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Incidence of Fern Contamination in Nodal Segment Cultures of *Shorea parvifolia* Dyer

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ABSTRACT

The greatest drawback in large scale micropropagation of tropical woody forest species is high contamination of cultures. In developing a sterilization protocol for micropropagation of *Shorea parvifolia* Dyer utilizing nodal segments excised from nursery-grown seedlings, it was found that washing 20% (v/v) with Clorox solution for 18 minutes was the best. After six weeks of culture in WPM media supplemented with 10^{-5} M BAP (apart from fungal and bacterial contamination), the nodal segments developed hair-like structures which were amenable to subculture. Upon subculture, green leafy structures developed from the mass of hairy structure after six weeks. These later developed into ferns which are normally found as epiphytes on older forest trees, known as *Asplenium nidus*.

Keywords: Shorea parvifolia, sterilization, contamination, *Asplenium nidus*

INTRODUCTION

Shorea parvifolia Dyer, or commonly known as *Meranti Sarang Punai* in Malaysia is an important timber species under the red Meranti group. It is one of the potential species for plantation purposes due to its multipurpose uses as interior joinery, domestic and light traffic flooring, utility furniture, domestic woodware, manufacture of plywood, railings ceiling framing, and lorry body work. It can grow in different sites and attain high percentage of survival. Propagation of this species is normally

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through seedlings raised from seeds and saplings. However, problems faced in propagating tropical species, including *S. parvifolia*, are related to seed production and seed viability. Its unpredictable flowering and seeding years, including obtaining large quantity of seeds, are becoming an ever increasing problem for large scale plantation programme. Moreover, it produces seeds which are recalcitrant in nature and thus possesses storage problem that will further hinder regular production of sufficient and quality planting materials. Nonetheless, the propagation of this species by stem cuttings is possible to a certain extent from juvenile plants (Aminah, 1991).

Propagation through tissue culture provides an alternative method of fulfilling this requirement. It is envisaged that development of successful micropropagation technique will be a boon to large-scale tropical plantation establishment. Micropropagation of tropical woody species with nodal segments as the initial explants have been reported in *Shorea stenoptera* (Sakai & Yamamoto, 1992), *Dryabalanops lanceolata* (Ishii *et al*., 1992; Sakai & Yamamto, 1992; Yamamoto *et al*., 1993), *S. platyclados*, *S. selanica*, *S. pauciflora*, and *S. laevis* (Yamamoto *et al*., 1993), *Dyera costulata* (Aziah & Darus, 1995) and *Aquilaria malaccensis* (Aziah *et al*., 2000). There are numerous culture methods available for timber tree species and some of the methods are successful in culturing certain species of tree (Pijut *et al*., 2012). Plant tissue culture has been exploited for numerous applications, which include both research and commercial applications. Even though it is possible to mass propagate plants by micropropagation, the greatest problem in this technique is contamination (Altan *et al*., 2010; Cassells, 2012). It is exceptionally very difficult to maintain and grow cultures under *in vitro* conditions that are free from any biological contaminants. The biological contaminants may or may not be pathogens but will influence the growth of the tissue by producing metabolites. This is because the growth of these biological contaminations will compete with the growth of the culture (Cassells, 2012). In most cases, contaminants in plant tissue cultures have been identified to vary in a wide range of microorganisms, including filamentous fungi, yeasts, bacteria, viruses, viroids, mites and even thrips. They may be introduced with the explant, during manipulation in the laboratory, via micro-arthropod vectors (Leifert & Cassells, 2001) or by endophytic bacteria (Pereira *et al*., 2003). On the other hand, fungus contaminants may arrive from the explant or airborne, or enter a culture (Babaoglu *et al*., 2001). Frequent contaminations by bacteria and fungus have lead researchers to incorporate the uses of antibiotics and fungicides to combat the problems (George, 1993; Cassells, 2012). Nevertheless, to date, there has been no report on contamination by epiphytic fern or its allied species on micropropagation work on plants.

Development of suitable sterilisation protocol is needed to eradicate contaminants on the outer surfaces of the explants. However, tissue culturists must address

issues that are associated with random endophytic contamination of stock plant tissues by environmental microorganisms (Cassells, 2012). There is a sceptic amongst scientists on the existence of sterile cultures and whether plant cultures can be microorganism free (Leifert, 1990). Thus, this study was undertaken to identify all possible contaminants and to examine the effects of different sterilants and their combinations in the micropropagation of *S. parvifolia*. In addition, this paper records and discusses the incidence of an epiphytic contamination by fern, *Asplenium nidus* in *S. parvifolia.*

MATERIALS AND METHODS

Plant Sources

The explants used for the sterilization protocols were shoot tips and nodal segments. They were obtained from shoots of about 8-9 cm length that were excised from three-year-old *S. parvifolia* seedlings raised in the FRIM's nursery by the Tree Improvement Unit and Seed Technology unit of Forest Research Institute (FRIM) for the purpose of progeny testing. About 100 seedlings were made available for micropropagation studies. They were raised from seeds collected from plus trees of Ulu Tranum Forest Reserve, Pahang. Thus, it was ensured that the fruit/seeds were carefully collected manually by skill climbers and were kept in plastic bags.

These seeds were initially germinated in 2.5cm x 2.5cm polybags containing a mixture of 1:3 (v/v) proportions of sand and soil. They were watered twice daily and maintained in the nursery under 50% shade by covering with a netting for four weeks until the seedlings developed two-leaf stage. They were then transferred into 9cm diameter by 17cm height polybags containing one litre potting medium that was made up of equal volumes of a mixture of forest soil and paddy husk. The seedlings were also watered twice daily and they were fertilised at monthly intervals with commercial fertilizer NPK (12:12:117). Insecticides and fungicides were also applied whenever necessary. The seedlings/ saplings were kept initially under 33% light, which was gradually increased to 50% and finally to 70% light.

Development of Sterilization Protocol

The sterilization protocols utilised in this study are as described and summarized in Table 1. In each protocol, the explants were placed in a 500ml Erlenmeyer flask and the volume of each solution was 250ml. Several sterilization methods were adopted and sterilants used include mercuric chloride ($HgCl_2$), benlate (with and without streptomycin), and Clorox (with and without pH) that was adjusted (pH6-pH11). After being subjected to the sterilization procedure, these shoots were further apportioned into 1-1.5 cm stem segments, which included the nodal segments and shoot tips and were utilised as the initial explants. The explants were then cultured in culture tubes (125mm X 25mm) containing 20ml of Woody Plant Medium (WPM) (McCown & Lloyd, 1981) that was supplemented with concentrations of 10^{-4} M to 10^{-7} BAP. A total of 40 explants were used for each treatment.

TABLE 1
The sterilisation protocols developed for nodal segment explants excised from nursery raised seedlings of *S. parvifolia.* Sterilants used include mercuric chloride ($HgCl_2$), benlate, with or without streptomycin, Clorox with pH adjusted (pH6-pH11) and also clorox with pH unadjusted.

Types of Sterilant	Sterilisation Procedure
Mercuric chloride ($HgCl_2$)	Three methods utilised are as follow: i) Method (a): The 8-9 cm shoot-portions were immersed in 70% ethanol for 30 seconds. This was then followed by washing in a mixture of 0.1% $HgCl_2$ mixed with Tween 20 for 10 minutes. The shoot segments were then rinsed in several rinses of sterile distilled water. ii) Method (b) is similar to method (a) except that instead of rinsing in a mixture of 0.1% $HgCl_2$ mixed with Tween 20 for 10 mins, the duration was 15 minutes. iii) Method (c) is similar to Method (b) except that after washing in mixture of 0.1% $HgCl_2$ and Tween 20 for 15 minutes, the shoots were segmented into 1-1.5cm portions and washed in 10% Clorox prior to the last step of rinsing with sterile distilled water.
Benlate	Two methods utilised are as follow: i) Method (d), 8-9 cm shoot segments were immersed in 0.05% Benlate with 200mg/l streptomycin for 2 days. This was then followed by washing in 10% Clorox and subsequently with several rinses of sterile distilled water ii) Method (e) is similar to method (d) but without streptomycin. This was then followed by washing in 10% Clorox and subsequently with several rinses of sterile distilled water.
Clorox with pH adjusted	Method (f), 8-9 cm shoot segments were immersed in 20% Clorox pH adjusted to i) pH 6, ii) pH 7, iii) pH 8, iv) pH 11 for 15 minutes. This was then followed by several rinses of sterile distilled water.
Clorox	Method (g), 8-9 cm shoot segments were immersed in sterile distilled water mixed with Tween 20 for 5 minutes. The Tween 20 solution was then poured off and 20% Clorox was poured into the flask until the solution spills over and was left standing for 18 minutes. Half of the 20% Clorox was poured off and the flask was then hand shaken for 2 minutes. Finally the shoots were rinsed with sterile distilled water 8 to 10 times.

The effects of sterilization protocols on these explants were observed after four weeks in the culture. The contamination percentage for each sterilization method was enumerated by calculating the number of tubes contaminated divided by the total number of tube for the particular sterilization protocol. The data obtained was analyzed using a complete randomised design that was run by the Statistical Analysis System (SAS). Further mean separation tests were evaluated using the Duncan's Multiple Range Test. Means differing at a probability of $\leq 0.05\%$ were considered to be significantly different.

Identification of Microbial Contaminants

Prior to identification, the fungi were isolated onto Potato Dextrose Agar (PDA) (DIFCO No. 0013-01-4), which was prepared by suspending 39g in 1 litre distilled or ionised water and boiled to dissolve completely. The

PDA was sterilised by autoclaving at 105 kPa for 15 min. After cooling to approximately 40°C, the molten medium was poured into 9cm Petri dishes in the laminar flow cabinet. The identification of fungi was carried out using the morphological characteristics by the FRIM forest pathologist.

For the identification of the bacteria, samples were first cultured from the explants in Lennox broth (LB) that was prepared by dissolving 20g in 1 litre distilled or de-ionised water and 15 ml volumes dispensed into 25 ml McCartney bottles prior to autoclaving at 105 kPa for 15 min. A loopful of the broth was then streaked onto a 9cm Petri dish containing Nutrient agar (NA) (Merck) and incubated overnight at 30±1°C. The NA media was prepared by dissolving 20g in 1 litre distilled or de-ionised water and sterilised by autoclaving at 105 kPa for 15 min. After cooling to approximately 40°C, the molten medium was poured into 9cm Petri dishes in the laminar flow cabinet at 17 ml per Petri dish.

Single bacterial colonies were isolated and streaked for a second time onto NA to further purify the culture with incubation overnight at 30±1°C. The bacterial cultures were stored at 4°C before despatching to the Malaysian Agriculture Research and Development Institute (MARDI) for identification using the HP5898A Microbial Identification System (MIS). MIS utilises qualitative and quantitative analyses of the fatty acid composition of the organisms (MIS Operating Manual, 1984).

RESULTS AND DISCUSSION

The effects of sterilisation protocols on the nodal segments described above were observed after 4 weeks in culture. The contamination percentage was recorded and tabulated in Table 2. The most effective results were obtained with method (g) using Clorox that recorded only 38.7% contamination. The main contaminants found after four weeks of culture were fungus and bacteria. The fungal contamination observed included feathery white contaminants that were identified as *Collectotrycum* spp. This particular fungus is known to cause damping-off and leaf disease in *S. parvifolia* and infections were observed at the seed, seedling and sapling stages (Elouard & Zakaria, 2000). The bacterial contaminants, which were slimy white or red colonies, were identified using the MIDI Microbial Identification System (MIS). They were identified as *Kleibsella planticola*, *Enterobacter agglomerans*, *Erwin auredora, Erwina herbicola*, *Serratia odorifera*, *Serratia marcesens*, *Serratia proteomaculans*, *Morganella moragnii*, and *Kluyera ascorbata.*

Apart from contamination due to microbial contaminants, the nodal segments derived from the nursery-grown seedlings developed hair-like structures, which were amenable to subculture (Fig.1). After six weeks in the culture, green leafy structures developed from the mass of hairy structure (Fig.2). These later developed into ferns that were normally found as epiphytes on older trees, known as *A. nidus*. The ferns could be multiplied further and spontaneous

TABLE 2
The effects of different sterilization protocols on the nodal and shoot tip explants excised from nursery raised seedlings of *S. parvifolia.* Percentage contamination was observed after 4 weeks in the WPM medium

Types of Sterilent	Code	Sterilisation procedure	Contamination* (%)
$HgCl_2$	a	• 70% ethanol 30 seconds • 0.1% $HgCl_2$+ Tween 20, 10 minutes • several rinses of sterile distilled water	90.7[a]
	b	• 70% ethanol 30 seconds • 0.1% $HgCl_2$+ Tween 20, 15 minutes • several rinses of sterile distilled water	92.0[ab]
	c	• 70% ethanol 30 seconds • 0.1% $HgCl_2$+ Tween 20, 15 minutes • segmented shoots washed in 10% Clorox • several rinses of sterile distilled water	88.0[ab]
Benlate	d	• 0.05% Benlate (Immersed for 2 days) • 10% Clorox • several rinses of sterile distilled water	65.3[c]
	e	• 0.05% Benlate +200mg/l streptomycin (Immersed for 2 days) • 10% Clorox • several rinses of sterile distilled water	70.7[c]
Clorox (pH adjusted)	f i	• 20% Clorox adjusted to pH 6 for 15 minutes • several rinses of sterile distilled water	98.7[ab]
	ii	• 20% Clorox adjusted to pH 7 for 15 minutes • several rinses of sterile distilled water	100[a]
	iii	• 20% Clorox adjusted to pH 8 for 15 minutes • several rinses of sterile distilled water	98.7[ab]
	iv	• 20% Clorox adjusted to pH 11 for 15 minutes • several rinses of sterile distilled water	100[a]
Clorox	g	• sterile distilled water with Tween 20 for 5 minutes • 20% Clorox poured into flask until solution spills over and left standing for 18 minutes • pour out half of solution and shake flask for 2 minutes • rinse with sterile distilled water 8 to 10 times	38.7[d]

*Means with the same letters are not significantly different at a= 0.05 according to Duncan's Multiple Range Test

rooting occurred. However, none of these ferns survived upon acclimatization. On the contrary, Mazumder *et al*. (2011) reported that a medicinal fern *Drynaria quercifolia* can be successfully micropropagated only when it was cultured on MS-Z4 medium (Full MS+1mg/L IAA+5mg/L Kinetin+20%CM+300mg/L CH). This indicates the difficulty of constructing a suitable medium for the propagation of fern which is nevertheless a contamination in the present experiment despite the use of the WPM medium.

Fig.1: Development of hairy-like structures (possibly rhizoids) on the nodal segment explants excised from nursery raised seedlings (the diameter of the vial is 25mm)

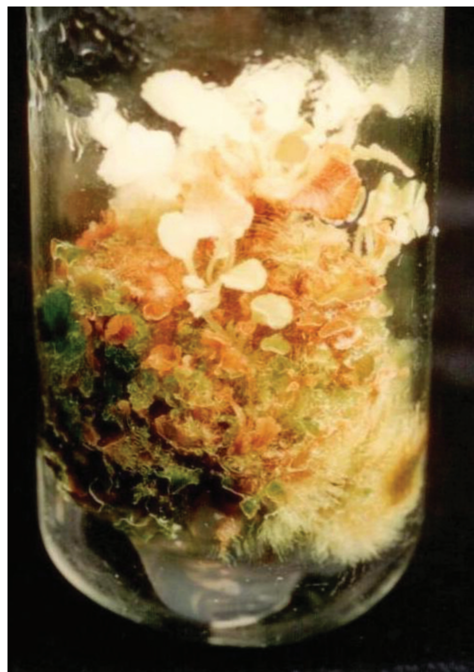

Fig.2: The development of leafy structures, young sporophytes in the mass of hairy-like structures which later developed into ferns (the diameter of the vial is 25mm)

Leifert *et al.* (1991) suggested that plant cultures should not be described as aseptic, sterile or free of contaminants, but instead as "index negative" or "free of detectable contaminants". The term index negative cultures described the state of cultures that have undergone assessment(s) by one of more microbial indexing method. The term "free of detectable contaminants" should be used for situations where cultures were visibly assessed. In most cases, the microbial contaminants were mycelia fungi, yeasts or bacteria (Leifert, 1990). Contamination may also arise in the cultures that were contaminant-free for several subcultures but became contaminated with bacteria after a long period in the culture due to occurrences of endogenous, endophtytic or internal bacteria that became pathogenic after a prolonged period of latency in the plants (Bastiaen, 1983). Meanwhile, the existence of bacteria in *Pinussyvestris* L. bud was detected by 16S rRNA *in situ* hybridisation and it was found that the bacteria inhabited the buds of every tree examined, primarily colonizing the cells of scale primordium and resin ducts (Pirttila *et al*., 2000). Sudden outbreaks of the bacterial contamination resulting in the loss of whole crops and high contamination rates in commercial concerns make production of some plants uneconomical. Most yeasts, mycelia fungi and bacteria isolated from the plant tissue cultures are not known to be pathogenic to plants in the field (Leifert *et al*., 1994). Laboratory environments, which include the indoor air, have been known to contribute to contamination (Odutayo *et al*., 2007).

However, reports on the bacteria associated with Dipterocarpaceae are not available. In the study conducted by Ramesh *et al*. (2012), no bacterial contamination was observed after sterilization of the explants. However, members of some genus of the bacteria identified have been reported to be associated with some tropical plants. *K. planticola* is a common soil bacteria and its genetically engineered strain has been utilised to assess the potential effects on soil biota and plant growth (Holmes *et al*., 1999). Although *K. planticola* has not been identified as a pathogen of any woody tropical species, its relative *Klebsiella* spp. has been associated with the bark necrosis of *Hevea brasiliensis* (Chee, 1976) and isolated from wilted plant of tomatoes (Williams & Liu, 1976). Similarly, *E. agglomerans* has not been reported to be a pathogen in woody species. However, *E. aerogense* has been associated with bud rot in *Cocos nucifera* (Williams & Liu, 1976), found on the bark of brown bast tree of *H. brasiliensis* (Chee, 1976), and associated with the root rot of *Mangifera indica* (Williams & Liu, 1976). Another member of the genus, *E. cloacae,* has been associated with the trunk canker of *H. brasiliensis* (Anon, 1967) and stem canker in *Lansium domesticum* (Williams & Liu, 1976). *Enterobacter* spp. has also been reported to be associated with stem canker (Williams & Liu, 1976). Khan and Doty (2009) identified the strains belonging to the genus *Enterobacter* as one of the endophtyic bacteria associated with sweet potato plants. Members of the bacteria genera *Erwina* have been known to cause diseases in the *Citrus*

genera. *E. citrimaculans* (Doidge) Magrou has been isolated from the branches of *C. mitis* (Blanco), *E. herbicola* (Lohnis) Dye from leaves of *C. aurantifolia* and *E. lathyri* (Manns and Taubenhaus), Magrau from the scaly bark of *C. grandis* (Williams & Liu, 1976). Meanwhile, *Serrattia marcesens* has been isolated from the brown bast tree of *H. brasiliensis*, (Johnston, 1960), whereas S. *odorifera* has not been reported to be a pathogen of any woody species. *Morganella morganii* and *Kluyvera ascorbata* have not been reported to be isolated from any of the tropical agricultural crop.

One interesting observation in this study is the incidence of fern contamination. The fern *A. nidus,* an epiphyte, was found to have contaminated the nodal segment explants from the nursery-raised seedlings. The ferns only live on the surface of the barks and gather all its water from its roots, which usually form a mat and gradually collect humus. It absorbs the moisture during rain and from dew at nights. The trees with these epiphytes are normally unharmed, except that a large mass of them may have a smouldering effect or breaking a branch due to its excessive weight. Food supply is restricted, except for those that have special humus gathering capabilities. The amount of food needed by ferns is surprisingly small and it can be obtained from the decaying bark, fallen leaves, dust, and debris, which the rain may wash down from higher parts of the tree (Holttum, 1954). *A. nidus* is also known as birds nest fern that is commonly found both in lowlands and the mountains; it is a large epiphyte and found throughout the tropics of the Old World. It is frequently found on roadside trees, as well as in plantations of tree crops and on jungle trees. Long narrow sori are produced along the veins of the upper parts of the fronds and they reach from near the midrib to halfway or more towards the edge. These ferns are found aplenty on trees in the surrounding areas of FRIM's open nursery. The spores could have flown off the fronds of the ferns and landed onto the seedlings of *S. parvifolia* planted in polybags which were left in the open. At the point of explant excision, the spores could have been embedded onto the nodal segments and developed further in the media as conditions are the most suitable for its development.

The hair-like structures formed on the *S. parvifolia* nodal segment, which later developed into leafy structure, is the prothallus of gametophyte stage of the fern. They were amenable to subculture and being confirmed as fern, identified as *A.nidus* by a botanist in FRIM. However, these ferns did not survive upon acclimatization. On the other hand, Khan *et al*. (2008) successfully developed micropropagation techniques for *A. nidus*, whereby an addition of sodium dihydrogen sulphate resulted in differentiation to the sporophytic stage.

CONCLUSION

Loss of cultures caused by a contamination of microbes, which includes fungus, bacteria and yeast, is a major hindrance in the *in vitro* establishment of *S. parvifolia* and other tropical forest species cultures. However, this study has shown that epiphytic ferns such as

A. nidus is a new source of contamination to *in vitro* cultures.

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