© by PSP Volume 26 – No. 12A/2017 pages 8239-8245 Fresenius Environmental Bulletin

THE EFFECTS OF CO-CULTIVATION IN WHEAT (TRITICUM SP.) AND FLAX (LINUM USITATISSIMUM L.) ON SHOOT REGENERATION CAPACITY OF EXPLANTS

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ABSTRACT

This study was carried out to investigate the relationship between explants from two different genotypes (wheat and flax) cultured together and determine the effects of co-cultivation on tissue culture response of mature embryo explants of wheat (*Triticum* sp.) and hypocotyl explants of flax (*Linum usitatissimum* L.). One winter bread wheat (*T. aestivum* L.) cv. 'Bezostaja 1', one winter durum wheat (*Triticum durum* Desf.) cv. 'Cakmak 79', and two flax cvs. 'Madaras' and '1886 Sel.' were used in the study. Mature embryos excised from sterilized seeds of wheat cultivars were placed scutellum downwards on a medium containing 3 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and incubated at 24±1°C in total darkness for 14 days for callus induction. Hypocotyl segments in 5 mm length were excised from 7-day-old *in vitro*-grown seedlings of flax. Explants (callus for wheat and hypocotyl for flax) of both genotypes were cultured on MS medium supplemented with 0.20 mg l^{-1} 6-benzylaminopurine (BAP) and 0.01 mg l^{-1} naphthalene acetic acid (NAA) for regeneration at 24±1ºC under cool white fluorescent light (27 μmol m^{-2} s^{-1}) with a 16-h light/8-h dark photoperiod for 4 weeks. Sixteen explants were cultured in a 4 x 4 matrix in a Petri dish. Eight out of 16 explants for each genotype were cultured together for co-cultivation. 16 explants from each genotype were cultured alone as control. The results showed that co-cultivation between wheat and flax gave rise to the highest scores in comparison with culturing explants alone.

KEYWORDS:
Co-cultivation, flax, wheat, shoot regeneration

INTRODUCTION

Flax is an important crop all over the world in terms of its aspects as a source of natural fiber and industrial oil, and has the potential of meeting edible oil and protein deficiency [1]. Moreover, it has been used as a model system for genetic manipulation studies due to its small nuclear genome. Wheat (*Triticum aestivum* L.) is the main nutrition source for two thirds of the world population [2, 3]. Yield of wheat has gradually increased in the last century by using conventional breeding methods. However, these methods have some limitations such as long time requirement and limited gene pool [4]. It is well known that cereals' breeding depends on the use of tissue culture techniques which are used to overcome the problems of conventional breeding methods [5].

Flax regenerates more easily from hypocotyl explants *in vitro* [6, 7, 8, 9, 10]. Although the highest frequencies of callus and plant regeneration have been reported to be obtained from the culture of immature embryos in wheat [11, 12, 13] mature embryos which are readily available at all times have been successfully used in callus induction and plant regeneration [14, 15, 16, 17].

Plant tissue culture techniques help us propagate plants vegetatively in a large amount by using small parts of a tissue and by using the ability of cells to form a whole, fertile plant which is called totipotency. Explants regenerate shoots and roots, and consequently whole fertile plants under certain cultural conditions. Tissue culture studies aim to obtain high-frequency shoot regeneration, which is also a prerequisite for an efficient transformation system and clonal propagation of plants. Introduction of foreign genes coding agronomically important traits into plant cells has no meaning unless transgenic plants are regenerated from the genetically modified cell(s). The types and concentrations of plant growth regulators in plant cell culture significantly affect growth and morphogenesis. In order to obtain high frequency adventitious shoot regeneration for a related genotype, correct explant type, concentrations and combinations of auxins and cytokinins should be determined. However, determining the explant type, and correct concentrations and combinations of growth regulators is not enough for high-frequency shoot regeneration. Since every cell has an ability of forming a whole fertile plant under *in vitro* conditions, shoot regeneration frequency can always be higher than we obtain in theory. Many factors affecting the regeneration capacity of explants have not been found out yet. For instance, a recently reported technique utilizing competition among explants was

very effective in increasing shoot regeneration capacity [18]. Thus, this study was conducted to develop an efficient method to obtain higher shoot regeneration frequency via culturing explants together, which we called 'co-cultivation'. The method we described in this study was based on culturing explants from two different genotypes together and consequently increasing regeneration capacity of mature embryo explants of wheat (*Triticum* sp.) and hypocotyl explants of flax (*Linum usitatissimum* L.).

MATERIALS AND METHODS

Wheat. One winter bread wheat (*T. aestivum* L.) cv. 'Bezostaja 1' and one winter durum wheat (*Triticum durum* Desf.) cv. 'Cakmak 79' as sources of mature embryos were used in the study. Mature seeds of wheat cultivars were surface-sterilized with 70% (v/v) ethanol for 5 min, washed several times with sterile distilled water, immersed in commercial bleach (containing 5% sodium hypochlorite) for 25 min, and rinsed at least 7 times with sterile distilled water. Then, the seeds were imbibed in sterile distilled water for 24 h at 4ºC. For callus induction, mature embryos from imbibed and dehulled seeds were aseptically excised slightly with a scalpel. Excised embryos were placed scutellum downwards on a basal medium containing the mineral salts and vitamins of Murashige and Skoog (MS) [19], 3 mg l^{-1} 2,4-D, 2% (w/v) sucrose and 0.7% (w/v) agar. The Petri dishes were incubated at 24±1ºC in total darkness for 14 days. Developed calli were transferred to a shoot induction medium containing MS mineral salts and vitamins, 0.20 mg l^{-1} BAP and 0.01 mg l^{-1} NAA, glycine (2 mg l^{-1}), 2% (w/v) sucrose and 0.7% (w/v) agar at pH 5.8 in Petri dishes. The transferred callus cultures were incubated at 24±1ºC under cool white fluorescent light (27 μmol m^{-2} s^{-1}) with a 16-h light/8-h dark photoperiod for 4 weeks. At the end of the culture, culture efficiency in percentage, number of regenerated shoots and shoot lengths were determined.

Flax. Flax (*Linum usitatissimum* L. cvs. 'Madaras' and '1886 Sel.') seeds obtained from the Northern Crop Science Laboratories in North Dakota, USA were surface-sterilized with 40% commercial bleach containing 5% sodium hypochlorite at 10ºC for 20 min with continuous stirring and they were then washed three times with sterile distilled water at the same temperature according to the protocol described by Yildiz and Er [15]. Sterilized seeds were germinated on a basal Murashige and Skoog [19] medium containing the mineral salts and vitamins, 3% (w/v) sucrose and 0.7% (w/v) agar. All cultures were incubated at 25±1ºC under cool white fluorescent tubes (27 µmol m^{-2} s^{-1}) with a 16h light/8h dark photoperiod. The pH of the medium was adjusted to 5.8 prior to autoclaving. Hypocotyl segments in 5-mm lengths were excised from 7-d-old seedlings and imbibed in sterile distilled water with gentle shaking for 20 min. Then, the segments were cultured on an MS medium supplemented with 0.20 mg l^{-1} BAP and 0.01 mg l^{-1} NAA for regeneration. Shoot regeneration percentage, shoot number per explant, shoot length and total shoot number per petri dish were recorded 4 weeks after culture initiation. Sixteen explants were cultured in a 4 x 4 matrix in a Petri dish. Eight out of 16 explants for each genotype were cultured for co-cultivation. 16 explants from each genotype were cultured alone as control (Figure 1).

Statistical Analysis. Four replicates were tested. Petri dish was considered as the unit of replication. The number of explants per replication for co-cultivation was 8 for each genotype, and for cultivation alone, it was 16 for each genotype. All experiments were repeated two times. The data were statistically analyzed by Duncan's multiple range test using the 'SPSS for Windows' computer program [20].

RESULTS

The Effects of Co-cultivation on Tissue Culture Response of Wheat Cultivars. The culture efficiency of mature embryo-derived callus of wheat cvs. 'Cakmak 79' and 'Bezostaja 1' co-cultured with the hypocotyl explants of flax cvs. 'Madaras' and '1886 Sel.' was significantly higher ($P<0.01$) than that of callus cultured alone (Table 1). Culture efficiency of callus cultured alone was recorded as 59.38% and 53.13% while it was 96.88% and 100.00% in co-cultivation with 'Madaras'; 100.00% and 100.00% in co-cultivation with '1886 Sel.' in cv. 'Cakmak 79' and in cv. 'Bezostaja 1', respectively (Table 1). 9.50 and 8.50 shoots were regenerated out of 16 explants cultured alone in cv. 'Cakmak 79' and in cv. 'Bezostaja 1', respectively (Figure 2B and b). 7.75 and 8.00 shoots regenerated from 8 callus explants in cv. 'Cakmak 79' when callus was cultured together with hypocotyl explants of flax cvs. 'Madaras' and '1886 Sel.', respectively (Table 1) (Figure 2A, a1 and a2). In cv. 'Bezostaja 1', 8.00 and 8.00 shoots were regenerated from callus where they were cultured together with hypocotyls of both flax cultivars. The results clearly showed that co-cultivation between wheat and flax genotypes increased regeneration capacity significantly. Although only 8 explants from each cultivar were cultured in co-cultivation, the number of regenerated shoots was almost two times more than that in the explants cultured alone. Higher shoot lengths in co-cultivation could be attributed to the effect of explants from different genotypes on each other. The mean shoot length was recorded as 7.50 in callus cultured alone while it was about 10.50 in co-cultivation.

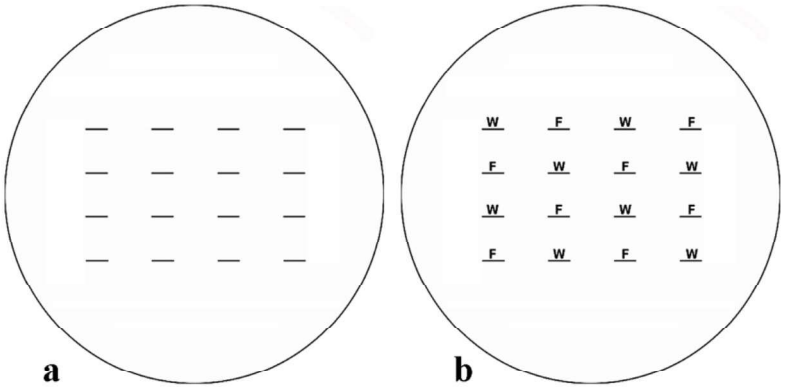

FIGURE 1
Schematic plan of explants cultured at '1.0 x1.0' distance for shoot regeneration. a) culture alone, b) co-cultivation between flax and wheat explants. F=Flax, W= Wheat

TABLE 1
Culture responses of mature embryo-derived callus of wheat (*Triticum* sp.) cvs. 'Cakmak 79' and 'Bezostaja 1' cultured alone and co-cultured with flax (*Linum usitatissimum* L.) cvs. 'Madaras' and '1886 Sel.'

Cultivars	Culture efficiency (%)[1]		
	Cultured alone	Co-cultured with 'Madaras'	Co-cultured with '1886 Sel.'
'Cakmak 79'	59.38 b±7.33	96.88 a±3.83	100.00 a±0.00
'Bezostaja 1'	53.13 b±4.94	100.00 a±0.00	100.00 a±0.00
Mean	**56.26**	**98.44**	**100.00**
Cultivars	**No. of regenerated shoots / No. of calli cultured**		
	Cultured alone	**Co-cultured with 'Madaras'**	**Co-Cultured with '1886 Sel.'**
'Cakmak 79'	9.50/16 b±0.07	7.75/8.00 a±0.04	8.00/8.00 a±0.00
'Bezostaja 1'	8.50/16 b±0.05	8.00/8.00 a±0.00	8.00/8.00 a±0.00
Mean	**9.00/16.00**	**7.88/8.00**	**8.00/8.00**
Cultivars	**Shoot length (cm)**		
	Cultured alone	**Co-cultured with 'Madaras'**	**Co-cultured with '1886 Sel.'**
'Cakmak 79'	7.21 b±0.45	10.24 a±1.58	10.94 a±1.10
'Bezostaja 1'	7.68 b±0.68	10.34 a±1.47	10.81 a±0.85
Mean	**7.45**	**10.29**	**10.88**

[1](Number of calli regenerated/Number of calli cultured) x 100
Means followed by different letters in a row for each cultivar are significantly different at 0.01 level

The Effects of Co-cultivation on Tissue Culture Response of Flax Cultivars. The culture efficiency of hypocotyl explants of flax cvs. 'Madaras' and '1886 Sel.' co-cultured with mature embryo-derived callus of wheat cvs. 'Cakmak 79' and 'Bezostaja 1' was significantly higher ($P<0.01$) than that of hypocotyls cultured alone (Table 2). Shoot regeneration percentage of hypocotyls cultured alone was 70.31% and 92.19% while it was 100.00% and 100.00% in co-cultivation with 'Cakmak 79' and with 'Bezostaja 1'. The data related to shoot number per explants was recorded as 1.64 and 2.00 in explants cultured alone in cv. 'Madaras' and in cv. '1996 Sel.', respectively (Figure 2C and c). 3.81 and 4.35 shoots regenerated from single explants in cv. 'Madaras' when hypocotyls were cultured together with callus of wheat cvs. 'Cakmak 79' and 'Bezostaja 1', respectively (Table 2) (Figure 2A, a1 and a2). 5.07 and 5.03 shoots regenerated from single explants when cv. '1886 Sel.' was cultured together with callus of both wheat cultivars. Shoot number per explant was significantly higher ($P<0.01$) in co-cultivation application than cultivation alone in both flax cultivars. The stimulatory effect of co-cultivation application could be seen in shoot length. The shoot lengths regenerated in hypocotyl explants cultured alone were lower than in the explants from two genotypes cultured together (Table 2). The mean shoot length in explants cultured alone was about 1.50 cm while it was approximately 3.50 cm in the co-cultivation application. The total shoot number per petri dish increased to 29.75 and 34.75 in hypocotyls of cv. 'Madaras' co-cultured with cv. 'Cakmak 79' and with cv. 'Bezostaja 1', respectively, while it was 22.75 in explants cultured alone. Similarly, total shoot number per petri dish increased to 40.50 and

40.25 in hypocotyls of cv. '1886 Sel.' cultured with cv. 'Cakmak 79' and with cv. 'Bezostaja 1' from 32.00 in explants cultured alone (Table 2).

DISCUSSION

High-frequency shoot regeneration is the main target of tissue culture studies. High-frequency shoot regeneration is also a prerequisite for an efficient transformation system. Shoot regeneration capacity of cells or tissues to be used in transformation studies affects the success of genetic transformation significantly [9, 21]. Viability and age of explant, and the tissue source from which the explant is excised are very important parameters for high-frequency shoot regeneration [22]. Fatima et al. [23] reported that growth and morphogenesis are controlled by the types and concentrations of plant growth regulators in plant cell culture. In order to obtain high frequency adventitious shoot regeneration for a related genotype, correct concentrations and combinations of auxins and cytokinins have been aimed to be determined.

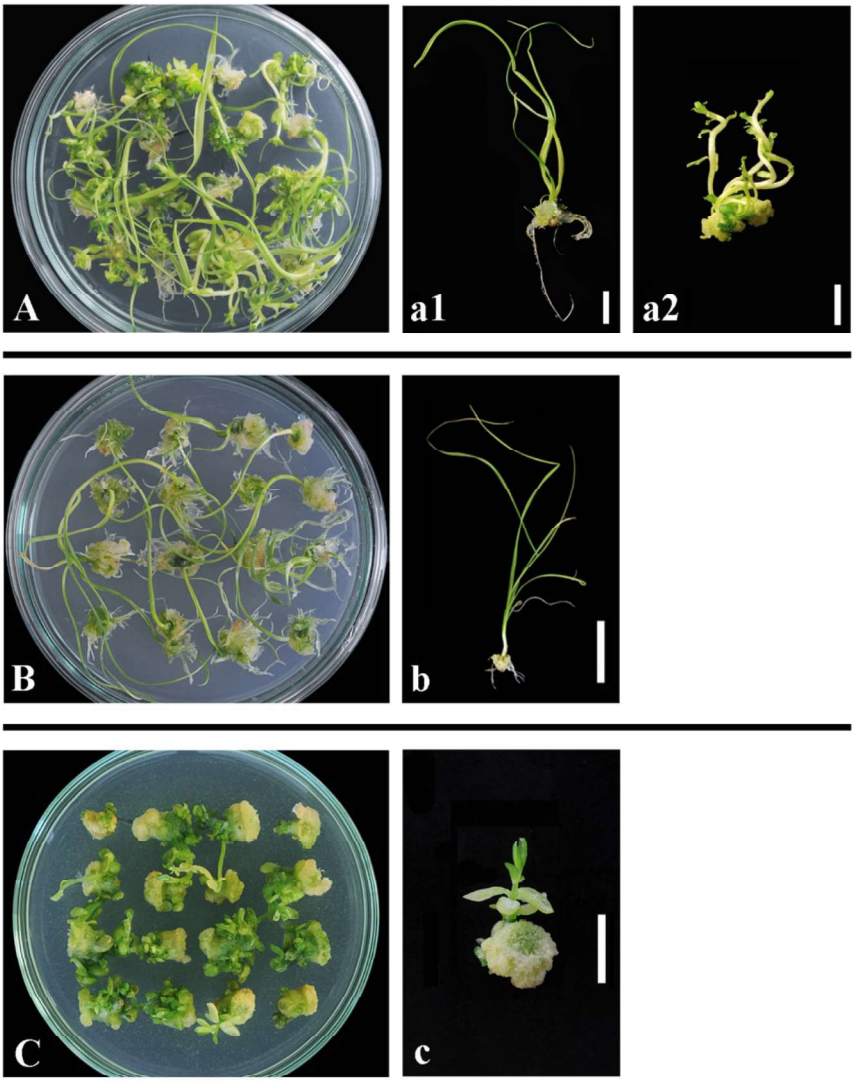

FIGURE 2
Shoot regeneration from flax hypocotyl and wheat callus explants 4 weeks after culture initiation. A) Tissue culture responses of flax hypocotyl and wheat callus explants cultured together, a1) Shoot formation from a wheat callus explant cultured together with flax hypocotyl explant, a2) Shoot development from flax hypocotyl explant cultured together with wheat callus explant, B-b) Tissue culture response of wheat callus explants cultured alone, C-c) Tissue culture response of flax hypocotyl explants cultured alone. Bars = 1.0 cm

TABLE 2
Culture responses of hypocotyl explants of flax (*Linum usitatissimum* L.) cvs. 'Madaras' and '1886 Sel.' cultured alone and co-cultured with mature embryo-derived callus of wheat (*Triticum* sp.) cvs. 'Cakmak 79' and 'Bezostaja 1'

Cultivars	Shoot regeneration (%)			Shoot number per explant		
	Cultured alone	Co-cultured with 'Cakmak 79'	Co-cultured with 'Bezostaja 1'	Cultured alone	Co-cultured with 'Cakmak 79'	Co-cultured with 'Bezostaja 1'
'Madaras'	70.31 b±6.54	100.00 a±0.00	100.00 a±0.00	1.64 b±0.29	3.81 a±0.32	4.35 a±0.49
'1886 Sel.'	92.19 b±7.25	100.00 a±0.00	100.00 a±0.00	2.00 b±0.17	5.07 a±0.13	5.03 a±0.25
Mean	**81.25**	**100.00**	**100.00**	**1.82**	**4.44**	**4.69**
Cultivars	**Shoot length (cm)**			**Total shoot number per petri dish**		
	Cultured alone	Co-cultured with 'Cakmak 79'	Co-cultured with 'Bezostaja 1'	Cultured alone	Co-cultured with 'Cakmak 79'	Co-cultured with 'Bezostaja 1'
'Madaras'	1.25 b±0.08	3.27 a±0.16	3.07 a±0.31	22.75 b±3.06	29.75 a±2.61	34.75 a±3.92
'1886 Sel.'	1.72 b±0.50	3.81 a±0.26	3.75 a±0.46	32.00 b±2.78	40.50 a±1.06	40.25 a±2.02
Mean	**1.49**	**3.54**	**3.41**	**27.38**	**35.13**	**37.50**

Means followed by different letters in a row for each cultivar are significantly different at 0.01 level

Plants compete each other with respect to water, nutrients and light. There are many studies reporting such competition among plants in field conditions [24, 25]. Plant density was reported as a biotic stress factor in natural conditions and this was one of the reasons for competition among plants [21]. Plant development and yield of many vegetable crops were affected significantly by plant density [26]. It was noted that fruit yield is increased by increasing plant density [28, 29, 30]. However, if plant density causes competition, plant yield starts to decrease. Early and total yield per pepper plant decreased by increasing plant density caused by higher interplant competition due to less spacing [31]. Abubaker [32] reported that the highest planting density caused the lowest yield in beans because of the high competition among plants for water and minerals. Asghari et al. [33] reported that the chicory plant had increased root diameter for increased absorption of water under high density due to high competition between plants.

Although many studies about the effects of plant density and competition have been conducted in field conditions, such competition has firstly been reported by Yildiz [10] regarding *in vitro* culture. In these studies, hypocotyl explants of different flax (*Linum usitatissimum* L.) cultivars were cultured at four different distances as '0.5 x 0.5', '1.0 x 1.0', '1.5 x 1.5' and '2.0 x 2.0' cm in order to determine the effects of competition among explants for constant amount of water and nutrients on shoot regeneration capacity. In all genotypes, the highest results with respect to tissue culture response were recorded at a culture distance of '1.0 x 1.0' cm. From the results, it could be concluded that 1.0 cm culture distance encouraged explants to compete each other for constant amount of water, sucrose and nutrients in the growth medium. Yildiz [10] showed that shoot regeneration capacity could be increased not only by determination of correct concentrations and combinations of auxins and cytokinins in the growth medium, but also by encouraging explants into competition. This is why, in this study, explants from both genotypes were cultured alone or together at a culture distance of '1.0 x 1.0' cm.

To our knowledge, this is the first study reporting that shoot regeneration frequency could be increased by culturing explants from two separate genotypes together. Finding two separate genotypes that could be cultured together is the main obstacle in front of the protocol described in this study.

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Received: **09.08.2017**
Accepted: **25.09.2017**

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