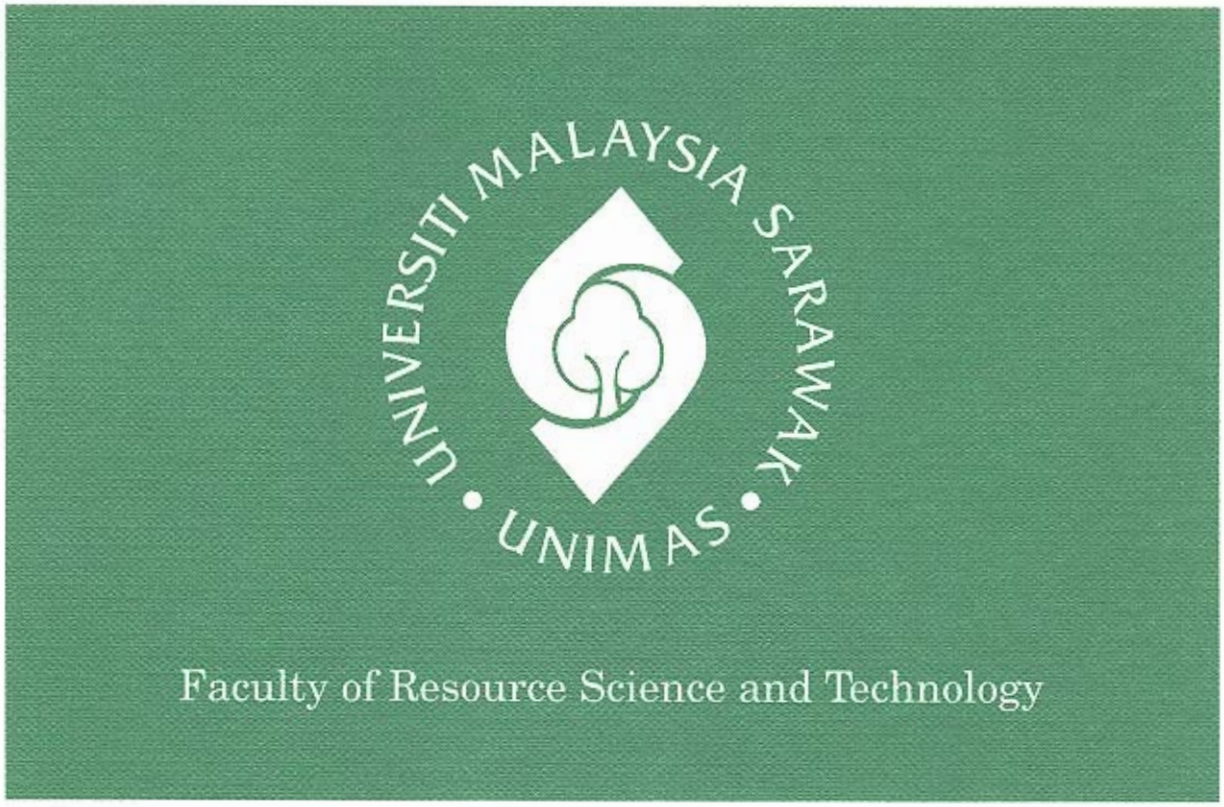

IN-VITRO AND CONVENTIONAL PROPAGATION OF *NEPENTHES MIRABILIS* (LOUR) DRUCE

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LIST OF ABBREVIATIONS

2, 4-D	2, 4-Dichlorophenocyl Acetic Acid
BAP	6-Benzyl Amino Purine
IBA	Indole 3-Butyric Acid
NAA	Napthalene Acetic Acid
MS	Murashige & Skoog
PPM	Plant Preservative Mixture

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In-vitro and Conventional Propagation of *Nepenthes mirabilis* (Lour) Druce

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Abstrack

The study is aimed at developing methods of *in-vitro* and conventional propagation of *N. mirabilis* (Lour) Druce which can help in the effort of conversation of the species to prevent extinction. Besides, tissue culture may facilitate the study of bioactive compounds in this species. The propagation by cuttings is used because of the low seeds germination of *Nepenthes*. Three types of rooting hormones have been tested; commercial rooting hormone, Seradix 2 and two solutions of plant growth regulators, Napthalene-Acetic Acid (1g/l) and combination of Napthalene Acetic-Acid with Indole-3 Butyric Acid (1g/l each). Distilled water was served as control. Explants for *in-vitro* culture are lamina, stems and buds. The results showed that *Nepenthes* could be propagated by means of rooting of stem cuttings. Over all, 47% of the cuttings produced new shoots and 25% developed roots. Seradix 2 is the most appropriate treatment with 48% rooting success. Meanwhile, surface sterilization treatment for *N. mirabilis* explants was most effective when 15% clorox concentration with 20 minutes exposure time and 20% clorox concentration with 30 minutes exposure time were used. The major problem encountered in this research was the contaminantion from fungus and bacteria.

Key words: *in-vitro*, stem cuttings, explants

Abstrak

Kajian ini bertujuan untuk menperkenalkan kaedah in-vitro dan 'conventional' Nepenthes mirabilis (Lour) Druce di dalam usaha untuk memelihara spesis tersebut daripada kepupusan. Di samping itu, kaedah kultur tisu juga memudahkan kajian ke atas kompoun bioaktif yang terdapat pada spesis ini. Teknik keratan digunakan kerana Nepenthes sukar dibiakkan melalui biji. Tiga jenis hormon pengakaran digunakan iaitu hormon komersial, Seradix 2 dan dua jenis larutan pengalak pertumbuhan pokok, Napthalene-Acetic Acid (1g/l) serta campuran Napthalene Acetic-Acid dan Indole-3 Butyric Acid (masing-masing 1g/l). Rawatan kawalan ialah menggunakan air suling. Eksplan untuk kultur in-vitro ialah daun, mata tunas dan batang lembut. Keputusan menunjukkan Nepenthes boleh dibiakkan secara keratan batang. Secara keseluruhan, 47% keratan menghasilkan tunas dan 25% telah berakar. Seradix 2 merupakan rawatan terbaik dengan 48% kejayaan pengakaran. Sementara itu, rawatan pensterilan permukaan yang paling efektif bagi N. mirabilis eksplan ialah 15% kepekatan klorox dengan 20 minit masa rendaman dan 20% kepekatan klorox dengan 30 minit masa rendaman. Masalah utama kajian ini ialah serangan kulat dan bakteria.

Kata kunci: in-vitro, keratan batang, eksplan

CHAPTER ONE

INTRODUCTION

1.1. General Introduction

Nepenthes is a carnivorous perennial plant producing jug-shaped leaves extent from the tendrils. It is thus called the pitcher plant. The plant grows only in the tropics (Kurata, 1976). The generic name, *Nepenthes* is said to be associated with a Greek word which means "banishing sorrow". It is because the pitcher is likened to a medicine bottle containing some drug or medicine. Several vernacular names are reported in various areas; "Periok kera (monkey's rice pot)" and "Gendi kera (monkey's pitcher)" are common names in Malaya and Borneo; "Tahul-tahul (small kettle)" and "Kantong semar (sack of semar)" are popular names in Sumatra.

The pitcher evolved to attract, trap and in many cases, digest small insects for nutritional benefit. Those species which do not produce their own digestive enzymes may rely upon bacteria or other organisms to digest the prey for them. The products of digestion are then absorbed through the walls of pitchers and transported to other parts of the plant to assist in growth and reproduction (Clarke, 1997). This is to make up for the deficiency of normal plant foods in poor soil habitats. Thus, *Nepenthes* also called an insectivorous or carnivorous plant (Kurata, 1976).

1.2. Background

Nepenthes were first recorded by E. de Flacourt, a colonial governor of French Madagascar in 1658, under the name of *Amramitico*. This species was described as *N. madagascariensis* Poiret by Poiret in 1797 (Kurata, 1976).

The pitcher plant comprises of seven genera, distributed in America, Asia and Australia (Clarke, 1997). Of the five American genera, two are bromeliads (one species of *Catopsis* and at least one species of *Brocchinia*), while the remainder all belong to family Sarraceniaceae (*Heliamphora, Sarracenia* and *Darlingtonia*).

The monotypic *Cephalotus* (Cephalotaceae) grows only in south-western Australia, whereas the largest genus, *Nepenthes* (Nepenthaceae) is distributed from northern Australia throughout South-east Asia to southern China. Outlying species of *Nepenthes* occur in Sri Lanka, India, the Seychelles, Madagascar and New Caledonia, while the vast majority occurs on the islands of Borneo and Sumatra (Clarke, 1997).

Nepenthes species favour nutrient deficient soils with high acidity and grows in wide range of altitudes (1500-3000m) and inhabits a variety of different terrain (Clarke, 1997). Kurata (1976) classified *Nepenthes* species into two according to their habitat; highland-those usually occur above 1000m above sea level (68%) and lowland-those that grow primarily from sea level up to 1000m (32%).

1.3. Problem Statement

Nepenthes are dioecious plants, which mean that a plant is either male or female and it has low percentage of natural pollination. There is no report on the exact time for the seeds to develop to maturity. Dispersal of seeds is by wind. Therefore, there are difficulties to collect and get the fertile seeds for germination. Furthermore, many fruits are destroyed by caterpillars, which bore through the capsule walls to eat the developing seeds (Clarke, 1997).

As an alternative to solve the problem on propagation of *Nepenthes*, propagation by stem cutting and *in vitro* culture could be attempted. Presently, there is lack information on the vegetative propagation of *Nepenthes* through conventional propagation and *in vitro* culture. Conventional vegetative propagation and in vitro culture methods would be reliable method for mass production of *Nepenthes*.

1.4. Objectives

The main objectives of this study are:

a) To develop a suitable vegetative propagation method for *Nepenthes mirabilis*.

b) To attempt the development of a protocol for mass propagation of *Nepenthes mirabilis*.

CHAPTER TWO

LITERATURE REVIEW

2.1. Economic Importance of *Nepenthes* sp.

Nepenthes is reported as one of the traditional medicinal plants. According to Kurata (1976), in Sumatra, a Batak use the fluid of *N. tobaica* as a remedy for burns, eye inflammation and for coughs. A record from Bangka says that the leaf of *N. reinwardtiana* is useful for skin diseases, and its extract serve as styptic. Malays at Pulau Lingga and Malacca believes that the roots of *N. gracilis* are to be boiled and given in case of dysentery and stomach-ache and an exact is used in case of mouth-sore and swollen tongue. *N. mirabilis* and *N. ampullaria* now have a high medicinal value. China and Hong Kong used it for treatment of high blood pressure and urine infection.

The pitcher of *Nepenthes* can be used in the field as an instant container of water. Several records from the Philippines and Borneo also relate that large pitchers are used for cooking rice or vegetables. This could be a good use of natural resources; container made of natural material rather than plastic which require more energy to produce and causes environment pollution. The Singapore Herbarium mentions that *N. ampullaria* is used for tying fence and it is very durable (Kurata, 1976).

Over the last few years, *Nepenthes* species has become popular as ornamental plants because of its unique modification of leaves and colourful pitchers. There is an amazing variety of shape, size and colour within the pitchers themselves (Phillipps & Lamb, 1996). In

Hong Kong and Taiwan, *Nepenthes* are introduced as indoor ornamental plants and for landscaping purposes (Meekiong, 1998). Nowadays, these plants have high economic value due to its high demand in the ornamental sector not only in Asia but in Europe and America.

2.2. Current Status of *Nepenthes* sp.

Nepenthes are difficult group of plants to monitor and protect. Many of the lowland species colonies disturbed areas, and as there are a lot of these, they appear to be under no immediate threat from the activities of people. In contrast, a number of other species have very restricted distributions and require greater protection.

In Sarawak for instance, all *Nepenthes* species are protected under Wildlife Protection Ordinance 1958, and it is illegal to collect any part of a plant without a permit.

2.3. Propagation of *Nepenthes* sp.

For the last few decades, the interest in exotic tropical plants, including pitcher-plants and orchids led the botanists such as Frederick Burbidge, William Jack, Hugh Low and the others who travelled throughout the tropical forest. When they return home, they brought along some seeds and seedlings for propagation purposes (Phillipps & Lamb, 1996).

Since that, many attempt made to propagate the plants. The first nursery to cultivate the amazing *Nepenthes* commercially and make the plants available to the public was Messrs Loddiges of Hackney (Phillipps & Lamp, 1996).

There are two main ways of propagating *Nepenthes*. The first and easiest is by taking cuttings and the second is to grow them from seeds. Many *Nepenthes* are easy to propagate from cuttings, particularly lowland species. Most highland species will strike quite easily. Roots appear around four to six weeks after planted (Meekiong, 1998).

Seeds of *Nepenthes* take three weeks to three months to germinate. They grow very slowly and take years to mature. There are other difficulties in propagating *Nepenthes* by seed. Firstly the seed of many *Nepenthes* species has a very short time of viability and the second reason is that the difficulties to collect the fertile and mature seeds (Meekiong, 1998).

2.4. Other Research on *Nepenthes* sp.

Previous research on vegetative propagation of *Nepenthes* species includes those reported by Meekiong (1998) on vegetative propagation through stem cutting of *N. ampullaria, N. mirabilis* and *N. rafflesiana*; Lee (1998) reported the work on micropropagation of *N. ampullaria* while Sobri (1999) on micropropagation of *N. mirabilis* and subculture of *N. ampullaria.*. *In vitro* propagation of *N. macfarlanei* has been attempted by Chua and Henshaw (1999).

Vegetative propagation through stem cuttings of *N. ampullaria, N. mirabilis* and *N. rafflesiana* by Meekiong (1998) showed that *Nepenthes* can be propagated by means of rooting of stem cuttings. The result showed that sand was the best rooting medium compared to the liquid medium. 26.4% of the cutting developed roots in the sand medium while 16.5%

cuttings formed roots in the liquid medium. Seradix 2 is the most appropriate rooting hormone with 48.9% success.

Lee (1998) in a study of *in-vitro* propagation using tissue culture on *N. ampullaria* showed that ½ MS (half strength of full MS formulation) with high sucrose level enhance seed germination compared to other media (formulation Knudson-C and Vain and Went). Seedlings grown on MS media supplemented with 1.0mg/l IBA enhanced leave and root growth as well as pitcher production.

Sobri (1999) used seeds of *N. mirabilis* to induce callus formation and subsequently induced formation of shoots. Different concentration of cytokinin(BAP) and auxin (IBA) were used to produce various combinations of hormone treatments. The results showed that *N. mirabilis* seeds germinate successfully in ½ MS with the stimulation of 5.0mg/l BAP, 2.0g/l IBA and 0.2g/l of activated charcoal. This condition has created a high germination rate with a uniform formation of leaves, pitcher and roots. The subculture of *N. ampullaria* was successfully produced plantlets from the original explants with a high survival rate of 81%.

Chua and Henshaw (1999) found that half-strength Murashige and Skoog medium supplemented with at least 5×10^{-6} M BAP was required for shoot multiplication from cotyledonary seedlings of *N. macfarlanei.* Cotyledonary seedlings produced higher numbers of shoot buds compared to apical shoots and nodal segments. Half-strength Murashige and Skoog medium supplemented with various concentrations of NAA was favourable for rooting of shoot buds.

Other studies on *Nepenthes* include different explant reaction in *in-vitro culture* from species *N. gracilis* was reported by Normah (1994), morphological development of *N. marlarlanei, N. gracillima, N. sanguinea* and *N. gracilis* by Habsah (1981); morphological description and isoenzym of *N. ampullaria and N. mirabilis* by Fariza (1997) and alkaloid and hydrocarbon identification by Norizah (1998).

2.5. Description of Plant Used in This Study

Nepenthes mirabilis (Lour) Druce.

Synonyms : *Phyllamphora mirabilis* Lour., *N. phyllamphora* Willd., *N. macrostachya* Blume., *N. fimbriata* Blume., *N. distillatoria* Wall., *N. kennedyana* F. Muell., *N. kennedyi* Benth., *N. echinostoma* Hook. F., *N. bernaysii* Bail., *N. obrieniana* Linden et Rodigas, *N. jardinei* Bail., *N. albo-lineata* Bail., *N. moorei* Bail., *N. alicae* Bail., *N. cholmondeleyi* Bail., *N. pascoensis* Bail., *N. armbrustae* Bail., *N. garrawayae* Bail., *N. tubulosa* Macf., *N. beccariana* Macf.

Distribution : Sourthern China, Indo-China, Malaya, Sumatra, Borneo, Philippines, Celebes, Moluccas, Palau, New Guinea and Australia.

Vertical Distribution : 0-5000m alt.

Habitats : Open and swampy sites, stream sides, sterile ground or sand fields.

Description

Stems : Cylindrical, climbing up to 10m high, 6-10mm thick.

Leaves : Petiole, the leaf blade elliptic or oblong-lanceolate, minutely fimbriate or denticulate at the margin, 20-40cm long, 4-10cm broad, rounded or acute at the apex, the base abruptly contracted into the petiole; longitudinal nerves originating from the base of the midrib, running 4-8 on each side: the petiole 10-20cm long,

with wings, forming a short sheath at the base; tendrils 1.5 times as long as the leaf blade.

Lower pitchers : 5-10cm high, 1.5-3cm wide, with 2 fringed wings; mouth orbiculate, oblique; peristome flattened, 2-3mm broad, ribs about 0.2mm apart; inner surface of the pitcher glandular in the ventricose part; lid orbiculate, 3cm broad, without glandular crest on the lower surface.

Upper pitchers : Tubulate-infundibulate, 12-16cm high, 2-3cm wide, with 2 prominents ribs over the whole lengths; mouth 0.2mm apart; inner surface of the pitcher glandular in the lower half; lid and spur like those of the lower pitchers.

Inflorescence : Raceme, the axis 25-40cm long, the pedicels about 15mm long, 1- flowered without bract.

Fruits : Fusiform, 100-500 seeds.

Seeds : Filiform, 8-18mm long.

Plate 1. *N. mirabilis* in the wild.

CHAPTER THREE

MATERIALS AND METHODS.

3.1. Materials

The materials used for this experiment were fresh leaves and stems of *Nepenthes mirabilis*. Samples were collected from various areas such as Kuching-Serian road and at secondary forest near UNIMAS campus.

3.2. Methods

3.2.1. Vegetative Propagation through Stem Cuttings.

In this experiment, river sand is used as rooting medium. The sand bed was covered with transparent plastic sheet to maintain the moisture in the atmosphere. Three different rooting hormone were used; Seradix 2 (a commercial rooting powder), plant growth regulators, α-NAA (α-Napthalene-Acetic Acid) at 1g/l and a combination of IBA (Indole-Butyric Acid) and α-NAA each at 1g/l. Cutting treated with distilled water was served as the control. The plant growth regulator solutions were prepared the day before experiment.

The cuttings were taken from the main stem from the position where the green stem was turning brown downwards. It is important to get the cuttings early in the morning, around 7.00am to 11.00 am to make sure the cuttings are still fresh and have high water content (Hartmann *et al*, 1997). Two-node cuttings were obtained. There were 25 cuttings for each treatment and a total of 100 cuttings were prepared for the experiment. The leaf of the lower node was removed and the leaf of the top node then trimmed to one third their size to reduce the leaf surface area. This is to help reducing transpirational water loss and allows closer

spacing in the sand bed. This also make the weekly inspection of the roots much easier without disturbed the other cutting.

The lower node of the cutting was immersed into the PGR solution, air-dried for 5 minutes and then inserted into the sand bed. Completely Randomized Design (CRD) is applied in this experiment. The cutting was planted 4-5cm apart to allow root inspection which was done every week after planting.

The cutting was watered daily using sprinkler to maintain the moisture of the atmosphere. Care and protection from pest and diseases was also done. The observation was made once a week until the twelve week to check the emergence of shoots and the production of roots. For checking the presence of root, the cutting was removed carefully. The cutting is considered as produced root when a root is at least 0.5cm long. The rooted cutting was marked with rubber band after inserted it again into the sand bed.

After twelve weeks, all the cuttings were harvested. The rooted cutting was separated according to the treatment. The number of roots for each cutting was counted. The roots then cut off and put inside the oven. The temperature oven was set to 60°C. Overall, the observation included:

1) The emergence of new shoots
2) The production of roots
3) Number of roots
4) Time rooting
5) Dry weight of roots