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Effects of genotype, explant age and growth regulators on callus induction and direct shoot regeneration of Lettuce (*Lactuca sativa* L.)

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Abstract

The genetic engineering of Lettuce (*Lactuca sativa* L.) requires a reliable and efficient tissue culture method. Callus induction and direct shoot regeneration of lettuce using cotyledon explants was investigated by studying the effects of genotype, explant age and different combinations of plant growth regulators. The landrace genotypes were collected from central (Yazd) and southwest (Ahvaz) of Iran. Cotyledon explants of Lettuce with different explant age and genotypes were cultured on MS medium supplemented with different concentrations of NAA and BA. The highest frequency of callus induction was obtained on 2.7 µM NAA and 4.4 µM BA from 3-day-old explants of Yazd genotype. In addition, the effect of explant age for callus induction was a genotype-dependent characteristic. The highest number of direct shoot regenerations was obtained at low BA concentrations (<1 µM). Pearson's correlation coefficient identified that callus induction correlated significantly and positively (0.408) with direct shoot regeneration. It was also shown that the region near the petiole of cotyledon was the best for direct shoot regeneration from cotyledon explants of lettuce. Direct shoot regeneration from the region near to petiole of cotyledons may aid the use of genetic engineering to improve important characteristics of lettuce.

Keywords: *Lactuca sativa*, Genotype, Cotyledon, Shoot regeneration, Growth regulators.
Abbreviations: BA – 6-benzylaminopurine; IAA – indole 3-acetic acid; IBA – indole-3-butyric acid; NAA – naphthaleneacetic acid; MS – Murashige and Skoog medium

Introduction

Lettuce (*Lactuca sativa* L.) is a major fresh leafy vegetable and belongs to the Asteraceae family (Compositae), tribe Cichoreae and genus *Lactuca*. Lettuce has been cultivated all over the world and is a self-pollinated annual plant with 2n=2x=18 chromosomes. In some eastern countries like China and Egypt, stems of lettuce are consumed. The leaves of Lettuce are important source of vitamins A, C and Lactupicrin which are known to prevent cancers (Ryder, 2002; Resh, 2001). Lactucarium, the dried latex produced from a wild Lettuce relative, *Lactuca virosa* L., is used to make a sleep- inducing medicine (Ryder, 1986). About two-third of the total production area (about 1 million hectare) in the world is resided in Asia. Iran is one of the major lettuce and chicory- producing countries (90000 metric tons) in this continent (FAO, 2007). Notable accomplishments of modern lettuce breeding have been made in the areas of disease/insect resistance, improved quality, and increased yield. These characteristics may be improved using biotechnology and gene transfer strategies. For example, 'Evola' lettuce transformed with an *ipt* gene, significantly delayed development and leaf senescence in mature heads (McCabe et al., 2001). Lettuce plants expressing a nitrate reductase gene from tobacco had lower nitrate accumulation in the leaves (Curtis et al., 1999). But genetic engineering requires that specific genotype can be regenerated from transformed cells in large numbers and in a reliable manner. Regenerated shoots from lettuce have been produced by culturing cotyledon explants. The first successful shoot regeneration was reported when cotyledon explants were culture on media containing 5 mgl^{-1} IAA and 0.5 mgl^{-1} kinetin (Doerschug and Miller, 1967). Xinrun and Conner (1992) and Ampomah-Dwamena et al. (1997) evaluated the effect of lettuce genotypes on shoot regeneration from cotyledon explants using a media containing 0.1 mgl^{-1} IAA, 0.5 mgl^{-1} kinetin plus 0.05 mgl^{-1} zeatin. Vanjildorj et al. (2005) also reported successful shoot regeneration from cotyledon explants of lettuce using medium containing 0.5 mgl^{-1} kinetin and 0.05 mgl^{-1} NAA. Some researchers have also reported about the effect of genotype and explant age on callus induction and shoot regeneration from cotyledon explants. Hunter and Burritt (2002) reported the percentage of explants producing shoots and the mean number of shoots per explants doubled by culturing cotyledon explants isolated 3-14 days following germination on media containing 0.54 µM NAA and 0.44 µM BA compared to other combinations of auxins and cytokinins (1- 28.54 µM IAA, 32 µM kinetin, 0.23 µM zeatin; 2- 0.57 µM IAA, 2.32 µM kinetin; 3- 0.57 µM IAA, 0.44 µM BA). In the other study, five different genotypes were tested on several types of media and high shoot regeneration (more than 2 shoot per cotyledon explant) was obtained on Murashige and Skoog (1962) medium including 0.1 mgl^{-1} NAA and 0.1 mgl^{-1} BA (Kanamoto et al., 2006).

Table 1. Seven combination of the Plant growth regulators in MS medium used for callus induction and direct shoot regeneration of Lettuce

Medium	Plant growth regulators concentrations	
	NAA(µM)	BA(µM)
M1	0.54	0.44
M2	2.70	2.20
M3	5.40	2.20
M4	2.70	4.40
M5	5.40	4.40
M6	0.27	1.76
M7	0.10	0.44

Dias et al. (2006) cultured cotyledons from 48 hr germinated seeds on MS medium supplemented with 0.1 mgl^{-1} BA and 0.1 mgl^{-1} IBA for callus induction and callus was transferred to MS medium containing 0.1 mgl^{-1} BA for indirect shoot regeneration. In this investigation, the effects of explant factors (time of explants excision and genotype) and culture medium (exogenous growth regulators) for callus induction and direct shoot regeneration of lettuce are described. Also, the other aim of this study was to establish a suitable method for callus induction and direct shoot regeneration of lettuce (*Lactuca sativa* L.). The two used genotypes are landraces from central (Yazd) and southwest (Ahvaz) of Iran which had good potential for biotic and abiotic stress.

Materials and methods

Preparation of cotyledon explants

Mature seeds of two lettuce (*Lactuca sativa* L.) genotypes (Ahvaz and Yazd, two Lettuce landrace genotypes) were used in the experiments. Seeds were surface-sterilized with 5% sodium hypochlorite for 10 min. To remove sodium hypochlorite residues, the seeds were then washed at least three times with sterile distilled water. Seeds were dried by blotting on sterile filter paper for about 3 min, after which 25 seeds were placed in each 90 mm plate containing germination medium (2.15 gl^{-1} MS salt and vitamins, 10 gl^{-1} sucrose and 8 gl^{-1} agar without growth regulators). The medium was adjusted to pH 5.7. The medium was sterilized by autoclaving at 121°C for 20 min. Seeds were incubated at a temperature of 25±2°C for 3 and 7 days under 16/8 hrs (day/night) photoperiod provided by cool-white fluorescent tubes (50 $\mu molm^{-2}s^{-1}$).

Callus induction and direct shoot regeneration

The cotyledon explants were aseptically excised from 3 and 7-day-old *in vitro* raised lettuce seedlings. Cotyledon explants were transferred on MS medium supplemented with various concentrations of plant growth regulators (NAA and BA). The concentrations of plant growth regulators are shown in Table 1. The MS media was fortified with 3% sucrose (w/v), and solidified with 0.8% plant agar. Explant was placed abaxial side down on 25 ml of MS medium in 90 mm plates. All plates were incubated in a controlled environment room as for seed germination.

Statistical analysis

Experiments were established in a completely randomized design, and three replicates per treatment with 20 explants for each replicate were used and the experiment was repeated twice. The reported data are the means of the two experiments. The percentage of callus induction and direct shoot regeneration was calculated for the cotyledon explants that had been cultured for 3 weeks. Three-way ANOVA was used to test the significance of effects of genotype, explant age, plant growth regulators, and their interactions for callus induction and direct shoot regeneration. The means for treatment combinations was compared by using the Waller-Duncan multiple range test (Type I/Type II error ratio 100). Data was subjected for analysis of variance and compare means using the statistical analysis system (SAS) program version 9.1 (SAS, 2003). Normality test of the data was assessed using the Anderson-Darling normality test (Minitab 14.0, 2005) and homogeneity test of variances with Levene test (SPSS 14.0, 2005).

Results and Discussion

The results of Anderson-Darling normality and Levene test for homogeneity of variances indicate that variances were homogenous and dataset was normal (data not shown). All explants after 3 days of culture initiation, showed symptoms of swelling and extending. The effect of genotype, explant age and different concentrations of growth regulators on the average callus percentage are shown in Table 2. Callus formation was initiated within 7 days of culture initiation and was observed in all media. The highest number of callus was obtained using 2.7 µM NAA and 4.4 µM BA from 3 days old explants of Yazd genotype (95%). The lowest number of callus was achieved when 3-day-old explants of Ahvaz genotype was cultured on MS medium with 0.54 µM NAA and 0.44 µM BA (10%). According to experimental results, the effect of combination of growth regulators for callus induction only showed genotype and explant age-dependent. Yazd genotype showed a high value of responding from 3-day-old cotyledon explants in all the growth regulator combinations of media, used a combination of growth regulators containing 0.1 µM NAA and 0.44 µM BA suitable for callus induction in two genotypes and explant ages. It is well known that genotype can influence a species capacity to respond to tissue culture conditions. Callus induction in lettuce is also genotype-dependent. Our results are consistent with those reported by Xinrun and Conner (1992); Curtis et al. (1994) and Ampomah-Dwamena et al. (1997) who concluded that marked genotypic differences occurred in callus initiation from a wide range of lettuce genotypes. This genotypic difference with respect to callus initiation was also observed in many other plants, such as *Dieffenbachia*, flax (*Linum usitatissimum* L.), cotton (*Gossypium hirsutum* L.), wheat (*Triticum aestivum* L) and *Hibiscus sabdariffa* L. (Shen et al., 2008; Burbulis et al., 2007; Michel et al., 2008; Miroshnichenko et al., 2009; Sié et al., 2010). In this study, explant age had an important influence on callus initiation in Yazd genotype. Yazd genotype produced high percentages of callus (up to 56%) in all media containing different growth regulators concentrations. To our Knowledge this is the first report of a genotype–dependent effect of explants age on callus induction in lettuce. The effects of genotype, explant age and different concentrations of growth regulators on direct shoot regeneration are shown in Table 3. Direct shoot regeneration was observed within 14 days of culture. Shoot buds emerged directly from the cut end of the region near to petiole on cotyledon explants. Multiple shoot was formed directly within 21 day of culture initiation (Fig. 1). According to percentage of direct shoot regeneration , different plant

Table 2. Effects of genotype, explant age and growth regulators on callus induction (%) of Lettuce (*Lactuca sativa* L.) after 3 weeks of culture

Medium	Ahvaz		Yazd	
	3 day	7 day	3 day	7 day
M1	10^{i}	73^{bcd}	60^{de}	86^{ab}
M2	22^{ghi}	32^{gh}	89^{ab}	37^{g}
M3	40^{fg}	37^{g}	56^{ef}	34^{gh}
M4	19^{hi}	27^{ghi}	95^{a}	23^{ghi}
M5	23^{ghi}	36^{gh}	73^{bcd}	28^{gh}
M6	38^{g}	56^{ef}	60^{de}	74^{bcd}
M7	66^{cde}	62^{de}	81^{ab}	83^{abc}

Means followed by the same letters are not significantly different (Waller-Duncan multiple range test with Type I/Type II error ratio 100)

Table 3. Effects of genotype, explant age and growth regulators on direct shoot regeneration (%) of lettuce (*Lactuca sativa* L.) after 3 weeks of culture

Medium	Ahvaz		Yazd	
	3 day	7 day	3 day	7 day
M1	65^{a}	72^{a}	67^{a}	46^{c}
M2	15^{fghi}	7^{hi}	13^{fghi}	14^{fghi}
M3	17^{fghi}	9^{ghi}	16^{fghi}	16^{fghi}
M4	23^{def}	6^{i}	12^{fghi}	18^{defgh}
M5	17^{fghi}	9^{ghi}	20^{defg}	18^{defgh}
M6	29^{d}	29^{d}	51^{bc}	53^{bc}
M7	65^{a}	61^{ab}	61^{ab}	64^{a}

Means followed by the same letters are not significantly different (Waller-Duncan multiple range test with Type I/Type II error ratio 100)

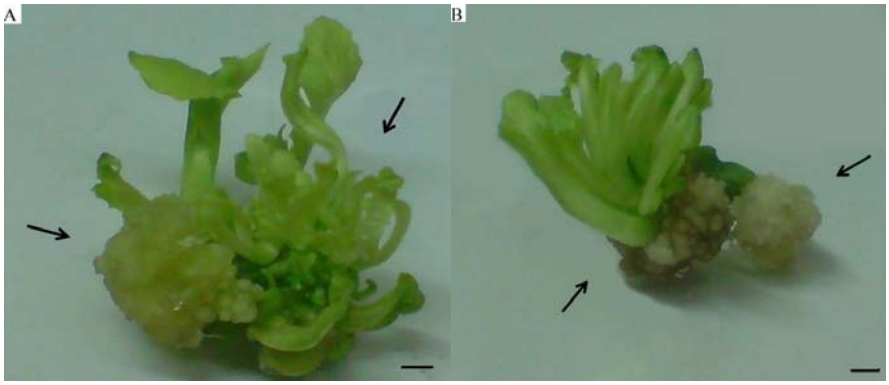

Fig 1. The best region for direct shoot regeneration on cotyledon explants of lettuce is near to petiole of cotyledons. **(A)** Left arrow is middle of cotyledon; right arrow is the region near to petiole of cotyledon (Ahvaz genotype). Bar=2 mm, **(B)** left arrow is the region near to petiole of cotyledon; right arrow is middle of cotyledon (Yazd genotype). Bar=2 mm, Multiple direct shoots were formed at the region near to petiole of cotyledons within 21 days of culture initiation

growth regulators combinations showed different responses. Thus, the best result was obtained in M1 and M7 medium with 0.54 µM NAA, 0.44 µM BA (72% direct shoot regeneration) and 0.1 µM NAA, 0.44 µM BA (65% direct shoot regeneration), respectively. Higher concentrations of NAA and BA did not increase the number of direct shoot regeneration. These results are similar to those obtained by Hunter and Burritt (2002), who produced the greatest number of shoots when 0.54 µM of NAA used in combination with 0.44 µM of BA. Similarly, Kanamoto et al. (2006) obtained high shoot regeneration from leafy explants cultivated on medium supplemented with 0.1 mgl^{-1} NAA and 0.1 mgl^{-1} BA. Webb et al. (1984) showed that cotyledon age can influence the regeneration response; with older cotyledons has less ability for direct shoot regeneration than younger ones. According to Hunter and Burritt (2002), cotyledon age influences the shoot-forming ability of cotyledon explants but in a genotype manner. But in the present study, explants age did not have a significant effect on direct shoot regeneration in two lettuce landraces. The present study also showed that lettuce genotypes did not have a significant effect on direct shoot regeneration. The relative influence of the genotypes on tissue response has been a matter of controversy. Vasil and Vasil (1986) suggested that the differential response of the genotypes may be due to differential expression, which in turn, depends upon the spatial and temporal distribution of their physiological and developmental stages. To identify the relationship between callus induction and direct shoot

regeneration, Pearson's correlation coefficient was computed. Pearson's correlation coefficient identified that callus induction was correlated with direct shoot regeneration. These variables were positively correlated (0.408), and the correlation was statistically significant at the 0.05 level. This result is not similar to that of Ampomah-Dwamena et al. (1997), who reported no correlation between callus production and shoot regeneration. Three regions (middle, near to petiole and petiole) on cotyledon explants were also tested to identify the best region for direct shoot regeneration. According to obtained results to identify the best region for direct shoot regeneration, petiole of cotyledons did not show significant response to growth regulators. On the other hand, middle parts of cotyledon showed significant response to growth regulators and produced callus. But the best region for direct shoot regeneration in lettuce was the region near to petiole on cotyledon explants which showed high significant response to growth regulators (Fig. 1). In conclusion, our study revealed that the effect of growth regulators combinations on callus induction was genotype and explant age-dependent. The highest number of callus was obtained using 2.7 µM NAA and 4.4 µM BA. It also showed that direct shoot regeneration significantly decreased with increasing BA concentration. However, the use of BA less than 1 µM increased direct shoot regeneration in two genotypes. Positively significant correlation was observed between callus induction and direct shoot regeneration. It was also shown in our study that the best region for direct shoot regeneration on cotyledon explants of lettuce is near to petiole. This adventitious origin of shoots directly from the region near to petiole of cotyledons may aid the use of genetic engineering to improve horticultural characteristics. If propagation through tissue culture can be done efficiently on a large scale in plant breeding programs, it may be used to produce F_1 hybrids to use the hybrid vigour. This regeneration protocol will be useful not only for further research studies such as *Agrobacterium* mediated genetic transformation or protoplast fusion studies, but also for commercial nurseries to produce virus-free plants and agricultural practices that could reduce pesticides application and increase production.

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