UNIVERSITI TEKNOLOGI MALAYSIA

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LAPORAN AKHIR PENYELIDIKAN

TAJUK PROJEK : IN VITRO REGENERATION OF GARDEN BALSAM, IMPATIENS BALSAMINA USING COTYLEDONS DERIVED FROM SEEDLINGS

Saya FAHRUL ZAMAN HUYOP

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IN VITRO REGENERATION OF GARDEN BALSAM, *IMPATIENS BALSAMINA* USING COTYLEDONS DERIVED FROM SEEDLINGS

(REGENERASI SECARA *IN VITRO* BALSAM TAMAN OLEH *IMPATIENS BALSAMINA* MENGGUNAKAN KOTILEDON YANG DIDAPATI DARI BIJI BENIH)

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Dedication

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IN VITRO REGENERATION OF GARDEN BALSAM, _IMPATIENS BALSAMINA_ USING COTYLEDONS DERIVED FROM SEEDLINGS

(*Keywords: plant tissue culture, cotyledons and hypocotyls explants, Impatiens balsamina)*

Garden balsam, *Impatiens balsamina*, is one of an important ornamental plant in Malaysia. An effort to develop a reliable tissue culture system for this *Impatiens* species was carried out for future genetic manipulation of this plant for useful traits. Parts of cotyledons and hypocotyl derived from different age of seedlings, from surface sterilized seeds, were subjected to shoot induction on MS media containing BAP, TDZ or combination of BAP and NAA in different ratios. Roots were developed from the above shoots on full- or half-strength MS media containing IAA, IBA or NAA of different concentrations. Finally fully-developed plantlets were produced on hormone free MS media or full- or half-strength MS media containing specific hormones. It was observed that the most efficient shoot induction (93%) was obtained from proximal part of cotyledon derived from 7 days old seedlings on MS media supplemented with 1mg/L BAP. Roots were most efficiently produced (92%), in term of percentage and morphology, on half-strength MS media supplemented with 0.1 mg/L IAA. Further regeneration of plantlets, approximately 8-10 cm in height, was achieved after 3 weeks on hormone-free MS media. Finally, full regeneration system of *Impatiens balsamina* was developed, using MS media supplemented with 1 mg/L BAP for shoot and 0.1 mg/L IAA for roots, within 8 weeks in culture.

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REGENERASI SECARA *IN VITRO* BALSAM TAMAN OLEH *IMPATIENS BALSAMINA* MENGGUNAKAN KOTILEDON YANG DIDAPATI DARI BIJI BENIH

(*Kata kunci: plant tissue culture, cotyledons and hypocotyls explants, Impatiens balsamina)*

Balsam taman, *Impatiens balsamina*, merupakan salah satu tumbuhan perhiasan yang penting di Malaysia. Usaha untuk mengembangkan teknik kultur tisu untuk spesis ini telah dilaksanakan bagi tujuan menghasilkan tumbuhan terubah genetik pada masa hadapan sebagai tanaman berguna. Bahagian kotiledon dan hipokotil dari pelbagai umur biji benih yang telah di sterilkan permukaannya telah diuja untuk pembentukan pucuk atas media MS mengandungi BAP, TDZ atau campuran kedua BAP dan NAA dalam nisbah yang berbeza. Akar dan pucuk telah tumbuh dari kultur yang sama atas media MS penuh dan separa yang mengandungi IAA, IBA atau NAA pada kepekatan berbeza. Pada akhirnya, anak pokok dapat dibentuk atas media MS penuh dan separa tanpa kehadiran hormon yang spesifik. Pemerhatian lanjut ialah induksi pucuk sangat baik (93%) telah didapati dari bahagian proksimal kotiledon pada umur 7 hari atas media MS yang dibekalkan dengan 1 mg/L BAP. Akar juga tumbuh dengan baik (92%), dari segi peratusannya atas media MS separa yang di bekalkan dengam 0.1 mg/L IAA. Regenerasi selanjutnya ialah, anak pokok telah tumbuh pada ketinggian 8-10 cm selepas minggu ke 3 dalam media MS tanpa hormon. Akhirnya, proses regenarasi *Impatiens balsamina* telah berjaya sepenuhnya menggunakan media MS yang di bekalkan dengan 1 mg/L BAP untuk pembentukan pucuk dan 0.1 mg/L IAA untuk pembentukan akar selama 8 minggu dalam kultur.

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CHAPTER 1

1.0 General Introduction

Impatiens balsamina is locally known as Garden Balsam and are found growing throughout tropical Africa, India, southwest Asia, southern China, Japan, as well as parts of Europe, Russia, and North America (Grey-Wilson, 1980). It has also been cultivated as an ornamental plant in many parts of the world.

1.1 General Problem Statement or State of the Art

This research highlights the efforts to develop a reliable tissue culture technique for one of the Malaysian's balsam, *Impatiens balsamina.* Plant tissue culture is a fundamental knowledge before can proceed to more advance research. For example after establishing plant tissue culture technique we can proceed to plant transformation system study in order to develop a transgenic plant.

1.2 Objectives and scope of research

Tissue culture system for this *Impatiens* species was developed using cotyledons and hypocotyls derived from seedlings. The explants were regenerated through shoot and root inductions and followed by *in vitro* regeneration of whole plantlets. The effects of seedlings age and various concentrations of MS media in combination of different types and concentrations of cytokinins or auxins, on this *Impatiens* species were evaluated and will be discussed. Current study will focus on establishment the plant regeneration system of *Impatiens balsamina*.

CHAPTER 2

2.0 LITERATURE REVIEW

Impatiens balsamina is locally known as Garden Balsam and are found growing throughout tropical Africa, India, southwest Asia, southern China, Japan, as well as parts of Europe, Russia, and North America (Grey-Wilson, 1980). It has also been cultivated as an ornamental plant in many parts of the world. The genus *Impatiens* is estimated to contain between 400-850 species (Grey-Wilson, 1980). It is annual plant with a soft stem and produced four different color of flower, purple, pink, white and red. The flower of *Impatiens balsamina* is a very attractive model for analysing the flowering process as it has an absolute requirement for short day conditions for flowering, and flower reversion can be obtained in a predictable way after transfer to long day conditions (Pouteau et al., 1998). The parts of the plants has been traditionally claimed to have medicinal properties such as leaves used for treating warts and flowers used to cool burning skin or cooling fewer. An antimicrobial peptide from *Impatiens balsamina* has been shown to have antifungal activity against *Candida albicans* (Dong et al., 1980).

Impatiens is one of the host plants for Impatiens Necrotic Spot Virus (INSV), the virus that could infect the crops in many species. As this plant has ornamental value, the use of biotechnological techniques to produce

transgenic plants for resistant to INSV has a clear advantage (Daughtery *et al.*, 1997). However, in order to produce transgenic *Impatiens balsamina* plants, the plant regeneration system need to be established. Plant regeneration system of many *Impatiens* species was achieved using multiple shoots from variety of explants such as cotyledons of immature ovules of *Impatiens platypetala* Lindl (Kyungkul, 1993) and shoot tips from both *Impatiens* hybrids (Kyungkul and Stephens., 1987) and Java x New Guinea *Impatiens* (Stephens *et al.*, 1985). Multiple shoots were also successfully regenerated from cotyledon explants of other plants species such as *Pinus* (Sul and Korban, 2004), barley (Sharma *et al.*, 2004), squash (Ananthakrishnan *et al.*, 2003), Bambara groundnut (Lacroix *et al.*, 2003), *Terminalia chebula* (Shyamkumar *et al.*, 2003) and cotton (Agrawal, 1997). Cotyledons usually utilize the meristematic cells and were chosen to establish a plant regeneration system. Based on previous reports, cotyledons were proven to be the most responsive explants for many species (Kyungkul, 1993).

Plants growth regulators are media components that have an effect on growth and development of plant organs. There are five classes of plant growth regulator: auxins, cytokinins, gibberellins, abscisic acid and ethylene. Generally, auxins and cytokinins are the most widely used plant growth regulator in plant tissue culture which will determine the type of culture and regeneration of explants. Auxin in high ratio generally induces the roots whereas high cytokinin will induce the shoots, and the intermediate ratio favors on callus formation. Auxin promotes both the cell division and cell growth and cytokinins promote the cell division (Slater *et al.*, 2003).

Cytokinins has clearly played an important role in various plant development processes, including the promotion of cell division, the counteraction of senescence and the regulation of apical dominance. (Sakikabara, 2004). The endogenous hormones that are naturally synthesized are such as IAA and zeatin. IAA, is sensitive to both heat and light. However, currently more stable of synthetic auxins and cytokinins have been widely used in plant cell culture (Slater *et al.*, 2003).

Auxins are defined as organic substances that promote cell elongation when applied in low concentrations to plant tissue segments (Jennifer *et al.*, 2004). There is evidence on the cell elongation and growth of tobacco young shoot using GUS assay, where the presence of auxin on the lower side of the stem resulting in elongation of one side of the leaves and causing the leaf to bend. Endogenous auxin, IAA, is synthesized in young apical meristem and is transported basipetally to the growing zones of the stem, and more distantly to the root via polar transport system (Davies, 2004).

The auxin transport is a polar transport which requires energy driven by ATP. The mechanism of auxin transport is one of chemiosmosis system which dependent on the H^+ gradients generated from the proton pump. The proton pumps located in plasma membrane play a role in growth response of cells. The acid growth theory states that auxin stimulates the proton pump to lower the pH of the cell wall. This wall acidification will activate enzymes that break the hydrogen bonds between the cellulose microfibrils and loosening

the wall. These cells will have higher water uptake via osmosis, and the turgor pressure will push to loosen the cell wall. This pressure will enhance the elongation of cells and simultaniously the growth of plant cell (Lack and Evans, 2005).

This report highlights the efforts to develop a reliable tissue culture system for one of the Malaysia balsam, *Impatiens balsamina*. Tissue culture system for this *Impatiens* species was developed using cotyledons and hypocotyls derived from seedlings. The explants were regenerated through shoot and root inductions and followed by *in vitro* regeneration of whole plantlets. The effects of, seedlings age and various concentrations of MS media in combination of different types and concentrations of cytokinins or auxins, on this *Impatiens* species were evaluated and will be discussed.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Plant materials and seed sterilization

All seeds were washed under running tap water for 1 hour. This folloed by surface sterilization by soaking the seeds in 70 % (v/v) ethanol for 1 minute and 10% (v/v) commercial sodium hypochlorite solution for 10 minutes. Seeds were rinsed with sterile distilled water three times and blotted dry on sterile filter paper.

3.2 Germination of seeds

Seeds were germinated on MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, vitamins (glycine 2 mg/L; myo-inositol 100 mg/L; nicotinic acid 0.5 mg/L; pyridoxine HCL 0.5 mg/L and thiamine HCL 0.1 mg/L) and solidified with 0.8 % (w/v) Bacto Difco agar. The pH of media was adjusted to 5.7 with 0.1 M NaOH or 0.1 M HCL prior to sterilization.

3.3 Plant growth regulators and stock solutions

Plant growth regulator stock solutions were prepared at 1 mg/ml concentration by dissolving in appropriate solvent. All plant growth regulators except for IAA and IBA were added into the media before sterilization. IAA and IBA were filter sterilized using 0.2 µm filters (Whatman, USA) and were added into the media after autoclaving and cooling the media to 50˚C.

3.4 Explants preparations

Cotyledons and hypocotyls were used as source of explants. Cotyledons were excised from 7, 14 and 21 days old *in vitro* seedlings. Cotyledons were separated into proximal and distal parts. Hypocotyls were cut in three different sections for near base, center and near cotyledon. All explants with different ages and sections from cotyledons and hypocotyls were transferred onto shooting media containing MS (Murashige and Skoog, 1962) with 1 mg/L BAP and 3 mg/L BAP, respectively. Each experiment was carried out in three replicates with ten explants. The experiments were repeated three times for reconfirmation. Figure 1 showed examples of 7 days old seedling explants for both cotyledons and hypocotyls.

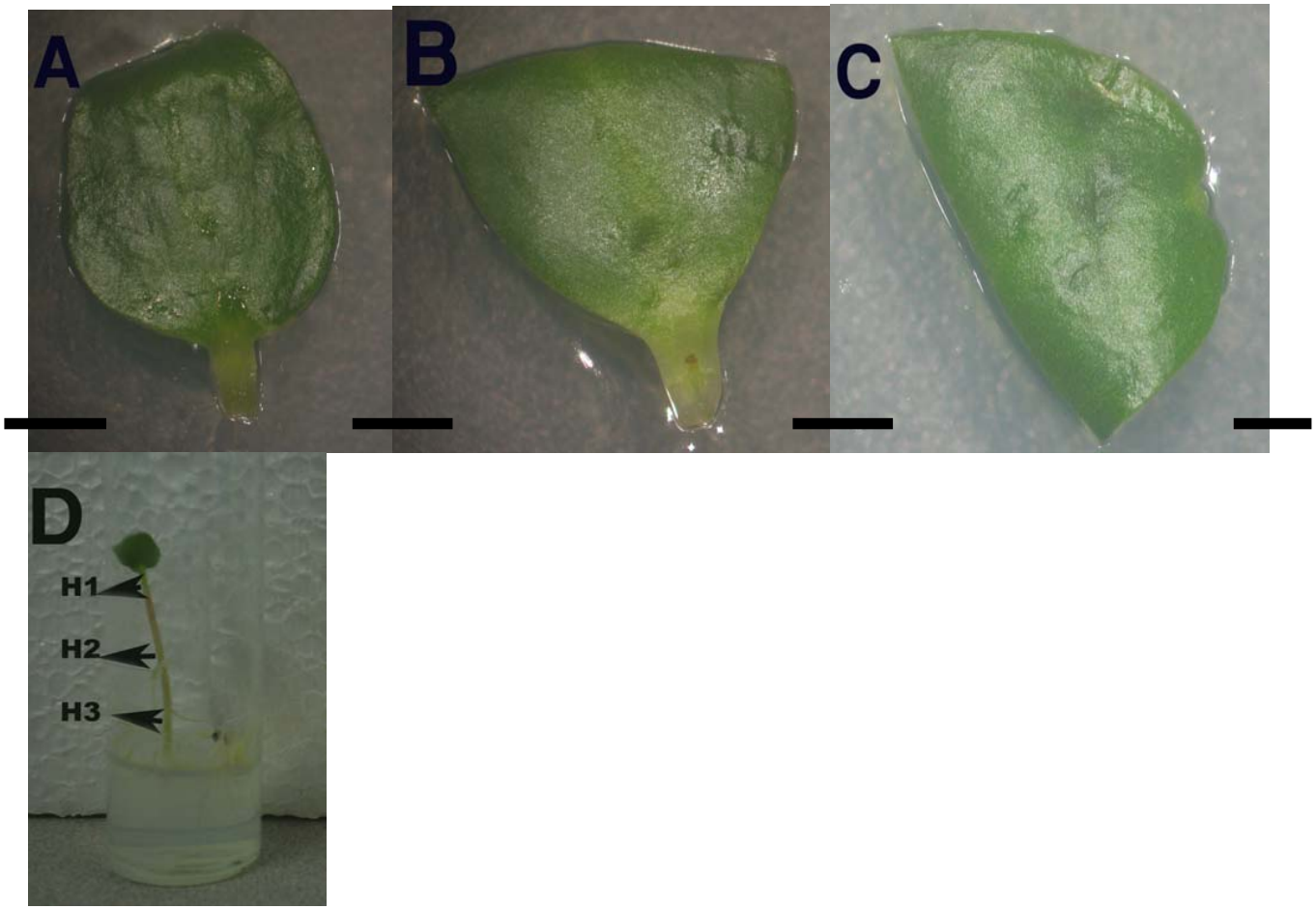
3.5 Shoot induction experiment

Explants were transferred to shooting media consisting of MS macro- and micronutrients supplemented with different concentration of cytokinin hormones TDZ, BAP alone or in combination with different ratio of NAA (Table 1). Each treatment was carried out with three replicates containing ten explants and the experiments were repeated three times. The explants on

media were cultured in an incubator at 25° C ± 2° C with 16 h photoperiod, 17 µmol m^{-2} s^{-1} supplied by "coolight" Daylight fluorescent tubes (LICOR, USA).

3.6 Root induction experiment

Explants were transferred onto rooting media containing MS macro- and micronutrient supplemented with different concentration of NAA, IAA or IBA (Table 2). The effect of media types on rooting was carried out using either full- or half strength MS mediums. Each experiment contained three replicates with ten explants and the experiments were repeated three times. The explants on media were cultured in an incubator at 25° C ± 2° C with 16 h photoperiod, 17 µmol m^{-2} s^{-1} supplied by "coolight" Daylight fluorescent tubes (LICOR, USA).


A
B
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H1
H2
H3

Figure 1 The different types and sections of 7 days old seedling explants.

Cotyledon used in the study. Bar= 0.5cm.

Proximal section of cotyledon. Bar= 0.2cm.

Distal section of cotyledon. Bar= 0.2cm.

Hypocotyls with different section in H1 (near cotyledon), H2 (center), and H3 (near roots). Bar=1 cm.

Table 1. The types and concentrations of cytokinins used in shoot induction media.

Cytokinins	**Concentrations (mg/L)**
BAP	1.0 3.0 5.0
BAP : NAA	: 0.5, and 1.0:1.0 3.0:0.5 and 3.0: 1.0 5.0: 0.5 and 3.0: 1.0
TDZ	0.5, 1.0, and 3.0

Table 2. The different types and concentrations of auxins on root induction.

Auxins	**Concentrations (mg/L)**
IAA	0.1, 0.5 and 1.0
IBA	0.5, 1.0 and 2.0
NAA	0.1, 0.5 and 1.0

3.7 *In vitro* regeneration.

Cotyledons from proximal section were cultured onto optimal shooting MS macro- and micronutrient supplemented with 1 mg/L BAP for 3 weeks. The shoots were transferred onto optimal rooting media in half strength MS media supplemented with 0.1 mg/L IAA. After 2 weeks on rooting media, plantlets were sub-cultured in different media for development observation. Half of the plantlets were sub-cultured onto half strength MS media supplemented with 0.1 mg/L IAA and the other half were transferred onto hormone-free MS media. Development of plantlets, measured as height, was observed up to 3 week. The plantlets were cultured in an incubator at 25° C ± 2° C with 16 h photoperiod, 17 µmol m^{-2} s^{-1} supplied by "coolight" Daylight fluorescent tubes (LICOR, USA).

CHAPTER 4

4.0 RESULTS

4.1 The effect of explants age, types and sections on shoots induction.

The morphological and biochemical characteristics of cotyledons change with age and affect on cytokinin uptake. The plant regeneration potential also exists from proximal and distal section within a cotyledon and different section of hypocotyls of *Impatiens balsamina*. Therefore, this experiment was carried out to evaluate the effects of age, types and sections of explants on shoot induction.

In the present study, the highest shooting percentage (88%) was obtained from 7 days old seedling of proximal cotyledon sections followed for 14 days (86%) and 21 days old seedling (81%), respectively (Table 3). No shoot was induced from distal section of the cotyledons tested. On the other hand, callus formation was only observed in distal cotyledon section with the highest percentage (76%) of white callusing obtained from 7 days old cotyledons explants (Table 3 and Figure 2). However, the callus failed to produce any shoot after four weeks of sub-culturing on shooting media due to necrosis.

Hypocotyls were also tested for shoot induction. However, when all ages and sections tested, hypocotyls only produced green to brown callus without any shoots after 2 weeks on MS media supplemented with 3 mg/L BAP (Figure 2).

From the above observation, it could be suggested that 7 days old seedling of proximal cotyledon section was the best to be used as an explant throughout the experiment for shoot induction.

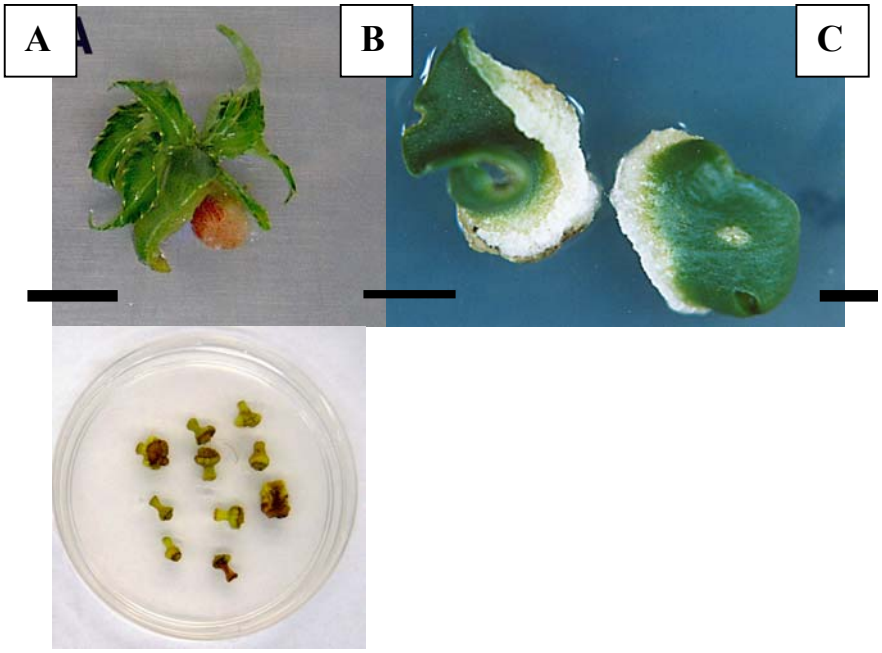

Figure 2. The effect of cytokinins in shooting media on different types of explants after 3 weeks in culture.

Proximal sections with shoots formation on MS media supplemented with 1 mg/L
BAP. Bar = 0.5cm.

Distal parts with white callus formation on MS media supplemented with 1mg/L
BAP. Bar = 0.2cm.

Hypocotyls with green to brown callus on MS media supplemented with 3 mg/ L
BAP. Bar = 2 cm.

Table 3. The effect of different explants age and parts of cotyledons on shoot induction after 3 weeks in MS media contained 1 mg/L BAP.

Explant ages	Parts of cotyledon	Average number of shoots per explants ± SE	Percentage shooting explants (%)	Percentage rooting (%)	Percentage callus formation (%)
7 days	Proximal	4.4 ± 0.21	88	0	0
	Distal	0	0	0	76
14 days	Proximal	3.0 ± 0.18	86	0	0
	Distal	0	0	0	54
21 days	Proximal	1.9 ± 0.18	81	0	0
	Distal	0	0	0	52

4.2 Shoot Induction

Cytokinin of different types and concentrations has been widely used for shoot induction in many plants. In the present study, proximal section of 7 days old seedling's cotyledon showed the highest percentage of shooting (93%) and average number of shoots (6.8) on MS media supplemented with 1 mg/L BAP alone after 3 weeks on culture (Table 4 and Figure 3 A). Development of multiple shoots is demonstrated in Figure 5 A-E. Multiple shoots induction was achieved in all treatments tested. However, MS media supplemented with BAP showed the best result.

Combination of BAP and NAA on MS media induced callus formation. However, the combination also inhibited shoots elongation as compared to BAP alone (Figure 3 B). On the other hand, TDZ treatment induced both shoots and roots. However, the effects of TDZ was not consistent when the shoots and roots induction was inhibited in certain times due to the intermediate actions of TDZ as cytokinin and auxin in culture system (Figure 3 C). High percentage of shooting was also observed for the control culture. However, the average number of shoots was lower compared to ctyokinin treatments. In addition, shoot was induced in control explants after two weeks in culture as compared to within one week for the treated.

Hormones	Concentrations (mg /L)	Average number of shoots ± SE	Percentage of shooting (%)	Percentage of rooting (%)	Percentage of callus formation (%)
BAP	1.0	6.8 ± 1.05	93	0	0
	3.0	3.6 ± 0.63	85	0	0
	5.0	3.6 ± 0.65	84	0	0
BAP: NAA	: 0.5	1.4 ± 0.25	60	77	99
	: 1.0	1.7 ± 0.40	60	78	96
	: 0.5	1.3 ± 0.26	63	80	100
	: 1.0	1.2 ± 0.47	50	82	100
	: 0.5	1.5 ± 0.32	70	65	100
	: 1.0	1.7 ± 0.59	50	76	100
TDZ	0.5	4.1 ± 0.86	87	97	0
	1.0	4.4 ± 1.09	83	99	0
	3.0	3.8 ± 0.97	82	100	0
Control	0	1.2 ± 0.02	80	100	0

Table 4. The effect of different concentrations of hormones on shoot induction
after 3 weeks in culture.

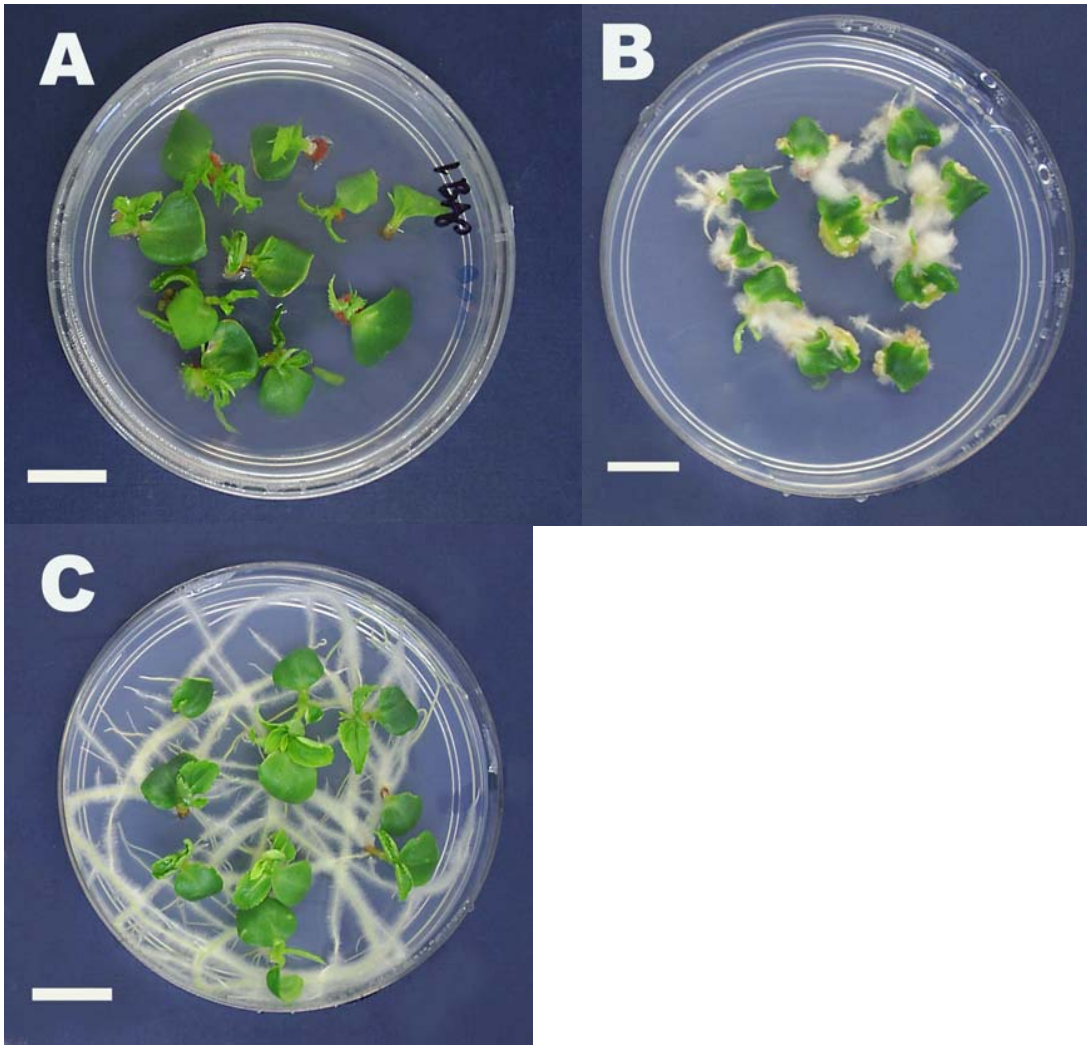
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Figure 3. Shoot induction from the proximal section of 7 days old seedling cotyledons in MS media supplemented with different concentrations of hormones after 3 weeks in culture.

1 mg/L BAP. Bar= 1.5 cm.

5 mg/L BAP with 0.5 mg/L NAA. Bar=1.5 cm.

0.5 mg/L TDZ. Bar= 1 cm.

4.3 Root Induction

Overall result showed that there was no significant difference for all types or concentration of auxin treatment tested for root induction for *Impatiens balsamina*. However, percentage of rooting was the highest for half-strength MS media supplemented with 0.5 mg/L IBA (96%) followed by 0.1 mg/L IAA (92%) or o.5 IAA for full-strength MS (942) (Table 5). Root morphology was also evaluated. Treatment with 0.1 mg/L IAA showed the production of more hairy and longer roots. The growth of roots was faster using IAA, i.e. within 4 days post-treatments as compared to IBA treatment (Figure 4). Therefore, in term of root morphology, 0.1 mg/L IAA was the best treatment and was used for rooting induction throughout the experiment.

The used of half MS media by diluting mineral solution in root induction media could reduce nitrogen concentration that effect on root induction. Therefore, the use of full and half-strength of MS media showed that half strength MS media gave better roots induction in terms of percentage and morphology. In the present study, half strength MS media induced rooting in at least 90% of the explants as compared to full strength MS media with showed a lowest percentage of rooting of 88% (Table 5 and Figure 4). In terms of morphology, the roots obtained from full strength MS media were less hairy as compared to the root obtained using half strength MS media were treated with auxin. However, for control treatment, roots induction was also observed in both concentrations of MS media with an average of 90% of rooting with less hairy roots (Table 5).

Table 5. The effect of media types and different concentrations of hormones on roots induction after 2 weeks in culture.

Hormones	Concentration	Full MS		Half strength MS	
		Average of Rooting ± SE	Percentage of rooting (%)	Average of rooting ± SE	Percentage of rooting (%)
IAA	0.1	9.0 ± 0.17	90	9.2 ± 0.15	92
	0.5	9.4 ± 0.18	94	9.2 ± 0.17	90
	1.0	8.8 ± 0.22	88	7.8 ± 0.22	78
IBA	0.5	9.0 ± 0.33	90	9.6 ± 0.18	96
	1.0	9.3 ± 0.24	93	9.1 ± 0.26	91
	2.0	9.0 ± 0.24	90	9.3 ± 0.16	90
NAA	0.1	8.4 ± 0.17	84	9.1± 0.2	91
	0.5	9.0 ± 0.24	90	9.0 ± 0.29	90
	1.0	9.0 ± 0.24	90	9.0 ± 0.17	90
Control	0	9.0 ± 0.23	90	9.2 ± 0.32	92

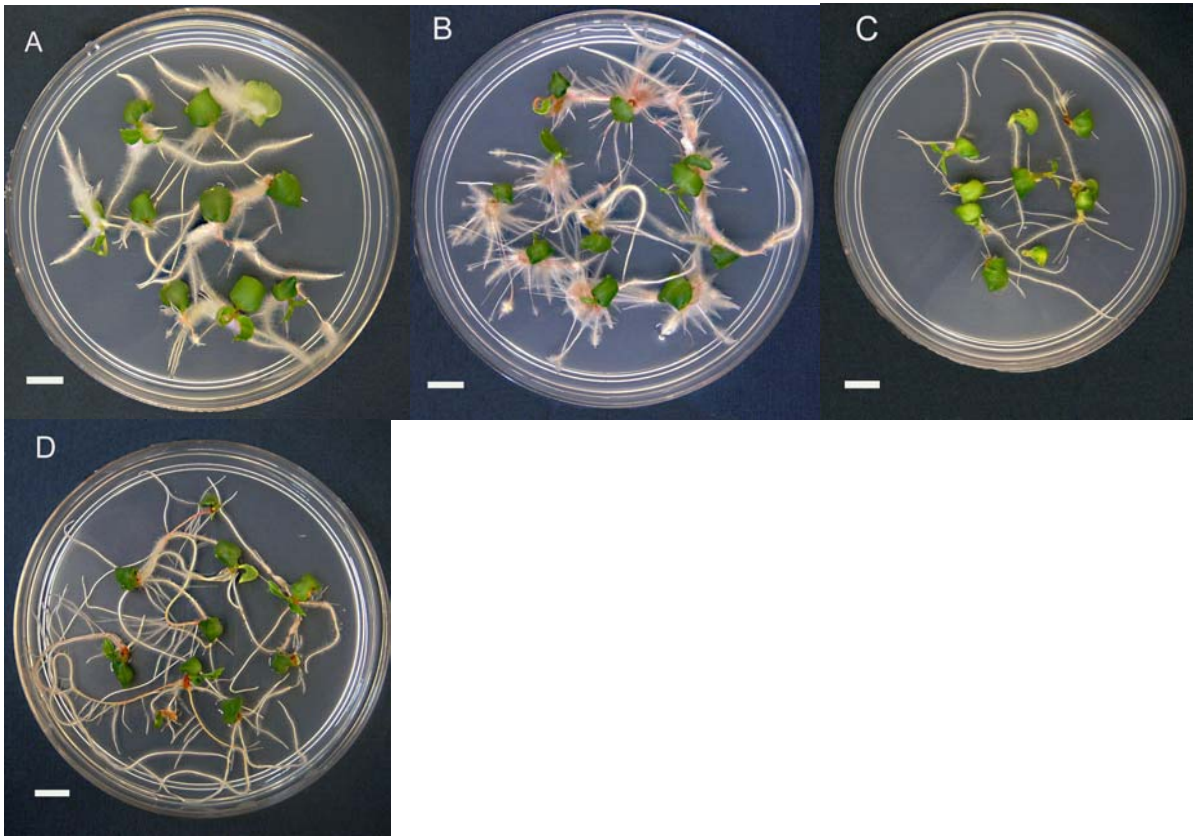
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Figure 4. Comparison of root morphology from different auxins treatment in different concentrations of MS media after 2 weeks in culture.

Root induction in half strength MS media supplemented with 0.1 mg/L IAA. Bar=0.1cm.

Root induction in half strength MS media supplemented with 0.5 mg/L IBA. Bar=0.1cm.

Control in half strength MS media. Bar=0.1cm.

Control in full strength MS media. Bar=0.1 cm.

4.4 *In vitro* regeneration.

After 8 weeks in culture, *in vitro* plantlets were achieved from various explants and treatments (Figure 5 H). The plantlets were later transferred to a different media for development comparison. The plantlets were sub-cultured on hormone-free MS media for another three weeks. After three weeks further growth of plantlets approximately 8 to 10 cm were observed. In contrast, slower growth of plantlets with 5 cm in height, was observed when plantlets were continuously cultured on rooting media containing half strength MS media and supplemented with 0.1 mg/L IAA. Slower growth of plantlets in half strength MS medium with 0.1 mg/L IAA possibly affect on growth of plantlets because it was under pressure by hormones that could interferes with elongation and development of plantlets.

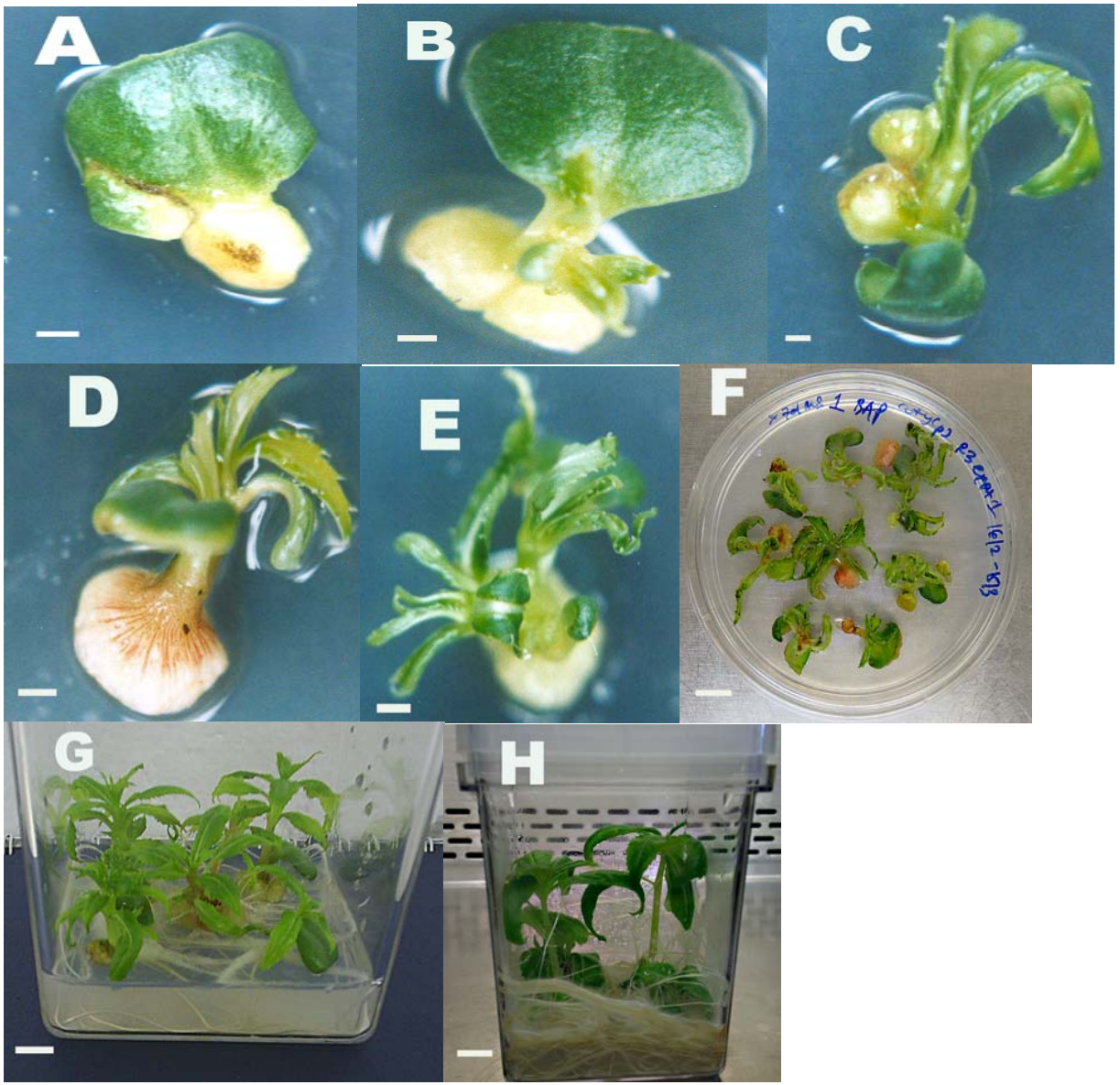
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Figure 5. *In vitro* regeneration of *Impatiens balsamina* using 7 days old seedling cotyledons within 8 weeks in culture.

A-E. Multiple shoots development within 2 weeks in culture. Bar=0.1cm.

Proximal section of 7 days old seedling cotyledon on 1 mg/L (BAP) after 3 weeks in culture. Bar=0.8 cm.

Roots formation on half strength MS media supplemented with 0.1 mg/L IAA after 5 weeks in culture. Bar= 1cm.

Well rooted plantlets sub-cultured on hormone-free MS media after 8 weeks on culture. Bar= 1cm.

CHAPTER 5

5.0 DISCUSSIONS

In this study, highest rate or percentage offshoot induction was obtained for proximal cotyledons from 7 days old seedlings on MS medium containing 1mg/L BAP. The proximal cotyledons also include the axillary meristem region. In agreement to this observation, highest shoot induction using proximal cotyledons has also been reported for various plants. In plant, generally, the meristematic regions are exclusively constitutive to the regions of dividing cells (Luc and Harry, 2004). It has been reported for bootle guard and squash that the inclusion of proximal region showed maximum shoot regeneration while removal of this region led to the reduction of direct shoot development (Han *et al.*, 2004; Ananthakrisnan *et al.*, 2003). The greater induction of organogenic tissues was obtained from whole and proximal halves of cotyledons as compared to the distal halves. Whole cotyledon explants were also not significantly different from proximal half explants for the induction percentage of organogenic tissue. In *Impatiens platypetala,* whole and proximal half explants were also reported to result in fewer explant death and larger diameter of organogenic tissue as compared to the distal half (Kyungkul, 1993).

The distal half cotyledon explant also failed to induce any organogenic tissues in *Impatiens platypetala* when exposed to any BAP concentrations (Kyungkul, 1993). Similar results were observed in the present study where no shoots

were induced from all explants age of distal section on the shoot induction media. Such differences in response among explant types suggests that a unidirectional polarity exists in the cotyledon explants in which a certain gradient of plant regeneration potential exists from proximal to the distal region within a cotyledon (Kyungkul, 1993). However, the distal sections resulted in callus formation rather than shoot induction. There were also no reports previously on shoot induction from callus derived from distal sections. Therefore, in this study, although the callus was sub-cultured onto shooting media continuously, there were no shoot induced and the entire white callus turned into necrosis of explants.

Similar result was also observed for hypocotyls explants with white callus turning into brown callus after eight weeks of continuous sub-culturing. This finding suggested that hypocotyls as a non responsive explants for *in vitro* regeneration system of *Impatiens balsamina*. Moreover, there was no other report on shoot induction from hypocotyls of *Impatiens balsamina*. In contrast, shoot induction from hypocotyls were reported in other species such as *Arabis gunnisoniana* (Taskin *et al.*, 2003) and niger, *Guazotia abysinica* (Murthy *et al.*, 2003).

The age of explant is one of the factors that could influence the shooting percentage. In this present study, the youngest cotyledons, 7 day old, showed the highest shooting percentage as compared to other older ages, 14 and 21 days. The highest induction of shoots with younger cotyledon explants has also been reported previously for other *Impatiens* species. The greater

induction of shoots with less callus formation was obtained from 13 days old cotyledons from *Impatiens platypetala* Lindl (Kyungkul, 1993). Similarly, in other plants, cotyledon explants of sunflower as showed higher shoot regeneration frequency from four days old seedling cotyledon as compared to six days old seedling cotyledon (Baker *et al*, 1999). Therefore, in agreement with other plant species, the ages of Impatiens cotyledon explant plays an important role in the induction of organogenic tissues and shoot regeneration.

The benefit of BAP over the other cytokinins in *Impatiens* shoot regeneration has been reported previously in *Impatiens platypetala* Lindl (Kyungkul, 1993) and *Impatiens* L. interspesific hybrids T63-1, a Java (J) x New Guinea (NG) (Kyungkul and Stephens, 1987). In the present study, the shoot formation was observed in all types and concentrations of cytokinin tested (Table 4). The highest average number of shoots (6.8) and the highest frequency (93%) of explants producing shoots was obtained after three weeks in culture using 1 mg/L BAP. This finding were similar to Kyungkul (1993) who reported that highest frequency of *Impatiens platypetala* explants producing shoots were achieved from cotyledon explants on media supplemented with BAP. On the other hand, cytokinins generally inhibited shoot elongation when the concentration is increased (Kyungkul and Stephens, 1987). The higher concentrations of BAP showed lower number of shoots per explants for *Impatiens platypetala* due to delay in organogenic tissue induction and overall regeneration process (Kyungkul, 1993). Similar result was demonstrated in the present study where concentrations higher than 1 mg/L BAP showed lower number of shoots and inhibition of shoot elongation

The combination of BAP with NAA in the present study showed lower percentage of shooting. However, this combination also resulted in the unintended callus and root production. In agreement to this finding, *Impatiens* hybrid of 'T63-1' and 'Star Fire', does not dependent on NAA for shoot multiplication (Stephens *et al.*, 1985). However, NAA was reported to stimulated shoot elongation of T63-1 at the lowest concentration, but inhibited shoot elongation of 'Star Fire' at all concentrations of NAA, regardless of the NAA combination (Kyungkul and Stephens, 1987). Combination of BAP with NAA or IAA treatment resulted in lower shoot regeneration frequency, suggesting that only BAP play an important role in shoot induction for bottle gourd (Han *et al.*, 2004). The combination of BAP and NAA also led to increased callus formation in the present study and continuous sub-culturing of the callus led to the necrosis of explants. Previous studies also reported that callus formation from cotyledon explants of *Lagenaria siceraria* Standl (Han *et al.*, 2004) and sunflower (Baker *et al*, 1999) after the cotyledon explants were cultured with BAP and NAA.

Non-purine based chemicals, such as substituted phenylureas are also used as cytokinins in plant cell culture media. These substituted phenylureas can also substitute for auxin in some culture system (Slater *et al.*, 2003). Therefore, in the present study, shoot and root formation was also found in all concentrations of TDZ but the percentage was lower compared to BAP treatments. Even though TDZ percentage was lower compared to BAP, there was no significant difference in both treatments on shoot induction. However,

in the present study, TDZ was not consistent in producing high percentage of shoots and roots because the shoots and roots induction were inhibited at certain times during development due to the intermediate actions of TDZ as cytokinin and auxin in culture systems. In other plant system, both BAP and TDZ were effective in inducing shoot regeneration of *Spartina alterniflora* (Wang *et al.*, 2003). However, treatment with TDZ in the shoot regeneration medium inhibited the root regeneration of *Spartina alterniflora*. Other than the above, there are no other report on TDZ occurring as endogenous hormone of cytokinins as compared to BAP. Therefore, BAP was effective in uptake and led to the high shoot induction compared to TDZ (Sakikabara, 2004). In contrast, cotyledon of *Swainsola salsula* Taubert was found to produce higher number of shoots (average 9.3) after TDZ treatment (Yang *et al.*, 2001). For the control explant without any cytokinins treatments, lower shoots induction was observed in this study compared to cytokinins treatments. The shoots induced without any treatment was achieved due to the effect of the endogenous cytokinin which naturally present in the explants (Sakikabara, 2004).

In this study, root induction was obtained using all the auxins tested with the highest percentage of rooting obtained when using IBA treatment. However, in terms of root morphology, IAA showed more hairy roots and longer roots formation. In addition, the rapid growth of roots in the present study was observed within four days. The other treatments with IBA and NAA showed slower growth of roots of more than four days. The endogenous auxin that has been reported to occur in plant is IAA (Jennifer *et al.*, 2004). Therefore,

the presence of IAA as the endogenous auxin and the addition of the exogenous IAA in this study described the longer and rapid growth of the roots as compared to other treatments. The presence of endogenous auxin also induced roots for control culture in both concentrations of MS media in this study with average of 90% of rooting. It was reported that the endogenous auxin, IAA, is synthesized in young apical meristems (Davies, 2004). This finding agrees with Kyungkul (1993) who found that multiple shoots of *Impatiens platypetala* Lindl successfully rooted in hormone-free medium. However, in the present study, the roots in control culture were only induced after more than six days as compared to auxin treatments within four to six days. In terms of morphology, roots in control culture were less hairy and reduced the effectiveness of auxins uptake into the roots.

The concentrations of MS media also affect the roots induction as the results showed differences in root formation in terms of morphology. The roots obtained were less hairy in full strength MS media compared to half strength MS media with auxin treatment. The high rooting percentage from cotyledons explants in terms of morphology was reported previously when half-strength MS media containing auxin were used in bottle gourd (Han *et al.*, 2004) and *Swainsola salsula* Taubert (Yang *et al.*, 2001). The favorable effects of a lower concentration (diluted) mineral solution on rooting can be explained by the reduction of nitrogen concentration. The reduction of the mineral concentration of MS media to half increased the rooting percentage in *Prunus persica* with IBA treatments (Fotopoulus and Sotiropoulus, 2005). Therefore, in

this study, the highest rooting percentage in terms of morphology was obtained when half-strength MS were used and supplemented with 0.1 mg/L IAA.

After successfully rooting the shoots, plantlets were transferred into different MS media. The plantlets which were cultured in hormone-free MS media showed faster rate of growth, 8 to 10 cm in height, compared to plantlets produced after sub-cultured in half-strength MS media supplemented with 0.1 mg/L IAA which showed slow rate of growth, 5 cm in height, after three weeks in culture. Therefor for further explants, half-strength MS media will be used to include the growth of *Impatiens balsamina* plantlets.

CHAPTER 6

6.0 CONCLUSIONS

A reliable and efficient tissue culture system for *Impatiens* species has been successfully developed. Most efficient shoot induction was obtained from proximal part of cotyledon derived from 7 days old seedlings on MS media supplemented with 1mg/L BAP. Roots were most efficiently produced on half-strength MS media supplemented with 0.1 mg/L IAA. Further regeneration of plantlets was achieved after 3 weeks on hormone-free MS media. Full regeneration system of *Impatiens balsamina* has been achieved within 8 weeks in culture. Efforts to develop a reliable transformation system for this plant for genetic manipulation could now be carried out.

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In vitro Regeneration of Garden Balsam, *Impatiens balsamina* Using Cotyledons Derived from Seedlings

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Abstract: In this study, an effort to develop a reliable tissue culture system for *Impatiens* species was carried out for eventually to genetically engineer this plant for useful traits. Parts of cotyledons and hypocotyl derived from different age of seedlings, from surface sterilized seeds, were subjected to shoot induction on MS media containing BAP, TDZ or combination of BAP and NAA in different ratios. Roots were developed from the above shoots on full-or half-strength MS media containing IAA, IBA or NAA of different concentrations. Finally fully-developed plantlets were produced on hormone free MS media or full- or half-strength MS media containing specific hormones. It was observed that the most efficient shoot induction (93%) was obtained from proximal part of cotyledon derived from 7 days old seedlings on MS media supplemented with 1 mg L^{-1} BAP. Roots were most efficiently produced (92%), in term of percentage and morphology, on half-strength MS media supplemented with 0.1 mg L^{-1} IAA. Further regeneration of plantlets, approximately 8-10 cm in height, was achieved after 3 weeks on hormone-free MS media. In conclusion, full regeneration system of *Impatiens balsamina* was developed, using proximal part of cotyledon derived from 7 days old seedlings on MS media supplemented with 1 mg L^{-1} BAP for shoot regeneration and 0.1 mg L^{-1} IAA for roots regeneration. Full regeneration system has been achieved within 8 weeks in culture.

Key words: Plant tissue culture, cotyledons and hypocotyl explants, *Impatiens balsamina*

INTRODUCTION

Impatiens balsamina is locally known as Garden Balsam and are found growing throughout tropical Africa, India, Southwest Asia, Southern China, Japan, as well as parts of Europe, Russia and North America (Grey-Wilson, 1980). It has also been cultivated as an ornamental plant in many parts of the world. The genus *Impatiens* is estimated to contain between 400-850 species (Grey-Wilson, 1980). It is annual plant with a soft stem and produced four different color of flower, purple, pink, white and red. The flower of *Impatiens balsamina* is a very attractive model for analysing the flowering process as it has an absolute requirement for short day conditions for flowering and flower reversion can be obtained in a predictable way after transfer to long day conditions. The parts of the plants has been traditionally claimed to have medicinal properties such as leaves used for treating warts and flowers used to cool burning skin or cooling fewer. An antimicrobial peptide from *Impatiens balsamina* has been shown to have antifungal activity against *Candida albicans* (Dong *et al*., 1980).

Impatiens is one of the host plants for Impatiens Necrotic Spot Virus (INSV), the virus that could infect the crops in many species. As this plant has ornamental value, the use of biotechnological techniques to produce transgenic plants for resistant to INSV has a clear advantage (Daughtrey *et al*., 1997). However, in order to produce transgenic *Impatiens balsamina* plants, the plant regeneration system need to be established. Plant regeneration system of many *Impatiens* species was achieved using multiple shoots from variety of explants such as cotyledons of immature ovules of *Impatiens platypetala* Lindl (Kyungkul, 1993) and shoot tips from both *Impatiens* hybrids (Kyungkul and Stephens, 1987) and Java x New Guinea *Impatiens* (Stephens *et al*., 1985).

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Multiple shoots were also successfully regenerated from cotyledon explants of other plants species such as *Pinus* (Sul and Korban, 2004), barley (Sharma *et al*., 2004), squash (Ananthakrishnan *et al*., 2003), Bambara groundnut (Lackroix *et al*., 2003), *Terminalia chebula* (Syhamkumar *et al*., 2003) and cotton (Agrawal *et al*., 1997). Cotyledons usually utilize the meristematic cells and were chosen to establish a plant regeneration system. Based on earlier reports, cotyledons were proven to be the most responsive explants for many species (Kyungkul, 1993).

Plants growth regulators are media components that have an effect on growth and development of plant organs. There are five classes of plant growth regulator: auxins, cytokinins, gibberellins, abscisic acid and ethylene. Generally, auxins and cytokinins are the most widely used plant growth regulator in plant tissue culture which will determine the type of culture and regeneration of explants. Auxin in high ratio generally induces the roots, whereas high cytokinin will induce the shoots and the intermediate ratio favors on callus formation. Auxin promotes both the cell division and cell growth and cytokinins promote the cell division (Slater *et al*., 2003).

Cytokinins has clearly played an important role in various plant development processes, including the promotion of cell division, the counteraction of senescence and the regulation of apical dominance. (Sakikabara, 2004). The endogenous hormones that are naturally synthesized are such as IAA and zeatin. IAA, is sensitive to both heat and light. However, currently more stable form of synthetic auxins and cytokinins have been widely used in plant cell culture (Slater *et al*., 2003).

Auxins are defined as organic substances that promote cell elongation when applied in low concentrations to plant tissue segments (Jennifer *et al*., 2004). There is evidence on the cell elongation and growth of tobacco young shoot using GUS assay, where the presence of auxin on the lower side of the stem resulting in elongation of one side of the leaves and causing the leaf to bend. Endogenous auxin, IAA, is synthesized in young apical meristem and is transported basipetally to the growing zones of the stem and more distantly to the root via polar transport system (Davies, 2004).

The auxin transport is a polar transport which requires energy driven by ATP. The mechanism of auxin transport is one of chemiosmosis system which dependent on the H^+ gradients generated from the proton pump. The proton pumps located in plasma membrane play a role in growth response of cells. The acid growth theory states that auxin stimulates the proton pump to lower the pH of the cell wall. This wall acidification will activate enzymes that break the hydrogen bonds between the cellulose microfibrils and loosening the wall. These cells will have higher water uptake via osmosis and the turgor pressure will push to loosen the cell wall. This pressure will enhance the elongation of cells and simultaneously the growth of plant cell (Lack and Evans, 2005).

This study highlights the efforts to develop a reliable tissue culture system for one of the Malaysia balsam, *Impatiens balsamina*. Tissue culture system for this *Impatiens* species was developed using cotyledons and hypocotyls derived from seedlings. The explants were regenerated through shoot and root inductions and followed by *in vitro* regeneration of whole plantlets. The effects of seedlings age and various concentrations of MS media in combination of different types and concentrations of cytokinins or auxins on this *Impatiens* species were evaluated and will be discussed.

MATERIALS AND METHODS

Plant materials and seed sterilisation: All seeds were washed under running tap water for 1 h. This followed by surface sterilisation by soaking the seeds in 70% (v/v) ethanol for 1 min and 10% (v/v) commercial sodium hypochlorite solution for 10 min. Seeds were rinsed with sterile distilled water three times and blotted dry on sterile filter paper.

Germination of seeds: Seeds were germinated on MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, IB vitamins (2 glycine; 100 myo-inositol; 0.5 nicotinic acid; 0.5 pyridoxine HCl and 0.1 mg L^{-1} thiamine HCl) and solidified with 0.8% (w/v) Bacto Difco agar. The pH of media was adjusted to 5.7 with 0.1 M NaOH or 0.1 M HCl prior to sterilisation.

Plant growth regulators and stock solutions: Plant growth regulator stock solutions were prepared at 1 mg mL^{-1} concentration by dissolving in appropriate solvent. All plant growth regulators except for IAA and IBA were added into the media before sterilisation. IAA and IBA were filter sterilised using 0.2 μm filters (Whatman, USA) and were added into the media after autoclaving and cooling the media to 50°C.

Explants preparations: Cotyledons and hypocotyls were used as source of explants. Cotyledons were excised from 7, 14 and 21 days old *in vitro* seedlings. Cotyledons were separated into proximal and distal parts. Hypocotyls were cut in three different sections for near base, center and near cotyledon. All explants with different ages and sections from cotyledons and hypocotyls were

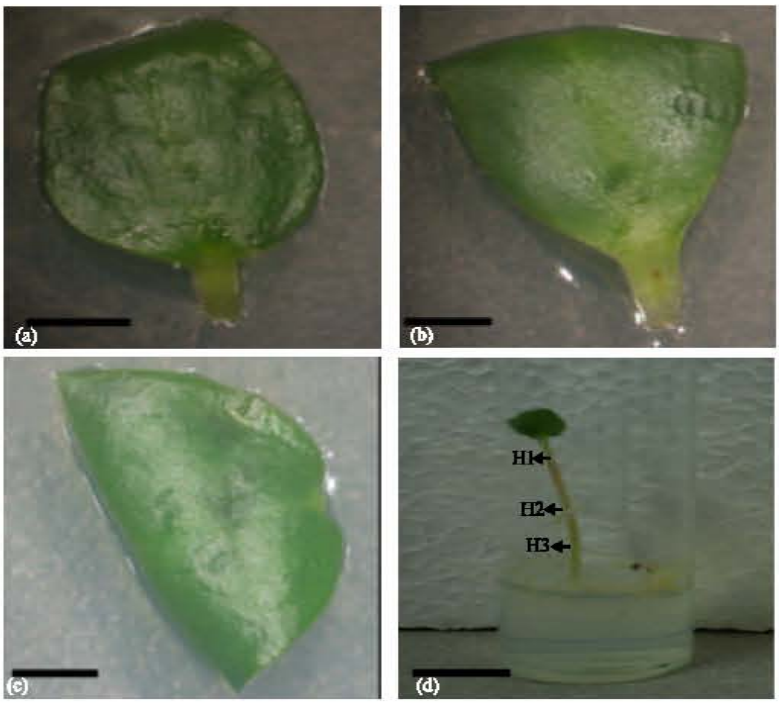

Fig. 1: The different types and sections of 7 days old seedling explants. (a) Cotyledon used in the study. Bar = 0.5 cm, (b) Proximal section of cotyledon. Bar = 0.2 cm, (c) Distal section of cotyledon. Bar = 0.2 cm and (d) Hypocotyls with different section in H1 (near cotyledon), H2 (center) and H3 (near roots). Bar =1 cm

Table 1: The types and concentrations of cytokinins used in shoot induction media

Cytokinins	Concentrations (mg L^{-1})
BAP	1.0
	3.0
	5.0
BAP:NAA	1.0:0.5 and 1.0:1.0
	3.0:0.5 and 3.0:1.0
	5.0:0.5 and 3.0:1.0
TDZ	0.5, 1.0 and 3.0
IAA	0.1, 0.5 and 1.0
IBA	0.5, 1.0 and 2.0
NAA	0.1, 0.5 and 1.0

transferred onto shooting media containing MS (Murashige and Skoog, 1962) with 1 mg L^{-1} BAP and 3 mg L^{-1} BAP, respectively. Each experiment was carried out in three replicates with ten explants. The experiments were repeated three times for reconfirmation. Figure 1a-d showed examples of 7 days old seedling explants for both cotyledons and hypocotyls.

Shoot induction experiment: Explants were transferred to shooting media consisting of MS macro- and micronutrients supplemented with different concentrations of cytokinin hormones TDZ, BAP alone or in combination with different ratio of NAA (Table 1). Each treatment was carried out with three replicates containing ten explants and the experiments were repeated three times. The explants on media were cultured in an incubator at 25±2°C with 16 h photoperiod, 17 µmol/m^2/sec supplied by coolight daylight fluorescent tubes (LICOR, USA).

Root induction experiment: Explants were transferred onto rooting media containing MS macro- and micronutrient supplemented with different concentration of NAA, IAA or IBA (Table 1). The effect of media types on rooting was carried out using either full- or half strength MS mediums. Each experiment contained three replicates with ten explants and the experiments were repeated three times. The explants on media were cultured in an incubator at 25±2°C with 16 h photoperiod, 17 µmol/m^2/sec supplied by coolight daylight fluorescent tubes (LICOR, USA).

***In vitro* regeneration:** Cotyledons from proximal section were cultured onto optimal shooting MS macro- and micronutrient supplemented with 1 mg L^{-1} BAP for 3 weeks. The shoots were transferred onto optimal rooting media in half strength MS media supplemented with 0.1 mg L^{-1} IAA. After 2 weeks on rooting media, plantlets were sub-cultured in different media for development observation. Half of the plantlets were sub-cultured onto half strength MS media supplemented with 0.1 mg L^{-1} IAA and the other half were transferred onto hormone-

free MS media. Development of plantlets, measured as height, was observed up to 3 weeks. The plantlets were cultured in an incubator at 25±2°C with 16 h photoperiod, 17 μmol/m^2/sec supplied by coolight daylight fluorescent tubes (LICOR, USA).

RESULTS

The effect of explants age, types and sections on shoots induction: The morphological and biochemical characteristics of cotyledons change with age and affect on cytokinin uptake. The plant regeneration potential also exists from proximal and distal section within a cotyledon and different section of hypocotyls of *Impatiens balsamina*. Therefore, this experiment was carried out to evaluate the effects of age, types and sections of explants on shoot induction.

In the present study, the highest shooting percentage (88%) was obtained from 7 days old seedling of proximal cotyledon sections followed by 14 days (86%) and 21 days old seedling (81%), respectively (Table 2). No shoot was induced from distal section of the cotyledons tested. On the other hand, callus formation was only observed in distal cotyledon section with the highest percentage (76%) of white callusing obtained from 7 days old cotyledons explants (Table 2, Fig. 2a-c). However, the callus failed to produce any shoot after 4 weeks of sub-culturing on shooting media due to necrosis.

Hypocotyls were also tested for shoot induction. However, when all ages and sections tested, hypocotyls only produced green to brown callus without any shoots after 2 weeks on MS media supplemented with 3 mg L^{-1} BAP (Fig. 2). From the above observation, it could be suggested that 7 days old seedling of proximal cotyledon section was the best to be used as an explant throughout the experiment for shoot induction.

Shoot induction: Cytokinin of different types and concentrations has been widely used for shoot induction in many plants. In the present study, proximal section of 7 days old seedling's cotyledon showed the highest percentage of shooting (93%) and average number of shoots (6.8) on MS media supplemented with 1 mg L^{-1} BAP after 3 weeks on culture (Table 3, Fig. 3a). Development of multiple shoots is shown in Fig. 5a-h. Multiple shoots induction was achieved in all treatments tested. However, MS media supplemented with BAP showed the best result.

Combination of BAP and NAA on MS media induced callus formation. However, the combination also inhibited shoots elongation as compared to MS media

Table 2: The effect of different explants age and parts of cotyledons on shoot induction after 3 weeks on MS media containing 1 mg L^{-1} BAP

Explant ages (days)	Parts of cotyledon	Shooting explants (%)	Rooting (%)	Callus formation (%)
7	Proximal	88	0	0
	Distal	0	0	76
14	Proximal	86	0	0
	Distal	0	0	54
21	Proximal	81	0	0
	Distal	0	0	52

Table 3: The effect of different concentrations of hormones on shoot induction after 3 weeks in culture

Hormones	Concentrations (mg L^{-1})	Shooting (%)	Rooting (%)	Callus formation (%)
BAP	1.0	93	0	0
	3.0	85	0	0
	5.0	84	0	0
BAP: NAA	1.0:0.5	60	77	99
	1.0:1.0	60	78	96
	3.0:0.5	63	80	100
	3.0:1.0	50	82	100
	5.0:0.5	70	65	100
	5.0:1.0	50	76	100
TDZ	0.5	87	97	0
	1.0	83	99	0
	3.0	82	100	0
Control	0.0	80	100	0

Fig. 2: The effect of cytokinins in shooting media on different types of explants after 3 weeks in culture.(a) Proximal sections with shoots formation on MS media supplemented with 1 mg L^{-1} BAP. Bar = 0.5 cm, (b) Distal parts with white callus formation on MS media supplemented with 1 mg L^{-1} BAP. Bar = 0.2 cm and (c) Hypocotyls with green to brown callus on MS media supplemented with 3 mg L^{-1} BAP. Bar = 2 cm

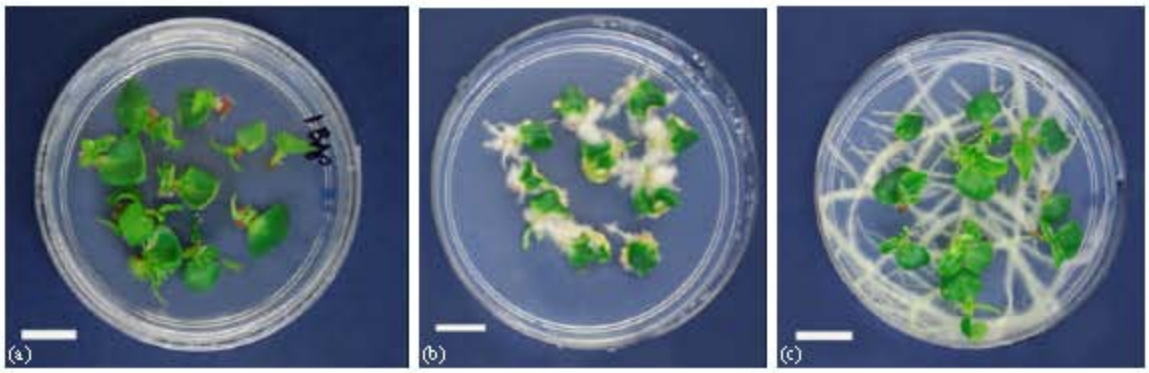

Fig. 3: Shoot induction from the proximal section of 7 days old seedling cotyledons on MS media supplemented with different concentrations of hormones after 3 weeks in culture. (a) 1 mg L^{-1} BAP. Bar = 1.5 cm, (b) 5 mg L^{-1} BAP with 0.5 mg L^{-1} NAA. Bar = 1.5 cm and (c) 0.5 mg L^{-1} TDZ. Bar = 1 cm

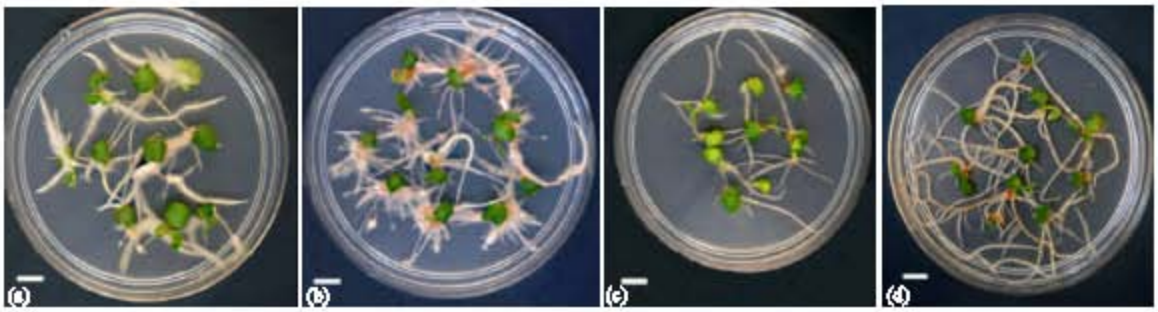

Fig. 4: Comparison of root morphology from different auxins treatment on different concentrations of MS media after 2 weeks in culture. (a) Root induction in half strength MS media supplemented with 0.1 mg L^{-1} IAA. Bar = 0.1 cm, (b) Root induction in half strength MS media supplemented with 0.5 mg L^{-1} IBA. Bar = 0.1 cm, (c) Control in half strength MS media. Bar = 0.1 cm and (d) Control in full strength MS media. Bar = 0.1 cm

supplemented with BAP (Fig. 3b). On the other hand, TDZ treatment induced both shoots and roots. However, the effects of TDZ was not consistent when the shoots and roots induction was inhibited in certain times due to the intermediate actions of TDZ as cytokinin and auxin in culture system (Fig. 3c). High percentage of shooting was also observed for the control culture. However, the average number of shoots was lower compared to ctyokinin treatments. In addition, shoot was induced in control explants after 2 weeks in culture as compared to within one week for the treated.

Root induction: Overall results showed that there was no significant difference for all types or concentration of auxin treatment tested for root induction for *Impatiens balsamina*. However, percentage of rooting was the highest for half-strength MS media supplemented with 0.5 mg L^{-1} IBA (96%) followed by 0.1 mg L^{-1} IAA (92%) or 0.5 IAA for full-strength MS (94%) (Table 4). Root morphology was also evaluated. Treatment with 0.1 mg L^{-1} IAA showed the production of more hairy and longer roots. The growth of roots was faster using IAA,

Table 4: The effect of media types and different concentrations of hormones on roots induction after 2 weeks in culture

		Full MS	Half strength MS
Hormones	Concentration	Rooting (%)	Rooting (%)
IAA	0.1	90	92
	0.5	94	90
	1.0	88	78
IBA	0.5	90	96
	1.0	93	91
	2.0	90	90
NAA	0.1	84	91
	0.5	90	90
	1.0	90	90
Control	0.0	90	92

i.e., within 4 days post-treatments as compared to IBA treatment (Fig. 4a-d). Therefore, in term of root morphology, 0.1 mg L^{-1} IAA was the best treatment and was used for rooting induction throughout the experiment.

The used of half MS media by diluting mineral solution in root induction media could reduce nitrogen concentration that effect on root induction. Therefore,

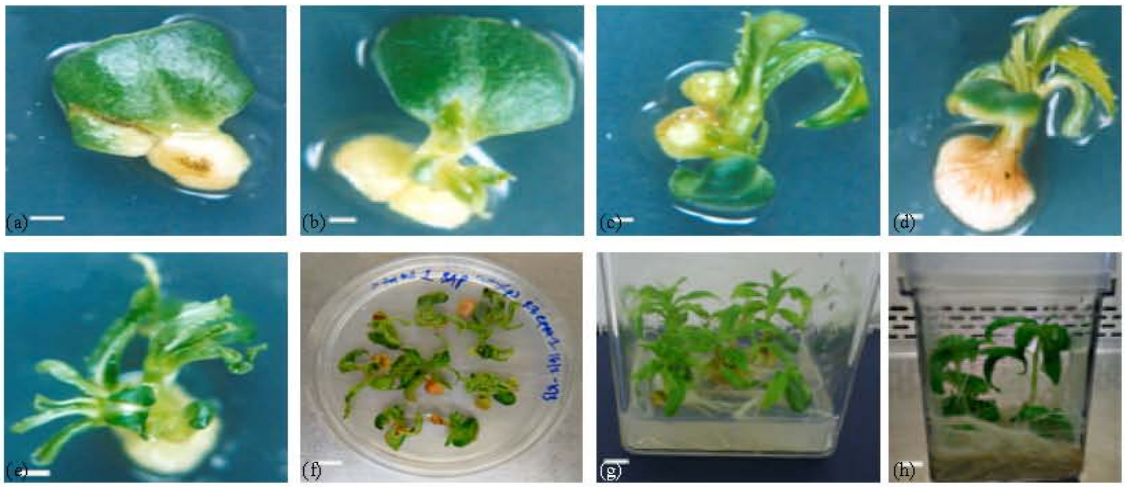

Fig. 5: *In vitro* regeneration of *Impatiens balsamina* using 7 days old seedling cotyledons within 8 weeks in culture. (a-e) Multiple shoots development within 2 weeks in culture. Bar = 0.1 cm, (f) Proximal section of 7 days old seedling cotyledon on 1 mg L^{-1} (BAP) after 3 weeks in culture. Bar = 0.8 cm, (g) Roots formation on half strength MS media supplemented with 0.1 mg L^{-1} IAA after 5 weeks in culture. Bar = 1 cm and (h) Well rooted plantlets sub-cultured on hormone-free MS media after 8 weeks on culture. Bar = 1 cm

the use of full and half-strength of MS media showed that half strength MS media gave better roots induction in terms of percentage and morphology. In the present study, half strength MS media induced rooting in at least 90% of the explants as compared to full strength MS media which showed a lowest percentage of rooting of 88% (Table 4, Fig. 4). In terms of morphology, the roots obtained from full strength MS media were less hairy as compared to the root obtained using half strength MS media which were treated with auxin. However, for control treatment, roots induction was also observed in both concentrations of MS media with an average of 90% of rooting with less hairy roots (Table 4).

***In vitro* regeneration:** After 8 weeks in culture, *in vitro* plantlets were achieved from various explants and treatments (Fig. 5h). The plantlets were later transferred to a different media for development comparison. The plantlets were sub-cultured on hormone-free MS media for another three weeks. After three weeks, further growth of plantlets with approximately 8-10 cm in height were observed. In contrast, slower growth of plantlets with 5 cm in height, was observed when plantlets were continuously cultured on rooting media containing half strength MS media and supplemented with 0.1 mg L^{-1} IAA. Slower growth of plantlets in half strength MS medium supplemented with 0.1 mg L^{-1} IAA possibly would affect the plantlets growth. This is because they were pressured by hormones which could interferes with their elongation and development process.

DISCUSSION

In this study, highest rate or percentage of shoot induction was obtained for proximal cotyledons from 7 days old seedlings on MS medium containing 1 mg L^{-1} BAP. The proximal cotyledons also include the axillary meristem region. In agreement to this observation, highest shoot induction using proximal cotyledons has also been reported for various plants. In plant, generally, the meristematic regions are exclusively constitutive to the regions of dividing cells (Luc and Harry, 2004). It has been reported for bottle guard and squash that the inclusion of proximal region showed maximum shoot regeneration while removal of this region led to the reduction of direct shoot development (Han *et al*., 2004; Ananthakrisnan *et al*., 2003). The greater induction of organogenic tissues was obtained from whole and proximal halves of cotyledons as compared to the distal halves. Whole cotyledon explants were also not significantly different from proximal half explants for the induction percentage of organogenic tissue. In *Impatiens platypetala*, whole and proximal half explants were also reported to result in fewer explant death and larger diameter of organogenic tissue as compared to the distal half (Kyungkul, 1993).

The distal half cotyledon explant also failed to induce any organogenic tissues in *Impatiens platypetala* when exposed to any BAP concentrations (Kyungkul, 1993). Similar results were observed in the present study, where, no shoots were induced from all explants age of distal section on the shoot induction media. Such differences in

response among explant types suggests that a unidirectional polarity exists in the cotyledon explants in which a certain gradient of plant regeneration potential exists from proximal to the distal region within a cotyledon (Kyungkul, 1993). However, the distal sections resulted in callus formation rather than shoot induction. There were also no previously reports on shoot induction from callus derived from distal sections. Therefore, in this study, although the callus was sub-cultured onto shooting media continuously, there were no shoot induced and the entire white callus turned into necrosis of explants.

Similar result was also observed for hypocotyls explants with white callus turning into brown callus after eight weeks of continuous sub-culturing. This finding suggested that hypocotyls as a non responsive explants for *in vitro* regeneration system of *Impatiens balsamina*. Moreover, there was no other report on shoot induction from hypocotyls of *Impatiens balsamina*. In contrast, shoot induction from hypocotyls were reported in other species such as *Arabis gunnisoniana* (Taskin *et al*., 2003) and niger, *Guazotia abysinica* (Murthy *et al*., 2003).

The age of explant is one of the factors that could influence the shooting percentage. In this present study, the youngest cotyledons, 7 day old, showed the highest shooting percentage as compared to other older ages, 14 and 21 days. The highest induction of shoots with younger cotyledon explants has also been reported previously for other *Impatiens* species. The greater induction of shoots with less callus formation was obtained from 13 days old cotyledons from *Impatiens platypetala* Lindl (Kyungkul, 1993). Similarly, in other plants, cotyledon explants of sunflower from four days old seedling showed higher shoot regeneration frequency as compared to six days old seedling cotyledon (Baker *et al*., 1999). Therefore, in agreement with other plant species, the ages of *Impatiens* cotyledon explant plays an important role in the induction of organogenic tissues and shoot regeneration.

The benefit of BAP over the other cytokinins in *Impatiens* shoot regeneration has been reported previously in *Impatiens platypetala* Lindl (Kyungkul, 1993) and *Impatiens* L. interspesific hybrids T63-1, a Java (J) x New Guinea (NG) (Kyungkul and Stephens, 1987). In the present study, the shoot formation was observed in all types and concentrations of cytokinin tested (Table 3). The highest frequency (93%) of explants producing shoots was obtained after three weeks in culture using 1 mg L^{-1} BAP. This finding were similar to Kyungkul (1993), who reported that highest frequency of *Impatiens platypetala* explants producing shoots were achieved from cotyledon explants on media supplemented with BAP. On the other hand, cytokinins generally inhibited shoot elongation when the concentration is increased (Kyungkul and Stephens, 1987). The higher concentrations of BAP showed lower number of shoots per explants for *Impatiens platypetala* due to delay in organogenic tissue induction and overall regeneration process (Kyungkul, 1993).

The combination of BAP with NAA in the present study showed lower percentage of shooting. However, this combination also resulted in the unintended callus and root production. In agreement to this finding, *Impatiens* hybrid of T63-1 and Star Fire, does not dependent on NAA for shoot multiplication (Stephens *et al*., 1985). However, NAA was reported to stimulate shoot elongation of T63-1 at the lowest concentration, but inhibited shoot elongation of Star Fire at all concentrations of NAA, regardless of the NAA combination (Kyungkul and Stephens, 1987). Combination of BAP with NAA or IAA treatment resulted in lower shoot regeneration frequency, suggesting that only BAP play an important role in shoot induction for bottle gourd (Han *et al*., 2004). The combination of BAP and NAA also led to increase callus formation in the present study and continuous sub-culturing of the callus led to the necrosis of explants. Earlier studies also reported that callus formation from cotyledon explants of *Lagenaria siceraria* Standl (Han *et al*., 2004) and sunflower (Baker *et al*., 1999) after the cotyledon explants were cultured with BAP and NAA.

Non-purine based chemicals, such as substituted phenylureas are also used as cytokinins in plant cell culture media. These substituted phenylureas can also substitute for auxin in some culture system (Slater *et al*., 2003). Therefore, in the present study, shoot and root formation was also obtained in all concentrations of TDZ but the percentage was lower compared to BAP treatments. Even though TDZ percentage was lower compared to BAP, there was no significant difference in both treatments on shoot induction. However, in the present study, TDZ was not consistent in producing high percentage of shoots and roots because the shoots and roots induction were inhibited at certain times during development due to the intermediate actions of TDZ as cytokinin and auxin in culture systems. In other plant system, both BAP and TDZ were effective in inducing shoot regeneration of *Spartina alterniflora* (Wang *et al*., 2003). However, treatment with TDZ in the shoot regeneration medium inhibited the root regeneration of *Spartina alterniflora*. Other than the above, there are no other report on TDZ occurring as endogenous hormone of cytokinins as compared to BAP. Therefore, BAP was effective in uptake and led to the high shoot induction compared to TDZ (Sakikabara, 2004). In contrast, cotyledon of *Swainsola salsula* Taubert was found to

produce higher number of shoots (average 9.3) after TDZ treatment (Yang *et al*., 2001). For the control explant without any cytokinins treatments, lower shoots induction was observed in this study compared to cytokinins treatments. The shoots induced without any treatment was achieved due to the effect of the endogenous cytokinin which naturally present in the explants (Sakikabara, 2004).

In this study, root induction was obtained using all the auxins tested with the highest percentage of rooting obtained when using IBA treatment. However, in terms of root morphology, IAA showed more hairy roots and longer roots formation. In addition, the rapid growth of roots in the present study was observed within four days. The other treatments with IBA and NAA showed slower growth of roots of more than four days. The endogenous auxin that has been reported to occur in plant is IAA (Jennifer *et al*., 2004). Therefore, the presence of IAA as the endogenous auxin and the addition of the exogenous IAA in this study described the longer and rapid growth of the roots as compared to other treatments. The presence of endogenous auxin also induced roots for control culture in both concentrations of MS media in this study with average of 90% of rooting. It was reported that the endogenous auxin, IAA, is synthesized in young apical meristems (Davies, 2004). This finding agrees with Kyungkul (1993), who found that multiple shoots of *Impatiens platypetala* Lindl successfully rooted in hormone-free medium. However, in the present study, the roots in control culture were only induced after more than six days as compared to auxin treatments within four to six days. In terms of morphology, roots in control culture were less hairy which reduced the effectiveness of auxins uptake into the roots.

The concentrations of MS media also affect the roots induction as the results showed differences in root formation in terms of morphology. The roots obtained were less hairy in full strength MS media compared to half strength MS media with auxin treatment. The high rooting percentage from cotyledons explants in terms of morphology was reported previously when half-strength MS media containing auxin were used in bottle gourd (Han *et al*., 2004) and *Swainsola salsula* Taubert (Yang *et al*., 2001). The favorable effects of a lower concentration (diluted) mineral solution on rooting can be explained by the reduction of nitrogen concentration. The reduction of the mineral concentration of MS media to half increased the rooting percentage in *Prunus persica* with IBA treatments (Fotopoulus and Sotiropoulus, 2005). Therefore, in this study, the highest rooting percentage in terms of morphology was obtained when half-strength MS were used and supplemented with 0.1 mg L^{-1} IAA.

After successfully rooting the shoots, plantlets were transferred into different MS media. The plantlets which were cultured in hormone-free MS media showed faster rate of growth, 8-10 cm in height, compared to plantlets produced after sub-cultured in half-strength MS media supplemented with 0.1 mg L^{-1} IAA which showed slow rate of growth, 5 cm in height, after three weeks in culture. Therefore for further explants, half-strength MS media will be used to induce the growth of *Impatiens balsamina* plantlets.

CONCLUSION

A reliable and efficient tissue culture system for *Impatiens* species has been successfully developed. Most efficient shoot induction was obtained from proximal part of cotyledon derived from 7 days old seedlings on MS media supplemented with 1 mg L^{-1} BAP. Roots were most efficiently produced on half-strength MS media supplemented with 0.1 mg L^{-1} IAA. Further regeneration of plantlets was achieved after 3 weeks on hormone-free MS media. Full regeneration system of *Impatiens balsamina* has been achieved within 8 weeks in culture. Efforts to develop a reliable transformation system for this plant for genetic manipulation could now be carried out.

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