



Effect of Explant Type, Benzyl amino purine (BAP), Type of Media and Light Intensity on In Vitro Shoot Multiplication of *Chrysanthemum morifolium* Ramat

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ABSTRACT

This study was conducted to evaluate the effect of explant type (shoot tips and lateral buds); different concentrations of the cytokinin 6-Benzyl amino purine (BAP) at 1, 2, 4, 6, 8 mg/l; the organic acid (ascorbic acid) at 20 and 40 mg/l; the amino acid L-glycine at 30 and 60 mg/l; liquid medium overlay onto solid media (0, 4, 8, 12, 16 ml of liquid medium) and light intensity (0, 1.4, 7, 14 $\mu\text{mol m}^{-2} \text{s}^{-1}$) on in vitro shoot multiplication in *Chrysanthemum morifolium*. The completely randomized design was used in these experiments. The multiplication parameters collected were percentage of explants with shoots, number of shoots/explant, shoot length, number of leaves/explant and number of nodes/shoot. Data were subjected to analysis of variance. Means were separated by Duncan's multiple range test, and T-test. With regard to the parameter percentage of explants with shoots, all levels of all factors tested resulted in 100% explants with shoots. For the other parameters there was no significant difference between shoot tips and lateral buds. Significant differences were recorded as a result of BAP concentrations. The low concentrations of BAP (1 and 2 mg/l) significantly stimulated multiplication parameters. The supplement of ascorbic acid at low concentration (20 mg/l) significantly enhanced multiplication. Incorporation of L-glycine at the concentration of 30 or 60 mg/l in the medium enhanced multiplication significantly compared to the control. Addition of liquid medium onto the surface of solid medium stimulated all growth parameters significantly. Cultures incubated under dark condition resulted in significant increase in multiplication but the resultant shoots exhibited clear symptoms of etiolation. The study concluded that the use of lateral buds as explants, BAP at 1 mg/l, ascorbic acid at 20 mg/l, glycine at 30 mg/l, 16 mls of liquid medium as overlays onto solid medium and light intensity of 14 $\mu\text{mol m}^{-2} \text{s}^{-1}$ is suitable within a protocol for micropropagation of *Chrysanthemum morifolium*. *Guava, Pakistani, Physico-chemical, Shendi*

KEYWORDS:

In Vitro multiplication; *Chrysanthemum morifolium*; Benzylaminopurine; Organic acids; Amino acids; Light intensity.

INTRODUCTION

Chrysanthemum (*Chrysanthemum morifolium* Ramat) belongs to the family Asteraceae (Arora, 1990). Today, it is the world's second most economically important floricultural crop following the rose. It is popular for flower arrangements due to its long vase life (Ilahi *et al*, 2007). The flower is widely used as a healthy beverage, a useful food and folk medicine in China (Ostrovsky, 2015). Yellow or white *Chrysanthemum* flowers are boiled to make a sweet drink in some parts of Asia especially in China. It is known as *Chrysanthemum* tea. This tea is often used to cure some diseases such as influenza. The leaves are steamed or boiled and used as greens. The flowers are important as natural source of insecticides called pyrethrins. *Chrysanthemum* is propagated by seeds and vegetatively either through suckers, root divisions or terminal cuttings (Bhaskar, 2011). The conventional process of shoot cutting is very slow. Clonal propagation through *in vitro* culture can enhance multiplication many folds (Nalini, 2012). Regeneration through *in vitro* culture has become a viable alternative to the conventional propagation methods. Due to the high popularity and demand, *Chrysanthemum* has become one of the first commercial targets for micropropagation, and thus tissue culture can be utilized for its large scale production (Levin *et al.*, 1988). Khan *et al.* (1994) observed shoot proliferation onto MS medium supplemented with 0.5 and 1.0 mg/l BAP. At 2.0 mg/l BAP shoot regeneration was higher but they were compact and stunted in growth. Karim *et al.* (2002) reported that frequency of multiple shoot regeneration response was 95% and 91% for *Chrysanthemum* nodal segments and shoot tips, respectively, when cultured on the medium containing MS with 0.1 mg/l BAP. Mohamed (2010) observed that in *Chrysanthemum* cultures, 0.1 mg/l BAP and 0.1, 0.2 mg/l of Kinetin were the best with regard to shoots initiation as they resulted in the highest number of shoots per explant. Growth regulators - free medium gave significantly the highest length of shoot. Organic acids can have three roles in plant culture media: they may act as chelating agents, improving the availability of some micronutrients, they can buffer the medium against pH changes and they may act as nutrients (George *et al*, 2008). Dougall *et al.* (1979) found that 20 mM succinate, malate or fumarate supported maximum growth of wild carrot cells when the medium was initially adjusted to pH 4.5. Although cultured cells are normally capable of synthesizing all of the required amino acids, the addition of certain amino acids or amino acid mixtures may be used to further stimulate cell growth. Abdelmuttalib (2009) reported that the amino acids are sources of organic nitrogen. Amino acids are not essential ingredients of media, but beneficial responses were reported upon their incorporation in media of lime. According to Mohammed (2012), addition of either L-glycine or L-asparagine at low concentration promoted growth and proliferation of ginger cultures. The correct choice of explants material can have an important effect on the success of tissue culture (Chaddha, 2011). The three features of light which influence *in vitro* growth, are wavelength, flux density and the duration of light exposure or photoperiod (George, 1993). Shoot cultures of *Pelargonium* have been established and encouraged to form multiple shoots under continuous fluorescent light of $7 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Debergh and Maene, 1977). Aika (2010), obtained multiplication of tissue cultured banana upon incubation under light intensity of only $1.4 \mu\text{mol m}^{-2} \text{s}^{-1}$. The objective this study was to evaluate the effect of explant type, different concentrations of the cytokinin BAP, the organic acid (ascorbic acids), the amino acid L-glycine, liquid medium overlay onto solid media and light intensity on *in vitro* shoot multiplication in *Chrysanthemum morifolium*.

MATERIALS AND METHODS

This study was conducted at the plant tissue culture laboratory of the College of Agricultural Studies, Sudan University of Science and Technology during the year 2013. Greenhouse *Chrysanthemum morifolium* plants were used as primary explant source. The medium used for primary culture initiation was MS (Murashige and Skoog, 1962) medium with Thiamine-HCl at 0.4 mg/l, Sucrose at 30 g/l, BAP at 0.5 mg/l, NAA (Naphthalene acetic acid) at 0.1mg/l, Agar at 7 g/l and Activated charcoal at 1 g/l. The pH of all media was adjusted to 5.6. Twenty five mls of medium were distributed in glass culture tubes capped with medical cotton plugs and aluminum foil for culture initiation and 50 ml in Jam glass jars for the experiments. The media were then autoclaved at 121°C and a pressure of 1.05 kg/ cm² for 20 minutes. For primary culture initiation nodal buds were taken from the middle portion of the *Chrysanthemum* shoots, washed in running tap water for 1 hour and immersed in 15% commercial bleach (Clorox) with 2 drops/100 ml of Tween 20 for 15 minutes with continuous shaking. They were then rinsed 3 times with autoclaved distilled water. The cultures were placed at 25 ±1°C under continuous fluorescent light intensity of 14 μmol m⁻² s⁻¹ with 16 h / 8 h light / dark cycle. Six experiments were performed using segments of sterile *in vitro* raised shoots resulting from the primary culture.

Influence of explant type:

Two types of explant were tested in the first experiment which were shoot tips and lateral buds.

Explants were inoculated onto MS medium with Thiamine-HC at 1 0.4 mg/l, Sucrose at 30 g/l, BAP at 0.5 mg/l, NAA at 0.1 mg/l, Agar at 7 g/l and Activated charcoal at 1 g/l.

Effect of BAP:

Effect of different concentrations of BAP was tested in the second experiment. Explants were inoculated onto MS medium with Thiamine-HCl at 0.4 mg/l, Sucrose at 30 g/l, NAA at 0.1 mg/l, Agar at 7 g/l and Activated charcoal at 1 g/l. The BAP concentrations tested were: 0, 1, 2, 4, 8 mg/l.

Influence of ascorbic acid:

Different concentrations of ascorbic acid were tested in the third experiment. Explants were inoculated onto MS medium with Thiamine-HCl at 0.4 mg/l, Sucrose at 30 g/l, BAP at 1 mg/l, NAA at 0.1 mg/l, Agar at 7 g/l and Activated charcoal at 0.5 g/l. The concentrations of ascorbic acid tested were: 0, 20, 40 mg/l.

Influence of glycine:

Different concentrations of glycine were tested in the third experiment. Explants were inoculated onto MS medium with Thiamine-HCl at 0.4 mg/l, Sucrose at 30 g/l, BAP at 1 mg/l, NAA at 0.1 mg/l, Agar at 7 g/l and Activated charcoal at 0.5 g/l. The concentrations of glycine tested were: 0, 30, 60 mg/l.

5. Effect of adding liquid medium as overlay onto solid medium:

Different concentrations of liquid medium as overlay onto solid medium were tested in the fifth experiment. Explants were inoculated onto MS medium with Thiamine-HCl at 0.4 mg/l, Sucrose at 30 g/l, BAP at 1 mg/l, NAA at 0.1 mg/l, Agar at 7 g/l and Activated charcoal at 1 g/l. The overlay liquid medium was MS medium with Thiamine-HCl at 0.4 mg/L, Sucrose at 20 g/l, BAP at 1 mg/l, NAA at 0.1 mg/l. The pH was adjusted to 5.2. The different concentrations of medium for overlay tested were 0, 4, 8, 12, 16 ml/ GA7 culture vessel.

Influence of different levels of light intensity:

Different light intensities were tested in the sixth experiment. Explants were inoculated on MS medium with Thiamine-HCl at 0.4 mg/l, Sucrose at 30 g/l, BAP at 1 mg/l, NAA at 0.1 mg/l, Agar at 7 g/l and Activated charcoal at 0.5 g/l. The levels of light intensity tested were: 0, 1.4, 7, 14 $\mu\text{mol m}^{-2} \text{s}^{-1}$. A completely randomized design with 10 replications was used for all experiments. Each culture vessel contained 2 explants. Statistical analysis was performed using Mstat-C computer program. Mean separation was done using Duncan's multiple range test (DMRT) at 5% level of significance. The T-test was used for means comparison in the first experiment (explant type). The Parameters measured in all experiments were percentage of explants with shoots, number of shoots/explant, shoot length, number of leaves/explant and number of nodes/shoot. The parameters were measured 6 weeks after culture initiation.

RESULTS AND DISCUSSION

With regard to the parameter percentage of explants with shoots, all levels of all factors tested resulted in 100% explant response (tables 1, 2, 3, 4, 5, 6). This might be due to the use of sterile *in vitro* raised shoots as explant source in this study. In a study conducted in Brazil by Victório *et al* (2012), nodal segments from apical, intermediate and basal region were excised from 45-day-old clonal (*in vitro*) plantlets of *Calendula officinalis* and placed on MS medium. They obtained 100% shoot regeneration from all explant sources. For the rest parameters measured in this study results were as follows:-

Influence of explant type:

According to Table 1, no significant difference was observed between shoot tip and lateral bud explants. This indicates no influence of explant on cultures performance. Hence, the use of lateral buds seems to be of preference due to its abundance. The result is in line with that reported by Karim *et al.* (2002) who found that frequency of multiple shoot regeneration response was 95% and 91% for *Chrysanthemum* nodal segments and shoot tips, respectively, when cultured onto a medium containing MS + 0.1 mg/l BAP.

Table 1. Effect of type of explant on performance of *in vitro* cultures of *Chrysanthemum morifolium* after 6 weeks.

Types of explant	% of explants with shoots	Shoots no.	Shoot length (cm)	Leaves no.	Nodes no.
Shoot tip	100	2.1a	6.2a	12.3a	11.3a
Lateral bud	100	2.5a	5.6a	19.7a	18.7a

Means within column with the same letter(s) are not significantly different at P= 0.05 according to t-test.

Effect of BAP:

As shown in table 2, application of different concentrations of BAP resulted in significant differences in number of shoots/explant, shoot length, number of leaves/explant and number of nodes/shoot. The use of 2 mg/l BAP excelled all other treatments for shoot number although it did not differ significantly from the 1 mg/l BAP treatment. The least shoot number was obtained from the control which was devoid of BAP. The control shoots were the longest with significant difference from all cultures containing cytokinins. The shortest shoots resulted from the concentration 4 mg/l of BAP. The leaf number was significantly enhanced by the 1 mg/l BAP treatment. The minimum leaf number was observed in 4 mg/l BAP. Similarly, the node number was significantly increased by 1 mg/l BAP treatment. The 4 mg/l BAP treatment resulted in the least node number compared with the other treatments. Results indicate that proliferation of axillary shoots was remarkably influenced by the concentrations of BAP. The response was enhanced by low rather than high concentrations. Such results had been reported by Khan *et al.* (1994) who observed shoot proliferations upon use of MS medium containing 2.0 mg/l BAP, but shoots were compact and stunted in growth. Mohamed (2010) observed that 0.1 mg/l of BAP and 0.1- 0.2 mg/l of Kinetin were the best for shoot initiation as they resulted in the highest number of shoots per explant.

Table 2. Effect of BAP concentration on performance of *in vitro* cultures of *Chrysanthemum morifolium* after 6 weeks.

BAP conc. (mg/l)	% of explants with shoots	Shoots no.	Shoot length (cm)	Leaves no.	Nodes no.
Control	100	2.6e	7.4a	24.0de	23.0de
1	100	4.0ab	6.5c	27.4a	26.4a
2	100	4.3a	6.8bc	26.8ab	25.8ab
4	100	3.4cd	5.1f	23.5e	22.5e
8	100	3.0de	6.8b	26.4b	25.4b

Means within column with the same letter(s) are not significantly different at P= 0.05 according to DMRT.

Influence of ascorbic acid:

As shown in table 3 application of different concentrations of ascorbic acid resulted in significant differences in number of shoots/explant, shoot length, number of leaves/explant and number of nodes/shoot. The use of 20 mg/l ascorbic acid excelled all other treatments for shoot number. The least shoot number was obtained from the control which was devoid of ascorbic acid. Regarding shoot

length, the control resulted in the longest shoot although it did not differ significantly from the 20 mg/l treatment. The shortest shoots resulted from the 40 mg/l treatment differing non significantly from the 20 mg/l treatment. With regard to leaf number, both 20 mg/l and 40 mg/l resulted in significantly higher values than the control with 20 mg/l occupying the highest rank. The node number followed the same trend as the leaf number. Incorporation of organic acids proved to be beneficial. This might be attributed to the fact that organic acids are respireable substrates that add to energy available from sucrose. Gamborg and Shyluk (1970) found that some organic acids could promote ammonium utilisation. The incorporation of small quantities of sodium pyruvate, citric, malic and fumaric acids into the medium was one factor which enabled Kao and Michayluk (1975) to culture *Vicia hajastana* cells at low density. Beside its role as an antioxidant, ascorbic acid was reported to be involved in division and elongation of tobacco cells (De Pinto *et al.*, 1999). According to Joy *et al.*, (1988), enhanced shoot formation in both young and old tobacco callus was observed upon incorporation of ascorbic acid in culture media. Moreover, Mohammed (2012) reported that the addition of citric acid at low concentration to ginger medium improved growth and enhanced multiplication.

Table 3. Effect of different concentrations of Ascorbic acid on performance of *in vitro* cultures of *Chrysanthemum morifolium* after 6 weeks.

Ascorbic acid conc. (mg/l)	% of explants with shoots	Shoots no.	Shoot length (cm)	Leaves no.	Nodes no.
Control	100	2.6e	6.0b	24.0d	23.0d
20	100	6.4a	5.7bc	38.6a	37.6a
40	100	5.0c	5.6c	29.8c	28.8c

Means within column with the same letter(s) are not significantly different at P= 0.05 according to DMRT.

Influence of glycine:

As shown in table 4 application of different concentrations of glycine resulted in significant differences in number of shoots/explant, shoot length, number of leaves/explant and number of nodes/shoot. Both concentrations of glycine (30 and 60 mg/l) resulted in significantly higher values of all measured parameters than the control with no significant differences among themselves. Amino acids are sources of organic nitrogen. Their incorporation in media is not essential especially in high salt media like MS. Nevertheless, supplements of amino acids were found beneficial in frequent cases. Amino acids have been used as organic nitrogen sources in *in vitro* cultures of several species such as alfalfa, maize, sorghum, pineapple, rice and other monocots to enhance somatic embryogenesis and regeneration (Skokut *et al.*, 1985; Claparols *et al.*, 1993; Rao *et al.*, 1995; Hamasaki *et al.*, 2005; Grewel *et al.*, 2006). It has been suggested that positive effect of organic nitrogen, in comparison to that of inorganic sources is associated to enhanced mobility of the former at a lower energy cost than the later (Kim and Moon, 2007). Abdelmuttalib (2009) reported that the amino acids are sources of organic nitrogen. Amino acids are not essential ingredients of media, but beneficial responses were reported upon their incorporation in media of lime. According to Mohammed (2012), the addition of either L-glycine or L-asparagine at low concentration promoted growth and proliferation of ginger cultures.

Table 4. Effect of glycine concentration on performance of *in vitro* cultures of *Chrysanthemum morifolium* after 6 weeks.

glycine conc. (mg/l)	% of explants with shoots	Shoots no.	Shoot length (cm)	Leaves no.	Nodes no.
Control	100	2.5b	6.6b	20.1c	19.1c
30	100	3.2a	6.1c	23.5a	22.5a
60	100	3.5a	6.2c	23.4a	22.4a

Means within column with the same letter(s) are not significantly different at P= 0.05 according to DMRT.

Effect of aliquots of liquid medium as overlays onto solid medium:

Table 5 shows that application of different aliquots of liquid multiplication media as overlays onto solid multiplication media resulted in significant differences in number of shoots/explant, shoot length, number of leaves/explant and number of nodes/shoot. The application of 16 ml aliquot was the best for all parameters. All other applications of liquid media enhanced the growth parameters significantly over the control except for shoot length as the 4 ml treatment equaled the control. In general, the enhancement of growth was positively correlated with the increase of aliquots of liquid medium. The supply of different aliquots enhanced all measured parameters. This might be attributed to better availability of nutrients and growth regulators. Similar results were reported by Idris (2002), who mounted pineapple cultures onto agar medium by a layer of liquid media and obtained significant enhancements in growth and multiplication of cultures compared to those on agar solidified media. He attributed the enhancement to better availability of nutrients and growth regulators. The result is also in line with that reported by Ugool (2007) who mounted ginger cultures onto agar solidified medium by a layer of liquid media and obtained improvements in shoot multiplication rate.

Table 5. Effect of different aliquots of liquid medium as overlays onto solid medium on performance of *in vitro* cultures of *Chrysanthemum morifolium* after 4 weeks.

Liquid overlay aliquots (ml)	% of explants with shoots	Shoots no.	Shoot length (cm)	Leaves no.	Nodes no.
0	100	2.0d	3.1c	18.1e	17.1e
4	100	2.4c	3.3c	20.4d	19.4d
8	100	2.8b	3.7b	26.0c	25.0c
12	100	2.9b	3.9b	29.0b	28.0b
16	100	3.9a	5.6a	36.8a	35.8a

Means within column with the same letter(s) are not significantly different at P= 0.05 according to DMRT.

Influence of light intensity:

As shown in table 6, the use of different levels of illumination resulted in significant differences in number of shoots/explant, shoot length, number of leaves/explant and number of nodes/shoot. The dark ($0.0 \mu\text{mol m}^{-2} \text{s}^{-1}$) treatment resulted in significant increase in shoot number compared to the 7 and $14 \mu\text{mol m}^{-2} \text{s}^{-1}$ intensities. The longest shoot was obtained under the conditions of full darkness with significant difference from the other treatments. The shortest shoots were obtained from the $7 \mu\text{mol m}^{-2} \text{s}^{-1}$ treatment. The leaf number increased with the increase of light intensity. Significant differences were observed between each light intensity and the succeeding one, with the $14 \mu\text{mol m}^{-2}$

s⁻¹ treatment ranking top. Similarly, the node number was affected by the different light intensities in a similar pattern as reported for leaf number. The universal use of illumination for incubated tissue cultures is at an intensity of 14 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent or incandescent tubes. In this study, dark incubation treatment resulted in significant increase in shoot number, but the plants were albino and etiolated. Culture deprivation from light might have created a status of stress that encouraged multiple shoot formation for survival. To normalize growth, such plants should be subjected to illumination to restore greening. Shoot cultures of *Pelargonium* have been established and encouraged to form multiple shoots under continuous 7 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from fluorescent light (Debergh and Maene, 1977). Nevertheless, the result obtained here is similar to that reported by Aika (2010), who obtained better multiplication of tissue cultured banana upon incubation under very low illumination of only 1.4 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The result is matching with that of Mohammed (2012), who obtained better performance in ginger cultures when exposed to low light intensity of about 1.4 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent lamps, while shoot length was highest in dark incubated cultures. In conclusion, the use of lateral buds as explants, BAP at 1 mg/l, ascorbic acid at 20 mg/l, glycine at 30 mg/l, 16 mls of liquid medium as overlays onto solid medium and light intensity of 14 $\mu\text{mol m}^{-2} \text{s}^{-1}$ seems to be suitable within a protocol for micropropagation of *Chrysanthemum morifolium*.

Table 6. Effect of different levels of light intensity on performance of *in vitro* cultures of *Chrysanthemum morifolium* after 6 weeks.

Light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	% of explants with shoots	Shoots no.	Shoot length (cm)	Leaves no.	Nodes no.
0	100	3.1a	6.6a	12.0d	11.0d
1.4	100	2.8ab	4.8d	19.0c	18.0c
7	100	2.3c	5.5c	20.4b	19.4b
14	100	2.6bc	6.0b	23.5a	22.5a

Means within column with the same letter(s) are not significantly different at P= 0.05 according to DMRT.

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**تأثير الجزء النباتي المنفصل و بنزائل أمينوبيورين والاضافات الكيميائية
و شدة الاضاءة على تضاعف الأفرع خارج الجسم الحي لنبات الأراولا**

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المستخلص

أجريت هذه الدراسة بهدف تقييم أثر الجزء النباتي المنفصل (القمم النامية والبراعم الجانبية) و التراكيز المختلفة من الساييتوكاينين بنزائل أمينوبيورين (BAP) بالتراكيز 1، 2، 4، 6، 8 ملجم/لتر والحمض العضوي (حمض الأسكوربيك) بالتركيزين 20 و 40 ملجم/لتر والحمض الأميني الجللايسين بالتركيزين 30 و 60 ملجم/لتر ووضع وسط سائل على الوسط الصلب (0، 4، 8، 12، 16 مل وسط سائل) و شدة الإضاءة (0، 1.4، 7، 14 $\mu\text{mol m}^{-2} \text{s}^{-1}$) على تضاعف الأفرع خارج الجسم الحي لنبات الأراولا. أستخدم التصميم كامل العشوائية في هذه التجارب. قياسات التضاعف كانت نسبة الأجزاء النباتية المنفصلة التي كونت أفرع، عدد الأفرع بالجزء النباتي المنفصل، طول الفرع، عدد الأوراق بالجزء النباتي المنفصل وعدد العقد بالفرع. أخضعت القراءات لتحليل التباين وتم الفصل بين المتوسطات باستخدام اختبار دنكن واختبار T. بخصوص المعيار نسبة الأجزاء النباتية المنفصلة التي كونت أفرع فكل مستويات كل العوامل التي اختبرت نتج عنها أجزاء نباتية منفصلة مكونة أفرع بنسبة 100%. بالنسبة لبقية المعايير لم يكن هناك فرق معنوي بين البراعم القمية والبراعم الجانبية. رصدت فروقات معنوية نتيجة لمعاملات الساييتوكاينين، اذ أحدثت التركيزات المنخفضة من البنزائل أمينوبيورين (1 و 2 ملجم/لتر) حفزا معنويا لمقاييس التضاعف. حدثت زيادة معنوية في التضاعف نتيجة لإضافة حمض الأسكوربيك بتركيز منخفض (20 ملجم/لتر) للوسط الغذائي. اضافة الجللايسين بتركيز 30 أو 60 ملجم/لتر حفز التضاعف معنويا مقارنة بالشاهد. اضافة الوسط الغذائي السائل على سطح الوسط الغذائي الصلب حفز كل مقاييس التضاعف بصورة معنوية. حضانة المزارع تحت ظروف الإظلام التام أدت لرفع معدل التضاعف معنويا الا أن مظاهر الشحوب الظلامي كانت واضحة في الأفرع الناتجة. خلصت الدراسة الى أن استعمال البراعم الجانبية كأجزاء نباتية منفصلة و البنزائل أمينوبيورين بتركيز 1 ملجم/لتر و حمض الأسكوربيك بتركيز 20 ملجم/لتر و الجللايسين بتركيز 30 ملجم/لتر و 16 مل من الوسط السائل على الوسط الصلب و شدة الاضاءة $14 \mu\text{mol m}^{-2} \text{s}^{-1}$ يعتبر مناسباً داخل طريقة للاكثار الدقيق لنبات الأراولا.