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The effect of drying and submersion pretreatment on adventitious shoot regeneration from hypocotyl explants of flax (*Linum usitatissimum* L.)

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Abstract: Hypocotyl explants of 3 flax cultivars were cultured for adventitious shoot regeneration in 3 different ways. Two pretreatment applications were compared with the routinely applied conventional regeneration protocol of culturing explants directly on Murashige and Skoog (MS) medium containing 1 mg L^{-1} 6-benzylaminopurine (BAP) and 0.02 mg L^{-1} naphthalene acetic acid (NAA). In the first pretreatment application, explants kept in a sterile cabin under an air flow for 30 min were immersed in MS solution containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA for 15 min; then pretreated explants were cultured on MS medium without any growth regulators (MS0). In the second pretreatment application, explants were kept in a sterile cabin under air flow for 30 min and then immersed in MS solution containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA for 15 min. The pretreated explants were then transferred to a culture medium enriched with 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA. Fresh and dry weights of hypocotyl explants, shoot regeneration percentage, shoot number per hypocotyl, shoot length, and total chlorophyll content were recorded. From the results, it could be seen that treating explants before culture initiation for regeneration with a liquid MS medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA for 15 min after keeping hypocotyls under an air flow in a sterile cabin for 30 min gave rise to the highest scores for tissue culture response.

Key words: In vitro shoot regeneration, *Linum usitatissimum*, pretreatment application

Keten bitkisi (*Linum usitatissimum* L.) hipokotil eksplantlarından adventif sürgün rejenerasyonu üzerine kurutma ve daldırma ön muamelesinin etkisi

Özet: Bu çalışmada, üç keten çeşidinin hipokotil eksplantları adventif sürgün rejenerasyonu için üç farklı şekilde kültüre alınmıştır. İki ön muamele uygulaması, rutin olarak uygulanan ve eksplantların doğrudan 1 mg L^{-1} 6-benzylaminopurine (BAP) ve 0,02 mg L^{-1} naphthalene acetic acid (NAA) içeren Murashige ve Skoog (MS) ortamında kültüre alınmasını kapsayan geleneksel rejenerasyon protokolü ile karşılaştırılmıştır. Birinci ön muamele uygulamasında, steril kabinde hava akımı altında 30 dak. bekletilen eksplantlar 1 mg L^{-1} BAP ve 0,02 mg L^{-1} NAA içeren solüsyona 15 dak. daldırılmış ve sonuçta ön muamele edilmiş eksplantlar büyüme düzenleyicileri içermeyen MS ortamında kültüre alınmıştır. İkinci ön muamele uygulamasında, eksplantlar steril kabinde hava akımı altında 30 dak. bekletildikten sonra 1 mg L^{-1} BAP ve 0,02 mg L^{-1} NAA içeren solüsyona 15 dak. daldırılmış ve sonra ön muamele edilmiş eksplantlar 1 mg L^{-1} BAP ve 0,02 mg L^{-1} NAA ile zenginleştirilmiş kültür ortamına aktarılmıştır. Hipokotil eksplantlarının yaş ve kuru ağırlıkları, sürgün

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rejenerasyon yüzdesi, hipokotil başına sürgün sayısı, sürgün uzunluğu ve toplam klorofil kapsamı belirlenmiştir. Sonuçlardan, rejenerasyon için kültür başlangıcından önce eksplantların steril kabinde hava akımı altında 30 dak. tutulmasından sonra 1 mg L^{-1} BAP ve 0,02 mg L^{-1} NAA içeren MS sıvı ortamında 15 dak. muamele edilmesinin doku kültürü tepkisi bakımından en yüksek değerleri verdiği görülebilir.

Anahtar sözcükler: İn vitro sürgün rejenerasyonu, *Linum usitatissimum*, ön muamele uygulaması

Introduction

Flax (*Linum usitatissimum* L.), a dicotyledonous plant from the family Linaceae, is an important crop that is widely used all over the world as a source of natural fibres and industrial oil, and it has the potential of meeting edible oil and protein deficiencies (Green & Marshall, 1984). Furthermore, it has been used as a medicinal plant for thousands of years, and its components, such as lignans and α-linolenic acid, have been used in many drugs (Tolkachev & Zhuchenko, 2000; Spence et al., 2003). Flax has also been used as a model system for genetic manipulation studies due to its small nuclear genome (Millam et al., 1992).

Flax regenerates more easily from hypocotyl explants in vitro (Gamborg & Shyluk, 1976; Friedt, 1990; Millam et al., 1992; Dong & McHughen, 1993), and medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA has been successfully used for shoot regeneration (Jordan & McHughen, 1988; Dong & McHughen, 1993; Yıldız & Özgen, 2004). At doses of BAP higher than 1 mg L^{-1}, callus growth and adventitious shoot formation were affected negatively (Xiang-can et al., 1989).

Since high frequency shoot regeneration from cultured cells, tissues, and organs is a prerequisite for an efficient transformation system, in vitro studies have been carried out continually.

This study aimed to obtain a higher shoot regeneration frequency from hypocotyls of flax by the drying and submersion pretreatment of explants before culturing on a regeneration medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA.

Materials and methods

Seeds of 'Omega', 'Fakel,' and 'Ariane' flax cultivars obtained from Northern Crop Science Laboratories, in North Dakota, USA, were used in the study. Seeds were surface sterilised with 40% commercial bleach containing 5% sodium hypochlorite at 10 °C for 20 min with continuous stirring, and then were washed 3 times with sterile water at the same temperature, according to the protocol described by Yildiz and Er (2002). Sterilised seeds were germinated on a basal medium containing the mineral salts and vitamins of Murashige and Skoog (1962), 3% (w/v) sucrose, and 0.7% (w/v) agar.

All cultures were incubated at 25 ± 1 °C under cool white fluorescent light (27 μmol m^{-2} s^{-1}) with a 16:8 photoperiod. The pH of the medium was adjusted to 5.8 prior to autoclaving. Hypocotyl segments of 5 mm in length were excised from 7-day-old seedlings as reported by Yıldız et al. (2003).

Explants were pretreated before culturing for adventitious shoot regeneration in 2 different ways; these 2 pretreatment applications were compared to the non-pretreatment application as a control.

First pretreatment application

Explants were kept in a sterile cabin under an air flow for 30 min. They were then immersed in a solution containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA for 15 min. Finally, pretreated explants were cultured on MS medium without any growth regulators (MS0).

Second pretreatment application

Explants were kept in a sterile cabin under an air flow for 30 min. Then they were immersed in a solution containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA for 15 min. Finally, the pretreated explants were transferred to a culture medium enriched with 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA.

Non-pretreatment application

Excised hypocotyl segments were directly cultured on MS medium including 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA.

Fresh and dry weights, shoot regeneration percentage, shoot number per hypocotyl, shoot length, and total chlorophyll content were recorded 6

weeks after culture initiation. Explants with shoots were weighed to determine the fresh weight. The dry weight was obtained after drying explants at 105 °C for 2 h. All measurements were made using an analytical scale, with a precision of 0.001 g.

Total chlorophyll content was determined in the leaves of plantlets regenerated from hypocotyls according to the protocol of Curtis and Shetty (1996). Fresh leaf tissue of 50 mg was put into 3 mL of methanol and kept in total darkness at 23 °C for 2 h. In this way, the chlorophyll in the fresh tissue passed through into the methanol. After 2 h, absorbancies were determined at 665 and 650 nm. Total chlorophyll content was calculated as micrograms of chlorophyll per gram of fresh tissue.

Three replicates were tested. Petri dishes were considered the units of replication. The number of explants per replication was 31. All experiments were repeated 2 times. Data were statistically analysed with Duncan's multiple range test using SPSS for Windows. Data presented in percentages were subjected to arcsine ($\sqrt{X}$) transformation before statistical analysis (Snedecor & Cochran, 1967).

Results and discussion

In an earlier study conducted by Yıldız et al. (2002), the regeneration capacities of flax hypocotyls and stem explants excised from both greenhouse-grown and in vitro seedlings were investigated. At the end of the study, the best results were obtained from the hypocotyl explants of in vitro seedlings.

In another study, the effect of different doses (0.5, 1, 2, 4, and 8 mg L^{-1}) of thidiazuron on shoot regeneration of flax was compared with combinations of BAP (1 mg L^{-1}) and NAA (0.02 or 0.2 mg L^{-1}). The highest values in all parameters of both genotypes were recorded from the medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA (Yildiz & Özgen, 2006), and this was verified as the optimum growth medium for flax by previous studies (Jordan & McHughen, 1988; Millam et al., 1992; Dong & McHughen, 1993; Yıldız et al., 2005).

Flax hypocotyls of 5 mm in length, excised from 7-, 12-, and 17-day-old seedlings, were cultured on MS and Gamborg's B-5 (Gamborg et al., 1968) media containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA and solidified with agar and Phytagel for shoot regeneration. According to the results, the highest scores for shoot regeneration percentage, shoot number per hypocotyl, and total shoot number per petri dish were obtained from the hypocotyls excised from 7-day-old seedlings and cultured on MS medium solidified with agar (Yıldız et al., 2003).

We used hypocotyls as the explants and 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA as the combination of growth regulators for shoot regeneration in parallel with the above studies, which reported that the most suitable explant for the in vitro culturing of flax was hypocotyl and the most suitable combination of plant growth regulators was 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA.

In the current study, pretreated and non-pretreated hypocotyl explants of 3 flax cultivars ('Omega', 'Fakel,' and 'Ariane') were cultured for adventitious shoot regeneration. Two different pretreatment applications were compared to the conventional regeneration protocol with respect to hypocotyl fresh and dry weights, shoot regeneration percentage, shoot number per hypocotyl, shoot length, and total chlorophyll content.

In the first and second pretreatment applications, hypocotyl explants were kept in a sterile cabin under an air flow for 30 min in order to dry them, as reported by Christmann et al. (2005), in order to decrease the tissue water content and to improve the explants' ability to uptake increased amounts of water, all solutes, and plant growth regulators by using osmotic pressure in consequent applications. Explants were then treated with MS solution containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA for 15 min. Finally, all explants were cultured on MS0 medium in the first pretreatment application and on MS medium enriched with 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA in the second pretreatment application. It was expected that immersing explants into liquid regeneration medium after drying would enable all cells to absorb more growth regulators along with water in both pretreatment applications. However, this was only the case in the second pretreatment application, in which explants were cultured on MS medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA, which means that tissues maintained an increased uptake of water and growth regulators from the regeneration medium,

which led to higher results in all parameters studied, as reported by Yildiz and Özgen (2004). Likewise, Okubo et al. (1991) have reported that endogenous hormone levels of tissue affected regeneration capacity in vitro significantly. Fatima et al. (2009) have also reported that internal factors such as chemicals and mineral nutrients affect in vitro plant growth. The required amount of exogenous plant growth regulators for cultured tissues depends on the endogenous levels that plant tissues have (Fatima et al., 2009). It was formerly reported that keeping explants in sterile distilled water for 20 min before culturing on MS medium enriched with 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA increased the regeneration capacity of hypocotyls of flax tremendously by increasing the permeability of the epidermis layer and the tissue's water content, and enabling water, all solutes, and growth regulators to transfer into the tissue more easily (Yildiz & Özgen, 2004).

According to the results, there were statistically significant differences among pretreated and non-pretreated hypocotyls in all cultivars (Table). The highest results for both fresh and dry weights of hypocotyls of all cultivars were obtained from the second pretreatment application, followed by the non-pretreated hypocotyls. The lowest results were recorded from the first pretreatment application in all cultivars studied (Table). From the results, it could be concluded that increases in the fresh and dry weights were chiefly due to an increase in the absorption of water and growth regulators from the medium in which explants were first pretreated and then cultured. When the results of the second pretreatment application were examined, it could be easily seen that culturing explants on MS medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA after treating them with liquid MS medium supplemented with 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA clearly enriched the tissue's levels of growth regulators, which caused higher fresh and dry weights (Table). In the first pretreatment application, transferring explants to MS0 medium after treating them with liquid MS with 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA, growth regulators of tissues did not seem to provide high scores with respect to fresh and dry weights. In the non-pretreatment application, culturing explants directly on MS medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA again failed to produce higher scores. Dale (1988) has reported that fresh weight increase was mainly due to cell enlargement by water absorption, cell vacuolation, and turgor-driven wall expansion. It was noted that an increase in dry weight was closely related to cell division and new material synthesis (Sunderland, 1960).

Table. Tissue culture response from pretreated and non-pretreated hypocotyls of 3 flax cultivars 6 weeks after culture initiation.

Cultivar	Application number	Hypocotyl		Shoot regeneration* (%)	Shoot number per hypocotyl	Shoot length (cm)	Total chlorophyll content (µg/g fresh tissue)
		Fresh weight (g)	Dry weight (g)				
'Omega'	1	0.25** ± 0.020 c	0.014 ± 0.0019 b	82.40 ± 1.07 b	6.76 ± 0.46 b	1.93 ± 0.13 b	217.1 ±10.40 c
	2	0.48 ± 0.023 a	0.034 ± 0.0023 a	100.00 ± 0.00 a	11.38 ± 0.69 a	2.82 ± 0.14 a	380.6 ± 26.91 a
	3	0.37 ± 0.017 b	0.018 ± 0.0017 b	90.00 ± 5.77 ab	7.99 ± 0.74 b	1.51 ± 0.17 b	286.2 ±10.45 b
'Fakel'	1	0.21 ± 0.027 b	0.016 ± 0.0030 b	72.03 ±7.53 b	6.42 ± 0.19 c	1.05 ± 0.11 b	197.0 ±15.40 c
	2	0.42 ± 0.006 a	0.032 ± 0.0046 a	100.00 ± 0.00 a	8.89 ± 0.37 a	1.61 ± 0.18 a	316.5 ±14.37 a
	3	0.31 ± 0.045 b	0.024 ± 0.0031 ab	80.60 ±7.45 a	7.49 ± 0.10 b	0.96 ± 0.10 b	252.1 ± 9.89 b
'Ariane'	1	0.19 ± 0.024 b	0.014 ± 0.0023 b	46.23 ± 6.20 c	4.26 ± 0.18 b	1.27 ± 0.06 b	192.0 ± 11.25 c
	2	0.36 ± 0.055 a	0.030 ± 0.0012 a	100.00 ± 0.00 a	6.64 ± 0.25 a	2.00 ± 0.13 a	346.0 ± 18.62 a
	3	0.26 ± 0.026 ab	0.019 ± 0.0018 b	65.35 ± 3.76 b	4.59 ± 0.07 b	1.06 ± 0.09 b	268.2 ± 24.10 b

Applications:

1. First pretreatment: Hypocotyls were kept for 30 min in a sterile cabin under an air flow and for 15 min in a solution containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA, and finally cultured on MS0 medium.
2. Second pretreatment: Hypocotyls were kept for 30 min in a sterile cabin under an air flow and for 15 min in a solution containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA, and finally cultured on MS medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA.
3. Non-pretreatment: Hypocotyls were cultured directly on MS medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA.

* The values represent mean ± standard error of the mean.

** Values within a column for each cultivar followed by different letters are significantly different at the 0.01 level.

The results related to shoot regeneration percentage indicated that the lowest results were obtained from the first pretreatment application for all cultivars. Hypocotyl explants formed fewer roots and calluses in the first pretreatment application than in the others. All explants regenerated successfully in the second pretreatment application, and consequently, the shoot regeneration percentage was recorded as 100% for all cultivars (Table, Figure).

The highest results for shoot number per hypocotyl and shoot length were obtained from the second pretreatment application for all cultivars. The highest shoot number per hypocotyl was recorded as 11.38 for 'Omega,' 8.89 for 'Fakel,' and 6.64 for 'Ariane.' The highest scores related to shoot length were 2.82, 1.61, and 2.00 cm for 'Omega,' 'Fakel,' and 'Ariane,' respectively (Table). The shoot regeneration capacity of the hypocotyls increased significantly in the second pretreatment application.

The best results for total chlorophyll content were obtained from the second pretreatment application for all cultivars. The highest scores for total chlorophyll content were recorded as 380.6 in 'Omega,' 316.5 in 'Fakel,' and 346.0 in 'Ariane' (Table). The explants that received the second pretreatment application were more vital, grew better, and more capable of regeneration (Figure). Emerson (1929) reported a close relation between photosynthesis and chlorophyll content. Chlorophyll content affects photosynthesis directly (Rensburg & Krüger, 1994; Kyparissis et al., 1995; Jagtap et al., 1998). The chlorophyll content of a leaf is accepted as an indicator of the photosynthetic capacity of tissues (Pal & Laloraya, 1972; Wright et al., 1994; Nageswara Rao et al., 2001).

From the results of the present study, it could be concluded that the lower levels of all parameters recorded in the first pretreatment and the non-pretreatment applications were directly due to a decreased uptake of water and growth regulators from the medium. Tissue culture response has been affected significantly by tissue water content (Yildiz & Özgen, 2004). Treatment of explants with liquid MS medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA before culture initiation enabled water, all solutes, and plant growth regulators to transfer into the tissue much more, providing all cells with a high regeneration capacity and consequently increasing the explant's tissue culture response.

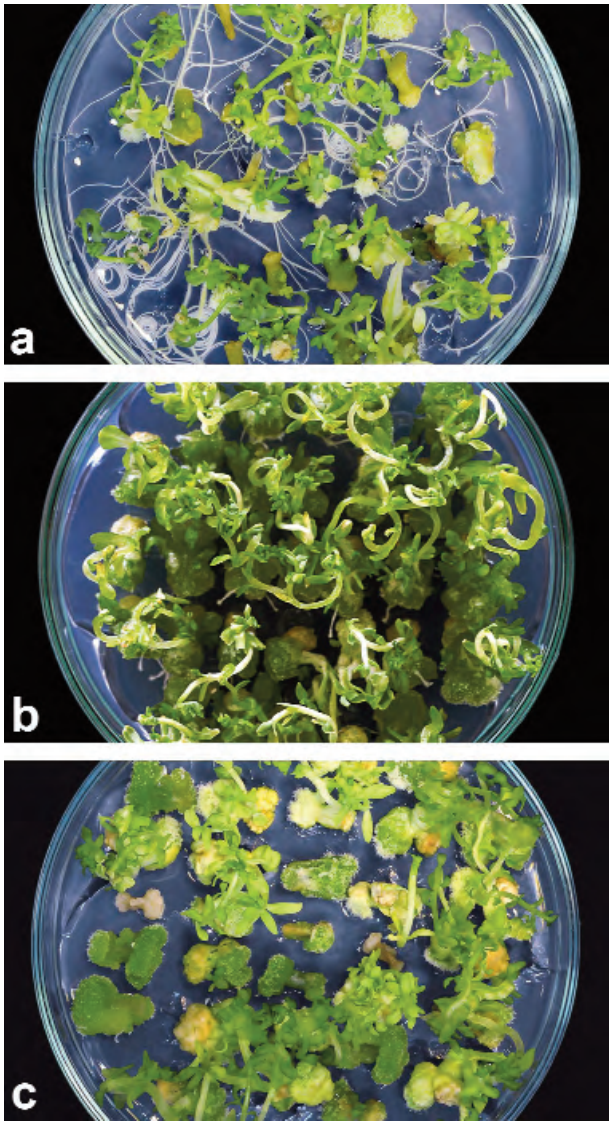

Figure. In vitro shoot regeneration of pretreated and non-pretreated hypocotyls of flax (*Linum usitatissimum*) cv. 'Omega.'
(a) First pretreatment application: Hypocotyls were kept for 30 min in a sterile cabin under an air flow, treated with solution containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA for 15 min, and finally cultured on MS0 medium.
(b) Second pretreatment application: Hypocotyls were kept for 30 min in a sterile cabin under an air flow, treated with solution containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA for 15 min, and finally cultured on MS medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA.
(c) Non-pretreatment application: Hypocotyls were directly cultured on MS medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA.

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