IN VITRO PLANT REGENERATION OF TOBACCO
(*Nicotiana tabaccum* TAPM 26)

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ABSTRACT

In the present research, a reproducible procedure has been developed for efficient plant regeneration of shoot from cotyledon explants of *Nicotiana tabaccum* TAPM 26. *In vitro* regeneration of *Nicotiana tabaccum* TAPM 26 was efficiently achieved applying combination of growth hormones supplemented into MS medium using cotyledon from 5-day-old and 7-day-old as explants. The growth hormones used were IAA for auxin (0.0, 0.1, 1, 10 mgL^{-1}) and BAP for cytokinin (0.0, 0.1, 1, 10 mgL^{-1}). The effects of growth hormones combination were assessed based on number of shoot and roots formation. Maximum number of shoots produced per explant was found when media continued with 10 µM BAP +0.0 µM IAA after 4 weeks in culture. Maximum number of root produced per explant was reached when media continued 10 µM IAA after 4 weeks in MS media. The current techniques proved that *Nicotiana tabaccum* was successfully propagated by direct organogenesis and can be applied for further research in making as transgenic *Nicotiana tabaccum*.

ABSTRAKT

Dalam kajian ini, suatu prosedur keboleh-ulang telah dibangunkan untuk regenerasi tanaman dari pucuk eksplan kotiledon pokok *Nicotiana tabaccum* TAPM 26. Regenerasi *in vitro Nicotiana tabaccum* TAPM 26 amat baik melalui kombinasi hormone pertumbuhan ke dalam media MS pada usia kotiledon 5-hari dan 7-hari. Hormon pertumbuhan yang digunakan adalah auksin iaitu IAA (0,0, 0,1, 1, 10 mg/l^{-1}) dan sitokinin BAP (0,0, 0,1, 1, 10 mg/l^{-1}). Pengaruh gabungan hormon pertumbuhan dikaji berdasarkan jumlah tunas dan pembentukan akar. Jumlah maksimum tunas yang dihasilkan per eksplan dijumpai pada media kepekaten 10 μM BAP dan 0 μM IAA selepas 4 minggu. Jumlah maksimum akar telah dihasilkan per eksplan pada media kepekatan 10 μM IAA selepas 4 minggu dalam media MS. Teknik ini membuktikan bahawa *Nicotiana tabaccum* telah berjaya ditumbuhkan melalui organogenesis langsung dan boleh dilaksanakan untuk kajian lebih lanjut seperti kajia transgenik *Nicotiana tabaccum.*

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LIST OF SYMBOLS/ ABBREVIATIONS

2, 4-D	2,4- Dichlorophenoxyacetic acid
BAP	6- benzylaminopurine
IAA	Indole-3-acetic acid

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The term of plant tissue culture is commonly applied for the sterile culture of organs, tissues, cells and their components under defined physical and chemical condition *in vitro*. Tissue culture usually applied as a medium for propagation, production of virus free plants, genetics transformation (Ali *et al*., 2007) and it is also proved more efficient in the production of secondary metabolites, for examples, nicotine and pigment. The plant tissue culture is based on the idea that a plant body can be dissected into small parts and the small part which can be grown into a whole plant, and the smaller part of plant is called explants. In plant tissue culture, scientists have accomplished success to many different problems in basic science. The culture has supplied the traditional techniques of forestry, agriculture and horticulture (Timir, 2005; Roberta, 1992; Indro 1999; Bhojwani 1992).

Plant tissue culture, broadly refers to technique of growing plants cells, tissues, organs, seed in a sterile environment on a nutrient medium, and the culture depend on three essential abilities of plant which are totipotency, dedifferentatiation, and competency. Totipotency is the ability of a single cell, divides and manufactures all differentiated cells in an organism such as extraembryonic tissues. Totipotent cells are creator during asexual and sexual reproduction, and it contains spores and zygotes. In some group organism, cells may dedifferentiate and regain totipotency. For example, a plant cutting or callus becomes an entire plant. Dedifferentiation is the capacity of mature cells to return to merismatic condition and development of a

new growing point. And competency is described the endogenous potential of given cell to develop in a particular way (Adrinal *et.al*., 2003; Roberta, 1992; Bhojwani 1992).

Nicotiana tabaccum belongs to Solanaceae family, and it is originated from South America. In the world, *N. tabaccum* is possibly the most broadly cultivated non-food crop. *N. tabaccum* has been used widely as medicinal herb and trade commodity in many different cultures and cultivated by human being for thousands of years. In the world, Over 33 million people engaged themselves in tobacco production. This drug crop commonly consumed as a snuff, a chew or smoke for its stimulant alkaloid, nicotine.

1.2 Research Objectives

The objective of the research is to study sterilization method and plant regeneration system from *Nicotiana tabacum* TAPM 26. The establishment of this technique may be used for future research involving plant genetic engineering.

1.3 Scope of the Study

1- Development of efficient *in vitro* plant regeneration from *Nicotiana tabaccum* cotyledon by optimization of plant growth hormones, explant sizes and explant ages.

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APPENDICES

APPENDIX A: COMPOSITION AND PREPARATION OF MS MEDIUM

	Constituent	Molarities in medium	Concentration of stock solution(mg/L)	Volume of stock per litre of medium(ml)	Storage of stock solution
A	MAJOR INORGANIC NUTRIENTS				
	NH_4NO_3	$2.06x10^{-2}$	33000		
	KNO_3	$1.88x10^{-2}$	38000		
	$CaCl_2.2H_2O$	$3.00x10^{-3}$	8800	50	-4°C
	$MgSO_4.7H_2O$	$1.50x10^{-3}$	7400		
	KH_2PO_4	$1.25x10^{-3}$	3400		
B	TRACE ELEMENTS				
	KI	$5.00X10^{-6}$	166		
	H_3BO_3	$1.00X10^{-4}$	1240		
	$MnSO_4.4H_2O$	$9.99X10^{-5}$	4460		
	$ZnSO_4.7H_2O$	$2.00X10^{-5}$	1720	5	+4°C
	$Na_2MoO_4.2H_2O$	$1.00X10^{-6}$	50		
	$CuSO_4.5H_2O$	$1.00X10^{-7}$	5		
	$CoCl_2.6H_2O$	$1.00X10^{-7}$	5		
C	IRON SOURCES				
	$FeSO_4.7H2O$	$1.00X10^{-4}$	5560		
	$Na_2EDTA.2H_20$	$1.00X10^{-4}$	7460	5	+4°C, DARK
D	ORGANIC SUPPLEMENTS				
	Myo-Inositol	$4.90x10^{-4}$	20000		
	Nicotinic acid	$4.66x10^{-6}$	100		
	Pyridoxine-HCL	$2.40x10^{-6}$	100	5	+4°C
	Thiamine-HCL	$3.00x10^{-7}$	100		
	Glycine	$3.00x10^{-5}$	400		
E	CARBON SOURCE				
	sucrose	8.80x10-2	-	Add as solid (30 g/L)	-

APPENDIX B

Solvent used for Plant Growth Hormones stock

Plant Growth Hormones	Solvents
BAP	EtOH
IAA	1 NaOH

APPENDIX C

Two types of sterilization of plant growth hormones

A) Heat stable plant growth hormones

- BAP
- The heat stable PGR was added into media before autoclaving

B) Non heat stable PGR

- IAA
- The hormones was filter-sterilized through a 0.2 µm filter unit sterile bottle and added to following autoclaved sterile medium.

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MQT 2900: Master Project

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Content

1. Introduction
2. Literature review
3. Research methodology
4. Result
5. Discussion

1-Inroduction

- Plant tissue culture, broadly refers to technique of growing plants cells, tissues, organs, seed in a sterile environment on a nutrient medium, and the culture depend on three essential abilities of plant which are totipotency, dedifferentatiation, and competency.
- Totipotency is the ability of a single cell, divides and manufactures all differentiated cells in an organism such as extraembryonic tissues.

- Dedifferentiation is the capacity of mature cells to return to merismatic condition and development of a new growing point.
- competency is described the endogenous potential of given cell to develop in a particular way (Adrinal *et.al*., 2003; Roberta, 1992; Bhojwani 1992).

2-Literature review

- The genus of *Nicotiana tabaccum* is member of the family Solanaceae that contains 75 species. Some of important species are: *Nicotiana rustica, Nicotiana clevelandi, Nicotiana otta* and *Nicotiana tabaccum. N. tabaccum* is the well-known species which spreads all around the world.

Taxonomy of *Nicotiana tabaccum*	
Kingdom	Plantae
Subkingdom	Tracheobionta -- vascular plants
Division	Magnoliophyta -- angiospermes, angiosperms, flowering plants,
Class	Magnoliopsida -- dicots, dicotylédones, dicotyledons
Subclass	Asteridae
Order	Solanales
Family	Solanaceae -- nightshades, solanacées
Genus	*Nicotiana L.* – tobacco
Species	*tabacum L.* -- cultivated tobacco

Tobacco plants have been applied as a model plant system; they have the advantage of short generation time and their DNA has been extensively mapped over the years.

- **Plant growth hormones**

Auxins

The hormone promotes both cell division and cell growth. The most important member Auxins family is indole-3-acetic acid (IAA). On the other hand IAA applies in plant culture media is limited. IAA is unstable in both heat and light (Davis, 2004). Other Auxins are described in Table 2.3.

Cytokinin

Cytokinins are a group of phytohormone substance active in promoting cell division, differentiation and some other physiological processes. They are most complex class of plant growth substances, and the hormone has two forms; natural and synthetic.

Research Objectives

- The objective of the research is to establish an easy method in tissue culture of *Nicotiana tabaccum TAPM 26.* The establishment of this technique may be used for future research involving plant genetic engineering.

Scope of the Study

- Establishment of efficient *in vitro* plant regeneration from *Nicotiana tabaccum* cotyledon by optimization of plant growth hormones, explant sizes and explant ages.

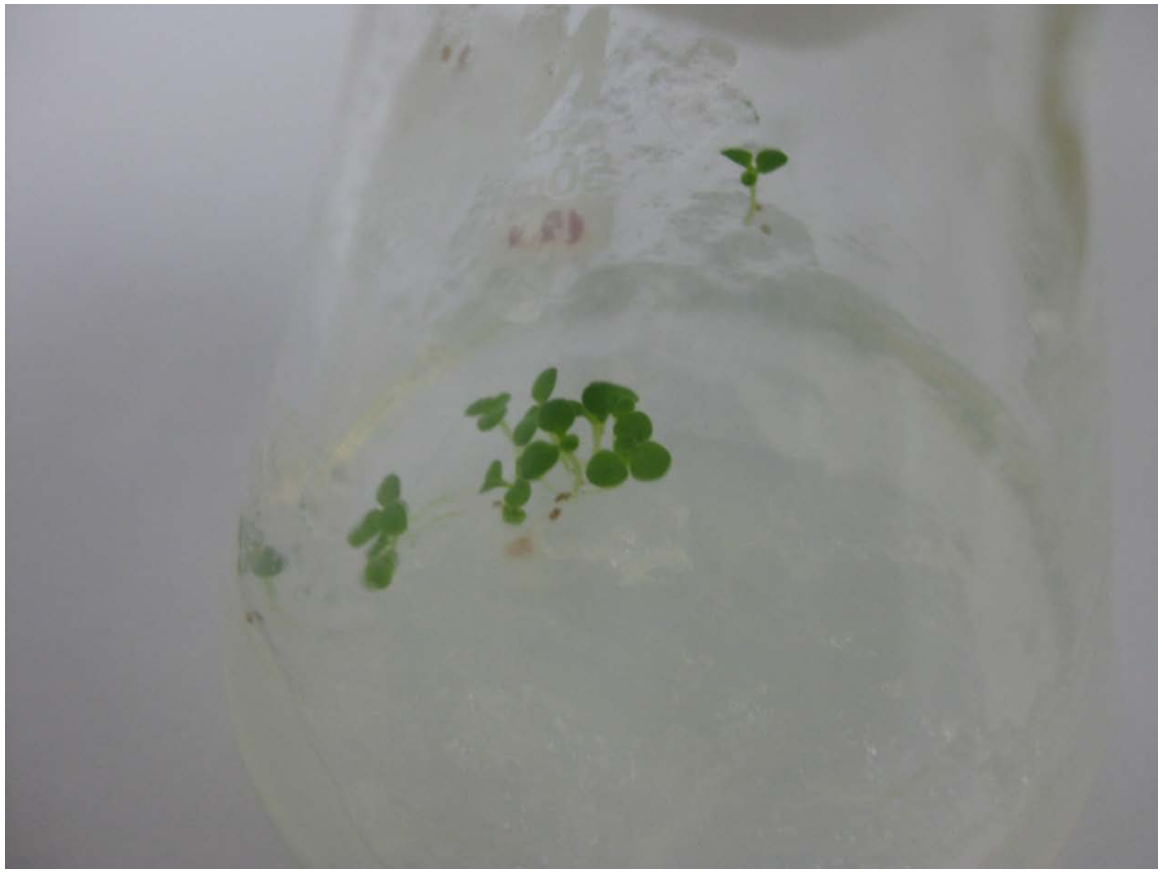
3-Research methodology

1. Aseptic condition
2. Media preparation
3. Plant growth hormones preparation
4. Seed sterilization and germination
5. Preliminary research of effect of age, size and plant hormone on shoot regeneration
6. The effect of growth hormones on shooting
7. The effect of growth hormones on rooting

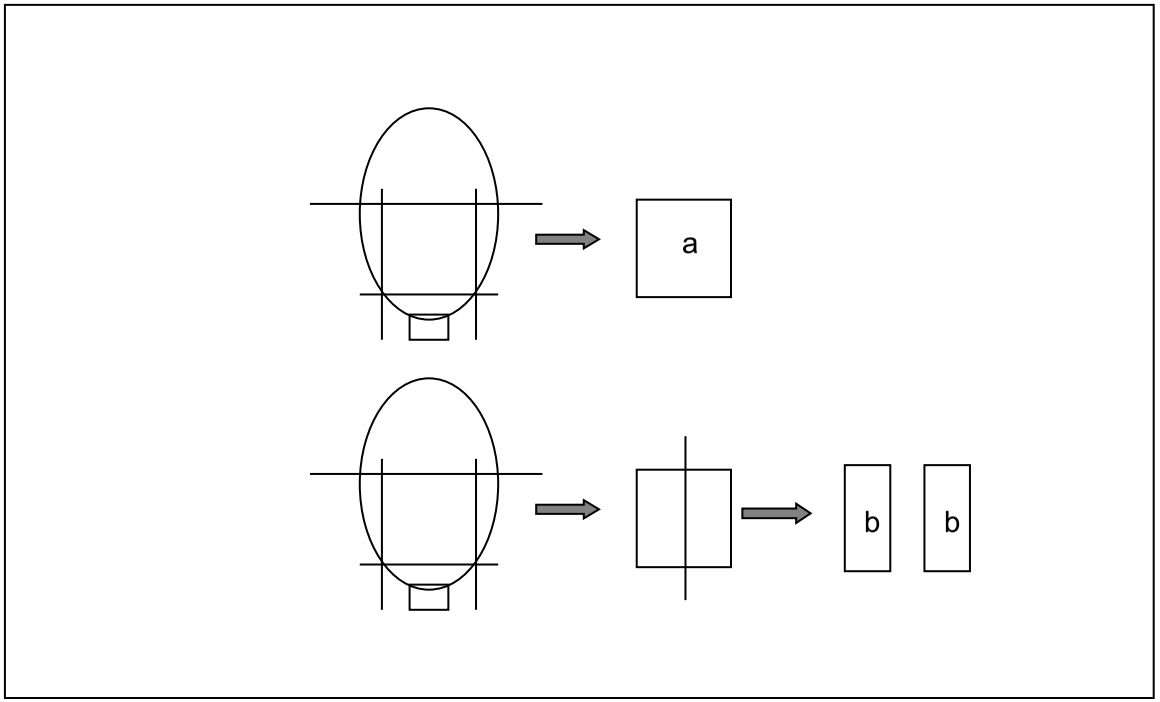
4-Results

Times of imbibitions	seed case	Germination (%)	Contamination (%)
0 h	dry-seed	73	16
	wet-seed	50	36
2 h	dry-seed	83	26
	wet-seed	36.5	40
4 h	dry-seed	80	26.6
	wet-seed	29.8	45

Seed sterilization and germination



Seed germination



Dissection of cotyledon explant from a 5 and 7-day-old seedlings of *Nicotiana tabacum.* Explant type 'a' is the cotyledon proximal with 1mm margin removed, explant type 'b' is the half cotyledon proximal.

Explants age			
5 day-old			
Type	Concentration of BAP (µM)	Mean number of shoot per explants	Shoot regeneration (%)
	0	0	0
	0.5	6.33±0.88	54
a	1	7.66±0.88	44
	2	8.33±0.80	77
	4	8.50±0.50	55
	0	0	0
	0.5	6.0±0.57	54
b	1	3.66±0.66	77
	2	9±0.57	100
	4	8.6±0.87	44
7-day-old			
Type			
	Concentration of BAP (µM)	Mean number of shoot per explants	Shoot regeneration (%)
	0	0.00	0
	0.5	5.33±0.33	33
a	1	3.0 ±0.57	54
	2	6.66±0.88	88
	4	3.66±0.88	44
	0	0.0	0
	0.5	4.0±0.56	66
b	1	5.0 ±0.59	55
	2	3.0±0.50	66
	4	5.3±0.88	44

Preliminary research of effect of age, size and plant hormone on shoot regeneration

Growth regulators (µM)		Mean number of shoot per cotyledon ± SE	Percentage of shooting (%)
BAP	IAA		
0	0	0.00±0.00	0
0.1	0	4.3±0.66	55
1	0	8.33±2.4	66
10	0	9.83±0.54	44
0.1	0.1	5.0±1.15	66
1	0.1	9.66±1.2	98
10	0.1	7.0±1.5	100
0.1	10	7.66±1.4	66
1	10	9.0±2.5	44
10	10	5.6±0.5	55

The effect of growth hormones on shooting

Type of hormones		Percentage of Rooting (%)
IAA(μM)	BAP(μM)	
0	0	0
0,1	0	100
1	0	66
10	0	66
0,1	0,1	55
1	0,1	77
10	0,1	66
0	0,1	0
0	1	0
0	10	0

The effect of growth hormones on rooting

5-discussion

1- Germination of *Nicotiana tabacum*

- The experiments were carried under aseptic conditions.
- The seeds of *Nicotiana tabaccum* were germinated on solid MS media without any growth hormones (Vissenberg *et.al*., 2001).
- The seed germination was best on Murashige and Skoog (1962) media mainly due to the availability of nutrients in sufficient amount as compared to agar media consisting of only 30 g/l sucrose, 8 g/l agar and distilled water.
- Temperature around 25 ^{0}C and photoperiod of 16 h light and 8 h dark were favorable for germination and further growth of seedling (Ekrum, 2001).

- The seed germination in the presence of light confirmed the result of Bradbeer (1988) reported that light is a requirement for germination of *Nicotiana tabaccum* seed (Harbinder, 1988).
- In this research, *Nicotiana tabaccum TAPM 26 of* seeds were disinfected for 20 minutes in 10 % (v/v) bleach solution and 1 minute for 70% (v/v) ethanol
- These result are in agreement with those of Harbinder (1988) and Ekrem (2001).

2 **Preliminary Studies on Shoot Regeneration of Cotyledon Section**

- In the current studies, the best treatment for shoot production was the addition of BAP at the concentration of 2µM (b type) of 5-day-seedling. The present work result was different from other reports because of difference of cultivar (different genotype), age of donor explants, and size of explants

- The explants of age and size have illustrated to be key for morphogenesis induction (Harbinder, 1988). Cytokinin-induced shoot organogenesis in *Nicotiana tabacum* was well documented (Skoog and Miller 1957; Prabhudesai and Narayanswamy 1974; Brown and Thorpe 1986). My results of shoot induction by BAP were not in agreement with these findings.

3-Comparison of Different Combination of Auxin (IAA) and Cytokinin (BAP) for Shoot Induction

- In current study, for shoot production, the best treatment was the addition of (BAP) at concentration of 1 µM with 0.0 µM IAA.
- These results are in agreement with those of Attfield and Evans (1991a) in a different cultivar of *Nicotiana tabacum.* BAP was very effective in shoot formation from cotyledon explants as shown in Table 4.4.

4 Plant Growth Regulator (IAA and BAP) Effect on Rooting on *Nicotiana tabaccum*

- For rooting, the shoot *Nicotiana tabaccum* were cultured onto MS medium supplemented with IAA and BAP.
- All explants cultured on the media supplemented with IAA demonstrated good rooting within a period of three weeks.
- For root regeneration IAA at 10 µM alone were obtained better among all combinations tested.
- These results arent in agreement with Harbinder (1988).
- The IAA response to root organogenesis was different in different experiments. It was most probably due to unstable nature of IAA. And in addition studied done by Nissen and Sutter (1990) showed that IAA was not stable and IAA was readily degraded during autoclaving and culture

conclusion

- The result from this study had shown that the development of tissue culture of *Nicotiana tabaccum TAPM 26* through cotyledon regeneration was influenced by some reasons, such as source of explants, age of explants, and type of growth regulators supplemented to the MS medium.
- For cotyledon explants, IAA with BAP was optimal for shoot culture.

- The present study shown that the production of shoot from cotyledon explants of the seed, using BAP at concentration of 10 µM was found the most effective among the IAA X BAP combination tested.
- The best root formation from cotyledon explants was found when Indole-3-butyric (IAA) was at 10 µM.
- Plant growth hormones promote the optimum growth explants and also induced the shoot formation. For the development of protocols for regeneration the use of IAA should be avoided because of its unstable nature during its sterilization, such as autoclaving.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Thank you

Seed germination (After 15 days)

5-day old type a (1 µM)(after 31 days)

7-DAY SEEDLING TYPE B (2 µM) (after 31 days)

Shoot-BAP 2 µM (31 days)

Root induction (after 31 days)