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Research Note

The effect of *in vitro* spacing competition on shoot regeneration from cotyledon node explants of *Lathyrus chrysanthus* Boiss

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Abstract

This study was aimed to determine the effect of *in vitro* spacing competition among explants for water and for both macro and micro nutrients in the growth medium on tissue culture response of *Lathyrus chrysanthus* Boiss. cotyledon node explants. Cotyledon node explants were excised from 14-day-old sterile seedlings and cultured in a petri dish at '0.5 × 0.5', '1.0 × 1.0' and '2.0 × 2.0' cm distance for 4 weeks. At the end of the culture, fresh and dry weights of explant, water content of explant, shoot regeneration percentage, shoot number per explant, shoot length, total shoot number per petri dish and total chlorophyll content in leaves of regenerated shoots were recorded. The positive effect of spacing competition among explants was observed in all characters examined at '1.0 × 1.0' cm culture distance. Shoot number per cotyledon node explant and total shoot number per petri dish were obtained the highest as 2.99 and 30.67, respectively, at '1.0 × 1.0' cm culture distance. According to the results, it could be concluded that encouraging explants for spacing competition by decreasing the culture distances among them from '2.0 × 2.0' cm to '1.0 × 1.0' cm caused to a significant increases in all characters examined. This study showed that the spacing competition among explants under *in vitro* conditions could be evaluated to increase the success of tissue culture studies for related genotype.

Keywords: *Lathyrus chrysanthus* Boiss., cotyledon node, *in vitro* spacing competition, shoot regeneration.
Abbreviations: BAP_6-benzylaminopurine; MS_Murashige and Skoog; NAA_Naphthalene acetic acid.

Introduction

Lathyrus chrysanthus as an ornamental plant, from the family *Fabaceae*, is sturdy annual, stems, erect, winged, 15–45 cm; leaves with tendrils, leaflets 1-paired, elliptic; peduncles 2–4 flowered, much longer than the leaves; corolla 18–25 mm, golden; legume linear oblong, densely tuberculate-pilose, 2–7 seeded (Davis, 1970). It was reported that cotyledon nodes were the most suitable explant type in *Lathyrus* species (Malik et al., 1992; Barik et al., 2004) and the combination of BAP at 0.25 mg l^{-1} and NAA at 0.05 mg l^{-1} has been effective on shoot regeneration of *Lathyrus chrysanthus* Boiss. (Telci, 2012). Tissue culture studies aim to obtain high-frequency shoot regeneration, which is also a prerequisite for an efficient transformation system and a clonal propagation of plants. The recovery of transgenic plants carrying genes coding agronomically important traits depends on shoot regeneration from the genetically modified cell(s) (Jordan and Mc Hughen, 1988; Dong and Mc Hughen, 1993). Explant health and the seedling from which the explant is excised are very important for high-frequency shoot regeneration (Yildiz and Er, 2002). Fatima et al. (2009) have 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 related genotype, correct concentrations and combinations of auxins and cytokinins should be determined. Is determining the explant type, and correct concentrations and combinations of growth regulators enough for high frequency shoot regeneration? How can we know that the shoot regeneration frequency we obtain is the highest for the genotype of interest? Since every cell has an ability of forming a complete plant under *in vitro* conditions, shoot regeneration frequency can always be higher than we obtain in theory. So, more factors affecting *in vitro* regeneration capacity of explant should be found out. This study was conducted to test whether the method of Yildiz et al. (2011) and Yildiz (2011) reporting that the spacing competition among explants could be utilized to increase *in vitro* shoot regeneration capacity, worked in *Lathyrus chrysanthus*.

Results

The current study was aimed to obtain high frequency shoot regeneration by utilizing *in vitro* spacing competition among cotyledon node explants of *Lathyrus chrysanthus* Boiss. One-way analysis of variance (ANOVA) for shoot regeneration from cotyledon node explants of *Lathyrus chrysanthus* Boiss. cultured at different culture distances showed highly significant difference (Table 1).

Explant growth

Explants cultured at '1.0 × 1.0' cm distance were found to be heavier than the ones cultured at '2.0 × 2.0' cm from the

Table 1. One-way analysis of variance (ANOVA) for shoot regeneration from cotyledon node explants of *Lathyrus chrysanthus* Boiss. cultured at different culture distances four weeks after culture initiation on MS medium containing 0.25 mg l^{-1} BAP and 0.05 mg l^{-1} NAA.

		Cotyledon node explant							
	Degrees of Freedom	Fresh weight (g)		Dry weight (g)		Water content (g)		Shoot regeneration (%)	
		Sum of Squares	F	Sum of Squares	F	Sum of Squares	F	Sum of Squares	F
Culture Distances	2	0.013	24.057**	0.000	9.814**	0.011	25.316**	154.981	3.260
Error	6	0.002		0.000		0.001		142.607	
Total	8								

	Degrees of Freedom	Shoot number per explant		Shoot length (cm)		Total shoot number per petri dish		Total chlorophyll content (µg g^{-1} fresh tissue)	
		Sum of Squares	F	Sum of Squares	F	Sum of Squares	F	Sum of Squares	F
Culture Distances	2	4.018	26.296**	3.423	86.979**	468.222	22.179**	95635.717	36.625**
Error	6	0.458		0.118		63.333		7833.645	
Total	8								

** Significantly different at 0.01 level

Table 2. Shoot regeneration from cotyledon node explants of *Lathyrus chrysanthus* Boiss. cultured at different culture distances four weeks after culture initiation on MS medium containing 0.25 mg l^{-1} BAP and 0.05 mg l^{-1} NAA.

Culture distance (cm)	Cotyledon node explant			Shoot regeneration (%)	Shoot number per explant	Shoot length (cm)	Total shoot number per petri dish	Total chlorophyll content (µg g^{-1} fresh tissue)
	Fresh weight (g)	Dry weight (g)	Water content (g)					
'0.5 × 0.5'	0.108 C±0.016	0.015A±0.002	0.093 B±0.014	83.33	1.36 C±0.070	2.12 A±0.065	13.67 B±1.453	286.45 C±20.568
'1.0 × 1.0'	0.172 A±0.003	0.016 A±0.001	0.156 A±0.003	85.00	2.99 A±0.237	1.85 A±0.074	30.67 A±2.848	536.82 A±25.356
'2.0 × 2.0'	0.082 B±0.003	0.007 B±0.001	0.075 B±0.004	72.22	2.18 B±0.124	0.70 B±0.100	18.00 B±0.577	383.28 B±15.480

Each value is the mean of 3 replications containing 12 explants per replication. The study was set in two parallels.
Means followed by different letters in a column were significantly different at P = 0.01.

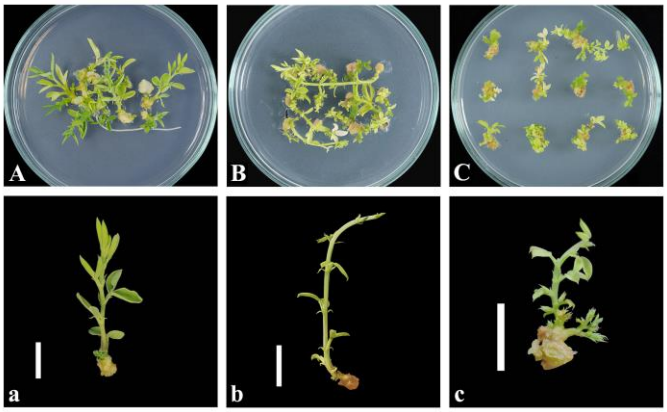

Fig 1. Shoot regeneration from cotyledon node explants cultured at (A-a) '0.5 × 0.5' cm, (B-b) '1.0 × 1.0' cm and (C-c) '2.0 × 2.0' cm culture distance (bars = 1 cm)

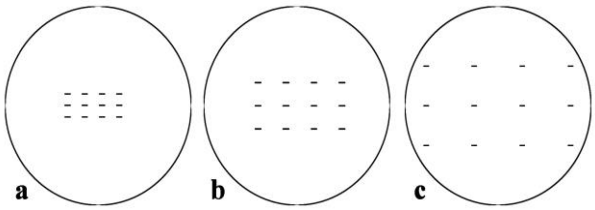

Fig 2. Schematic plan of explants cultured at (a) '0.5 x 0.5' cm (b) '1.0 x 1.0' cm and (c) '2.0 x 2.0' cm distances in a petri dish

aspect of fresh weight as 0.172 g (Table 2). The difference in fresh weight between the explants cultured at different distances was found to be statistically significant ($P<0.01$). At the end of four weeks, it was observed that explants cultured at '1.0 × 1.0' cm distance formed more dry matter than the ones cultured at '2.0 × 2.0' cm as 0.016 g (Table 2). The differences in dry weight between the explants cultured at different distances were found to be statistically significant ($P<0.01$). Water content was also determined to be higher in explants cultured at '1.0 × 1.0' cm distance as 0.156 g (Table 2). Increase in tissue water content of explants was found to be statistically significant ($P<0.01$). From these results of our study, '1.0 × 1.0' cm culture distance increased explant's water absorption capacity and the fresh weight increase of explants cultured at '1.0 × 1.0' cm distance was chiefly because of an increase in tissue water content.

Tissue culture response

In the characters of shoot regeneration percentage, shoot number per explant, shoot length and total shoot number per petri dish, the highest values were obtained as 85.00%, 2.99, 1.85 cm and 30.67 from explants cultured at '1.0 × 1.0' cm distance while the lowest results were recorded as 72.22%, 2.18, 0.70 cm and 18.00 in explants cultured at '2.0 × 2.0' cm distance (Table 2, Fig. 1). In our study, '1.0 × 1.0' cm culture distance induced explants to compete each other for constant amount of water and nutrient in the growth medium and consequently the highest values were recorded in all characters examined.

Chlorophyll content

In total chlorophyll content, the highest values were obtained at '1.0 × 1.0' cm culture distance. The highest value in total chlorophyll content was recorded as 536.82 µg g^{-1} fresh tissue in leaves of regenerated shoots grown from explants cultured at '1.0 × 1.0' cm culture distance (Table 2).

Discussion

Many studies have reported that plants compete each other for water, nutrient and light in field conditions (Wilson, 1988; McPhee and Aarssen, 2001). Plant density as a biotic stress factor was considered one of the reasons of spacing competition among plants in natural conditions (De Klerk, 2007). It was reported that plant development and yield of many vegetable crops have been affected by plant density (Stoffella and Bryan, 1988) and fruit yield increased by increasing plant density (Decoteau and Graham, 1994; Jolliffe and Gaye, 1995; Morgade and Willey, 2003). Increasing plant density caused to a decrease in early and total yield per pepper plant (Daşgan and Abak, 2003). The highest planting density gave rise to the lowest yield in bean due to the high spacing competition among plants for water and minerals (Abubaker, 2008). High competition between plants under high density conditions caused to increase in root diameter of the chicory plant for increased absoption of water (Asghari et al., 2009). Although many research studies about the effect of plant density and spacing competition have been conducted in field conditions, there are no studies reporting such spacing competition under *in vitro* conditions except the ones of Yildiz et al. (2011) and Yildiz (2011). Higher mass production of explants could be attributed to an increased absorption of water and other components from the growth medium via higher metabolic activity (Yildiz and Ozgen, 2004; Dale, 1988; Sunderland, 1960). The results of the current study were compatible with the ones of Asghari et al. (2009) reporting that the average total fresh weight per chicory plant was higher at the lower plant density. The reason for the fresh weight increase was cell enlargement by water absorption, cell vacuolation, and turgor-driven wall expansion (Dale, 1988). Increase in dry weight was caused by higher mass production via cell division and new material synthesis (Sunderland, 1960). Water content of the tissue is accepted as an indicator of higher metabolic activity (Yildiz and Ozgen, 2004). Prado et al. (1995) reported that decreasing water absorption resulted in reduced fresh weight. In our case, spacing competition increased explant's growth by increasing tissue metabolic activity. In a study conducted by Yildiz et al. (2011), hypocotyl explants of two flax (*Linum usitatissimum* L.) cultivars were cultured at four different distances as '0.5 × 0.5', '1.0 × 1.0', '1.5 × 1.5' and '2.0 × 2.0' cm in order to determine the effect of spacing competition among explants for constant amount of water and nutrient on shoot regeneration capacity. It was reported that shoot number per hypocotyl, total shoot number per petri dish and total chlorophyll content increased to 4.03, 44.3 and 332.20 at '1.0 × 1.0' cm culture distance from 2.10, 22.0 and 287.30 at '2.0 x 2.0'cm distance in cv. 'Ariane' and to 3.09, 34.0 and 326.30 at '1.0 x 1.0'cm culture distance from 2.60, 18.7 and 268.40 at '2.0 × 2.0' cm distance in cv. 'Verne', respectively.

Although the same growth regulators composition was used as before, by encouraging the explants for spacing competition, shoot number per hypocotyl, total shoot number per petri dish and total chlorophyll content were recorded to be the highest in both cultivars. Yildiz et al. (2011) have reported that neither shoot regeneration percentage nor shoot number per explant is lonely an indicator of the success of tissue culture studies but 'total shoot number per petri dish' is the character indicating the success of both shoot regeneration percentage and shoot number per explant for related genotype under *in vitro* conditions. Our study showed that culture distance could be used as a way of increasing shoot regeneration capacity by encouraging explants into competition. Chlorophyll content of leaf is considered as a sign of photosynthetic capacity of tissues (Emerson, 1929; Pal and Laloraya, 1972; Wright et al., 1994; Nageswara et al., 2001) which plays a critical role in plant growth and development (Yang et al., 2010) and its amount changes under stress conditions (Rensburg and Kruger, 1994; Kyparissis et al., 1995; Jagtap et al., 1998). Chlorophyll content can be used to measure growth (Gireesh, 2009). In the study of Yildiz (2011), physical microenvironment was manipulated by changing distances among explants cultured which caused positive spacing competition among them that resulted in increased shoot regeneration capacity, root formation and plantlet establishment. The inhibition of growth under water stress conditions was the result of inhibition of cell division and cell elongation (Hsiao, 1973). According to the results, the promising method of Yildiz et al. (2011) and Yildiz (2011) was tested and approved that it also worked very well in *Lathyrus chrysanthus* Boiss. to obtain high shoot regeneration frequency.

Materials and Methods

Plant material

Lathyrus chrysanthus seeds of an ecotype (Diyarbakir) found in southeast of Turkey were surface disinfected according to the protocol of Telci et al. (2011).

Surface sterilization and germination of seeds

The seeds were disinfected using 75% commercial bleach (containing 5% sodium hypochlorite) at 35ºC for 15 min. with continuous stirring and were then rinsed three times with sterile distilled water. The temperature of the rinse water was the same as that of the disinfectant as reported by Yildiz and Er (2002), and Telci et.al. (2011). Sterilized seeds were germinated on a basal medium of Murashige and Skoog's (MS) mineral salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, and 0.7% agar in Magenta vessels (15×15 cm).

Culture conditions

All cultures were incubated at 25±1ºC under cool white fluorescent light (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. Cotyledon node explants were excised from 14-day-old seedlings and cultured in a petri dish at '0.5 × 0.5', '1.0 × 1.0' and '2.0 × 2.0' cm distances (Fig. 2) for 4 weeks on MS medium supplemented with 0.25 mg l^{-1} 6-benzylaminopurine (BAP) and 0.05 mg l^{-1} naphthalene acetic acid (NAA) for regeneration.

Observations

Fresh and dry weights of cotyledon node explant, shoot regeneration percentage, shoot number per explant, shoot length and total shoot number per petri dish were recorded at the end of culture. Cotyledon node explants were weighed to determine the fresh weight. The dry weight was obtained after drying explants at 105ºC for 2 h. All measurements were made using an analytical scale, with precision of 0.001 g. Water content of cotyledon node explants was the difference between fresh and dry weights.

Total chlorophyll content was determined in leaves of seedlings according to the protocol of Curtis and Shetty (1996). Fresh tissue of 50 mg from leaf was put in 3 ml methanol and kept in total dark at 23ºC for 2 hours. By this way, chlorophyll in fresh tissue passed through into methanol. After 2 hours, absorbancies were determined at 665 and 650 nm. Total chlorophyll content was calculated as "µg/g fresh tissue".

Statistical analysis

All statistical analyses were performed by "SPSS 15.0 for Windows" computer program. Three replicates were tested. Petri dishes (100 × 10 mm) were considered as units of replication. The number of explants per replication was 12. The study was set in two parallels each with three replicates to confirm the accuracy of the study. One-way Analysis of Variance (ANOVA) was used to test the effect of different culture distances on tissue culture response of cotyledon node explants of *Lathyrus chrysanthus* Boiss. Data were statistically analyzed by Duncan's multiple range test using "SPSS for Windows" program. Data presented in percentages were subjected to arcsine ($\sqrt{X}$) transformation before statistical analysis (Snedecor and Cochran, 1967).

Conclusion

In our study, the lower levels of all characters of explants cultured at '2.0 × 2.0' cm distance could be attributed to a decreasing amount of water uptake from the environment and consequently, a reduced mobilization of plant growth regulators which promote cell division and cell elongation. The lowest values were noted at 0.5 × 0.5 cm distance in the characters of shoot number per explant, total shoot number per petri dish and total chlorophyll content which could be result from *in vitro* stress against lower amount of sucrose and nutrients in the growth medium. Establishment of an efficient regeneration protocol is a prerequisite for the application of biotechnology to crop improvement. Plant growth regulators are perhaps the most important media components affecting shoot regeneration capacity of explant. In order to obtain high frequency adventitious shoot regeneration for related genotype, correct concentrations and combinations of auxins and cytokinins should be determined. The current study verified that shoot regeneration frequency of *Lathyrus chrysanthus* Boiss. cotyledon node explants could be increased not only by determination of explant type, and correct concentrations and combinations of auxins and cytokinins in growth medium but also by encouraging explants into spacing competition.

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