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IN VITRO **STUDIES ON TARO, *Colocasia esculenta* var. *esculenta* (L) Schott.**

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ABSTRACT

An effective method (protocol) for micropropagation of taro (*Colocasia esculenta* var. *esculenta* cv. Egyptian) was developed. This method comprised of four successive stages. In stage I "callus induction", the results showed that the highest percentage of callus induction was obtained by culturing the axillary buds explants on AZ-medium and on both half and full strength of MS-medium. Supplementation of MS-medium with 0.45 mg/l dicamba (potent auxin) obviously encouraged callus induction percentage comparing to the other concentrations. In stage II "callus growth and shoot formation", the highest percentage of shoot formation was obtained when the initiated calli were subcultured onto MS-medium supplemented with 0.4 mg/l dimethylallylamino purine (2iP) + 0.45 mg/l dicamba. The highest percentage of green meristematic regions production was obtained in case of adding 0.1 mg/l 2iP + 0.45 mg/l dicamba to the culture medium. Also, in this stage, the highest percentage of plantlets formation (70%) was obtained by culluring the initiated calli on MS-medium containing 0.60 mg/l thidiazuron (TDZ) alone. Supplementation of MS-medium with 1.05 mg/l TDZ gave the highest number of shoots per responded cultures. In stage III " plantlet formation and elongation", the obtained friable callus alone or along with green meristematic regions which derived in the previously stage were used as plant material. When they subcultured on MS-medium supplemented with 3.0 mg/l kinetin + 0.3 mg/l IAA + 20 mg/l adenine hemisulfate, the highest percentage of clusters of taro plantlets (77.5%) was obtained. In stage IV "acclimatization", plantlets derived *in vitro* with well developed roots were successfully acclimatized when potted in a peat moos – vermiculite (1:1 w/w) mixture.

Keywords: Taro, *Colocasia esculenta* var. *esculenta* (L) Schott, *Araceae* family, callus culture, micropropagation, tissue culture.

INTRODUCTION

Colocasia esculenta var. *esculenta* (L) Schott (taro) is a member of the family *Araceae*. This plant is mainly grown in Egypt, and its corms have a high nutritional value and good flavor. It is vegetatively propagated through the corm pieces and the small cormels. However this method is difficult and slow for the large scale cultivation of new cultivars and clones. This method of propagation is also ineffective for maintaining pathogen-free stock plants, resulting in the dissemination of diseased or pest-infested planting materials. Taro plants are very liable to be infected with virus diseases, in particular Dasheen Mosaic virus. However, virus-free plants can be obtained from shoot tips explants using tissue culture techniques. These explants generally give cultures producing axillary shoots (Jackson *et al*., 1977).

In vitro techniques offer an alternative, reliable method for the production of planting material, but rates of multiplication have generally been modest (Yam *et al*., 1990b, 1991; Malamug *et al*., 1992b).

The purpose of this work was to establish an applicable protocol for the *in vitro* production of large numbers of taro plants in a relatively short time.

MATERIALS AND METHODS

This work was carried out during the period from 2005 to 2006 in the tissue culture laboratory and greenhouse, Horticulture Research Institute (HRI), Agriculture Research Centre, Ministry of Agriculture and Land Reclamation, Giza, Egypt.

Plant material and preparation of explants:

The Egyptian taro corms were obtained from Potato and Vegetatively Propagated Vegetable Department, HRI. These corms were used as source of explants. Corms were cut longitudinally into small pieces each with buds and weight about 120-150 g, and left to sprout in planting pots containing mixture of peat moss and vermiculite 1:1 (v/v). They were grown and maintained in a greenhouse. The plants obtained from sprouted corm pieces were used as a source for leaf pieces and petiole sections explants. Also, axillary buds were directly excised from dry un-sprouted taro corms. Explants *i.e.*, axillary buds, leaf and petiole were washed under running tap water for 1 hr. Then they were superficially disinfected with 20% (v/v) commercial bleach (1.05% NaOCl) plus Tween 20 (2 drops / 100 ml) for 20 min with agitation. Explants were then rinsed three times using sterile distilled water and trimmed. Axillary buds were re-disinfected with 10% (v/v) bleach (0.525% NaOCl) for 5 min, rinsed three times in sterile distilled water before culturing on the nutrient media.

Different experiments were conducted in the different stages of the protocol which was developed for mass propagation of taro *i.e.*, I- callus induction stage, II- callus growth and shoot formation stage, III- plantlet formation and elongation stage and IV- acclimatization stage.

I. Callus induction stage:

This stage included two experiments:

Experiment (1):

This experiment was carried out to investigate the effects of five different types of culture media on callus induction percentage from the axillary buds. The five types of media were MS (Murashige and Skoog, 1962), AZ (Abo El-Nil and Zettler, 1976), LS (Linsmaier and Skoog, 1965), half-strength (macro-and micro elements) MS-medium (HMS) and MS-medium containing 20 ml/l taro extract[(*)] (MSTE). All culture media were supplemented with 30 g/l sucrose, 7 g/l agar and 5 mg/l each of naphthaleneacetic acid (NAA) and benzyladenine (BA). The media were adjusted to pH 5.7 prior autoclaving. Prepared media (30 ml) were dispensed into 200 ml glass jars, and autoclaved at 121°C for 20 min.

Axillary buds explants were prepared and sterilized as previously mentioned and then individually placed in culture jars containing the various culture media. Cultures were maintained at 25±2°C and 16 hr/day photoperiods provided by white fluorescent lights at an intensity of 1000 Lux.

[(*)] The taro extract was prepared according to Yam *et al.* (1900a) by boiling 600 g of 1cm^3 corms cubes for 5 minutes in 1 liter water, and then simmering the mixture for 1 hour. The liquor obtained after filtering through a Whatman No. 1 paper was used immediately, or frozen.

Each treatment consisted of at least 30 culture jars. Data of callus induction percentage were recorded after 6 weeks of culturing.

Experiment (2):

This experiment was conducted to study the effect of different concentrations (0.3, 0.45, 0.6 and 0.75 mg/l) of dicamba (3, 6 – dichloroanisic acid), added to the culture medium as auxin, on callus induction percentage from axillary buds, leaf pieces and petiole sections explants. The three explant types were dissected, prepared and sterilized as previously described, then individually placed in 200 ml culture jars containing 30 ml of solid MS medium supplemented with the different concentrations of dicamba. Each treatment consisted of 20 culture jars. After 8 weeks of culturing, callus induction percentage were recorded.

II. Callus growth and shoot formation stage:

The experiments conducted at this stage aimed to improve the growth of callus as well as increase the rate of callus derived shoots. This stage contained two experiments:

Experiment (1):

This experiment was conducted to study the morphognic responses of calli, which were initiated in the previous stage, to four different concentrations of 2iP (0.1, 0.2, 0.3 and 0.4 mg/l) added to nutrient media. The solid MS nutrient medium supplemented with 0.45 mg/l dicamba was used in this part of study. Calli were individually placed in 200 ml culture jars containing 30 ml of the various four media. Each treatment consisted of 20 culture jars. Data were recorded after 8 weeks of culturing as percentage of cultures which gave callus formation, green meristematic regions, shoot formation and non organogenic brown tissue mass as well as average shoot number per-responded cultures. In cases of percentages of each green meristematic regions, shoot formation and non organogenic brown tissue, the original data were logarithmatic-transformed to make the values more amenable to analysis of variance.

Experiment (2):

This experiment aimed to investigate the effect of thidiazuron (TDZ), has cytokinin-like activities, on the morphogenic responses of calli initiated in the previous stage. Callus was subcultured onto solid MS nutrient medium supplemented with the different concentrations of TDZ (0.15, 0.60 and 1.05 mg/l). Each treatment consisted of 20 culture jars (200 ml) and each jar contained 30 ml medium. Data were recorded after 6 weeks of culturing as percentage of cultures which gave callus formation, green meristematic regions, plantlet formation and non organogenic brown tissue mass as well as average shoot number per-responded cultures. In cases of percentages of each callus formation, green meristematic regions and non organogenic brown tissue, the original data were logarithmatic-transformed prior to statistical analysis.

In order to obtain sufficient stocks of calli with green meristematic regions, needed for the followed experiment in the next stage, the cultures which gave callus formation in this experiment were subcultured for another 6 weeks on fresh MS medium contained the best TDZ treatment (0.60 mg/l).

III. Plantlet formation and elongation stage:

The objective of the experiment conducted in this stage was the formation of clusters of taro plantlets as well as enhancement the growth of the proliferated plantlets. The MS medium supplemented with 50 mg/l $NaH_2PO_4.2H_2O$ and four combinations of some plant growth regulators and some other addition were used in this experiment as follows:

a. 3 mg/l kinetin + 0.3 mg/l IAA + 20 mg/l adenine hemisulfate.
b. 3 mg/l kinetin + 0.3 mg/l IAA + 0.026 mg/l TDZ.
c. 4 mg/l kinetin + 0.4 mg/l IAA + 20 mg/l adenine hemisulfate.
d. 4 mg/l kinetin + 0.4 mg/l IAA + 0.026 mg/l TDZ.

Cultures which gave callus formation (friable callus) alone or along with green meristematic regions obtained in the previous stage were taken and divided in equal pieces and individually subcultured on the different four types of culture media.

Each treatment consisted of 20 culture jars (400 ml) and each jar contained 50 ml medium. Data were recorded after 8 weeks of culturing as percentage of cultures which gave clusters of plantlets.

IV. Acclimatization stage:

Plantlets with well developed roots were taken out of the culture jars and carefully cleaned from agar by washing in current tap water, and then individually transplanted in plastic pots containing peat moss-vermiculite mixture (1:1 w/w). Pots were maintained in the greenhouse and covered with transparent polyethylene sheets which were gradually removed. The plantlets were irrigated whenever needed and fertilized using nutrient solution containing macro and micro nutrients similar to that used in MS medium, to be ready for planting in the open field.

The treatments of all experiments were arranged in a complete randomize design. The obtained data were subjected to the analysis of variance according to Snedecor and Cochran (1980). The differences between various treatment means were tested by L.S.D. In addition, standard error was computed for the responded cultures according to Heath (1970).

RESULTS AND DISCUSSION

I. Callus induction stage:

Experiment (1): Effect of type of medium on callus induction. Data presented in Table (1) showed the influences of the five types of media *i.e.*, MS, AZ, LS, MSTE and HMS on callus induction from axillary buds explants after 6 weeks of culture *in vitro*.

Table (1): Effect of type of medium on callus induction (%) from axillary buds, after 6 weeks of culture.

Type of medium	MS	AZ	LS	MSTE	HMS
Callus induction (%)	51.50	68.53	48.63	30.93	56.90

L.S.D. 5% = 18.83

The obtained results revealed that, the highest percentage of callus induction was obtained via using AZ-medium which came in the first rank, followed by HMS-medium and MS-medium, respectively. No significant

difference was observed among these three culture media in this measurement. On the contrary, using MSTE medium recorded the lowest value of callus induction.

In this respect, many media combinations were tested for callus induction in a number of studies. Abo El-Nil and Zettler (1976) developed their own medium for this purpose. They initiated callus from shoot tip explants on this medium. Yam *et al*. (1990a) produced a fast growing, friable and morphogenetically active callus from axillary bud explants of *Colocasia esculenta* var. *esculenta* when cultured on half-strength MS medium containing taro corm extract. In another study, Yam *et al*. (1990b) used modified MS medium with full-strength and containing taro corm extract. In addition, Yam *et al*. (1991) studied the effects of five modified basal media on the induction of callus, one of them (MS) at several macroelement concentrations. Microelements in all media were the same as in MS, but at one-tenth the concentration. They found that the differences between basal media concerning the weight of callus were not pronounced during the first month of culture. After four months (four subcultures on the same medium), the weight increase on half-strength macroelements of MS medium plus taro extract was higher than those growing on the other culture media. Esenowo (1986) initiated callus from tuber segments of *C. esculenta* on a medium containing Linsmaier and Skoog's salt mixture. Also, Nyman and Arditti (1984) and Nyman *et al*. (1987) used Linsmaier and Skoog medium for callus culture of *C. esculenta* var. *antiquorum*.

The superior effect of AZ-medium on the percentage of callus induction in the present study could be related to the appropriate balance between the macro and micro - elements, particularly the absence of ammonium nitrate NH_4NO_3 in this medium. In this connection, Malamug *et al*. (1992a) stated that by reducing the concentration of NH_4NO_3 from 720 mg/l to 200 mg/l in modified Nitsch medium, the degree of taro callus formation and percent of explants forming callus was increased.

Experiment (2): Effect of dicamba concentrations on callus induction.

Data in Table (2) cleared that the percent of callus induction from axillary bud explants were significantly affected by dicamba concentrations. The highest percentage of callus induction was more achieved via supplementation of medium with 0.45 mg/l dicamba, followed by the treatment of 0.30 mg/l. Such Table also, revealed that leaf pieces and petiole sections explants did not produce any callus tissue on the four tested media and the culture completely dead during 8 weeks. Concerning axillary bud explants, the obtained results were similar to those published by Nyochembeng and Garton (1998) while the results of leaf and petiole explants were in contrast with the findings of the same authors who stated that, calli were initiated from shoot tip and petiole explants of cocoyam (*Xanthosoma sagittifolium*) on medium containing dicamba at 1.36 μM (0.3 mg/l), this concentration produced significantly more callus than the other tested concentrations (0.45, 4.52 and 13.5 μM).

The differences observed among explant types in their responses to callus induction could be due to the mitotically active cells in such explants. The tissue from axillary bud is more active than tissue obtained either from

leaf or petiole, because it contains specific meristematic tissue which is characterized by rapid cell division. This could be related to the increase in the volume of nucleus and the cell is full of cytoplasm with no vacuole (Esau, 1979).

Table (2): Effect of dicamba concentrations on callus induction (%) from axillary buds, leaf pieces and petiole sections explants, after 8 weeks from culture.

Dicamba (mg/l)	Callus induction (%) from		
	Axillary buds	Leaf pieces	Petiole sections
0.30	46.67	0.0[m]	0.0[m]
0.45	56.67	0.0[m]	0.0[m]
0.60	28.87	0.0[m]	0.0[m]
0.75	27.30	0.0[m]	0.0[m]
L.S.D 5%	12.31		

[m] culture dead during 8 weeks.

II. Callus growth and shoot formation stage:

Experiment (1): Effect of 2iP on the morphogenic responses of calli.

Data in Table (3) showed that, the percentage of green meristematic regions and shoot formation were significantly affected by the different 2iP concentrations. However, callus formation and non-organogenic brown tissue mass were not significantly affected by these treatments. The treatment of 0.1 mg/l 2iP gave pronounced percentages of green meristematic regions as compared with the other concentrations. Concerning shoot formation percentage, the highest value was more achieved via supplementation of culture medium with 0.4 mg/l 2iP. In case of callus formation, all 2iP concentrations used in culture media gave callus formation with an average approximately 53% over the entire concentrations. Concerning the average of shoot number, no significant differences were observed between these four concentrations of 2iP. In addition, all 2iP concentrations produced a few number of shoot.

Table (3): Effect of 2iP on percentage of callus formation, green meristematic regions, shoot formation and non organogenic brown tissue mass and average shoot number per responded cultures, after 8 weeks of culturing the initial callus.

2iP concen. (mg/l)	Percentages of cultures which gave				Average shoot number per-responded cultures ± S.E.[l]
	Callus formation	Green meristematic regions[k]	Shoot formation[k]	Non organogenic brown tissue mass[k]	
0.1	57.37	51.67[a]	20.00[ab]	18.75[a]	1.25 ± 0.25
0.2	43.23	18.33[b]	6.25[b]	30.83[a]	1.0 ± 0.0
0.3	53.54	17.78[b]	30.42[a]	24.58[a]	1.0 ± 0.0
0.4	59.79	11.11[b]	41.25[a]	35.00[a]	1.12 ± 0.12
L.S.D.5%	N.S.				

k = Means of green meristematic regions, shoot formation and non organogenic brown tissue mass presented are calculated from the original data. Means followed by the same letter are not significantly different by L.S.D. $_{0.05}$, based on the ANOVA which was performed on the logarithmatic transformed data.

l = S.E.: Standard error.

The present results concerning callus formation can be supported by the findings of Murakami and Matsubara (1998) who found that callus proliferation occurred with 2.0 mg/l 2iP and 2.0 mg/l NAA. Also, Murakami *et al*. (1998) noted that many green compact cell masses and a small number of yellow friable calli were formed by culturing stem segments on MS medium supplemented with 1.0mg/l each of 2iP and 2,4- D. Friable calli were proliferated by subculturing on the same fresh medium.

From the previously mentioned results, it could be suggested that 2iP at low concentration (0.1mg/l) stimulated callus formation and formed green meristematic regions on it. In this regard, Gamborg *et al*. (1976) mentioned that cell division are stimulated by addition of cytokinins to the culture medium.

Experiment (2): Effect of thidiazuron (TDZ) on the morphogenic responses of calli.

Data in Table (4) showed that, the percentage of plantlets formation was significantly affected by TDZ concentrations. However, among all TDZ concentrations used in culture medium no significant differences on the percentage of callus formation, green meristematic regions and non-organogenic brown tissue mass were recorded. It was found that, supplementation of MS-medium with 0.60 mg/l TDZ gave the highest percentage of plantlets formation (70%). All TDZ concentrations added to culture medium produced callus formation with an average 70% over the entire concentrations. Such data also, revealed that the average of shoot number was significantly affected by TDZ concentrations. The highest number of shoots was obtained by adding 1.05 mg/l TDZ to culture medium. In general, TDZ treatments showed a gradual increase in shoot number as the concentration of TDZ increased in the culture medium.

Table (4): Effect of thidiazuron (TDZ) on percentage of callus formation, green meristematic regions, plantlets formation and non organogenic brown tissue mass and average shoot number per responded cultures, after 6 weeks of culturing the initial callus.

TDZ concen. (mg/l)	Percentages of cultures which gave				Average shoot number per-responded cultures ± S.E.[l]
	Callus formation[k]	Green meristematic regions[k]	Plantlets formation	Non organogenic brown tissue mass[k]	
0.15	78.75[a]	12.50[a]	52.5	15.00[a]	1.0 ± 0.0
0.60	63.75[a]	18.75[a]	70.0	18.75[a]	2.08 ± 0.38
1.05	67.50[a]	18.75[a]	47.5	27.50[a]	2.57 ± 0.29
L.S.D.5%			14.1		

k = Means of callus formation, green meristematic regions and non organogenic brown tissue mass presented are calculated from the original data. Means followed by the same letter are not significantly different by L.S.D. $_{0.05}$, based on the ANOVA which was performed on the logarithmatic transformed data.
l = S.E.: Standard error.

It is well kown that thidiazuron has cytokinin – like activities and has been shown to be effective in promoting shoot regeneration in a number of different systems (Compton *et al*., 1993; Kanakis and Demetriou, 1993).

The results concerning plantlets formation and shoot number can be supported by the findings of Chand *et al.* (1999) who used modified MS medium supplemented with TDZ at concentrations of 0.0, 0.03, 0.13, 0.63, 1.13, 2.0 and 3.13 mg/l for multiplication of shoot tip of taro cv. Niue. They found that the main shoot grew vigorously in TDZ 0.13 – 1.13 mg/l during the first four weeks. At the end of the second subculture on the same fresh medium (20 weeks from starting) TDZ at 1.13 mg/l produced the maximum shoot number (7.5 shoots per single explant). Furthermore, they added that, in another experiment, shoot tips of cv. Niue were initially cultured on a modified MS medium plus 0.6 mg/l TDZ for 4 weeks, and then transferred to modified MS plus 1.0 mg/l TDZ. Subculturing to fresh medium of the same composition occurred every 4 weeks. At the end of the third subculture all of the plantlets had produced roots. At the later transfers (fifth subculture) TDZ – treated explants were increasing at rates of up to 25 fold per four weeks period. In this connection, Palupe (1999) in three successive – stage culture system used modified MS medium with 0.025 mg/l TDZ, 1.0 mg/l BA and 0.005 mg/l TDZ, respectively. Each stage tested 4 weeks and produced about 55 shoots per taro shoot tip explant.

The obtained results concerning the effect of TDZ on callus formation are similar to those published by Nyochembeng and Garton (1998) who noted that thidiazuron (0.045 µM) had a promotive effect on cocoyam callus proliferation only at higher dicamba concentrations (4.52 and 13.5 µM). Callus derived from media containing TDZ were compact and light green with visible shoot initials and bud aggregates.

According to the previously mentioned results in this stage (Tables 3 and 4), it could be suggested that thidiazuron alone in MS medium stimulated callus proliferation and shoot formation more than 2iP in combination with dicamba.

III. Plantlet formation and elongation stage:

Effect of different combinations of kinetin (kin.), indole-3-acetic acid (IAA), thidiazuron (TDZ) and adenine hemisulfate (AdS) on the production of clusters of taro plantlets is presented in Table (5). The results revealed that the four different tested combinations were varied significantly. The highest percentage of this character was resulted from supplementation of MS – medium with the combination of 3.0 mg/l kin. + 0.3 mg/l IAA + 20 mg/l AdS (Fig.1).

Table (5): Effect of different combinations of kinetin (kin.), indole-3-acetic acid (IAA), thidiazuron (TDZ) and adenine hemisulfate (AdS) on the production of clusters of taro plantlets, after 8 weeks of culturing the friable callus.

Treatments	Percentages of cultures which gave clusters of taro plantlets
Kin. 3 mg/l + IAA 0.3 mg/l + AdS 20 mg/l	77.56
Kin. 3 mg/l + IAA 0.3 mg/l + TDZ 0.026 mg/l	45.24
Kin. 4 mg/l + IAA 0.4 mg/l + AdS 20 mg/l	72.70
Kin. 4 mg/l + IAA 0.4 mg/l + TDZ 0.026 mg/l	50.00
L.S.D. 5%	10.31

(A)

(B)

Fig. (1) : (A) Clusters of taro plantlets produced from friable callus, after subculturing 8 weeks on MS + 3.0 mg/l kin. + 0.3 mg/l IAA + 20 mg/l adenine hemisulfate. (B) Individual taro plantlets separated from a part of cluster similar to those in panel A..

Such results were in conformity with the findings of Malamug *et al.* (1992b) who investigated the regeneration of shoots from taro callus and found that proliferation of regenerated shoots was enhanced with the addition of cytokinins (BA) and auxins (NAA) at 1mg/l to culture medium. also, Murakami *et al.* (1998) stated that taro shoots were regenerated from many green compact cell masses (calloids), friable calluses and protoplast derived – calluses by transferring them to MS medium supplemented with 0-2 mg/l NAA and 2 mg/l BA or 2iP. The present results also revealed that media containing adenine hemisulfate (20 mg/l) stimulated clusters production more than those containing TDZ (0.026 mg/l). In this connection, Okasha *et al.* (1996) stated that supplementation of the culture medium by 60 mg/l adenine sulfate obviously enhanced the *in vitro* derived shoots of globe artichoke.

The results obtained in the present study may be explained on the basis that a high concentration of cytokinin and low concentration of auxin in culture medium promotes abundant plantlets proliferation.

IV. Acclimatization stage:

The plantlets with well developed roots were easily separated and transplanted in plastic pots containing peat moss – vermiculite mixture (1:1 w/w) for acclimatization (Fig.2).

Finally, an effective protocol for multiplication of Egyptian taro plantlets was established through callus initiation and proliferation from axillary buds explants. Tissue culture derived plantlets were successfully acclimatized before transferring to the open field.

Fig. (2): Poted plantlets of taro Egyption cv. 4 weeks after transplanting on mixture of peat moss: vermiculite (1: 1 w/w).

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دراسات على مزارع الأنسجة فى القلقاس

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الهدف الرئيسى من هذه الدراسة هو الوصول إلى طريقة فعالة (بروتوكول) للإكثار المعملى الدقيق للقلقاس (الصنف المصرى). للوصول إلى هذه الطريقة أجريت عدة تجارب على مدى أربعة مراحل متعاقبة:

المرحلة الأولى (تنشئة الكالس)، أوضحت النتائج أن أعلى نسبة لتنشئة الكالس تحققت عند زراعة البراعم الجانبية للقلقاس على بيئة (AZ) أو على بيئة موراشيج وسكوج بكامل قوتها (MS) أو بنصف قوتها (HMS). إمداد بيئة موارشيج وسكوج بـ ٠,٤٥ ملجم/لتر dicamba (أوكسين قوى) شجع كثيراً من تنشئة الكالس مقارنة بالتركيزات الأخرى.

المرحلة الثانية (نمو الكالس وتكون الأفرع)، أعلى نسبة لتكوين الأفرع تحققت عندما أعيدت زراعة الكالس (المنشأ) على بيئة (MS) المحتوية على ٠,٤ ملجم/لتر (2iP) + ٠,٤٥ ملجم/لتر (dicamba). أعلى نسبة مئوية لإنتاج المناطق المرستيمية الخضراء تحققت فى حالة إضافة ٠,١ ملجم/لتر (2iP) + ٠,٤٥ ملجم/لتر (dicamba). أيضاً فى هذه المرحلة، أعلى نسبة لتكوين النباتات الصغيرة (٧٠%) نتجت من زراعة الكالس (المنشأ) على بيئة (MS) المحتوية على ٠,٦ ملجم/لتر (thidiazuron) بمفردة. إمداد بيئة (MS) بـ ٠٥.١ ملجم/لتر (thidiazuron) أعطى أعلى عدد من الأفرع فى المزارع التى حدث بها استجابة.

المرحلة الثالثة (تكوين النباتات الصغيرة واستطالتها)، الكالس المتكون (الحبيبى) بمفردة أو به المناطق المرستيمية الخضراء والذى أنتج فالمرحلة السابقة قد تم استخدامه كمادة نباتية فى هذه المرحلة. عندما تم إعادة زراعة هذا الكالس على بيئة (MS) المحتوية على توليفة من ٣,٠ ملجم/لتر كينتين + ٠,٣ ملجم/لتر اندول حمض الخليك (IAA) + ٢٠ ملجم/لتر أدينين همى سلفات أنتج أعلى نسبة من نباتات القلقاس الصغيرة المتجمعة (clusters) ٧٧,٥%.

المرحلة الرابعة (الأقلمة)، النبيتات ذات الجذور الجيدة قد تم أقلمتها بنجاح وذلك بزراعتها فى أصص تحتوى على مخلوط من بيت موس + فرميكيوليت (بنسبة ١ : ١ وزن / وزن).