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Indirect Micropropagation of *Gerbera jamesonii* Bolus cv. Winter Queen through Callus Redifferentiation

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ABSTRACT

This research was achieved in the Laboratory of Plant Tissue Culture, Vegetable and Floriculture Department, Mansoura University, Egypt during 2015/2016 on *Gerbera jamesonii* Bolus cv. Winter Queen for studying the indirect micropropagation of this important pot and cut flower plant in Egypt. For callus induction, unopened flower buds(10 days old) were cultured after preparation on MS medium fortified with either 2,4-D or NAA (0.5, 1.0, 2.0, 4.0 and 6.0 mg/l) each alone. The calli were transferred on media supplemented with either BAP or *meta*-topolin (0.0, 0.3, 0.6, 1.2 and 2.4 mg/l) each alone with 0.5 mg/l NAA as a fixed concentration for redifferentiation. Moreover, shoots were transferred to media supplemented with NAA (0.0, 0.5, 1.0, 1.5 and 2.0 mg/l) with 0.3 mg/l *meta*-topolin for the *in vitro* rooting. Results showed that 6.0 mg/l 2,4-D produced the heaviest callus fresh weight (7.22 g), and decreased the callus induction time (17.50 days), but the callus texture was compact and creamish to brown in color. In addition, the lower concentrations from either 2,4-D (1.0 and 2.0 mg/l) or NAA (0.5 to 1.0 mg/l) produced a nodular greenish calli with greenish color. Also, supplying the redifferentiation media with 1.2 and 2.4 mg/l *meta*-topolin or 2.4 mg/l BAP, significantly reduced the redifferentiation time, increased the multiplication percentage and micro-shoots number. Moreover, ½ strength MS medium fortified with 2.0 mg/l NAA+0.3 mg/l *meta*-topolin gave the maximum roots number, rooting% and reduced the roots initiation time. Finally, well rooted plantlets were transferred to a mixture of peatmoss+perlite+clay (2:1:1 v/v) produced a survival percentage of 92.90% with a plantlets height of 4.61cm and 4.50 leaves / plantlet on average.

Abbreviations: MS; (Murashige and Skoog medium, 1962), 2,4-D; (2,4-Dichlorophenoxyacetic acid), NAA; (1-Naphthaleneacetic acid), BAP; (6-Benzylaminopurine), *meta*-topolin; [6-(3-hydroxybenzylamino)purine]

Keywords: callus dedifferentiation, callus redifferentiation, callus induction, multiplication, *in vitro* rooting.

INTRODUCTION

African daisy or Transvaal daisy (*Gerbera jamesonii* Bolus) is an important pot plant and cut flower with attractive colors. It is native to the Family Asteraceae and takes the fifth positions in the global cut flower exchange (FloraHolland Fact and Figures, 2015). The flowers are tough and strong enough to the freight and transport operations with very good postharvest characteristics which make it brings a decent market value (Chung *et al*., 2016). The gerbera species are perennial herbs which local to Asia and South Africa. It is growing and developing all through the world in an extensive variety of climatic conditions. Gerbera is generally utilized as a decorative garden plant, as well as cut flowers or potted plants because of its magnificence, colors and long shelf life. Routinely, gerbera is propagated through the sexual or the vegetative propagation (Kanwar and Kumar, 2008). Although the sexual propagation process through the seeds gives the maximum number of regenerants, yet it isn't favored as the plants show heterozygosity non-consistency and this protocol can't be utilized to build up the gerbera for commercial propagation (Winarto and Yufdy, 2017). On the other side, rhizomes division as a vegetative propagation produces and keeps up the genetic purity and uniformity, but it is arduous and tedious with less outcomes (Son *et al*., 2011). Along these lines clonal proliferation through plant tissue culture works are imperatively tended to a large vigorous plants number, pathogen free and uniform (Debergh and Maene, 1981; Van Den Dries *et al*., 2013). As of late, micropropagation has been perceived as the most effective way for the commercial propagation scale of the important plants within a short period in restricted space with all the advantages of the vegetative propagation which prompting methodical advancement of the floriculture business.

Several protocols for micropropagation of gerbera with differed results were performed by many researchers through using different explants of *gerbera jamesonii* cultivars; like using the capitulum (Ray *et al.*, 2005; Akter *et al.*, 2012), shoot tip (Gantait *et al.*, 2010; Cardoso and Teixeira da Silva, 2013; Nazari *et al.*, 2014), petal (Kumar and Kanwar, 2006), leaf (Kumar *et al.*, 2004; Altaf *et al.*, 2009), seeds (Nazari *et al.*, 2014), apical meristems and vegetative buds (Naz *et al.*, 2012), auxillary bud (Kadu, 2013).

The aim of the present research was to developing an indirect micropropagation protocol for African daisy (*Gerbera jamesonii* Bolus cv. Winter Queen) through callus induction from unopened flower buds with using some auxin types and concentrations, as well as improving the redifferentiation process of it by using some cytokinin types and concentrations. In addition, study the *in vitro* rooting and the acclimatization of this important pot and cut flower plant. As, the micropropagation protocols described by various scientists need to be improve, in addition to create a micropropagation protocol in line with Egypt conditions.

MATERIALS AND METHODS

This study was conducted during 2015/2016 in the Biotechnology Laboratory of Plant Cell and Tissue Culture, Vegetable and Floriculture Department, Faculty of Agriculture, Mansoura University, Egypt on *Gerbera jamesonii* Bolus cv. Winter Queen, plant.

Explant source and sterilization

G. *jamesonii* cv. Winter Queen growing in a polyethylene greenhouse in the Nursery of Ornamental and Floriculture plants, Faculty of Agriculture, Mansoura University, was the source of the explants (Figure, 1a). Young unopened flower buds (approximately 0.8 cm in diameter, 10 days old) were cut from the donor plants (Figure, 1b). Likewise, flower buds were pre-treated under running faucet water for 60 min., then immersing in 1% Tween 20 solution on magnetic stirrer for 30 min. After that 1% pesticide solution of Rizolix 50% (Tolclofos-methyl) was used for 30 min and rinsing 4-5 times (3 min each) by sterilized distilled water to remove all residuals disinfection materials. The explants were then moved to the laminar air flow cabinet for sterilization. The main

sterilizer was $HgCl_2$ at 0. 1% solution for 5min according to Kharrazi *et al.*, (2018) with adding 5 drops of Tween 20 for 5 min, followed by washing for 4-5 times (3 min each) through sterile distilled water.

Figure 1. Explants source, a; G. *jamesonii* cv. Winter Queen (mother plant), b; explant type (unopened flower bud of approximately 0.8 cm in diameter)

Callus induction treatments

Sterilized flower buds explants were cut from all of its sides for enhancing callus cells formation to reach 0.5 cm3 approximately. Then, the treated flower buds were transferred on MS (Murashige and Skoog, 1962) basal medium fortified with either 2,4-D or NAA (0.5, 1.0, 2.0, 4.0 and 6.0 mg/l) each alone for callus induction. It is worth to mention that the hormone-free medium did not induce any callus. So, it was omitted from the statistical analysis. All the media were fortified with 8 g/l agar and 30 g/l sucrose with a pH of 5.8 before sterilization in the autoclave. Data were recorded for callus induction period (days), callus induction percentage, callus fresh weight and callus morphology characteristics (texture and color).

Callus redifferentiation treatments

Nodular calli obtained from the previous experiment (1.0 and 2.0 mg/l 2,4-D or 0.5 and 1.0 mg/l NAA) were transferred on MS free hormone medium for 4 weeks to eliminate any residual hormones from the previous experiment. Then, callus pieces of approximately 1 cm3 were transferred on MS solid medium fortified with either BAP or *meta*-topolin each alone at different concentrations (0.0, 0.3, 0.6, 1.2 and 2.4 mg/l), in addition to a fixed concentration from NAA (0.5 mg/l) as recommended by Gantait *et al.*, (2010) on G. *jamesonii* cv. Sciella. Three subcultures were carried out after every 4 weeks interval. All the media were supplemented with 8 g/l agar and 30 g/l sucrose. Data were recorded after the third subculture for shoot initiation time (days), multiplication percentage, microshoots number/callus, microshoots length (cm) and microshoots total chlorophylls (mg/gfw.) according to Lichtenthaler (1987), since leaf chlorophylls was extracted by soaking 0.5 g of plantlets leaf tissues in 20 ml of 80% acetone. The absorbance of the extract was measured for chlorophyll a and b at 663.2 and 646.8 nm, respectively by a spectrophotometer. Then, total chlorophylls were calculated by using the following equations;

$$\textbf{Chlorophyll a (mg/gfw)} = 12.25\ A_{663.2} - 2.79\ A_{646.8}$$

$$\textbf{Chlorophyll b (mg/gfw)} = 21.50\ A_{646.8} - 5.10\ A_{663.2}$$

$$\textbf{Total chlorophylls (mg/gfw)} = 7.15\ A_{663.2} - 18.71\ A_{646.8}$$

***In vitro* rooting**

Microshoots with approximately 3cm long which derived from the previous treatment (callus redifferentiation) were excised and subjected to ½ MS basal medium supplemented with various concentrations of NAA (0.0, 0.5, 1.0, 1.5 and 2.0 mg/l) with a fixed concentration from *meta*-topolin (0.3 mg/l) for rooting. Data were recorded for the rooting initiation time (days), rooting percentage, roots number/plantlet and roots length (cm).

Incubation condition

All experiments were incubated at 25 ± 3°C and were exposed to a photoperiod of 16/8 hours light and dark cycling under 2000 Lux intensity provided by cool day light fluorescent tubes with 70% ± 3% relative humidity maintained in the culture room.

Acclimatization of the *in vitro* rooting plantlets

Plantlet acclimatization was prepared by pulling out well-rooted plantlets from the culture jars gently using forceps. Plantlet roots were put under running faucet water to remove the media and agar residual attaching them. The plantlet roots were then immersed in 1% Rizolix 50% (Tolclofos-methyl) for 5 min and air-dried on paper, then cultured in pre-sterilized 5cm plastic pots containing a mixture of peatmoss + perlite + clay (2:1:1 v/v). The cultured pots were watered with sufficient amount of ¼ strength MS salts medium, and then placed in a glass box covered with a polyethylene transparent for 15 days. In addition, after the first week, the polyethylene sheet was exposed gradually every day during the second week. These plantlets were then transferred to a polyethylene greenhouse in the nursery.

Statistical analysis

A completely randomized design (one way) was used with all the experiments. Numbers in each table represent the mean of twice repeated experiments of 4 replicates each contains 4 jars. COSTAT v.63 statistical software was used for analysis of variance (ANOVA) and subsequently Least Significant Differences (LSD) method according to Steel and Torrie (1980) was done for means comparison at $P \leq 0.05$.

RESULTS AND DISCUSSION

Callus induction

No callus was induced in hormone-free medium (control treatment), so it was omitted from the statistical analysis

Callus induction period (days)

As for auxin type and concentration on the callus induction period, data in Table (1) showed a relationship between increasing the concentrations of the two tested auxins (2,4-D and NAA) and the required time for starting callus induction. Since the least significant periods for the callus induction (17.50 and 18.75 days) were recorded for MS media supplemented with either 2,4-D or NAA at 6.0 mg/l each alone, respectively. Moreover, media fortified with either 2,4-D or NAA at the lowest concentration of 0.5 and 1.0 mg/l significantly retarded the time for the callus induction (42.75, 36.00, 45.25 and 33.50 days, respectively). In general, 2,4-D was superior for reducing the required number of days for starting callus induction comparing with the NAA one. This result was in the same trend which obtained by Akter *et al*., (2012) on G. *jamesonii* who revealed that callus initiation could appeared within 22 to 25 days from culturing the flower buds, but in case of our findings the required time was

more less and this might be according to the different in gerbera cultivars, growth regulators types and concentration. Subsequently, Arumugam *et al*., (2009) confirmed that the callus induction be based on many factors such as the culture environment, explant nature, and the growth regulator.

Table 1. Impact of auxin type and concentration on callus initiation characters from unopened bud flowers of G. *jamesonii* cv. Winter Qween after 6 weeks.

Auxin type and Conc.(mg/l)		Callus induction period (days)	Callus fresh weight (g)	Callus morphology	
				Texture	Color
2,4-D	0.5	42.75^{a}	4.17^{f}	friable	Creamish white
	1.0	36.00^{b}	4.41^{e}	nodular	Greenish white
	2.0	30.00^{c}	5.74^{b}	nodular	Greenish yellow
	4.0	22.00^{d}	5.85^{b}	compact	Creamish green
	6.0	17.50^{e}	7.22^{a}	compact	Creamish brown
NAA	0.5	45.25^{a}	3.16^{h}	nodular	Brownish green
	1.0	33.50^{b}	3.09^{h}	nodular	Greenish yellow
	2.0	29.75^{c}	3.67^{g}	compact	Greenish white
	4.0	20.50^{de}	4.59^{d}	compact	Greenish white
	6.0	18.75^{e}	5.34^{c}	compact	Greenish brown

Data in table represent means of twice repeated experiments and each number have the same letter in the same column indicate statistically non-significant difference at p < 0.05.

Callus induction percentage

Data illustrated in Figure (2) cleared that the highest callus induction percentages were obtained when pieces of closed flower buds were cultured on MS media fortified with 2,4-D at 4.0 and 6.0 mg/l or NAA at 6.0 mg/l, as it were 87.50, 93.75 and 81.25, respectively with non-significant differences between them. Similar results were obtained by Shabbir *et al*., (2012) on G. *jamesonii* cv. Sunglow, since they cleared that among the tested auxins 2,4-D was more effective in producing the highest callus induction percentage (100%), followed by NAA which produced 83.33%. On the other hand, the lower concentration from 2,4-D or NAA (0.5 and 1.0 mg/l), minimized the callus induction percentages for a range from 31.25 to 43.75%. These results were in harmony with Mohlakola *et al*., (2017) on G. *jamesonii* cv. Daxueju who revealed that among the plant growth hormone 2,4-D was more effective in increasing and producing the highest callus induction percentage of 96.70%. Also, they indicated that 2,4-D at the lowest concentration, negatively affected the callus formation and when the 2,4-D concentrations gradually increased, there was a positive increase in callus formation with the different in the explant type (petiole) and 2,4-D concentration (1.5 mg/l), and this might be a cultivar dependent.

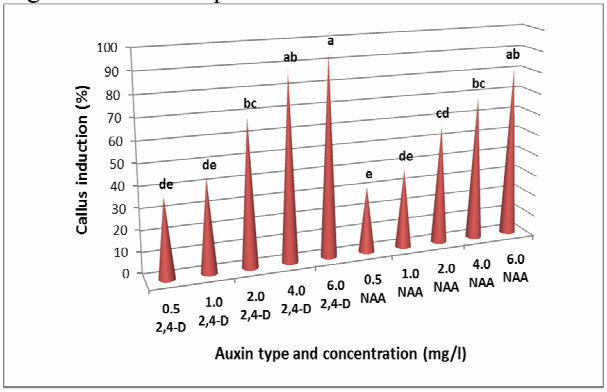

Figure 2. Impact of auxin type and concentration on callus induction % of G. *jamesonii* cv. Winter Queen after 6 weeks. Columns with the same letters indicate statistically non-significant difference at p < 0.05.

Koroch *et al*., (2003) on *Echinacea pallida* compared between auxins and cytokinins in callus induction percentages and found that auxins like (2,4-D and NAA) was more effective, since auxins stimulate the metabolism of RNA and improve the mRNA transcription of which produce the required proteins for callus formation and cell proliferation. Moreover, the optimal concentrations of 2,4-D or NAA on *Chrysanthemum morifolium* improve the enlargement and cells division which increase the synthetic enzymes and the autolytic activities through synthesis of cell wall components and its effects on cell wall plasticity (Nahid *et al*., 2007).

Callus fresh weight

From the data in Table (1), it was clear that 2,4-D at 6.0 mg/l significantly produced the heaviest callus fresh weight of 7.22g, when compared with all of the other treatments. This result emphasizes the result of Karimian *et al*., (2014) on *Taxus Brevifolia*, who found that 1.5 mg/l 2, 4-D was more effective on callus growth especially the callus fresh weight, but the 2,4-D optimal concentration varied with respect to explant types. In addition, media fortified with 2.0 and 4.0 mg/l 2,4-D or 6.0 mg/l NAA came in the second and the third order in that respect, since they were 5.85, 5.74 and 5.34g, respectively. The lightest callus fresh weight recorded for medium supplemented with NAA at concentrations of 0.5, 1.0 and 2.0 mg/l, as it were 3.16, 3.00 and 3.67g, respectively.

Auxins (NAA and 2,4-D) stimulate the cell elongation through activating the transportation process of the hydrogen ions away from the plant cells and decreasing the pH surrounding the cells. The acidification process surrounding the cell walls enhances and improves the cellulose micro-fibrils slippage and breaking the bonds of polysaccharides in the cell wall, which make the cell wall more flexible and then a vigorous callus growth will obtain (Taiz and Zieger, 2002). In addition, Can *et al*., (2008) stated that auxins increase the methylation of the nuclear DNA which makes the cells to enter in the redifferentiation process again and finally improve the cell division.

Callus morphology

Data in Table (1) and illustrated in Figure (3) cleared that a friable calli were formed with adding 2,4-D at the lowest concentrations of 0.5 mg/l. In addition, nodular calli were formed on MS medium fortified with 1.0 and 2.0 mg/l 2,4-D or 0.5 and 1.0 mg/l NAA. Moreover, the highest concentrations from either 2,4-D or NAA produced a

compact calli. As for callus color, it was noticed that the nodular calli which formed when 2,4-D or NAA were added to the growth media, have a greenish color mixed partially with another color as white, yellow or brown. Also, the friable or the compact calli which formed in case of using 2,4-D were creamish mixed with white, green and brown. Clearly, in most cases NAA concentrations a greenish calli contained some yellow, white or brown tissues were formed. This may be due to the findings of Shabbier *et al.* (2012) who reported that increasing 2,4-D concentrations turned the callus color of G. *jamesonii* cv. Sunglow to brown or dark brown when it pass the optimal level. In addition, Shirin *et al.*, (2007) revealed that the browning of the formed callus might be as a result of 2,4-D high concentration which inhibit the cell division and suppress synthesis of protein. Also, Mohlakola *et al.*, (2017) revealed that increasing the concentrations of 2,4-D derived a necrosis calli which made the calli tissues take a dark brownish color.

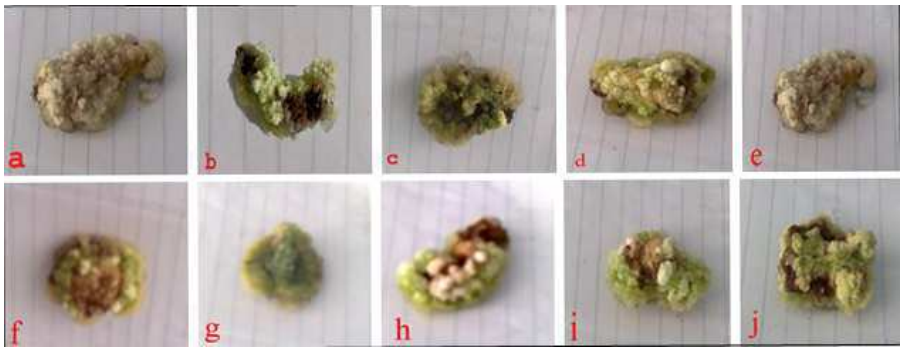

Figure 3. Impact of auxin type and concentration on callus morphology of G. *jamesonii* cv. Winter Queen after 6 weeks, as figures from a to e; represent effects of 2,4-D concentrations (0.5, 1.0, 2.0, 4.0 and 6.0 mg/l, respectively), and figures from f to j; represent effects of NAA concentrations (0.5, 1.0, 2.0, 4.0 and 6.0 mg/l, respectively).

Callus redifferentiation

Micro-shoots initiation time (days)

Impact of BAP and *meta*-topolin on micro-shoots initiation time from the callus tissues were recorded in Table (2). It can be observed that *meta*-topolin had the upper hand in minimizing the required time for micro-shoots initiation with its different concentrations comparing with the concentrations of BAP. Moreover, supplying the redifferentiation media by *meta*-topolin at 1.2 and 2.4 mg/l or 2.4 mg/l BAP, came in the first order in that respect, since they recorded 14.75, 15.00 and 15.25 days, respectively. On the other side, media fortified with 1.2 mg/l BAP + 0.5 mg/l NAA or 0.6 mg/l BAP + 0.5 mg/l NAA delayed the required time for micro-shoots initiation to reach 24.50 and 23.25 days, respectively. Therefore, Shabanpour *et al.*, (2011) indicated that the shoot regeneration process in gerbera is depended on plant growth regulators combinations and cultivars, so the regeneration procedure and the growth medium should be optimize for each cultivar.

Multiplication percentage

Data illustrated in Figure (4) showed that the highest multiplication percentages of 93.75% were recorded for MS medium fortified with 2.4 mg/l BAP + 0.5 mg/l NAA, followed by 87.50% for redifferentiation media supplemented with 1.2 or 2.4 mg/l *meta*-topolin. This finding was in a similar trend with Altaf *et al.*, (2009) who crushed a germinated seeds of G. *jamesonii* and cultured it on MS medium fortified with BA at 3.0 mg/l and obtained the highest multiplication percentage. In addition, Koszeghi *et al.*, (2014) on *Ocimum basilicum* cleared that fortifying the multiplication medium by BAP at 1.0 mg/l or *meta*-topolin at 0.5 improved the new shoots development characteristics, but *meta*-topolin still showing an increase comparing with the BAP one. Moreover, Bairu *et al*., (2006) on *Aloe polyphylla* used *meta*-topolin for inducing shoots multiplication and found that it increased the multiplication percentage and reduced the hyperhydricity. Also, in the current research adding 1.2 mg/l BAP + 0.5 mg/l NAA recorded 81.25% with non-significant differences between all the previous treatments. On contrary, the control medium (MS free hormone medium) failed to regenerate any micro-shoots from the cultured calli.

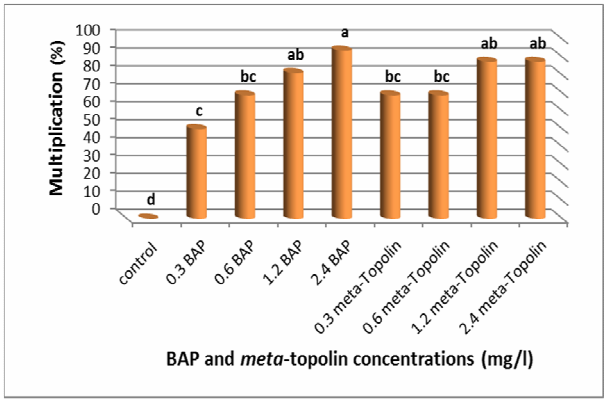

Figure 4. Impact of BAP and *meta*-topolin on multiplication % of G. *jamesonii* cv. Winter Queen after 6 weeks from culturing. Columns with the same letters indicate statistically non-significant difference at $p < 0.05$.

Micro-shoots number

As for the impact of BAP and *meta*-topolin on micro-shoots number derived from the redifferentiated calli of G. *jamesonii* Bolus cv. Winter Queen, data in Table (2) and illustrated in Figure (5) cleared that *meta*-topolin showed superiority than BAP especially with the higher concentrations (1.2 and 2.4 mg/l), when compared with all the other treatments. This result confirm the finding of Bairu *et al.*, (2006) who found a positive relationship between increasing the *meta*-topolin concentrations and the shoots number of *Aloe polyphylla*. Moreover, the lowest concentrations from BAP (0.3 and 0.6 mg/l) derived a lower micro-shoots number of 4.00 and 5.75 micro-shoots, respectively.

Table 2. Impact of BAP and *meta*-topolin on callus redifferentiation of G. *jamesonii* cv. Winter Queen after 6 weeks from culturing.

Cytokinin type and Conc.(mg/l)		Microshoots initiation time (days)	Microshoots number	Microshoots length (cm)	Total chlorophylls in microshoots(mg/gfw
Control		0.00^{f}	0.00^{f}	0.00^{c}	0.00^{h}
BAP	0.3+ 0.5 NAA	19.75^{c}	4.00^{e}	2.40^{b}	7.90^{d}
	0.6+ 0.5 NAA	23.25^{b}	5.75^{d}	4.15^{a}	6.29^{e}
	1.2+ 0.5 NAA	24.50^{a}	8.25^{c}	2.33^{b}	5.85^{f}
	2.4+ 0.5 NAA	15.25^{e}	13.00^{b}	1.35^{bc}	5.02^{g}
meta-topolin	0.3+ 0.5 NAA	22.50^{b}	6.50^{d}	2.45^{b}	9.43^{a}
	0.6+ 0.5 NAA	17.00^{d}	11.75^{b}	2.23^{b}	9.23^{ab}
	1.2+ 0.5 NAA	14.75^{e}	18.25^{a}	2.45^{b}	8.96^{b}
	2.4+ 0.5 NAA	15.00^{e}	17.25^{a}	2.12^{b}	8.57^{c}

Data in table represent means of twice repeated experiments and each number have the same letter in the same column indicate statistically non-significant difference at p < 0.05.

Figure 5. Impact of BAP and *meta*-topolin on micro-shoots number of G. *jamesonii* cv. Winter Queen after 6 weeks from culturing, as letters from a to d represent effects of BAP concentrations (0.3, 0.6, 1.2 and 2.4 mg/l, respectively) and letters from e to h represent effects of *meta*-topolin concentrations (0.3, 0.6, 1.2 and 2.4 mg/l, respectively).

Micro-shoots length (cm)

A negative effect was shown in Table (2) as a result of increasing the concentrations of BAP. The matter was different with the other cytokinin one (*meta*-topolin) since there was non-significant differences between all its tested concentrations, but it still give a shortest micro-shoots. On the other side, the optimal concentration of BAP which gave the tallest micro-shoots (4.15 cm) was 0.6 mg/l + 0.5 mg/l NAA. In contrast, MS medium supplemented with 2.4 mg/l BAP + 0.5 mg/l NAA produced the shortest micro-shoots of 1.35 cm. As, supplementing the growth medium by BAP, improve and increase the biosynthesis of cytokinins through coding the enzymes which enhance conversion of adenosine-monophosphate (AMP) and dimethylallyl-diphosphate (DMAPP) to isopentenyladenosine-5-monophosphate (iPMP) and this improve the micro-shoots length (Haberer and Kieber, 2002). Also, the high doses from cytokinins may inhibit the shoots elongation because of increasing ethylene production in the cell which increases the inhibition of protein synthesis, giving a shortened shoots (Werner *et al.*, 2001).

Total chlorophylls in micro-shoots

Total chlorophylls in micro-shoots were analyzed as an indicator for the vitrification problem and the data was shown in Table (2). All over the tested concentrations from BAP and *meta*-Topolin, it was obvious that all the *meta*-topolin concentrations still giving higher values for that respect as it ranged from 8.57 to 9.43 mg/gfw, comparing with BAP concentrations which ranged from 5.02 to 7.90 mg/gfw. Also, Mutui *et al.*, (2012) sprayed various growth regulators on *Pelargonium×hortorum* and found that *meta*-topolin had significant effects in increasing the total chlorophylls and it is very active in reducing the leaf senescence. Moreover, Anuraj *et al.*, (2017) stated that supplemented the *in vitro* growth medium with 2.5 mg/l *meta*-topolin improved the tolerance of *Gracilaria corticata* var. cylindrica to the salinity condition and increased the total chlorophylls and carotenoids. In general, our results showed that the superior treatment in increasing the total chlorophylls and then decreasing the vitrification in the micro-shoots was recorded for MS medium fortified with either 0.3 or 0.6 mg/l *meta*-topolin which showed a significant differences comparing with all the other cases.

***In vitro* rooting process**

Rooting initiation time (days)

Impact of NAA concentrations with a fixed concentration from *meta*-Topolin (0.3 mg/l) in the rooting initiation time was recorded in Table (3). There was a relationship between NAA concentrations and the rooting initiation time, since increasing concentrations of NAA directly decreased the essential time (days) which needed for the roots initiation. So, ½ strength MS medium contained 2.0 mg/l NAA + 0.3 mg/l *meta*-topolin significantly minimized the required period for the rooting initiation to 13.00 days comparing with the control which delayed the essential period to reach 29.75 days.

Table 3. Impact of NAA concentrations with *meta*-topolin at a fixed concentration (0.3 mg/l) in half strength MS medium on rooting characters of G. *jamesonii* cv. Winter Queen after 4 weeks from culturing.

Rooting treatments (mg/l)	Rooting initiation time (days)	Rooting %	Roots number/shoot	Roots length (cm)
Control	29.75^{a}	18.75^{c}	2.25^{e}	4.55^{a}
NAA 0.5+ 0.3 *meta*-topolin	24.00^{b}	31.25^{c}	4.29^{d}	3.88^{ab}
NAA 1.0+ 0.3 *meta*-topolin	17.25^{c}	50.00^{b}	5.50^{c}	4.73^{a}
NAA 1.5+ 0.3 *meta*-topolin	13.50^{d}	81.25^{a}	8.27^{b}	4.23^{a}
NAA 2.0+ 0.3 *meta*-topolin	13.00^{d}	93.75^{a}	9.75^{a}	3.02^{b}

Data in table represent means of twice repeated experiments and each number have the same letter in the same column indicate statistically non-significant difference at p < 0.05.

Rooting percentage

Data illustrated in Figure (6) indicated that the highest rooting percentage of 93.75 and 81.25% resulted from supplying 2.0 mg/l NAA + 0.3 mg/l *meta*-topolin or 1.5 mg/l NAA + 0.3 mg/l *meta*-topolin into ½ strength MS medium. The least rooting percentage of 18.75% derived from the control medium (½ strength MS free hormone medium). Gentile *et al.*, (2014) cleared that fortifying the rooting medium by *meta*-topolin produced a higher rooting percentage, so in the current research we combined *meta*-topolin with NAA for increasing the rooting percentage.

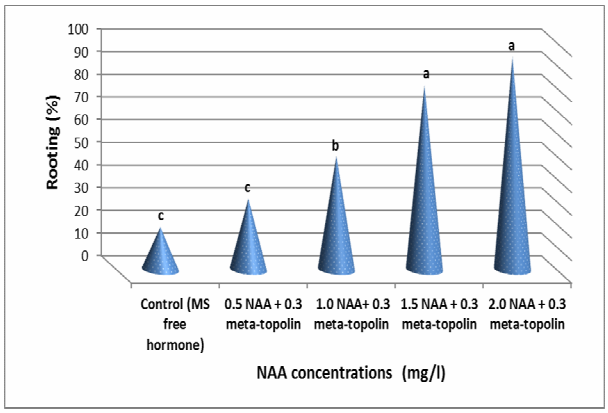

Figure 6. Impact of NAA concentrations with *meta*-topolin at a fixed concentration (0.3 mg/l) on the rooting % of G. *jamesonii* cv. Winter Queen after 4 weeks from culturing. Columns with the same letters indicate statistically non-significant difference at $p < 0.05$.

Roots number

In a similar way, Figure (7) showed that the superior treatments in increasing the rooting percentage also increased the roots number per plantlet. As, medium fortified with 2.0 mg/l NAA + 0.3 mg/l *meta*-topolin significantly gave the maximum number of roots (9.75 roots/plantlet), followed by 8.27 roots/ plantlet for medium contained 1.5 mg/l NAA+ 0.3 mg/l *meta*-topolin. In addition, the control medium recorded the least value of 2.25 roots/ plantlet. A different result was obtained by Warar *et al.*, (2008) who reported healthy roots of G. *jamesonii* cv. Sciella formed on MS medium fortified with 0.5 mg/l NAA, but Hasbullah *et al.*, (2008) confirmed our findings, but with replacing the fixed concentration of *meta*-topolin by using 0.1 mg/l BAP in combination with 2.0 mg/l NAA, since this treatment increased the roots number value. In addition, Werbrouck *et al.*, (1995) on *Spathiphylum flouribundum* found that micro-shoots which treated by *meta*-topolin significantly produced a higher roots number and length.

Roots length (cm)

As for roots length, data in Table (3) and illustrated in Figure (7) indicate that non-significant difference was shown between most of the tested NAA concentrations, but it was obvious that the control medium and media supplemented with 1.0 mg/l NAA + 0.3 mg/l *meta*-topolin and 1.5 mg/l NAA + 0.3 mg/l *meta*-topolin still improving the roots length, since they recorded 4.55, 4.73 and 4.23 cm, respectively. Clearly, the highest NAA concentration of 2.0 mg/l + 0.3 mg/l *meta*-topolin produced the shortest roots length of 3.02 cm. This result was agreed with the finding of Nazari *et al.*, (2014) who showed non-significant differences between using NAA at 0.5 and 1.0 mg/l on roots length. In addition, higher concentration of NAA reduced the growth of roots as a result from increasing biosynthesis of endogenous ethylene (Salisbury and Ross, 2005).

Figure 7. Impact of NAA concentrations with *meta*-topolin at a fixed concentration (0.3 mg/l) on *in vitro* roots number of G. *jamesonii* cv. Winter Queen after 4 weeks from culturing, as a; represent the rooting control medium (MS free hormone), figures from b to e; represent NAA concentration (0.5, 1.0, 1.5 and 2.0 mg/l, respectively).

Acclimatization process

Individual rooted plantlets were transferred to the mixture of Peatmoss + perlite + clay (2:1:1 v/v) gave a survival percentage of 92.90% with a plantlets height of 4.61cm and 4.50 leaves/plantlet (Figure, 8). This high response could be a result of the mixture ability to provide enough aeration and moisture to the plantlet. This confirm the findings of Weaam *et al.*, (2015) on *Holmskioldia sanguinea* who cleared that mixing several acclimatization growing medium is very important to produce a vigor plantlets with a good characteristics. In addition, they illustrated that the adaptation media must provide a good water balance with suitable water supply. Also, Ravanfar *et al.*, (2011) cleared that perlite is a vital commodity in the adaptation potting mixture if it mixed with peatmoss, since adding the perlite to the peatmoss improving the air amounts (oxygen) which held in the peatmoss beside the suitable water content in the peatmoss.

Figure 8. Acclimatized G. *jamesonii* cv. Winter Queen plantlet cultured in a mixture of peatmoss + perlite + clay (2:1:1 v/v) after 4 weeks from the adaptation process.

CONCLUSION AND RECOMMENDATION

Indirect micropropagation procedure of *Gerbera jamesonii* Bolus cv. Winter Queen through callus induction and redifferentiation was achieved following the next stages. Unopened flower buds (10 days old) were taken from pot plants inside a polyethylene greenhouse and placed under running tap water for one hour, then soaked in 0.1% fungicide solution (Rizolix 50%,Tolclofos-methyl) for 30 min., the main sterilizer was $Hgcl_2$ at 0. 1% for 5min. For obtaining the highest green and nodular callus induction, we economically recommend to use MS full strength medium fortified with 1.0 mg/l 2,4-D or 0.5 mg/l NAA. After transferring these calli on solid MS free hormone medium for 4 weeks to eliminate any auxins residuals, callus pieces of approximately 1 cm^3 cultured on MS medium fortified with 1.2 mg/l *meta*-topolin for obtaining a higher multiplication percentage (87.50%) and micro-shoots number (18.25), as it also decreased the hyperhydricity through increasing the total chlorophylls (8.96 mg/gfw.) comparing with the BAP one. The best rooting medium for increasing the rooting percentage (93.75%), roots number (9.75 roots/plantlet) and decreased the required time for rooting initiation was ½ MS basal medium augmented with 2.0 mg/l NAA + 0.3 mg/l *meta*-topolin. Plantlets were acclimatized in a mixture of peatmoss + perlite + clay (2:1:1 v/v) produced a survival percentage of 92.90%, plantlet height of 4.61cm and 4.50 leaves/plantlet. Finally, for further studies a finger print for the donor plant (mother plant) and the derived plantlets from the callus redifferentiation must be done, as the culture condition and the plant growth regulators may effect on the cultivar stability which could use in the breeding programs for that important pot and cut flower plant.

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الإكثار الدقيق الغير مباشر للجربيرا من خلال إعادة تميز الكالس

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تم تنفيذ البحث بمعمل زراعة الانسجة النباتية بقسم الخضر والزينة كلية الزراعة جامعة المنصورة، مصر خلال الفترة 2016/2015 على نبات الجربيرا صنف ملكة الشتاء بهدف الإكثار الدقيق الغير مباشر لهذا النبات المستخدم كنبات أصص مزهر أو زهرة قطف مهمة تحت الظروف المصرية. لحث وإنتاج الكالس, تم زراعة براعم زهريه غير متفتحة (عمر عشر ايام) بعد تعقيمها وتجهيزها على بيئة موراشيج وسكوج كاملة القوى والمزوده بتركيزات مختلفه من 2,4-D أو NAA (0.5 , 1.0 ,2.0 ,4.0 و6.0 ملليجرام/ لتر) كل على حده . ولإعادة التميز أو التكشف للكالس فقد تم نقله الى بيئات محتويه على تركيزات مختلفه لكل من BAP أو *meta*-topolin (0.0، 0.3، 0.6، 1.2و 2.4 ملليجرام/اللتر) كل على حده بالإضافة إلى تركيز ثابت من NAA (0.5 ملليجرام/اللتر). علاوة على ذلك فقد تم نقل النموات الخضرية (بطول 3سم) الى بيئات التجذير والمحتوية على تركيزات مختلفة من NAA (0.0، 0.5، 1.0، 1.5 و 2.0 ملليجرام/اللتر) بالإضافة إلى تركيز ثابت من *meta*-topolin (0.3 ملليجرام/اللتر). أظهرت النتائج أن تزويد بيئة إنتاج الكالس بـ 6.0 ملليجرام/اللتر 2,4-D قد اعطت أعلى وزن طازج للكالس، كما قللت الوقت اللازم لحث وإنتاج الكالس ولكن كان الكالس المتكون مندمج وذو لون كريمى إلى بنى. بالإضافة إلى ذلك فإن التركيزات المنخفضة لكل من 2,4-D (1.0 و 2.0 ملليجرام/اللتر) او NAA (0.5 و 1.0 ملليجرام/اللتر) قد أنتجت كالس ذو عقد أو مبادئ نموات وباللون الأخضر. كذلك فإن تزويد بيئة التميز أو التكشف بـ *meta*-topolin بتركيزات 1.2 ، 2.4 ملليجرام/اللتر أو BAP بتركيز 2.4 ملليجرام/اللتر بالإضافة للتركيز الثابت من NAA (0.5 ملليجرام/اللتر) أدت الى تقليل الوقت اللازم للتميز وزيادة النسبة المئوية للتضاعف وكذلك عدد النموات الخضرية. علاوة على ذلك فإن تزويد بيئة موراشيج وسكوج ذات النصف قوى ب 2.0 ملليجرام على اللتر NAA + 0.3 ملليجرام/اللتر *meta*-topolin قد أعطت أكبر عدد للجذور ، أعلى نسبة مئوية للتجذير وكذلك خفضت الوقت اللازم لبدء التجذير. وأخيرا فقد تم أقلمة النبيتات بمخلوط أقلمة مكون من البيتموس + البرليت + التربة الطينية (2: 1: 1 بالحجم) بنسبة مئوية للبقاء92.90% ، طول النبيتات4.61 سم و عدد 4.50 ورقة/ النبيتة.