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FACTORS AFFECTING MICROPROPAGATION OF *NEPETA SEPTEMECRENATA* PLANT AND VITRIFICATION

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ABSTRCT: *Nepeta septemcrenata Benth. is endangered endemic Egyptian medicinal plant which is a member of the mint family (Lamiaceae). Nepeta septemcrenata Benth. was recorded in Saint Catherine mountains at Gebel Catherina, Gebel Musa, Ras Safsafa, and Wadi Razana. The main aim of this study is to establishment a successful protocol for micropropagation of N. septemcrenata in order to save plant of extinction. The effect of different concentrations of cytokinin types (BAP and TDZ) single or in combination with activated charcoal (AC) were tested, also the effect of puclobutrazol concentrations (PP_{333}) individual or in combination with AC on overcome vitrification was examined. Also, effect of different concentrations of IBA on rooting and growth parameters was investigated. The best medium for multiplication of Nepeta septemcrenata Benth was MS medium supplemented with 30 g/l sucrose, 6 g/l agar, 1 mg/l TDZ and 2 g/l Ac and 1 mg/l PP_{333}. For rooting, MS medium supplemented with 30 g/l sucrose, 6 g/l agar, 2 mg/l IBA was effective in induction healthy roots.*

Key words: *Nepeta septemcrenata, micropropagation, cytokinin, BAP,TDZ, AC, IBA, PP_{333} and vitrification*

INTRODUCTION

About 250 species of Nepeta plants were reported (Evans and Evans 1996), but only one species of this genus was recorded in Egypt (*Nepeta septemcrenata* Benth.), which commonly known as Zaeeta. It is a rare endemic Egyptian medicinal plant with a restricted habitat, which is a member of the mint family (Lamiaceae) and has a considerable folkloric reputation (Abd El-Moaty, 2009). *Nepeta septemcrenata* Benth. was recorded in Saint Catherine mountains at Gebel Catherina, Gebel Musa, Ras Safsafa, and Wadi Razana. It is more common in rocky crevices. Its distribution is limited to elevations of 2200-2500m (Täckholm, 1974 and Abdel-Raouf and Kamel, 1995). Genetic variability of *Nepeta septemcrenata* Benth populations from six locations in Saint Katherine Protectorate was determined by RAPD marker (Elkholy *et al*., 2011). In traditional Bedouin use, Zaeeta is believed to be sedative, carminative, and antispasmodic agent. It has also been used traditionally to treat colds, flu, cough, spasms and fevers (Abd El-Moaty, 2009). Also, it has antimicrobial and antifungal activity. The constituents of Zaeeta have been investigated and several classes of secondary metabolites have been isolated such as flavonoids, essential oil-containing monoterpenes, terpenoids, and sterols (Abd El-Moaty, 2010). Efforts to preserve this species should be carried out to prevent its extinction and ensure its survival, and to take advantage of its valuable biotechnological potential. Measures to develop micropropagation protocols for this endangered shrub with high field survival are essential (Boulos, 2002).

In vitro culture can have major advantages over conventional propagation techniques in the management of some wild plants species. Rapid multiplication under controlled, pathogen-free conditions can be achieved by the inclusion of plant growth regulators or hormones. In this technology, large numbers of shoots can be produced from small quantities of initial material, in some cases, as little as one bud or seed (Fay, 1994). *In vitro* clonal propagation of many plant species through tissue cultures has been frequently based on the prosperous adjustment of the type and combination of plant growth hormones. Most cytokinins are adenine derivatives. The

adjustment of exogenous auxins and cytokinins levels play an important role in shoot induction and plant regeneration in most plant species and also may stimulate cell division. Thidiazuron has been used with considerable success in the promotion of plant regeneration in woody species (Murashige, 1990; Huetteman and Preece,1993, Rout *et al*., 2000, Uranbey *et al*., 2005). *In vitro* shoot regeneration of native spearmint (*Mentha spicata* L.) from internodes was evaluated on MS media supplemented with individual cytokinins TDZ, BAP or Kn. The highest regeneration was achieved by the second internodes on a medium containing MS basal salts with B5 vitamins, 10% coconut water and 1.0 mg/l TDZ (Poovaiah *et al.* (2006)). In a study on *Nepeta septemcrenata* Benth., benzyl adenin showed the highest multiplication rate (3.11) at 1 mg/l concentration among the different cytokinins tested (BA, Kn and 2iP) (Maksoud, 2012).

Vitrification phenomena (hyperhydricity) is a physiological disorder, expression involves excess water uptake and inhibition of lignin and cellulose synthesis. This condition appears to be a consequence of water relationships between the matrix potential in the medium and developing shoots, as well as a low nitrate to ammonium ratio. Vitrification is more prevalent if plants are grown in liquid media or with low agar concentrations, high humidity, and high ammonium concentrations. (as in MS medium). Some relief from this problem include increasing the agar concentration, changing brands of agar, modification of inorganic ingredients in the medium, changing cytokinin concentration, and the addition of antivitrification agents (Pierik, 1987 and Ziv, 1991). Physiological disorder; hyperhydricity, was affected by MS salt strength. Adding 0.2 mg/l PP_{333} to MS medium at full salt strength was superior to overcome hyperhydricity (Maksoud, 2012).

Shoots of *Teucrium fruticans* cultured on MS medium supplemented with 2.5 µM IBA showed the highest average root number (Frabetti *et al.* 2009). Also, shoots obtained from nodal cuttings of *Justicia gendarussa* Burm were rooted on MS medium augmented with 9.8 µM IBA. (Thomas and Yoichiro, 2010).

The present investigation was undertaken to identify the suitable cytokinin types and concentrations for micropropagation of *Nepeta septemcrenata* Benth and to find out suitable medium for overcome vitrification to establishment a successful protocol for micropropagation of *Nepeta septemcrenata* in order to save plant of extinction.

MATERIALS AND METHODS

Shoot tips of *Nepeta septemcrenata* Benth. were harvested from its germplasm growing in greenhouse of Genetic Engineering and Biotechnology Research Institute (GEBRI), Minufiya University, Sadat City, Egypt. Explants were washed by rinsing in running tap water and detergent for one minute, then, explants were surface-sterilized in laminar air flow chamber with 1.5% (v/v) sodium hypochlorite (NaOCl) solution for 7 min, and then rinsed three times with sterilized distilled water. Explants were cultured in Sigma culture tubes contain 12.5 ml of basal MS medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose and 6 g/l agar. After 21 days from culturing, shoots free contamination causes (Fig.1, a). were divided into nodal segments containing two nodes, then two explants were cultivated in jars (250 ml) contained 50 ml MS medium supplemented with different concentrations of 6benzylaminopurine (BAP) or Thidiazuron (TDZ) as cytokinin at (0.0, 1.0 and 2.0 mg/l) single or in combination with 2 g/l activated charcoal (AC). Shoots number, shoot length (cm) and nodes number were recorded at the end of subculture (30 days of cultivation), also vitrification (Fig.1, b) and growth vigor were measured according to the methods described by Pottino (1981). Physiological disorder phenomena (vitrification which cleared in Fig.1, b) was observed, so the resulted shoots were transferred to MS medium supplemented with 30 g/l sucrose, 1mg/l TDZ, 6 g/l agar and different concentrations of puclobutrazol (PP_{333}) at (0.0, 0.05, 0.50, 1.00 and 2.00 mg/l)

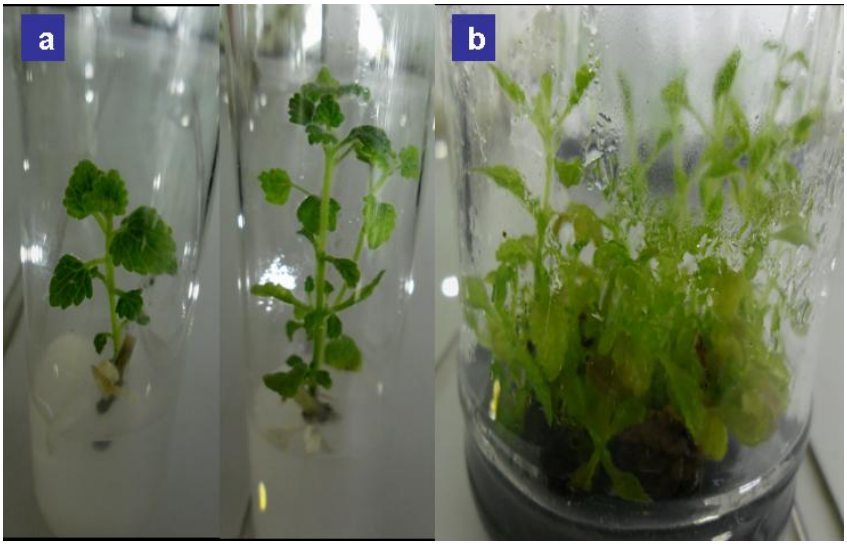

Figure (1): a) Shoots free contamination causes in sigma culture tube after 21days of culture.
b) Vitrification phenomena appeared in multiplication stage with increasing cytokinin concentrations

individual or in combination with 2g/l AC to study the effect of both puclobutrazol and. activated charcoal on overcome vitrification. Shoot number, shoot length, vitrification and growth vigor were recorded after about 30days. The resulted shoots were transferred to rooting MS medium containing 30g/l sucrose, 1mg/l PP_{333}, 2g/l AC, 6g/l agar and different concentrations of indole buteric acid (IBA). Shoot number, shoot length (cm), root number and root length (cm) were recorded. The pH of all media was adjusted to 5.8 prior to steam sterilized in an autoclave under pressure of 1.2Kg/cm2 (121^{o}C) over a period of 20minutes. The cultures were incubated at a temperature of 25±2°C and 70-80% relative humidity with 16h photoperiod and light intensity 2000lux.

Statistical analysis: Data were statically analyzed by one or two factorial complete randomized design using SAS (1988) package. Each treatment was represented as six replicate jars with two explants per jar. Differences among various treatments were compared using the least significant differences (LSD) test at 5% level according to Steel and Torrie (1980).

RESULTS AND DISCUSSIONS

Nepeta septemcrenata Benth. is a rare endangered endemic Egyptian medicinal plant with a restricted habitat. There are few researches carried out on its finger print, chemical components and biological effects. *Nepeta septemcrenata* Benth is a rare plant, so, establishment a successful protocol for micropropagation of *Nepeta septemcrenata* Benth. is essential to obtain a huge number of plantlets. Many factors affect multiplication of any plant especially growth regulators. The effect of different concentrations of cytokinin types (6benzylaminopurine (BAP) or Thidiazuron (TDZ) were discussed in Table (1) and Fig (2). Data cleared that shoot proliferation positively related to cytokinin concentrations, the highest shoot number was resulted when the highest BAP and TDZ concentration (2 mg/l) was used (9.17 and 10.50 shoot/explant, respectively) with no significant difference between BAP and TDZ. Also, AC affected shoot proliferation positively (13.00 shoot/explant). Interaction between different concentrations of cytokinin types and activated charcoal revealed that 1 and 2 mg/l TDZ in combination with AC

Table 1

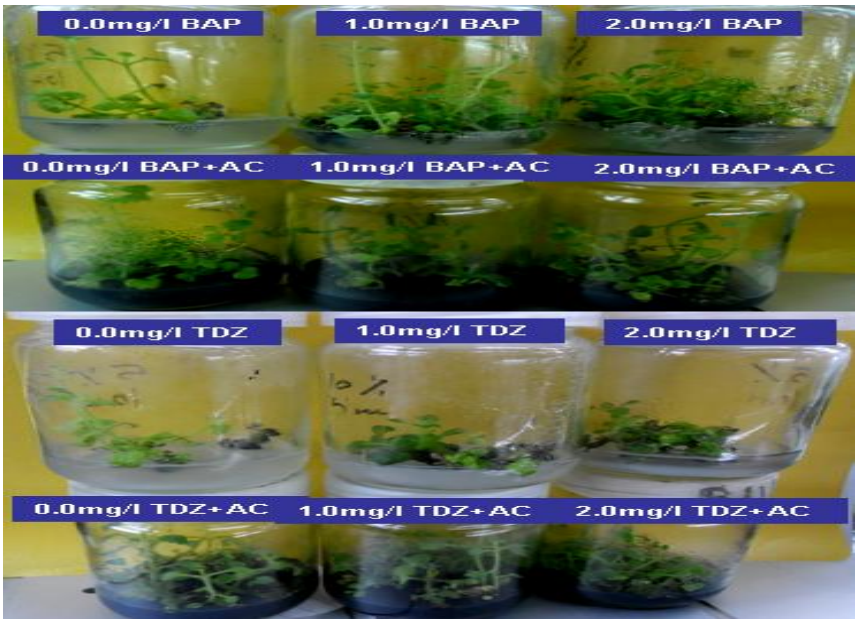

Figure (2): Effect of different concentrations of cytokinin types (BAP and TDZ) and activated charcoal (AC) on growth parameters and vitrification of *Nepeta septemcrenata* Benth. during multiplication stage

significantly increased shoot number (13.00 and 13.00 shoot/explant, respectively). Regarding shoot length, TDZ at 2.0mg/l significantly maximized shoot length (6.83 cm). Also, AC significantly enhanced shoot length (5.52 cm). Interaction between different concentrations of cytokinin types and activated charcoal showed that 2.0 mg/l of TDZ in combination with AC resulted in the highest shoots length (8.40cm). Nodes number and growth vigor were also significantly affected by different concentrations of cytokinin types and activated charcoal. The maximum values of nodes number and growth vigor were obtained at 2 mg/l TDZ in combination with AC (6.00 and 5.00, respectively). Vitrification was significantly increased with increasing cytokinin concentrations. Presence of AC in significantly decreased vitrification. Results came in line with Ziv, 1991 who reported that increasing cytokinin concentrations lead to maximum vitrification appearance.

Data in Table (2) and Fig.(3) showed the effect of different concentrations of puclobutrazol (PP_{333}) single or in combination with activated charcoal (AC) on growth parameters and overcome vitrification. Data revealed that no significant differences were observed in shoot number as a result of PP_{333} concentrations, adding AC to MS medium or interaction between them. While shoot length was negatively affected by increasing PP_{333} concentrations and positively affected by presence of AC in MS medium. Interaction between PP_{333} concentrations and AC showed that 1mg/l PP_{333} in combination with AC resulted in slight decrease in shoot length (3.73 cm). Regarding vitrification, it was shown that the high concentration of PP_{333} the low value of vitrification, also, data cleared that adding AC led to decrease of vitrification. Interaction between PP_{333} and AC cleared that 1 and 2 mg/l with or with-out AC showed overcome vitrification phenomena, this result may be due to the effect of both PP_{333} and AC on synthesis or accumulation of ethylene which affects vitrification. Growth vigor was maximized with increase PP_{333} concentrations and Adding AC. The highest growth vigor obtained at 1 mg/l PP_{333} in the presence of AC (5.00) and 2 mg/l PP_{333} without AC (5.00). This result may be due to enhancing storage as well as growth vigor. Data came in agreement with Pierik, 1987, Ziv, 1991 and Maksoud, 2012.

Table 2

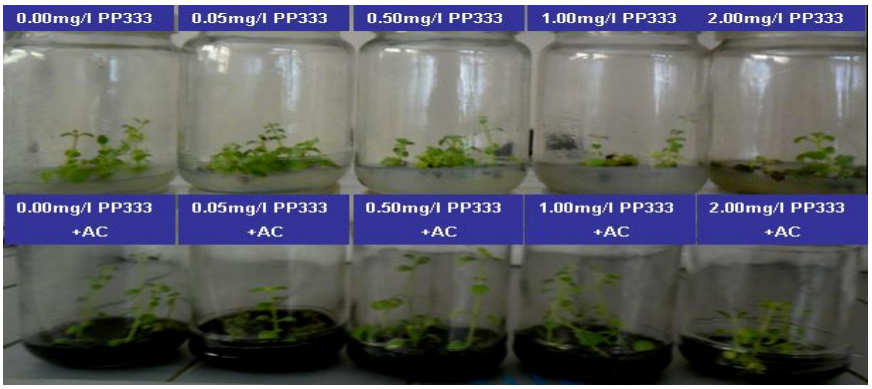

Figure (3): Effect of puclobutrazol (PP_{333}) concentrations and activated charcoal (AC) on growth parameters and vitrification of *Nepeta septemcrenata* Benth. during multiplication stage

Data in Table (3) and Fig (4) cleared the effect of IBA concentrations on growth parameters of *Nepeta septemcrenata* Benth. Results revealed that increasing IBA concentrations resulted in decreasing shoot number and shoot length, but the differences were not significant. Root number was promoted with increasing IBA concentration but the differences were not significant. While root length was significantly enhanced with increasing IBA concentrations. Results agree with *Thomas and Yoichiro, 2010* who used IBA in rooting of *Justicia gendarussa*.

Conclusion

Nepeta septemcrenata Benth is a rare endangered endemic Egyptian medicinal plant, so establishment a successful protocol for Micropropagation is very important issue. This investigation is a primary step to propagate and save this important plant and prevent its extinction. The best medium for multiplication of *Nepeta septemcrenata* Benth is MS medium supplemented with 30 g/l sucrose, 6 g/l agar, 1 mg/l TDZ, 2 g/l Ac and 1 mg/l PP_{333}. For rooting, MS medium supplemented with 30 g/l sucrose, 6 g/l agar, 1 mg/l PP_{333}, 2 gm/l AC and 2 mg/l IBA.

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Table (3): Effect of indole buteric acid (IBA) on growth parameters of *Nepeta septemcrenata* Benth. during rooting stage

IBA concentration (mg/l)	Shoot NO./plantlet	Shoot length (cm)	Root NO./ plantlet	Root length (cm)
0.0	2.00	11.0	7.00	4.53
0.5	2.33	10.0	6.33	2.90
1.0	1.67	11.0	7.00	3.30
1.5	1.67	9.3	6.33	4.23
LSD at 5%	NS	NS	NS	0.68

Figure (4): Effect of indole buteric acid (IBA) concentrations on growth parameters of *Nepeta septemcrenata* Benth. during rooting stage

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العوامل المؤثرة على الإكثار الدقيق وظاهرة التزجج لنبات نبيتا سيبتماكريناتا بنث

ابتسام مبارك حمزة

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الملخص العربى

نبات نبيتا سيبتماكريناتا من النباتات المصرية المهددة بالانقراض. ينتمى نبات النبيتا سيبتماكريناتا الى العائلة الشفوية والتى منها النعناع. ينمو نبات النبيتا سيبتماكريناتا فى منطقة سانت كاترين بسيناء بطريقة برية فى جبل سانت كاترين وجبل موسى و رأس صفصافا و وادى رازان. الهدف الاساسى للدراسة هو تأسيس بروتوكول ناجح لإكثار نبات النبيتا سيبتماكريناتا وحمايتها من الانقراض. تمت دراسة تأثير التركيزات المختلفة للأنواع المختلفة للسيتوكينينات (BAP&TDZ) بمفردها او بالاضافة الى الفحم النشط (AC). كذلك تم دراسة تأثير التركيزات المختلفة للبكلوبترازول (PP_{333}) بمفرده او بالاضافة الى الفحم النشط (AC) فى التغلب على ظاهرة التزجج كما اختبر تأثير التركيزات المختلفة لل IBA على التجذير ومواصفات النمو. وكانت أفضل بيئة للتضاعف هى بيئة موراشيجى وسكوج (MS) والمضاف إليها 30جم/لتر سكروز+ 6جم/لتر آجار+1ملجم/لتر TDZ+2جم/لتر AC+1ملجم/لتر PP_{333}. وكانت أفضل بيئة للتجذير هى بيئة موراشيجى وسكوج (MS) والمضاف إليها 30جم/لتر سكروز+ 6جم/لتر آجار+2ملجم/لتر IBA+2جم/لتر AC والتى اظهرت فاعلية فى الحث على تكوين جذور.

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Table (1): Effect of different concentrations of cytokinin types (BAP and TDZ) and activated charcoal (AC) on growth parameters and vitrification of *Nepeta septemcrenata* Benth. during multiplication stage.

Cytokinin (A)	Parameters	Shoot NO./explant			Shoot length (cm)			Nodes NO./shoot			Vitrification*			Growth vigor**		
	AC (B) / Conc.(mg/l)	Without AC	With AC	Mean (A)	Without AC	With AC	Mean (A)	Without AC	With AC	Mean (A)	Without AC	With AC	Mean (A)	Without AC	With AC	Mean (A)
BAP	0	6.33	6.33	6.33	3.87	4.27	4.07	4.33	4.07	4.00	2.00	2.67	2.33	2.67	3.00	2.83
	1	10.00	8.33	9.17	3.70	5.27	4.48	3.67	4.00	3.83	1.33	1.67	1.50	3.00	5.00	4.00
	2	9.00	9.33	9.17	5.10	5.27	5.18	4.33	4.67	4.50	1.00	2.00	1.50	3.00	5.00	4.00
TDZ	0	6.33	6.53	6.43	3.87	4.27	4.07	4.33	3.00	3.67	2.00	2.67	2.33	2.67	3.00	2.83
	1	5.00	13.00	9.00	3.27	5.63	4.45	3.00	5.00	4.00	3.33	1.67	2.50	3.00	3.67	3..33
	2	8.00	13.00	10.50	5.27	8.40	6.83	5.33	6.00	5.67	4.33	1.33	2.83	3.33	5.00	4.17
Mean (B)		7.44	9.42		4.18	5.52		4.17	4.39		2.33	2.00		2.94	4.11	
LSD at 5%		A	B	AxB	A	B	AxB	A	B	AxB	A	B	AxB	A	B	AxB
		1.55	0.897	2.19	0.92	0.53	1.304				0.83	NS	1.18	0.41	0.24	0.58

*Vitrification and growth vigor were measured as described by Pottino (1981)

Table (2): Effect of puclobutrazol (PP_{333}) and activated charcoal (AC) on growth parameters and vitrification of *Nepeta septemcrenata* Benth. during multiplication stage

PP_{333} Conc. (mg/l)(A)	Shoot NO./explant			Shoot length (cm)			Vitrification*			Growth vigor		
	AC (B)		Mean (A)	AC (B)		Mean (A)	AC (B)		Mean (A)	AC (B)		Mean (A)
	Without AC	With AC		Without AC	With AC		Without AC	With AC		Without AC	With AC	
0.0	4.33	4.00	4.17	3.77	6.10	4.93	3.27	2.00	2.63	2.00	3.00	2.50
0.05	4.00	4.00	4.00	3.27	3.90	3.58	3.33	2.00	2.52	2.67	3.33	3.00
0.5	4.67	4.33	4.50	2.77	3.50	3.13	2.57	1.00	1.78	2.67	4.00	3.33
1.0	4.00	4.33	4.17	2.23	3.73	3.48	1.00	1.00	1.00	4.00	5.00	4.50
2.0	4.00	4.00	4.00	3.10	3.10	3.10	1.00	1.00	1.00	5.00	3.00	4.00
Mean (B)	4.20	4.13		3.23	4.07		2.17	1.40		3.27	3.67	
LSD at 5%	A	B	AxB	A	B	AxB	A	B	AxB	A	B	AxB
	NS	NS	NS	0.31	0.19	0.43	0.11	0.07	0.15	0.35	0.22	0.50

*Vitrification and growth vigor were measured as described by Pottino (1981)