Propagation of *Faucaria tuberculosa by in vitro* culture

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ABSTRACT

The study was carried in 2004 and 2005. The aim of this study was to establish a protocol for micropropagation of Faucaria tuberculosa plants in vitro. Chlorox and mercuric chloride were used for sterelization treatments. Multiplication of shoots was done by using MS-medium fortified with benzyladenine (BA) or kinetin. Also, full strength Murashige and Skoog (MS) or Woody plant medium (WPM) with addition of BA, Zeatin or 2iP were studied. Different agar concentrations were added to WPM to promote shoot proliferation and reduce the shoot vitrification. To promote rooting on Faucaria tuberculosa, different strengthes of MS media with or without activated charcoal (AC) were examined.The results showed that the best treatment, to be recommended to obtain free of contamination explants with the highest survival, was by using 40% chlorox with 0.2% mercuric chloride (MC). Addition of 3 mg/l BA to full strength MS medium produced the highest shootlet number/explant and respectively reduced vitrification of shootlets/explant as compared with 3 mg/l kin in the 3th subculture. No significant differences in shootlets number were found by using full strength MS or WPM with 3 mg/l BA, Zeatin or 2iP, while addition of 3 mg/l BA to full strength WPM reduced vitrificated shootlets/explant. Increasing agar concentration from 7 to 11 g/l in WPM gradually reduced shootlet number and vitrificated shootlet/ explant. In rooting stage, using full strength MS medium with 1 g/l activated charcoal increased rooting percentage and root length, while for root number no significant differences could be observed. In acclimatization of plantlets, all treatments gave 100% survival.

Key words: *Sterilization, micropropagation, in vitro culture, Fucaria tuberculosa, multiplication, vitrification, acclimatization.*

INTRODUCTION

Faucaria tuberculosa Tiger-Jaws, belonge to the family Alizoaceae. The rosettelike leaves, which stand opposite each other like crosses, have white patterns and stand densely together. The yellow blossoms last several days. The plants are suited for indoor cultivation without problems (Hans Hecht, 1997).

In vitro culture plants are submitted to particular environmental conditions which are the source of injuries and stress: explantation, high osmosis of the culture medium (high sucrose and ammonium content), high relative humidity and gas accumulation in the atmosphere of the jar, light filtration through the flask, unusual hormonal treatment (high cytokinin application), etc. are constrains to modify organogenesis of the explants. Although most plants can adapt to these

environmental conditions, some of them become abnormal with translucent aspect due to chlorophyll deficiency and high water content (Gaspar, 1991). This phenomenon (previously known as vitrification) is called hyperhydricity (Debergh *et al*., 1992).

For sterilization of the explants, clorox and murcuric chloride can be used as reported by Chiwon *et al.* (1994) on *Coreopsis lanceolata,* Sujatha (1997) on *Guizotia abyssinica*, Abou Dahab *et al*. (2005) on *Russcus hypoglossum* and Sayed *et al*. (2005) on *Cereus peruvianus. In vitro* shoot regeneration and subsequent plant micropropagation continuous supply of growth regulators to the culture medium is needed.

In the multiplication stage, different kinds of cytokinins had a great effect on shootlet proliferation and shootlet vitrification as reported by Dencso (1987) on *Conifers* and *Carnation*, Leonhardt and Kandeler (1987) on *Cacti species*, Chiwon *et al.* (1994) on *Coreopsis laceolata*, Kushel *et al.* (1994) on *Chrysanthemum motifolium* cv. "Riot" and Pramod-Dikishit *et al.* (1997) on *Chrysanthemum* cv. "Maghi". Many workers such as Daguin and Letouze (1986) on Plum, Ziv *et al*. (1987) on *Carnation*, Leonhardt and Kandeler (1987) on *Cacti species* studied the effect of different types of media on shootlet proliferation and shootlet vitrification. Different concentrations of agar had a great effect on multiplication of shootlets and shootlet vitrification as reported by (Debergh, 1983 and Debergh *et al*., 1981) on *Cynara scolymus,* (Ziv *et al*., 1983 and Hakkaart and Versluijs 1983) on *Carnations* and Rady (2005) on *Gypsophyila paniculata*.

For rooting stage, the concentration of macro-and micro elements of culture medium with or without activated charcoal remarkedly affected the rooting rate, root number and root length of rooted shootlet as showed by George and Pavishankar (1997) on *Vanilla planifolia*, LÊ (1998) on *Artimesia umbelliforms,* Sakr *et al*. (1999) on *Yucca elephantipes* and Sayed *et al.* (2005) on *Cereus peruvianus.* For acclimatization stage, transfer of rooted shootlets that were grown under different concentrations of macro- and micro-elements of culture medium with or without activated charcoal to greenhouse showed different effects on the survival as revealed by Wawrosh *et al*. (1994) on *Achilla asplenifolia.*

The aim of present study was to improve the different stages of *in vitro* propagation of *Faucaria tuberculosa* for commerical production of this plant in Egypt through using different kinds of cytokinins, different type of media and different agar concentrations for initiation of shootlets as well as different strengthes of media for rooting shootlets and acclimatization of plantlets.

MATERIALS AND METHODS

The expermintes of this study were carried out at the Tissue Culture and Germplasm Conservation Research Laboratory, Horticulture Research Institute, Agricultural Research Center, during the years of 2004 and 2005. The objective of this study was to establish a protocol for micropropagation of *Faucaria tuberculosa*. Therefore, clorox and mercuric chloride were used for sterilization treatments. Multiplication of the shootlets was studied by using MS-medium fortified with benzyladenine (BA) or kinetin (Kin). Also, the effect of full strength Murashige and Skoog (MS) or Woody Plant Medium (WPM) with addition of BA, Zeatin or 2iP was investigated. Different agar concentrations added to WPM to promote shootlet proliferation and reduce the shootlet vitrification were tested. To promote rooting and for acclimatization survival, different strenghts of MS-medium with or without

activated charcoal (AC) were studied. Therefore, five experiments were carried out.

Plant material

Seedlings of *Fucaria tuberculosa* (three-month-old) with 3-4 leaves (1.5-2.0 cm height) obtained from the Cactus International Farm (Tahanoub, Shebien El-Kanater,Qulubia) were used as a source of plant materials.

Surface sterilization of explant

The seedlings were surface sterilized with 70% (v/v) ethanol solution for one min and then they were treated with 20, 30, 40, 50% (v/v) clorox with a few drops of tween-20 for ten min, followed by rinsing three times with a sterile distilled water. After that, they were immersed in different concentrations of mercuric chloride (0.1, 0.2 and 0.3%) with a few drops of tween-20 for ten min. Finally they were rinsed three times with sterile-distilled water. The culture media under trails consisted of macro-and micro-elements and vitamins of MS-medium (Murashige and Skoog, 1962) or WPM (Llyod and McCown, 1981) enriched with 25 g/l sucrose and 7 g/l agar.

For seedling sterilization, shootlet proliferation and rooting treatments, all the used culture media were adjusted to pH 5.7±0.1 and autocalved at 121°C and 1.2 kg/cm^2 for 20 min before using. The shootlet explants were placed vertically in 200 ml capacity glass jars containing 25 ml media.

Experiment 1: Effect of different concentrations of clorox and murcuric chloride on free of contamination explants and survival capacity

Four concentrations of clorox (20, 30, 40 or 50% v/v) and three concentrations of murcuric chloride (0.1, 0.2 or 0.3%) were examined. The seedlings were sterilized, cultured on MS-medium free of hormones and incubated for three weeks. After this period the percentage of explants free of contamination and survival capacity were recorded. In this experminet 12 treatments were done.

Experiment 2: Effect of different concentrations of cytokinins (BA or kin) and number of subcultures on shootlet number/explant and vitrification of shootlet percentage

The free of contamination explants were cultured on MS-medium fortified with different concentrations of benzyladenine (BA) or kinetin (kin) at the rate of (0.0, 0.5, 1.0, 2.0 or 3.0 mg/l). The data (shootlet number and shootlet vitrification percentage) were recorded after 4, 8 and 12 weeks (representing 3 subcultures). Therefore 9 treatments in each subculture for 3 subcultures were performed.

Experirment 3: Effect of different types of media (MS or WPM) with different kinds of cytokinins (BA, Zeatin or 2iP) on shootlet number/explant and shootlet vitrification percentage

The shootlets were cultured on MS or WPM supplemented with BA, Zeatin or 2iP (3.0 mg/l). The data (shootlet number and shootlet vitrification percentage) were recorded after one month. Therefore, 6 treatments were done.

Experiment 4: Effect of different levels of agar on shootlet number/explant and shootlet vitrification percentage

The shootlets were cultured on WPM and were gelled with different levels of agar (7.0, 8.0, 9.0, 10.0 or 11 g/l). The data (shootlet number and shootlet vitrification percentage) were recorded after one month. Therefore, 5 treatments were done.

Experiment 5: Effect of different strengthes of MS-medium with or without activated charcoal on rooting behaviour and acclimatization survival

The non-vitrified shootlets were cultured on MS-medium free of hormones at full, ¾ or half strength of macro-and micro-elements with or without 1g/l activated charcoal. After one month rooting percentage, rooting number and root length (cm) were determined.

In the acclimatization stage, the rooted shootlets resulting from rooting treatments were transffered to plastic pots (0.2 liter) containing peat moss which was adjusted to pH 6.2 and irrigated with a solution of 0.2% Topsin-M70 fungicide, pots were covered by transprent polyethylene bags. The acclimatized vitroplants were kept in acclimatized galss-house for four weeks before transplanting outdoors. After one month, survival capacity was determined. Therefore, 6 treatments were done.

Anatomical studies

A microscopical study was carried out on vitreous and normal leaves of *Faucaria tuberculosa* to elucidate the histological variation between them. The leaf represented by its middle portion of the blade was subjected to a microtechnique developed by Nassar and El-Sahhar (1998). Specimens were killed and fixed for at least 48 hrs. in F. A. A. (10 ml formalin, 5 ml glacial acetic acid, 25 ml distilled water, 60 ml ethyl alcohol 95%). After fixation, the materials were washed in 50% ehtyl alcohol and dehydrated in a normal butyl alcohol series before being embedded in paraffin wax (melting point 56-58°C). Transverse sections which were cut on a rotary microtone to a thickness of 20 micron (μ) were stained with safranin before mounting in Canada balsam and covering. Slides were analyzed microscopically and photomicrographed.

Statistical analysis

A completely randomized design was employed in all of the executed experiments. Each treatment was replicated five times, each replication was represented by four explants. Analysis of variance was used to show statistical differences between treatments according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

Effect of different concentrations of clorox and mercuric chloride on free of contamination explants and survival capacity

Data in (Table 1) revealed that using different concentrations of clorox and mercuric chloride for sterilization of explants had significant effects on explants free of contamination and survival percentage. Using clorox at 40% (v/v) gave the highest percentage of explants free of contamination (73.0%) and the highest percentage of survival (66.67%). Moreover, using (MC) at the rate of 0.2 and 0.3% (w/v) gave the highest percentage of explants free of contamination (51.25 and 56.25%, respectively), no significant differences were found between different concentrations of MC on survival percentage. Also, treated explants with 40% clorox and 0.2% (MC) gave the highest percentage of explants free of contamination (89.0%) and survival percentage (85%). These findings are in agreement with those reported by Abou Dahab *et al.,* (2005) on *Ruscus hypoglossum* and Sayed *et al.,* (2005) on *Cereus peruvianus.* They concluded that using clorox or mercuric chloride in different concentrations had a great effect on sterile explants.

Table (1): Effect of clorox and mercuric chloride (MC) on free of contamintion and survived explants of Faucaria tuberculosa in vitro.

	Free of contamination %					Survival %				
	Clorox %									
MC%	20	30	40	50	Mean B	20	30	40	50	Mean B
0.1	5.00	15.00	50.00	90.00	40.00	5.00	15.00	50.00	60.00	32.50
0.2	10.00	15.00	89.00	95.00	51.25	10.00	25.00	85.00	40.00	40.00
0.3	10.00	30.00	85.00	100.00	56.25	10.00	30.00	65.00	20.00	31.26
Mean A	8.33	20.00	73.00	95.00		8.33	23.35	66.67	40.00	
LSD at 5% A		11.49				13.20				
LSD at 5% B		9.948				11.43				
LSD at 5% A×B		19.90				22.86				

A: Clorox B: Mercuric chloride (MC) A×B: Interaction

Effect of different concentrations of cytokinins (BA or kin) on shootlet number/explant and vitrification of shootlet percentage at different number of subcultures

Shootlet number/explant

Data presented in Table (2) clearly show that addition of BA at the rate of 3 mg/l to the MS-medium gave significantly the highest number of shootlets/explant (5.66) as compared with MS-medium free of hormones or other concentrations of BA. A significant and gradual increase was shown in number of shootlets/explants by increasing the number of subcultures. The highest value was resulted from the 3rd subculture (3.84) as compared with the first subculture, which gave the lowest number of shootlets/explant (1.963). Concerning the interaction between concentrations of BA or Kin and number of subcultures, the highest number of shootlets/explant (7.50) was obtained when adding BA at 3.0 mg/l in the 3rd subculture as compared with the control (free of hormones) in 1st and 2nd subculture and when adding BA (0.5 mg/l) and Kin (0.5 mg/l) in 1st which gave 1.00, 1.08, 1.08 and 1.00 shootlets/explant, respectively. As for cytokinins and number of subcultures, Chiwon *et al.* (1994) on *Coreopsis lanceolata*, Kushel *et al.*, (1994) on *Chrysanthemum morifolium* cv. "Riot", Pramod-Dikishit *et al.* (1997) on *Chrysanthemum morifolium* cv. "Maghi" and Abou Dahab *et al.* (2005) on *Ruscus hypoglossum*. They revealed that different concentrations of BA or Kin and different subcultures had significant effects on the number of shootlets.

Shootlet vitrification percentage

Data in Table (2) clearly indicated that, the percentage of shootlet vitrification was highest when MS-medium was containing Kin at the rate of 3 mg/l (60.62%) as compared with MS-medium free of hormones, which gave (0.0%), shootlet vitrification. Increasing the number of subculture from 1st to 3rd subculture increased shootlet vitrification percentage (which gave 10.17 to 27.41% respectively). For the interaction between different concentrations of BA and Kin and number of subcultures, the highest percentage of shootlet vitrification was found in the 2nd and 3rd subculture with adding Kin at the rate of 3.0 mg/l (72.83 and 75.00%). But using MS-medium free of hormones in 1st, 2nd and 3rd subculture and when adding BA and Kin at 0.5 mg/l in 1st subculture decreased the shootlet vitrification percentage to the lowest value (0.00%). In this respect, Zuccherelli (1979) on peacj, Dencso (1987) on conifers and carnation and Leonhardt and Kandeler

(1987) on *Cacti species* concluded that reduction of cytokinins decreased shootlet vitrification percentage.

From the above-mentioned results it could be concluded that the most effective treatment in increasing the shootlet number and reducing the percentage of shootlet vitrification is supplementing BA (3.0 mg/l) to MS-medium.

Table (2): Effect of different concentrations of BA and Kin at different of subcultures on number of shootlets and shootlet vitrification percentage of Faucaria tuberculosa in vitro.

Treatments	Shootlet number/explant				Shootlet vitrification%			
	Sub 1	Sub 2	Sub 3	Mean B	Sub 1	Sub 2	Sub 3	Mean B
Control	1.00	1.08	1.25	1.11	0.00	0.00	0.00	0.00
BA 0.5 mg/l	1.08	1.42	2.00	1.50	0.00	4.16	6.27	3.48
BA 1.0 mg/l	1.83	3.66	4.41	3.30	6.27	17.66	17.72	14.55
BA 2.0 mg/l	2.83	5.75	6.08	4.89	14.17	16.69	25.96	18.94
BA 3.0 mg/l	3.00	6.50	7.50	5.66	15.83	18.74	25.96	20.18
Kin 0.5 mg/l	1.00	1.25	1.66	1.30	0.00	4.16	6.27	3.48
Kin 1.0 mg/l	1.58	1.41	2.16	1.72	6.33	20.83	29.17	18.78
Kin 2.0 mg/l	2.66	2.41	3.33	2.80	19.72	40.28	58.33	37.44
Kin 3.0 mg/l	2.66	4.75	6.17	4.53	34.02	72.83	75.00	60.62
Mean A	1.96	3.14	3.84		10.71	21.71	27.41	
LSD at 5% A	0.498				4.675			
LSD at 5% B	0.863				8.097			
LSD at 5% A×B	1.496				14.020			

A: Number of subcultures B: Treatments A×B: Interaction

Effect of different types of media (MS or WPM) with different kinds of cytokinins (BA, Zeatin or 2iP) on shootlet number/explant and shootlet vitrification percentage

Shootlet number/explant

Data in Table (3) revealed that, no significant differences were found between using MS and WPM in the number of shootlets. The same trend was observed on the effect of different cytokinins (BA, Zeatin and 2iP). Moreover, for the interaction between the kinds of cytokinins and types of media no significant effect was found on shootlet number/explant.

Shootlet vitrification percentage

As shown in Table (3) the percentage of shootlet vitrification was affected by using different types of media. Using MS-medium gave the highest percentage of shootlet vitrification (62.42%) as compared to WPM medium (39.58%). But using different kinds of cytokinins (BA, Zeatin and 2iP) had no significant effect. For the interaction between different types of media and different kinds of cytokinins the data revealed that, the highest percentage of vitrification of shootlets was obtained when adding BA at 3.0 mg/l to MS-medium (73.93%), while adding BA at 3.0 mg/l to WPM gave the lowest percentage of shootlet vitrification (22.08%). This subject was studied by Paques and Boxus (1987), Ziv *et al.* (1987), Daguin and Letouze (1986) and Leonhardt and Kandeler (1987) on rootstok apple M.26, carnation, Plum and *Cacti species*. They found that media rich in mineral nutrients, such as MS promote vitrification, but reduction the levels of NH_4^+ in the medium caused increase in lignification and reduction in vitrification.

In conclusion, the best shootlet number/explant and lowest percentage of shootlet vitrification were recorded when adding BA at 3.0 mg/l to WPM.

Table (3): Effect of type of media (MS and WPM) and different kinds of cytokinins (BA, Zeatin and 2iP) on shootlet number/explant and vitrification percentage of Faucaria tuberculosa in vitro.

Treatments	Shootlet number/explant			Shootlet vitrification %		
	MS	WPM	Mean B	MS	WPM	Mean B
BA 3.0 mg/l	7.667	8.167	7.917	73.93	22.08	48.01
Zeatin 3.0 mg/l	6.667	6.833	6.750	50.00	41.67	45.83
2iP 3.0 mg/l	6.667	6.500	6.586	63.33	55.00	59.17
Mean A	7.00	7.167		62.42	39.58	
LSD at 5% A	NS			14.11		
LSD at 5% B	NS			NS		
LSD at 5% A×B	NS			24.45		

A: Type of media B: Treatments A×B: Interaction

Effect of different levels of agar on shootlet number/explant and shootlet vitrification percentage

Shootlet number/explant

The data in (Table 4) indicated that adding different levels of agar to WPM medium containing BA at 3.0 mg/l had a significant effect on shootlet number/explant. Adding 7.0 or 8.0 g/l agar resulted in the greatest number of shootlets/explant (7.833 and 6.500, respectively), but using 11.0 g/l agar caused reduction in the shootlet number/explant and gave the lowest number (1.27). In this regard Debergh (1983) showed that increasing the concentration of agar or other gelling agents had a great effect on the non-availability of variuos medium components, in particular cytokinins.

Shootlet vitrification percentage

Data in Table (4) revealed that, adding 7.0 g/l agar to WPM containing BA at 3.0 mg/l increased the shootlet vitrification to the highest percentage (22.08%), while increasing ager in the medium to 11.0 g/l decreased the percentage of shootlet vitrification to the lowest percentage (2.11%). This subject was studied by Debergh (1983) and Debergh *et al.* (1981) on *Cynara scolymus*, Ziv *et al.* (1983) and Hakkaart and Versluijs (1983) on carnation, Von Arnold and Eriksson (1984) on *Picea abies* and Rady (2005) on *Gypsophyila paniculata*. They reported that, increasing the agar concentration reduced vitrification but very often also lowered the propagation ratio.

Microscopic examination of vitrification leaves

Leaves of some *Fucaria tuberculosa* shootlets showed virtification. Such leaves were glossy and succulent. Microscopic analysis of leaf transverse sections showed great differences between vitreous and normal leaves. Core cells of the vitreous leaves were undetected. Sapholes in vitreous leaves were more numerous and larger than in normal leaves, although their contents were lesser in the vitreous. The mesophyll mainly consisted of spongy tissue, rich in intercellular sporces. The vitreous leaves showed cells with thinner walls as compared with normal leaves. In the mean time the cells of vitreous leaves contained a relatively poor, largely vacuolated cytoplasm. The vitreous leaf was more sinuous in outline, having thin cuticle and irrigular stomata as compared with normal leaf. The edisermis was thin and necrosis was observed (Figures 1 and 2).

Table (4):Effect of different agar concentrations on shootlet number/explant and shootlet vitrification percentage of Faucaria tuberculosa in vitro.

Agar concentration	Shootlet number	Vitrification %
Agar 7.0 g/l	7.83	22.08
Agar 8.0 g/l	6.50	14.17
Agar 9.0 g/l	3.83	8.39
Agar 10.0 g/l	2.33	4.22
Agar 11.0 g/l	1.26	2.11
LSD at 5%	2.54	7.33

Effect of different strengthes of MS-medium with or without activated charcoal on rooting behaviour and acclimatization survival.

Rooting percentage (%)

Data presented in Table (5) revealed that, the highest percentage of rooting was obtained with using full strength MS-medium supplemented with 1 g/l activated charcoal (100%), while using ¾ strength of MS-medium without activated charcoal and half strength of MS-medium with or without activated charcoal gave the lowest percentage of rooting (60.0%). This subject was studied by LE (1998) on *Artimesia umbelliforma*, Sakr *et al.*(1999) on *Yucca elephyantipes* and Saito and Nakano (2000) on *Hemerocallis hybrida*. They found that, transferring young growing shoots to growth regulators free medium easily developed roots.

Root number

Data in Table (5) revealed that all the tested strengths of MS-medium gave a number of roots varied from2.66 to 5.00 roots/shootlet without any significant differences between the treatments. Thus, the root number was not affected by the culture medium strength. In this respect George and Ravishankar (1997) on *Vanilla planifolia*, found that the best root formation was observed in half-strength of MS-medium containing activated charcoal. Also, Sakr *et al.* (1999) on *Yucca elephantipes* and Sayed *et al.* (2005) on *Cereus peruvianus*, obtained more root developmet when full-strength-MS medium was used.

Root length (cm)

As shown in Table (5) the root length was significantly influnced by the different strength of MS-medium (full, ¾ and half). Using full strength MS with 1 g/l activated charcoal gave the longest root length (5.684 cm) as compared with all strengthes of MS-medium (full, ¾ and half) without activated charcoal, which gave 0.722, 0.550 and 0.810 cm, respectively.

Survival of acclimatization

Data in Table (5) revealed that the survival percentage of vitroplant was not affected by different strengths of MS-medium (full, ¾ and half with or without 1 g/l activated charcoal. All vitroplant showed the maximum survival percentage (100%). In this regard, Wawrosch *et al.* (1994) on *Achillea asplenifolia* found that shootlets were easily rooted on hormone-free MS basal medium and subsequently acclimatized to greenhouse and field conditions with 100% survival.

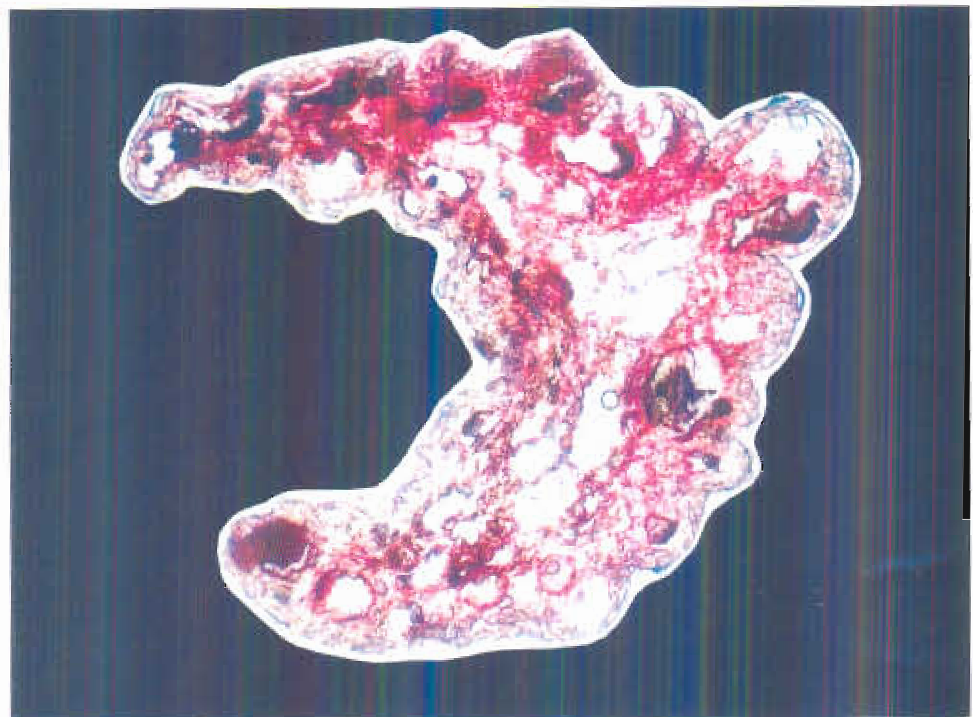

Fig. (1): Normal Leaf of Fucaria tuberculosa in vitro

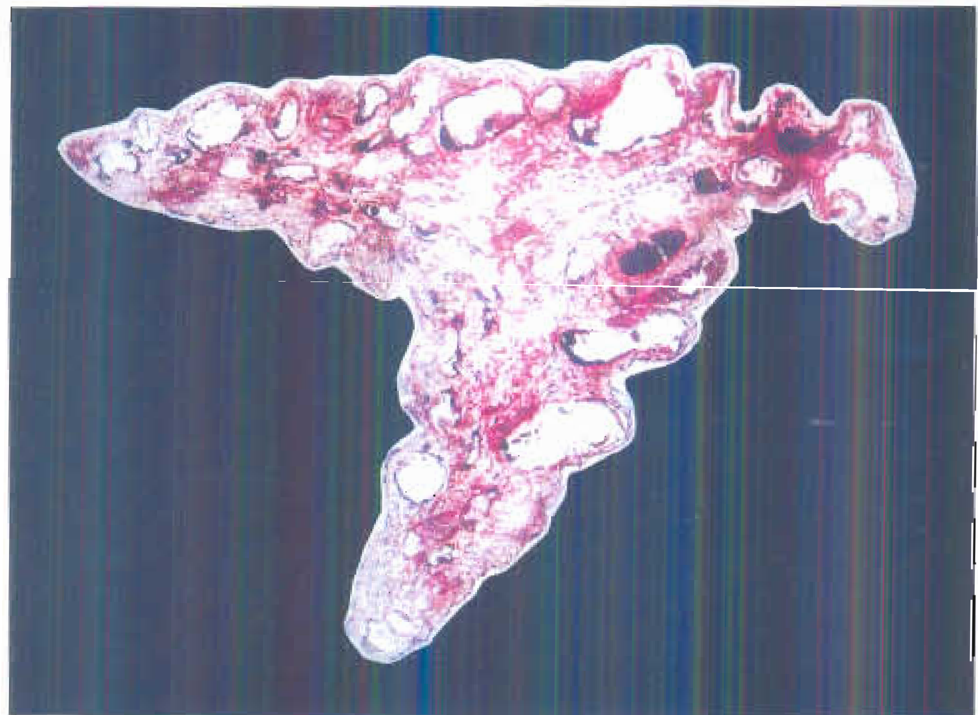

Fig. (2): Vitreous Leaf of Fucaria tuberculosa in vitro

Table (5): Effect of different strengthes of MS medium with or without activated charcoal on rooting behaviour and acclimatization survival of Faucaria tuberculosa in vitro.

Treatments	Rooting (%)	Root number	Root length (cm)	Survival (%)
Full MA	80.0	4.8	0.722	100.0
¾ MS	60.0	4.0	0.550	100.0
Half MS	60.0	2.6	0.810	100.0
Full MA + 1g/l AC	100.0	3.0	5.684	100.0
¾ MS + 1g/l AC	80.0	5.0	1.836	100.0
Half MS + 1g/l AC	60.0	3.0	2.968	100.0
LSD at 5%	38.08	NS	1.976	NS

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الملخص العربي

اكثار نبات الفيوكاريا تيبركلوزا بواسطة زراعة الانسجة

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اجريت هذه الدراسة فى معمل زراعة الانسجة وحفظ الاصول الوراثية – معهد بحوث البساتين – مركز البحوث الزراعية خلال الاعوام 2004 ، 2005 بهدف عمل بروتوكول لبداية الاكثار الدقيق باستخدام الكلوروكس وكلوريد الزئبقيق فى مرحلة التعقيم. بالاضافة لذلك تم اختبار زيادة عدد الافرع باستخدام املاح MS مضاف اليها تركيزات مختلفة من البنزيل ادنين او الكينتين. ايضا تم اختبار تركيز كامل من املاح MS او WPM مضاف اليها بنزيل ادنين او ذياتين او 2iP. وايضا استخدام تركيزات مختلفة من الاجار مضافة الى بيئة WPM وذلك لزيادة عدد النبيتات وتقليل نسبتها التى بها تزجج. وفى مرحله التجذير تم استخدام تركيزات مختلفه من املاح MS مضاف اليها او خالية من الفحم النشط. وجد ان احسن المعاملات التى ينصح باستخدامها للحصول على اجزاء نباتيه خاليه من التلوث وفى حاله حيه هى باستخدام 40% كلوروكس مع 0.2% كلوريد زئبقيق. اضافه البنزيل ادنين بتركيز 3 مليجرام/لتر الى بيئه املاح كاملة التركيز من MS نتج عنه اعلى معدل لتكوين النبيتات واقل نسبه من النبيتات التى بها تذجج بالمقارنه باستخدام 3 مليجرام/لتر كينتين فى النقله الثالثة. كما وجد انه عند استخدام بيئه املاح كاملة التركيز من MS او WPM مضاف الى اى منها 3 مليجرام/لتر بنزيل ادنين او ذياتين او 2iP لم توجد اى فروق معنوية فى عدد النبيتات المتكونه بينما استخدام بيئة WPM مضاف اليها 3 مليجرام/لتر بنزيل ادنين قلل من النبيتات التى بها تزجج. وان زيادة تركيز الاجار فى بيئة WPM من 7 الى 11 جرام/لتر قلل بالتدريج من عدد النبيتات بينما زاد من عدد النبيتات الخاليه من التزجج. كما وجد فى مرحلة التجذير ان استخدام تركيز كامل من املاح MS مضاف اليها 1 جرام/لتر فحم نشط زاد من نسبه تكوين الجذور وطول الجذور بينما لم يوجد فروق معنويه فى عدد الجذور بين المعاملات المختلفه. بالنسبة لمرحلة الاقلمة بالصوبه تم نقل كل النبيتات المتكونة من معاملات التجذير الى الصوبه فكانت نسبة بقاء النبتات حية 100%.