Use of Biotechnology for Multiplication of *Curcuma longa* L. plant during six subcultures

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ABSTRACT: The aim of this study was to set a protocol for the *in vitro* culture of *Curcuma longa* L. Sprouted bud explants of *C. longa* L. were explanted on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzyladenine (BA) 0, 1, 3, 5, and 7 mg l^{-1} during six subcultures. The maximum number of multiplied shoots (8.60 and 8.70 shoots $explant^{-1}$), was obtained in the medium containing 7 mg l^{-1} BA at 4th and 5thsubculture with (90 and 100% multiplication frequency) respectively. The optimum BA concentrations for maximum number of multiplication varied across subcultures. The 7 mg l^{-1} BA concentration at the late subculture 4th, 5th and 6th produce highest values of (number of shoots, longest shoot, number of leaves and fresh weight).The maximum number of roots (6.38 roots $explant^{-1}$) was induced from 3 mg l^{-1}IBA. The tallest roots (40.6 and 36.5 cm) were obtained on MS medium supplemented with 1 mg l^{-1} NAA and 3 mg l^{-1} IBA. Also, the heaviest fresh weight was obtained from medium containing 2 and 3 mg l^{-1} IBA and all concentrations of NAA. *In vitro* plantlets immediately acclimatized to greenhouse conditions, showing 100% survival rates in a peat moss and vermiculite (1:3) medium.

Keywords: Turmeric, *in vitro*, Mass production, subculture

INTRODUCTION

Zingiberaceae is comprised of about 52 genera and over than 1,600 species that is widely distributed in the humid tropics subtropics and some seasonably dry regions of the world (Ravindran *et al.,* 2007; Christenhusz and Byng, 2016). Of these genera, *C. longa* L. is one of the most important plants that is represented by 120 species (Sumathi, 2014; Chen *et al.,* 2015). Turmeric (*C. longa* L.) has been an important source of spice, dye and medicine for worldwide in the present and for traditional people throughout the ages (Deb and Chakraborty, 2017; Lamo and Rao, 2017). It is extremely nutritious, containing vitamins such as Betaine, Vitamin A and C, Folate and Choline and the minerals such as calcium, phosphorus, iron, zinc, magnesium, potassium and sodium (Yadav and Tarun, 2017). Besides, turmeric contains over than 235 compounds. Traditionally, turmericis vegetatively propagated from axillary branches "fingers or rhizomes", which commercially are called seed rhizomes. Propagation from seed rhizomes faced several problems for many reasons:

- Seed rhizomes have 1 or 2 buds, takes from 8 to 10 months to fully mature and become dormant over winter, even in tropical climates (Ghosh *et al.,* 2013; Ravindran, 2017).
- About 20 to 30% of whole production was needed for the next season with high cost, low productivity and disease susceptibility of seed rhizomes (Goyal *et al.,* 2010; Antoniazzi *et al.,* 2016).
- Flowers don't produce viable seeds because of their natural sterility (triploid 2n=63), mutations over period of the time, low fertility, natural seed set and environmental problems (Cheethaparambil *et al.,* 2014; Raju *et al.,* 2015).

Biotechnology is excellent tool for solving of turmeric production problems because it improves the crop and evolves the conventional breeding programs. In last few years, using of biotechnological tools have a quantum jump in commercial propagation and opened many possible ways in agriculture. Tools of agriculture biotechnology are solving the different problems, growing organisms, somaclonal variation, novel varieties and genetically engineered plants through tissue culture (Herdt, 2006; Ravindran *et al.,* 2007; Ravindran and Babu, 2016).

Obtaining an aseptic culture from underground explant is difficult due to high contamination. Rhizomes and leaves are the major parts affected by microbial diseases causing economic and yield losses ranged from 50 to 60 % in turmeric and their control is difficult. Like *Zingiberaceae* family, turmeric suffers from some disease problems in all turmeric-growing countries depending upon region and environmental condition from generation to other (Ravindran *et al.,* 2007; Jasim *et al.,* 2014; Sarathi *et al.,* 2014; Ilyas *et al.,* 2016).

Unfortunately, *C. longa* L. often presents a challenge to producers since it may be infected with many pathogens. Fungi such as *Pythium* and *Fusarium*, bacteria such as *Ralstonia* Sp., *Pseudomonas* Sp., *Bacillus* sp., *Staphylococcus* sp. and sot rot bacteria (*Erwinia carotovora*) and nematodes such as *Meloidogyne* spp. are the most frequent pathogens found on the surface and inside underground plant parts (Anoop *et al.,* 2014; Ajitomi *et al.,* 2015; Prabhu *et al.,* 2018).

The use of plant tissue culture technique as a biotechnological application of agriculture is more promising than other conventional propagation methods of *C. longa* L. plant. The objective of this study is to establish a micropropagation protocol for *C. longa* L. plants in Egypt.

MATERIALS AND METHODS

This work was carried out in the Plant Tissue Culture Laboratory, of Horticulture Department, Faculty of Agriculture, Suez Canal University (SCU), Ismailia Governorate, Egypt, during the period from 2013 to 2015.

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Plant materials

Turmeric rhizomes (seed rhizome) were collected from local market and used as a plant material. Morphological identification was performed at the Herbarium of Botany Dep., Fac. of Science, SCU., then confirmed using molecular identification tools ~~by~~ in Institute of Biotechnology for Postgraduate Studies and Research, SCU, Egypt.

Establishment of aseptic explant

Sprout bud explants were surface cleaned. Explants were excised with blade and washed with running tap water for 30 minutes. Disinfestation was performed with commercial systematic fungicide known as Sogaat™ 72% wet powder (SO)containing Mancozeb 64% + Metalaxyl 8% WP (Al-Ezz Group Comp.) 45 min. and mercury chloride $HgCl_2$for 7 min. and finally washed 3 times with distilled sterilized water.

Explants were cultured vertically into MS medium fortified with different concentrations of 6-benzyladenine (BA) at 0, 1, 3, 5, and 7 mg l^{-1} on multiplication during six subcultures.

Shoot initiation and multiplication

This experiment was designed to investigate the effect of BA concentrations (0, 1, 3, 5, and 7mg l^{-1}) on multiplication during six subcultures. One sprout buds were used as explants in each culture jars, and the *in vitro* multiplied shoots were sub-cultured every 8 weeks. Each jar contained 40 ml MS medium (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose, solidified with 7 g l^{-1} agar and pH was adjusted to 5.7±0.1. The cultural jars were immediately capped with polypropylene closure and autoclaved at 121 °C at 1.5 Kg/ cm^2 for 20 min. The jars were then placed in growth room at 16/8-hour photoperiod with 3000 lux (45 µmol $m^{-2}s^{-1}$) light at 23±2ºC day temperature. The data were record every 8 weeks after each subculture.

***In vitro* rooting of *C. longa* L.**

This experiment was carried out to examine the optimal auxin from IAA, IBA and NAA and its concentrations for the *in vitro* rooting of multiplied shoot of *C. longa* L. Multiplied shoot were cultured onto MS medium supplemented with sucrose at 30g l^{-1} and solidified with agar at 6 g l^{-1}. IAA, IBA and NAA were added individually to the medium at concentration of 0, 1, 2 and 3 mg l^{-1}. The cultures were incubated in the growth room under photo periodic cycle of 16/ 8 hrs. as light/ dark and 23±2 °C. The data were record after 4 weeks.

Acclimatization

Rooted plantlets were washed under running tap water to remove agar on the roots. Thoroughly, plantlets were soaked in fungicide solution contains 2.0 g l^{-1} Sogaatfor 10 seconds. The plantlets were individually transplanted into 5.0 cm plastic pots containing peatmoss + vermiculite (1:3). Pots were covered with transparent plastic sheets and mist irrigated daily for a week. Misting was decreased until plantlets were fully acclimatized after four weeks. Plants were re-cultured into large plastic pots (20 cm) containing sand. During this period, the plants were watered when needed with tap water and once per week with compound fertilizer which consisted of N: P: K (20:20:20) + 1 MgO + micro elements with irrigation water.

Data analysis or Statistical analysis:

Data were computerized and subjected to statistical analysis using SPSS "version 19" statistical software according to McDonald (2014).Statistical differences between the means were tested using LSD at 5% level of probability according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

The main effects of BA concentrations

The main effect of BA concentrations had a significant effect on the number of shoots, longest shoot, number of leaves, fresh weight and base diameter.

Data in Table (1a) indicate that 7 mg l^{-1} BA significantly produced the greatest number of shoots (5.78 shoots $explant^{-1}$), longest shoot (10.8 cm), number of leaves (20.8 leaves $explant^{-1}$), plantlet fresh weight (7.50 g) and base diameter (4.12 mm). In addition, the control has lower values of all parameters.

The main effects of six subcultures

The cultures were sub-cultured every 8 weeks using the same type of media, until the 6th subculture and the effect of six subcultures were analyzed with all parameters. The data in Table (1a) revealed that the 5thsubculture significantly increased the number of shoots (5.42 shoots $explant^{-1}$) and fresh weight (5.56 g). On the other hand, the 6thsubculture produced the highest values of the shoot length (12.04 cm), number of leaves (23.0 leaves $explant^{-1}$) and base diameter 4.31 mm. The results could then be used to guide later experiments to study the stability of every subculture genome.

The interaction effects between BA concentrations and subcultures

The same mother explant was subculture on MS medium supplemented with different concentrations of BA until six subcultures. Statistical analysis showed significant differences for interaction effects between BA concentration and subcultures for all studies characters as shown in Table (1b) and Fig. (1). Multiplication frequency % was calculated for each BA concentration during six subcultures is shown in Table (1b). The Multiplication frequencies are in the range of 75 to 100 %.

Absence of BA produces minimum number of multiple shoots in the range 1.0 to 1.4 shoots $explant^{-1}$, through all six subcultures. On other hand, the maximum number of multiplied shoots (8.6 and 8.7 shoots $explant^{-1}$), was obtained in the medium containing 7 mg l^{-1} BA at 4th and 5thsubculture with 90 and 100% multiplication frequency, respectively.

Data in same Table (1b) show that the minimum number of leaves was produced in the medium without BA in all subcultures, but the maximum number of leaves (34.6 leaves $explant^{-1}$) was obtained in the medium supplemented with 5 mg l^{-1} BA in 6thsubculture without any differences than medium containing 7 mg l^{-}

^{1} BA in 5th, 4th and 6thsubcultures (30.2, 29.8 and 29.8 leaves explant^{-1}) respectively. The BA concentrations at 0, 1, 3, and 7 mg l^{-1} in the first subculture produced the shortest shoot than 5 mg l^{-1} BA in the same subculture. In contrast, the tallest shoots (13.7 and 13.80 cm) was obtained in the medium supplemented with 1 and 7 mg l^{-1} BA in 5rdsubculture respectively as shown in Table (1b).

Absence of BA resulted in least values of fresh weight at all subcultures while the highest concentration 7 mg l^{-1} BA produce heaviest fresh weight at the last subcultures (4th, 5th and 6thsubculture). MS medium supplemented with 7 mg l^{-1} BA was found to be the most effective concentration for fresh weight 11.0 gat the 5thsubculture and least value 1.55 gat absence of BA in the first subculture as shown in Table (1b).

Concerning shoot base diameter, there was a significant difference between BA concentrations at different subcultures. clearly the data showed that the biggest base diameters were produced as follow: at the 6thsubcultures in medium supplemented with 0, 5 and 7 mg l^{-1} BA (3.50, 5.56 and 4.93 mm) respectively and at the 3rdsubculture in medium containing 1 and 3 mg l^{-1} BA (4.24 and 4.83 mm) respectively. The greatest value of multiple shoot base diameter (5.35 mm) were obtained from MS medium supplemented with 5 mg l^{-1} BA in 6th generation and the least values at the first subculture in absence of BA.

The optimum BA concentrations for maximum multiplication varied across subcultures or different subcultures. In this process the tissue or explant is first subdivide, then transferred into fresh culture medium. The 7 mg l^{-1} BA concentration at the subcultures 4th, 5th and 6th produce highest values of number of shoots, tallest shoots, number of leaves and heaviest fresh weight. This observation was confirmed by Sunitibala *et al.* (2001), who found that plantlets obtained after each subculture (as total 20 subculture) appeared healthy even after eleven or twelve subcultures. Besides, results in an average tenfold increase in shoot numbers per monthly culture subculture. On the same hand, Yusuf *et al.* (2011) found that the number of multiple shoots was low during first subculture but increased in third subculture and slightly decreased after fourth subculture for *Boesenbergia rotunda* L. (Zingiberaceae family) from sprouted bud explant.

Another successful result has been obtained by Khalafalla *et al.* (2011). They found that the maximum mean number of shoots per explant (86.5±3.6) was produced after three multiplication cycles on 3 mg/L BA-supplemented medium. *In vitro* induced shoots were excised and rooted on half strength MS medium fortified with 0.25 mg l^{-1}IBA to obtain complete plantlets of *Boscia senegalensis* regenerated plantlets obtained *in vitro* for the first time, were hardened and 95% survived under greenhouse conditions. Also, Selvakkumar *et al.* (2007) when cultured the explants excised from sub-culturing shoots of *Alpinia officinaru*m on the same fresh medium, found that during the 5-6 subculture the percentage of shoot development as well as the number of shoots per explant retained the same value (8.5 and 5.6 shoot explant^{-1}) and (3.3 and 4.2 cm explant^{-1}) for shoot length. Such type of simultaneous production of multiple shoot was reported earlier for a few medicinal plant species (Mohanty *et al.,* 2013). In addition, similar results were reported on banana by Al-Amin *et al.* (2009) and Bhosale *et al.* (2011) who found that addition of BA at 7.5 mg l^{-1} to MS medium enhanced the production of multiple shoots and maximum number of leaves.

Table (1a): Main effects of BA concentrations and subcultures on number of shoots, length of shoot, number of leaves, fresh weight and base diameter of *C. longa* L. during six subcultures (sprout bud explants)

	No. of shoots explant^{-1}	Length of shoot (cm)	No. of leaves explant^{-1}	Fresh weight (g)	Base diameter (mm)
			BA (mg l^{-1})		
0	1.25	9.95	3.35	2.00	2.90
1	4.12	10.2	17.0	4.35	3.69
3	4.48	9.22	17.7	4.46	3.71
5	3.78	9.67	17.1	4.19	3.82
7	**5.78**	**10.7**	**20.9**	**7.50**	**4.12**
			subcultures		
1	2.46	5.40	8.88	2.74	3.22
2	2.70	10.3	11.8	4.06	3.64
3	3.18	10.5	11.0	4.16	3.67
4	5.00	10.0	17.0	5.25	3.15
5	**5.42**	11.4	19.6	**5.56**	3.90
6	4.54	**12.0**	**23.0**	5.23	**4.31**
LSD 5% 1st factor	**0.40**	**0.59**	**1.82**	**0.48**	**0.32**
LSD 5% 2nd factor	**0.44**	**0.64**	**1.99**	**0.53**	**0.35**

Means with the same letters in the same column are not significantly different according to least significant difference test (LSD) 5%

Table (1b): Interaction effects of BA concentrations and subcultures on number of shoots, length of longest shoot, number of leaves, fresh weight and base diameter of *C. longa* L. during six subcultures (sprout bud explants)

BA(mg l^{-1})	Subcultures	multiplication frequency %	No. of shoots explant^{-1}	Length of longest shoot (cm)	No. of leaves explant^{-1}	Fresh weight (g)	Base diameter (mm)
0	1st	75	1.00	5.50	2.40	1.55	2.20
	2nd	75	1.20	9.30	3.40	2.01	2.55
	3rd	75	1.20	11.8	3.00	1.83	2.60
	4th	100	1.40	11.7	3.00	2.14	3.22
	5th	100	1.40	10.0	3.80	2.14	3.30
	6th	100	1.30	11.4	4.50	2.32	3.50
1	1st	80	3.00	5.00	9.90	2.29	3.05
	2nd	80	3.20	10.4	17.3	4.93	3.67
	3rd	100	3.30	8.90	14.5	4.46	4.24
	4th	100	5.20	11.5	16.6	4.76	3.39
	5th	100	6.60	13.7	26.4	5.53	3.75
	6th	100	3.40	11.7	17.5	4.14	4.02
3	1st	80	2.50	3.30	10.6	2.87	2.95
	2nd	80	2.70	10.1	11.5	4.31	4.12
	3rd	80	2.90	7.70	11.9	3.88	4.83
	4th	80	6.30	7.90	21.0	5.78	2.89
	5th	100	6.80	13.2	22.6	5.56	3.95
	6th	100	5.70	13.1	28.8	4.35	3.54
5	1st	80	2.80	8.70	11.2	3.57	3.42
	2nd	80	3.10	10.6	14.9	3.42	3.56
	3rd	100	3.10	11.7	12.1	4.08	3.52
	4th	90	3.50	8.40	14.5	3.78	2.81
	5th	90	3.60	6.20	15.1	3.57	4.07
	6th	100	6.60	12.4	34.6	6.72	5.56
7	1st	70	3.00	4.50	10.3	3.43	4.48
	2nd	80	3.30	11.1	11.9	5.63	4.32
	3rd	90	5.40	12.6	13.3	6.54	3.16
	4th	80	8.60	10.6	29.8	9.80	3.44
	5th	100	8.70	13.8	30.2	11.0	4.41
	6th	80	5.70	11.6	29.8	8.60	4.93
LSD 5%			**0.98**	**1.44**	**4.46**	**1.19**	**0.78**

Means with the same letters in the same column are not significantly different according to least significant difference test (LSD) 5%

***In vitro* rooting of *C. longa* L.**

Multiplied plantlets of *C. longa* L. were cultured individually on MS medium supplemented with different concentrations of auxin types IAA, IBA and NAA at the concentrations of 0, 1, 2 and 3 mg l^{-1}. Presented data in Table (2) show that MS medium supplemented with or without auxin types produced 100% of rooting.

Data present in Table (2) show that the maximum number of roots (6.38 roots/explant) was induced from medium supplemented with 3 mg l^{-1} IBA. In addition, NAA at 1 mg l^{-1} and IBA at 2 and 3 mg l^{-1}produced the tallest plantlets as 20.0, 20.2 and 19.4 cm, and longest roots as 40.9, 36.5 and 34.7 cm, respectively. The tallest roots (40.6 and 36.5 cm) were obtained from MS medium supplemented with 1 mg l^{-1} NAA and 3 mg l^{-1} IBA. Also, the heaviest fresh weight was obtained from medium containing 2 and 3 mg l^{-1} IBA and all concentration of NAA.

Generally, in multiplied shoots of *C. longa* L. there was no significant effect between both IBA and IAA and the control treatment. So, we support that using of free-hormone medium to *in vitro* rooting stage. The plantlets rooted with IAA and IBA and also those produced without auxins survived well in soil as long and hardy. In respect to the studies of Das *et al.* (2010)Ghosh *et al.*(2013) and Babu *et al.* (2016) and others who mentioned to use NAA and IBA for *in vitro* rooting. Several studies reported that irrespective BA concentrations resulted in prolific root system in culture medium as mentioned by Ravindran *et al.*(2007). Also, Bhattacharya *et al.* (2014) mentioned that plant growth regulator may or may not be required for *in vitro* rooting and absence of PGRs in medium rooted well of *C. zedoria, Z. zerumbet, Z. zedoaria, A. galangal*, and others.

Acclimatization

Plantlets were washed under running tap water to remove agar on the roots. Thoroughly, plantlets were soaked in fungicide solution (0.2%). Plantlets were acclimatized in the greenhouse for 30 days and 100% of plants survived and had normal growth after transferring them to the field during the planting season.

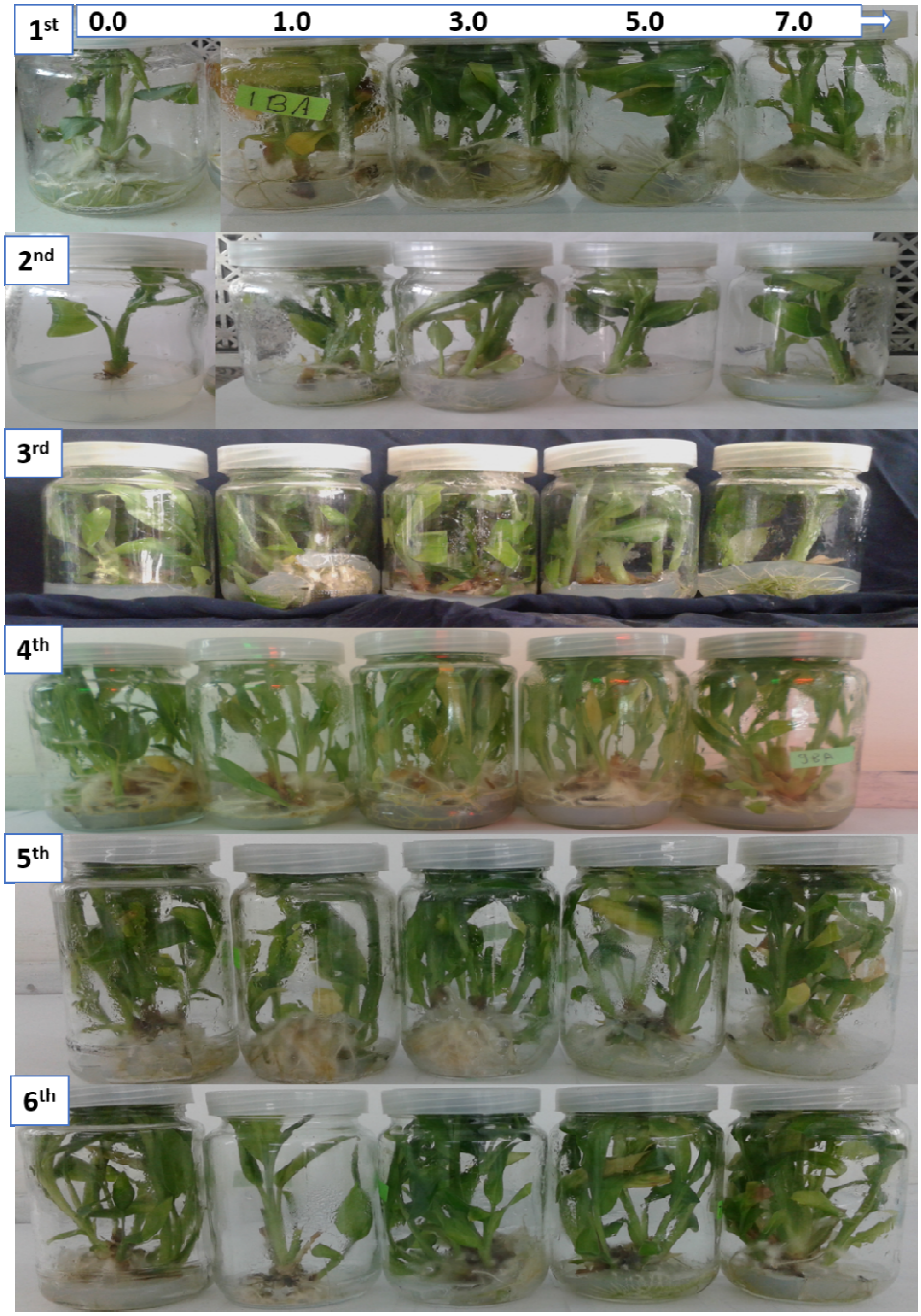

Figure (1): Multiplied shoots induction of *C. longa* L. (a) first subculture (1st) (b) second subculture (2nd) (c) third subculture (3rd) (d) fourth subculture (4th) (e) fifth subculture (5th) (f) sixth subculture (6th)

Table (2): Effects of IAA, IBA and NAA concentrations on *in vitro* rooting of *C. longa* L

IAA mg l^{-1}	IBA mg l^{-1}	NAA mg l^{-1}	Rooting %	No. of roots/explant	Root length (cm)	Plantlets height (cm)	Fresh weight (g)
0	**0**	**0**	100	5.20	23.9	15.0	7.94
1	**0**	**0**		4.25	20.8	17.0	8.65
2	**0**	**0**		3.38	16.5	13.8	7.05
3	**0**	**0**		3.58	17.6	13.2	7.52
0	**1**	**0**		4.50	27.2	17.3	10.9
0	**2**	**0**		5.30	34.7	20.2	16.3
0	**3**	**0**		6.38	36.5	19.4	18.7
0	**0**	**1**		5.60	40.6	20.0	16.2
0	**0**	**2**		4.22	20.4	17.6	16.7
0	**0**	**3**		5.13	22.6	17.4	18.6
LSD 5%				**2.09**	**16.3**	**5.43**	**4.20**

Means with the same letters in the same column are not significantly different according to least significant difference test (LSD) 5%

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استخدام التكنولوجيا الحيوية لتضاعف نبات الكركم خلال ستة أجيال

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الهدف من هذه الدراسة هو تأسيس بروتوكول لإنتاج نبات الكركم *Curcuma longa* L. معمليا. تم استخدام البراعم المنبتة من نبات الكركم كمنفصل نباتي وزراعتها على بيئة موراشيج وسكوج مضافا اليها تركيزات مختلفة من هرمون BA (0.0 و1.0 و3.0 و5.0 و7ملجم/لتر) خلال ستة أجيال. وجد ان أكبر عدد من الأفرع المتضاعفة (8.7 و8.6 فرع/منفصل نباتي) تم الحصول عليها في بيئة تحتوي على 7 ملجم/لتر BA خلال المرحلة الرابعة والخامسة لإعادة الزراعة على التوالي (90 و100% لمعدل تكرار التضاعف). كان التركيزات الأمثل من هرمون BA للحصول على أكبر عدد من الأفرع المتضاعفة متنوعة خلال المراحل المختلفة من إعادة الزراعة. وكان لتركيز 7 ملجم/لتر BA خلال المرحلة الرابعة والخامسة والسادسة لإعادة الزراعة أعطت أعلى القيم لكل من (عدد الأفرع وأطول الأفرع وعدد الأوراق والوزن الطازج للأفرع المتضاعفة). كان أكبر عدد للجذور (6.38 جذر/نبتة) تم الحصول عليها من 3 ملجم/لتر IBA. كما ان اطول الجذور (40.9 – 36.5 سم) تم الحصول من بيئة موراشيج وسكوج مضافا اليها تركيز 1 ملجم/لتر NAA و3 ملجم/لتر IBAعلى التوالي. بالإضافة للحصول على أثقل وزن طازج من بيئة تحتوي على 2 و3 ملجم/لتر IBAوجميع تركيزات NAA. النبيتات المنتجة معمليا تم أقلمتها مباشرة تحت ظروف الصوبة وأظهرت النتائج معدل نجاح 100% في بيئة بيت موس وفيرموكليت (1:3).