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Micro-Propagation of *Nepenthes* Species through Seed Culture

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ABSTRACT

***Nepenthes* is a carnivorous species which consists of a special structure called pitchers to trap organisms like insects. Most of these species have very attractive pitcher with different colour, size, and pattern making them as an ornamental plant species. *Nepenthes* species produce very small seeds with poor germination. Therefore, alternative propagation techniques would be desired for propagation. In vitro seed culture is one of the suitable micropropagation methods for *Nepenthes*. Objective of this study was to establish suitable in vitro seed propagation technique by identifying proper *Nepenthes* pod sterilization method and suitable culture medium. All the pods were treated with Iso propyl alchol (IPA), teepol, clorox (Sodium hypochlorite) and distilled water and then subjected to four different types of sterilization procedures consisting of a) lightly flamed b) 0.1% $Hgcl_2$ c) lightly flamed and treated with Streptomycin sulphate d) 0.1% $Hgcl_2$ and treated with Streptomycin sulphate. Then these seeds were inoculated to vessels containing media with five different compositions such as a Murashige and Skoog (MS); MS+1mg/l BAP (N-6-Benzylaminopurine);1/2 MS; 1/2 MS+ 0.5 mg/l Gibberellic acid; distilled water alone without nutrient medium. The culture vessels were kept in culture room at 29 ^{0}C under florescent light. The results indicated that, sterilization methods (c) and (d) were the best among these methods with equal percentage (8.33%) having the lowest contamination. When health, economical and environment aspects are considered, the method that can be highly recommended was method (c). As medium e showed higher germination percentage (87.23%) it was the most suitable culture medium.**

KEYWORDS: Culture Media, Micropropagation, *Nepenthes*, Sterilization Method

INTRODUCTION

Nepenthe is a plant genus in the family Nepenthaceae that comprises over 100 species. They are insectivorous plants found in tropical forests ranging from South China, Indonesia, Malaysia and the Philippines; westward to Madagascar and Seychelles, southward to Australia and New Caledonia, and northward to India and Sri Lanka. There are two types of *Nepenthes* species as highland species and lowland species. The majority of *Nepenthes* are highland or mountain plants, which prefer habitats with both high humidity and rainfall. Only 30 percent of the species are found in lowland areas where the days are hot and the nights are warm (D'amato, 1998).

Nepenthes species grow in acidic and nitrogen deficient soil experiencing high rainfall and warm climate (Rathore *et al.*, 1991). Therefore, they carry special structure called Pitcher. *Nepenthes'* uniqueness is attributed to its pitcher which is the true leaf. The pitcher is supported by a tendril loop to the leaf petiole. It is the natural cup used by the plant to capture organism like insects. Most *Nepenthes* species produce two types of pitchers. Young stems produce short, squat pitchers called terrestrial or lower pitchers. On climbing stems, narrow, funnel shaped pitcher called aerial or upper pitchers are produced. Although *Nepenthes* pitchers share the basic structure, they vary in size, shape, and colour. This variation in the pitcher morphology provides useful guides in identifying different species of *Nepenthes* (Clarke, 1997).

Nepenthes distillatoria is found in Sinharaja rain forest in Sri Lanka. This is a lowland endemic species which is native to Sri Lanka (Anon, 2000). This species has been included in red list of International Union for Conservation of Nature and Natural Resources (IUCN) as endangered species since 2000 (Anon, 2012). Further, *Nepenthes distillatoria* is named as conserved plant in the Convention on International Trade in Endangered Species of wild fauna and flora (CITES) in appendix II.

Pitcher plant has many potential uses, which are not fully explored (Phillips and Lamb, 1989). These include the species potential for ornamental plant (both indoor and outdoor) since the different shape and colour of the pitcher can be very attractive (Sani *et al.*, 2000). Furthermore, these species have medicinal value as well.

Nepenthes plant produce very small seeds which are known to have poor germination rate, thus, natural regeneration through seeds have become slow in effort to conserve the

plant ex situ (Sani *et al.*, 2000). High growing demand of this plant as ornamental and medicinal plant value are cause to increase the market demand of the plant (Damayanthi *et al.*, 2010). The genus *Nepenthes* includes number of species which become highly endangered due to habitat destruction (Clarke, 1997). Therefore, to ensure the species existence and expansions, it is important to look for an alternative propagation technique through micro propagation such as tissue culture, seed culture or rooting of stem cuttings. Among these methods, in vitro seed culture is one of good method for *nepenthes*. However, research of this nature on *nepenthes* seed culture is scarce (Sani *et al.*, 2000). Therefore, this study is designed to determine suitable protocol for in vitro seed culture by determining suitable sterilization method and suitable culture medium.

MATERIALS AND METHODS

Location

The experiment was carried out in the plant tissue culture laboratory of the Forestry Research Centre, Kumbalpola, Kurunagala from January to April 2013.

Seeds Collection

Nepenthes distillatoria infructescence were collected from Weddagala which is an adjacent village to the Sinharaja Rain Forest in Kalawana area of Ratnapura district and transported to the tissue culture laboratory on the same day under high humidity condition to reduce the evaporation. Samples were kept in the refrigerator at 4 ^{0}C until culture.

Experimental Design and Treatment

Comparatively same colour, shape, size and fully matured 480 pods were separated from the bunch of pods by visual observation and a type of common treatment (CT) was applied as follows. Surface of the seeds pods were sterilized with Iso propyl alcohol (IPA) and cleaned with teepol. Then pods were washed from series of distilled water and brought to the laminar flow. Pods were washed with sterilized distilled water thoroughly and dipped in 5 % Clorox (Sodium hypochlorite) solution for 5 min. Then pods were washed with series of sterilized distilled water again.

Part 1: Determination of Suitable Sterilization Method

Four different surface sterilization methods were used in this experiment (Table 1). Four hundred and eighty of commonly treated pods were divided into 4 equal size (number) groups and each group was surface sterilized within the laminar flow using 4 types of surface sterilization methods as given in Table 1.

Table 1. Four different types of sterilization methods

Sterilization method (STM)	Procedure
Method 1	CT + Pod lightly flamed
Method 2	CT + Pod dipped in 0.1% $Hgcl_2$ Solution for 3 minutes
Method 3	CT + Pod lightly flamed +Seeds dipped in1g/100ml Of Streptomycin sulphate 20 minutes
Method 4	CT + Pod dipped in 0.1%$Hgcl_2$ Solution for 3minutes + Seeds dipped in1g/100ml of Streptomycin sulphate 20 minutes

CT: Common treatment

Part 2: Determination of the Suitable Medium

Five types of solid (Agar) culture media were used (Table 2). pH of the media were maintained at 5.7 and Media sterilization was done by autoclaving at 121 ^{0}C for 25 min. All other tools used were sterilized by heat.

Table 2. Composition of media

Type of media	Composition
Medium 1	Murashige and skoog (MS) media + Agar
Medium 2	MS + 1mg/l N-6-Benzyl amino purine (BAP) + Agar
Medium 3	½ MS + Agar
Medium 4	½ MS + 0.5mg/l of Gibberellic acid + Agar
Medium 5	Distilled water only (Without Nutrient) + Agar

All media were prepared using distilled water and sterilized glassware

In vitro Seed Culture and Maintenance

Seeds were inoculated in vessels containing media within the laminar flow cabinet under aseptic conditions.

For one medium 48 culture vessels were prepared and all those 48 vessels were inoculated with 96 seeds pods being 2 pods per vessels. Those pods were sterilized using 4 types of different sterilization methods (Table 1). Twenty four pods (12 vessels) per each treatment were used. Same procedure was

applied for each media and finally 240 vessels were produced.

Culture vessels were kept in the culture room under florescent light (16 hr light and 8 hr darkness within 24 hr). Temperature of the culture room was maintained at 29^{0}C.

Data Recording

Total numbers of contaminated and germinated vessels were taken separately. Data were recorded weekly, from 3rd week to 12th week and finally grand total was taken. Contaminated vessels were removed and rest of the vessels was kept for germination.

Statistical Analysis

All data were statistically analyzed using CATMOD procedure by using (SAS 9.2 software, SAS Inc., Cary, USA). Non-parametric analytical tool was used as the method. ($P < 0.05$) since all data were in categorical form.

RESULTS AND DISCUSSION

Sterilization Method

Contaminated and uncontaminated vessels percentages were calculated (Table 3). Contaminations of all contaminated vessels were caused by bacteria. It was observed that survival (uncontaminated) percentages were significantly higher than that of contaminated percentage in four types of sterilization methods. STM 3 and 4 both had 91.67% of uncontaminated percentage and those values were higher than the values obtained for STM 1 (60%) and STM 2 (68%).

Table 3. Percentage of not contaminated and contaminated vessels in different types of sterilization method

STM	Not contaminate	Contaminate
Method 1	60.00%	40.00%
Method 2	68.33%	31.67%
Method 3	91.67%	8.33%
Method 4	91.67%	8.33%

It also showed that there was a significant difference in contamination percentage among treatment methods (Table 4). However, no significant interaction was found between type of media and contaminations, and the sterilization method and culture media as well. Chi square value was 6.16 with 12 df (P= 0.9077) suggesting that there was no 3 way interaction among sterilization method, culture media and contaminations.

Table 4. Effect of sterilization method and culture media on contamination

Source	Probability
Sterilized method (STM)	0.0352
Media	0.6767
STM × media	1.0000
Contamination	0.0001
STM × contamination	0.0001
Media× contamination	0.2626
Chi- square	0.9077

Note: Probability value< 0.05-significantly different

Anti-bacterial chemical, Streptomycin sulphate used in STM 3 and STM 4 effectively reduced the bacterial contamination. It indicates that STM3 and STM 4 are suitable for surface sterilization of *Nepenthes* seed pods than STM 1 and STM2.

$Hgcl_2$ used in STM 4 is a heavy metal, carcinogenic and highly toxic. Therefore, it may influence the viability of the seeds as well as it may pollute the environment if contact with ground water. When considering health, economic and environmental aspects STM 3 can be recommended. However, this method may depend on the exposure time and amount of flame. As *Nepenthes distillatoria* seed pod coat is thin high flame and duration may damage the seeds.

Culture Media

Germinated vessels percentages were taken after removing contaminated vessels (Table 5).

Table 5. Percentage of germinated vessels in different types of media

Type of media	Germination %
Medium 1	10.00
Medium 2	19.51
Medium 3	41.03
Medium 4	60.00
Medium 5	87.23

There was a significant effect of culture medium on germination of the seeds (Table 6). However, significant interaction was not found between STM and germination of seeds.

Table 6. Effect of sterilization method and culture media on germination

Source	Probability
STM	0.9994
Media	0.2128
STM × media	1.0000
Germination	0.0007
STM × germination	0.9673
Media × germination	0.0001
Chi- square	0.9725

Note: Probability value< 0.05-significantly different

Germination percentages of all medium were significantly different. Moreover, high germination percentage was observed in medium 5 (87.23%) which was significantly higher than that of other media. Second highest percentage was observed in the medium 4 (60%) as shown in (Table 6).

According to (Peries, 2005) *Nepenthes* seeds must be cultured in nutrient or sugar free agar medium first and it must be transferred to other nutrient medium after seed germination. Seedling after germination may suffer from nutrient deficiency due to lack of nutrient in the medium. However, when seedlings are transferred, contaminations can occur. This problem can be avoided by culturing seeds in medium with nutrient and sugar.

In medium 4, germination percentage (60%) was significantly higher than other media except for medium 5. Therefore, medium 4 can be recommended to avoid contaminations during seedling transfer process. According to results MS media (medium 1) had the lowest percentage (10%) of germination while medium 2 had the 2nd lowest (19.51%). ½ MS media (media 3) had comparatively high (41.3%) percentage of germination over more nutrient and sugar consisting medium (Table 5). This revealed that high nutrient percentage of medium reduces the germination percentage of *Nepenthes* seeds.

CONCLUSIONS

These results indicated that lightly flamed and treated with Streptomycin sulphate (STM 3) and 0.1% $Hgcl_2$ and treated with Streptomycin sulphate (STM 4) are most suitable surface sterilization methods among used methods in the present study. However, due to health and economical concern lightly flamed and treated with Streptomycin sulphate was the most suitable technique.

Distilled water alone without nutrient medium is the best for seed germination studies while medium 4 can also be used in continuous monitoring of growth characteristics and commercial production.

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