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...
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 & Yamamoto, 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 microarthropod 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 (HgCl₂), 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.
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
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 *Klebsella 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
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.

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

<table>
<thead>
<tr>
<th>Types of Sterilent</th>
<th>Code</th>
<th>Sterilisation procedure</th>
<th>Contamination* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HgCl₂</td>
<td>a</td>
<td>70% ethanol 30 seconds</td>
<td>90.7&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>0.1% HgCl₂+ Tween 20, 10 minutes</td>
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<td></td>
<td></td>
<td>several rinses of sterile distilled water</td>
<td></td>
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<tr>
<td></td>
<td>b</td>
<td>70% ethanol 30 seconds</td>
<td>92.0&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>0.1% HgCl₂+ Tween 20, 15 minutes</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>several rinses of sterile distilled water</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>70% ethanol 30 seconds</td>
<td>88.0&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>0.1% HgCl₂+ Tween 20, 15 minutes</td>
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<tr>
<td></td>
<td></td>
<td>segmented shoots washed in 10% Clorox</td>
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<td></td>
<td></td>
<td>several rinses of sterile distilled water</td>
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<tr>
<td>Benlate</td>
<td>d</td>
<td>0.05% Benlate (Immersed for 2 days)</td>
<td>65.3&lt;sup&gt;c&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>10% Clorox</td>
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<td></td>
<td></td>
<td>several rinses of sterile distilled water</td>
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<tr>
<td></td>
<td>e</td>
<td>0.05% Benlate +200mg/L streptomycin (Immersed for 2 days)</td>
<td>70.7&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>10% Clorox</td>
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<td></td>
<td></td>
<td>several rinses of sterile distilled water</td>
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<tr>
<td>Clorox (pH adjusted)</td>
<td>f</td>
<td>20% Clorox adjusted to pH 6 for 15 minutes</td>
<td>98.7&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>several rinses of sterile distilled water</td>
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<tr>
<td></td>
<td>i</td>
<td>20% Clorox adjusted to pH 7 for 15 minutes</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>several rinses of sterile distilled water</td>
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<tr>
<td></td>
<td>ii</td>
<td>20% Clorox adjusted to pH 8 for 15 minutes</td>
<td>98.7&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>several rinses of sterile distilled water</td>
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<tr>
<td></td>
<td>iii</td>
<td>20% Clorox adjusted to pH 11 for 15 minutes</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>several rinses of sterile distilled water</td>
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<tr>
<td>Clorox</td>
<td>g</td>
<td>sterile distilled water with Tween 20 for 5 minutes</td>
<td>38.7&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>20% Clorox poured into flask until solution spills over and left standing for 18 minutes</td>
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<td></td>
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<td>pour out half of solution and shake flask for 2 minutes</td>
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<td></td>
<td></td>
<td>rinse with sterile distilled water 8 to 10 times</td>
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</tbody>
</table>

*Means with the same letters are not significantly different at a = 0.05 according to Duncan’s Multiple Range Test
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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, endophytic or internal bacteria that became pathogenic after a prolonged period of latency in the plants (Bastiaen, 1983). Meanwhile, the existence of bacteria in Pinussylvestris 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. aerogenes 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 endophytic bacteria associated with sweet potato plants. Members of the bacteria genera Erwina have been known to cause diseases in the Citrus
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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.

REFERENCES


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