MICROPROPAGATION OF *Crotalaria pallida*

FONG KHAR KEI

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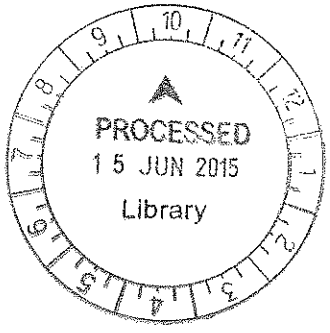

FACULTY OF SCIENCE, TECHNOLOGY,
ENGINEERING AND MATHEMATICS
INTI INTERNATIONAL UNIVERSITY
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ABSTRACT

Crotalaria pallida is a medicinal herb with various beneficial properties such as antibacterial properties, anti-inflammatory properties, antipyretic properties, estrogenic properties, to list a few. In order to realise its benefits, an attempt to successfully multiply *C. pallida* via micropropagation was conducted. Explants were obtained from shoots germinated from sterilised seeds and cultured in varying concentrations of IBA and zeatin in factorial design. Concentrations used were 0.0 mg/L, 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L, and 5.0 mg/L. The optimum plant growth medium for shoot induction was the control medium, MS medium while for root induction it was MS medium supplemented with 5.0 mg/L IBA. Microprogation could serve as an alternative reproduction system for *C. pallida*.

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%	percent
°C	degree Celsius
µM	micro Molarity
2,4-D	2,4-dichlorophenoxyacetic acid
2-iP	2-isopentenyladenine
ANOVA	analysis of variance
BAP	6-benzylaminopurine
$CaCl_2$	calcium chloride
cm	centimetre
$CoCl_2 \cdot 6H_2O$	cobalt(II) chloride hexahydrate
Cp-AMP	*Crotalaria pallida* antimicrobial peptide
$CuSO_4 \cdot 5H_2O$	copper(II) sulfate pentahydrate
Fe	ferum
$FeSO_4$	iron(II) sulfate
g	gram
H_3PO_4	phosphoric acid
HCl	hydrochloric acid
HIV	human immunodeficiency virus
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
KH_2PO_4	monopotassium phosphate
KI	potassium iodide
KNO_3	potassium nitrate
L	litre

LAF	Laminar Air Flow
M	Molarity
mg/L	milligrams per litre
mg/mL	milligrams per millilitre
$MgSO_4$	magnesium sulphate
mL	millilitre
mM	milli Molarity
$MnSO_4 \cdot 4H2O$	manganese(II) sulfate tetrahydrate
MS	Murashige and Skoog
Na_2EDTA	disodium 2,2'-{1,2-ethanediylbis[(carboxymethyl)imino]}diacetate
$Na_2MoO_4 \cdot 2H_2O$	sodium molybdate
NAA	1-naphthaleneacetic acid
NaOH	sodium hydroxide
NH_4NO_3	ammonium nitrate
PGR	plant growth regulator
pH	negative log of the activity of the hydrogen ions
$ZnSO_4 \cdot 7H_2O$	zinc sulfate heptahydrate

1.0 CHAPTER 1:

INTRODUCTION

Crotalaria pallida is a herb with many documented traditional uses, including treating rheumatism (Rahman, Uddin, & Wilcock, 2007), curing common skin diseases, treating eczema (Acharya & Pokhrel, 2007; Ayyanar & Ignacimuthu, 2005), and in the treatment of indigestion (Mithra & Mukherjee, 2005). Recent research has identified multiple beneficial properties of *C. pallida* such as antimicrobial properties, anti-inflammatory properties (Govindappa, 2011), analgestic properties, antipyretic properties (Panda, Das, & Tripthathy, 2015), and estrogenic properties (Boldrin et al., 2013). Extracts of *C. pallida* were also shown to have strong antioxidant properties (Govindappa, 2011). A HIV-protease inhibitor has also been identified in *C. pallida* (Boldrin et al., 2013).

Plant cell and tissue culture is a technique used to propagate plant cells, tissues, and organs under aseptic conditions on artificial growth mediums (Evans, Coleman & Kearns, 2003; Yildiz, 2012). This is a crucial prerequisite for any wide scope biotechnology application of plants (Das, Hasan, Hossain & Rahman, 2013). As reported by Hellwig, Drossard, Twyman & Fischer (2004), plant cell and tissue culture was developed when people realized that it had the potential to synthesize various valuable molecules. In order to obtain adequate clean samples for scientific studies and industrial uses, it is necessary to establish proper conditions required for plant cell and tissue culture. Once the proper procedures have been established, *C. pallida* may be propagated in large quantities, without the use of seeds in order to produce sufficient samples for studies and conservation (Yildiz, 2012).

The composition of the growth medium used for plant cell and tissue culture is important as it affects the growth and development of the explants (Yildiz, 2012). Important components of the growth mediums for plants include macronutrients, micronutrients, carbon source, vitamins, nitrogen supplements, organic supplements, plant growth regulators (PGR), and solidifying agents (Yildiz, 2012). PGRs are

known to be important in regulating plant growth rate and growth pattern, including organogenesis (Gaspar et al., 1996). There are five groups of PGRs, namely auxins, cytokinins, ethylene, gibberellins, and inhibitors (Nickell, 1982). Among these groups, auxin and cytokinins are believed to cause plant development, prominently shoot and root development, depending on their combination ratios (Nordstrom et al., 2004). Since both PGRs seem to work in tandem, it is difficult to isolate their individual effect on plant development (Nordstrom et al., 2004).

With human urbanization and deforestation progressing at an alarming rate, the habitat of *C. pallida* is gradually decreasing. As the loss of habitat is one of the major factors causing decrease in biodiversity (Breggin, George & Pencak, 2003), it is important to find conservation friendly ways to obtain *C. pallida*, be it for research or for medical treatment, without further damaging the natural balance of wild populations. Micropropagation of *C. pallida* would be beneficial as it allows mass production of the plant (Yildiz, 2012), making it more readily available in large quantities. The plants produced by this method have the advantage of being disease free and sterile (Yildiz, 2012), thereby easing the production of plant extracts. Therefore, the objectives of this study were to determine the germination rate of *C. pallida* seeds and the effect of different concentrations of zeatin and indole-3-butyric acid (IBA) on the growth of *C. pallida*.

2.0 CHAPTER 2:

LITERATURE REVIEW

2.1 *Crotalaria pallida*-ITS MEDICINAL USAGE

C. pallida is a perennial herb, found in grasslands and unearthed sandy zones. It typically has three-foliate leaves that range from oblong to elliptic, with distinct veins that can be observed on both sides. The plant sports inflorescence of small yellow flowers and glabrescent legumes that are oblong in shape (Hong, Raven & Wu, 2010).

C. pallida has its many uses in traditional medicine documented. Among them, *C. pallida's* seeds, when taken with ripe banana leaves, may be used to treat rheumatism (Rahman et al., 2007). A paste made of the plant's powdered root and leaves is used as a topical treatment to cure common skin diseases and treat eczema (Acharya & Pokhrel, 2007; Ayyanar & Ignacimuthu, 2005). The paste may be taken orally to cure body swelling (Rai, 2006). Aqueous root extracts may also be mixed with goats' milk and honey to create a tonic for indigestion and overall weakness (Mithra & Mukherjee, 2005).

Scientists, recognizing *C. pallida's* potential as a herb, have conducted many studies to identify its possible utilization. So far, *C. pallida* has been found to have antimicrobial properties (Govindappa, 2011) and contain an antimicrobial peptide, *Crotalaria pallida* antimicrobial peptide (*Cp*-AMP) (Pelegrini et al., 2009). *C. pallida* has anti-inflammatory properties, which could inhibit a spectrum of inflammatory activities, such as proteinase activity, xanthine oxidase activity, and acetylcholinesterase activity, and lipoxygenase activity (Govindappa, 2011). Accompanying that fact, *C. pallida* extracts are shown to be analgestic and antipyretic (Panda et al., 2015). A HIV-protease inhibitor has been detected in *C. pallida's* flowers and stems (Govindappa, Kumar, & Santoyo, 2011). A recent study showed that *C. pallida* exhibits estrogenic activity as it contains stigmasterol and thus may be used in hormone replacement therapy (Boldrin et al., 2013). As with many herbs, *C.*

pallida extract is a strong antioxidant even when compared to ascorbic acid and butylated hydroxytoluene (Govindappa, 2011).

2.2 PLANT CELL AND TISSUE CULTURE

Plant cell and tissue culture is a technique that is applied to propagate, under aseptic conditions, plant cells, tissues, and organs from a mother plant on artificial growth mediums (Evans et al., 2003; Yildiz, 2012). This technique is based on totipotency, in which a cell is able to differentiate into other cells in order to generate an entire whole plant (Yildiz, 2012). Aside from propagation, plant cell cultures may be used as an expression system (Hellwig et al., 2004).

After discovering the technique could be used to synthesize valuable low molecular weight molecules, this technique was brought into development (Hellwig et al., 2004). Using this method, many different molecules such as enzymes and growth hormones have been successfully produced, including secondary metabolites as well as recombinant proteins (Hellwig et al., 2004). Recent refinements of the technique have also allowed a greater range of reproducible and quantitative experiments involving such cultures (Street, 1973).

Plant cell and tissue culture can also be used in commercial agricultural industries. Usually micropropagation, the in vitro vegetative propagation of plants, is used (Pierik, 1987). Compared to traditional propagation methods, this technique allows the production of new plants in larger quantities, in a shorter amount of time. This technique also eases the process of artificial selection as the cultures may be more easily observed and handled. The technique may be used for the recovery of plants that are infected by diseases or pathogens as the produced plants are clinically sterile. Issues of germination or growth may be overcome as well since plant cell tissue culture bypasses the use of seeds for propagation and the need for germination (Yildiz, 2012).

Micropropagation of plants via direct organogenesis is generally preferred over indirect organogenesis and somatic embryogenesis (Arockiasamy, Prakash & Ignacimuthu, 2002). In indirect organogenesis, the genotype of the produced plants

may not be uniform due to somaclonal variations in the produced callus culture (Khachatourians, 2002). As direct organogenesis is the formation of plant organs directly from the excised explants, the formation of callus is thus avoided.

2.3 PLANT GROWTH REGULATORS

Plant growth regulators (PGRs) are simple, small molecules that are fundamental for plant growth and development (Gaspar et al., 1996). They are classified into five groups, specifically auxins, cytokinins, ethylene, gibberellins, and inhibitors (Nickell, 1982). As cited by Rahimi, Naderi, Ghaemaghami, Kalatejari & Farham (2013), the development of auxiliary meristems is dependent on their presence in the culture medium.

Notably, auxins and cytokinins have been found to work antagonistically, affecting shoot and root development in plants, depending on their ratios (Nordstrom et al., 2004). As their overall effect on the plant growth is integrated, their individual effects are challenging to isolate (Nordstrom et al., 2004). Another example of antagonistic interactions is between gibberillin and abscisic acid in causing or delaying germination (Gray, 2004).

Other interactions between PGRs to control other processes are synergistic (Gray, 2004). Such processes include defensive mechanisms, developmental processes, cell expansion, and wounding response (Gray, 2004).

2.3.1 Auxins

Auxins are a class of PGRs that are produced in both the plants' roots and shoots (Overvoorde, Fukaki & Beeckman, 2010). Examples of auxins include indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-1-acetic acid (NAA) (Simon & Petrášek, 2011). In higher plants, IAA is the main auxin present (Zhao, 2010). They affect the growth of cells in general by initiating cellular division, promoting cell growth and expansion, acidification of cell walls, organogenesis, and differentiation of vascular tissue (Gaspar, 1996). It induces increase in the length of roots and the number of lateral root primodia (Overvoorde et al, 2010). It also