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PROPAGATION OF *COTONEASTER HORIZONTALIS,* DECNE THROUGH *IN VITRO* CULTURE

BY

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ABSTRACT

This study has been executed on *Cotoneaster horizontalis,* Decne at the Tissue Culture and Germplasm Conservation, Research Laboratory, Horticulture Research Institute, Agriculture Research Center during the years of 2006 and 2007. The goal of this research was to establish a protocol for micropropagation of Cotoneasters plants.

The data revealed that for sterilization explants, using (15% Clorox for 10 min) gave the best result for free contamination explants and the highest survival percentage.

For shooting behaviour, adding (3 mg/l BA) to MS-medium increased the shootlet number/explant; fresh and dry weights of shootlets. However shootlet length increased by adding (2 mg/l BA) to MS-medium, while the number of leaves increased by adding (1 mg/l 2iP) to MS-medium. For the type of media, culture the shootlet on MS-medium increased the number and length of shootlets, number of leaves, fresh and dry weight compared to culture the shootlets on WPM, B_5 or LS media.

Concerning to chemical composition, the highest amount of chlorophyll-a was recorded with MS-medium supplemented with 4 mg/l BA, while the highest amount of chlorophyll-b and carotenoids was recorded with MS-medium supplemented with 2 mg/l kin. Total indoles increased by adding 1 mg/l kin and total soluble phenols increased when using 3 mg/l 2iP.

Regarding to rooting behaviour, the highest percentage of rooted shootlet was recorded when culture the shootlets on full MS-strength with 1 mg/l IAA. For the number and the length of roots, no significant differences between all treatments on number of roots per shootlet and length of root (cm). For acclimatization, the survival percentage was 70%.

Key words: *Cotoneaster horizntalis, in vitro* culture, BA, Kin, 2iP, MS, WPM, B_5, LS

INTRODUCTION

Cotoneaster horizontalis, Decne family Rosaceae are ornamental shrubs, many of them with decorative fruit remaining usually through the whole winter.

Cotoneaster horizontalis, Decne low shrub: branches almost horizontal and densely distichously branched, Baily (1976).

Cotoneaster horizontalis, is very rare in Egypt, it is found only in Orman, Giza (Khalifa and Loutify, 2006).

There are many techniques available for the conservation of plant genetic resources of rare and endangered species. These include micropropagation, seed germination, regeneration from callus, micrografting and cryopreservation (Nitzsche, 1983; Rick, 1984; Stanilova *et al.,* 1994). Micropropagation in Cotoneaster plants as an alternative to conventional methods for vegetative propagation attracts much attention, because of its advantages. It increases many times the multiplication level (Novak and Petra, 1981; Takayama and Misawa, 1982 and 1983; Van Aartijk and Blom-Barnhoorn, 1981; Van Aartijk *et al.*, 1990 and Wickremesinhe *et al.*,1994) and enables material free virus and other diseases to be obtained (Blom-Barnhoorn and Van Aartijk, 1985 and Van Aartijk *et al.*, 1990).

The success of tissue culture in propagation of ornamental plants is greatly influenced by the nature of culture media. The nutrient media has two major functions, the first is to supply the basic nutritional ingredients for continued growth of isolated explants and subsequent propagules; the second function is to direct growth and development through hormonal control (George and Sherrington, 1994).

The aim of this study was to investigate the effect of some sterilization treatments, some growth regulators, and different types of media on shooting behaviour and strength of salt MS-medium with or without auxin and with or without activated charcoal on rooting behaviour of *Cotoneaster horizontalis,*.

MATERIALS AND METHODS

This investigation was carried out in Tissue Culture and Germplasm Conservation Research Laboratory, Horticulture Research Institute, Agriculture Research Center during the years of 2006 and 2007 on *Cotoneaster horizontalis,* Decne.

The aim of this study was to establish a protocol for micropropagation of *C. horizontalis,* Decne. Therefore, clorox and period of disinfection were used for sterilization treatments. Multiplication of shootlet was studied by using different kinds of cytokinins (BA, kin or 2iP) with different concentrations adding to MS-medium. Moreover, studying the effect of different type of media (MS, WPM, B_5 or LS media). The rooted shootlets were studied by using strength of MS-medium with different kinds of auxine (free hormone, IAA, NAA and IBA) with or without activated charcoal.

Plant material

The explant used for these experiments were taken from *C. horizontalis*, Decne shrub at Orman Garden, Giza

Culture media

Using MS-medium only or with different types of media (WPM, B_5 and LS) enriched with sucrose 25 g/l and agar 7 g/l. The media were adjusted to pH 5.7 ± 0.1, then poured at 25 ml in 200 ml capacity glass jars before autoclaving at 121°c and 1.2 kg/cm^2 for 15 min.

Culture conditions

The cultures were incubated in growth chamber at 24 ±1°c under 16 hr photoperiod (day light fluorescent tube) at 3 k lux.

Effect of clorox concentrations and periods of disinfection on sterilization explant

The explants were surface sterilized with 70% (v/v) ethanol solution for one min and then they were treated with 5, 10, 15 and 20% (v/v) clorox with a few drops of tween 20 for 5, 10 and 15 min followed by rinsing three times with a sterile distilled water. After that, they were immersed in 0.1% mercuric chloride (MC) with a few drops of tween-20 for ten min. Finally there were rinsed three times with sterile distilled water and cultured on MS-medium free hormones for two weeks (twenty explants in five replicated). After that, free contamination and survival percentage of explant were calculated.

Effect of different concentrations of cytokinins (BA, kin or 2iP)

The free of contamination explants were cultured on MS-medium supplemented with different concentrations of benzyladenine (BA), kinetine (kin), 6-γ-γ-dimethylallyl aminopurin (2iP) at the rate of (0, 1, 2, 3 or 4 mg/l). The explants were cultured incubated for one month (twenty explants were cultured in five replicate). The shootlets were subcultured three times, after that shootlets number/explant, shootlets length, number of leaves, fresh and dry weights were calculated and determine chemical composition.

For chemical composition, in three replicates, shootlet resulting from different concentrations of cytokinins were cut into small pieces and served for pigments, indoles and phenol analysis. For pigments determination, the ethanolic extraction was submitted to procedures of Saric *et al.* (1976) to qualitatively determine the endogenous chlorophyll-a,-b and carotenoids. For total indoles determination, the method of Selim *et al.* (1978) was applied. For total soluble phenols determination, the procedure of Daniel and George (1972) was used.

Effect of different media types (MS, WPM, B_5 or LS)

The shootlets produced from culture on MS-medium with different kinds of cytokinins with different concentration (the best kind and concentration 2 mg/l BA) were added to different types of media. The media were used MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1980), B_5 (Gamborg *et al.*, 1968) and LS (Linsmaier and Skoog, 1965). The shootlet (twenty shootlets

in five replicate) were culture on each medium used for one month and subcultured for three times. After this period shootlets number, shootlets length (cm), number of leaves, fresh and dry weight were calculated.

Effect of salt strength of MS-medium, types of auxin and activated charcoal

The resulted shootlets were cultured on different salt strength of MS-medium (full, half or quarter) free hormones or provided with 1 mg/l IAA, NAA or IBA without or with 1 g/l activated charcoal. The shootlets (twenty shootlet in five replicate) were incubated for 45 days. After this period rooting percentage, roots number per shootlet and root length (cm) was recorded.

Acclimatization rooted shootlets

The rooted shootlets resulting from rooting treatments were transferred to plastic pots containing peat moss irrigated with solution of 0.2 Topsin-M70 fungicides and covered by transparent polyethylene bags. The acclimatized vitroplants were kept in acclimatized galss house for four weeks before transplanting out-of-door after that survival capacity was calculated.

Experimental design and data analysis

The lay-out of the experiments was designed in completely randomized design and the test of LSD was used for comparison among means according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

Effect of clorox concentrations and periods of disinfection on sterilization explant

The data in Table (1) indicated that the increasing clorox concentrations increased percentage of free contaminated explants and decreased survival percentage of explants. The highest percentage of free contamination explants (73.33%) was recorded by using 20% clorox and decreased the survival percentage to the lowest percentage (46.67%).

On the other hand, the data indicated that increasing the time of immersed explants increased percentage of free contaminated but decreased the survival percentage of explant. The highest percentage of free contaminated explant (57.5%) was recorded by immersio ı the explant for 15 min, but gave the lowest percentage of survival explants (38.75%).

The data on the interaction between the concentration of clorox and immersion time indicated that the best percentage of free contaminated and survival percentage (70.0 and 60.0%) were observed when explants immersed in 10 min with 15% clorox.

Our results on sterilization of explants are in line with those of Hosni *et al.*, (2000) on *Limonium sinnuatum* "Citron Mountion: and Hussein (2002) on *Aglaeonema* plants.

Table (1): Effect of clorox concentrations and periods of disinfection on free contamination and survival percentage of *Cotoneaster horizontalis*, explants.

Treatments	Free contamination				Survival percentage			
	5 min	10 min	15 min	Mean B	5 min	10 min	15 min	Mean B
Clorox 5%	5.0	15.0	25.0	**15.00**	100.0	85.0	60.0	**81.67**
Clorox 10%	25.0	40.0	40.0	**35.00**	95.0	75.0	45.0	**70.00**
Clorox 15%	65.0	70.0	75.0	**70.00**	85.0	60.0	35.0	**60.00**
Clorox 20%	60.0	85.0	90.0	**78.33**	65.0	60.0	15.0	**46.67**
Mean A	**38.75**	**52.50**	**57.50**		**86.25**	**68.75**	**38.75**	
LSD 5% A	9.88				8.15			
B	11.41				9.41			
A×B	19.77				16.92			

Effect of different concentrations of cytokinins (BA, kin or 2iP) on shooting behaviour of *Cotoneaster horizontals* explant

The data in Table (2) indicated that significant fluctuations in the behaviour of micropropagation of explants in vitro (i.e. number of the shootlets/explant, length of shootlet (cm), number of leaves/shootlet, fresh and dry weights) were attributed to the effect of cytockinins (BA, kin and 2iP) treatments.

For the number of shootlets/explant, the addition of BA at 3 or 4 mg/l BA in MS-medium produced the highest number of shootlets/explants (6.40 or 5.95). While the control (free hormone), 1 mg/l BA and all concentration of kin or 2iP gave the lowest number of shootlets/ explant ranging from (1.0 to 2.95).

Regarding to shootlet length, the longest shootlet (2.13 cm) was recorded for explants grown on MS-medium supplemented with 1 mg/l BA. But culture the explants on MS-medium containing 3 mg/l BA reduced the shootlet length to the shortest length (0.84 cm).

Concerning to number of leaves/shootlet, the highest number of leaves/shootlet (7.63) was recorded when adding 1 mg/l 2iP. Whereas, the lowest number of leaves/shootlet (3.96) was recorded for explants grown on MS-medium free hormone.

As for fresh and dry weights, culturing the shootlets on MS-medium supplemented with 3 mg/l BA produced the highest fresh and dry weights of shootlets (1.03 and 0.252 g, respectively). On the other hand, culture the shootlets on MS-medium with 2 mg/l kin gave the lowest fresh weight (0.24 g). While all treatments except 3 mg/l BA had no significant effect on dry weight which ranging from (0.032 to 0.092 g).

From the above results, it could be generally suggested that adding 3 mg/l BA increased shootlets number/explant, fresh and dry weights of shootlets. Shootlet length increased by adding 2 mg/l BA, while number of leaves increased by adding 1 mg/l 2iP to MS-medium. Approving results were reported by Singh *et al.* (1994) on *chrysanthemum morifolium*; Monier (1995) on five Cotoneaster genotype; Sharma *et al.* (2003) on *Crataeva adansonii* and Parasharami *et al.* (2003) on *Pinus roxburghii*

Table (2): Effect of different concentrations of cytokinins (BA, kin or 2iP) on shooting behaviour of *Cotoneaster horizontals* explant *in vitro*.

Treatments	Number of shootlet	Length of shootlet (cm)	Number of leaves	Fresh weight (g)	Dry weight (g)
Control	1.23	0.87	3.96	0.27	0.032
1 mg/l BA	1.68	2.13	5.89	0.71	0.092
2 mg/l BA	2.95	1.46	5.24	0.85	0.078
3 mg/l BA	6.40	0.84	4.90	1.03	0.252
4 mg/l BA	5.95	0.99	5.82	0.95	0.082
1 mg/l kin	1.20	1.03	5.63	0.42	0.052
2 mg/l kin	1.08	1.52	5.19	0.24	0.036
3 mg/l kin	1.20	1.50	5.19	0.49	0.058
4 mg/l kin	1.00	1.59	5.85	0.41	0.058
1 mg/l 2iP	1.00	1.71	7.63	0.56	0.046
2 mg/l 2iP	1.38	1.30	4.86	0.55	0.066
3 mg/l 2iP	1.50	1.56	5.56	0.37	0.048
4 mg/l 2ip	1.30	1.82	6.33	0.54	0.052
LSD (5%)	**0.882**	**0.433**	**1.025**	**0.267**	**0.1216**

Effect of different concentrations of cytokinins (BA, kin or 2iP) on chemical composition

The results in Table (3) indicated that the amounts of chlorophyll-a, -b, carotenoids, total indoles and total soluble phenols which were mined in the shootlets tissues were significantly affected by different concentrations of cytokinins (BA, kin or 2iP).

For chlorophyll-a, the data showed that using MS-medium with 4 mg/l BA produced the highest amount (287.2 mg/100g fw). While adding 4 mg/l kin, 1, 2, 3 or 4 mg/l 2iP to MS-medium reduced the amount of chlorophyll-a to the lowest amounts (85.56, 85.52, 83.90, 101.9 or 89.50 mg/100g fw, respectively).

As for chlorophyll-b, the highest amounts (45.96 mg/100g fw) was recorded by using MS-medium supplemented with 2 mg/l kin, while the lowest values (18.22 or 15.16 mg/100g fw) was recorded by using MS-medium supplemented with 1 or 4 mg/l 2iP.

Concerning carotenoids content, adding kin at concentration 2 mg/l increased the amount of carotenoids to the highest value (45.35 mg/100g fw). But using 3 or 4 mg/l kin or 1, 2, 3 or 4 mg/l 2iP decreased the amounts of

carotenoids to the lowest values (12.56, 10.72, 10.88, 8.28, 11.88 or 11.86 mg/100g fw, respectively).

Regarding the total indole, using MS-medium containing 1 mg/l kin increased the total indole to a maximum amount (187.9 mg/100g fw). While adding 3 mg/l 2iP decreased the content of total indole to minimum amount (18.32 mg/100g fw)

Relating to the total soluble phenols, MS-medium contain 3 mg/l 2iP produced the highest amount of total soluble phenols (195.0 mg/100g fw). But culture shootlet on MS-medium free hormones (control) decreased the content of total soluble phenols to the minimum amount (112.5 mg/100g fw).

In conclusion, the highest content of chlorophyll-a was recorded with MS-medium supplemented with 4 mg/l BA, while the highest amount of chlorophyll-b and carotenoids was recorded with MS-medium supplemented with 2 mg/l kin. Total indoles increased by adding 1 mg/l kin and total soluble phenols increased when using 3 mg/l 2iP. In this respect Tung (1997) on Cucumber seedling studied the effect of different concentrations of cytokinins BA, BA-ribosid (BAR), BA-3G, BA-7G, BA-9G, Zeatin (Z), Z-riboside, dihydrozeatin and 2iP on the formation capacity of chlorophyll-a,-b and carotenoids. He declared that the production of such pigments was negative related with the concentrations of the other cytokinins examined.

Table (3): Effect of different concentrations of cytokinins (BA kin or 2iP) on chemical composition (mg/100g fw) of *Cotoneas er horizontals* shootlets *in vitro*.

Treatments	Chloro-phyll –a	Chloro-phyll -b	Carote-noids	Total indole	Total soluble phenols
Control	199.80	33.64	29.77	132.80	112.50
1 mg/l BA	220.10	37.93	26.67	181.50	147.60
2 mg/l BA	167.40	27.20	20.82	175.30	182.90
3 mg/l BA	227.80	37.13	30.69	165.00	119.80
4 mg/l BA	287.20	33.47	27.23	181.50	140.40
1 mg/l kin	212.90	38.16	28.33	187.90	141.90
2 mg/l kin	245.50	45.96	45.35	172.00	177.40
3 mg/l kin	114.30	25.59	12.56	117.90	163.9
4 mg/l kin	85.56	23.31	10.72	26.07	120.40
1 mg/l 2iP	85.52	18.22	10.88	88.42	180.50
2 mg/l 2iP	83.90	23.43	8.28	58.80	181.50
3 mg/l 2iP	101.90	21.60	11.88	18.32	195.00
4 mg/l 2ip	89.50	15.16	11.86	52.71	158.40
LSD (5%)	53.19	12.36	13.29	53.07	49.23

Effect of different media types (MS, WPM, B_5 or LS) on shooting behaviour of *Cotoneaster horizontals* explants

The behaviour of shooting due to media type including the number of shootlets/explant, length of shootlet (cm), number of leaves/shootlet, fresh and dry weights (g) were illustrated in Table (4).

For number of shootlets/explant and number of leaves/shootlet the data indicated that all types of media (MS, WPM, B_5 or LS) had no significant effect on these characters.

Relating to the length of the formed shootlet, the data declared that the tallest shootlets was found by using MS or LS media, which gave (1.28 or 1.25 cm). But the shortest length (0.93 cm) was observed when culture the shootlet on WPM medium.

Concerning fresh weight of shootlets, culture the shootlet on MS-medium increased fresh weight of shootlet (1.36 g) compared to culture the shootlet on WPM, B_5 or LS, which decreased the fresh weight (0.63, 0.62 or 0.68 g, respectively).

Regarding to dry weight of shootlet, the shootlet culture on MS-medium gave the highest dry weight (0.31 g), while shootlet culture on LS-medium decreased dry weight (0.062 g) of shootlet.

A wide survey of the obtained data indicated that culture the shootlet on MS-medium increased the number and length of shootlets, the number of leaves, fresh and dry weights compared to culture the shootlet on WPM, B_5 or LS media. Approving results were reported by Monier (1995) on five Cotoneaster genotypes, Youssef (1996) on *Melaleuca armillars*, Ipekci *et al.* (2001) on *Paulownia elongata* and Brum *et al.* (2003) on *Ficus carica*.

Table (4): Effect of different media types (MS, WPM, B_5 or LS) on shooting behaviour of *Cotoneaster horizontals* explant *in vitro*.

Treatments	Number of shootlet	Length of shootlet (cm)	Number of leaves	Fresh weight (g)	Dry weight (g)
MS	5.90	1.28	6.20	1.36	0.310
WPM	4.81	0.93	5.66	0.63	0.088
B_5	5.43	1.07	6.27	0.62	0.070
LS	4.13	1.25	6.13	0.68	0.062
LSD (5%)	NS	0.279	NS	0.304	0.242

Effect of salt strength of MS-medium, types of auxins and activated charcoal on rooting behaviour of *Cotoneaster horizontals* shootlets *in vitro*.

It is quite clear from the data in Table (5) that the various treatments caused a significant influence on rooting percentage, while they had no significant effect on the number and the length of root.

For rooting percentage, full strength of MS-medium with 1 mg/l IAA produced the highest percentage of rooting shootlets (30.0%). On the other hand, the different strength of MS-medium (full, half or quarter) without auxine and without or with 1 g/l activated charcoal failed to form roots (0.0%).

Regarding to the root number/shootlet and root length (cm), the different strengths of MS-medium (full, half or quarter) without auxine or with 1 g/l IAA, NAA or IBA without or with 1 g/l activated charcoal had no significant effect on the roots number which ranged from (0.25 to 1.25 root/shootlet) and length of roots which ranged from (0.63 to 1.88 cm).

These findings go in line with those reviewed by Rajendra *et al.* (1998) on *Artocarpus heterophyllus;* Sakr *et al.* (1999) on *Yucca elephantips*; Hussein (2002) *Aglaeonema* plants and Sayed and Abou-Dahab. (2006) on *Faucaria tuberculosa.*

Table (5): Effect of strength of salt MS-medium, types of auxine and activated charcoal on rooting behaviour of *Cotoneaster horizontals* shootlets *in vitro.*

Treatments	Rooting (%)	Number of root	Length of root (cm)
F. MS	0.0	---	--
H. MS	0.0	--	--
Q. MS	0.0	--	--
F. MS+IAA	30.0	1.25	1.88
H. MS+IAA	15.0	0.25	0.75
Q. MS+IAA	15.0	0.75	1.63
F. MS+NAA	20.0	0.25	0.63
H. MS+NAA	5.0	0.75	1.38
Q. MS+NAA	10.0	0.75	1.50
F. MS+IBA	20.0	0.75	0.63
H. MS+IBA	15.0	0.75	1.25
Q. MS+IBA	10.0	1.00	1.38
F. MS+AC	0.0	--	--
H. MS+AC	0.0	--	--
Q. MS+AC	0.0	--	--
F. MS+IAA+AC	10.0	0.50	0.63
H. MS+IAA+AC	10.0	0.50	0.75
Q. MS+IAA+AC	10.0	1.25	1.50
F. MS+NAA+AC	15.0	0.50	1.13
H. MS+NAA+AC	5.0	0.25	0.75
Q. MS+NAA+AC	5.0	1.50	0.88
F. MS+IBA+AC	10.0	1.00	1.38
H. MS+IBA+AC	15.0	0.50	0.75
Q. MS+IBA+AC	10.0	1.00	1.38
LSD (5%)	18.49	NS	NS

F.: Full **H.:** Half **Q.:** Quarter

Acclimatization rooted shootlets

All rooted shootlets were acclimatized on peat moss. The survival of plantlet after one mother were calculated and gained 70% percentage

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إكثار الكوتنيستر هوريزنتالس من خلال زراعة الأنسجة

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أجرى هذا البحث بمعمل زراعة الانسجة وحفظ الاصول الوراثيـة بمعهـد بحوث البساتين– مركز البحوث الزراعية خـلال الاعـوام ٢٠٠٦و ٢٠٠٧ ويهـدف البحث الى عمل بروتوكول للاكثار الدقيق لنبات الكوتنيستر وقد اظهرت النتائج:

بالنسبة لمرحلة التعقيم: فقد ادى استخدام تركيز ١٥% كلـوركس لمـدة ١٠ دقيقة للحصول على احسن نسبة من الاجزاء النباتية الحية و الخالية من التلوث.

بالنسبة لمرحلة تكوين اللافرع فقد ادى اضافة ٣ ملليجرام/لتر BA الى زيادة عدد الفريعات والوزن الطازج والجاف للفريعات. وقد ادى اضافة ٢ ملليجرام/لتر BA الى زيادة طول الفريعات. بينما عدد الاوراق زاد باضافة ١ ملليجرام/لتـر 2iP. أمـا بالنسبة لانواع البيئات المختلفة فان زراعة الفريعات على بيئة MS زاد من عدد وطول الفريعات كما زاد عدد الاوراق و الوزن الطازج والجاف للنبيتات.

بالنسبة للمحتوى الكيماوى فان اعلى محتوى من الكلوروفيل –أ تم تقديره فى بيئة بها ٤ ملليجرام/لتر BA بينما اعلى كمية من كلوروفيـل – ب والكـاروتين تـم تقديرها فى النبيتات المزروعة على بيئة MS بها ٢ ملليجرام/لترkin اما الاندولات فقد زادت بأضافة ١ ملليجرام/لتر kin والفينولات الكليـة الذائبـة زادت عنـد اضـافة ٣ ملليجرام/لتر 2iP.

بالنسبة لمرحلة التحذير فان أعلى نسبة لتكـوين الجـذور كـان للفريعـات المزروعة على بيئة MS كاملة التركيز مضاف إليها ١ ملليجرام/لتر IAA أمـا طـول وعدد الجذور فلم يوجد بين المعاملات فروق معنوية. وقد وصلت نسبة نجاح الأقلمـة إلى ٧٠% فى النبيتات المتكون عليها جذور