Direct somatic embryogenesis from leaf primordia in date palm *(Phoenix dactylifera L.)* cv. Zaghloul

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ABSTRACT

The present work was conducted in the Central Laboratory for Date Palm Research and Development, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. The aim of this study was to enhance somatic embryogenesis directly from leaf primordia in Date palm (Phoenix dactylifera L.) cv. Zaghloul by using dichlorophenoxy acetic acid at concentrations (2.5 , 5.0 ; 7.5 and 10.0 mg/l). Results revealed that the high rate of direct somatic embryos was obtained from leaf primordia explants cultured individually each on 3/4 strength MS medium supplemented with 2,4-D levels (2.5; 5.0; 7.5 and 10 mg/l) either solely or combined with 3 mg/l 2ip +0.4 mg/l thiamin-HCl + 170 mg/l KH_2PO_4 + 7 g/l agar + 200 mg/l glutamine + 3 g/l activated charcoal and 30 g/l sucrose. The lowest concentration of 2,4-D (2.5 mg/l) induced somatic embryos directly, compared to the other three investigated levels. Embryos were transferred to medium supplemented with 3/4 MS+ 3.0 mg/l kinetin + 170 mg/l KH_2PO_4 + 200 mg/l glutamine + (1.5g/l) charcoal. Multiplication medium amended with kinetin (6-furfuryl amino purine) and 2ip (N6-2isopenteyl adenine) each at two concentrations (0.1 & 1.0 mg/l) combined with 0.1 or 0.5 NAA mg/l. The increase in number of proliferated shootlets was exhibited by 2ip over kinetin, However higher 2ip level (0.5mg/l) and NAA (0.5 mg/l) tended to be relatively more effective. For rooting formation, several levels (20, 40 and 80 mg/l) of phloroglcionl (PG) were added to the rooting. The superior concentration which exhibited the tallest and greatest number of rootlets per each plantlet was 20 mg/l. Acclimatization was achieved by transferring the plantlets into pots contained equal volumes of peat moss and vermiculite(1:1by volume) under high (85-90%) humidity, 27°C ± 1 and 10,000 LUX (Light intensity).

***Key words**: Date palm, direct somatic Embryogenesis, dichlorophenoxy acetic acid, 2ip kinetin, NAA, cv.Zaghloul.*

INTRODUCTION

Plant micropropagation has been greatly helped by development of somatic embryogenesis technique (Steward *et al*., (1958). Researchers believed that auxins such as 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), Picloram, Dicamba, 2,4,5-tricholorophenoxy acetic acid (2,4,5-T) and endogenous hormone metabolism which is controlled by genetic, physiological and environmental factors play roles in somatic embryogenesis in different plant species (Dodema *et al*., 1996, Rao *et al*., 1997; Feher, 2006). But, other studies reported the importance of cytokinins in inducing and developing somatic embryos (Chen and Chang, 2001; Jiménez, 2005).Tissue culture micropropagation, has been employed to aid in the clonal propagation of numerous plant species (De-ossard, 1976). The inherent advantage of tissue culture over field propagation is the greatest plant production potential from a single plant. Tissue culture

techniques, may offer a plausible method to produce large numbers of genetically uniform palms in a short period of time. In date palm, there are two pathways in production of somatic embryos. The first pathway is indirect method which is based on the induction of embryogeneic callus, and the second pathway is direct somatic embryogenesis (somatic embryos without visible callus) (Sudhersan *et al* 1993; Al-Khayri 2005). The first occurrence of direct somatic embryogenesis in date palm was observed on leaf of in vitro plant Sudhersan *et al (*1993). Later, Othmani *et al (*2009) found that direct somatic embryos were formed on the base of young leaf explants when cultured on MS medium enriched with 10mg/l 2,4-D. Since, the first successful demonstration of induction of direct somatic embryogenesis was made, studies were conducted to examine the effect of auxins, cytokinins, and explants to induce direct somatic embryogenesis. In this respect, the aim of the present work was to enhance somatic embryogenesis directly from leaf primordial in date palm *(Phoenix dactylifera* L.) cv. Zaghloul by using 2,4-D concentrations (2.5, 5.0 ; 7.5 and 10.0 mg/l).

MATERIALS AND METHODS

The present work was conducted in the Central Laboratory for Date Palm Research and Development, Agriculture Research Center, Ministry of Agriculture, Giza Governorate, It was aimed to enhanc somatic Embryogenesis directly from leaf primordia in one of the most important soft date palm cultivar grown in Egypt i.e., Zaghloul

Preparation of explants

Young offshoots 2-3 years old about 7-10 kg in weight of date palm cv. Zaghloul were detached from mother palm.. These offshoots were detached from adult fruitful Zaghloul date palms grown in a private orchard located at Rashid region, El-Behara Governorate. The outer large leaves and fibers were carefully and gradually removed from offshoots by sharp knife until the appearance of the shoot tip zone. About 3-4 leaves primordia from each offshoot were excised each with a thin layer of shoot tissue, also apical meristem was divided into 4-8 portions (as a shoot tip explants), and each was approximately 0.5-1.0 cm length.

Culture media Establishment stage

In this stage, leaf primordia were cultured on Murashige and Skoog (1962) (MS) medium supplemented with +100 mg/l 2,4- D +3 mg/l 2ip +30g/l sucros+ 3g/l activated charcoal+0.4 mg/l theamin-HCl +200 g/l glutamin+ 170 mg/l, KH_2PO_4.and 7.0 g/l agar. Cultures were incubated in the dark condition to reduce phenolic secretions from the explants. Leaf primordia were subcultured 8 times (at 4 weeks intervals).

Direct formation somatic embryogenesis

Leaves were transferred to 3/4 MS supplemented with 2.5; 5.0; 7.5 and 10 mg/l 2,4-D either solely or combined with 3 mg/l 2ip. Besides, the culture medium was also supplemented with 0.4 mg/l thiamin-HCl + 170 mg/l, KH_2PO_4 + 7 g/l agar + 200 mg/l glutamine + 3 g/l activated charcoal and 30 g/l sucrose. Leaves primordia were subcultured three time (4 weeks intervals) on the same culture madia. At the end of the third subculture, number of somatic embryos (globular like structure) formed per each treatment was counted.

Direct Somatic embryo germination.

Direct somatic embryos were easily separated at the end of the 3rd subculture. They were immediately transferred individually on 3/4 MS medium supplemented with 3.0 mg/l Kinetin + 170 mg/l KH_2PO_4 + 200 mg/l glutamine +1.5g charcoal. For further

development through four subcultures at four weeks interval, taking into consideration that all cultured jars were incubated through the four subcultures for germination of somatic embryos at 27°C ± 1 with 16 hrs light of 3000 LUX followed by 8 hrs dark periods.

Multiplication stage

Effect of cytokinin type and concentration

The direct somatic embryos resulted from previous stage were used as a mother stock explants for multiplication experiments. In this. Kinetin (6-furfuryl amino purine) and 2ip (N6-2isopenteyl adenine) each at two concentrations (0.1 & 1.0 mg/l) combined with 0.1or 0.5 NAAmg/l were used to study their effects on the multiplication process, number of proliferated shootlets and their average length at the end of each subculture.The experiment consisted of 3 subcultures each for four weeks.

Every treatment was replicated three times, whereas each replicate was represented by 3 cultured jars containing 3/4 MS medium supplemented with 3 g/l activated charcoal +30/l g sucrose + 7.0 g/l agar + 170 mg/l KH_2PO_4 + 200 mg/l glutamine + 0.4 mg/l thiamine HCl. Cultured jars were incubated at 27°C ± 1 and exposed to (16 hrs light of 3000 LUX followed by 8 hrs dark).

Rooting stage

Shoots from previous stage (2-3 leaves) were transferred to rooting medium, which consists of ½ MS inorganic salts supplemented with 60 g/l sucrose + 170 mg/l KH_2PO_4 + 200 mg/l glutamine + 100 mg/l myo-inositol + 0.4 mg/l thiamine-HCl + 0.1 mg/l NAA + 7 g/l agar. The influence of providing culture media with phloroglucinol (PG) at 3 levels, i.e. 20; 40 and 80 mg per liter, as well as control medium, Each treatment was replicated three times and every replicate was represented by three cultured tubes each with only one cultured shoot. All cultured tubes were incubated in growth room at 27°C ± 1 and light intensity of 3000 LUX illumination for 16 hrs daily through the three successive subcultures (4 weeks interval) included in this stage. At the end of each subculture the following measurements were recorded:

1- Number of developed rootlets per each cultured shoot (plantlet).
2- Average length of root (cm).

Statistical analysis

The analytical statics was performed in a completely randomized design with three replicates. All obtained results were determined by subjection to statistical analysis of variance, according to the method described by Snedecor and Cochran (1982) using the LSD test at 5%.

RESULTS AND DISCUSSION

Effect of 2,4 – D leavel and Direct somatic embryo formation

Table (1) displays the obvious superiority of the lowest level (2.5 mg/l) over the three other investigated levels (5.0; 7.5 and 10.0 mg/l) for inducing somatic embryos directly. However, number of somatic embryos was gradually increased by increasing subculture number as averages were 6.17; 8.83 and 12.0, respectively. On the contrary, three other 2,4-D levels (5.0; 7.5 and 10.0 mg/l) failed completely to stimulate direct somatic embryogenesis process during all subcultures. As for the specific effect of providing 3/4 MS medium with 3 mg/l 2ip on number of somatic embryos formation, Table (1) reveals clearly that the 3 mg/l 2ip supplemented medium surpassed statistically the corresponding ones (2ip omitted medium). The difference between presence and absence of 3mg/l 2ip was significant through the three subcultures however rate of variance was increased with aging.

A. Interaction effect

With regard to the interaction effect of 2,4-D levels (2.5; 5.0 ; 7.5 and 10.0 mg/l) and omission or presence of 3 mg/l 2ip, Table (1) show obviously the great variance in response. Anyhow, 3/4 MS medium supplemented with the least 2,4-D level (2.5 mg/l) + 3 mg/1 2ip was statistically the superior. However differences between the aforesaid two combinations was gradually increased as the number of subculture was advanced. On the contrary, 6 other combinations failed completely to force direct formation of somatic embryos by Zaghloul date palm leaf primordial. The present result pertaining the stimulative effect of lower auxin level goes in line with the finding of Abo EL-Soaud *et al*. (2002-b), who reported the possibility of direct embryogenesis from cells of parent tissues by transferring the oldest explants from high auxin media to low auxin ones. Moreover, finding of Abo EL-Soaud and Ibrahim (2002) gave support to the present result regarding the beneficial effect of using 2,4-D and 2ip combination on stimulating the direct somatic embryos production in date palm. Khierallah *et al*. (2007). revealed that MS modified medium supplemented with 2.0 mg/l 2ip, 1.0 mg/l BA, 1.0 mg/l NAA and 1.0 mg/l NOA was the best for bud formation from shoot tip after 16 weeks. Furthermore, about 70% of adventitious buds were formed on Murashige and Skoog (MS) medium supplemented with 2 mg/l naphthalene acetic acid (NAA), 4 mg/l benzyl amino purine (BAP), and 40 g/l sucrose Khierallah *et al*. (2017).

Table (1): The Effect of 2, 4-D levels in the present or absent of 2ip on number of direct somatic embryos through three subcultures of Zaghloul date palm cv.

Auxin & cytokinin treatments(mg/l)	No. of somatic embryos directly formed per leaf primordium through three successive subcultures								
2ip / 2,4-D	Subculture I			Subculture II			Subculture III		
	Without 2ip	3.0 mg 2ip	Mean*	Without 2ip	3.0 mg 2ip	Mean *	Without 2ip	3.0 mg 2ip	Mean *
2.5 mg/l	5.33 b	7.00 a	6.17 A	7.33 b	10.33 a	8.83A	9.33 b	14.67 a	12.00 A
5.0 mg/l	0.00 c	0.00 c	0.00 B	0.00 c	0.00 c	0.00 B	0.00 c	0.00 c	0.00 B
7.5 mg/l	0.00 c	0.00 c	0.00 B	0.00 c	0.00 c	0.00 B	0.00 c	0.00 c	0.00 B
10.0 mg/l	0.00 c	0.00 c	0.00 B	0.00 c	0.00 c	0.00 B	0.00 c	0.00 c	0.00 B
Mean **	1.34 B	1.76 A		1.84 B	2.59 A		2.34 B	3.67 A	

*; ** refer to specific effect of 2, 4-D and 2ip level added to MS medium, respectively.Capital and small letter/s were used for distinguishing between specific and interaction effect values, respectively. Mean followed by the same letter/s did not significantly differ at 5% level. Developed somatic embryos directly formed as shown in (Fig.1) after culturing on MS medium.

Fig.(1): Developed somatic embryos directly formed after culturing on MS medium supplemented with 2.5 mg/l 2,4-D + 3 mg/l 2ip

Multiplication stage

Effect of cytokinin type and NAA concentration

Number of proliferated shootlets

As for the specific effect of cytokinin type, data in **Table (2)** show the superiority of 2ip over kinetin as the results were 4.5and 3.79. On the other hand, number of proliferated shootlets was gradually increased by advancement of subculture number. Hence, kinetin supplemented MS medium resulted in 3.79; 5.17 and 6.04 shootlets per explent, while numbers of shootlets exhibited by 2ip supplied medium were 4.54 ; 8.46 and 9.73 per each explent during 1st ; 2nd and 3rd subcultures, respectively. Referring the specific effect of NAA concentration on number of shootlets, Table (2) reveals that no significant differences could be observed between 0.1 and 0.5 mg/l NAA at the first and second subculture, however 0.5mg/l NAA surpassed 0.1mg/l after the third subculture.

B. Interaction effect

The greatest number of proliferated shootlets was of Zaghloul date palm cultured on 3/4 MS medium supplemented with both 2ip and NAA each at 0.5 mg/l. The superiority of such combination over other investigated ones was significant during the three successive subcultures. Moreover, 3/4 MS medium supplemented with 0.1 mg/l. 2ip + 0.5 mg/l NAA, especially during both 2nd and 3rd subcultures ranked statistically second as the number of proliferated shootlets was concerned. On the contrary, four and NAA kinetin combinations exhibited generally the least number of proliferated shootlets, especially during all subculture tested 0.5 mg/l kinetin x 0.1 mg/l. Results regarding the effect of cytokinin concentrations goes generally in line with the early findings of several investigators, i.e. Abd-El-Baky. (2001) Abd- El-Hamid *et al.* (2001). Khierallah and Bader *et al*. (2007) reported that the best multiplication rate was achieved with 3 mg/l 2ip and 2 mg/l; for shoot elongation, the best

medium was MS containing 0.5 mg/l BAP, 0.5 mg/l 2ip, and 1 mg/l GA_3. Well-developed shoots were cultured for rooting on half MS medium amended with 1 mg/l NAA and 45 g/l sucrose.

Table (2): The effects of cytokinin type (Kin. & 2ip levels) and NAA rate on number of proliferated shootlets of Zaghloul date palm during multiplication stage .

Cytokinin treatments		Number of proliferated shootlets per jar cultured with indirectly formed somatic embryos through 3 subcultures of multiplication stage								
Type	**concentration. mg/l**	**Subculture I**			**Subculture II**			**Subculture III**		
		NAA mg/l			**NAA mg/l**			**NAA mg/l**		
		0.1 mg/l	0.5 mg/l	**Mean***	0.1 mg/l	0.5 mg/l	**Mean***	0.1 mg/l	0.5 mg/l	**Mean***
Kinetin	0.1 mg/l	4.00 cde	3.83 de	**3.79 B**	6.33 d	4.17 g	**5.17 B**	7.17 d	6.00 e	**6.04 B**
	0.5 mg/l	3.66 e	3.66 e		4.67 f	5.50 e		5.17 f	5.83 e	
2ip	0.1 mg/	4.66 ab	4.16 cd	**4.5 4 A**	8.00 c	9.08 b	**8.46 A**	9.50 c	10.17 b	**9.73 A**
	0.5 mg/l	4.33 bc	5.00 a		6.75 d	10.00 a		7.08 d	12.17 a	
Mean**		**4.16 A**	**4.16 A**		**6.44 A**	**7.19 A**		**7.23 B**	**8.54 A**	
Mean* for 0.1 & 0.5 mg/l**		**4.16 A & 4.12 A**			**6.90 A & 6.73 A**			**8.21 A & 7.56 A**		

*; ** and *** refer to specific effect of cytokinin type; NAA concentration and cytokinin concentration, respectively. Capital and small letters were used for distinguishing between specific and interaction effect values, respectively. Values followed by the same letter/s did not significantly differ at 5% level.

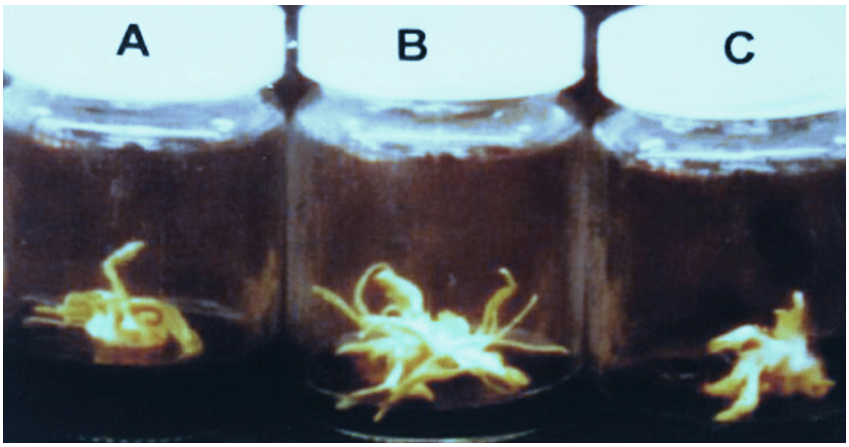

Fig.(2): Effect of two cytokinintypes (kinetin and 2ip) each at two concentration (0.1 and 1.0mg/l)on number of proliferated shootlets per each somatic embryodirectly induced (control) B . 0.1 mg/l 2ip C. 1.0mg/l kinetin

The effect of kinetin and 2ip on number of proliferated shootlets per each somatic embryo directly induced is demonstrated clearly in Fig.(2). The present results regarding the influence of cytokinin x NAA combinations go generally in line with the findings of several investigators, i.e. Nasir *et al.* (1994); AL-Khayri and AL-Maarri (1997); Bekheet and Saker (1998); Saker *et al.* (1998) and Ahmed (1999). All demonstrated the necessity of the proper rate of cytokinin x auxin combinations for the best shoots proliferation in date palm. Moreover, the suitability of 2iP than kinetin as cytokinin resource, data obtained in this regard is in

accordance with the findings of Abd EL-Baky (2001); Abd EL-Hamid *et al.* (2001) and Taha *et al.* (2001). All indicated the superiority of 2iP. However, Zaid (2003) separated the superiority of kinetin over 2iP.

Rooting stage

Effect of phloroglycinol on rooting

Rooting percentage

Table (3) and Fig. (3). show that the 3 PG levels (20 and 40 and 80 mg/l) resulted in significant increase in rooting percentage over control, but the intermediate level (40 mg/l PG) surpassed both 20 and 80 mg/l PG. On the other hand, rooting % was increased with advancement of subculturing number for any of the investigated PG treatments. However, rooting % reached its peak (100%) during 2nd subculture for cultured shootlets on either 20 or 40 mg/l PG supplemented MS rooting media. However, both 20 and 40 mg/l PG concentration were the most effective especially during 2nd and 3rd subcultures, whereas both resulted in 100 % rooting. From the other side, the lowest PG level (20 mg/l) was the most preferable as the economic standpoint was taken into consideration.

Number of rootlets / plantlet

Data in Table (3) reveal that both control and PG at the highest level (80 mg/l) were statistically the inferior, whereas the least number of rootlets per each plantlets was found (1.6 & 1.99 rootlets/plantlet). However, 40 mg/l PG was statistically the superior and resulted in the greatest number of rootlets per each plantlet (6.44). In addition, 20 mg/l PG supplemented MS rooting medium was statistically more effective as compared to those of either control or provided with 80 mg/l PG**.**

Average length of rootlets

Table (3) displays that ½ MS rooting medium provided with the moderate phloroglucinol (PG) level (40 mg/l) was statistically the superior and exhibited the tallest rootlets, i.e. 1.87; 2.72 and 3.00 cm during 1st , 2nd and 3rd subcultures, respectively.

Table (3): Effect of phloroglucinol (PG) on rooting formation of proliferated shootlets of Zaghloul cv. through three subcultures.

Treatments / Subculture / PG mg/ L	Rooting %			No. of rootlets/plantlet			Average rootlets length (cm)		
	Subculture I	Subculture II	Subculture III	Subculture I	Subculture II	Subculture III	Subculture I	Subculture II	Subculture III
0.0(control)	55.55 C	66.66 C	66.66 B	1.00 C	1.53 B	1.60 C	1.04 B	1.90 B	2.00 C
20.0	77.77 B	100.0 A	100.0 A	1.44 B	1.66 B	3.05 B	1.72 A	1.72 B	2.50 B
40.0	88.89 A	100.0 A	100.0 A	1.99 A	3.99 A	6.44 A	1.87 A	2.72 A	3.00 A
80.0	77.77 B	88.89 B	100.0 A	1.00 C	1.00 B	1.99 C	1.30 AB	1.64 B	2.61 B

Within each column, mean followed by the same letter/s did not significantly differ at 5% level.

The stimulative effect of providing MS rooting medium with phloroglucinol on rooting measurements is supported by the earlier findings of several investigators. However, most of these researches were carried out on other fruit species, i.e. Chongshum *et al.* (1998) on apricot ; Zanol *et al.,* (1998) on apple ; Madgi *et al.* (1999) on

apple ; Schmildt *et al.* (2000) on *Citrus sinesis*, Erbenova *et al.* (2001) on sweet cherry and Mona, (2005) on date palm. In addition, the beneficial effect of phloroglucinol (PG) on improving two rooting measurements (No and length of developed rootlets) goes generally in line with Madgi *et al.* (1999) on apple. Khierallah *et al*. (2017) cultured well-developed shoots for rooting in half MS medium amended with 1 mg/l NAA and 45 g/l sucrose and plantlets with well-developed roots were successfully hardened in the greenhouse. As shown in (Fig.3) the superior and tallest and greatest numbers of rootlets per each plantlet were found in 40 mg / l PG.

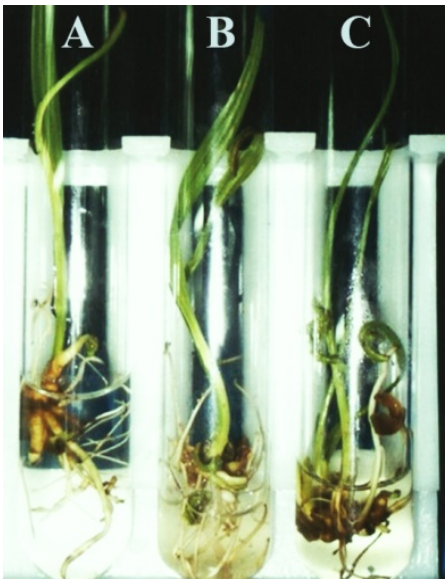

Fig. (3): Effect of phloroglycinol (PG) in the different level one rooting stage (A) 20mg/l (PG), (B) 40mg/l (PG) and (C) 80mg/l (PG) supplemented MS rooting medium.

CONCLUSION

The study highlighted on the auxin 2,4-D as important factor in inducing direct somatic embryogenesis in date palm shoot tip and leaf primordial explants. It could be recommended to use (2.5 mg/l 2.4-D) to induce somatic embryos directly from leaf primordia and use 2ip type at 0.5mg/ l in presence of 0.5 mg/l NAA to obtain the highest number of proliferated shootlets. Moreover, we can use phloroglcionl (PG) at concentration 20mg/l to get the tallest and greatest numbers of rootlets per each plantlet in rooting stage.

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الملخص العربي

انتاج الأجنة الجسدية المباشرة من الاوراق الأولية لنخيل البلح (صنف زغلول)

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المعمل المركزي لأبحاث وتطوير نخيل البلح مركز البحوث الزراعية -جيزة

الغرض من هذه الدراسه هو إستحثاث انتاج الاجنه الجسديه المباشره فى نخيل البلح صنف زغلول. أثبتت النتائج انه يمكن الحصول على أعلى نسبه من الاجنه الجسديه المباشره من الاوراق الاوليه على بيئه مكونه من ¾ ms (موراشيجى وسكوج) +2.4-D بتركيزات (٢.٥ و ٥.٠ و٧.٥ و١٠ ملليجرام/لتر) tفى وجود او عدم وجود 2ip+ 170 +0.4 mg/l thiamin-HCl g/l activated charcoal 30 g/l sucrose +7 g/l agar+200 mg glutamine+٣mg/l, KH2PO47 g/l وأوضحت النتائج ان التركيز الاقل من 2.4-D (٢.٥) أدى الى أنتاج نسبه أعلى من الآجنه الجسديه المباشره .تم نقل الاجنه المباشره على البيئه المكونه من 3.0 mg/l Kinetin + 170 mg/l KH2PO4 + 200 (1.5g) activated charcoal + 3/4 M S mg/l glutamine بهدف الاستطاله . من اجل التضاعف يضاف الى بيئه الزراعه (N6-2isopenteyl adenine) 2ipو Kinetin (6-furfuryl كلاهما بتركيز (0.1 & 1.0 mg/l) بمصاحبه NAA٠.٥ و٠.١ مليجرام على اللتر ولقد أوضحت النتائج ان الزياده فى عدد الافرع المتكشفه فى معاملات 2ip أعلا مقارنه Kinetin بينما كان التركيز الاعلى من (1.0 mg/l 2ipو تركيز٠.٥ NAA كان أكثر تأثيرو من أجل الحصول على تجذير جيد أضيف الى بيئه التجذير الفلوروجليسنول بتركيزات ٢٠ ،٤٠ و٨٠ ملليجرام على اللتر وكان أفضل تركيز ادى الى الحصول على اطول وأكبر عدد من الجذور ٢٠ ملليجرام على اللتر.عند الوصول الى مرحله الاقلمه تنقل النباتات الى قصارى من البلاستك على خليط من البيت موس + بيرليت بنسب متساويه تحت ظروف رطوبه عاليه ودرجه حرارة ٢٧ درجه مئويه وشده اضاءه ١٠٠٠٠ لاكس .