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Influence of plant growth regulators on shoot development of Chrysanthemums

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Abstract Plant nodes were cultured on MS mediums supplemented with multiple concentrations of plant growth regulators. Sunny Snow produced the largest callus of 1.19×1.19 cm and obtained 93.33% of new shoots with 10.00 shoots per callus, and 0.84 g callus fresh weight, when grew in MS medium supplemented with 1 mg/l BA + 0.1 mg/l IAA as compared to the control which no callus was produced. After 20 weeks of culturing, the second set showed that the control treatment of Sunny Snow gave the highest stem growth and the best canopy size of 9.00 and 5.60 cm respectively. Chompoo Phan gave the highest scores of chlorophyll content, i.e., chl a 652.14 µg/g FW, chl b 332.47 µg/g FW and carotenoid 165.92 µg/g FW. Besides, MDA appeared to be highest, 17.37 nmol/g FW when cultured on MS medium supplemented with 1 mg/l BA + 0.1 mg/l IAA.

Keywords: Shoot regeneration, Callus induction, Plant growth regulator

Introduction

Chrysanthemum (*Dendranthemum grandiflora*) belongs to Asteraceae. Chrysanthemums are of considerable importance as a prominent type of cut flower and economically valuable flowering plant on a global scale. The global flower industry demonstrates a significant demand for the flower rated second, which closely follows the top-ranked rose (Kumar *et al.,* 2006). The natives are in China and Japan. The plant exhibits aesthetically pleasing floral characteristics. The blossoms possess vibrant hues. Moreover, a wide array of breeds exists (Krasaechai, 1992). The propagation of chrysanthemums can be accomplished using various methods, such as the use of root suckers or terminal cuttings. However, it must be mentioned that these procedures necessitate a protracted period of growth. In addition, the transmission of viruses and other diseases to cultivated plants seems to persist as a recurring challenge in the cultivation of disease-free chrysanthemum mother stocks for replication. The

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application of tissue culture techniques in plant propagation methods has the potential to enhance the general efficacy of the plant propagation process and can expedite the growth of plants (Teixeira da Silva *et al.,* 2013). In contrast to conventional vegetative propagation methods, direct organogenesis involves the regeneration of plants directly from pre-existing meristems or non-meristematic regions, such as internodes, leaves, and roots. This process yields new plants that closely resemble the original plant (Satish *et al.,* 2015). Furthermore, tissue culture serves as a valuable tool in the field of plant breeding, and within the tissue culture room, it maintains the ability of the breed to regulate environmental factors, such as temperature and light quality, within the tissue culture. The medium can employ plant growth regulators to support plant development and growth as well (Kaweeta, 1998). The application of plant growth regulators (PGRs) is necessary in these procedures since they play a crucial role in promoting the successful growth of diverse species under in vitro conditions (Teixeira da Silva, 2014). The callus induction process often involves the use of growth regulators from the auxin and cytokinin groups. The initiation of shoot regeneration from the callus involves the use of growth regulators from the cytokinin group. However, in the utilization of growth regulators within the identical category, several types of chemicals are utilized. Furthermore, varying concentrations are also employed. The efficacy of callus and shoot induction is contingent upon the composition of the culture medium and the inclusion of growth regulators. The impact of the plant's tissue type and genetics is especially noteworthy (Gahan and George, 2008). Numerous academic investigations have examined the impact of plant growth regulators on the process of callus induction as well as the subsequent effective shoot regeneration from callus tissues in chrysanthemum. Such as Chen *et al.* (1985), whereby the researchers documented the process of propagation utilizing chrysanthemum leaves. The cultures that were cultivated in MS medium supplemented with a concentration of 3-5 mg/l of BA, together with 2 mg/l of NAA, exhibited the most efficacy in promoting shoot formation. Additionally, it was observed that MS medium supplied with a concentration of 0.1 mg/l of NAA resulted in the most pronounced induction of shoot formation. The roots are considered to be superior. Limsanguan *et al.* (2017) The findings of the study indicated that the hormone-free medium, as well as the media containing only 2,4-D or BA, did not successfully promote callus formation in any of the tested cultivars. In the meantime, the medium consisting of a combination of 2,4-D and BA exhibited the formation of verdant and densely packed callus tissue. In their study, Shahidul *et al.* (2020) employed nodal segments of Chrysanthemum as explants for their experiments. The experimental treatment, which involved a concentration of 2.0 mg/l BA combined with 1.0 mg/l 2,4-D, yielded the highest

number of shoots, with an average of 3.20. In a study conducted by Nasri *et al.* (2018), it was shown that the leaves of chrysanthemum cultivars 'Homa' and 'Delkash' exhibited growth when cultivated on a MS medium supplemented with 2 mg/l BAP and 0.05 NAA. The shoot regeneration rates observed were 13.78 and 8.89 shoots, respectively. Nahid *et al.* (2007) conducted a study. The petal was cultivated on a MS medium supplemented with plant growth regulators, specifically cytokinin or auxin-cytokinin, at different combinations and concentrations. It was investigated the creation of calluses and the induction of shoots. The maximum callus production, reaching a percentage of 96%, was seen when using MS medium supplemented with 2 mg/l BA and 0.1 mg/l NAA. Therefore, the research finding was examined the influence of a combination of auxin, IAA (indole-3-acetic acid), and cytokinin, BA (6-benzyladenine), and a medium containing only one plant growth regulator in Murashige and Skoog (1962) medium for callus induction and shoot development of three chrysanthemum varieties.

Materials and methods

Plant materials

The study used chrysanthemum nodes as explants. The purpose of the examination focused on three different varieties of chrysanthemum, namely Canter (Figure 1A), Chompoo Phan (Figure 1B), and Sunny Snow (Figure 1C). The canter is classified as a purple spray type, while Chompoo Phan is classified as a pink spray type, both being under the same category. Sunny Snow is a decorative-flowered cultivar characterized by its green inner white coloration. The provided samples consisted of three different types obtained from the tissue culture laboratory. Department of Plant Production Technology, School of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

Figure 1. Flower of chrysanthemum varieties: (A) Canter, (B) Chompoo Phan, (C) Sunny Snow

Callus induction and shoot regeneration

Three different varieties of nodes, measuring approximately 0.3-0.5cm in length, were cultured on MS medium supplemented with 3% sucrose, 8 g/l agar, 3 mg/l 6-benzyladenine (BA), and 1 mg/l BA with 0.1 mg/l indole-3-acetic acid (IAA) for callus induction. The control treatment used a basic medium consisting of MS medium. The pH of the solution was carefully regulated to a range of 5.5–5.7 before subjecting it to autoclaving at a temperature of 121°C for a duration of 20 min. The cultures were placed under white LED light with a photosynthetic photon flux density (PPFD) of 40 µmol m^{-2} s^{-1} for 16 hr/day at a temperature of 25±3 °C. The subculture was done at 4 weeks intervals. After an 8 weeks period of callus culture, the callus was transferred to the MS medium for shoot induction. The experiment consisted of 9 treatments with each treatment being 3 replicates. Each replication had 5 explants.

Determinations of Chlorophyll a, chlorophyll b and carotenoids

The determination of chlorophyll a (chl a), chlorophyll b (chl b), and carotenoid concentrations in the callus and shoot samples was carried out using a modified procedure outlined by Lichtenthaler and Buschmann (2001). A specimen with a mass of 0.2 g was pulverized and subjected to extraction using 5 ml of an acetone solution with an 80% concentration (w/v). Subsequently, the resulting mixture was enveloped with aluminum foil and subjected to a period of 3 hr in darkness before undergoing the filtration process. The experiment involved the utilization of filter paper No. 93, with a diameter of 125 mm, as the medium for filtration. Subsequently, the filtrate was subjected to centrifugation at a speed of 10,000 rpm for a duration of 20 min. The resulting absorbance values were then recorded at wavelengths of 470 nm, 647 nm, and 663 nm. The evaluations were conducted by calculating the value.

chlorophyll a (µg/ml) = 12.25 (A_{663}) - 2.79 (A647),

chlorophyll b (µg/ml) = 21.50 (A_{647}) - 5.10 (A663), and

carotenoids (µg/ml) = [1000 (A_{470}) - 1.82 (chl a) - 85.02 (chl b)] 198

The results were expressed as chlorophyll and carotenoid contents in the tissue (µg/ g FW).

Determinations of MDA content

The method of experimentation Heath and Packer (1968) employed this method for the quantification of thiobarbituric acid-reactive compounds (TBARs), which are byproducts of lipid peroxidation. The process of

homogenization was performed on callus and shoot samples weighing 0.2 g each, using 3 mL of a trichloroacetic acid (TCA) solution with a concentration of 0.1% (w/v). Following centrifugation at a temperature of 4 °C and a rotational speed of 10,000 rpm for a duration of 20 minutes, a volume of 1 mL of the upper-layer solution was combined with 4 mL of a solution consisting of 20% trichloroacetic acid (TCA) and 0.5% (w/v) thiobarbituric acid (TBA). The mixture was subjected to a 30-minute duration of heating in a boiling water bath, followed by a rapid cooling process. The samples were subjected to centrifugation at a speed of 10,000 rpm for a duration of 10 minutes, and subsequently, the absorbance was quantified at a wavelength of 532 nanometers. The subtraction of the value corresponding to non-specific absorption at a wavelength of 600 nm was performed. The quantification of malondialdehyde (MDA) content was performed by employing an extinction value of 155 mM/cm. The findings were reported in units of nanomoles per gram of fresh weight.

Data collection and analysis

The collected data includeds callus growth measurements such as callus fresh weight, size of callus, percentage of shoot regeneration, and number of shoots. After a period of 14 weeks, shoot growth, stem height, and canopy size were measured. The evaluation of chlorophyll and carotenoid contents, and the measurement of malondialdehyde (MDA) content were done for 3 times with 5 explants in each treatment. The data were subjected to analysis of variance (ANOVA) and Duncan's multiple range tests using the SAS program with a significance level of $p \leq 0.05$.

Results

Callus induction and shoot regeneration

Nodes from three different varieties of chrysanthemum were exposed to cultivation on a MS medium supplemented with 3 mg/l BA, and 1 mg/l BA with 0.1 mg/l IAA. Results showed that Sunny Snow variety, when cultured on MS medium supplemented with 1 mg/l BA + 0.1 mg/l IAA for 8 weeks of experiment exhibited the highest average callus fresh weight and size of callus 0.84 g and 1.19×1.19 cm, respectively (Figure 2) The highest among all the treatments. Consequently, the average values of the percentage of shoot regeneration and the number of shoots 93.33% and 10.00 shoots per callus increased as well (Figure 3) On the other hand, Chompoo Phan variety, when callus induction on MS medium supplemented with 1 mg/l BA + 0.1 mg/l IAA. The lowest average

callus fresh weight and size of callus 0.40 g and 0.89×0.88 cm, respectively. Besides, Chompoo Phan variety, when callus induction on MS medium supplemented with 3 mg/l BA. The lowest average of the percentage of shoot regeneration and the number of shoots 5 3.33% and 6 .00 shoots per callus decreased as well (Table 1).

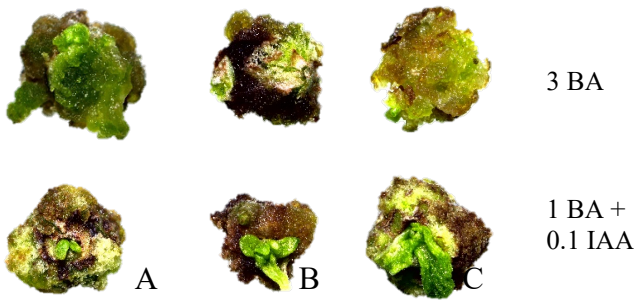

Figure 2. Callus from nodes of different varieties of three chrysanthemum cultured on MS medium supplemented with 3 mg/l BA and 1 mg/l BA + 0.1 mg/l IAA after 8 weeks of culturing: (A) Canter, (B) Chompoo Phan, (C) Sunny Snow

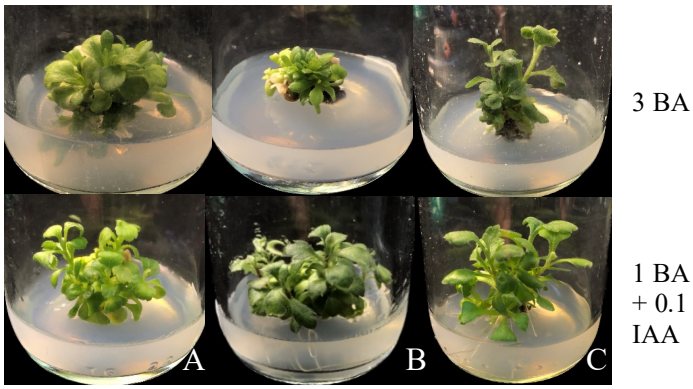

Figure 3. Callus 8 weeks of different varieties of three chrysanthemum, cultured on MS medium supplemented with 3 mg/l BA and 1 mg/l BA + 0.1 mg/l IAA. Transferred to the MS medium for shoot induction. After a duration of 12 weeks (A) Canter, (B) Chompoo Phan, (C) Sunny Snow

Table 1. Effects of plant growth regulators and chrysanthemum varieties on percentage of shoot regeneration, shoot number, callus fresh weight and size of callus for 8 weeks

Varieties	MS medium (mg/l)	%Shoot regeneration	Shoot number	Callus fresh weight (g)	Size of callus (cm)	
					Width	Height
Canter	MS	100.00±0.00^{a}	5.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{d}
	3 BA	60.00±20.00^{cd}	7.00±1.00^{bc}	0.58±0.07^{abc}	1.23±0.20^{a}	0.83±0.10^{c}
	1 BA + 0.1 IAA	73.33±11.55^{bc}	8.33±0.58^{b}	0.49±0.19^{bc}	1.25±0.09^{a}	1.07±0.27^{ab}
Chompoo Phan	MS	100.00±0.00^{a}	5.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{d}
	3 BA	53.33±11.55^{d}	6.00±1.00^{cd}	0.41±0.09^{c}	0.93±0.15^{b}	0.83±0.19^{c}
	1 BA + 0.1 IAA	60.00±0.00^{cd}	7.67±0.58^{b}	0.40±0.03^{c}	0.89±0.10^{b}	0.88±0.03^{bc}
Sunny snow	MS	100.00±0.00^{a}	5.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{d}
	3 BA	86.67±11.55^{ab}	8.00±1.00^{b}	0.71±0.31^{ab}	1.06±0.21^{ab}	0.95±0.12^{bc}
	1 BA + 0.1 IAA	93.33±11.55^{a}	10.00±1.00^{a}	0.84±0.25^{a}	1.19±0.08^{a}	1.19±0.01^{a}
F-test A		**	**	**	**	**
F-test B		**	**	**	**	**
F-test A*B		**	**	**	**	**
CV%		12.61	10.45	39.64	16.79	19.19

** Significant different at P≤0.01. Means within column followed by the same letter are not significant different as determined by Duncan's multiple range test.

Chlorophyll a, chlorophyll b and carotenoids contents

Nodes from three different varieties of chrysanthemum were exposed for cultivation on a MS medium supplemented with 3 mg/l BA, and 1 mg/l BA with 0.1 mg/l IAA. Results showed that callus of Chompoo Phan variety, when cultured on MS medium supplemented with 1 mg/l BA + 0.1 mg/l IAA for 8 weeks of experiment exhibited the highest average chlorophyll a, chlorophyll b and carotenoids contents of 24.71, 28.29 and 21.68 µg/g FW, respectively (Table 2) which showed the highest among all the treatments. On the other hand, the control treatments were developed for shoot of all varieties. Consequently, the level of chlorophyll present is greater compared to other treatments. This disparity arised due to a higher concentration of pigments in shoot compared to callus.

Development of shoot regeneration

Chrysanthemum shoots from three different varieties were cultured on MS medium. Results showed that callus induction of Sunny Snow variety on MS medium supplemented with 1 mg/l BA + 0.1 mg/l IAA for 20 weeks exhibited the highest averaged stem height and canopy size of 9.00 and 5.60 cm, respectively which were the highest among all the treatments. On the other hand, the callus induction of Chompoo Phan variety on MS medium supplemented with 3 mg/l BA showed. the lowest averaged stem height and canopy size of 6.93 and 4.03 cm, respectively (Figure 4), but there were shown the highest averaged chlorophyll a, chlorophyll b and carotenoids contents of 652.14, 332.47 and 165.92 µg/g FW, respectively. The highest one showed among all the treatments. Whereas, the callus induction of Canter variety on MS medium showed the lowest averaged chlorophyll a, chlorophyll b and carotenoids contents of 350.75, 125.25 and 51.84 µg/g FW, respectively (Figure 5). Thus, it is revealed that the culture medium did not exert any influence on shoot growth (Figure 6).

Table 2. Effects of plant growth regulators and chrysanthemum varieties on chlorophyll a, chlorophyll b, and carotenoid contents for 8 weeks

Varieties	**MS medium (mg/l)**	**Chlorophyll a (µg/g FW)**	**Chlorophyll b (µg/g FW)**	**Carotenoid (µg/g FW)**
Canter	MS	201.43±43.75^{a}	85.62±16.20^{a}	42.70±8.85^{a}
	3 BA	14.58±2.91^{b}	13.42±3.35^{b}	7.48±1.02^{b}
	1 BA + 0.1 IAA	24.71±4.65^{b}	14.97±3.08^{b}	10.39±1.53^{b}
Chompoo Phan	MS	212.10±8.75^{a}	92.03±4.30^{a}	41.74±5.56^{a}
	3 BA	18.60±1.93^{b}	14.46±2.51^{b}	8.05±0.64^{b}
	1 BA + 0.1 IAA	28.29±5.11^{b}	19.41±5.62^{b}	9.63±1.16^{b}
Sunny snow	MS	208.84±15.46^{a}	92.54±6.86^{a}	47.95±3.64^{a}
	3 BA	23.86±4.19^{b}	14.81±3.11^{b}	8.70±1.33^{b}
	1 BA + 0.1 IAA	27.68±5.08^{b}	12.21±1.56^{b}	9.82±1.25^{b}
F-test A		**	**	**
F-test B		**	**	**
F-test A*B		**	**	**
CV%		19.06	16.67	18.41

** Significant different at P≤0.01. Means within column followed by the same letter are not significant different as determined by Duncan's multiple range test.

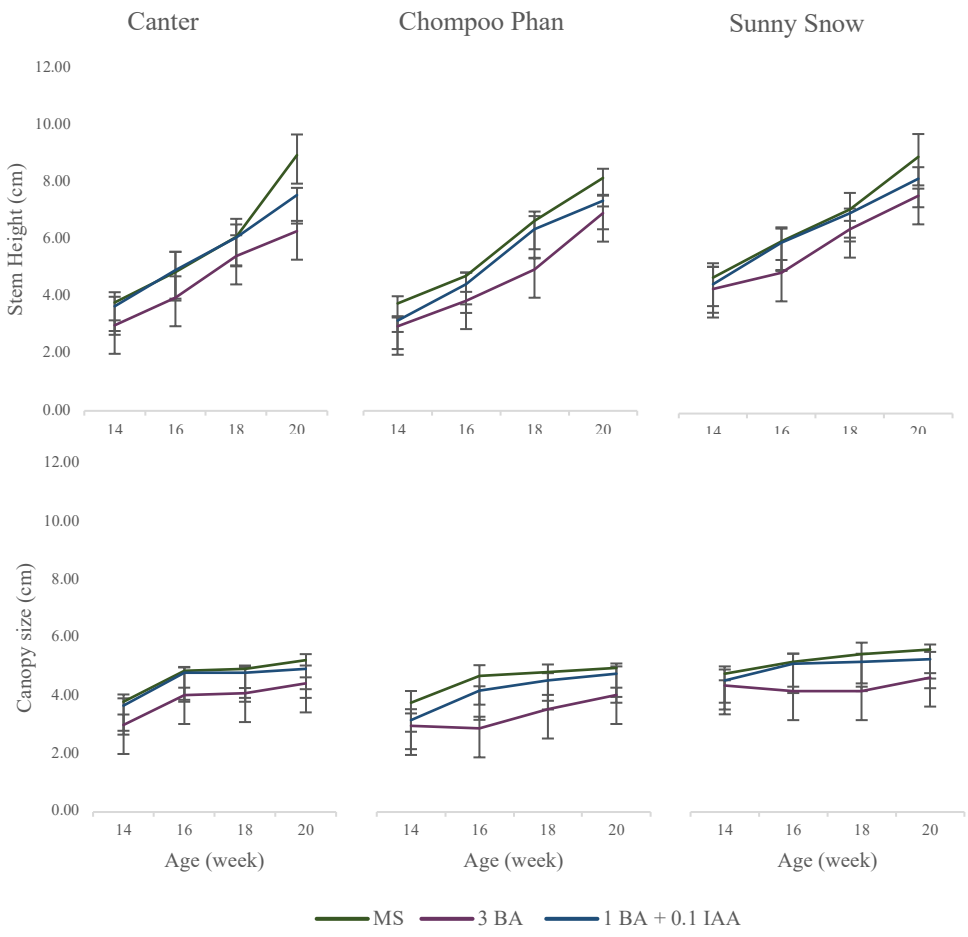

Figure 4. The average number (±SD) of stem heights and canopy size for three chrysanthemums induced from callus induction on MS medium supplemented with 3 mg/l BA and 1 mg/l BA + 0.1 mg/l IAA and the callus transferred to the MS medium for shoot induction. After a duration of 20 weeks

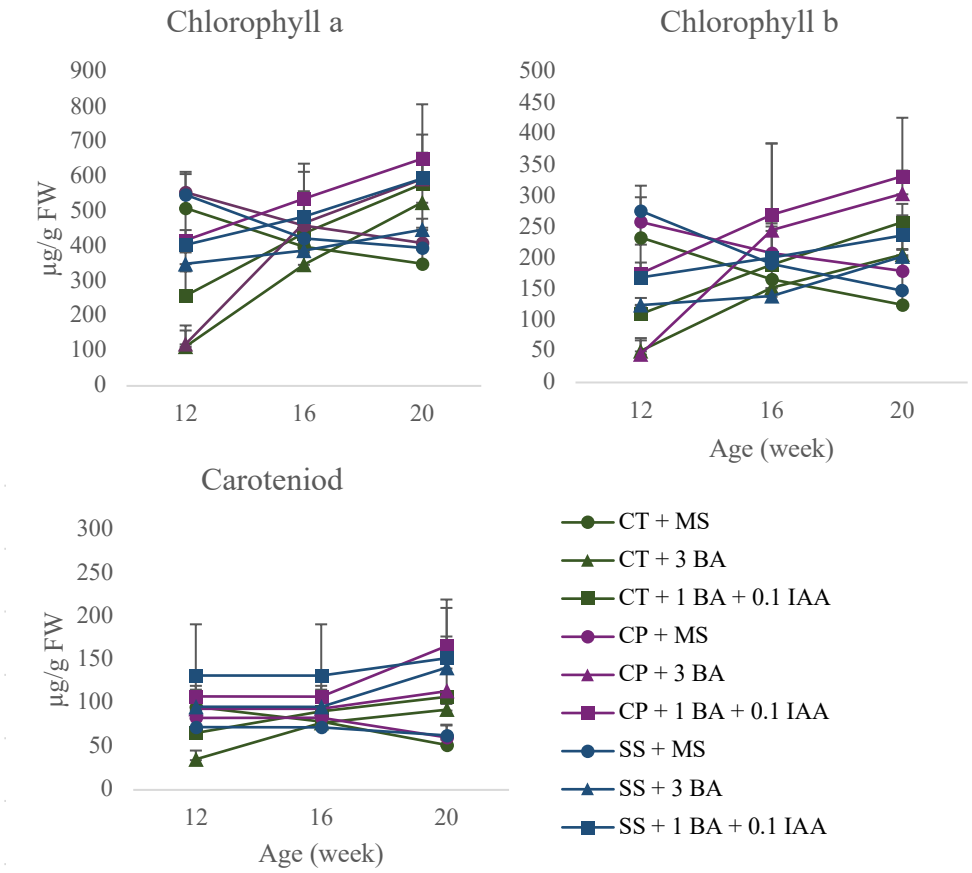

Figure 5. The average number (±SD) of chlorophyll a, chlorophyll b, and carotenoid contents for Canter (CT), Chompoo Phan (CP), and Sunny Snow (SS) induced from callus induction on MS media supplemented with 3 mg/l BA and 1 mg/l BA + 0.1 mg/l IAA and the callus transferred to the MS medium for shoot induction

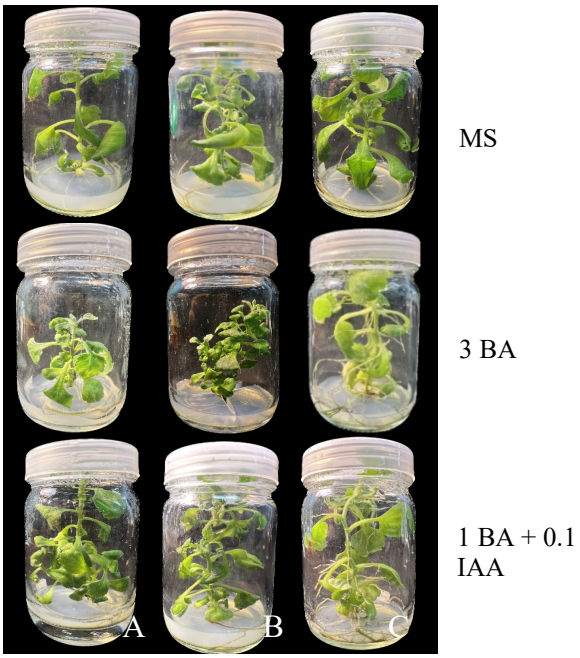

Figure 6. Shoots of different varieties of three chrysanthemum, cultured on MS medium supplemented with 3 mg/l BA and 1 mg/l BA + 0.1 mg/l IAA and they transferred to the MS medium for shoot induction. After a duration of 20 weeks (A) Canter, (B) Chompoo Phan, (C) Sunny Snow

MDA contents

Chrysanthemum shoots from three different varieties were cultured on MS medium. Results showed that the Chompoo Phan variety was cultured on MS medium supplemented with 1 mg/l BA + 0.1 mg/l IAA exhibited the highest averaged MDA content of 17.37 nmol/g FW. This MDA content was observed to be the highest among all the treatments. The efficiency of growth was impacted resulting in reduced stem height and bush diameter as compared to other varieties. In contrast, Canter and Sunny Snow had the lowest mean MDA concents of 8.24 and 9.62 nmol/g FW respectively under the control treatment (Figure 7). Thus, the treatment of plant growth regulators had a significant effect on the growth and increase of MDA concentrations.

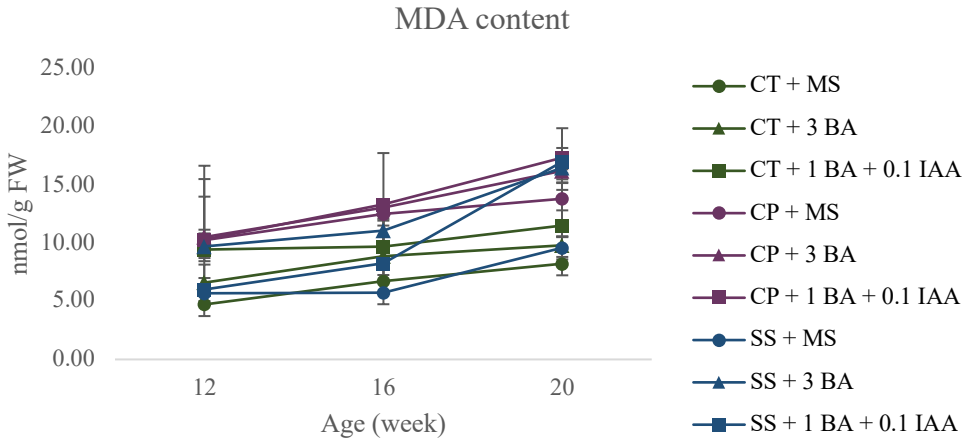

Figure 7. The average number (±SD) of Malondialdehyde (MDA) content for Canter (CT), Chompoo Phan (CP), and Sunny Snow (SS) induced from callus induction on MS medium supplemented with 3 BA mg/l and 1 mg/l BA + 0.1 mg/l IAA and the callus transferred to the MS medium for shoot induction

Discussion

This experiment investigated the node cultured of three chrysanthemum varieties cultured on a MS medium supplemented with BA and IAA. It was observed that this medium was more effective in callus induction and promoting shoot regeneration compared to the MS medium supplemented with BA alone or the MS medium without plant growth regulators. This result supports that the concentration of plant growth regulators, specifically the ratio of auxins/cytokinins are important factors that regulate callus induction and shoot regeneration. Brock and Kaufman (1991) reported that BA, classified as a cytokinin, has a role in regulating cellular division. Promoting crest induction and stimulating lateral bud growth in plants. Jafrai *et al.* (2016) reported that the IAA is categorized as a phytohormone that falls within the auxin category and demonstrates a stimulatory effect on various biological processes, including cell growth, cell elongation, and root development. Momoko *et al.* (2013) reported that the introduction of exogenous auxin and cytokinin has been observed to stimulate the formation of calluses in several plant species. The induction of callus is facilitated by an intermediate ratio of auxin and cytokinin, but a high ratio of either auxin-to-cytokinin or cytokinin-to-auxin stimulates the regeneration of roots or shoots, respectively. Therefore, the tissue cultivated on a medium supplemented just with BA or IAA did not effectively stimulate callus development in the present investigation. In a similar vein, Nasri *et al.* (2018)

observed that the cultivation of chrysanthemum 'Homa' and 'Delkash' leaves on the MS medium supplemented just with BA did not yield any significant improvement in callus development. Bhattacharya *et al.* (1990) reported that the most optimal conditions for the callus induction of chrysanthemum were obtained by using a combination of 0.1 mg/l of IAA and 0.2 mg/l of BAP. The growth and development of plants are dependent on the interaction between the concentration of plant growth regulators (PGRs) and the plant cultivars. Sun *et al.* (2007) reported that the most effective shoot regeneration from leaf explants occurs through the formation of callus in a chrysanthemum medium containing 0.2 mg/l NAA and 1 mg/l BA. Thangmanee and Kanchanapoom (2011) reported that the regeneration of shoots or plantlets is primarily influenced by the concentration and type of cytokinins, which are particularly effective in stimulating shoot regeneration from the callus of *Chrysanthemum morifolium*. Tymoszuk and Zalewska (2014) reported that shoot development is evident in a culture medium with a substantial cytokinin content and a relatively low auxin concentration. In contrast, the process of root formation occurs inside a culture medium characterized by a substantial presence of auxins and a relatively limited presence of cytokinins. Xu *et al.* (2008) reported that cytokinin plays a crucial role in the shoot regeneration process from the callus. Nahid *et al.* (2007) reported that the determination of the optimal dosage of plant growth regulators (PGRs) for inducing the regeneration of adventitious shoots should be customized to the specific plant cultivars. Shahidul *et al.* (2020) reported that a concentration of 2.0 mg/l BA combined with 1.0 mg/l 2,4-D had a significant effect on the node of the chrysanthemum, exhibited the highest occurrence of callus production, and produced the greatest number of shoots. Conversely, the MS medium without plant growth regulators did not show any observable callus formation.

The addition of growth regulators to the MS medium resulted in an increase in chlorophyll and carotenoid content. The Chompoo Phan variety was cultured on MS medium supplemented with 1 mg/l BA + 0.1 mg/l IAA exhibited the highest amounts of chlorophyll and carotenoid content, resulting in augmented photosynthetic activity in comparison to other treatments. Alsoufi *et al.* (2021) reported that cytokines have been found to offer safeguarding effects on chlorophyll, enzymes that facilitate photosynthesis, and the breakdown of proteins. Akaneme and Eneobong (2008) reported that in the same plant species, compact callus has the ability to produce chlorophyll over friable callus due to the dense cell aggregation, thus producing a large amount of chloroplast. George and Sherrington (1984) reported that the fact that the green color of the callus involves the development of various organs. Scott (2008) reported that chlorophyll a, chlorophyll b, and carotenoids are major pigments found in the

chloroplasts of plants, responsible for the absorption of light energy. The pigments are recognized as having a vital role in the process of photosynthesis, as they assist in the transformation of light energy into chemical energy. This conversion enables the synthesis of food and promotes the overall development of plants.

Lipid peroxidation pertains to the oxidative degradation process of lipids. Malondialdehyde (MDA) is the ultimate end product that arises from the process of lipid peroxidation. The TBARS assay is commonly utilized as the primary technique for measuring the amount of malondialdehyde (MDA) (Marnett, 1999). Considering the well-established role of fatty acids and lipids as essential components of cellular membranes, it is reasonable to propose that the degradation of these membranes might potentially result in the accumulation of free lipids within the cytoplasm of certain cells. The lipids present in the cytoplasm, which are not attached to any specific structures, have the potential to undergo oxidative reactions (Scrivanti *et al.,* 2003). The study findings indicated a significant and positive relationship between the age of chrysanthemum plants and the increase in MDA content.

In conclusion, the findings of this study indicated that the effectiveness of the Sunny Snow variety was enhanced when cultured on an MS medium supplemented with 1 mg/l BA + 0.1 mg/l IAA. This combination of growth regulators facilitated the prompt initiation of callus induction and subsequent shoot regeneration. The Chompoo Phan variety was cultured on a MS medium enriched with 1 mg/l BA and 0.1 mg/l IAA. The application of this medium resulted in a notable augmentation of the chlorophyll and carotenoid levels in both callus and shoots. Furthermore, it exhibited a higher concentration of MDA content in comparison to alternative cultivars.

Acknowledgements

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