THE EFFICACY OF AMINO ACIDS ON REGENERATION AND ANALYSIS OF GENETIC FIDELITY OF REGENERATED
Chrysanthemum morifolium cv. ZEMBLA

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THE EFFICACY OF AMINO ACIDS ON REGENERATION AND

ANALYSIS OF GENETIC FIDELITY OF REGENERATED

Chrysanthemum morifolium **cv. ZEMBLA**

ABSTRACT

Tissue culture technique has been widely applied for the propagation of *Chrysanthemum morifolium*, a high value ornamental plant. However, somaclonal variation is the biggest challenge for tissue culture industry due to generation of "off-types" plants. This study aims to investigate the effects of supplements in order to improve callus and shoot induction media composition and evaluate the clonal fidelity in regenerated plantlets using retrotransposon-based markers. Leaves and nodal explant materials of *Chrysanthemum morifolium* "Zembla" were used for callus induction on Murashige and Skoog (MS) media containing 30 g L^{-1} sucrose, 2 g L^{-1} gelrite, 3 mg L^{-1} benzylaminopurine (BAP), 1 mg L^{-1} naphthaleneacetic acid (NAA) supplemented with amino acids (glutamine and arginine) and casein hydrolysate at concentrations of 250, 500 and 750 mg L^{-1}. Addition of amino acids and casein hydrolysate individually to the media showed no significant effect in callus induction efficiency. However, arginine (500 and 750 mg L^{-1}) and casein hydrolysate (500 mg L^{-1}) individually produced the highest number of shoots (4.6-4.8 shoots per explant) in calli induced from the nodal explants. Shoots with calli induced from leaf and nodal explants were transferred to elongation media. Elongation media supplemented with glutamine (750 mg L^{-1}) produced the highest number of shoots at 1 cm within 4 weeks (1.4 shoots per explants) when nodal explants were used. Thirty regenerants of *Chrysanthemum morifolium* "Zembla", (17 plants of white flower variety, and 13 plants of yellow flower variety,) were assessed for their genetic variation using Inter Retrotransposon Amplified Polymorphism (IRAP) marker. Out of nine primer combinations, only Sukkula primer was able to produce clear IRAP banding pattern in *Chrysanthemum morifolium*. Sukkula primer was able to differentiate

between the white and yellow variants of *Chrysanthemum morifolium* "Zembla". Furthermore, IRAP revealed polymorphic bands within the varieties. White and yellow flower varieties showed 20.0 % and 15.4% genetic variations, respectively. Further analysis is needed to confirm the linkage between these genetic variations and somaclonal variations.

Keywords: *Chrysanthemum morifolium,* amino acids, Inter Retrotransposon Amplified Polymorphism, genetic fidelity, somaclonal variation

KEBERKESANAN ASID AMINO TERHADAP RGENERASI DAN EVALUASI

KEDEKATAN KLON *Chrysanthemum morifolium* cv. ZEMBLA

ABSTRAK

Teknik kultur tisu telah digunakan secara meluas untuk pembiakan pokok *Chrysanthemum morifolium*, salah satu pokok hiasan yang mempunyai nilai yang tinggi. Walau bagaimanapun, variasi somaklon adalah cabaran terbesar untuk industri kultur tisu disebabkan oleh generasi pokok yang tidak normal. Dalam kajian ini, kami ingin mengenalpasti kesan bahan tambahan pokok untuk menambahbaikan komposisi media pembiakan chrysanthemum dan menilai kedekatan klon menggunakan penanda molekular. Eksplan seperti daun dan nod *Chrysanthemum morifolium* "Zembla" telah dikultur di atas media Murashige dan Skoog (MS) yang mengandungi 30 g L^{-1} sukrosa, 2 g L^{-1} gelrite, 3 mg L^{-1} benzylaminopurin (BAP), 1 mg L^{-1} asid naphthaleneacetic (NAA) dan beberapa jenis bahan tambahan pokok, iaitu, glutamin, arginin dan kasein hidrolisis dengan kandungan kepekatan yang berbeza-beza (250, 500 and 750 mg L^{-1}) untuk menghasilkan kalus. Penambahan bahan tambahan pokok secara individu kepada media tidak menunjukkan sebarang kesan signifikasi dalam efisiensi pertumbuhan kalus. Walaubagaimanpun, arginin (500 and 750 mg L^{-1}) dan kasein hidrolisis (500 mg L^{-1}) secara individu telah menghasilkan bilangan tunas yang paling banyak (4.6 - 4.8 tunas per eksplan) pada kalus yg dibiakkan dari eksplan nod. Tunas dan kalus yang dibiakkan dari eksplan daun dan nod telah dipindahkan ke media pemanjangan pokok. Dalam masa empat minggu, media pemanjangan pokok yang ditambah glutamin (750 mg L^{-1}) telah menghasilkan bilangan tunas berketinggian melebihi 1 cm yang paling banyak (1.4 tunas per eksplan) pada kalus yang dibiakkan menggunakan eksplan nod. Tiga puluh pokok *Chrysanthemum morifolium* "Zembla" (17 pokok varieti putih dan 13 pokok varieti kuning) telah dinilai variasi genetik dengan menggunakan penanda Inter Retrotransposon Amplified Polymorphism (IRAP). Dari Sembilan gabungan primer, hanya primer

Sukkula yang boleh menghasilkan jalur IRAP yang jelas. Primer ini jugak boleh mengenalpasti varieti putih dan kuning *Chrysanthemum morifolium* "Zembla". Tambahan pula, IRAP telah mendedahkan jalur polimorfik dalam kalangan varieti pokok. Varieti putih dan kuning masing-masing menunjukkan 20.0 % *dan* 15.4% variasi genetik. Analisis yang lebih lanjut diperlukan untuk memastikan hubungan variasi gentik dan variasi somaklon ini.

Kata kunci: *Chrysanthemum morifolium,* asid amino, Inter Retrotransposon Amplified Polymorphism, kedekatan klon, variasi somaklon

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	degree celcius
%	:	percent
2,4-D	:	2,4-Dichlorophenoxyacetic acid
AFLP	:	Amplified Fragment Length Polymorphism
BAP	:	Benzyl-amino purine
cm	:	centimeter
cv.	:	cultivar
DNA	:	deoxyribonucleic acid
$g\,L^{-1}$	:	gram per liter
IBA	:	Indole-butyric acid
IRAP	:	Inter Retrotransposon Amplified Polymorphism
ISSR	:	Inter Simple Sequence Repeat
LTR	:	long-terminal repeats
$MgCl_2$	:	Magnesium chloride
µM	:	micro molar
µl	:	micro-liter
mg	:	milligram
$mg\,L^{-1}$	:	milligram per liter
MS	:	Murashige and Skoog
NAA	:	Naphthaleneacetic acid
NN	:	Nitsch and Nitsch
PCR	:	Polymerase Chain Reaction
RAPD	:	Random Amplified Polymorphic DNA

RFLP	:	Restriction Fragment Length Polymorphism
rpm	:	revolutions per minute
RNA	:	ribonucleic acid
s	:	second
SRAP	:	Sequence-related Amplified Polymorphism
SSR	:	Simple Sequence Repeat
TBE	:	Tris-Borate-EDTA
w/v	:	weight per volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Chrysanthemum morifolium is one of the most important temperate flowers in the world and commercially produced as cut flowers and potted plants. In 2018, the Malaysian floriculture market was valued at USD 87.1 million. With 44.8%, chrysanthemum accounted for the major share among the temperate flower types, followed by rose (25.8%) and carnation (12.1%). Chrysanthemums are primarily planted in highland cultivation areas such as Cameron Highland, Gua Musang and Ranau. Article from Mordor Intelligence stated that the major countries importing cut flowers from Malaysia are Japan, Thailand, Singapore and Australia ("Malaysia Floriculture Market," n.d). It is very important to establish a very efficient method to produce chrysanthemums to meet the demand. Previously, they were planted commercially using root suckers and stem cuttings. Since chrysanthemum is vegetatively propagated, diseases are carried over from one generation to the next which can contribute to quality deterioration and limited supply of clonal planting materials. To overcome these limitations, an alternative propagation method using plant tissue culture technique is indispensable as it could produce a large number of uniform disease-free plantlets within a short period. In this study, Murasighe and Skoog (MS) media supplemented with different amino acids and casein hydrolysate were investigated to improve the regeneration capacity of *Chrysanthemum morifolium* "Zembla" and minimizing somaclonal variation. Plant regeneration via callus, type of tissue, explant source, media components and culture cycle duration are the major factors causing somaclonal variation or the production of "off-types" plants (Sarmah et al., 2017). Since the regeneration system via callus intermediary was used in this study, the regenerants were evaluated for their variation to assess clonal fidelity. PCR-based technique using inter retrotransposon amplified polymorphism (IRAP) marker was used for the assessment by detecting new retrotransposon insertions into the genome. IRAP can detect a high level of polymorphism

without the need of DNA ligations, digestion, or probe hybridization. Furthermore, the marker can be used for any crops (Teo et al., 2005).

The objective of the study was to evaluate the effect of arginine, glutamine and casein hydrolysate on callus induction, shoot regeneration, and shoot elongation and clonal assessment of the regenerants.

Specific objectives:

- To investigate the effects of different types and concentration of amino acids and casein hydrolysate on callus induction, shoot regeneration, and shoot elongation in *Chrysanthemum morifolium* cv. Zembla.
- To assess clonal fidelity in *C. morifolium* cv. Zembla using IRAP markers.

CHAPTER 2: LITERATURE REVIEW

2.1   General introduction

Chrysanthemum belongs to the Asteracea family and has been bred for more than 2000 years (Ohmiya et al., 2009). It is a short-day plant that can be either an annual or perennial flowering herb. The height ranging from one to three meters, chrysanthemum has alternate and toothed leaves. Having adventitious roots and woody solid stem, the flowers bloom in early winter with varieties of colors (Nalini, 2012). This genus has many hybrids and thousands of cultivars developed for horticultural purposes. Modern cultivated chrysanthemums are more attractive than their wild relatives with different forms of flower heads and colors. The flower heads are either daisy-like, pompons or buttons in various colors besides traditional yellow in color. The most important hybrid is Chrysanthemum × morifolium (syn. C. × grandiflorum), derived primarily from *C. indicum*, but also involving other species.

According to He et al. (2013) chrysanthemum is one of the most important ornamental plants in the world, commercially available as potted plants and cut flowers due to its long vase life (Imtiaz et al., 2019). In an article from Mordor Intelligence, in Malaysia, the floriculture market comprising of tropical and temperate flowers was valued at USD 87.1 million in 2018. Among the temperate flower types, chrysanthemum is the biggest contributor (44.8%), followed by rose (25.8%) and carnation (12.1%). The major countries importing cut flowers from Malaysia are Japan, Thailand, Singapore and Australia ("Malaysia Floriculture Market," n.d.). In Japan, it is the most popular cut flower where their production and sale accounts for more than 30% of the Japanese domestic flower market (Noda et al., 2013).

2.2 Medicinal benefits of chrysanthemum

Besides being commercialized as ornamental plants, food and beverage, it also has many useful medicinal benefits (Imtiaz et al., 2019). Traditionally, the roots can be used as a remedy for headache and flowers for blood purification, relieving hypertension, liver problem, infections on skin and expel wind. The dried petals are used as tea to help treat influenza, acne, sore throat and fever (Imtiaz et al., 2019). According to Yang et al., (2019), there are many reports regarding the biological activities of the chrysanthemum such as cardiovascular protectant, anti-oxidant, anti-human immune deficiency (HIV) viruses, anti-inflammatory, vasorelaxant, neuroprotectant, anti-cancer, hepatoprotectant, aldose reductase inhibitor and anti-mutagenesis.

Different types of phytochemical have been extracted from chrysanthemum. The accumulation of phytochemicals is specific in leaves and flowers as shown in Table 2.1 (Chae, 2016a).

Table 2.1: The chemical constituents and bioactive compounds from leaves and flowers of chrysanthemum.

Plant parts	Chemical constituents	Activities
Leaves	Octa-cosyl alcohol, ^{2}-sitosterol, lupeol, ±amyrin, daucosterol, ineupatorolide B, syringin, chlorogenic acid, petasiphenol, physcion, acacetin, eupatilin, quercetin, diosmetin, luteolin, apigenin, apigenin-7-O-^{2}-D-glucopyranoside, quercetin-3-O-^{2}-D-glucopyranoside, luteolin-7-O-^{2}-D-gluco pyranoside, apigenin-7-O-^{2}-D-neospheroside, and acacetin-7-O-^{2}-D-glucoside	Antibacterial effect against *Bacillus subtili*s and *Staphalycoccus epidermidis.*
Flowers	Anthocyanins, cyanidin 3-glucoside and cyaniding 3-(3"-malonoyl) glucoside and carotenoids:lutein, zeaxanthin, 2-cryptoxanthin, 13-cis-2-carotene, ±carotene, trans-2-carotene, and 9-cis-2-carotene	1.Antiallergic effects 2.Neuroprotective activity 3.Neuroprotective effects against hydrogen peroxide-induced neurotoxicity in human

2.3 Propagation of chrysanthemum

Chrysanthemum is propagated both by sexual and asexual methods. According to (Song et al., 2011), chrysanthemums are propagated asexually by stem cuttings, divisions, emergent and non-emergent rhizomes for commercial production. The use of propagation by stem cuttings has declined due to the risk of disease transmission. This method also produced low multiplication rate and low quality of plants (Sultana Nehid et al., 2007). On the other hand, conventional propagation by using suckers and terminal cuttings is not suitable for large scale production of chrysanthemums due to inefficient production volumes (Pant et al., 2015). Moreover, cuttings can only be established in early spring until early summer (DeNinno, 2014). To overcome the limitation, another method of propagating chrysanthemum is by using division. When planted, chrysanthemum will produce underground suckers which can be dug up, divided and replanted. However, by using this method, the production quality of the plants will be compromised if crowding was not well taken care of during the first cycle. Chrysanthemum can also be propagated by seeds, which will not produce clonal seedlings. This method is commonly used to produce new cultivars although seed viability is low and is a practise in the temperate climate.

2.4 Development of novel varieties

Crossbreeding and mutation breeding are the common methods used to develop novel varieties for example different colours in chrysanthemum (Noda et al., 2013; Su et al., 2019). Crossbreeding is a very simple and effective method to develop. Two parents with different target traits will produce F1 progeny that shows a wide phenotypic variation in chrysanthemum. Nonetheless, factors such as hybrid fertility of the cross combination, qualitative analysis of the target traits and selection of superior hybrid progeny are critical considerations in chrysanthemum crossbreeding. Moreover, non-desirable traits in the hybrid progeny are eliminated through backcrossing for several generations. This process

is costly, laborious and time consuming. An example of the cultivar that was been developed by crossbreeding is "Lavender daisy" (Su et al., 2019).

Mutation breeding which can occur spontaneously or induced by physical and chemical methods has been used to modify one or few traits in chrysanthemum. The cultivar Anna has been developed through natural mutations (Stewart & Dermen, 1970). Gamma, microwave, ion-beam and X-radiation have been used to produce novel traits such as new colors and shapes in chrysanthemums. Mutations erratically occur throughout the genome, sometimes producing phenotypic and genotypic changes. Several factors need to be taken into account, for instance, suitable genotype, type of explants used, method of induction mutation and the optimal irradiation dose (Su et al., 2019). The mutation rate can be increased by producing mutant varieties within a considerable period of time because of the high heterozygosity of chrysanthemums. In mutagenic treatment, physical treatment is uncommon while chemical mutagenic agents which are frequently used can cause harm to the environment and breeders. In addition, other disadvantages are large number of field trials, labour intensive, huge number of mutants to handle in order to select a desirable mutant (Su et al., 2019).

Alternatively, plant tissue culture methods are used to develop new varieties through stress and mutagenic factors such as culture condition and types of explants used. When extensively applied, these factors may affect the plant genome causing somaclonal variations and mutations (Miler & Zalewska, 2014). Somaclonal variation phenomena could result in stable or transient genetic change depending on the tissue culture stress effect on plant DNA.

2.5   Plant tissue culture

Plant tissue culture is a technique that can be used to propagate plants from different explants in a sterile controlled condition on a culture media. This technique can mass produce clonal and uniform plants within a reasonable period irrespective of seasons (Pant et al., 2015). However, variations may occur due to source of explants, regeneration pathways and culture procedures (Krishna et al., 2016). The composition of components in the culture media is optimised for different types of plants and explants as it is crucial to support the growth and regeneration of the explants in cultures. There are many basic media formulations published and sometimes specific for certain types of plants. For example, Gamborg's medium (B5) was used for *Dysosma pleiantha* (Hance) Woodson (Karuppaiya & Tsay, 2020) and Nitsch and Nitsch (NN) medium was used in *Cattleya trianae* (Br Sembiring, 2017).

The most widely used basic media is Murashige and Skoog (MS) which consists of mineral salts, amino acids, a carbon source (sucrose) and vitamins (Murashige and Skoog, 1962). Growth regulators, organic and inorganic supplements are reported to improve *in vitro* plant regeneration. Variation in the strength of the basic media was also shown to affect plant regeneration in cultures (Pawar et al., 2015).

Plant growth regulators or hormones are added to the basic formulation to promote either direct or indirect plant regeneration pathways. Organic supplements such as casein hydrolysate, vitamins, yeast extract, coconut milk, potato extract and malt extract are commonly used in tissue culture media formulations (George et al., 2008). According to Ageel & Elmeer (2011), casein hydrolysate contained a mixture of up to 18 amino acids, calcium, phosphate, several micro-elements and vitamins. Casein hydrolysate could replace glutamine when phosphorus is insufficient for biosynthesis. In addition, Daniel et al., (2018) has proven that the addition of glutamine and casein

hydrolysate could enhance the rate of somatic embryogenesis and plant regeneration in *Abelmoschus esculentus* (L.) Monech (okra). Inorganic supplements are also added to plant tissue culture media. Silicon was shown to improve organogenesis, embryogenesis, growth traits, morphological, anatomical, and physiological characteristics of leaves, enhanced tolerance to low temperature and salinity, protected cells and against metal toxicity, oxidative phenolic browning and reduced the incidence of hyperhydricity in various plants (Sivanesan & Park, 2014).

Many publications have reported the use of amino acids in plant tissue culture to improve the regeneration rate of the explants, promoted shoots growth, boost the quality and growth of callus, enhanced organogenesis, and assisted in rooting of the plantlets (El-Sharabasy et al., 2012; Pawar et al., 2015; Samarina et al., 2016). Glutamine, arginine, proline and tyrosine are some of the amino acids that have been reported to influence growth and regeneration of explants when exogenously added into the culture media. When directly absorbed by the roots, the plants do not need to metabolize the mineral nitrogen present in the media as nitrates and ammonium ions (El-Sharabasy et al., 2012). Amino acids will provide an additional source of organic nitrogen and is readily available for assimilation in *invitro* cultures (Samarina et al., 2016).

The role of amino acid as an inducer to tissue culture responses were seen in many plants. In rice, the regeneration rate was improved when proline and glutamine were supplied into the regeneration media (Pawar et al., 2015). Tyrosine was also proven to induce the regeneration of explants in strawberry during the multiplication stage (El-Sharabasy et al., 2012).

El-Sharabasy et al., (2012) also claimed that tyrosine, arginine and glutamine improved induction and development of maximum number of multiple shoots on strawberry. Arginine has been proven to increase the number of shoots of *in vitro* apple

rootstock (Sotiropoulos et al., 2005) and sugarcane (Asad et al., 2009). Shoot proliferation in *Citrus reticulata* (Siwach et al., 2012) was enhanced in culture media supplemented with glutamine.

Besides organogenesis, amino acids also improved callus growth as shown in rice and oil palm. In rice, proline and glutamine were shown to increase *in vitro* callus induction and subsequent regeneration (Pawar et al., 2015). In addition, glutamine was shown to increase storage protein accumulation during the maturation of oil palm somatic embryos and subsequently the germination of the embryos (Mariani et al., 2015).

In combination with plant growth regulators, amino acid improved rooting in many regenerated plants. For example, *Artemisia vulgaris* produced maximum number of roots per explant in medium containing a combination of plant growth regulator, silver nitrate and glutamine (Kumar & Kumari, 2010). Tyrosine has induced the rooting stage and acclimatization stage of strawberry (El-Sharabasy et al., 2012). Root number and root length of cherry rootstock was improved in media supplemented with a combination of indole-3-butyric acid and L-arginine (Sarropoulou et al., 2014).

2.6 Chrysanthemum tissue culture

The establishment of the chrysanthemum tissue culture was dependent on the source of explant, type and concentration of plant growth regulators and genotypes. Micropropagation of chrysanthemum was successfully reported using different types of explants. Even though Murashige and Skoog (MS) was commonly reported as the basic media for chrysanthemum tissue culture (Murashige & Skoog, 1962), the types and concentrations of plant growth regulators (PGR) were dependent on the source of explants (Table 2.2).

Plants were regenerated from these explants through either direct organogenesis or callus intermediary. For direct organogenesis, (Yesmin et al., 2014) have shown an efficient *in vitro* regeneration of chrysanthemum using nodal explants in MS media with benzyl-amino purine (BAP) and indole-butyric acid (IBA). On the other hand, Imtiaz et al., (2019) used shoot bud explants to establish a rapid *in vitro* propagation in media with benzyl adenine (BA). Song et al., (2011) reported that leaf, petal, petiole & internodal stem explants were able to produce plantlets where petal explants gave the highest number of plantlets. For indirect organogenesis, petal explants were commonly used where high frequency shoot regeneration protocol was established by Nahid et al., (2007) and Thangmanee & Kanchanapoom, (2011).

It was also reported that PGR used for plant regeneration was dependent on different chrysanthemum genotypes (Nehid et al., 2007).

However, for rooting of regenerants both full and half strength MS media was used. The common plant regulator used for rooting was auxin such as IBA, IAA and NAA (Chae, 2016b). In contrast to shoot induction both cytokinin were used either alone or in combination with auxin.

Table 2.2: Culture media and types of explants used to propagate *in vitro* chrysanthemum

Chrysanthemum morifolium varieties	Type of explants	Culture media	References
	Nodal	**Shoot regeneration:** MS media supplemented with 1.0 mg/l BAP and 0.1 mg/l IAA. **Rooting:** Half-strength MS supplemented with 0.2 mg/l IBA	(Yesmin et al., 2014)

Chrysanthemum morifolium varieties	Type of explants	Culture media	References
	Shoot bud	**Shooting media:** MS media supplemented with 44.39 µM 6-BAP **Rooting media:** Half MS media without PGR	(Imtiaz et al., 2019)
	Petals	**Callus induction media:** MS media supplemented with 2.0 mg/l BAP and 0.1 mg/l NAA. **Shooting media:** MS media supplemented with 2.0 mg/l BAP and 0.1 mg/l kinetin **(Genotype 89)** MS media supplemented with 1.0 mg/l kinetin and 0.1 mg/l NAA **(Genotype 4037)**	(Sultana Nehid et al., 2007)
Brighton	Petals	**Shooting media:** MS media supplemented with 6.66 µM BAP, 8.56 µM IAA, and 0.46 µM Kin **Rooting media:** MS media supplemented with 2.85–8.56 µM IAA	(Song et al., 2011)

Chrysanthemum morifolium varieties	Type of explants	Culture media	References
Chrysanthemum × grandiflorum (Ramat.) Kitam	Ray florets	**Callus induction:** MS medium supplemented with 13.3 µM BAP and 0.5 µM 2,4-D **Shooting media:** MS medium supplemented with 9.3 µM kinetin and 4.9 µM IBA **Rooting media:** MS medium supplemented with 12.3 µM IBA	(Thangmanee & Kanchanapoom, 2011)

2.7 Somaclonal variation

In vitro technology assures true-to-type clones, however tissue culture-induced variations are also common in plants and this phenomenon is known as somaclonal variation (Larkin & Scowcroft, 1981). Somaclonal variation includes protoclonal, gametoclonal and mericlonal tissue culture variations (Kaeppler et al., 2000). These variations are phenotypic and genetic changes which could be chimerical, temporary, or physiological and both heritable and non-heritable variation in nature. However, it is sometimes difficult to determine heritability in plants that are asexually propagated, sexually incompatible, seedless, polyploidy and long generation cycles (Skirvin et al., 1994).

It has been reported that the cause of variation, detection mode and mechanism are related to karyotypic alterations, sequence change, DNA methylation and the involvement of transposable elements with respect to cultural variation (Mujib et al., 2013).

The major factors contributing to this are related to sources of explants, culture media, cultural practices, cultural conditions, and plant regeneration pathways. Stress factors such as wounding, exposure to sterilizing agents, non-optimized media components, lighting conditions, imbalance relationship between high humidity and transpiration could trigger variation (Krishna et al., 2016).

Somaclonal variation is a flaw in any micropropagation program that is required to produce true-to-type plant materials. In contrast, it is an alternative way to the breeders in order to obtain genetic variability within a considerable short period and without using complex technology for crops that are either difficult to breed or have a narrow genetic base. Several strategies have been established to determine the genetic fidelity of the in vitro cultured plants such as morpho-physiological, biochemical, cytological and DNA-based molecular markers approaches (Krishna et al., 2016).

2.8   Somaclonal variation in chrysanthemum

Variations in chrysanthemum are obtained either naturally as sports or induced using chemicals and physical methods (Su et al., 2019). These methods are associated with challenges such as environment toxicity, accessibility to the technologies, expensive equipment, and low variants. Somaclonal variation induced during plant tissue culture is an alternative approach although the effectiveness of forming new variants is still low (Krishna et al., 2016).

The emergence of the somaclonal variation in chrysanthemum is enhanced by the usage of non-meristematic explants and adding mutagenic plant growth regulators (PGRs) to the medium as well as the induction of callus intermediary regeneration (Karp, 1995). Work has been published to induce somaclonal variation in chrysanthemum using various explants.

Petal induced somaclones have been successfully established ; Kengkarj et al., 2008). In chrysanthemum, somaclonal variation may be presented in regenerants that were derived from ray (ligulate) or disc (tubular) florets, which resulted in the production of plants with modified morphology of the plants such as the variations in colours (Teixeira da Silva et al., 2015). Vilasini & Latipah (2001) showed variation in vegetative growth and flowering in five *Chrysanthemum morifolium* varieties. One of the novel chrysanthemums*, Dendranthema grandiflora* cv. Kitam which was a somaclone was obtained from ray explants (Kengkarj et al., 2008).

To overcome the seasonal availability of chrysanthemum flowers, leaf and internode explants (Miler & Zalewska, 2014) as well as ovaries (Miler & Jedrzejczyk, 2018) were successfully used to induce somaclonal variants. New chrysanthemum variants with changes in their inflorescence colors and presence of carotenoids in the ligulate florets of the somaclones were obtained. From ovary cultures of chrysanthemum, multi-colored, marble-like and lighter green colors of the leaves, modified morphology of inflorescences and ligulate florets and also the different shape of corymb was observed from the regenerants.

2.9 Method for the detection of genetic fidelity in tissue cultured plants

According to Butiuc-Keul et al. (2016), there are several molecular techniques that can be used to evaluate the genetic stability and the somaclonal variation in plant tissue culture; Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Inter Simple Sequence Repeat (ISR) and Sequence-related Amplified Polymorphism (SRAP). Molecular markers such as RAPD and ISSR are simple, not costly and do not require prior knowledge of the DNA sequence for primer design. Adding to that, SSR can be considered as cheap, but it does need prior knowledge

on the sequence of interest for primer development. Because of the cost effectiveness, and the amount of DNA required are low, RAPD, SSR and ISSR are frequently used in assessment of *in vitro* plants fidelity (Butiuc-Keul et al., 2016).

2.10 Inter Retrotransposon Amplified Polymorphism (IRAP) Marker

The separation of repetitive sequences consists of a large fraction in many eukaryotic organisms' genomes which mainly consist of transposable elements. These elements are split into two major classes, class 1 retrotransposon and class 2 transposons. Retrotransposons are further classified into two major classes which differ in their transposition cycle and structure, that are, long terminal repeat (LTR) retrotransposons and the non-LTR retrotransposons (Kalendar et al., 2011). According to Teo et al., (2005), retrotransposons which are flanked by long-terminal repeats (LTR retrotransposons) are abundant in plants. This mobile element can be propagated within the genome through RNA intermediates in the cycle of transcription, reverse transcription and integration. Unlike the DNA transposons, once an active copy of retrotransposon is inserted into a new genome location, it will be stably integrated and maintained in the host organism. Genomic diversification will be caused by the accumulation, fixation and incomplete excision of retrotransposons over time. The wide distribution, high copy number and widespread chromosomal dispersion of LTR retrotransposons provided an excellent potential to develop a DNA-based marker system. Various PCR-based techniques can detect retrotransposons insertional polymorphisms. One of the techniques available is the Inter Retrotransposon Amplified Polymorphism (IRAP) marker, which can detect high levels of polymorphisms without the need of DNA digestion. This PCR-based assay is amenable to screen breeding populations and also can be applied for any crop. In the previous research, Teo et al., (2005) applied this technique to characterize the banana genome constitution and classifying various cultivars of banana.

According to Li et al., (2011), retrotransposon insertional polymorphisms can be detected by amplifying the portion of DNA between two retroelements, using one or two primers that point outward from an LTR. This method is simpler than the other because it can be carried out only by using one primer to screen the whole genome. More than that, it is economic and convenient. The method also does not need the sequencing apparatus in order to detect the product. It detects the products by using conventional agarose gel electrophoresis.

CHAPTER 3: METHODOLOGY

3.1   Plant material

White and yellow *Chrysanthemum morifolium* "Zembla" supplied by Kimagri Corporation Sdn Bhd, Kampar, Perak, were grown in Cameron Highlands, Pahang through stem cuttings.

3.2   Surface sterilization

The young leaves of the plants were surface sterilized with 70% ethanol for 30 seconds, 30% (v/v) Clorox (containing 5.25% w/v sodium hypochlorite) solution supplemented with 1-2 drops of Tween 20 for 15 minutes before being excised into smaller parts (0.4 cm × 0.4 cm).

3.3   Callus induction

The cleaned explants were cultured on Murashige and Skoog (MS) media containing 30 g L^{-1} sucrose (Systerm, Malaysia) and 2 g L^{-1} gelrite (Duchefa Biochemie, Netherlands) supplemented with 3 mg L^{-1} benzylaminopurine (BAP) (Duchefa Biochemie, Netherlands) and 1 mg L^{-1} naphthaleneacetic acid (NAA) (Duchefa Biochemie, Netherlands) for callus induction. Media pH was adjusted to 5.7 before autoclaved at 14.5 psi, 120°C for 20 minutes. The cultures were maintained at Plant Biotechnology Research Laboratory (PBRL), University of Malaya, at 26°C under 16-hour photoperiod.

3.4   Shoot induction and proliferation

The generated shoots were cultured on MS media containing 30 g L^{-1} sucrose (Systerm, Malaysia), 2 g L^{-1} gelrite (Duchefa Biochemie, Netherlands), 3 mg L^{-1} BAP (Duchefa Biochemie, Netherlands) and 0.1 mg L^{-1} NAA (Duchefa Biochemie, Netherlands) for shoot elongation. The shoots were then transferred to MS media

containing 30 g L^{-1} sucrose (Systerm, Malaysia) and 2 g L^{-1} gelrite (Duchefa Biochemie, Netherlands) for rooting. The cultures were maintained at Plant Biotechnology Research Laboratory (PBRL), University of Malaya, at 26°C under 16-hour photoperiod and were sub-cultured every 2-3 weeks.

3.5   Effects on different amino acids and casein hydrolysate on callus induction

The leaves (0.4 cm × 0.4 cm) and nodes (0.4 cm long) of 6-month-old *in vitro* chrysanthemum cultures were excised and transferred to MS media containing 30 g L^{-1} sucrose (Systerm, Malaysia), 2 g L^{-1} gelrite (Duchefa Biochemie, Netherlands), 3 mg L^{-1} BAP (Duchefa Biochemie, Netherlands), 1 mg L^{-1} NAA (Duchefa Biochemie, Netherlands) and different concentrations of glutamine, arginine and casein hydrolysate (250, 500 and 750 mg L^{-1}). The control explants were cultured on the similar MS media without any amino acids and casein hydrolysate. The pH of the media was adjusted to 5.7 before autoclaved at 14.5 psi, 120°C for 20 minutes. The cultures were maintained at PBRL at 26°C under 16-hour photoperiod. A total of 200 explants were cultured with 5 explants per treatment and the experiment was repeated twice. The morphology of the generated callus were recorded after five weeks of culture.

3.6   Effects on different amino acids and casein hydrolysate on shoot elongation

Total number of shoots was recorded after 8 weeks of culture. After 10 weeks of culture, the calli with shoots were transferred to MS media containing 30 g L^{-1} sucrose (Systerm, Malaysia), 2 g L^{-1} gelrite (Duchefa Biochemie, Netherlands), 3 mg L^{-1} BAP (Duchefa Biochemie, Netherlands), 0.1 mg L^{-1} NAA (Duchefa Biochemie, Netherlands) and different concentrations of glutamine, arginine and casein hydrolysate (250, 500 and 750 mg L^{-1}) for shoot elongation. After four weeks on the elongation media, the single shoots regenerated with more than 1 cm were counted and recorded.

3.7 DNA extraction

Sixty of *in vitro Chrysanthemum morifolium* "Zembla" were harvested. The total genomic DNA of *Chrysanthemum morifolium* was extracted from young leaves and stems of the *in vitro* plants. The extraction was done by using Plant DNA Rapid Extraction Kit (Bioteke, China). About 100 mg of plant tissue was ground into powder in liquid nitrogen by using sterilized pestle and mortar. A total of 550 ml pre-warmed Buffer P1 (65 °C) together with 4 µl RNaseA were added into the ground powder in 1.5 mL centrifuge tube. The mixture was vortexed for 1 minute and incubated at room temperature for 10 minutes. Buffer P2 (130 µL) was added and mixed thoroughly. The mixture was then centrifuged at 12,000 rpm for 3 minutes. The supernatant was carefully transferred to a separation column, centrifuged at 12,000 rpm for 1 minute and the flow through was collected. About 1.5 volumes of Buffer P3 was added to the flow through and mixed thoroughly. The mixture including the precipitate was transferred into a spin-column AC that was placed to a collection tube and was centrifuged for 1 minute at 12,000 rpm. The flow through in the collection column was discarded. After that, 700 µL of buffer WB was added and the spin-column was centrifuged for another 1 minute at 13,000 rpm. The previous step was repeated by adding 500 µL buffer WB. After the flow through was discarded, the empty spin-column AC was then centrifuged at 13,000 rpm for 3-5 minutes. The spin-column AC was transferred to a clean 1.5 mL microcentrifuge tube and 50 µL Buffer EB was added for elution.

3.8 Inter Retrotransposon Amplified Polymorphism Polymerase chain reaction

In total, three Inter Retrotransposon Amplified Polymorphism (IRAP) primers were used in this experiment; Sukkula, Reverse TY1, and Reverse TY2 (Table 3.1).

Table 3.1: Three primers used for the experiment

Primer	Sequence	Melting temperature (T_m)
Sukkula	5'-GATAGGGTCGCATCTTGGGCGTGAC-3'	66°C
Reverse TY1	5'-CCYTGNAYYAANGCNGT-3'	42°C
Reverse TY2	5'-TRGTARAGRAGNTGRAT-3'	36°C

The IRAP PCR was performed in a 25 µL reaction mixture containing 50 ng DNA, 1X PCR buffer (Promega, USA), 2 mM $MgCl_2$, 5 pmol of IRAP primers, 200 µM dNTP mix and 1 U *Taq* polymerase (Promega, USA). Amplification was performed using a thermal cycler (T100 Thermal Cycler, Biorad). The PCR reaction parameters consisted of: 95°C, 2 minutes; 30 cycles of 95°C, 60 s, 40°C for 60 s, for 2 min plus 3 s per cycle and a final extension at 72°C for 10 minutes.

The PCR products were analyzed by electrophoresis on 1.5 % (w/v) agarose gel (Promega, USA) and imaged by Bio-Rad Chemidoc MP system (Bio-Rad, USA).

3.9 Analysis of PCR products via gel electrophoresis

1.5 % (w/v) agarose gel was prepared by mixing 0.9 g of agarose gel (Promega, USA) with 60 mL of 1 x Tris-Borate-EDTA (TBE) buffer (First-base, Singapore). The mixture was heated up in a microwave until the agarose dissolved for about two minutes. It was then cooled down until the running tap water and mixed with 0.5 µL of gel stain (TransGen, China). The agarose gel was casted in a tray with the well comb. After the agarose gel solidified at room temperature for about 20 minutes, it was then placed into an electrophoresis tank filled with 1 x TBE buffer (First-base, Singapore).

Genomic DNA and PCR products, 5 µL and 10 µL respectively was mixed with 1 µL of 6 x Loading Buffer (Thermo Fisher Scientific, USA) before loaded into the well of agarose gel. 5 µL of Bench top 1 kb DNA ladder (Promega, USA) was loaded as molecular weight ladder. The samples were run on electrophoresis at 100V for about 35 minutes. The gel was then imaged by Bio-Rad Chemidoc MP system (Bio-Rad, USA).

3.10   Statistical analysis

All experiments were conducted in a completely random design. Analysis of variance (ANOVA) and Duncan's multiple comparison test were conducted using IBM SPSS (Statistical Packages for Science Social) Version 25 software at 0.05% significance level.

CHAPTER 4: RESULTS

The media formulation for chrysanthemum callus induction and shoot elongation was established by (Md Aiani, 2016). In this work, casein hydrolysate and selected amino acids were added separately to evaluate the effects of these supplements at various concentrations on the regeneration of *in vitro Chrysanthemum morifolium*. Callus development and shoot regeneration from leaf and nodal explants were observed and recorded to determine regeneration efficiency. For both nodal and leaf explants cultured on these media, plants were regenerated from indirect pathways which may induce somaclonal variation. Inter Retrotransposon Amplified Polymorphism (IRAP) method was used to verify the clonal fidelity of the regenerated plants from both leaf and nodal explants.

4.1   Establishment of axenic culture

Young leaves of *in vivo* plants that were propagated through stem cuttings were used to establish the axenic culture. The young leaves were surface sterilized and cultured on callus induction media. After 10 weeks of culture, the callus with micro shoots were transferred on shoot elongation media. The shoots were then cultured on MS media for rooting. The cultures were maintained and sub-cultured every 2-3 weeks. The stages of establishment of axenic culture are shown in Figure 4.1.
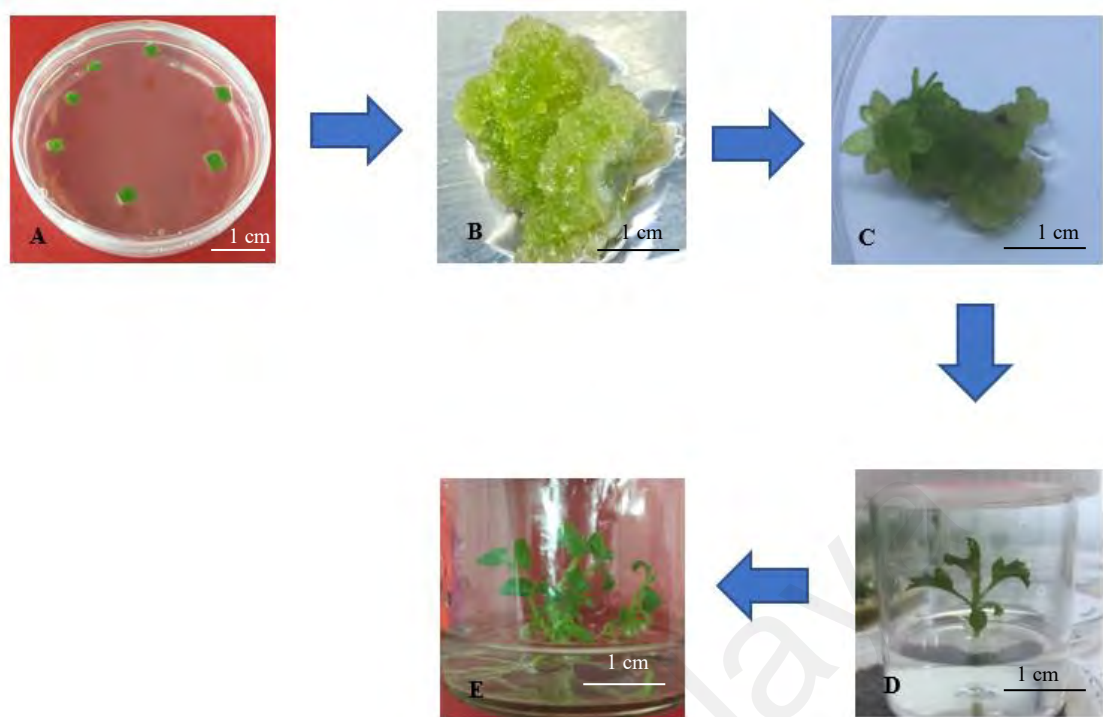
Figure 4.1: Establishment of chrysanthemum axenic cultures stages.

4.2   Effects of supplements on callus induction

Plant regeneration from leaf and nodal explants was successfully established via callus intermediary. Hence, it is crucial to optimize the media for callus induction to enhance shoot regeneration. In this experiment, casein hydrolysate, arginine and glutamine were added separately to the culture media to determine their effects on callus induction.

From the experiments carried out, it was shown that all (100%) leaf explants produced callus when cultured on MS medium supplemented with amino acids and casein hydrolysate (Table 4.1) including controls after 5 weeks in culture. However, it was observed that the initial callus induction period differed between each treatment. Callus was induced from leaf explants cultured on MS medium supplemented with glutamine after 2 weeks of culture followed by treatment with casein hydrolysate supplemented media and control at 3 weeks. The leaf explant response was slowest in MS medium

supplemented with arginine where callus was initiated only after almost 4 weeks in culture.

Table 4.1: The percentage of callus induced from leaf explants after 5 weeks of culture

Supplements	Concentration (mg L^{-1})	Percentage of callus (%)	Callus induction period (weeks of culture)
Control	0	100	3 weeks
Casein hydrolysate	250	100	3 weeks
	500	100	
	750	100	
Arginine	250	100	4 weeks
	500	100	
	750	100	
Glutamine	250	100	2 weeks
	500	100	
	750	100	

Five samples were used for each experiment and the experiment was done twice.

Similar observation was made for callus initiation from nodal explants (Figure 4.2). After 5 weeks of culture, 100% nodal explants produced callus when cultured on MS medium supplemented with amino acids and casein hydrolysate (Table 4.2) including controls. Similarly, it was observed that callus was initiated at different culture periods. On MS medium supplemented with glutamine, callus was initiated after 2 weeks of culture followed by casein supplemented media and control at 3 weeks. Nodal explants on arginine augmented MS medium started to produce callus after 3 and a half weeks in culture.

Table 4.2: The percentage of callus induced from nodal explants after 5 weeks of culture.

Supplements	Concentration (mg L^{-1})	Percentage of callus (%)	Callus induction period (weeks of culture)
Control	0	100	3 weeks
Casein hydrolysate	250	100	3 weeks
	500	100	
	750	100	
Arginine	250	100	3 and a half weeks
	500	100	
	750	100	
Glutamine	250	100	2 weeks
	500	100	
	750	100	

Five samples were used for each experiment and the experiment was done twice.
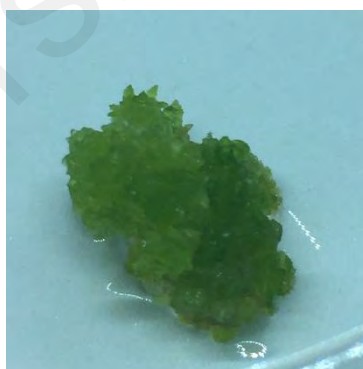

Figure 4.2: Callus of chrysanthemum induced on callus induction media.

4.3 Effects of supplements on shoot induction from leaf derived callus

In comparison to control, supplements were shown to enhance the number of shoots induced from callus derived leaf explants at certain concentration tested (Figure 4.3). The total number of shoots were recorded after 8 weeks of culture.

Shoot induction in media with casein hydrolysate at 250 and 750 mg L^{-1} were not significantly ($p<0.05$) different and did not improve shoot induction compared to the controls. Casein hydrolysate at 500 mg L^{-1} produced the highest number of shoots per explant among the three concentrations tested (Figure 4.3).

As for arginine, the number of shoots per explant were not significantly ($p<0.05$) different at 500 and 750 mg L^{-1} but improved significantly ($p<0.05$) at 250 mg L^{-1}. In comparison to controls and other supplements tested at all concentrations, arginine at 250 mg L^{-1} is the best enhancer for shoot induction (Figure 4.3).

Glutamine augmented media at 750 mg L^{-1} exhibited the highest number of shoots per explant compared to controls. This result is comparable to media supplemented with arginine at 250 mg L^{-1}) and casein hydrolysate (500 mg L^{-1}) where more than 2.6 shoots per explant were recorded.
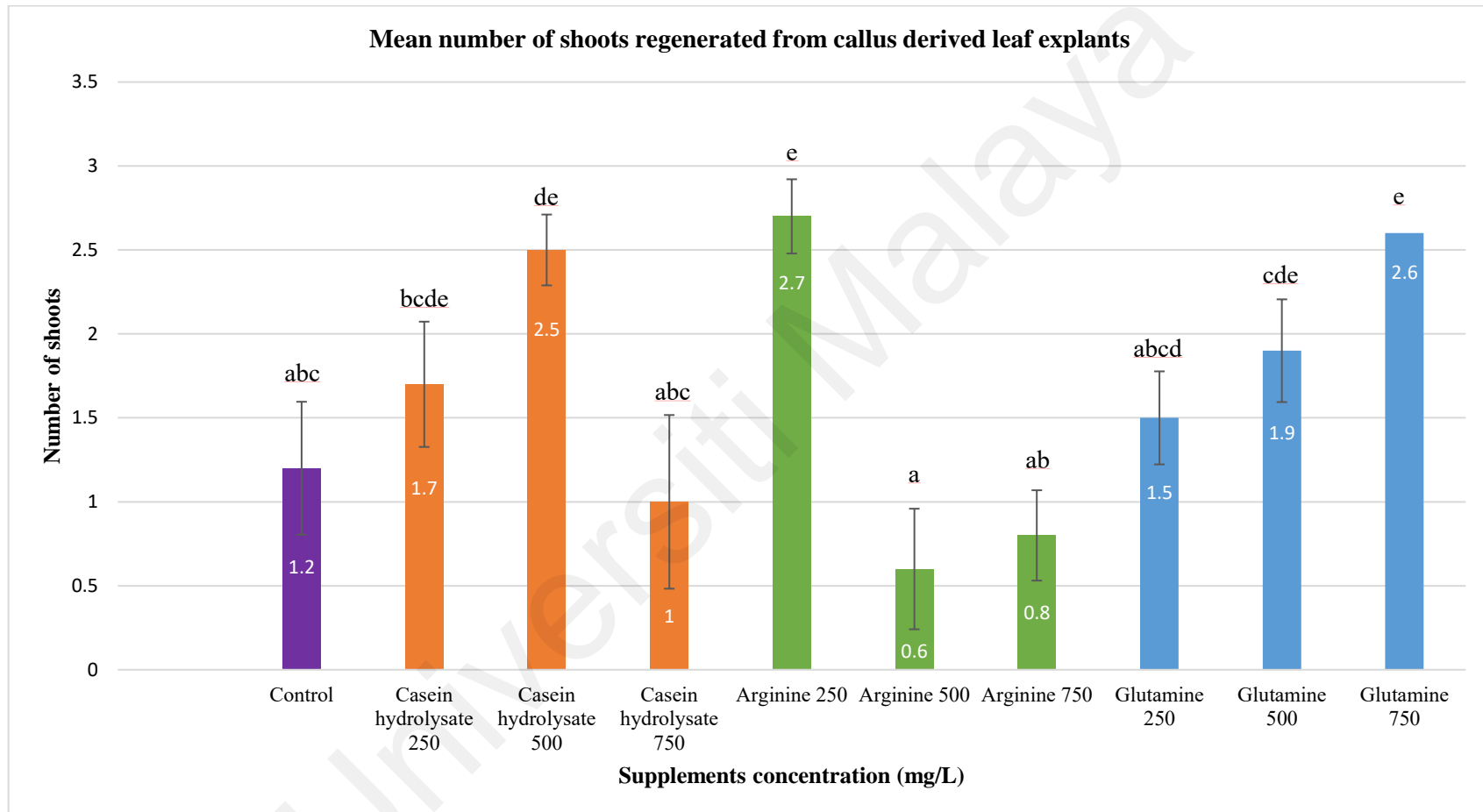
Figure 4.3: The number of shoots regenerated from leaf explants after 8 weeks of culture. Five samples were used for each experiment and the experiment was done twice.

4.4 Effects of supplements on shoot induction from callus derived nodal explant

Casein hydrolysate and arginine were shown to enhance the number of shoots induced from callus derived nodal explants at a certain concentration tested in comparison to control (Figure 4.4). Glutamine did not increase the number of shoots compared to control for all concentrations tested. Observation on the total number of shoots regenerated from callus was recorded after 8 weeks of culture.

Shoot induction in media with casein hydrolysate at 250 and 500 mg L^{-1} were not significantly ($p<0.05$) different between each other. However, casein hydrolysate at 500 mg L^{-1} produced the highest number of shoots per explant compared to controls. At 750 mg L^-1 casein were comparable to controls. (Figure 4.4).

Arginine had a positive effect as an enhancer for shoot induction where the highest number of shoots (4.6 and 4.7 shoots) was recorded in media supplemented with 500 and 750 mg L^{-1} arginine compared to controls. Arginine at 250 mg L^{-1} did not influence the number of shoots regenerated compared to controls. As for glutamine augmented media at all concentrations did not significantly ($p<0.05$) enhance shoot induction when compared to controls.

Arginine (500 mg L^{-1} and 750 mg L^{-1}) and casein hydrolysate (500 mg L^{-1}) significantly ($p<0.05$) produced the highest number of shoots (more than 4.6) per explant compared to control (Figure 4.4).
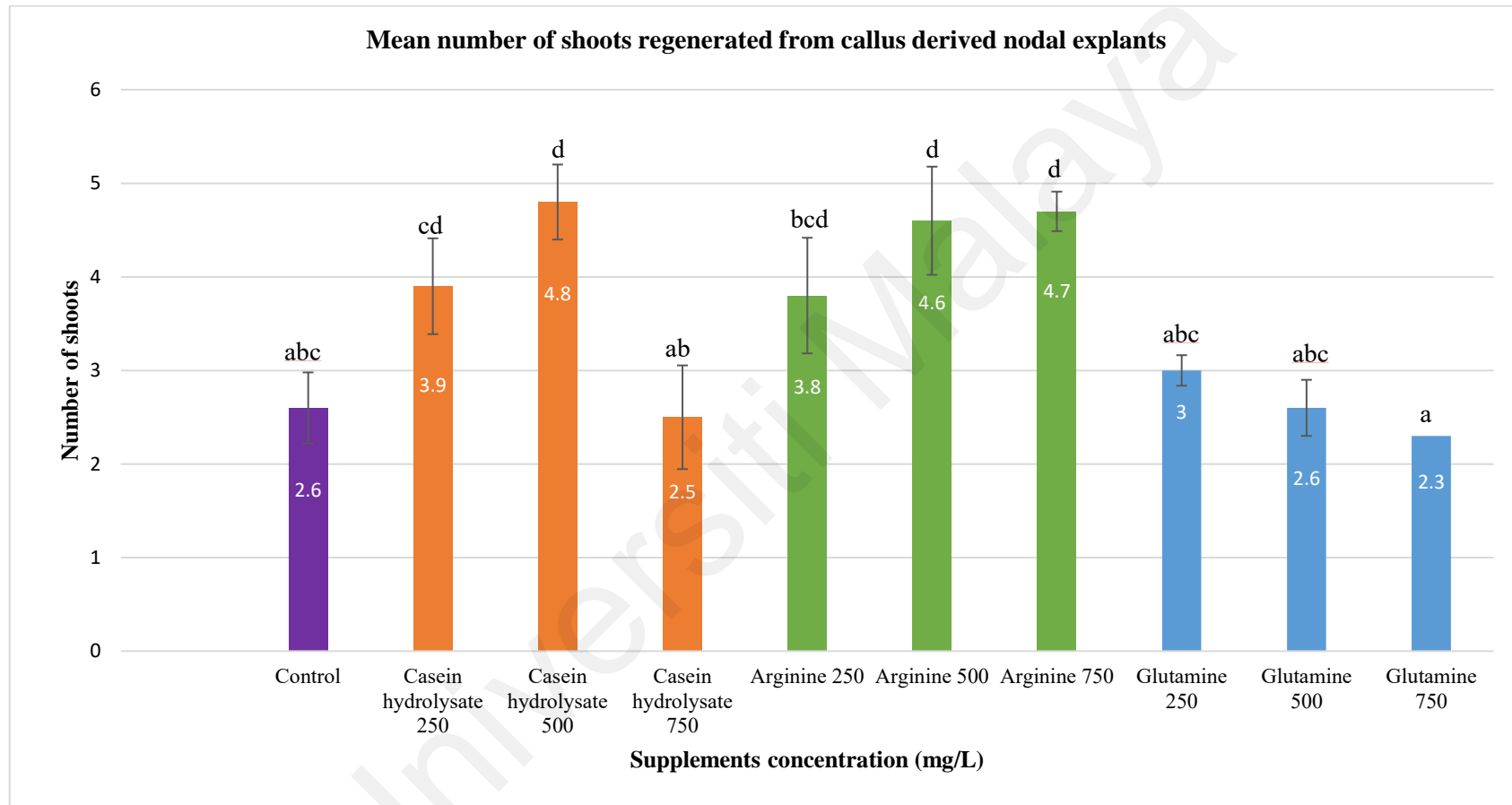
Figure 4.4: The number of shoots regenerated from callus derived nodal explants after 8 weeks of culture. Five samples were used for each experiment and the experiment was done twice.

4.5   Effects on different supplements on shoot elongation from callus derived leaf explants

Casein hydrolysate was shown to enhance the number of shoots elongated more than 1 cm from callus derived leaf explants at a certain concentration tested in comparison to control (Figure 4.5). Observation on the total number of shoots elongated more than 1 cm from was recorded after 4 weeks of culture in shoot elongation media.

Shoot elongation in media with casein hydrolysate at 250, 500 and 750 mg L^{-1} were not significantly ($p<0.05$) different between treatments. However, casein hydrolysate at 250 and 500 mg L^{-1} produced the highest number of shoots elongated per explant and significantly ($p<0.05$) different compared to controls. At 750 mg L^{-1} casein hydrolysate were not significantly ($p<0.05$) different compared to controls. Arginine and glutamine at 250, 500 and 750 mg L^{-1} did not significantly ($p<0.05$) increase the number of elongated shoots compared to controls. (Figure 4.5).

Among the promoting supplements assessed, casein hydrolysate at 250 mg L^{-1} and 500 mg L^{-1} significantly ($p<0.05$) produced the highest number of shoots elongated more than 1 cm per explant within 4 weeks observation when compared to control (Figure 4.5).
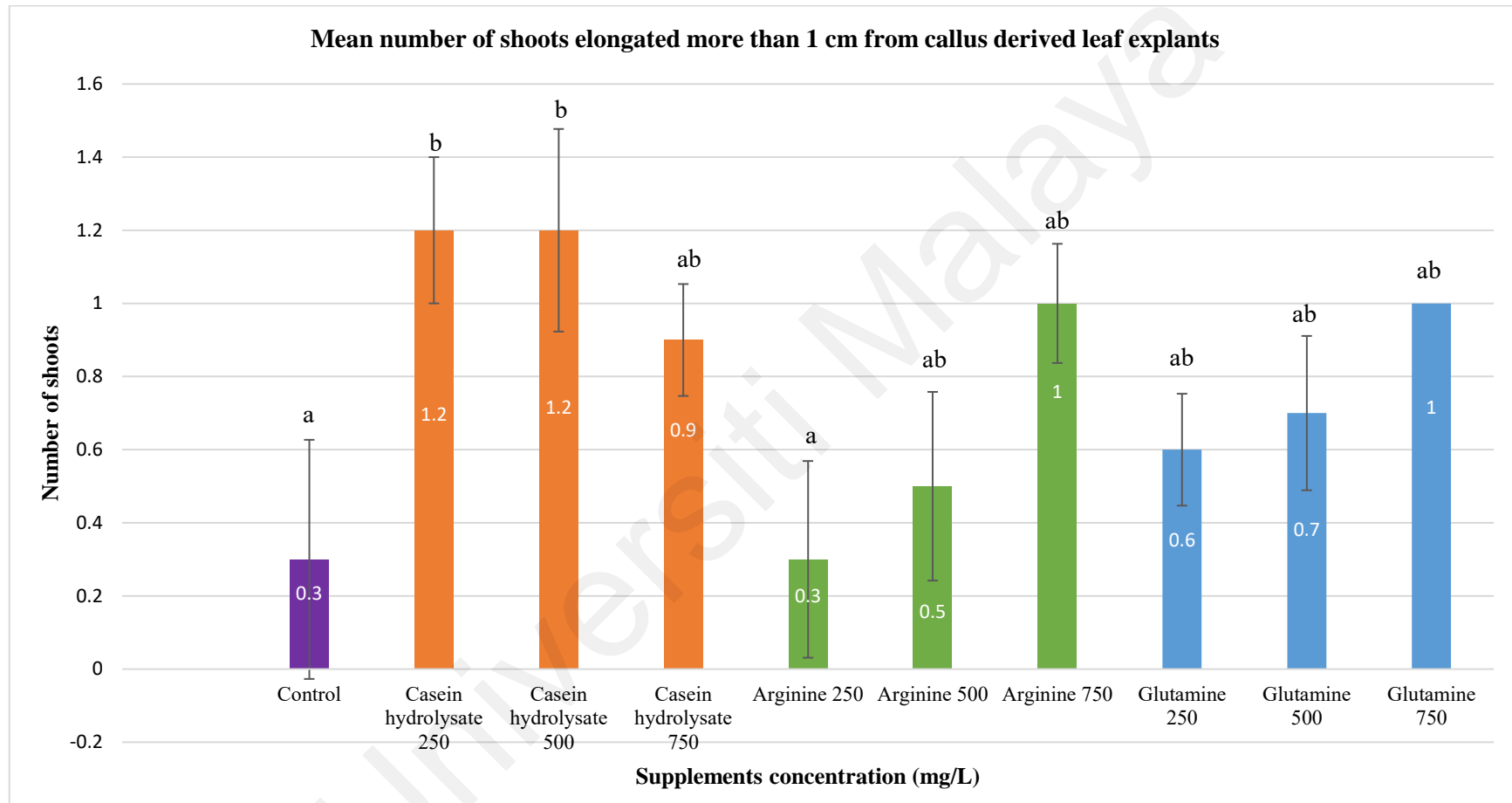
Figure 4.5: The number of shoots elongated more than 1 cm from callus derived leaf explants after 4 weeks of culture. Five samples were used for each experiment and the experiment was done twice.

4.6 Effects on different supplements on shoot elongation from callus derived nodal explants

As for shoot elongation from callus derived nodal explants, casein hydrolysate and glutamine at a certain concentration tested were shown to enhance the shoot elongation in comparison to control (Figure 4.6). Observation on the total number of shoots elongated more than 1 cm was recorded after 4 weeks of culture on elongation media.

Shoot elongation in media with casein hydrolysate at 250 and 500 mg L^{-1} were not significantly ($p<0.05$) different between these treatments. In comparison to control, casein hydrolysate at 500 mg L^{-1} produced a higher number of elongated shoots, while casein hydrolysate at 750 mg L^{-1} showed a contradictory effect. Elongation media supplemented with 250, 500 and 750 mg L^{-1} arginine produced the same number of elongated shoots as in controls. Glutamine at 250 and 500 mg L^{-1} showed no significant difference when compared to controls, but elongation media supplemented with glutamine at 750 mg L^{-1} significantly produced a higher number of shoots elongated more than 1 cm compared to controls (Figure 4.6).

The best promoting supplements were casein hydrolysate at 500 mg L^{-1} and glutamine at 750 mg L^{-1} which significantly ($p<0.05$) produced the highest number of shoots that elongated more than 1 cm per explants after 4 weeks in culture.
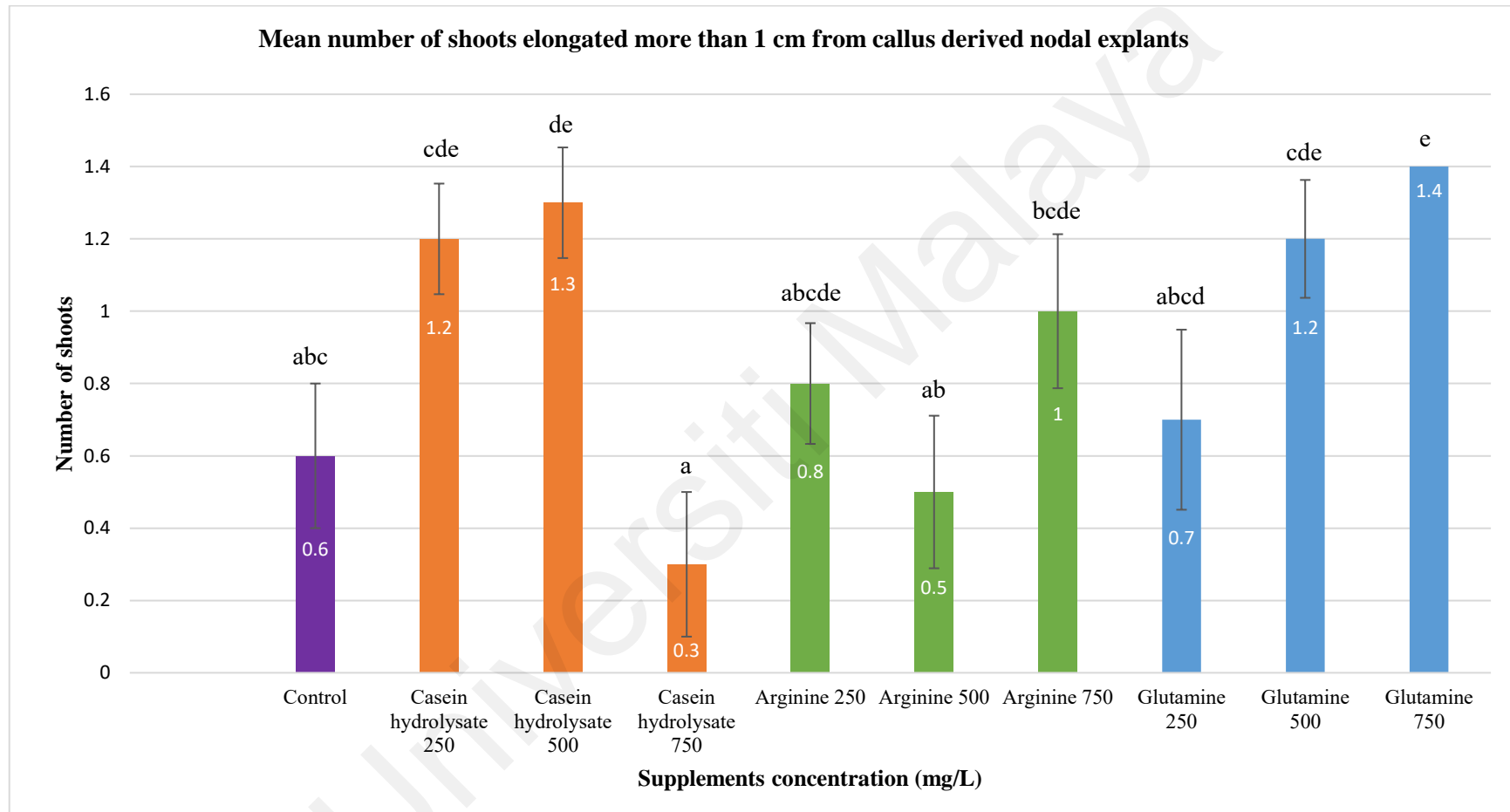
Figure 4.6: The number of shoots elongated more than 1 cm from callus derived nodal explant after 4 weeks of culture. Five samples were used for each experiment and the experiment was done twice.
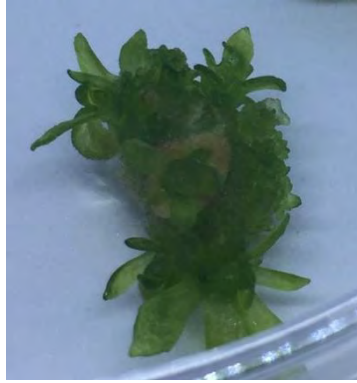
Figure 4.7: Micro shoots regenerated from callus after 10 weeks of culture.
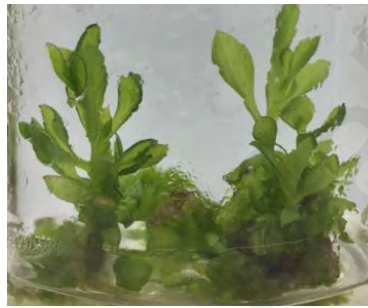
Figure 4.8: Shoots elongated more than 1 cm on shoot elongation media

4.7   DNA extraction

Total genomic DNA was extracted from sixty chrysanthemum regenerated plants by using Plant DNA Rapid Extraction Kit (Bioteke, China). The integrity of the extracted DNAs were then analyzed using gel electrophoresis and visualized by Bio-Rad Chemidoc MP system (Bio-Rad, USA) (Figure 4.9). A distinct band of more than 10kb in size was detected on gel for all the genomic DNA extracted (Figure 4.9). The band shows that the genomic DNA was successfully extracted from the chrysanthemum regenerated plants.

Figure 4.9 (a)

Figure 4.9 (b)
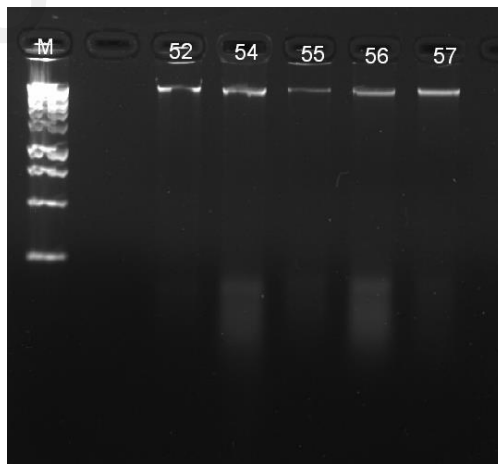
Figure 4.9 (c)

Figure 4.9: Extracted DNA of the chrysanthemum explants analyzed using gel electrophoresis. From left to right: M, 1kb DNA ladder, 41-45, 48-51 and 52- 57, the extracted DNA.

There are few wells of the gel electrophoresis showed some smearing towards the end of the gel. This might be due to DNA degradation. From Figure 4.9 (c), genomic DNA numbered with 52 and 54 showed an uneven band.
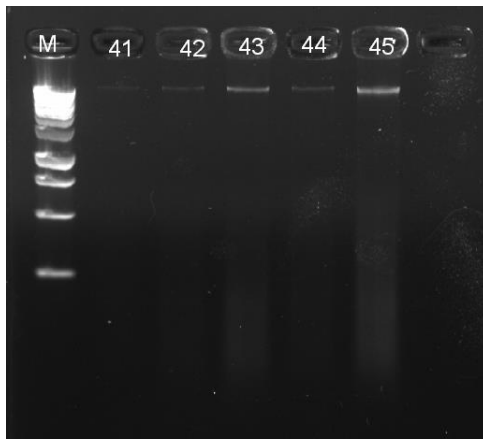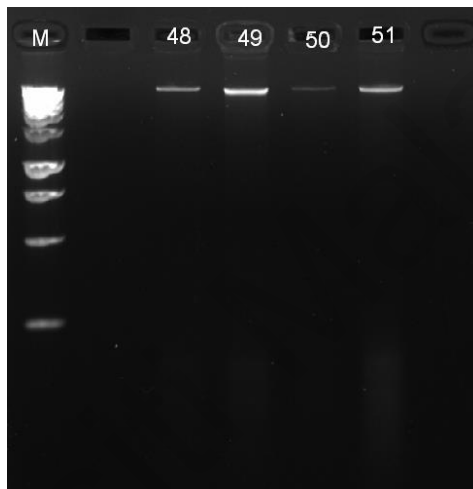
4.8 Inter Retrotransposon Amplified Polymorphism Polymerase Chain Reaction

Before performing the IRAP PCR, optimal annealing temperature of all the three IRAP primers, (Sukkula, RTY1 and RTY2), were determined by using gradient PCR technique. Four temperature conditions were tested to determine the optimal annealing temperature: 50°C, 47.9°C, 41.9°C, and 40°C.

For Sukkula primer, 40°C is the optimal temperature for IRAP PCR. Meanwhile, for the other two primers, RTY1 and RTY2, there was no band appeared on the electrophoresis gel (Figure 4.10). Unfortunately, only Sukkula primer produced the distinct bands at 40°C. The other two primers (RTY1 and RTY 2) did not produce distinct bands.

In Figure 4.10, lane number 8 shows one weak band. The annealing temperature for RTY2 was determined later at 40°C, but the primer seems not stable in producing distinct bands, hence, the use of RTY2 primer was excluded in this study.
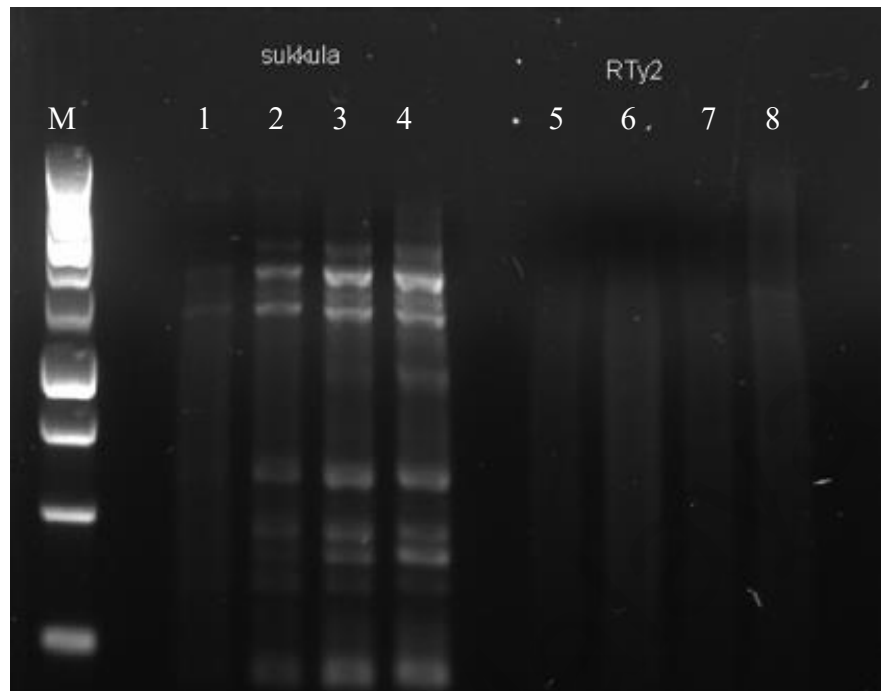
Figure 4.10: A gel visualization of the gradient PCR of the IRAP primers, Sukkula and RTY2 primers. From left to right: M, 1kb DNA ladder, 1, 50°C, 2, 47.9°C, 3, 41.9°C, 4, 40°C, 5, 50°C, 6, 47.9°C, 7, 41.9°C, and 8, 40°C.

Of all the three IRAP primers either single or in combinations, only a single Sukkula primer can be used for the clonal fidelity analysis. Table 4.3 summarized the primer combinations and optimal annealing temperatures used for the clonal fidelity analysis.

Table 4.3: IRAP primers combinations and the optimal annealing temperature.

IRAP MARKERS	SUKKULA	RTY1	RTY2
SUKKULA	40°C	🚫	🚫
RTY1	🚫	🚫	🚫
RTY2	🚫	🚫	🚫

🚫 indicates unsuccessful primers combination for the amplification screening.

Thirty out of sixty extracted DNAs were used to study the clonal fidelity analysis. Thirteen out of thirty extracted DNAs are from the yellow chrysanthemum regenerated plants while the other seventeen are from white chrysanthemum regenerated plants. By using sukkula primer, yellow and white chrysanthemum can be differentiates as shown in Figure 4.11. The arrow indicates the IRAP bands that can differentiated between yellow chrysanthemum and white chrysanthemum plants.
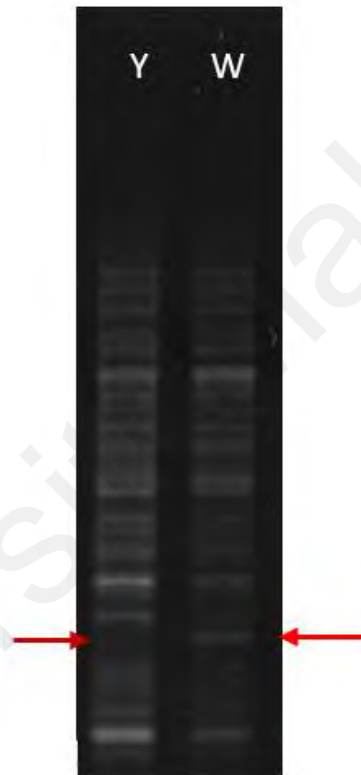
Figure 4.11: IRAP banding patterns of white and yellow chrysanthemum generated by using Sukkula primer. Lane from left to right: Y, yellow explant and W, white explant.

Figure 4.12 (a) and 4.12 (b) showed the IRAP banding patterns of the regenerated yellow chrysanthemum plants amplified using Sukkula primer. Out of thirteen yellow regenerated plants, two of them showed slightly different IRAP banding patterns (arrows; Figure 4.12 (a)). The red arrows showed the extra bands found in explant 28 and 31. These bands did not appear in other regenerated plants. This result indicates that 15.4% of thirteen yellow chrysanthemums have a different genotype and might be the somaclones of the yellow chrysanthemum.
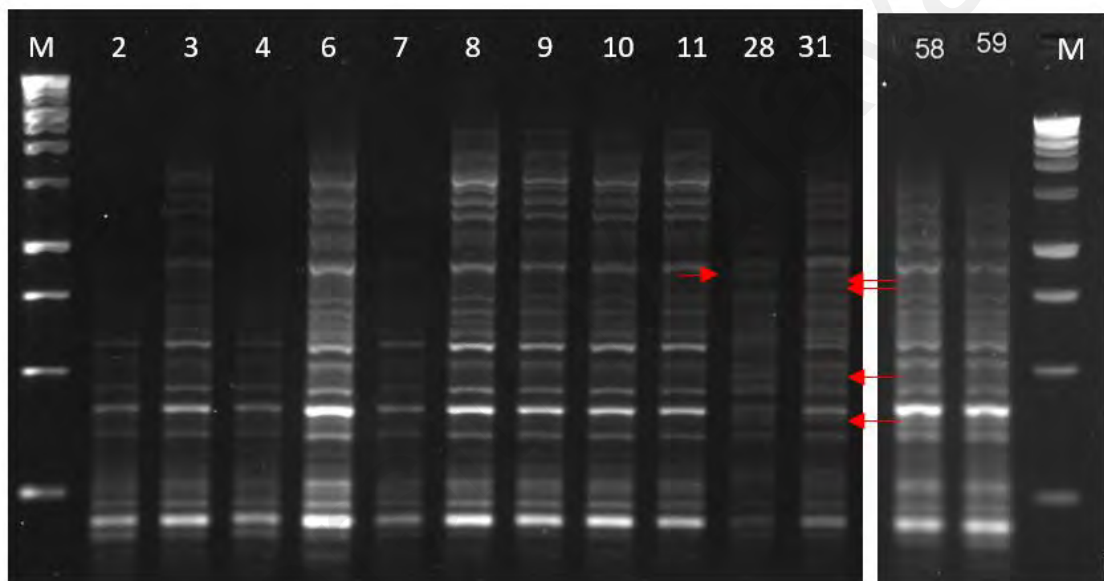
Figure 4.12 (a)   Figure 4.12 (b)

Figure 4.12: Gel visualization of the PCR product using Sukkula primer of yellow chrysanthemum regenerated plants. From left to right: M, 100bp ladder, 2, 3, 4, 6, 7, 8, 9, 10, 11, 28, 31, 58, and 59, PCR product from yellow chrysanthemum explants.

Seventeen white chrysanthemum regenerated plants were used to analyze the clonal fidelity using Sukkula primer. Two out of seventeen extracted DNAs (44 and 41) did not produce clear banding patterns and have been excluded from downstream analysis. IRAP analysis revealed that 20% of white chrysanthemum tested displayed different IRAP banding patterns when amplified using Sukkula primer (Figure 4.13).

Plants 36, 38 and 52 showed slightly different IRAP banding patterns than the others (red arrows, Figure 4.13). This indicates that these regenerated plants might be the putative somaclones of white chrysanthemum.
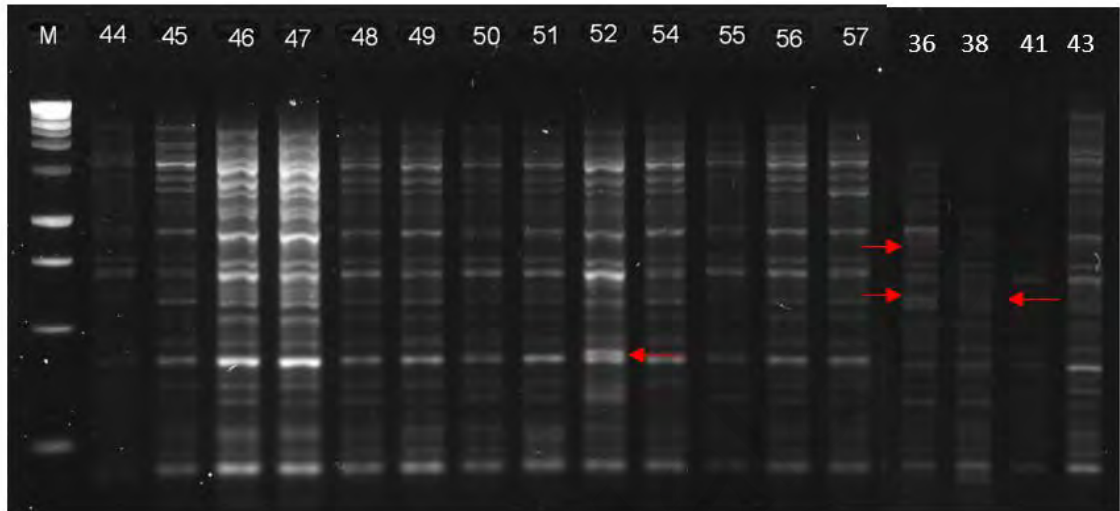
Figure 4.13 (a)   Figure 4.13 (b)

Figure 4.13: Gel visualization of the PCR product using Sukkula primer of white chrysanthemum regenerated plant. From left to right: M, 100bp ladder, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 55, 56, 57, 36, 38, 41 and 43, PCR product from white chrysanthemum explants.

CHAPTER 5: DISCUSSION

Axenic shoot cultures were established from leaf explants obtained from *in vivo* grown plants. These axenic cultures were used as a source of explants for subsequent experiments on the effect of organic supplements on the induction of callus and shoot elongation of *Chrysanthemum morifolium*. The callus induction and shoot elongation media used in this work was established by (Md Aiani, 2016) using similar plant growth regulators (PGR) established in other work (Kengkarj et al., 2008) but at different concentrations. The use of different PGR concentrations and media formulations have been reported to be specific to plant varieties (George et al., 2008). Casein hydrolysate, arginine, glutamine at different concentrations were added separately to the callus induction and shoot elongation media as organic supplements. The effect of these supplements on callus induction and shoot elongation from leaf and nodal explants were recorded.

Plants were regenerated via indirect organogenesis. The formation of callus prior to plant regeneration might prolong culture period and exposure to PGR which could increase the possibility of the incidence of somaclonal variation. Hence, a molecular assessment method, IRAP was conducted on the regenerants to confirm clonal fidelity among the plant population.

5.1 Response of explants towards plant growth regulators

Leaf and nodal explants were obtained from axenic cultures instead of *in vivo* plants to avoid the use of disinfectant (Clorox) which might affect the response of explants in tissue culture media. In addition, explants were consistently excised from uniformly grown 6 months old *in vitro* plants since culture period and number of subcultures has been reported to contribute to the incidence of somaclonal variation. The use of uniform aged *in vitro* grown plants as the source of explants was standardized.

A combination of plant growth regulators like NAA (auxin) and BAP (cytokinin) have been widely added into the media for *in vitro* chrysanthemum regeneration (Song et al., 2011; Naing et al., 2016; Sjahril et al., 2016). In this study, 100 % of the leaf and nodal explants produced callus after 5 weeks of culture on callus induction media (MS media supplemented with 3 mg L^{-1} BAP 1 mg L^{-1} NAA) with or without casein hydrolysate, arginine and glutamine. The same callus induction media has also been used by (Md Aiani, 2016) without the addition of any supplements. (Sjahril et al., 2016) concurred with this finding and reported that the best medium to induce callus in *Chrysanthemum* was MS medium containing 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BAP without any supplements. It can be concluded that supplements were not crucial to induce callus from leaf and nodal explants.

Both NAA and BAP were commonly used for callus induction and shoot regeneration in a few chrysanthemum varieties but at different concentrations. Others have reported on the use of other PGR for the induction of callus from leaf and nodal explants in chrysanthemum (Sultana Nehid et al., 2007; Thangmanee & Kanchanapoom, 2011; Kengkarj et al., 2008). This finding was anticipated since PGR used is dependent on plant variety (George et al., 2008).

It is noteworthy that the concentrations of auxin (NAA) and cytokinin (BAP) used in this study was reversed compared to work done by Sjahril et al., (2016) to induce callus in *Chrysanthemum*. It has been suggested that the concentration of auxin and cytokinin in the media is similar for callus induction in many plant species (George et al., 2008). The disparity in the concentrations of PGR used in the media to induce callus and differentiation may be due to the interplay between exogenous and endogenous PGR in the explants.

Nodal explants produced higher numbers of shoots compared to leaf explants. Many studies (Ilahi et al., 2007; Yesmin et al., 2014; Mohamed et al., 2015) have been using nodal explants for chrysanthemum in vitro propagation. Kaul et al., (1990) stated that stem explants were better compared to leaf explants in propagating chrysanthemum. In addition, (Song et al., 2011) also proved that shoot formation was better from nodal explants compared to leaf explants depending on the cultivars.

5.2   Response of explants towards organic supplements

In this work, arginine and glutamine are the amino acids chosen due to its effective use in callus development and shoot regeneration in diverse types of plants such as sugar cane (Asad et al., 2009), *Hibiscus moscheutos* (Greenwell & Ruter, 2018) and *Aquilaria malaccensis* (Borpuzari & Kachari, 2018). In addition to amino acids, casein hydrolysate was also used as an organic supplement. Its effective use for shoot regeneration has been described in various plants, for instance, *Citrus reticulata* L. (Badr-Elden, 2017), *Amorpha fruticosa* L. (Gao et al., 2003) and in banana (Silue et al., 2017). The effect of supplements varied depending on the plant species, synergistic effects with PGR and other inorganic or organic supplements used in the culture media.

From this study, although 100% of both leaf and nodal explants cultured on media with or without supplements produced callus, explants on media containing glutamine produced the earliest onset of callus initiation compared to other supplements. Similar findings were observed in rice where glutamine was used to increase the regeneration rate and fresh weight of callus (Pawar et al., 2015). The addition of glutamine to media has been shown to enhance shoot elongation compared to other amino acids. According to Samarina et al. (2016), it is an additional source of organic nitrogen which is directly assimilated by explants and plants cultured in tissue culture media. The addition of glutamine has also been shown to enhance cell division without any cytokinin (Samarina

et al., 2016). The additional source of nitrogen from glutamine in the media besides ammonium and nitrates ions is vital since nitrogen is an essential macro element and is involved with the synthesis of essential compounds for plant growth,

In this work, for both callus derived leaf and nodal explants, arginine and casein hydrolysate added separately at certain concentrations have shown to produce higher number of shoots per explant when compared to controls. Casein hydrolysate was also shown to enhance shoot elongation.

Many published works have reported the use of arginine in the regeneration media (Asad et al., 2009; Greenwell & Ruter, 2018; Liu et al., 2016). Greenwell & Ruter, (2018) found that arginine was better in enhancing growth of *Hibiscus moscheutos* than glutamine.   Liu et al., (2016) has also established an efficient protocol for plant regeneration from cotyledon petiole explants of *Jatropha curcas* with the addition of arginine to the regeneration media. This might be due to the two extra groups of nitrogen in the molecular structure of arginine which supported and improved the growth of shoots cultured on plant tissue culture media (Greenwell & Ruter, 2018).

As for casein hydrolysate supplemented media, shoot regeneration and elongation of chrysanthemum for both leaf and nodal explants were improved in this study. Other published work in agreement with these results included rice, banana, date palm and Kinnow mandarin (Ageel & Elmeer, 2011; Amer et al., 2018; Silue et al., 2017; Siwach et al., 2012). This could be due to the content of casein hydrolysate which has a mixture of up to 18 amino acids and is also a source of vitamins and microelements (Amer et al., 2018). Hence, this might suggest that shoot regeneration and elongation not only require amino acids, but also vitamins such as thiamine, nicotinic acid and pyridoxine as they are needed as vital intermediates or metabolic catalysts (George et al., 2008).

Casein hydrolysate at the highest concentration (750 mg/L) had a negative impact on shoot regeneration and elongation. From this study, the optimum concentration for casein hydrolysate was 500 mg/L. As for glutamine, at high concentration more elongated shoots from both leaf and nodal derived callus were produced and increased the number of shoots regenerated from leaf derived callus.

Thus, the optimal concentrations of different amino acids must be determined before incorporating into media formulations since they are genotype dependent (El-Sharabasy et al., 2012) (Hesami et al., 2019) and could affect the plant growth probably due to toxicity to the plants (Sjahril et al., 2016).

5.3   DNA extraction of *Chrysanthemum morifolium* cv. Zembla.

Sixty *in vitro* chrysanthemum regenerated plants were extracted for their total genomic DNA. Smearing towards the end of the gel were observed for some samples indicating DNA degradation in those samples. According to an article, the degradation of DNA samples may be the cause of the smearing effects on the agarose gel electrophoresis (Mayer, 2018). Adding to that, there are few factors that can also cause the smearing effect. For instance, too many samples loaded in the well and poorly prepared agarose gel (Mayer, 2018). Some of the DNAs showed uneven bands (sample no. 52 and 54 in Figure 4.8 (c)). This might be because of the bubbles produced during the sample loading that has hindered the migration of genomic DNA properly as suggested by Chauhan (2018) in an article from Genetic Education.

5.4   Optimization of IRAP marker

In an article on Genetic Education, the ability to amplify the DNA efficiently, with high precision and with a great yield are the main objective to perform the PCR (Chauhan, 2018). Annealing temperature is the most crucial part in PCR technique (Rychlik et al.,1991). A variation of the conventional PCR which can optimize PCR reaction by determining the exact annealing temperature is called gradient PCR (Chauhan, 2019). In order to determine the optimal annealing temperature for all the three IRAP primers (Sukkula, RTY1 and RTY2), gradient PCR technique was used.

The annealing temperature varies in different types of plants. For instance, in bananas, annealing temperature for sukkula primer used was 45.5°C (Teo et al., 2005). While in Pistachios, the annealing temperature used was 65°C (Pourian et al., 2019).

5.5   IRAP marker analysis of *Chrysanthemum morifolium* cv. Zembla

IRAP analysis using Sukkula primer in this study was able to distinguish yellow flower chrysanthemum cultivar "Zembla" from the white flower cultivar. There is a difference in both band patterns from white and yellow chrysanthemums. The white chrysanthemum has an extra band as shown in Figure 4.9. Teo et al., (2005) showed that IRAP markers can be used to distinguish closely related banana cultivars and tissue culture regenerants. Similar observation was observed in (Cheraghi et al., 2018) where IRAP clearly distinguish 34 varieties of *Lallemantia iberica.* In addition, IRAP markers were used to differentiate lemon cultivars into three cultivars namely 'Eureka', 'Fino', and 'Verna' (Bernet et al., 2004).

Besides that, IRAP markers can be a potential marker for the tissue culture industry to distinguish the somaclonal variation without the need to wait until the plants are fully grown which might cause time consuming. Moreover, as stated by (Rodrigues et al.,

1998), somaclonal variation may occur as the number of subculture increase, hence, in order to get clonal plants, this marker can also be used in detection of the time need to stop the subculture process.

Hordeum-specific IRAP markers used in this study allow identification of somaclonal variants where 15.4% of yellow flower chrysanthemum and 20% of white flower chrysanthemum showed slightly different IRAP banding patterns compared to their explants. Activation and integration of retrotransposons into host genome during tissue culture process have been reported in various plant species (Grandbastien & Spielmann, 1989; Hirochika et al., 1996; Mirani et al., 2020; Teo et al., 2005). Integrations of new retrotransposon in new genomic locations will produce new IRAP bands when compared to the explants. The new polymorphic IRAP bands can be used as the indicators of somaclonal variation. Mirani et al., (2020) showed that *Hordeum*-specific and *Phoenix*-specific IRAP together with *Phoenix*-specific miniature inverted-repeat transposable element (MITE) markers were able to detect somaclonal variants among tissue cultured date palms (*Phoenix dactylifera* L.) grown in open field trials (Mirani et al., 2020). Similar observation was detected in bananas where *Hordeum*-specific IRAP markers were able to detect new IRAP bands in tissue culture bananas (Teo et al., 2005).

CHAPTER 6: CONCLUSION

As the conclusion, addition of amino acids and casein hydrolysate individually to the media showed no significant effect in callus induction efficiency as all the explants produced callus including the control which has no supplements added. Nevertheless, arginine at 500 and 750 mg L^{-1} and casein hydrolysate at 500 mg L^{-1} individually produced the highest number of shoots (4.6-4.8 shoots per explant) in callus derived nodal explants. Elongation media supplemented with glutamine at 750 mg L^{-1} produced the highest number of shoots more than 1 cm within 4 weeks (1.4 shoots per explants) when nodal explants were used.

Out of nine primer combinations, only Sukkula primer was able to produce clear IRAP banding patterns in *Chrysanthemum morifolium*. Sukkula primer was able to differentiate between the white and yellow variants of *Chrysanthemum morifolium* "Zembla". Furthermore, IRAP revealed polymorphic bands within the varieties. White and yellow flower varieties showed 20.0 % and 15.4% genetic variations respectively. Further analysis is needed to confirm the linkage between these genetic variations and somaclonal variations.

In the future studies, few measures need to be taken into account in order to get more accurate and reliable results. The study could be extended to examine the synergistic effects of various combinations of organic supplements including organic and inorganic supplements. In addition, more studies could be done when adding more types of supplements such as vitamins (thiamine and myoinositol) with various concentrations of supplements. The study can be further investigated by narrowing the concentration range of the supplements to obtain accurate optimal results for tissue culture plants. In this study, the regenerated plants were observed up to the shoot elongation stage. In future work, the whole cycle of plant tissue culture including rooting of the plants might be

included in the study. As for the molecular part, there are many other molecular markers that can be used for the molecular assessment, including the RAPD, SSR and SCAR markers. More IRAP primers can be further optimized to analyze chrysanthemum plants.

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