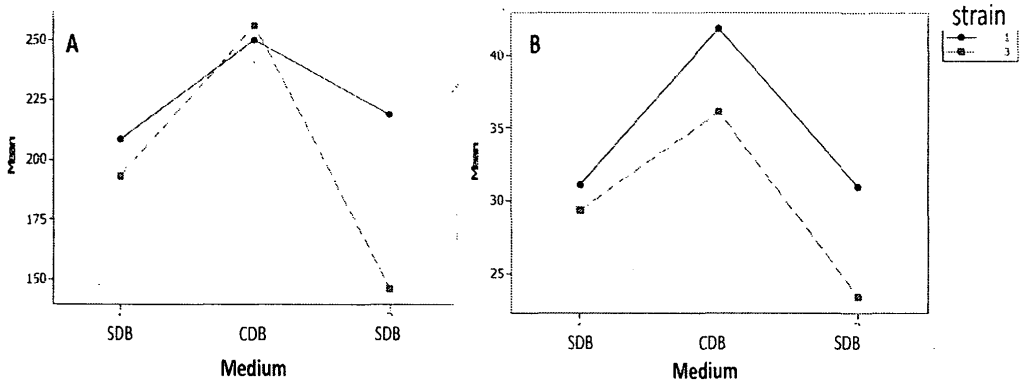

Figure 3: Fresh (A) and dry (B) weights of 14 day old mycelial mats produced by *Fusarium* isolates on different liquid media

Effect of light on in vitro proliferation of Fusarium

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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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