

## NOTE

# FORMULATION OF ALTERNATIVE MEDIA FOR FUNGAL GROWTH AND ITS APPLICATION AS AGARWOOD-INDUCING AGENT IN *AQUILARIA* TREES

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This study investigated the growth performance of *Fusarium proliferatum* isolated from agarwood, on different types of plated media prepared with liquid broth extracts of locally available agricultural products. The potential of *F. proliferatum* in selected media to induce agarwood formation in *Aquilaria* trees was also assessed. A total of 27 media formulations were evaluated as alternative media to potato dextrose agar (PDA) for *F. proliferatum*. The effects of culture media on the diameter growth of fungal mycelium were significant ( $F = 6.70$ ,  $df = 26$ ,  $p < 0.01$ ). The best growth was recorded on 50% soybean followed by PDA and 70% kidney bean with mean mycelium diameters of  $7.00 \pm 0.00$ ,  $6.63 \pm 0.83$  and  $6.50 \pm 0.06$  cm respectively. In the field trial, *F. proliferatum* in all three media successfully induced agarwood formation on *A. malaccensis* and *A. crassna* with significant discoloration zones compared with the uninoculated control trees ( $p \leq 0.05$ ) after five months. Fifty percent soybean in combination with *F. proliferatum* induced the largest discoloration zone, indicating potential as a cheaper and more accessible alternative to PDA. These results suggest that the combination of a chemical inducer in the form of a liquid media formulation and a biological inducer, in this case, an endophytic fungus could successfully induce agarwood formation in *Aquilaria* trees.

Keywords: *Fusarium proliferatum*, alternative media, inoculum, plantation, agarwood

## INTRODUCTION

*Aquilaria* spp. (family Thymelaeaceae) are known to produce agarwood or 'gaharu' in response to fungal infections and insect damage. Agarwood is a high-value non-timber product used in perfumes and pharmaceuticals. It is a valuable natural resource that can provide additional income for local communities but most of the *Aquilaria* species in Malaysia are now considered endangered due to overharvesting. To date, there are no obvious external signs that could be used to detect the presence of agarwood in wild *Aquilaria* trees. This is the reason why agarwood hunters/collectors slash the tree trunks to see if agarwood is present.

In general, only about 10% of *Aquilaria* trees in natural forest contain agarwood (Donovan & Puri 2004). In Malaysia, *Aquilaria malaccensis* and *A. crassna* are widely planted to meet the increasing demand for agarwood and reduce harvesting pressure on dwindling natural stands (Ismail & Mohd Zin 2010).

Inoculation of *Aquilaria* trees using physical, biological and chemical methods to induce agarwood production is an area of active research. An optimised media formulation and efficient inoculation technique that can produce large quantities of high quality agarwood is a most sought-after technology

nowadays. Commercially available liquid medium formulations for agarwood induction are limited and pricey, with tree genotype and environmental factors strongly influencing agarwood induction response and quality (Naziz et al. 2019). Endophytic fungi have been employed as a biological inducer for agarwood formation with promising results (Faizal et al. 2020, Justin et al. 2020). Various species of fungi including *Fusarium* sp. are present in the stems of *A. malaccensis* (Mohamed et al. 2010, Tian et al. 2013). *Fusarium* sp. is a popular biological inducer for agarwood formation (Sitepu et al. 2011, Faizal et al. 2020). To ensure the effectiveness of a biological inducer, the media on which it is cultured must contain the nutrients required to support its growth and reproduction. The culture media itself can act as a chemical inducer for agarwood formation. A properly formulated inducer can induce a good amount of high quality agarwood. While recent induction approaches have largely been based on chemicals applied using custom designed delivery methods (Tan et al. 2019), biological inducers using endophytic fungi cultured in standard media such as potato dextrose agar (PDA) have also shown potential (Faizal et al. 2020). For culturing these fungi, having alternative nutrient sources based on plant or animal extracts may be useful when addressing specific requirements of intended biotechnological applications and also reducing costs in large-scale fermentation (Basu et al. 2015). Studies of the effectiveness of alternative media on the growth performance of a fungus as biological inducer of agarwood formation are lacking. Our study aimed to evaluate the growth performance of an endophytic fungus, *Fusarium proliferatum* on a variety of plant-based and animal-based media formulations. The potential of the fungal inoculant to induce agarwood formation on *A. malaccensis* and *A. crassna* trees in the field was assessed.

## MATERIALS AND METHODS

### Culture media preparation

The liquid media, which form the base for the agar culture media and which were also used for fungal inoculation, were prepared using a variety of locally sourced vegetables, grains,

beef, fish and processed agricultural products that potentially support fungus growth. These main ingredients were pre-soaked with distilled water for 3 hours, boiled in 10% w/v water for 30 min then strained to collect the liquid broth extract. Large pieces, if present in the broth were ground to a paste using mortar and pestle then strained to obtain the liquid broth extract. The collected extract was used directly as liquid media at 100% concentration or diluted with distilled water to various concentrations (Table 1). The liquid media were supplemented with 5% (v/v) local honey and 2% (v/v) NaCl<sub>2</sub>. This supplemented liquid media was used together with the fungus, for inoculating *Aquilaria* spp. trees in the field study. For the fungal growth performance assessment in the laboratory, bacteriological-grade agar at 1.7% (w/v) was added to the liquid media and plated in petri dishes.

**Table 1** Media formulations plated for the fungal growth assessment

Medium	Concentration (% w/v)
PDA	100
Soybean	50
	70
	100
Kidney bean	50
	70
	100
Mung bean	50
	70
	100
Corn	5
	10
	15
Wheat flour	10
	15
Glutinous rice flour	50
	100
Beef extract powder	50
	70
	100
Bread, whole wheat	5
	15
Fish, fresh mackerel	100
Sweet potato	100
Nestum (a commercial cereal)	100
Almond	100
Peanut	100

## Assessing the growth of *F. proliferatum* on different media

The *F. proliferatum* isolate used in this study was obtained from the Mycology and Pathology Laboratory, Forest Biodiversity Division, Forest Research Institute Malaysia. The fungus was cultured on PDA and on the fifth day, mycelial discs measuring 0.5 cm across were extracted. One mycelial disc was placed at the centre of each petri dish containing the various media formulations. Commercial PDA was used as the control medium. Radial growth of the fungus was recorded daily for six days. The experiment was arranged in a completely randomised design with three replicates. Data were subjected to one-way analysis of variance (ANOVA) and mean separations were evaluated by Duncan's multiple range tests using the statistical software SPSS 17.0. The three media that supported the highest growth of *F. proliferatum* were selected as fungal inoculants for the subsequent field trial assessing their potential in inducing agarwood formation.

## Inoculation of field-grown *Aquilaria* spp.

In the field trial, three fungal inoculants chosen for best growth performance in the laboratory experiment were tested on *A. malaccensis* trees at Sungai Tekala, Semenyih, Selangor and *A. crassna* trees at Jasin, Melaka. The trees were about 3 years old with a mean diameter at breast height of  $12.7 \pm 1.7$  cm and mean height of  $7.5 \pm 0.8$  m. At each site, the fungal inoculant treatments were tested on 10 trees each, with 3 uninoculated trees as control, for a total of 33 trees per site. The bottle infusion method was used to administer 300 mL of fungal inoculant to each tree (FDPM 2012). Bottled inoculant was allowed to flow gravitationally through a plastic tube into a 1.5–2.0 cm hole drilled into the tree trunk. When the bottles were empty (after one or two days) the tubes were removed and each hole was plugged with a wooden stick. The inoculation was done in March 2018 and discoloration on the tree trunk indicating development of resins (agarwood) in response to wounding/infection was recorded in April, June and August 2018 using the formula:

$$\begin{aligned} &\text{Longitudinal discoloration length (L, in cm)} \\ &= \text{LU} \pm \text{LL} \end{aligned}$$

where, LU and LL are the upper and lower boundaries of visible discoloration. Data were subjected to one-way ANOVA, and any significant differences among treatments at 1, 3 and 5 months after treatment were compared by Duncan's multiple range tests using the statistical software SPSS 17.0.

## RESULTS

### Growth of *F. proliferatum* on different media

Most of the media formulations supported the growth of *F. proliferatum* (Table 2). The effects of media on fungal mycelium diameter were significant ( $F = 6.70$ ,  $df = 26$ ,  $p < 0.01$ ). Of these, three media, namely, 50% soybean, PDA and 70% kidney bean showed numerically higher support for the growth of *F. proliferatum* with mean mycelium diameters of  $7.00 \pm 0.00$ ,  $6.63 \pm 0.83$  and  $6.50 \pm 0.06$  cm respectively compared to the rest of the media. Soybean has been reported as an effective medium for the growth of *F. oxysporum* (Ikechi & Elenwo 2015), *F. virguliforme* and several other species of fungal pathogens (Xiang et al. 2014) as it contains carbohydrates, sugar, fibre, fat, protein, minerals and vitamins essential for the growth of fungi (Ikechi & Elenwo 2015). Protein, especially from legumes, is a good fungal culture medium (Ravimannan et al. 2014). Carbohydrates, lipids, proteins and nucleic acids are the most important nutrients needed for microbial growth (Basu et al. 2015). These compounds are abundant in commonly available legumes (Kumar & Xu 2017) and potato (Srivastava et al. 2011). In addition to needing organic compounds to grow, microbes also need Mn, Zn, Co, Mo, Ni and Cu for enzyme activity (Basu et al. 2015).

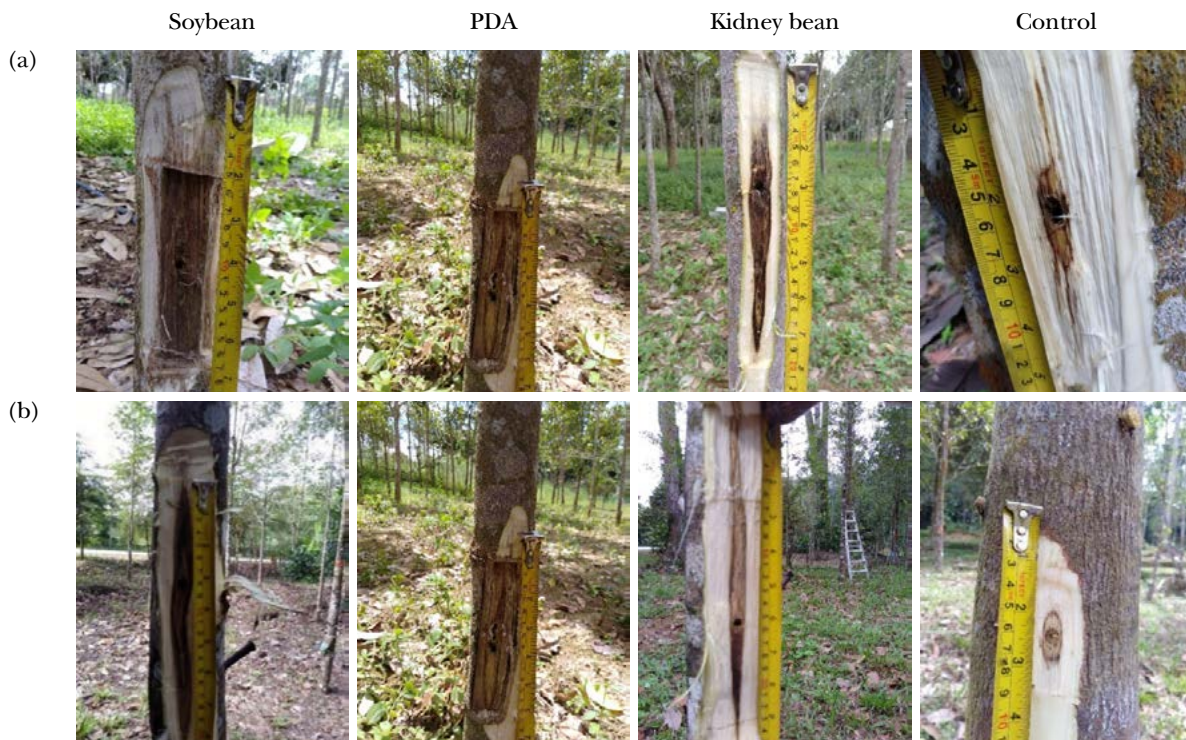
### Development of resinous zones in *Aquilaria* spp.

All three fungal inoculants (50% soybean, PDA and 70% kidney bean, all inoculated with *F. proliferatum*) induced the development of significantly larger resinous zones than the control ( $p < 0.05$ , Figure 1). The resinous zone sizes for inoculated trees were double that of control three months after inoculation, and

**Table 2** Mean growth ( $\pm$  SD) of *Fusarium proliferatum* on plated culture media prepared with liquid broth extracts of locally available agricultural products

Ingredient	Concentration (% w/v)	Diameter of mycelium (cm)
Soybean	50	7.00 $\pm$ 0.00 <sup>a</sup>
PDA	100	6.63 $\pm$ 0.68 <sup>a</sup>
Kidney bean	70	6.50 $\pm$ 0.06 <sup>a</sup>
Mung bean	100	6.00 $\pm$ 0.58 <sup>ab</sup>
Kidney bean	50	6.00 $\pm$ 0.00 <sup>ab</sup>
Kidney bean	100	5.76 $\pm$ 0.58 <sup>ab</sup>
Bread	15	5.75 $\pm$ 0.29 <sup>ab</sup>
Beef	50	5.52 $\pm$ 0.64 <sup>abc</sup>
Fish, fresh mackerel	100	5.29 $\pm$ 0.84 <sup>ab</sup>
Soybean	70	5.27 $\pm$ 0.13 <sup>abc</sup>
Soybean	100	5.25 $\pm$ 0.43 <sup>abc</sup>
Wheat flour	10	4.93 $\pm$ 0.35 <sup>abc</sup>
Corn	5	4.92 $\pm$ 2.46 <sup>abc</sup>
Corn	15	4.67 $\pm$ 2.34 <sup>abc</sup>
Corn	10	4.67 $\pm$ 2.34 <sup>abc</sup>
Sweet potato	100	4.67 $\pm$ 0.88 <sup>abc</sup>
Nestum (a commercial cereal)	100	4.42 $\pm$ 2.21 <sup>bc</sup>
Bread	5	4.12 $\pm$ 2.06 <sup>bc</sup>
Beef	70	3.72 $\pm$ 1.87 <sup>bc</sup>
Beef	100	3.58 $\pm$ 1.80 <sup>bc</sup>
Almond bean	100	3.58 $\pm$ 0.82 <sup>bc</sup>
Mung bean	70	3.35 $\pm$ 0.38 <sup>bc</sup>
Groundnut	100	3.03 $\pm$ 1.76 <sup>bc</sup>
Glutinous rice flour	50	2.35 $\pm$ 1.74 <sup>cd</sup>
Glutinous rice flour	100	2.35 $\pm$ 1.74 <sup>cd</sup>
Wheat flour	15	2.28 $\pm$ 2.28 <sup>cd</sup>
Mung bean	50	1.65 $\pm$ 0.03 <sup>d</sup>

\*Means followed by a different letter are significantly different ( $p < 0.05$ )



**Figure 1** Discoloration zones on (a) *Aquilaria crassna* and (b) *Aquilaria malaccensis* trees 5 months after inoculation with *Fusarium proliferatum* in PDA, 70% kidney bean and 50% soybean liquid media compared with uninoculated controls

triple that by the fifth month (Figures 1 and 2). The response of both *Aquilaria* species to the inoculants appeared to be similar. The extension of discoloration is related to the incubation period, whereby longer incubation time produces darker agarwood formation (Mohamed et al. 2014).

The size of resinous zones formed in the inoculated trees in the field did not differ significantly between inoculant treatments. The 50% soybean and 70% kidney bean media induced comparable discoloration to standard commercial PDA indicating that alternative media formulations could be used in place of standard culture media as fungal growth promoters and as chemical inducers in the early stages of agarwood formation.

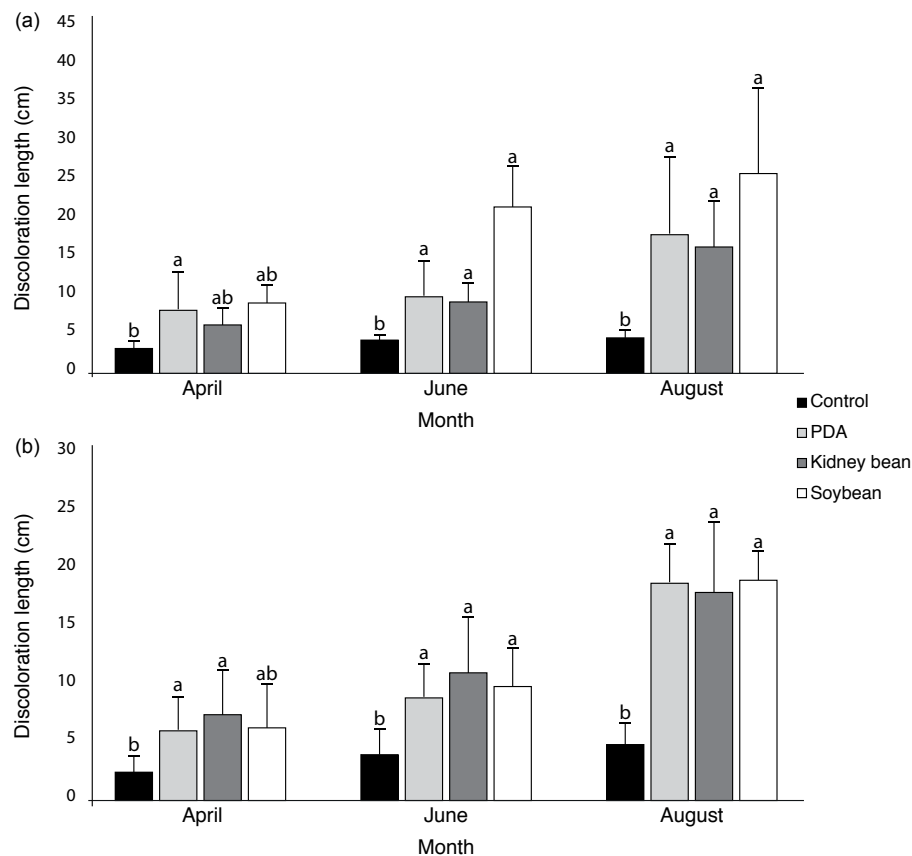
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**Figure 2** Mean size of discoloration zone ( $\pm$  SD) on (a) *Aquilaria crassna* and (b) *Aquilaria malaccensis* at 1, 3 and 5 months after inoculation with *Fusarium proliferatum* in PDA, 70% kidney bean and 50% soybean liquid media; different letters indicate significantly different means within month ( $p < 0.05$ )

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