BIOLOGIA PLANTARUM 55 (3): 541-544, 2011

BRIEF COMMUNICATION

Evaluation of the effect of *in vitro* stress and competition on tissue culture response of flax

M. YILDIZ*

Department of Field Crops, Faculty of Agriculture, University of Ankara, 06110 Diskapi, Ankara, Turkey

Abstract

This study was carried out to investigate the *in vitro* competition in tissue culture of three flax (*Linum usitatissimum* L.) cultivars using different distances among hypocotyl explants cultured. Hypocotyl fresh and dry masses, shoot regeneration percentage, shoot number per hypocotyl, regenerated shoot length and total chlorophyll content were examined during shoot regeneration, while plantlet height, number of roots and length of roots were recorded during rooting. With decreasing distance among explants we observed increased shoot regeneration and rooting till a certain point from where stress initiated and significant decreases in all parameters observed. Explants cultured at distance 1.0 cm were found to be at their optimum.

Additional key words: competition, *Linum usitatissimum*, plantlet establishment.

Flax (*Linum usitatissimum* L.) is a dicotyledonous plant of *Linaceae* family and it is an important source of natural fiber, high quality nutritious food and medicinal plant. Moreover, it has been used as a model crop in biotechnological studies due to its small nuclear genome (Basiran *et al.* 1987, Dong and McHughen 1991, Millam *et al.* 1992). It was reported that the most suitable explant for *in vitro* culture of flax was hypocotyl (Gamborg and Shyluk 1976, Jordan and Mc Hughen 1988a,b, Mc Hughen *et al.* 1989, Dong and Mc Hughen 1991, 1993, Millam *et al.* 1992) similarly as for many other species (Tavano *et al.* 2009). Shoot regeneration from hypocotyl explants and recovery of whole fertile flax plant were achieved by Murray *et al.* (1977). Plant growth regulators are more effective on *in vitro* shoot regeneration when they are used together than using alone (Dang and Wei 2009). For flax, the combination of 1 mg dm^{-3} 6-benzylaminopurine (BAP) and 0.02 mg dm^{-3} naphthalene acetic acid (NAA) was recommended (Jordan and McHughen 1988b, Dong and McHughen 1993). Callus growth and adventitious shoot formation in flax increases up to 1 mg dm^{-3} BAP while they were negatively affected at higher doses (Xiang *et al.* 1989).

Since high frequency shoot regeneration is a prerequisite for a successful transformation (Tavano *et al.* 2009), *in vitro* studies have been carried on perpetually. This study was aimed to obtain higher shoot regeneration frequency from hypocotyls of flax by utilizing the competition among explants *in vitro*.

Flax (*Linum usitatissimum* L. cvs. Madaras, 1886 Sel and Clarck) seeds, obtained from Northern Crop Science Laboratories, in North Dakota, USA, were surface sterilized with 40 % commercial bleach containing 5 % sodium hypochlorite at 10 ºC for 20 min with continuous stirring and then were washed three times with sterile distilled water at the same temperature according to the protocol described by Yildiz and Er (2002). Sterilized seeds were germinated on a basal Murashige and Skoog (1962; MS) medium containing the mineral salts and vitamins, 3 % (m/v) sucrose and 0.7 % (m/v) agar. All cultures were incubated at 25 ± 1 ºC under cool white fluorescent tubes (27 μmol m^{-2} s^{-1}) with a 16-h photoperiod. The pH of the medium was adjusted to 5.8 prior to autoclaving. Hypocotyl segments in 5 mm length were excised from 7-d-old seedlings and imbibed in sterile distilled water at a gentle shaking for 20 min. Then, the segments were cultured on MS medium supplemented with 1 mg dm^{-3} BAP and 0.02 mg dm^{-3} NAA for

Received 19 October 2009, *accepted* 8 March 2010.
Abbreviations: BAP - 6-benzylaminopurine; IBA - indolebutyric acid; MS - Murashige and Skoog; NAA - naphthalene acetic acid.
* Fax: +90 (312) 3182666, e-mail: myildiz@ankara.edu.tr

regeneration (Yildiz and Özgen 2004).

For shoot regeneration, hypocotyls were cultured in a Petri dish (90 × 90 mm) at 0.5, 1.0, 1.5 and 2.0 cm distances for 6 weeks. Fresh and dry masses, shoot regeneration percentage, shoot number per hypocotyl, the highest shoot length and chlorophyll *a+b* content were recorded at the end of the culture. The dry mass was obtained after drying explants at 105 ºC for 2 h. Total chlorophyll content was determined in leaves of plantlets according to Curtis and Shetty (1996) and absorbance in methanol extract was measured at 665 and 650 nm.

Regenerated shoots were transferred to rooting medium containing 3 mg dm^{-3} indole-butyric acid (IBA) at the same distances (0.5, 1.0, 1.5 and 2.0 cm) in *Magenta* vessels (60 × 60 mm). After 3 weeks, plantlet height, root number per plantlet and the highest root length were noted.

Three replicates were tested. Petri dish was considered as the units of replication. The number of explants per replication varied according to the distances. All experiments were repeated two times. Data were statistically analyzed by Duncan's multiple range test using *SPSS for Windows* computer program (Snedecor and Cochran 1967).

There are many studies about the plant competition in field conditions. Plant leaves compete for irradiance, and roots for water and nutrients (Wilson 1988, McPhee and Aarssen 2001). High plant density was accepted as a biotic stress factor (De Klerk 2007). This study was conducted to investigate if there was such competition among explants growing *in vitro*. It is thought that this is the first report evaluating competition among explants *in vitro*.

Results clearly showed that there were statistically significant differences in fresh and dry masses in all cultivars among explants cultured at different distances. The highest fresh and dry masses per explant of all cultivars were obtained at 2.0 cm distance of hypocotyls and they decreased by decreasing distances (Table 1). These findings were supported by Gersani *et al.* (2001) and Maina *et al.* (2002) who reported that plants grown alone produce more biomass or yield than those grown with the others. It could be concluded that the decreased distance in which explants were cultured induced stress caused likely by the deficiency of water, sucrose and nutrients. Increases in the fresh and dry masses were chiefly due to an increase in the absorption of water and other components from the basal medium (Yildiz and Özgen 2004). It was stated that the fresh mass increase was mainly due to cell enlargement by water absorption (Dale 1988) and increase in dry mass was closely related to cell division and new material synthesis (Sunderland 1960).

The highest shoot number per hypocotyl (20.70 in Madaras, 14.57 in 1886 Sel and 17.40 in Clarck) and the highest shoot length (3.10, 2.14 and 2.09 cm, respectively) were obtained at 1.0 cm distance in all cultivars studied (Table 1). It is thought that competition among explants cultured at 1.0 cm distance encouraged them to give higher results than at the other distances. Yildiz and Ozgen (2004) have previously reported 12.17, 11.20 and 10.83 shoots per hypocotyl while shoot length was 0.56, 0.58 and 0.60 cm in cultivars Madaras, 1886 Sel and Clarck, respectively. The higher values in the present study than in the previous one seems to be due to optimum competition among explants in this study.

Chlorophyll content of leaf is considered as a sign of photosynthetic capacity of tissues (Pal and Laloraya 1972, Wright *et al.* 1994, Nageswara *et al.* 2001). The highest chlorophyll *a+b* content was found at 1.5 cm distance in all cultivars. It was thought that this could be due to the fact that there were too many shoots at 1.0 cm

Table 1. Hypocotyl culture of three flax cultivars at 4 different distances 6 weeks after culture initiation on MS medium containing 1 mg dm^{-3} BAP and 0.02 mg dm^{-3} NAA. Means of 3 values from 3 independent replications ± SE. Values within a column for each cultivar followed by different letters are significantly different at the 0.01 level.

Cultivar	Distances [cm]	Hypocotyl FM [g]	Hypocotyl DM [g]	Regeneration [%]	Shoot number [hypocotyl^{-1}]	Shoot length [cm]	Total chlorophyll content [µg g^{-1}(FM)]
Madaras	0.5	0.24 ± 0.027c	0.02 ± 0.001c	100	4.37 ± 0.58d	0.52 ± 0.09d	264.5 ± 23.99b
	1.0	0.31 ± 0.022c	0.03 ± 0.002c	100	20.70 ± 1.41a	3.10 ± 0.12a	296.5 ± 10.06b
	1.5	0.46 ± 0.034b	0.04 ± 0.003b	100	11.87 ± 0.46b	1.82 ± 0.12b	417.9 ± 24.83a
	2.0	0.86 ± 0.027a	0.07 ± 0.005a	100	8.27 ± 0.38c	0.96 ± 0.08c	320.1 ± 24.95b
1886 Sel	0.5	0.15 ± 0.023c	0.01 ± 0.001d	100	4.64 ± 0.46c	0.41 ± 0.13c	227.1 ± 5.86c
	1.0	0.22 ± 0.018c	0.02 ± 0.001c	100	14.57 ± 0.67a	2.14 ± 0.10a	324.0 ± 15.66b
	1.5	0.33 ± 0.009b	0.04 ± 0.003b	100	10.77 ± 0.87b	1.54 ± 0.17b	480.2 ± 33.73a
	2.0	0.57 ± 0.058a	0.06 ± 0.002a	100	6.80 ± 0.78c	0.60 ± 0.09c	275.6 ± 22.35ab
Clarck	0.5	0.17 ± 0.019c	0.02 ± 0.001c	100	4.47 ± 1.07d	0.40 ± 0.10c	237.7 ± 15.52c
	1.0	0.23 ± 0.023c	0.02 ± 0.001c	100	17.40± 0.55a	2.09 ± 0.11a	523.4 ± 16.98a
	1.5	0.37 ± 0.013b	0.04 ± 0.003b	100	12.60 ± 0.38b	1.23 ± 0.07b	543.0 ± 32.11a
	2.0	0.62 ± 0.077a	0.06 ± 0.006a	100	9.00 ± 0.46c	0.52 ± 0.04c	423.4 ± 19.36c

Table 2. *In vitro* root development on shoots regenerated from hypocotyl explants cultured at different distances on rooting medium containing 3 mg dm^{-3} IBA determined 3 weeks after subculture initiation. Mean of 3 values from 3 independent replications ± SE. Values within a column for each cultivar followed by different letters are significantly different at the 0.01 level.

Cultivar	Distances [cm]	Plantlet height [cm]	Root number [plantlet^{-1}]	Root length [cm]
Madaras	0.5	1.27 ± 0.22d	8.00 ± 0.58d	0.97 ± 0.12d
	1.0	7.48 ± 0.49a	40.00 ± 2.30a	4.80 ± 0.38a
	1.5	4.43 ± 0.35b	27.00 ± 2.65c	3.10 ± 0.17b
	2.0	2.37 ± 0.12c	15.00 ± 1.73c	1.77 ± 0.09c
1886 Sel	0.5	1.43 ± 0.24d	11.00 ± 1.15d	1.20 ± 0.15d
	1.0	8.13 ± 0.54a	43.67 ± 2.60a	5.47 ± 0.35a
	1.5	5.20 ± 0.46b	32.67 ± 0.67b	3.40 ± 0.23b
	2.0	2.73 ± 0.19c	18.33 ± 1.45c	2.10 ± 0.15c
Clarck	0.5	1.40 ± 0.15d	10.00 ± 0.58d	0.90 ± 0.26d
	1.0	7.27 ± 0.13a	42.67 ± 2.40a	5.33 ± 0.26a
	1.5	4.97 ± 0.77b	31.67 ± 1.20b	3.37 ± 0.26b
	2.0	2.77 ± 0.12c	17.33 ± 0.88c	1.90 ± 0.12c

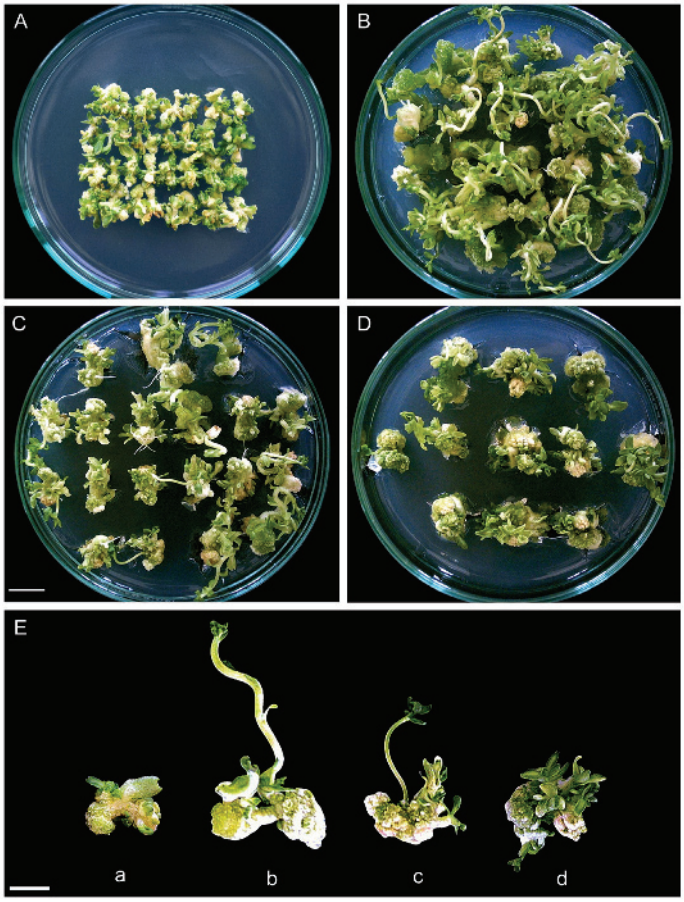

Fig. 1. Development of explants cultured at different distances 0.5 cm (*A*, *a*), 1.0 cm (*B*, *b*), 1.5 cm (*C*, *c*) and 2.0 cm (*D*, *d*); six weeks after culture initiation. *Bar* is 1.0 cm for Petri dishes and 0.5 cm for shoot regeneration.

distance in a Petri dish and shoots could easily be shaded each other. At 0.5 and 2.0 cm distances, the total chlorophyll content was lower (Table 2).

Regenerated shoots were rooted on MS medium containing 3 mg dm^3 IBA as reported by Yildiz and Özgen (2004) at the same distances (0.5, 1.0, 1.5 and 2.0 cm) in *Magenta* vessels for 3 weeks. Similar effects were recorded in rooting stage. This means that the

highest plantlet height, root number per plantlet and the highest root length were observed in shoots rooted at 1.0 cm distance (Table 2). The lowest values were noted at 0.5 cm distance in all cultivars which could be result from deficiency of water, sucrose and nutrients. At distances above 1.0 cm, plant development was getting lower again.

Mills (2009) has stated that sucrose in the growth medium improves biomass production and photosynthetic activity by increasing leaf area. Mingozzi and Morini (2009) have reported that physical microenvironment affects *in vitro* regeneration significantly. In the present study, physical microenvironment was manipulated by altering distances among explants cultured which caused positive competition among them that resulted in increased shoot regeneration capacity, root formation and plantlet establishment. We assume that the protocol presented in this study could be used for other crops to obtain high frequency shoot regeneration *in vitro*.

References

Basiran, N., Armitage, P., Scott, R.J., Draper. J.: Genetic transformation of flax (*Linum usitatissimum*) by *Agrobacterium tumefaciens*: regeneration of transformed shoots via a callus phase. - Plant Cell Rep. **6**: 396-399, 1987.

Curtis, O.F., Shetty, K.: Growth medium effects on vitrification, total phenolics, chlorophyll, and water content of *in vitro* propagated oregano clones. - Acta Hort. **426**: 498-503, 1996.

Dale, J.E.: The control of leaf expansion. - Annu. Rev. Plant Physiol. **39**: 267-295, 1988.

Dang, W., Wei, Z.M.: High frequency plant regeneration from the cotyledonary node of common bean. - Biol. Plant. **53**: 312-316, 2009.

De Klerk, G.J.: Stress in plants cultured *in vitro*. - Propag. Ornam. Plants **7**(3): 129-137, 2007.

Dong, J.Z., Mc Hughen, A.: Patterns of transformation intensity on flax hypocotyls inoculated with *Agrobacterium tumefaciens*. - Plant Cell Rep. **10**: 555-560, 1991.

Dong, J., Mc Hughen, A.: An improved procedure for production of transgenic flax plants using *Agrobacterium tumefaciens*. - Plant Sci. **88**: 61-71, 1993.

Gamborg, O.L., Shyluk, J.P.: Tissue culture, protoplasts, and morphogenesis in flax. - Bot. Gaz. **137**: 301-306, 1976.

Gersani, M., Brown, J.S., O'Brien, E., Mania, G.M., Abramsky, Z.: Tragedy of the commons as a result of root competition. - J. Ecol. **89**: 660-669, 2001.

Jordan, M.C., Mc Hughen, A.: Glyphosate tolerant flax plants from *Agrobacterium*-mediated gene transfer. - Plant Cell Rep. **7**: 281-284, 1988a.

Jordan, M.C., Mc Hughen, A.: Transformed calllus does not necessarily regenerate transformed shoots. - Plant Cell Rep. **7**: 285-287, 1988b.

Maina, G.G., Brown, J.S., Gersani, M.: Intra-plant *versus* inter-plant root competiton in beans: avoidance, resource matching or tragedy of the commons. - Plant Ecol. **160**: 235-247, 2002.

Mc Hughen, A., Jordan, M., Feist, G.: A preculture period prior to *Agrobacterium* inoculation increases production of transgenic plants. - J. Plant Physiol. **135**: 245-248, 1989.

McPhee, C.S., Aarssen, L.W.: The seperation of above- and below-ground competion in plants. A review and critique of methodology. - Plant Ecol. **152**: 119-136, 2001.

Millam, S., Davidson, D., Powell, W. The use of flax (*Linum usitatissimum*) as a model system for studies on organogenesis *in vitro*: the effect of different carbohydrates. - Plant Cell Tissue Organ Cult. **28**: 163-166, 1992.

Mills, D.: Effect of sucrose application, minerals, and irradiance on the *in vitro* growth of *Cistus incanus* seedlings and plantlets. - Biol. Plant. **53**: 415-421, 2009.

Mingozzi, M., Morini, S.: *In vitro* cultivation of donor quince shoots affects subsequent morphogenesis in leaf explants. - Biol. Plant. **53**: 141-144, 2009.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue culture. - Physiol. Plant. **15**: 473-479, 1962.

Murray, B.E., Handyside, R.J., Keller, W.A.: *In vitro* regeneration of shoots on stem explants of haploid and diploid flax (*Linum usitatissimum*). - Can. J. Genet. Cytol. **19**: 177-186, 1977.

Nageswara, R.R.C., Talwar, H.S., Wright, G.C.: Rapid assessment of specific leaf area and leaf nitrogen in peanut (*Arachis hypogaea* L.) using chlorophyll meter. - J. Agron. Crop Sci. **189**: 175-182, 2001.

Pal, R.N., Laloraya, M.M.: Effect of calcium levels on chlorophyll synthesis in peanut and linseed plants. - Biochem. Physiol. Pflanz. **163**: 443-449, 1972.

Snedecor, G.W., Cochran, W.G. (ed.): Statistical Methods. - Iowa State University Press, Ames 1967.

Sunderland, N.: Cell division and expansion in the growth of the leaf. - J. exp. Bot. **11**: 68-80, 1960.

Tavano, E.C.R., Stipp, L.C.L., Muniz, F.R., Mourão Filho, F.A.A., Mendes, B.M.J.: *In vitro* organogenesis of *Citrus volkameriana* and *Citrus aurantium*. - Biol. Plant. **53**:395-399, 2009.

Wilson, J.B.: Shoot competition and root competition. - J. appl. Ecol. **25**: 279-296, 1998.

Wright, G.C., Nageswara, R.R.C., Farquhar, G.D.: Water use efficiency and carbon isotope discrimination in peanut under water deficit conditions. - Crop Sci. **34**: 92-97, 1994.

Xiang, C.-Z., Jones, D.A., Kerr, A: Regeneration of shoots on root explants of flax. - Ann. Bot. **63**: 297-299, 1989.

Yildiz, M., Er, C.: The effect of sodium hypochlorite solutions on *in vitro* seedling growth and shoot regeneration of flax (*Linum usitatissimum*). - Naturwissenschaften **89**: 259-261, 2002.

Yildiz, M., Özgen, M.: The effect of a submersion pretreatment on *in vitro* explant growth and shoot regeneration from hypocotyls of flax (*Linum usitatissimum*). - Plant Cell Tissue Organ Cult. **77**: 111-115, 2004.