Lihat Sebelah

MICROPROPAGATION OF WETLAND MONOCOT CATTAIL

(*Typha latifolia*)

By
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the requirement for the award of the degree of
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DECLARATION

I hereby declare that this PITA research report entitled **Micropropagation of Wetland Monocot Cattail (*Typha latifolia*)** is the result of my own research except as cited in the references.

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MIKROPROPAGASI TUMBUHAN BERTANAH LEMBAB (*TYPHA LATIFOLIA*)

ABSTRAK

Typha latifolia adalah tumbuhan monokot yang mana ia biasanya tumbuh di kawasan bertanah lembab. Tumbuhan ini mempunyai kelebihan dalam tindak balas alleopathy, pembuatan racun tumbuhan, rawatan air tercemar serta aktiviti immun. Kajian ini dijalankan bertujuan untuk mengenalpasti kesan sitokinin ke atas pembiakan *T. latifolia* selain pemerhatian kepada jumlah flavon, flavonoid serta isipadu phenolik dalam tumbuhan. Hujung apeks atau rizom *T. latifolia* yang diperoleh daripada Simpang Pulai, Ipoh di kultur di antara enam atau sembilan minggu di dalam media MS dengan berbagai jenis sitokinin (1.0-5.0 mgl-1) iaitu 6-bezyladenine (BAP), 6-furfurylaminopurine (Kinetin/KN), Thidiazuron (TDZ) dan 6-4-hydroxy-3-methyl-but-2-enylaminopurine (Zeatin/ZEA). 1.0 mgl^{-1} dan 2.0 mgl^{-1} BAP dikenalpasti meransang pertumbuhan daun secara maksimum. Pemindahan rizom kepada media MS yang mempunyai kombinasi di antara BAP dan Indole-3-acetic asid (IAA), menunjukkan bahawa nisbah 1:1 BAP dan IAA diperlukan dalam pembiakan daun. Namun begitu, pemanjangan daun diperhatikan tinggi di dalam media tidak berhormon. 2.0 mgl^{-1} BAP dan 3.0 mgl^{-1} TDZ serta kombinasi di antara 5.0 mgl^{-1} BAP+3.0 mgl^{-1} IAA menghasilkan bilangan anak pokok baru yang lebih banyak. Pertumbuhan akar adalah rendah di dalam sitokinin dan tinggi di dalam auksin. 3.0 mgl^{-1} IAA meransang pertumbuhan akar yang lebih tinggi. Selain itu, jumlah flavon, flavonoid serta phenolik di dalam *T. latifolia* juga diukur. 1.0 mgl^{-1} BAP menghasilkan kandungan phenolik yang lebih tinggi. Kandungan flavonoid yang tinggi didapati daripada 3.0 mgl^{-} KN serta kombinasi diantara 1.0 mgl^{-1} BAP+3.0 mgl^{-1} IAA. Namun, kandungan flavon yang tinggi diperhatikan dalam *T. latifolia* yang tumbuh dalam 1.0 mgl^{-1} BAP+3.0 mgl^{-1} IAA media. Kesimpulannya, MS media yang dibekalkan dengan kepekatan sitokinin dan auksin yang berbeza akan mempengaruhi pertumbuhan serta bahan metabolic sekunder dalam *T. latifolia*.

MICROPROPAGATION OF WETLAND MONOCOT CATTAIL (*TYPHA LATIFOLIA*)

ABSTRACT

Typha latifolia is monocotyledonous plant which commonly grows in the wetland. It has the ability for alleopathy response, manufacture pesticides, treatment the polluted water and also stronger in immunosuppressive activity. This study was conducted to establish and determine the effect of cytokinin on proliferation of *T. latifolia* besides to observe the total flavone, flavonoid and phenolic content of plant. Apical tips and rhizome of *T. latifolia* were collected from Simpang Pulai, Ipoh was cultured for six to nine weeks in MS basal medium with various cytokinin (1.0-5.0 mgl^{-1}) of 6-bezyladenine (BAP), 6-furfurylaminopurine (Kinetin/KN), Thidiazuron (TDZ) and 6-4-hydroxy-3-methyl-but-2-enylaminopurine (Zeatin/ZEA). 1.0 mgl^{-1} and 2.0 mgl^{-1} BAP was induced maximum in leaves multiplication. Transfer of rhizome to MS medium supplemented with BAP and Indole-3-acetic acid (IAA), individually or combination, indicated that a combination 1:1 ratio of BAP and IAA was required in leaves multiplication. In contrast, the elongation of leaves was found highest in medium free hormone. The presence of a single 2.0 mgl^{-1} BAP and 3.0 mgl^{-1} TDZ and also combination of 5.0 mgl^{-1} BAP+3.0 mgl^{-1} IAA were produced the greater number of new plantlets. Root induction was lower in the presence of cytokinin but may induce higher with presence of auxin. 1.0 mgl^{-1} BAP+3.0 mgl^{-1} IAA was efficient in root development. The total flavone, flavonoid and phenolic content of *T. latifolia* were measured. 3.0 mgl^{-1} BAP gave the highest content of total phenolic compound. The highest content of flavonoids was obtained from 3.0 mgl^{-1} KN and combination between 1.0 mgl^{-1} BAP+3.0 mgl^{-1} IAA. However, the medium free hormone gave the highest content of total flavone than single treatment in *T. latifolia*. In contrast, combination between 1.0 mgl^{-1} BAP+3.0 mgl^{-1} IAA has the highest of total flavone content. On the basis of culture findings, we can conclude that MS media supplemented with different concentration of cytokinins and auxins may influence the growth and secondary metabolites in *T. latifolia*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

$AlCl_3$	Aluminium Chloride
ANOVA	Analysis of variance
BAP	6-benzyladenine
DMRT	Duncan's Multiple Range Test
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
KIN	Kinetin
nm	Nanometre
mgl^{-1}	miligram per litre
$mgml^{-1}$	milligram per mililitre
MS	Murashige and Skoog
NaOH	Sodium Hydroxide
TDZ	Thidiazuron
S.E	Standard Error
ZEA	Zeatin
μgml^{-1}	microgram per mililitre

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Typha latifolia is monocotyledonous plant which commonly grows in the wetland (Ye et al., 1997). It can colonize the wide range of wetland habitats, including heavy metal polluted areas (Taylor & Crowder, 1983) through seeds or by vegetative spread (Grace, 1987). Thus, *T. latifolia* has the ability for alleopathy response (Martin et al., 1997) to manufacture pesticide and also for treatment the polluted water (Dunbabin & Bowmer, 1992). The ethanol extract of Pollen *Typha* (EEPT) is a source of a number of bioactive compounds including flavonoids, steroids and volatile oils, which have the stronger immunosuppressive activity (Qin & Sun, 2005). It's improving the microcirculation raising cAMP level, preventing and curing coronary heart diseases, hyperlipidemia and myocardial infarction (Wang et al., 2003). *Typha* is also for manufacture of bedding, thatching, matting, baskets, boats and rafts, shoes, ropes and paper (Rook, 2002). Besides, it can be used as source of food due to higher of protein in this plant (Dewanji et al., 1997).

1.2 Problem Statement

Many secondary products produced from medicinal plants have been commercialized. However, there is problem on ensure the consistency of material with free microbial and heavy metal contamination. Although *T. latifolia* can spread aggressively and has

a higher reproductive potential (Maddison et al., 2009), the secondary products of the plant usually can produce only in small quantities and not always available. Plants are also endangered by other factors such as over-collecting, urbanization, pollution and climate change. Besides, the growth dynamics of these plants depend on environmental conditions which are water depth, competition with other species (Grace & Wetzel, 1981) and also nutrient conditions (Ulrich& Burton, 1988).

1.3 Significance of Study

Plant tissue culture techniques such micropopagation can be one alternative to maintain sustainability supply of plant materials for producing bioactive compound continuously under controlled conditions (Thorpe, 2006). Besides, it can regenerate a whole plant genetically to parental material and produce plant material all year round (Vancea et al., 2009). The plants that were collected from polluted site were found to contain heavy metal or toxic components such as mercury (Rai et al., 2005). As these plants will be utilized by human and in order to maintain sustainable supply of healthy and quality plants for human consumption, this technique should be used to produce disease free propagule and faster the growth rate (Kubota & Tadokoro, 1998) of explants.

However, there is no efficient tissue culture technique available for *T. latifolia*. Zimmerman and Read (1986) reported a low frequency of regeneration from callus derived from mature inflorescenes of *T. latifolia*. They encountered problem with contamination and poor callus production. Besides that, the immature explants they used were only available for a brief time in the summer. Although the mature seeds (Denchev & Conger, 1994) and immature embryo (Vasil &Vasil, 1980) of some monocot are good explants to initiate callus culture, the mature seeds of *T. latifolia* are too small making it difficult to isolate the embryos. Because of this practical problem, seed will be used as starting material for the micropopagation.

1.4 Objectives

Here are the objectives of the study:

1. To establish in vitro plantlet of *T. latifolia.*
2. To determine the effect of cytokinin and auxin on proliferation of *T. latifolia* plantlet.
3. To determine the total flavones, flavonoids and phenolic of cultures.

CHAPTER 2

LITERATURE REVIEW

2.1 Typha latifolia

Cattails are monocotyledonous flowering plant in the monogeneric family, Typhacea can be found in the wetland (Ye et al., 1997). Genus *Typha* consist of ten species and are probably the most familiar of all wetland plants in the world (Martin et al., 2002). *T. latifolia* is able to withstand fluctuations in hydrology although it generally occurs in shallower water that is less than two and half meters (Rook, 2002). It leaves are alternate and mostly basal to simple. The stem can range from 5 to 10 feet tall (Rook, 2002).

Grace (1987), reported that *T. latifolia* can reproduce by seed or vegetative via rhizome. These plants are wind-pollinated flower which developed in dense spikes. The heads of the female and male flowers are long, cylindrical like, usually with a dark-brown color, with the male flowers above the female flowers. The male flowers have narrow spikes that are not separated by more than 3 to 4 mm. The male portion is 5 to 15 cm long and 1.2 to 2 cm wide while the female flowers are 10 to 15 cm long and can be 2 to 3 cm wide when mature (Chadde, 2002). Each inflorescenes can contain 117,000 to 268,000 of seeds with 0.2 mm in long that attached to a fine hair. Their pericarp only opened to release the seeds when the inflorescenes contact with

water (Rook, 2002). These dense stands of the rhizome are often to exclude other plants.

2.2 Properties of *T. latifolia*

Although some people thought that *T. latifolia* is a nuisance plant because it grows rapidly and mostly dominant aquatic plant (Barton et al., 2002), this plant still possess its own benefit that can be used in manufacture of medicine, pesticides, food, treatment of water and bedding. Micropopagation technique is used to culture this plant because their inflorescene are only available in a brief of time (Zimmerman & Read, 1986) and this technique can produce the disease free propagule (Kubota & Tadokoro, 1999).

2.2.1 Medicinal

The main constituents of several species of the genus *Typha* have been shown as flavones and other phenolic compound, long chain hydrocarbon as well as various triterpenoids with a steroidal skeleton (Shode et al., 2002). There are eleven kinds of phenolic compound were detected in female flowers of *T. latifolia* (Ozawa & Imagawa, 1988). Wang et al. (2003) reported that the previous researches had proved that *Typha* had various pharmalogical functions such as improving the microcirculation, raising cAMP level, preventing and curing of coronary heart diseases, hyperlipidemia and myocardial infection. Pollen *Typha* is an important traditional Chinese herbal medicine which is used in the treatment of stranguria, hematuria, dyamenorrhea, methrorragia and injuries from falls (Qin & Sun, 2005). Arenas (2003) reported that *Typha* can treat tuberculosis and the nutrient content of the pollen is higher than that of pollen sold either at natural food stores in Argentina or manufactured as a pharmaceutical product.

Ishida et al. (1988), has isolated a new flavonol glucoside from *T. latifolia* pollen. This pollen used in Korean herbal medicine Sisosangami had been proved to exert anti-inflammatory effects related to inhibition of neutrophil functions (Park et al., 2004). However, the most important property of *Typha* is their immunosuppressive

activity. Immunosuppressive agent is a drug that uses to inhibit the activity of immune responseespecially those giving the autoimmune disease and transplant rejection (Qin & Sun, 2005). This plant also posses' side effect to the patient such as nephrotoxicity, hapatoxicity, induction of diabetes, induction of hypertension and also neuro toxicity (Serkova et al., 1996). As a consequence, there continues to be high demand for new immunosuppressants without any side effects. The ethanol extract of Pollen *Typha* (EEPT) possessed the stronger immunosuppressive activity (Qin and Sun, 2005). In addition, it also reduced the thymic weight, splenic weight and depressed the immune response in rats (Yuan & Xu, 1996). Chung et al. (2002) reported that linoleic acid and other unsaturated fatty acids isolated from pollen *Typha* had strong inhibitory effects on the binding of Myc-Max heterodimer to an E-box DNA site (CA(C/T) GTG).

2.2.2 Alleopathy

Alleopathy can be defined as the science that studies any process involving secondary metabolites produced by plants, algae, bacteria and fungi that influence the growth and development of agricultural and biological system (IAS, 1996). Alleopathy indicates inhibitory or stimulatory biochemical interactions between the two plant species. These biological phenomenon has influence the growth, survival and reproduction of other organism. Alleopathy response in manufacturing pesticides is widely used for agricultural pests. However, it have caused many problems such as the development of resistance in an organisms, environmental pollution, toxicity related health hazards in human and livestock.

Alleopathy is used to minimize or eliminate the use of present pesticides to control pests in field crops. *Typha* species has the ability for alleopathy because it likely to colonize stormwater wetlands (Schueler, 1994). The aqueous extract of *Typha domingensis* has phytotoxic properties inhibit the germination of lettuce and raddish seeds and reduce the oxygen production rates of the filamentous algae *Lyngbya majuscule* (Prindle & Martin, 1996). The autotoxic effect in *T. latifolia* is as much as aqueous extracts of various plant parts, particularly decaying leaves that playing role in inhibited the seed germination (McNaughton, 1968). This phytotoxin is refers to a

substance produced by a plant that is toxic to the plant. According Martin et al (1998), phenolic fraction may play a role in the phytotoxic action of *T. domingensis* extracts. From the research, fern *Salivinia minima* is sensitive to phenolic compounds in its growth medium even in low concentrations which is 2.5 ppm. Besides, *T. latifolia* possess alleochemicals which are sterols and fatty acis that involved in alleopathy (Macias et al., 2008). Dai et al. (1997) reported the algicidal palmitic acid and cholesteryl oleate are the type of fatty acids that composes in *T. latifolia*.

2.2.3 Nutrient/Food

T. latifolia is the edible food in which their roots have high starch and protein content (Vetayasupon, 2007). This plant can be make a gruel, thickened soup and also for coloring rice yellow (Pastor & Gustavo, 2003) while the young inflorescenes of this plant can be eats raw like asparagus. Besides, the young shoots of this plant can be cooked like vegetable and their pollen used in baked and roasted product (Vetayasupon, 2007). Prendergastet (2000) reported the Maori mixed the pollen with water and steamed to make the bread known as pua. Previous study by Harrington (1972) showed the flour from cattail core contains approximately 80% carbohydrate and 6% to 8% protein. According to Dewanji et al. (1997), the protein extraction from leaves of *T. latifolia* is highest among 30 freshwater aquatic plants in ponds at Calcutta India. Other than that, *Typha* also possess very high vitamin C content which is about four times higher than an average of citrus fruit (Pastor & Gustavo, 2003) According to Charpentier (1998) the vitamin is lasts for at least six months in the dry *Typha* pollen.

2.2.4 Ecology Important

Although *T. latifolia* was thought as a nuisance along lake margins, these plants plays an important functions that keep a lake healthy because *T. latifolia* can filter runoff as it flows into the lake. This helps to reduce the nutrients such as mud which enter lake from surrounding land. Besides that, this plant also helps to prevent shoreline erosion from waves created by wind or boats. According to Pip and Stepaniuk (1992), some aquatic plants are pollution indicators due to their ability to absorb and tolerate heavy metals. *T. latifolia* is an example species that important for ecology maintenance. This

is due to its ability to treat of polluted waters from industrial waste, agricultural runoff, municipals sewage, acid mine drainage and metal mining wastewaters (Dunbabin & Bowmer, 1992). Heavy metal contamination in aquatic and soil environments is a serious environmental problem because it is danger to aquatic ecosystems, agriculture and human health (Srivastav et al., 1994). *T. latifolia* has a high capacity for taking heavy metals such as manganese (Mn), copper (Cu), cadmium (Cd), cobalt (Co), zinc (Zn), lead (Pb), nickel (Ni) and chromium (Cr) in its tissue without giving serious physiological damage (McNaughton et al., 1974). Dunbabin and Bowmer (1992) reported that metal uptake by plants was highest in their roots.

2.2.5 Other Uses

T. latifolia has also provided people with building materials. It has been used as thatch for roofing. Their dried leaves are often woven to be made as furniture and mats, and their pulp and fibers can be made into paper and string (Rook, 2002). The fluff from seed heads also been used for padding, bedding and insulation. It has also been used in diapers. Besides that, *T. latifolia* has provides an important habitat for many species of wildlife and birds. The redwig blackbirds and many ducks and geese nest in *Typha* and some animal like muskrats eat this plant (Rook, 2002).

2.3 Micropopagation of *T. latifolia*

Micropopagation is used to multiplying the plant cells or organs in the culture vessels under highly controlled condition and the nutritive growth medium (Loberant & Altman 2010). The small piece of living tissues explants such as shoot tips, apical meristems, leaf sections, root tips and pollen grain cell is possible to grow into new plant in which it similar to their parent or totipotency (George et al., 2008). Besides, it can faster the growth rate of plant and produce the disease free propagule (Kubota & Tadokoro, 1998), produce plant for all year round (Vancea et al., 2009) and germinated the seeds with hundred percent contamination free (Rogers et al., 1998).
As consequence, this technique is widely use in agricultural industries. According to Kotahari et al. (2009), micropopagation and organogenesis is used in the development of chilli pepper plants or *Capsicum* to achieve the targets of pre-harvest and post-harvest improvement for value addition to this crop. Other than that, this technique

also helps in pharmaceuticals industries because the pharmaceuticals companies depend largely upon materials procured from naturally occurring stands that are being rapidly depleted. Therefore, micropopagation technique is used for a large number of plant species including many medicinal plants (Das et al., 2009).

Micropopagation can be form with technical advances, specialized equipment and sterile laboratory condition. *T. latifolia* is used as plant material in this experiment. The callus of *T. latifolia* had successfully regenerated within 9 weeks in Murashige and Skoog solid media (Rogers et al., 1998) while within 4 weeks in Gamborg B5 solid media (Estime et al., 2002). The different result present because the different media is used for the experiment. The selection for plant material is very important because not all plant material is suitable for initiating in tissue cultures. The underground organ of soil grown plants such as root and rhizome are not suitable as starting materials due to their highly contamination. Zimmerman and Read (1986) encountered problem with contamination poor callus production and low frequency in regeneration *T. latifolia* when the leaves, roots and rhizome was used as the starting material for induction the callus. However, *in vitro* seedlings are usually free from contaminations. Rogers et al. (1998) reported that 100% free contamination explants of *T. latifolia* was generated by using the seeds as starting material. Therefore, seed will be used as starting materials in this experiment. Genotype of the plant materials, environment and tissue dependent factors are the example factors that influencing the growth and morphogenesis of micropopagation.

2.3.1 Genotype

Genotype is one of the main factors that influence the growth in propagation of different plant species. The specific regeneration protocols are required for each cultivar because the existence of strong genotype specificity in the regeneration capacity of the different explants represents an important limiting factor that makes development of a standard regeneration protocol problem. According to Nhut et al. (2007), genotype affects the regeneration of explants in *Gerbera*. He was found that in propagation of plant *Gerbera*, the individually separated divisions root was better than undivided clusters. It showed that each genotype has its own *in vitro* response (Reynoird et al., 1993). Genotype factor also influences the organogenesis in

Capsicum cultures. Venkataiah et al. (2003) reported the response of the 10 pepper cultivars specifically depended upon its genotype.

2.3.2 Environment

Some environment plant propagation is varies for plant genera. The suitable condition in plant tissue cultures ensured the growth and morphogenesis of explants in high performance. Plant Growth Regulator (PGR), gaseous, container size, temperature, humidity and light intensity are the example of the environment condition that influencing in plant tissue cultures.

a) Plant Growth Regulator (PGR)

PGR is any chemicals including hormones and their synthetic analogs which able to influence plant growth and development of explants at low concentrations. Auxins, gibberellins, cytokinin absicic acid and ethylene are the example of PGRs. These components are important in determining the developmental pathway of plant cell. Auxins and cytokinins is the most usual PGRs had been used in plant tissue culture. The amount of PGRs in the culture medium was critical in controlling the growth and morphogenesis of the plant tissues (Skoog & Miller, 1957) and each play the different roles in plant development.

Auxins are important in promotes the formation of the callus growth, cell division, cell enlargement, adventitious buds and lateral rooting. Besides that, auxins also can inhibits leaf abscission and promote the lateral bud dormancy. Each of different plant cells needs the different amount of auxin in effective growth of the plant root formation. According to Pierik (1987), auxin alone or with the presence of a very low concentration of cytokinin was induced the root primordial.

On the other hand, cytokinins is important in promote the cell differentiation and stimulates the cell division with the combination of auxin. Cytokinins helps in promotes the growth of lateral buds into branches but it can inhibits the lateral growth

of roots. The combination of auxin and cytokinin are important because it influences each other. The low auxin and high cytokinins concentration in the medium resulted in the induction of shoot morphogenesis. Hariran et al. (1992) reported the highest number of shoots per explants was obtained with 13.9 µM kinetin and 2.2 µM BA in the micropopagation of *Kaempferia galangal*.

b) Light

The growth and morphogenesis of explants are influence by the interaction between shoot proliferation and regulative action of light quality. There are some qualities or types of light which influence in plant growth and morphogenesis which are blue, red, yellow and green light. The blue and red light are plays the important role on specific photoreceptor system in plant response while the blue and UV-A light increased the number of buds differentiated from the apical meristem without affecting apical dominance. Therefore, in limitating of light, it may inhibit the promotion of new shoot plant. According to Gopal and Sharma (1978), the germination of *T. latifolia* seeds was inhibit when it exposed to the blue light. He also observed that minimum light intensity is required for the seed germination. However, Bonnewell et al. (1983) observed that the *T. latifolia* seeds did not germinate without a light treatment and Rogers et al. (1998) reported that their seeds were germinated within two days in the presence of light.

2.3.3 Tissue Dependent Factor

a) Age of Explants

Stoutemeyer and Britt (1965) reported the differences in cell division and regenerative ability occur between juvenile and adult plants *in vitro* of *Hedera helix*. The explants from the juvenile plant tissue still can be generate to become the new plant because its cell still active and still can be differentiate (Nhut et al., 2007). However, when plant becomes older, their regenerative capacity often decreases and parts of juvenile plants are preferred than those of adults. The genetic information for totipotency is retained due to the loss of cells. The difference in cell division and regenerative ability between juvenile plants and adults plants *in vitro* were found in *Hedera helix*

(Stoutemeyer & Britt, 1965), *Lunaria annua* (Pierik, 1967) and *Anthrium andreanum* (Pierik et al., 1974), in which plant regeneration from juvenile plant parts occur more readily than those from adult plants. In the other hand, the different age of explants possess the different optimal regeneration of cells. As example, the 7-day-old *Gerbera* have not yet reached a sufficiently physiologically mature state for shoot regeneration as compared to10-day-old flower buds (Nhut et al., 2005). Its mean, the 10-day-old flower buds are optimal for shoot regeneration compared to 7-day-old flower buds in *Gerbera* propagation.

b) Size of explants

The rate growth also depends on size of explants in which the larger explants are sometimes more easier to regenerate than smaller size of explants (Nhut et al., 2007). This may due to the presence of more nutrient reserves. Larger explants have the larger surface areas which help in absorbing more nutrients. The small explants are being easily wounded, resulting in decreases the regeneration rate.

2.4 Plant Secondary Metabolites

2.4.1 Antioxidant in *T. latifolia*

Besides flavoring purposes, plants such as herbs have been used in folk medicine for centuries in most the cultures throughout the world. Generation of the free radicals or the reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system will give rise to the oxidative stress (Zima et al., 2001). Oxidative stress plays a role in heart disease, neurodegenerative disease, cancer and also the aging process (Astley, 2003). This is proved by the increasing evidence that the oxidative damage plays a role in the development of chronic and age related degenerative disease in which the dietary antioxidant can oppose this and lower the risk of disease (Atoui et al., 2005). Antioxidants are the substance that responsible to delay or prevent the oxidation of the substance when present in low concentrations compared to those of an oxidisable substrate (Halliwell & Gutteridge, 1989).

According to Hras et al. (2000), antioxidants are added in foods to prevent or delay the oxidation of food in which it initiated by free radicals formed during the exposure to environmental factors such as air, light and temperature. At present, there are most of the antioxidants are manufactured synthetically. However, it may give the side effect when taken in vivo (Chen et al., 1992). Therefore, plants are used as an alternative for the potential source of natural antioxidants that can be derived in secondary metabolites of the plant (Walton & Brown, 1999). The antioxidant compound can enhance the neurite outgrowth, promote fibroblast adhesion during wound healing and reduced the xenobitic-induced leukocyte hyperactivity and inflammatory damage due to its antioxidant effect (Weeks & Perex, 2007). Phenolic compound, (Ozawa & Imagawa, 1988), flavonoids, steroids and fatty acids (Aliotta 1990) are the example of phytochemical compound that can be found in genus *Typha*.

2.4.2 Phenolic Compounds

Re et al. (1999) reported that phenolic compound can be found as simple compound in most fresh fruits and vegetables or complex compounds which are present in bark, roots and leaves of plant. The structure of natural polyphenol are varies from simple molecules such as simple phenols or the volatile phenol to the highly polymerized compounds such as condensed tannins in which it depends on the basic skeleton of the polyphenols (Waterman & Mole, 1994). The antioxidant properties of phenolic compounds can act as free radical scavengers, hydrogen donators, metal chelators and singlet oxygen quencher due to their electron donating properties (Kahkonen, 1999). These phenolic compounds possess the different biological activities but most important are antioxidant activities in which it plays a major role in the protection of oxidation process. Ozawa and Imagawa (1988), isolated eleven phenolic compounds in the female inflorescenes of *T. latifolia*. This phenolic compound is very essential in healing the wounds due to their potential as an anti-inflammatory and antioxidants effects (Clark, 1991). The phenolic compound also has been found in female inflorescenes of *T. domingensis* in which it promote the healing process by preventing lipid peroxidation (Ysiladac et. al., 2011). Xanthones, flavonoids, isoflavonoids, lignins, acethophenons and *hydroxybenzoic* acids are the example of phenolic compound.

2.4.3 Flavonoids

Flavonoids are the most common and widely distributed group of plant phenolics that play the important role for normal growth development and defense against infection and injury (Kahkonenn, 1999). These flavonoids and other plant phenolics are commonly can be found in leaves, flowering tissues and woody parts such as stems and barks (Larson, 1988). Vuorela et al. (2000) reported that flavonoid constitutes a large group of plant secondary metabolites in the higher plants. As dietary compounds, flavonoid is widely known as antioxidants that inhibits the oxidation of low-density lipoproteins and reduced the thrombotic tendicies (Hertog et al., 1993). Flavonoids also important in anti-microbial activity, but there are no evidence in their effectiveness that has been reported (Mori et al., 1987; Tsuchiya et al., 1996). The major flavonoid classes include anthocyanins, leucoxanthin, flavonoidal and also anthoxanthins such as flavones, flavonols, flavanones, alkaloids, flavanols, chalcones and isoflavones (Houghton, 2002). Tannins are the polymeric flavonoids that divided into two groups which are condensed and hydrolysable.

2.4.4 Flavones and Flavonols

Flavonols consist of double-bonded oxygen atom that attached to position 4 of the C ring and a double bond in C ring. Flavones have the structure similar to flavonols, but there is no hydroxyl group attach to the 3 position of the C ring of flavones. In 1995, Hertog et al. was developed a specific and sensitive HPLC technique for the quantification of three major flavonols which are quercetin, myricetin and kaempferol and two major flavones which are luteolin and apigenin in foods. These flavonol and flavones intake was give the positive effects in pulmonary function and inversely associated with chronic cough (Sampson, 2002). There are five flavonol glucosides have been found in *T. latifolia* (Williams & Harbone, 1971) and several flavones were isolated from *T. capensis* and *T. domingensis* (Chapman & Hall, 1996).

CHAPTER 3

METHODOLOGY

3.1 Establishment of Cultures

3.1.2 Plant Material

Mature spikes of *T. latifolia* were collected from plants in single natural wetland site at Simpang Pulai, Ipoh, Perak. Seeds were harvested according to method by Rogers et al. (1998), the spike were transferred blender containing 500ml distilled water and blended at a low speed for 10 seconds. The mixture was poured into basin, seeds that settled at the bottom were collected using dropper, air dried, and stored at room temperature.

3.1.2. Seeds sterilization and Germination

Seeds were stirred in 100ml of sterilized distilled water few drops Tween 20 for 5 min. Solution then discarded and seeds then immersed 100 ml of 30% (v/v) commercial bleach Clorox with 0.1% Tween 20 for 30 minutes and stirrer on magnetic stirrer. Seeds then were rinsed three times with sterilized distilled water. Sterilized seeds were germinated in liquid MS basic salt (Murashige and Skoog, 1962) medium under continuous light or dark condition for one week. Seedling was transferred onto the solid MS media and allow for a month. MS medium used containing with 3% (w/v) sucrose and adjusted to pH 5.7-5.8. The medium was sterilized by autoclaving at 121^0C for 30 min.

3.1.3 Plant Growth and Proliferation of New Plantlet

a) Apical tips explant

Shoot tips obtained from five weeks old of *T. latifolia* cultures was transferred into MS medium with 0, 1.0, 2.0, 3.0 and 5.0 mgl^{-1} of Kinetin or N^6 –benzyladenine (BAP). Fifty explants were used per each treatment with five explants per each conical flask. The number of leaf, root and new plantlet produced were measured 7 days interval for nine weeks. The experiment was repeated three times.

b) Rhizhome explant

Rhizomes of three months old *T. latifolia* cultures was transferred into MS with 0, 1.0, 3.0 and 5.0 mgl^{-1} of Thidiazuron (TDZ), Zeatin (ZEA) or combination of N^6-benzyladenine (BAP) with Indole-3-acetic acid (IAA). Four explants were used per treatment, and the experiment was repeated three times. The number of leaves, root and new plantlet of plants was noted every week. The lengths and weight was measured. This experiment was carried out for six weeks.

3.2.Quantification of Total Phenolic, flavonoid and Flavone

3.2.1 Extraction

One g *T. latifolia* plantlet was homogenate in 10 ml of 95 % ethanol using the mortar and pestle with continous shaken at room temperature for 24 hours. Homogenate then was filtered using filter paper and the volume was made up to 10 ml with 80% ethanol.

3.2.2. Total Flavone Content

The flavones content was measured by spectrophotometric assay based on aluminium chloride complex formation (Popova et al., 2003). Two ml of the test extract solution, 20 ml methanol and 1 ml of 5% $AlCl_3$ were made up volume to 50 ml with distilled water.

The mixture solution was incubated at room temperature for 30 min and measured absorbance at 425 nm. Methanol was used as blank solution. Every assay was carried out in triplicate. Quercetin range 4-32 μg/ml was used to make the calibration curve. 10 ml quercetin was dissolved in 80% ethanol and then diluted into 5, 10, 15, 20, 25 and 30 μg/ml.

3.2.3 Total Flavonoid Content

Flavonoid content was measured using the procedure by Woisky and Saltino (1998). 0.5 ml of test solution was transferred to flask containing 1.5 ml of 95% ethanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1M potassium acetate and 2.8ml of distilled water. After 30 minutes, the absorbance was measured at 415 nm. For the blank, the amount of 10% $AlCl_3$ was substituted by the same amount of distilled water in the blank. Every assay was carried out in triplicate. Quercetin range 25-100 μg/ml was used to make the calibration curve.

3.2.4 Total Phenolics content

Phenolics were measured using Folin-Ciocalteu method (Woisky & Salatino, 1998). One ml test solution was added in flask containing 15 ml distilled water, 4 ml of the Follin-Ciocalteu reagent and 6 ml of 20% sodium carbonate. , the volume was made up tp 50 ml with distilled water. The absorbance was measured after 2 hours at 760 nm. Every assay was carried out in triplicate. Gallic acid range 37-326 μg/ml was used to make the calibration curve.

3.3 Statistical and Data Analysis

Data was subjected to analysis of variance (ANOVA) using SPSS version 16.0 software for Windows. Means differing significantly were compared using Duncan's Multiple Range Test (DMRT) at P=0.05. Data is expressed as the mean ± standard error (S.E)

CHAPTER 4

RESULTS

4.1 Growth of *Typha latifolia*

4.1.1 Leaves Number

Table 4.1 showed the effect of BAP and KN on number of *Typha latifolia* leaves established from the apical shoot tips. Result show no significant difference in week 3 (P=0.057) and week 6 (P=0.446) of the treatment. Highest number of leaves (10.67) was observed in 2.0 mgl^{-1} BAP after nine weeks. Rhizome in ZEA, TDZ and BAP (Table 4.2), exhibit no significance difference on number of leaves in week 2 (P=0.088) but differ in week 4 (P=0.008) and week 6 (P=0.013). In this treatment, 1.0 mgl^{-1} BAP (4.75) showed the highest number of leaves compared to others after six weeks of cultures. However, in treatment combination of BAP with IAA (Table 4.3), there has significance difference in week 2 (P=0.044) and week 6 (P=0.033) instead of week 4 (P= 0.498) from rhizome. There are showed that transfer of rhizome to MS medium supplemented with 1:1 ratio of BAP and IAA (6.27) was required in leaves multiplication. Compared to BAP, TDZ and ZEA alone, the combination of BAP with IAA in rhizome explant was enhanced leaves multiplication.

4.1.2 Leaves length

The length of leaves has significantly difference between the treatments. Result shows the highest length of apical tips (49.00cm) and rhizome (40.18cm) culture was observed in control treatment. Length of leaves did not increase in the combination BAP and IAA treatment. Table 4.3 show the longest leaf (40.18cm) was observed in control treatment when rhizome was used as the explants.

4.1.3 Fresh weight

Result show BAP and KN (Table 4.1) enhance the fresh weight of *Typha latifolia.* There are significantly difference in week 3 (P=0.003) and week 9 (P=0.007) instead of week 6 (P= 0.620) of culture. Maximum weight (0.66g) of plant was observed on 2.0 mgL^{-1} BAP after nine weeks of cultures. ZEA, TDZ and BAP enhanced the weight of *Typha latifolia* but there has no significance difference between each treatment (P= 0.082). In this treatment, 1.0 mgl^{-1} BAP (0.56g) showed the highest weight of plant compared to others after six weeks of cultures. 1.0 mgl^{-1} BAP+5.0 mgl^{-1} IAA was significantly (P= 0.011) gave the highest in weight of *Typha latifolia* (1.15g) in whole treatment besides BAP and IAA combination.

Table 4.1: Effect of BAP and KN on number, length and fresh weight of *Typha latifolia* leaf after nine weeks grown in tissue culture.

Treatment (mgl^{-1})	Number of leaves (±SE)			Length of leaves (cm)(±SE)			Weight (g) (±SE)		
	Week 3	Week 6	Week 9	Week 3	Week 6	Week 9	Week 1	Week 2	Week 3
Control	4.00 ±0.00^{a,b}	6.00±0.00^{a,b}	6.67±0.33^{a}	15.00±1.65^{c}	33.70±3.11^{d}	49.00±1.90^{d}	0.07±0.01^{a,b}	0.26±0.05^{a}	0.59±0.07^{c,d}
BAP 1.0	4.67±0.33^{a,b,c}	7.33±0.33^{b}	8.33±0.33^{a}	4.63±0.61^{a}	7.70±3.06^{a}	21.67±1.25^{a,b,c}	0.05±0.01^{a}	0.11±0.02^{a}	0.58±0.08^{c,d}
BAP 2.0	5.33±0.88^{b,c}	6.00±1.00^{a,b}	10.67±0.33^{b}	4.63±0.65^{a}	8.50±2.03^{a,b}	18.17±2.15^{a,b,c}	0.06±0.00^{a,b}	0.25±0.03^{a}	0.66±0.06^{d}
BAP 3.0	4.00±0.00^{a,b}	5.67±0.88^{a,b}	7.67±0.88^{a}	3.47±0.39^{a}	7.63±2.15^{a}	18.00±0.61^{a,b}	0.07±0.02^{a,b}	0.14±0.03^{a}	0.55±0.09^{b,c,d}
BAP 5.0	3.67±0.33^{a}	5.67±0.33^{a,b}	7.67±0.88^{a}	3.60±0.82^{a}	6.03±1.41^{a}	14.63±1.40^{a}	0.06±0.01^{a,b}	0.22±0.06^{a}	0.32±0.13^{a,b}
KN 1.0	4.67±0.33^{a,b,c}	5.33±0.33^{a,b}	7.33±0.33^{a}	12.40±1.22^{c}	19.60±1.36^{b,c}	21.60±0.72^{a,b,c}	0.07±0.01^{a,b}	0.15±0.02^{a}	0.26±0.03^{a}
KN 2.0	5.67±0.33^{c}	6.00±0.58^{a,b}	7.67±0.33^{a}	8.23±0.79^{b}	21.60±8.85^{c}	22.13±3.16^{a,b,c}	0.13±0.01^{c}	0.20±0.14^{a}	0.30±0.03^{a}
KN 3.0	4.33±0.67^{a,b}	5.33±0.33^{a,b}	8.33±0.88^{a}	6.67±0.4^{a,b}	19.97±1.63^{b,c}	25.17±4.10^{b,c}	0.09±0.06^{b,c}	0.17±0.03^{a}	0.37±0.05^{a,b,c}
KN 5.0	4.00±0.0^{a,b,c}	5.00±1.15^{a}	6.33±0.67^{a}	6.60±1.46^{a,b}	19.70±2.10^{b,c}	25.97±3.24^{c}	0.10±0.01^{b,c}	0.17±0.03^{a}	0.39±0.08^{a,b,c}

Note. Means followed by the same superscripts in same column do not differ significantly at P≤0.05 by Duncan multiple range test.

Table 4.2: Effects of ZEA, TDZ and BAP on number, length and fresh weight of *Typha latifolia* leaves after six weeks grown in tissue culture.

Treatment (mgl^{-1})			Number of Leaves (±SE)			Leaves Length (cm)(±SE)	Weight (g) (±SE)
ZEA	TDZ	BAP	Week 2	Week 4	Week 6	Week 6	Week 6
		0	$2.25\pm0.25^{a,b,c}$	3.25 ± 0.63^{c}	$4.25\pm0.48^{c,d}$	40.18 ± 3.99^{d}	$0.41\pm0.02^{a,b,c}$
		1	$2.50\pm0.29^{b,c}$	3.25 ± 0.25^{c}	4.75 ± 0.25^{d}	$22.45\pm4.81^{b,c}$	0.56 ± 0.06^{c}
		3	$1.75\pm0.25^{a,b,c}$	$2.50\pm0.65^{a,b,c}$	$4.00\pm1.22^{b,c,d}$	$14.20\pm3.45^{a,b}$	$0.48\pm0.09^{b,c}$
		5	$1.75\pm0.25^{a,b,c}$	$2.25\pm0.25^{a,b,c}$	$3.50\pm0.65^{a,b,c,d}$	$11.98\pm1.46^{a,b}$	$0.24\pm0.03^{a,b}$
	1		$1.33\pm0.33^{a,b,c}$	$1.67\pm0.33^{a,b}$	$2.00\pm0.58^{a,b}$	$11.20\pm0.75^{a,b}$	$0.31\pm0.04^{a,b,c}$
	3		1.00 ± 0.00^{a}	1.25 ± 0.25^{a}	$2.25\pm0.25^{a,b,c}$	6.38 ± 2.29^{a}	$0.47\pm0.18^{b,c}$
	5		$1.25\pm0.25^{a,b}$	1.50 ± 0.29^{a}	1.75 ± 0.48^{a}	5.88 ± 1.93^{a}	0.31 ± 0.08^{a}
1			$2.25\pm0.75^{a,b,c}$	3.25 ± 0.63^{c}	4.50 ± 0.65^{d}	27.33 ± 4.94^{c}	$0.39\pm0.12^{a,b,c}$
3			$1.50\pm0.65^{a,b,c}$	3.25 ± 0.48^{c}	$3.25\pm0.48^{a,b,c,d}$	$20.78\pm4.97^{b,c}$	$0.26\pm0.05^{a,b}$
5			2.67 ± 0.33^{c}	$3.00\pm0.00^{b,c}$	$3.33\pm0.33^{a,b,c,d}$	$16.50\pm0.50^{a,b,c}$	0.15 ± 0.00^{a}

Note. Means followed by the same superscripts in same column do not differ significantly at $P\leq0.05$ by Duncan multiple range test (DMRT).

Table 4.3: Effect of combination BAP with IAA on number, length and fresh weight of *Typha latifolia* leaves and weight after six weeks grown in tissue culture.

Treatment (mgl^{-1})		Number of Leaves (±SE)			Length of Leaves (cm) (±SE)	Weight (g) (±SE)
		Week 2	Week 4	Week 6	Week 6	Week 6
0	0	$2.25\pm0.25^{a,b,c}$	3.25 ± 0.63^{a}	$4.25\pm0.48^{a,b,c,d}$	40.18 ± 3.99^{e}	$0.41\pm0.02^{a,b}$
0	1	2.75 ± 0.25^{c}	3.25 ± 0.25^{a}	$5.25\pm0.75^{b,c,d}$	40.15 ± 4.04^{e}	$0.66\pm0.07^{a,b,c}$
0	3	$2.25\pm0.48^{a,b,c}$	3.00 ± 0.00^{a}	$5.50\pm0.50^{c,d}$	40.13 ± 4.62^{e}	$0.99\pm0.27^{b,c,d}$
0	5	$2.50\pm0.87^{b,c}$	3.00 ± 0.71^{a}	$4.75\pm0.85^{b,c,d}$	$31.98\pm2.85^{d,e}$	$0.38\pm0.05^{a,b}$
1	0	$2.50\pm0.29^{b,c}$	3.25 ± 0.25^{a}	$4.75\pm0.25^{b,c,d}$	$22.45\pm4.81^{a,b,c,d}$	$0.56\pm0.06^{a,b,c}$
1	1	$2.50\pm0.29^{b,c}$	3.50 ± 0.29^{a}	6.25 ± 0.25^{d}	$28.43\pm6.16^{c,d,e}$	$0.68\pm0.21^{a,b,c,d}$
1	3	$1.50\pm0.29^{a,b,c}$	2.75 ± 0.25^{a}	$4.25\pm0.48^{a,b,c,d}$	$25.10\pm4.82^{b,c,d}$	$0.73\pm0.19^{a,b,c,d}$
1	5	$1.33\pm0.67^{a,b,c}$	2.67 ± 0.33^{a}	$4.67\pm0.33^{b,c,d}$	$29.27\pm7.37^{c,d,e}$	1.15 ± 0.45^{d}
3	0	$1.75\pm0.25^{a,b,c}$	2.50 ± 0.65^{a}	$4.00\pm1.22^{a,b,c,d}$	$14.20\pm3.45^{a,b}$	$0.48\pm0.09^{a,b,c}$
3	1	$1.00\pm0.41^{a,b}$	2.25 ± 0.25^{a}	2.25 ± 0.25^{a}	$13.48\pm2.73^{a,b}$	$0.56\pm0.09^{a,b,c}$
3	3	2.75 ± 0.48^{c}	3.50 ± 0.29^{a}	$3.75\pm0.48^{a,b,c}$	10.80 ± 2.18^{a}	0.27 ± 0.03^{a}
3	5	$1.50\pm0.50^{a,b,c}$	3.75 ± 0.63^{a}	$4.50\pm0.96^{a,b,c,d}$	$13.03\pm3.25^{a,b}$	$0.73\pm0.26^{a,b,c,d}$
5	0	$1.75\pm0.25^{a,b,c}$	2.25 ± 0.25^{a}	$3.50\pm0.65^{b,c,d}$	$11.98\pm1.46^{a,b}$	0.24 ± 0.03^{a}
5	1	$2.50\pm0.65^{b,c}$	3.00 ± 0.58^{a}	$3.00\pm0.58^{a,b}$	$27.20\pm2.70^{c,d,e}$	$0.47\pm0.07^{a,b}$
5	3	$1.50\pm0.65^{a,b,c}$	3.25 ± 0.48^{a}	$3.75\pm0.48^{a,b,c}$	$16.59\pm4.31^{a,b,c}$	$0.67\pm0.13^{a,b,c,d}$
5	5	0.75 ± 0.25^{a}	3.00 ± 0.71^{a}	$4.25\pm1.03^{a,b,c,d}$	$17.15\pm1.53^{a,b,c}$	$0.86\pm0.07^{b,c,d}$

Note. Means followed by the same superscripts in same column do not differ significantly at $P\leq0.05$ by Duncan multiple range test.

4.1.4 Root Number

The BAP, KN, TDZ and ZEA inhibit the number of roots. The highest number of roots was in apical tips (20.00) and rhizome (9.00) in cultured. IAA and BAP (Table 4.6) show that 3.0 mgl^{-1} IAA has the maximum number of roots and there has significance difference between each week (Week 2: P= 0.044; Week 4=0.498; Week 6: P= 0.033).

4.1.5 Root Length

Table 4.4 and 4.5 showed the effect single cytokinin in the length of roots after nine weeks of cultures. Control showed significance difference (P=0.000) within each week of cultures when apical tips (6.30cm) and rhizome (2.75cm) was used as the explants. However, root length was significantly increased in additional of auxin. In the treatment combination between BAP and IAA (Table 4.6) showed that 3.0 mgl^{-1} IAA (6.68cm) has the maximum in root length.

Table 4.4: Effects of BAP and KN on number and length of *Typha latifolia* roots after nine weeks grown in tissue culture.

Treatment (mgl^{-1})	Number of Roots (±SE)			Length of Roots (cm) (±SE)		
	Week 3	Week 6	Week 9	Week 3	Week 6	Week 9
Control	10.67 ± 0.88^{d}	17.67 ± 0.67^{e}	20.00 ± 1.73^{c}	2.30 ± 0.25^{e}	3.70 ± 0.76^{d}	6.30 ± 0.66^{c}
BA 1.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
BA 2.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
BA 3.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
BA 5.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
KN 1.0	10.67 ± 0.88^{d}	13.67 ± 0.88^{d}	18.00 ± 2.08^{c}	$1.30\pm0.21^{c,d}$	3.00 ± 0.51^{d}	3.27 ± 0.29^{b}
KN 2.0	11.00 ± 0.58^{d}	12.33 ± 0.67^{d}	19.33 ± 1.86^{c}	1.43 ± 0.07^{d}	$1.70\pm0.40^{b,c}$	3.10 ± 0.30^{b}
KN 3.0	4.33 ± 0.88^{c}	9.00 ± 2.08^{c}	19.00 ± 2.00^{c}	0.83 ± 0.23^{b}	1.53 ± 0.20^{b}	3.13 ± 0.43^{b}
KN 5.0	2.67 ± 0.33^{b}	5.00 ± 1.15^{b}	10.33 ± 2.91^{b}	$0.90\pm0.12^{b,c}$	$2.63\pm0.24^{c,d}$	3.23 ± 0.26^{b}

Note. Means followed by the same superscripts in same column do not differ significantly at $P\leq0.05$ by Duncan multiple range test.

Table 4.5: Effects of ZEA, TDZ and BAP on number and length of *Typha latifolia* roots after six weeks grown in tissue culture.

Treatment (mgl^{-1})			Number of Roots (±SE)			Length of Roots (±SE)
ZEA	TDZ	BAP	Week 2	Week 4	Week 6	Week 6
		0	$2.25\pm0.63^{b,c}$	3.75 ± 1.03^{b}	9.00 ± 0.82^{b}	2.75 ± 0.40^{c}
		1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.05 ± 0.05^{a}
		3	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
		5	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	1		0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	3		0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	5		0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
1			$1.75\pm1.11^{a,b,c}$	3.75 ± 0.48^{b}	$7.50\pm1.19^{b,c}$	$2.12\pm0.56^{b,c}$
3			$1.25\pm1.25^{a,b,c}$	2.75 ± 2.14^{b}	6.00 ± 2.45^{b}	1.30 ± 0.59^{b}
5			0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Note. Means followed by the same superscripts in same column do not differ significantly at $P\leq0.05$ by Duncan multiple range test.

Table 4.6: Effect of BAP ,IAA and combination of BAP with IAA on number and length of *Typha latifolia* roots after six weeks grown in tissue culture.

Treatment(mgl^{-1})		Number of Roots (±SE)			Root Length (cm) (±SE)
BA	IAA	Week 2	Week 4	Week 6	Week 6
0	0	2.25 ± 0.63^{bc}	3.75 ± 1.03^{b}	9.00 ± 0.82^{b}	2.75 ± 0.40^{c}
0	1	2.75 ± 1.03^{c}	6.50 ± 1.66^{c}	14.00 ± 2.42^{c}	5.08 ± 0.33^{d}
0	3	1.75 ± 1.44^{abc}	9.75 ± 0.75^{d}	17.50 ± 2.96^{d}	6.68 ± 1.79^{e}
0	5	0.00 ± 0.00^{a}	2.75 ± 1.11^{bc}	7.50 ± 1.32^{b}	1.90 ± 0.23^{bc}
1	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.05 ± 0.05^{a}
1	1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
1	3	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
1	5	0.50 ± 0.50^{bc}	1.75 ± 0.63^{ab}	2.33 ± 0.33^{a}	0.32 ± 0.14^{ab}
3	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
3	1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.25 ± 0.25^{a}	0.18 ± 0.18^{a}
3	3	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
3	5	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
5	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
5	1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
5	3	0.00 ± 0.00^{a}	0.50 ± 0.50^{a}	0.75 ± 0.48^{a}	1.38 ± 0.94^{abc}
5	5	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Note. Means followed by the same superscripts in same column do not differ significantly at $P\leq0.05$ by Duncan multiple range test.

4.2 Proliferation of *Typha latifolia*

Table 4.7 showed increased the new plantlet of *Typha latifolia* after nine weeks of cultures in 2.0 mgl^{-1} BAP. However, in ZEA, TDZ or BAP, there is no significance difference. The maximum number of new plantlets (Table 4.7), was observed in the 3.0 mgl^{-1} TDZ (0.75) after six weeks of cultures. In the combination treatment, 5.0 mgl^{-1} BAP+3.0 mgl^{-1} IAA was enhance the new plantlet establishment (1.00). However, there is no increasing in new plantlets was observed after week 3 in this treatment.

Table 4.7: Effect of BAP and KN on number of new plantlet produced by *Typha latifolia* grown in tissue culture after nine weeks.

Treatment (mgl^{-1})	Control	BA 1.0	BA 2.0	BA 3.0	BA 5.0	KN 1.0	KN 2.0	KN 3.0	KN 5.0
Number of New Plantlet (±SE)	0.00 ± 0.00^{a}	$0.90\pm0.41^{a,b}$	$1.10\pm0.59^{a,b}$	$0.80\pm0.42^{a,b}$	$0.80\pm0.36^{a,b}$	0.00 ± 0.00^{a}	0.10 ± 0.10^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Table 4.8: Effect of ZEA, TDZ and BAP on number of new plantlet produced by *Typha latifolia* grown in tissue culture after six weeks.

Treatment (mgl^{-1})	Control	BA 1.0	BA 3.0	BA 5.0	BA 5.0	TDZ 1.0	TDZ 3.0	TDZ 5.0	ZEA 1.0	ZEA 3.0	ZEA 5.0
Number of New Plantlet (±SE)	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.25 ± 0.25^{a}	0.50 ± 0.50^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.75 ± 0.48^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Note. Means followed by the same superscripts in same column do not differ significantly at $P\leq0.05$ by Duncan multiple range test.

Table 4.9: Effect of BAP combine with IAA on number of new plantlet of *Typha latifolia* after six weeks grown in tissue culture.

Treatment (mgl^{-1})		Number of new plantlets		
BAP	IAA	Week 2	Week 4	Week 6
0	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
0	1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
0	3	0.50 ± 0.29^{ab}	0.50 ± 0.29^{ab}	0.75 ± 0.48^{a}
0	5	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
1	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
1	1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.50 ± 0.29^{a}
1	3	0.25 ± 0.25^{a}	0.25 ± 0.25^{ab}	0.25 ± 0.25^{a}
1	5	0.67 ± 0.67^{a}	0.67 ± 0.67^{ab}	0.67 ± 0.67^{a}
3	0	0.00 ± 0.00^{ab}	0.25 ± 0.25^{ab}	0.25 ± 0.25^{a}
3	1	0.25 ± 0.25^{a}	0.25 ± 0.25^{ab}	0.25 ± 0.25^{a}
3	3	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.75 ± 0.48^{a}
3	5	0.25 ± 0.25^{a}	0.50 ± 0.29^{ab}	0.50 ± 0.29^{a}
5	0	0.00 ± 0.00^{a}	0.25 ± 0.25^{ab}	0.50 ± 0.50^{a}
5	1	0.00 ± 0.00^{a}	0.50 ± 0.29^{ab}	0.50 ± 0.29^{a}
5	3	1.00 ± 0.41^{b}	1.00 ± 0.41^{c}	1.00 ± 0.41^{a}
5	5	0.25 ± 0.25^{a}	0.25 ± 0.25^{ab}	0.50 ± 0.29^{a}

Note. Means followed by the same superscripts in same column do not differ significantly at $P\leq0.05$ by Duncan multiple range test.

4.3 Polyphenol Content

4.3.1 Effect of BAP and KN

Figure 4.1 shows the total phenolic, flavonoid and flavone in *T. latifolia* culture in BAP or KN containing medium. Overall, there is no significance difference between the treatments (P=0.201) with 3.0 mgl^{-1} BAP gave the highest of total phenolics contents (66.67±2.51). Unlike the lowest value of phenolics content (45.17±4.08) was observed in the control medium. The total flavonoid content was found highest than total phenolic content. The total flavonoid content showed significant differences (P=0.019) among the treatments. 3.0 mgl^{-1} KN gave the high values of total flavonoid contents (87.58±10.45) in the plant. 5.0 mgl^{-1} BAP exhibit the lowest total flavonoid content (30.67±5.23). The total flavone content was not increased with the hormone combination in media. As shown in Figure 4.1, control has the highest total flavone content compare to in BAP or KN (60.17±8.21). 5.0 mgl^{-1} BAP had the lowest total flavone content (14.67±0.73).

4.3.2 Effect of Combination BAP, TDZ and ZEA

Figure 4.2 shows the total phenolic, flavonoid and flavone in *T. latifolia* in BAP, TDZ or ZEA containing media. Result show significance difference between the treatment (P=0.001), which 1.0 mgl^{-1} BAP gave the highest on total phenolic (83.42±8.74).5.0 mgl^{-1} BAP was lowest (28.83±2.09). The total flavonoid showed significance difference (P=0.000) between the treatments. Control exhibit the highest in total flavonoid content (81.25±7.28) (Figure 4.2) while 5.0 mgl^{-1} BAP gave the lowest in total flavonoid content (29.5±4.1). The total flavone showed significance difference (P=0.000) between each treatment. However, total flavone did not increase with hormone as control was found gave the highest in total flavone content (41.33±6.36).

4.3.3 Effect of Combination BAP and IAA on Phenolics, Flavonoid and Flavone

Figure 4.3 shows the effect of combination BAP and IAA on the total flavone, flavonoid and phenolic content of *T. latifolia*. There are significance difference in total phenolic between each treatment (P=0.042). The phenolic content was increase in the lower BAP concentration. From the figure, low BAP concentration, 1.0 mgl^{-1} BAP give the highest on total phenolic content (83.42±8.74), 1.0 mgl^{-1} IAA was lowest total phenolic content (47.50±5.48). The total flavonoid content showed significance difference (P=0.001) between each treatment. Combination between 1.0 mgl^{-1} BAP +3.0 mgl^{-1} IAA gave the highest of total flavonoid content (87.50±10.13). In contrast, the lowest value of phenolics content (28.17±3.81) was observed in the 5.0 mgl^{-1} BAP +3.0 mgl^{-1} medium. The lower IAA concentration with the higher BAP concentration was caused the reduction of total flavonoid content. The total flavone content shows the significance difference (P=0.001) between the treatment. The combination between 1.0 mgl^{-1} BAP+3.0 mgl^{-1} IAA has the highest of total flavone (67.50±9.37) content while 1.0 mgl^{-1} IAA (12.67±2.05) give the lowest of total flavone content.

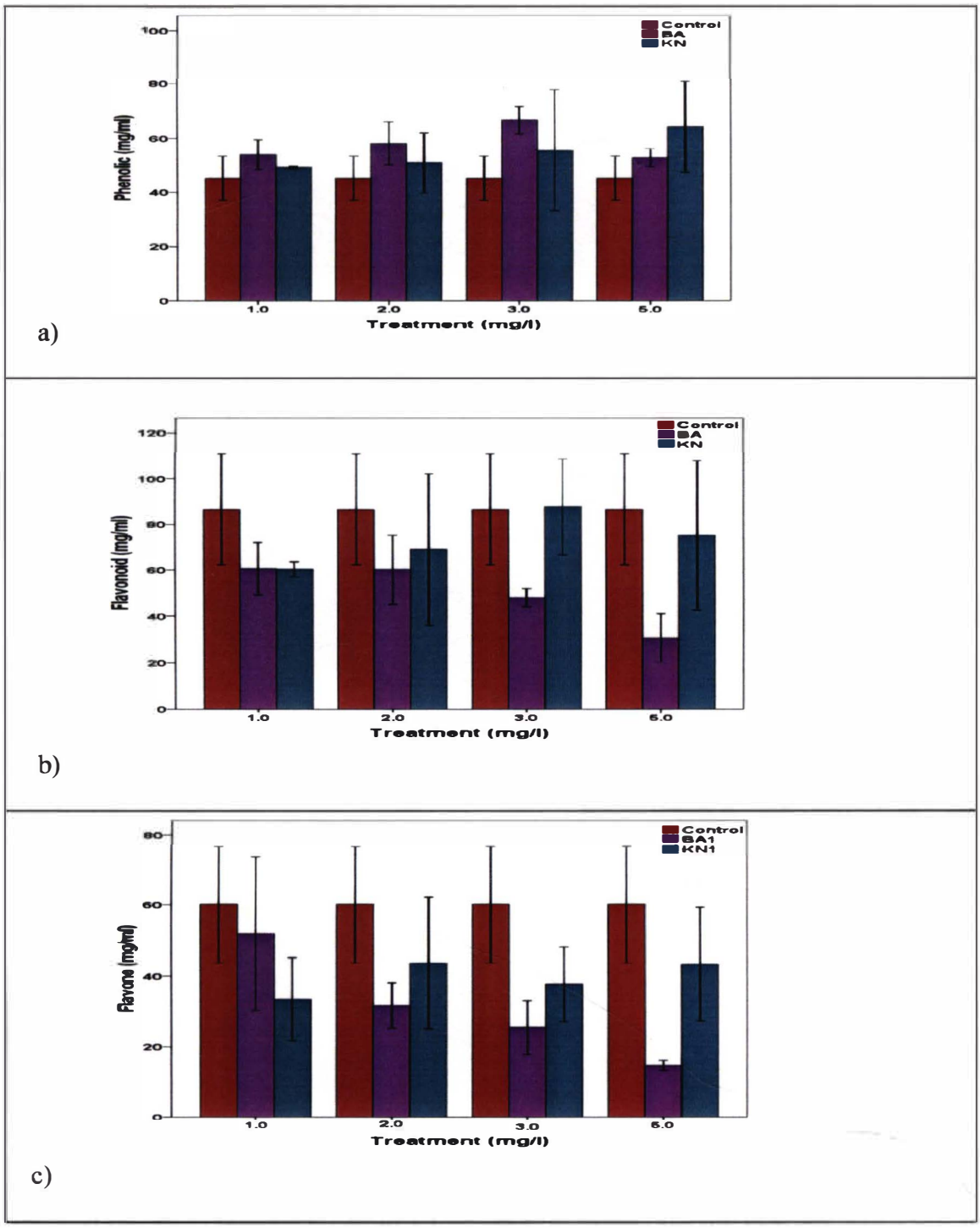

Figure 4.1: The effect of BAP and KN on total phenolics (a), flavonoid (b) and flavones (c) content in *Typha latifolia* after nine weeks grown in tissue culture. Bars on the column represent the standard error. (Means±S.E, n=3).

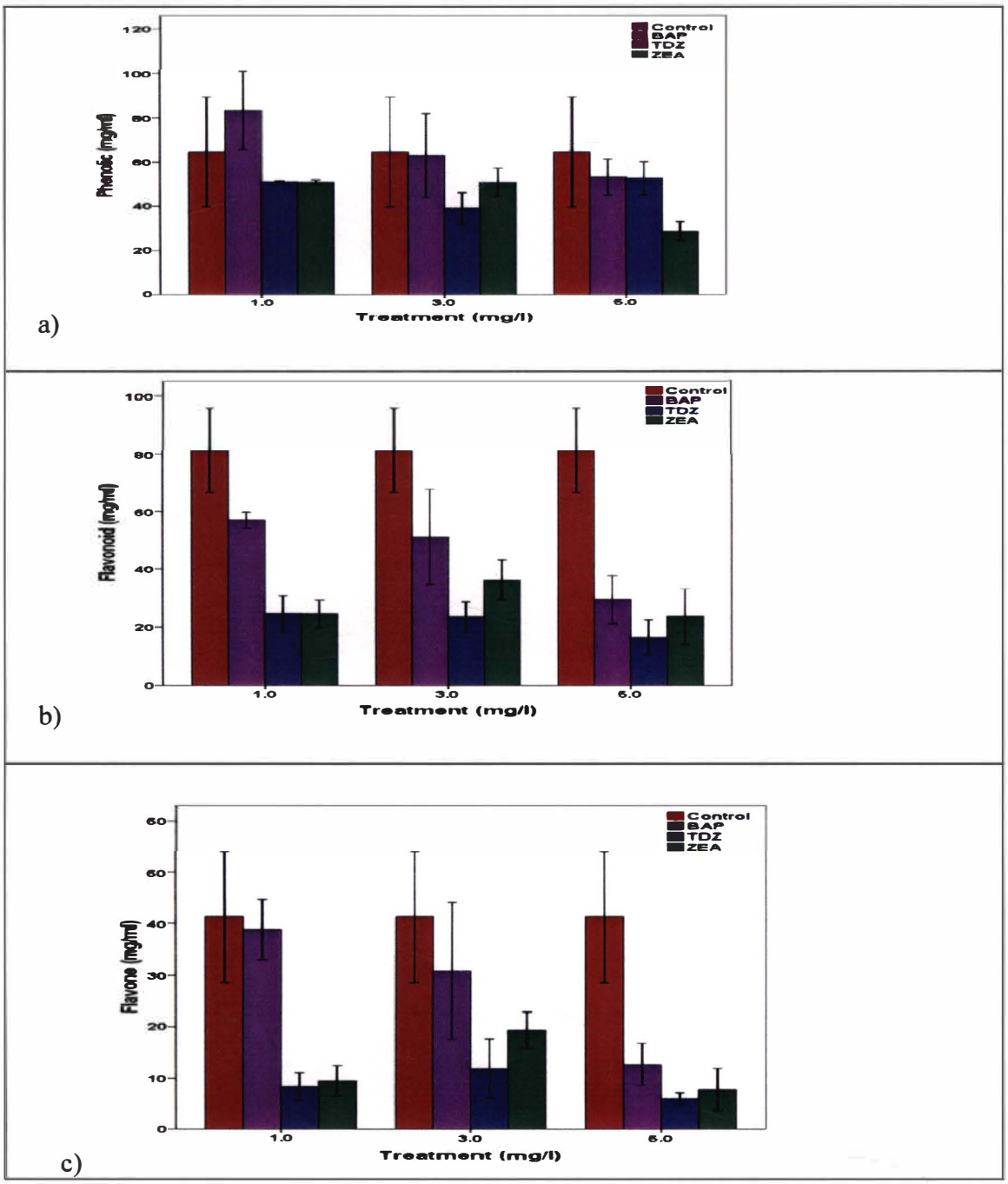

Figure 4.2: The effect of BAP, TDZ and ZEA on total phenolics (a), flavonoid (b) and flavones (c) content in *Typha latifolia* after six weeks grown in tissue culture. Bars on the column represent the standard error. (Means±S.E, n=3).

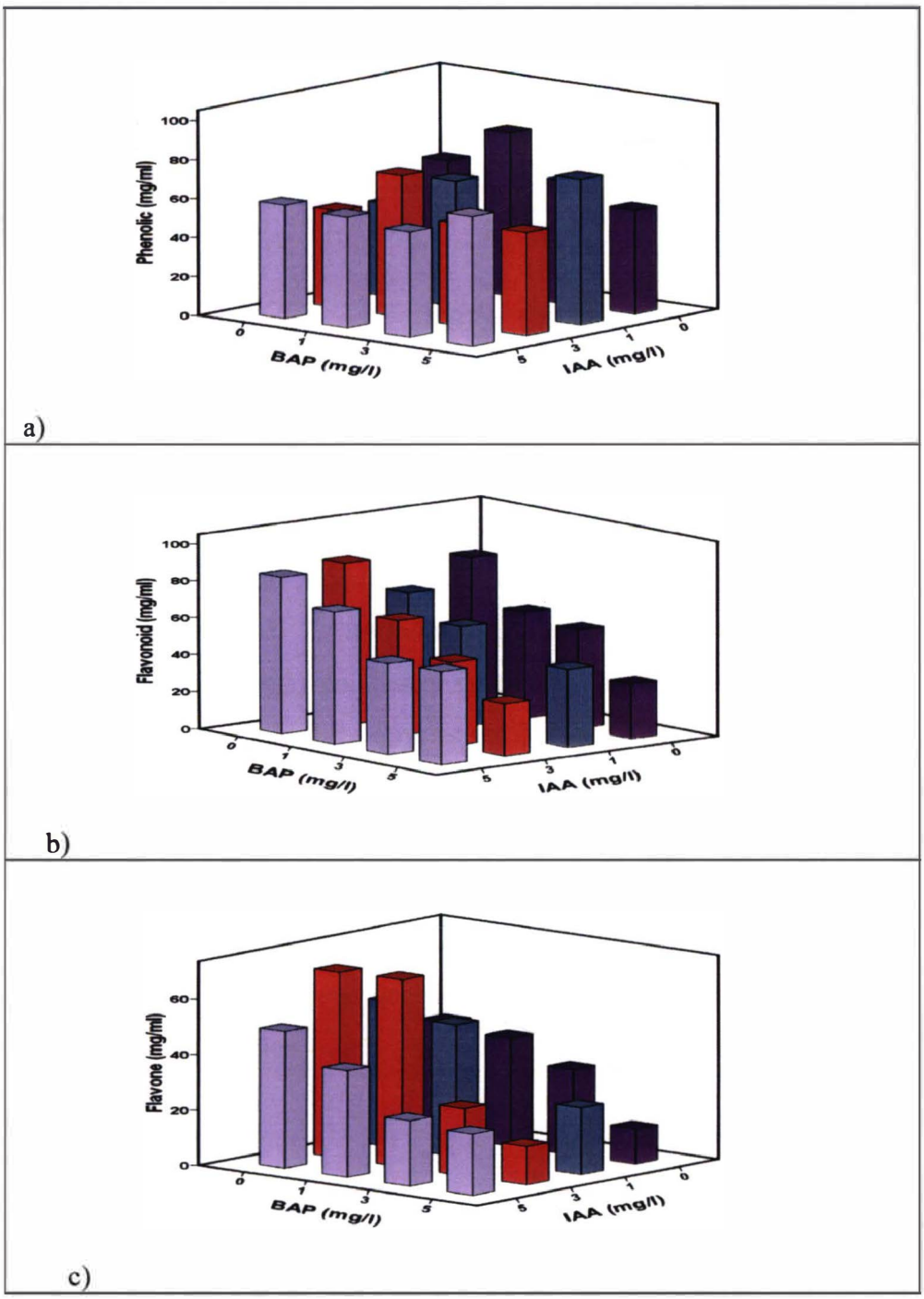

Figure 4.3: The effect of combination of BAP and IAA on total phenolics (a), flavono id (b) and flavones (c) content in *T. latifolia* after six weeks grown in tissue culture.

CHAPTER 5

DISCUSSION

The present show was developed efficient protocol for growth and proliferation of *T. latifolia* using apical tips and rhizome in different cytokinin and auxin. The disinfected seeds of *T. latifolia* did not germinate without a light treatment (Bonnewell et al., 1983). In present study also showed that seeds of *T. latifolia* can be germinated only within three days as similar with observed by Rogers et al. (1998). In tissue culture, cytokinin was incorporated mainly for cell division and differentiation of adventitious shoots from explants (Bojwani & Razdan, 1996). The concentration of cytokinin used significantly affected the leaves and roots multiplication and elongation besides proliferation of new plantlets. Rout et al. (2006) reported that cytokinin concentration was decisive for shoot proliferation and elongation of many medicinal plant species.

Both BAP and KN enhanced leaves multiplication but BAP induced more leaves, new plantlets and highest in weight than KN at 2.0 mgl^{-1}. The leaves multiplication was started grow rapid from initial until at the end (week 9) of which only 11 leaves per apical tips could be obtained. Geetha et al., (1998) reported BAP was more effective than KN in inducing shoot development and multiple shoot induction in all the explants of *Cajanus cajan* L. High multiplication of shoot regenerate from the cotyledonary nodes of *Vigna mungo* L. (Das et al., 1998) and the maximum multiple leaves (8 leaves per explants) in Safed musli (Biradar, 2005) was observed when

these plants were treated with 2.0 mgl^{-1} BAP.

The highest multiplication of leaves *T. latifolia* from rhizome was observed in 1.0 mgl^{-1} BAP compared to singly ZEA, TDZ and others concentration of BAP (1.0, 3.0 and 5.0 mgl^{-1}). Such an effect, BAP was induced most efficiently in shoot development of cassava compared to other cytokinin tests which are TDZ, ZEA and KN (Konan et al., 1997). This study showed that 63% of each nodal explants produced at least 25 shoots when it treated with 10 mgl^{-1} BAP. It has been reported that TDZ is the most effective in used for inducing adventitious and axillary shoot proliferation in several species (Kaneda et al., 1997). However, in the present study, TDZ was less effective than cytokinin BAP as reported in grass pea tissue culture (Barik et al., 2006). According to Fernando et al. (1998), high multiplication in shoot which that shoot length, multiple shoot and leaves would increased with increase in BAP concentration up to 2.0 mgl^{-1} BAP and decreased with further increase in BAP concentration. Based on his findings, 26 shoots per explants was observed in *Acorus Calamus* L. when it treated with 1.0 mgl^{-1} and 2.0 mgl^{-1} BAP.

Besides, combination hormone between 1:1 ratio BAP and IAA was induced multiplication of *T. latifolia* leaves. The additional of IAA into BAP was improved the leaves differentiation of *T. latifolia*. According to Bojwani and Razdan (1996), IBA and IAA with cytokinin interaction may enhance shoot proliferation. However, use of higher concentration of IAA and BAP (3.0 – 5.0 mgl^{-1}) reduced the number of leaves per explants as similar to the observation reported by Singh and Seghal (1999) in shoot regeneration of *Ocimum sanctum*. Findings showed that *O. sanctum* was produced maximum 11 shoot per explants in the lower concentration of IAA (0.05 mgl^{-1}) and BAP (1.0 mgl^{-1}). A high cytokinin concentration in combination with low auxin concentration will promotes shoot proliferation (Sharma et al., 1993).

In contrast, the elongation of leaves *T. latifolia* was not influence in single treatment of BAP and KN because it showed control has the maximum in length. Same observation was found by Kotahari and Dhaka (2005) in micropropagation of *Eclipta alba*. Based on his studied, the maximum length of leaves from the nodal explants of *E. alba* was 3.4 cm while from the shoot tips was 3.8cm. However, they reported that the shoot buds needed another subculture on medium containing BAP and GA_3 to enhance in shoot multiplication and elongation. Thus, it showed that BAP in the medium was sufficient for multiplication but it did not promote elongation of shoots or leaves. Control also was found as the best hormone in elongation of leaves from rhizome that treated with single cytokinin (BAP, TDZ and ZEA) and also combination hormone between BAP and IAA.

BAP in different concentration (1.0-5.0 mgl^{-1}) failed to induce rooting in *T. latifolia*. In contrast, KN had well development of rooting while control induced maximum of roots and highest in length. In addition, explants from rhizome did not develop roots in BAP and TDZ treatment except in control and ZEA range from 1.0 mgl^{-1} until 3.0 mgl^{-1}. The highest multiplication and elongation was observed in control treatment which is nine roots per rhizome. Such an effect of TDZ and BAP on root development is found in Nugget explants (Gurriaran et al., 1999). This study reported that rhizomes from Nugget plants did not develop roots in media with TDZ while ZEA and BAP rarely promoted the root formation. In contrast, single treatment of IAA and lower combination between BAP and higher concentration of IAA was induced roots regeneration. Auxins are involved in with elongation of stems and internodes, tropism, apical dominance, abscission and rooting in nature while in tissue culture, auxins have been used for cell division and root differentiation (Bojwani& Razdan, 1996). Besides, auxin would cause wall loosening, which in turn may reduce the turgor pressure and thus increase the water potential difference between the outside and the cell interior (Srivastava, 2002). Thus, IAA widely used for rooting. Findings showed that 3.0 mgl^{-1}.IAA induced maximum multiplication and elongation roots of *T. latifolia*.

Proliferation of new plantlets was observed in all BAP concentration and also 2.0 mgl^{-1} KN. Based on result, 2.0 mgl^{-1} BAP was induced high proliferation of new plantlet and the findings was similar to Geetha et al. in the shoot bud differentiation of *Cajanus cajan* L. BAP was found the best hormone in regeneration of shoot buds compared to KN. In this study, 2.0 mgl^{-1} BAP was found induced maximum of shoot bud differentiation. TDZ 3.0 mgl^{-1} was observed to give maximum of new plantlet regeneration compared to other single cytokinin treatment from the rhizome. According to Ernst (1994), the culture medium which using TDZ alone has a promotory effect on in vitro propagation of hybrid *Phalaenopsis dora* besides Chen and Chang (2000) reported that TDZ was effective in inducing embryos and buds of *Oncidium* from flower stalks directly from the wound surface and indirectly via white nodular callus. However, BAP and IAA combination promote higher of new plantlet compare to single cytokinin from rhizome. At higher concentrations of cytokinins and auxins, more number of new plantlets was observed. This study showed that combination between 5.0 mgl^{-1} BAP and 3.0 mgl^{-1} IAA generate maximum of new plantlets. Although BAP is effective in initiating multiple leaves proliferation, a combined effect of BAP and IAA was more efficient in new plantlet proliferation.

According to Bohidar et al. (2008), combination between BAP and IAA hormone was more effective in shoot bud initiation and subsequent proliferation in *Ruta Graveolens* L. compare to BAP alone although it enhance multiple shoot proliferation. *Typha* genus contains a number of antioxidants such as flavones, phenolic compound, long chain hydrocrarbon as well as various triterpenoids with steroidal skeleton (Shode et al., 2002). These natural antioxidants are secondary metabolites of the plant which play a major role in heart diseases, neurodegenerative diseases, cancer and in the aging process (Astley, 2003). According to Martin et al. (1998), two phenolic compound which are 2-chlorophenol and salicylaldehyde was extracts from *Typha domingenesis* that give alleophatic potential towards *Salvinia minima*. The phenolic compounds was gave the effect on the rates of oxygen production of *S. minima.* Thus, the effect of cytokinin and auxin towards secondary metabolites of *T. latifolia* was examined.

Control showed highest in total flavone content in single cytokinin (BAP, KN, TDZ and ZEA). Thus, it showed that the total flavone was not increase in the additional

cytokinin. In contrast, the combination hormone between 1.0 mgl^{-1} BAP+3.0 mgl^{-1} IAA had induced the production of flavone content. According to Mantell and Smith (1984), the type and concentration of auxin or cytokinin or the auxin and cytokinin ratio may alter dramatically both growth and the product formation in cultured plant cells. This because, auxin was responsible as the primary factor in controlling growth and morphology of roots, while cytokinin give the vary effect depending on secondary metabolites and the species (Rao & Ravishankar, 2002). As example, Seitz and Hinderer (1998) reported that kinetin stimulated the production of antocyanins in *Haplopappus gracilus* but inhibited the formation of antocyanins in *Populus* cell cultures. Thus, it showed that hormone can be stimulator or inhibitor in the production of plant secondary metabolites.

Flavonoids content differ in media with varied concentration of cytokinins. However, additional of cytokinin stimulated the proliferation of leaves was not stimulated the flavonoid production. In this study, the induction of flavonoid production in all tested concentration of single cytokinins was not achieved except in 3.0 mgl^{-1} of KN. Ionkova (2009) reported that KN was effective for flavonoid production in *Astragalus missouriensis Nutt.* compared to BAP. The additional of 2.0 mgl^{-1} KN to MS medium was favorable condition for flavonoid production. Besides, the production of flavonoid in *T. latifolia* was inverted with shootlet cultured in *Gardenia Jasminoides Ellis* which showed highest in all concentrations of cytokinins (BAP and KN) except to medium free hormones and mother plants which gave the lowest amount on total phenolics and flavonoid content (Sayd et al., 2010) . In contrast, the combination hormone between 1.0 mgl^{-1} BAP and 3.0 mgl^{-1} IAA gave the highest total flavonoid compound.

The total phenolic content showed that, 3.0 mgl^{-1} BAP was favorable for phenolic production in *T. latifolia* compared to KN and others concentration from nine weeks of cultured while 1.0 mgl^{-1} BAP was more efficient than other cytokinin (TDZ and ZEA) from six weeks of cultured. There showed that single BAP enhance the other constituents of total phenolic compound instead of flavonoid and flavone. Previous study showed that phenolic compound was highest in leaves compared to roots as

reported by Truong et al. (2007), that sweet potato leaves was about 8, 16 and 18 fold greater than the peel tissue, whole roots and flesh respectively. Among different concentration of BAP and IAA combination, this study of *T. latifolia* showed that no roots and high number of leaves 1.0 mgl^{-1} BAP was give the highest of total phenolic content compared to others combination hormone.

Phenolic compound is the major capacities of antioxidant in plant while flavonoids are a common and widely distributed group of plant phenolics that play the important role for normal growth development and defense against infection and injury (Kahkonen et al., 1999). Thus, it is well known that the concentration of phenolics compound usually higher than the concentration of flavanoid in most cases, but in this study, an inverse trend had been found. Based on result, the total flavonoid content was highest than total phenolic compound. Wu and Ng (2008) also reported that total flavonoid content (62 mg/g, d.w.) of *Momordica charantia* was higher than its total phenolic compound (51.6 mg/g, d.w.). This cause can be explained by the differences in the polarity of the extracting solvents could result in a wide variation in polyphenolic contents of the extract (El-Baz et al., 2010). Thus, the low total of phenolic compound may be possibly due to the fact that extraction with methanol was not release bound phenolics from the *T. latifolia*. Furthermore, the phenolic compound has different respond in the Folin Ciocalteu method besides rapid and widely used assay to detect the total phenolic compound (Kahkonen et al., 1999).

CHAPTER 6

CONCLUSION

BAP was found as the best hormone in leaves and new plantlet regeneration while auxin was found best in root induction. Combination BAP and IAA was efficient in plant growth and enhanced the production of phenolic, flavonoid and flavones content. However, the best hormone in plant growth does not influence in enhancing the total secondary metabolites in *T. latifolia*.

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APPENDICES

APPENDIX A

Culturing Media (Murashige and Skoog)

1. Stock for major elements (per Liter)	**g/l**
NH_4NO_3	16.50
KNO_3	19.0
CaCl	4.40
$MgSO_4.7H_2O$	3.70
KH_2PO_4	1.70

2. Stock for minor element	**g/500ml**
H_3BO_3	3.10
$MnSO_4.7H_2O$	11.15
$ZnSO_4.7H_2O$	4.30
KI	0.415
$Na_2MoO_4.2H_2O$	0.0125
$CuSO_4.5H_2O$	0.0125
$CoCl_3.6H_2O$	0.0125

2.2 Vitamin	**g/500ml**
Asid Nikotinik	0.50g
Thiamine.HCl	5.0g
Pyridoxine	0.50g

APPENDIX B

Statistical Analysis: Total Phenolic, Flavonoid and Flavone Content

Table 1: Total Phenolic, Flavonoid and Flavone in single BAP and KN treatment after nine weeks of cultures from apical tips

Treatment (mg/ml)	N	Total Phenolic Content(±SE)	Total Flavonoid Content(±SE)	Total Flavone Content(±SE)
Control	3	45.17 ± 4.08^{a}	86.58 ± 12.15^{c}	60.17 ± 8.21^{d}
BAP 1	3	$54.00\pm2.74^{a,b}$	$60.58\pm5.71^{a,b,c}$	$52.00\pm10.83^{c,d}$
BAP 2	3	$58.00\pm3.97^{a,b}$	$60.25\pm7.53^{a,b,c}$	$31.67\pm3.18^{a,b,c}$
BAP 3	3	66.67 ± 2.51^{b}	$48.00\pm2.01^{a,b}$	$25.50\pm3.82^{a,b}$
BAP 5	3	$52.75\pm1.70^{a,b}$	30.67 ± 5.23^{a}	14.67 ± 0.73^{a}
KN 1	3	$49.17\pm0.17^{a,b}$	$60.33\pm1.66^{a,b,c}$	$33.50\pm5.84^{a,b,c}$
KN 2	3	$50.92\pm5.56^{a,b}$	$69.17\pm16.49^{b,c}$	$43.67\pm9.28^{b,c,d}$
KN 3	3	$55.50\pm11.13^{a,b}$	87.58 ± 10.45^{c}	$37.67\pm5.26^{b,c}$
KN 5	3	64.17 ± 8.32^{b}	$75.17\pm4.37^{b,c}$	$43.33\pm7.99^{b,c,d}$

Note. Means followed by the same superscripts in same column do not differ significantly at P=0.05 by Duncan multiple range test

Table 2 (a,b,c): Analysis of Variance (ANOVA) in single BAP and KN after nine weeks of cultures

a)

ANOVA

Phenolic (mg/ml)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1147.949	8	143.494	1.576	.201
Within Groups	1638.958	18	91.053		
Total	2786.907	26			

b)

ANOVA

Flavonoid (mg/ml)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7869.769	8	983.721	3.214	.019
Within Groups	5509.542	18	306.086		
Total	13379.310	26			

c)

ANOVA

Flavone (mg/ml)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4527.241	8	565.905	4.027	.007
Within Groups	2529.500	18	140.528		
Total	7056.741	26			

Table 3: Total Phenolic, Flavonoid and Flavone in single BAP, TDZ and ZEA treatment after six weeks of cultures from rhizome

Treatment (mg/ml)	N	Total Phenolic Content(±SE)	Total Flavonoid Content(±SE)	Total Flavone Content(±SE)
Control	3	64.67±12.42^{c}	81.25±7.29^{d}	41.33±6.36^{c}
BAP 1	3	83.42±8.74^{d}	57.00±1.38^{c}	38.83±2.95^{c}
BAP 3	3	63.00±9.45^{c}	51.25±8.27^{c}	30.83±6.65^{c}
BAP 5	3	53.25±4.06^{b,c}	29.50±4.16	12.67±2.05^{a,b}
TDZ 1	3	51.17±0.17^{b,c}	24.58±3.10	8.33±1.36^{a,b}
TDZ 3	3	39.17±3.56^{a,b}	23.58±2.57^{a,b}	11.83±2.89^{a,b}
TDZ 5	3	52.75±3.74^{b,c}	16.50±3.02^{a}	6.00±0.58^{a}
ZEA 1	3	51.00±0.52^{b,c}	24.50±2.41^{a,b}	9.50±1.50^{a,b}
ZEA 3	3	50.92±3.17^{b,c}	36.33±3.48^{b}	19.33±1.76^{b}
ZEA 5	3	28.83±2.09^{a}	23.67±4.81^{a,b}	7.83±2.05^{a}

Note. Means followed by the same superscripts in same column do not differ significantly at P=0.05 by Duncan multiple range test

Table 4 (a,b,c): Analysis of Variance (ANOVA) in single BAP, TDZ and ZEA after six weeks of cultures

a)

ANOVA

Phenolic (mg/ml)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5825.492	9	647.277	5.708	.001
Within Groups	2268.000	20	113.400		
Total	8093.492	29			

b)

ANOVA

Flavonoid (mg/ml)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11117.825	9	1235.314	19.890	.000
Within Groups	1242.167	20	62.108		
Total	12359.992	29			

c)

ANOVA

Flavone (mg/ml)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4860.742	9	540.082	15.303	.000
Within Groups	705.833	20	35.292		
Total	5566.575	29			

Table 5: Total Phenolic, Flavonoid and Flavone in combination treatment between BAP and IAA treatment after six weeks of cultures from rhizome

Treatment (mg/ml)		N	Total Phenolic Content(±SE)	Total Flavonoid Content(±SE)	Total Flavone Content(±SE)
BAP	IAA				
0	0	3	64.67±12.42^{a,b,c,d}	81.25±7.29^{c,d,e}	41.33±6.36^{b,c}
0	1	3	47.50±5.48^{a}	66.67±12.12^{b,c,d,e}	52.33±10.43^{c,d}
0	3	3	49.58±4.75^{a,b}	87.50±10.13^{e}	66.83±3.66^{d}
0	5	3	58.08±6.58^{a,b,c}	84.50±18.04^{d,e}	49.33±11.94^{c,d}
1	0	3	83.42±8.74^{d}	57.00±1.38^{a,b,c,d}	38.83±2.95^{b,c}
1	1	3	64.25±3.36^{a,b,c,d}	53.75±7.07^{a,b,c}	47.50±11.45^{c,d}
1	3	3	72.50±9.06^{b,c,d}	61.67±11.46^{b,c,d,e}	67.50±9.37^{d}
1	5	3	56.92±10.22^{a,b,c}	71.08±10.91^{b,c,d,e}	38.50±2.93^{b,c}
3	0	3	63.00±9.45^{a,b,c,d}	52.92±9.31^{a,b,c}	30.83±6.65^{a,b,c}
3	3	3	52.25±0.90^{a,b,c}	44.75±8.71^{a,b}	24.33±3.42^{a,b}
3	5	3	53.92±7.61^{a,b,c}	48.75±5.50^{a,b}	23.83±3.98^{a,b}
5	0	3	53.25±4.06^{a,b,c}	29.50±4.16^{a}	12.67±2.05^{a}
5	1	3	74.67±2.57^{c,d}	41.92±2.81^{a,b}	24.17±4.34^{a,b}
5	3	3	52.83±7.07^{a,b,c}	28.17±3.81^{a}	14.00±1.26^{a}
5	5	3	66.67±0.82^{a,b,c,d}	50.08±7.78^{a,b}	22.33±2.49^{a,b}

Note. Means followed by the same superscripts in same column do not differ significantly at P=0.05 by Duncan multiple range test

Table 6 (a,b,c): Analysis of Variance (ANOVA) combination hormone between BAP and IAA after six weeks of cultures

a)

ANOVA

Phenolic (mg/ml)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4418.300	14	315.593	2.115	.042
Within Groups	4477.250	30	149.242		
Total	8895.550	44			

b)

ANOVA

Flavonoid (mg/ml)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14085.283	14	1006.092	4.114	.001
Within Groups	7337.417	30	244.581		
Total	21422.700	44			

c)

ANOVA

Flavone	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12644.578	14	903.184	7.015	.000
Within Groups	3862.333	30	128.744		
Total	16506.911	44			

APPENDIX C

Plant Observation

Figure 1: One week old seedling transferred to solid media

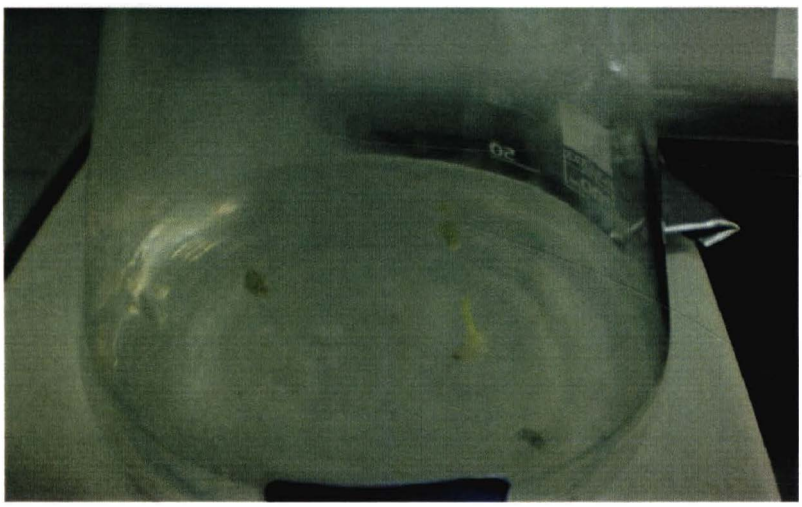

Figure 2: Two-days–old apical tips on MS solid media supplement 2.0 mg/l BAP

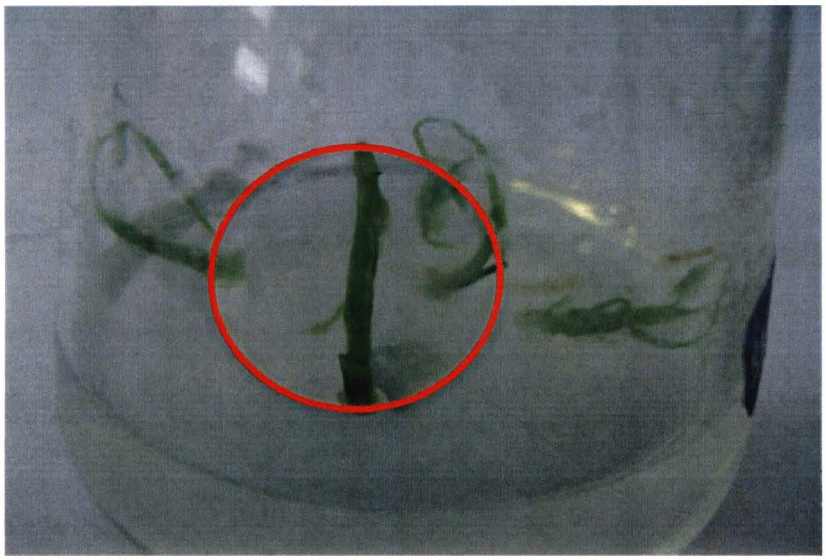

Figure 3: Shoot regeneration from apical tips cultured on MS solid media supplement 2.0 mg/L BA after one week.

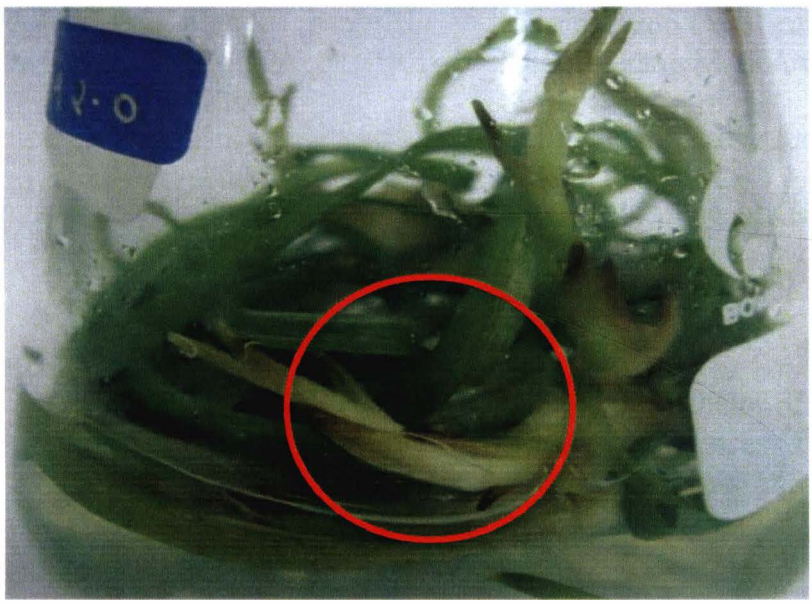

Figure 4: New plantlet regeneration on MS solid media supplement with 2.0 mg/L BA after nine weeks.

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