Performance of NAA, 2iP, BAP and TDZ on Callus Multiplication, Shoots Initiation and Growth for Efficient Plant Regeneration System in *Chrysanthemum* (*Chrysanthemum morifolium* Ramat.)

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Abstract: Three series of plant growth regulators experiments in *Chrysanthemum* tissue culture, each arranged in completely randomized design (CRD), were studied to formulate efficient plant regeneration system for developing *Chrysanthemum* plant cell engineering. In the first experiment, 100 mg callus were cultured in liquid Murashige and Skoog (MS) medium supplemented with different combinations of NAA and BAP concentrations treatments. After 30 days of culture, heaviest callus (1.55 g) was obtained in NAA (1.0 mg L^{-1}) + BAP (0.5 mg L^{-1}). Consequently, shoot regeneration experiment with different kinds and concentrations of auxin, *i.e*. NAA and 2iP were each combined with different concentrations of cytokine BAP. Callus grown on solid MS medium supplemented with both NAA and BAP at 0.5 mg L^{-1} showed fastest shoot initiation (30 days), largest number of shoots (5 shoots), longest shoot length (2.88 cm). While combination of 2iP (0.5 mg L^{-1}) and BAP (2.0 mg L^{-1}) also produced same response on shoot initiation but shorter (1.88 cm) and only one shoot. Largest number of shoots (3 shoots) was obtained in treatment BAP (0.5 mg L^{-1}) without 2iP, although shoot

initiation was slower (39 days) with shorter length (1 cm). In a separate experiment, effect of single treatment of potential synthetic cytokine for shoot regeneration, *Thidiazuron* (TDZ), at different concentrations was examined. However, callus grown on TDZ incorporated medium did not produce any shoots and changed from green to brown at end of study (90 days). It was assumed that TDZ inhibited formation of shoots in *Chrysanthemum* callus culture.

Keywords: *Chrysanthemum morifolium; Plant Cell Engineering*; NAA; 2iP; BAP; TDZ.

1. Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat. *syn. Dendranthema grandiflorum*) is one of the largest floriculture commodities in Indonesia. *Chrysanthemum* export volume in 2012 reached 50.92 tons, with a value of more than one million US dollars. The value is even greater than the value of exports of orchid that only reach about 600,000 US dollars. On the other hand, Indonesia also imported large amounts *Chrysanthemum*s, up to 8 tons imported in 2012. It has stayed at first rank of floriculture commodities most imported in Indonesia, compared with the volume of imports of orchids which only reached 4 tons in the same year (Directorate General of Horticulture, 2012).

The common commercially available *Chrysanthemum* in Indonesia is *Chrysanthemum morifolium* Ramat. It is native to the northern hemisphere, mainly Europe and Asia. A high interest in the Indonesian market is not without reason. This flower is known for its beauty and long vase life and durability as cut flowers. *Chrysanthemum* has the advantage of wide variable shape and flower color. This plant has thousands of different varieties with flower color ranges from white and cream to the shades of yellow, bronze, pink, red, green and deep purple.

Although it has a great potential for the country's income, the development of *Chrysanthemum* is still faced by several challenges. Among them is the lack of genetic diversity of C*hrysanthemum* in Indonesia. Consequently, large size grower or industry must always import seeds or mother plant from the Netherlands. Another important constraint is pests and plant diseases. Plants are very delicate to pests or diseases, hence easy to deteriorate making it difficult to market. Plants that are free of pests and diseases have been in crucial condition to meet the global export market.

One of the important diseases that attack *Chrysanthemum* is white rust (*Puccinia horiana* P. Henn.). Suhardi (2009) indicated that the yield losses due to white rust which could reached 30% from seedling born disease alone. The control of white rust disease is generally done with fungicide, but usually did not reach its target. As a result, the disease could not be eliminated. One potential alternative is to develop disease resistant *Chrysanthemum* plants.

Development of disease resistant plants can be made through conventional plant breeding although it may be difficult as it has lack of disease resistance varieties for tropical diseases, thus lack of available useful resistance genes. Alternatively, cell and genetic engineering technology can be

applied which allow the multiplication and selections as well as the insertion of genes through recombinant DNA from other organisms into the plant genome through *Agrobacterium*-mediated procedure using tissue culture technology (Hooykaas and Schilperoort, 1992; Takatsu *et al*., 1999; Sjahril *et* a*l*., 2006; Semiarti *et al.*, 2007; Khan *et al*., 2008).

Establishment of plant regeneration system in tissue culture is a prerequisite for high efficient production of transgenic plants in any *Agrobacterium*-mediated transformation protocol. Many research on increasing efficiency of regeneration system has been reported on *Chrysanthemum* (Teixeira da Silva, 2003[a], Teixeira da Silva, 2003[b]; Ilahi *et al.,* 2007; Keresa *et al.*, 2012) and other species such as *Petunia hybrida* (Thirukkumaran *et al*., 2009). The combination of plant growth regulator auxin and cytokine incorporation in the medium has been used extensively on plant regeneration in tissue culture.

The use of medium type and plant growth regulators as well as selection of appropriate protocol in *in vitro* culture can support the success of genetic engineering of *Chrysanthemum.* In this study the role of auxins, α-Naphthalenacetic Acid (NAA) and 2-Isopentyl Adenine (2iP), the cytokines Benzyl Amino Purine (BAP) and the new potent synthetic cytokine, Thidiazuron (TDZ) used in combination or sole plant growth regulators in inducing cells/callus and shoot regeneration in *Chrysanthemum* for an efficient protocol of *in vitro Chrysanthemum* plant regeneration system was investigated.

2. Materials and Method

This research was carried out in the Laboratory of Plant Bio-science and Reproduction Biotechnology, Department of Agronomy, Faculty of Agriculture, Hasanuddin University, Makassar. Experiments were conducted from March to November 2015.

Callus induction and culture were started from leaf explants of C*hrysanthemum* cultivar "Pasopati", obtained from the collection of the Tissue Cuture Laboratory Installation, UPTD – Horticultural Seed Center, Gowa Regency, South Sulawesi Province. Medium was made using the inorganic and organic media component for Murashige and Skoog (MS) medium, plant growth regulator (PGR) such as: α-Naphthalenacetic Acid (NAA), 2-Isopentyl Adenine (2iP), Benzyl Amino Purine (BAP), Thidiazuron (TDZ). Solidification was obtained by adding gellant gum (Gellex, Cica-Reagent). Other supporting materials and equipment as well as appliances, glassware and dissection kit for a standard plant tissue culture laboratory were also required.

Chrysanthemum cell/callus suspension culture experiment with liquid MS medium was arranged in a completely randomized design (CRD) with two treatment factors. The first factor was the concentration of NAA (0.1 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1}; 1.5 mg L^{-1}; 2.0 mg L^{-1}, 3 mg L^{-1} and without NAA). The second factor was concentration of BAP which consisted 0.5 mg L^{-1}, 1.0 mg L^{-1}; and 2.0 mg L^{-1} and without BAP. Effect of the combination of plant growth regulators, the auxin (NAA and 2iP) and the cytokine (BAP) were also carried out to study shoot and plant regeneration efficiency

of *Chrysanthemum* callus. The first factor is the type and concentration of auxin (NAA and 2iP) which consisted of four levels (0.0, 0.1, 0.5, and 1.0 mg L^{-1}), each of which was combined with the second factor, the cytokine (BAP), consisting of 4 levels (0.0, 0.5, 1.0, and 2.0 mg L^{-1}). Effect of single treatment of TDZ (0.0, 0.5, 1.0, 1.5, and 2.0 mg L^{-1}) incorporation into MS medium on shoot regeneration from callus explant was also investigated.

The PGRs were incorporated in to liquid MS medium for callus suspension culture and solidified with 1 g L^{-1} gellant gum for the second and third experiment. Callus inoculant (100 mg), were each placed either on the solid medium or in liquid culture medium. Sub-culture with new fresh medium was performed every two weeks. The experiments were carried out arranged in Completely Randomized Design with two-factors. All treatment combination in each experiment was repeated 4 times. Observation data on induction and growth of cell/callus suspension as well as regeneration of the plantlets were analyzed, both were visualized and analized by ANOVA and efficiency of plant regeneration was discussed.

3. Result and Discussion

3.1 Suspension Callus Cultures

Friable callus were subcultured suspended in liquid MS medium containing PGR according to treatments. Sub-cultures into fresh MS medium was repeated every 14 days. Results showed the rate of callus fresh weight (FW) gained after 30 days of treatment (Table 1). However, suspension cells took longger period to obtain during this study. Thus, result here only showed condition of friable callus culture turning slowly into cell suspension culture.

Table 1 showed that NAA concentration of 1.0 mg L^{-1} in combination with 0.5 mg L^{-1} BAP in liquid MS medium gave the best response on callus induction as shown by biomass weight gain (1.55 g) compared with other treatment combinations. Analysis of variance showed that the addition of NAA

Table 1. Callus Fresh Weight (FW) gain (g) 30 days of suspension culture in MS medium

Treatment	Callus FW (g)				Mean
	BAP 0 mg L^{-1}	BAP 0.5 mg L^{-1}	BAP 1.0 mg L^{-1}	BAP 2.0 mg L^{-1}	
NAA 0 mg L^{-1}	0.19	0.22	0.21	0.38	0.25 ^{c}
NAA 0.1 mg L^{-1}	0.38	0.20	0.22	0.20	0.25 ^{c}
NAA 0.5 mg L^{-1}	0.46	0.83	0.65	0.45	0.60 ^{b}
NAA 1.0 mg L^{-1}	0.15	1.55	1.32	0.59	0.90 ^{a}
NAA 1.5 mg L^{-1}	0.73	0.95	0.20	0.82	0.68 ^{b}
NAA 2.0 mg L^{-1}	0.32	1.38	0.60	0.65	0.73 ^{ab}
NAA 3.0 mg L^{-1}	0.45	0.65	0.82	0.53	0.61 ^{b}
Mean	0.38^{b}	0,83^{a}	0,57^{b}	0,52^{b}	

Remarks: Values followed by the same letter are not significantly different in Duncan's multiple range test (DMRT) level $\alpha = 0.01$ for mean FW in treatment of NAA and DMRT $\alpha = 0.05$ for FW mean in treatment of BAP.

as much as 1.0 mg L^{-1} gave the best callus weight gain (0.90 g), but it was not significantly different from the treatment of 2.0 mg L^{-1} NAA, although it was significantly different from other treatments. Furthermore, the addition of 0.5 mg L^{-1} BAP gave the best results on callus FW gain (0.83 g), and significantly different from other treatments.Cell division during *in vitro* culture is influenced by endogenic and exogenic PGR. According to George and Sherrington (1984) callus could be formed by a balance of auxin content in explant high enough or when content of cytokines were very low or non-existent, hence auxin alone could induce the formation of callus on explants.

The existence of plant growth regulators in plants medium influenced inhibition of adventitious and axillary buds formation, but its presence in tissue cuture medium is needed to improve somatic embryogenesis in cell suspension cultures. Smith (2013) explains that the use of appropriate auxin concentrations in *in vitro* culture can stimulate callus formation and suppress morphogenesis. Pierik (1997) suggests that auxin can increase cell elongation, cell division and formation of adventitious roots. Barakat *et al*. (2010) repoted the best medium to induce callus derived from *Chrysanthemum* leaf explants on MS medium was with the incoporation of 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BAP.

3.2 Shoots Regeneration

Cell aggregates formed in the best treatment during suspension culture, was grown on solid MS medium for shoot initiation and regeneration by incoporating appropriate PGR treatments. Shoots or plantlets regeneration experiments examined the influence of combination of some PGR namely NAA and BAP, 2iP and BAP and the effect of a single plant growth regulators (TDZ) when incorporated to the solid MS medium. Result of the experiment in Table 2 showed that the rate of formation or regeneration of shoots, as well as the number of shoots formed. Result on observations of the rate of formation of shoots, number of shoots formed on each PGR treatment combination is shown Table 2, 3 and 4.

Table 2 and Figure 1 above showed that the addition of NAA (0.5 mg L^{-1}) and BAP (0.5 mg L^{-1}) promoted the fastest of shoot initiation or formation *i.e.*: average of 30 days after planting. Analysis of variance in the number of shoots observations showed that the addition of 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} BAP in the MS medium gave the highest number of shoots (5 shoots) and shoot length (2.9 cm). These results did not differ significantly by treatment of NAA (0.1 mg L^{-1}) and BAP (0.5 mg L^{-1}), but significantly different from other treatments.

Auxin and cytokinin are two kinds of plant growth regulators are often used to induce morphogenetic plant (Zulkarnain, 2007). BAP is a type of cytokines that are often used in conjunction with NAA (auxin) to initiate plant shoot. The results obtained by Ilahi *et al.* (2007) showed that MS medium containing 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP was the best medium to induce callus in C*hrysanthemum* using explants derived from internodes.

Table 3 showed that the addition of 2iP and BAP in MS medium gave the best response in rate of formation of shoots and

Table 2. The average rate of shoot regeneration initiation, number of shoots formed and shoot height on combination treatment of NAA and BAP 90 days after callus inoculation

Treatment	Shoot initiation (days)	Number of shoots (shoot)	Shoot Length (cm)
MS 0	40 ^{ab}	1 ^{def}	0,50 ^{ef}
MS + BAP 0,5 mg L^{-1}	0 ^{c}	0 ^{g}	0,00 ^{f}
MS + BAP 1,0 mg L^{-1}	0 ^{c}	0 ^{g}	0,75 ^{de}
MS + BAP 2,0 mg L^{-1}	37 ^{ab}	1 ^{efg}	0,00 ^{f}
MS + NAA 0,1 mg L^{-1}	0 ^{c}	0 ^{g}	0,00 ^{f}
MS + NAA 0,1 mg L^{-1} + BAP 0,5 mg L^{-1}	34 ^{ab}	3 ^{c}	2,50 ^{a}
MS + NAA 0,1 mg L^{-1} + BAP 1,0 mg L^{-1}	46 ^{a}	1 ^{efg}	1,38 ^{cd}
MS + NAA 0,1 mg L^{-1} + BAP 2,0 mg L^{-1}	34 ^{ab}	2 ^{cd}	0,75 ^{de}
MS + NAA 0,5 mg L^{-1}	33 ^{ab}	2 ^{de}	2,25 ^{ab}
MS + NAA 0,5 mg L^{-1} + BAP 0,5 mg L^{-1}	30 ^{b}	5 ^{a}	2,88 ^{a}
MS + NAA 0,5 mg L^{-1} + BAP 1,0 mg L^{-1}	40 ^{ab}	1 ^{fg}	1,13 ^{cde}
MS + NAA 0,5 mg L^{-1} + BAP 2,0 mg L^{-1}	34 ^{ab}	4 ^{b}	1,75 ^{bc}
MS + NAA 2,0 mg L^{-1}	0 ^{c}	0 ^{g}	0,00 ^{f}
MS + NAA 2,0 mg L^{-1} + BAP 0,5 mg L^{-1}	0 ^{c}	0 ^{g}	0,00 ^{f}
MS + NAA 2,0 mg L^{-1} + BAP 1,0 mg L^{-1}	0 ^{c}	0 ^{g}	0,00 ^{f}
MS + NAA 2,0 mg L^{-1} + BAP 2,0 mg L^{-1}	0 ^{c}	0 ^{g}	0,00 ^{f}
CV (%)	18	11	36

Remarks: Values followed by the same letter in the same column are not significantly different in Duncan's multiple range test (DMRT) level $\alpha = 0.05$. Zero value = No growth after 90 days observation. Callus stay green and growing, but no shoots.

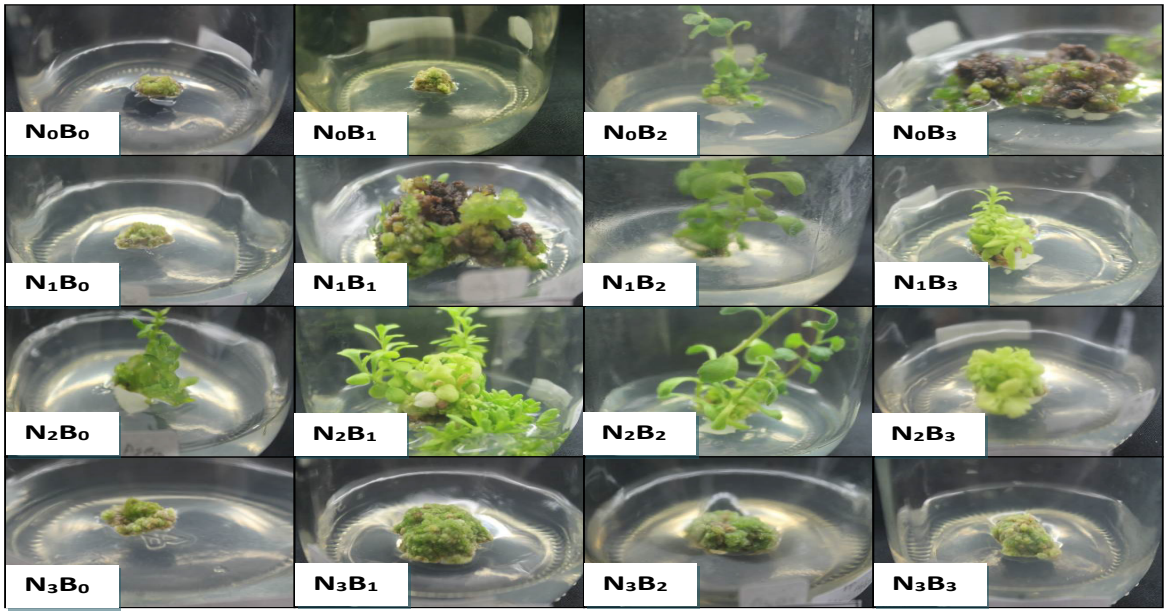

Figure 1. Shoots Regeneration on combination treatment of NAA and BAP, 90 days after callus inoculation. N_0 = without NAA; N_1 = NAA (0.1 mg L^{-1}); N_2 = NAA (0.5 mg L^{-1}); N_3 = NAA (1.0 mg L^{-1}). B_0 = without BAP; B_1 = BAP (0.5 mg L^{-1}); B_2 = BAP (1.0 mg L^{-1}); B_3 = BAP (2.0 mg L^{-1}).

shoots lenght. Shoots began to form on MS medium incorporated with 2iP (0.5 mg L^{-1}) and BAP (2.0 mg L^{-1}) at 30 days after inoculation giving highest shoot length (1.88 cm). Analysis of variance in the number of shoots observations indicated that the addition of 0.5 mg L^{-1} BAP without 2iP produced the highest number of shoots (3 shoots). The results showed no significant differences between the addition of 0.5 mg L^{-1} BAP or BAP 1.0 mg L^{-1}, but significantly different from other treatments.

Table 3. The average rate of shoot regeneration, number of shoots produced and shoot height on combination treatment of 2iP and BAP 90 days after inoculation

Treatment	Shoot initiation (days)	Number of shoots (shoot)	Shoot Length (cm)
MS 0	0 ^{b}	0 ^{b}	0,00 ^{c}
MS + BAP 0.5 mg L^{-1}	39 ^{a}	3 ^{a}	1,00 ^{ab}
MS + BAP 1.0 mg L^{-1}	44 ^{a}	2 ^{ab}	1,50 ^{ab}
MS + BAP 2.0 mg L^{-1}	0 ^{b}	0 ^{b}	0,00 ^{c}
MS + 2iP 0.1 mg L^{-1}	0 ^{b}	0 ^{b}	0,00 ^{c}
MS + 2iP 0.1 mg L^{-1} + BAP 0.5 mg L^{-1}	40 ^{a}	1 ^{ab}	0,50 ^{bc}
MS + 2iP 0.1 mg L^{-1} + BAP 1.0 mg L^{-1}	0 ^{b}	0 ^{b}	0,00 ^{c}
MS + 2iP 0.1 mg L^{-1} + BAP 2.0 mg L^{-1}	35 ^{a}	1 ^{ab}	0,50 ^{bc}
MS + 2iP 0.5 mg L^{-1}	0 ^{b}	0 ^{b}	0,00 ^{c}
MS + 2iP 0.5 mg L^{-1} + BAP 0.5 mg L^{-1}	0 ^{b}	0 ^{b}	0,00 ^{c}
MS + 2iP 0.5 mg L^{-1} + BAP 1.0 mg L^{-1}	0 ^{b}	0 ^{b}	0,00 ^{c}
MS + 2iP 0.5 mg L^{-1} + BAP 2.0 mg L^{-1}	30 ^{a}	1 ^{ab}	1,88 ^{a}
MS + 2iP 1.0 mg L^{-1}	0 ^{b}	0 ^{b}	0,00 ^{c}
MS + 2iP 1.0 mg L^{-1} + BAP 0.5 mg L^{-1}	0 ^{b}	0 ^{b}	0,00 ^{c}
MS + 2iP 1.0 mg L^{-1} + BAP 1.0 mg L^{-1}	0 ^{b}	0 ^{b}	0,00 ^{c}
MS + 2iP 1.0 mg L^{-1} + BAP 2.0 mg L^{-1}	42 ^{a}	0 ^{b}	0,00 ^{c}
CV (%)	20	24	19

Remarks: Values followed by the same letter in the same column are not significantly different in Duncan's multiple range test (DMRT) level α = 0.05. Zero value = No growth after 90 days observation. Callus stay green and growing, but no shoots.

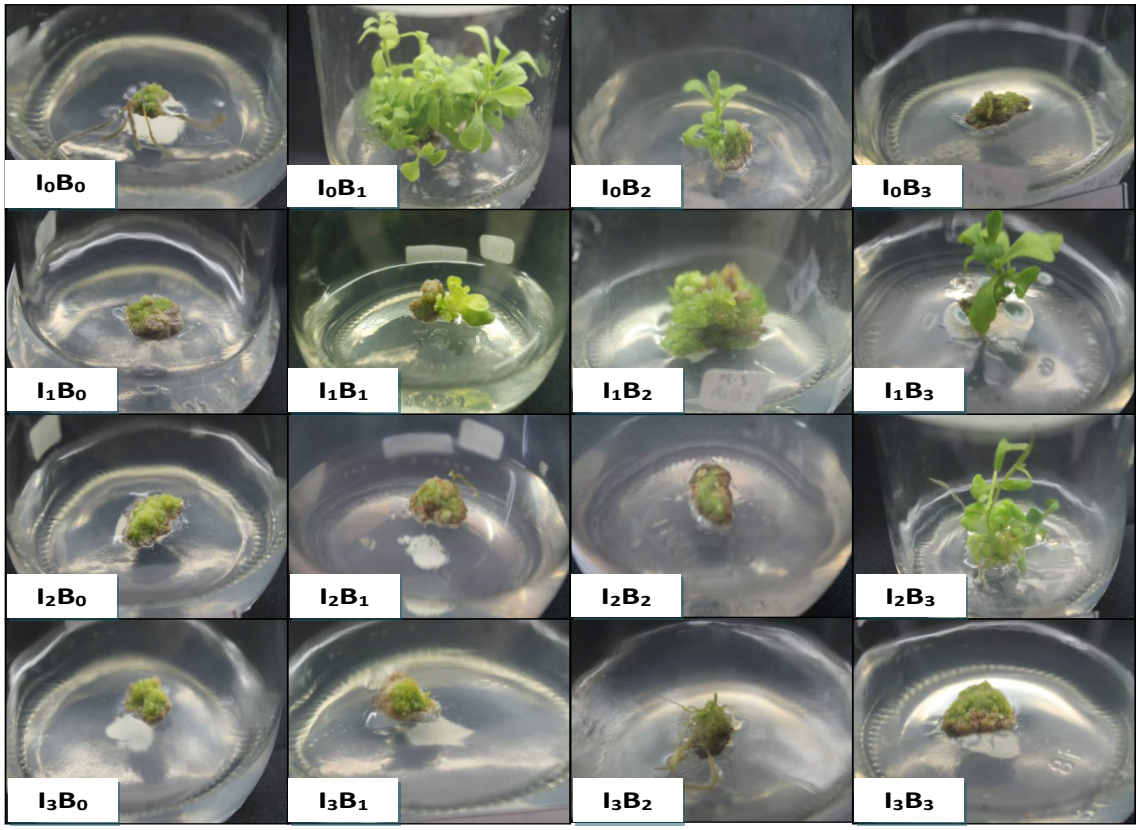

Figure 2. Plant regeneration experiment grown on MS medium supplemented with combinations of treatment of 2iP and BAP observed at 90 days after inoculation. I_0 = without 2iP; I_1 = 2iP (0.1 mg L^{-1}); I_2 = 2iP (0.5 mg L^{-1}); I_3 = 2iP (1.0 mg L^{-1}). B_0 = without BAP; B_1 = BAP (0.5 mg L^{-1}); B_2 = BAP (1.0 mg L^{-1}); B_3 = BAP (2.0 mg L^{-1}).

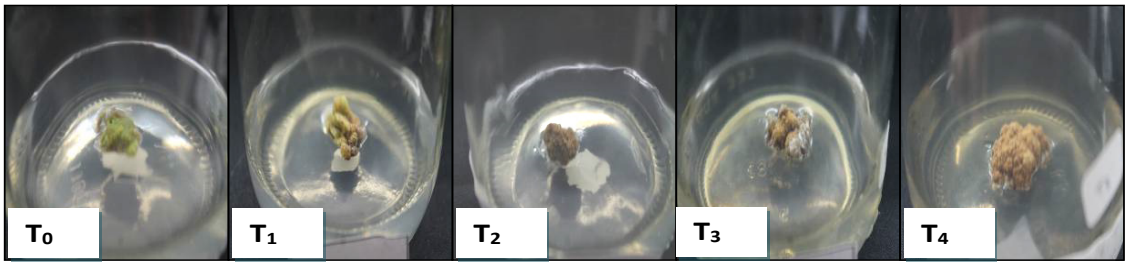

Figure 3. Regeneration in the treatment of TDZ on the observation of 90 days after planting. T_0 = without TDZ; T_1 = 0,5 mg L^{-1} TDZ; T_2 = 1,0 mg L^{-1} TDZ; T_3 = 1.5 mg L^{-1} TDZ; T_4 = 2,0 mg L^{-1} TDZ.

Where as for the parameters of the number of shoots, the best results were obtained in the treatment of the MS medium supplemented with BAP (0.5 mg L^{-1}) and without 2iP. However, the number of shoots produced was lowest, as many as 3 shoots. Results of this experiment showed that the use of media with different compositions lead to different responses to plant or shoot regeneration (Figure 2). Such conditions occured due to the interaction between 2iP and BAP as exogenous hormones found in plants which stimulated the formation of shoots, leaves and other morphological organ. According to Gunawan (1998), the interaction of growth regulators added in the medium and ones produced by the plant cells endogenously, determine the speed and direction of the development of a culture. Basri (2008) argued that a difference in the effectiveness of plant growth regulators on plant growth may be caused by two main aspects, namely the chemical base and its side chain groups.

The addition of TDZ in MS medium did not accelerate the emergence of shoots from callus explant (Table 4). Until the age of 3 months after inoculation the callus had not sprouted. Callus regenerated on media incoporated with 0.5-2.0 mg L^{-1} TDZ changed its original color from green to brownish color (Figure 3). Meaning that the addition of TDZ on regeneration medium inhibited the formation of shoots on the *Chrysanthemum* callus. At different concentrations, growth regulators can inhibit plant growth and even caused poisoning to the whole plantlet. Azwin *et al.* (2006) and Windujati (2011) confirmed that the addition of TDZ alone in the range hingher than to 0.25 mg L^{-1} in the planting medium may inhibit the formation of shoots and shoot length from leaf callus explants. Increased concentration above 0.5 mg L^{-1} as in this study may have corroborated the negative trend predicted (no growth or inhibited growth).

However, Thirukkumaran *et al.* (2009) have reported different reaction of TDZ on *Petunia hybrid* planted *in vitro.* Callus initiation occurred along the edges of explant within 2 weeks of culture in all media tested (0.5 – 5 mg L^{-1}) but not on medium without TDZ. Highest frequency of shoot regeneration (52.1%) was found on MS medium supplemented with 2 mg L^{-1} TDZ but was not significantly different with those observed at 1 or 3 mg L^{-1}. Furthermore, at lower (0.5 mg L^{-1}) or higher (4-5 mg L^{-1}) levels of TDZ, lower frequencies of shoot organogenesis were observed.

George and Sherrington (1984), stated that the effect of giving a concentration of growth regulators is different for each type

of crop different even between varieties within a species. Similarly, as for the concentration range. Improper concentration can cause unwanted effects, one of which can inhibit plant growth, can even cause toxicity in plants.

4. Conclusion

Addition of NAA 1.0 mg L^{-1} and BAP 0.5 mg L^{-1} to liquid suspension culture gives the best results in fresh weigh callus (1.55 g). While, the incorporation of NAA (0.5 mg L^{-1}) and BAP (0.5 mg L^{-1}) in the regeneration medium gave the best response to shoot initiation rate, growth and number of shoots formed. Similarly, the use of 2iP and BAP at 0.5 mg L^{-1} and 2.0 mg L^{-1} respectively, gave the best response to the early shoot initiation of *Chrysanthemum* and plant growth during *in vitro* culture but not on the number of shoots formed. Furthermore, the effect of TDZ inhibited plant regeneration when used in *Chrysanthemum* callus culture.

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