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Interactions of genotype and plant growth regulators affecting direct shoot regeneration of lettuce (*Lactuca sativa* L.)

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Abstract

Lettuce (*Lactuca sativa* L.) is one of the most important vegetable crops. The chloroplast transformation of lettuce requires a reproducible method to regenerate shoots directly from leafy explants. The direct shoot regeneration in lettuce is highly depends on genotype and the combinations of plant growth regulators. Callus induction, average number of regenerated shoots per leafy explant and percentage of shoot-producing explants were studied by investigating the effects of genotype and different concentrations of NAA and BAP. Orumia and American genotypes in media containing 0.1 mg l^{-1} NAA with either 0.1 mg l^{-1} or 0.2 mg l^{-1} BAP produced the highest number of regenerated shoots per explant. In addition, the minimum callus formation was observed in the same media from Ahwazi and Orumia genotypes. The results showed low callus formation and increased direct shoot regeneration from lettuce explants in low concentrations of NAA and BAP. The callus induction and direct shoot regeneration from leaf explants were correlated significantly and negatively. These data recommend a protocol for direct shoot regeneration in producing transplastomic lettuce.

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Introduction

Lettuce (*Lactuca sativa* L.) is a globally important leafy vegetable which belongs to the family Asteraceae (Compositae). Lettuce is an annual, self-fertile species that has been cultivated all around the world. The center of origin of lettuce is probably the Middle East and south-west Asia, while today, the main areas of production and consumption of lettuce are the United States and Europe (Ryder, 1986). The development of a gene transfer system for lettuce would be extremely valuable both in improving the genetic diversity of the crop and also for the transfer of useful agronomic traits.

Agrobacterium-mediated gene transfer into lettuce was achieved initially by Michelmore *et al.* (1987). Several important agronomic traits, including male sterility (Curtis *et al.*, 1996b), herbicide resistance (Mohapatra *et al.*, 1999), virus resistance (Pang *et al.*, 1996; Dinant *et al.*, 1997), extensive root development (Curtis *et al.*, 1996a), reducing bitterness (Sun *et al.*, 2006), drought, salinity and cold tolerance (Pileggi *et al.*, 2001) were introduced to lettuce by *Agrobacterium*-mediated gene transformation. Additionally, recombinant antibodies were produced in lettuce by this method (Negrouk *et al.*, 2005). Biolistic DNA delivery was reported to efficiently transform chloroplast genome and produce stable transplastomic lettuce plants (Kanamoto *et al.*, 2006). Some of valuable improvements, e.g. enhancing yield (Ichikawa *et al.*, 2010), carotenoid profile (Harada *et al.*, 2013), vitamin E quality (Yabuta *et al.*, 2013), and also the accumulation of pharmaceutical proteins (Ruhlman *et al.*, 2007; Davoodi-Semiromi *et al.*, 2010; Ruhlman *et al.*, 2010; Boyhan and Daniell, 2011; Lim *et al.*, 2011; Sharifi Tabar *et al.*, 2013) were achieved in lettuce by chloroplast transformation using particle bombardment.

The success of these genetic manipulation methods depend on that plants can be regenerated from transformed cells in a reliable and efficient manner. The first achievement in developing adventitious shoots *in vitro* was reported when cotyledon explants were cultured on media containing 5 mg l^{-1} indole acetic acid (IAA) and 0.5 mg l^{-1} kinetin (Doerschug and Miller, 1967). Also Webb *et al.* (1984) reported that benzylaminopurine (BAP) was more efficient than kinetin, to promote shoot regeneration from lettuce cotyledon explants. The effect of lettuce genotype on directly shoot regeneration from cotyledon explants cultured on media supplemented with 0.1 mg l^{-1} IAA, 0.5 mg l^{-1} kinetin, and 0.05 mg l^{-1} zeatin was investigated by Xinrun and Conner (1992) and Ampomah-Dwamena *et al.* (1997). The percentage of explants producing shoots and the mean number of shoots per cotyledon explant were doubled using media contained 0.54 µM naphthalene acetic acid (NAA) and 0.44 µM BAP comparing other combinations of auxins and cytokinins (Hunter and Burritt, 2002). The effects of different combinations of NAA and BAP on callus induction and direct shoot regeneration from cotyledon explants of lettuce were evaluated and it was shown that the highest number of direct shoot regeneration was obtained at low BA concentrations (Mohebodini *et al.*, 2011).

As mentioned above, cotyledon explants have frequently used for regeneration in the most cases, however, it may be of limited utility for the generation of plastid-transformed (transplastomic) plants as they usually underwent repeated rounds of regenerations to achieve homoplasmy; thereby there will be no cotyledon available for further rounds of regenerations. Therefore, it is vital to establish an efficient leaf-based regeneration system for attaining plastid transformation efficiently. Variable results in shoot regeneration from lettuce leaf explants were obtained in different investigations (Kanamoto *et al.*, 2006; Ruhlman *et al.*, 2010); furthermore, studies have shown that response to plant growth regulators (PGRs) is highly genotype dependent in lettuce (Ampomah-Dwamena *et al.*, 1997). These highlight the need to optimize for regeneration system in independent transformation experiments.

In this study, we investigated the effects of genotype (seven common lettuce landrace genotypes of Iran) and different concentrations of PGRs (NAA and BAP)

on average number of directly regenerated shoots per leafy explant, percentage of explants producing shoots, and two qualitative features including the amount of produced callus and the status of regenerated shoots. Our main objective was to select best genotypes and develop a reproducible method for direct shoot regeneration of lettuce, which will be valuable in chloroplast transformation experiments.

Materials and methods

Plant material and seed germination

Lettuce seeds of seven landrace genotypes were provided from various sources. Seeds were surface-sterilized by soaking in a 5% sodium hypochlorite solution containing two drops of Tween 20 per liter, for 10 min, and then rinsing three times with sterile distilled water.

Seeds were dried by placing on a sterile filter paper, and then germinated on medium containing MS salts and vitamins (Murashige and Skoog, 1962), supplemented with 30 g l^{-1} sucrose. The pH of the medium was adjusted to 5.8, and then solidified with 7 g l^{-1} agar. Planted seeds were incubated for 4 weeks at temperature of 24±1 ˚C under a 16 h light and 8 h dark photoperiod (50 µmol m^{-2} s^{-1}).

Shoot regeneration and culture conditions

The young and healthy leaves (~4 cm^2) were aseptically excised from *in vitro* grown seedlings, cut into small segments (~0.5 cm^2) and placed onto the regeneration medium (germination medium supplemented with different concentrations of PGRs). Six explants were placed adaxial side down onto the medium on each plate. All plates were incubated in controlled environmental circumstances as mentioned for seed germination. Different types of experimental treatments and corresponding coding numbers are shown in table 1.

Measured features

Average number of direcly regenerated shoots per explant and frequency of explants producing shoots were measured for each genotype and specific combination of PGRs after 4 weeks incubation of explants onto the regeneration medium. Two qualitative features including amount of produced callus (classified into: 1- limited, 2- moderate, and 3- extensive when < 20%, 20-50%, and > 50%, respectively, of explant surface is covered with callus) and status of regenerated shoots (classified into: 1- fragile, 2- weak, 3- mediocre, 4- good, and 5- vigor, corresponding to shoot's height < 1 cm, 1-2 cm, 2-3 cm, 3-4 cm, and > 4 cm respectively) were also measured.

Statistical analysis

The experiment was established in a completely randomized design with a 7 × 2 × 3 factorial arrangement of the following treatments: 1- seven lettuce genotypes namely Lorestani, American, Zirei, Ahwazi, Orumia, Suri, and Farangi, 2- NAA with two concentrations counting 0.1 and 0.2 mg l^{-1}, and 3- three concentrations of BAP including 0.1, 0.2 and 0.5 mg l^{-1}. Every treatment was triplicated with six explants for each replicate. The analysis of variances (ANOVA) was performed on data with a normal distribution to test the significance of effects of genotype, concentrations of PGRs (NAA and BAP), and their interactions for means of directly regenerated shoots and frequency of regenerated explants. For qualitative features, every combination of treatment were used as one group and the Kruskal-Wallis test were made for multiple group data. Probability values of $P < 0.05$ were considered significant. The statistical analyses were carried out using SPSS 19.0 and Minitab 15.0.

Results and discussion

Effects of genotype and PGRs on direct shoot regeneration

The normality of dataset and homogeny of variances were confirmed by Anderson-Darling and Levene tests respectively (data not shown). The variance analysis of three different factors' effects on the average of directly regenerated shoots and frequency of regenerated explants are shown in table 2. According to the results of ANOVA, the triplet interaction's effects of genotype and different concentrations of NAA and BAP were statistically

significant at the 0.01 level.

The average numbers of directly regenerated shoots in different treatments were compared by least significant difference (LSD) method (Fig. 1). Multiple shoot buds regenerated directly from the cut end of lettuce explants within 28 days of culture initiation. The highest numbers of regenerated shoots were obtained using 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} BAP from Orumia and American genotypes (treatments 511 and 211), and also combination of 0.1 mg l^{-1} NAA and 0.2 mg l^{-1} BAP from Orumia genotype (treatment 512). The lowest number of regenerated shoots was observed when Zirei genotype was cultured on medium containing 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} BAP (treatment 323). Orumia genotype showed a high value of responding to the all combinations of media, in order to direct shoot regeneration. The genotype and hormone composition of medium are important factors in plant tissue culture (Soltanmohammadi *et al.*, 2014). These results are similar to those obtained by (Kanamoto *et al.*, 2006; Ruhlman *et al.*, 2007; Ichikawa *et al.*, 2010; Lim *et al.*, 2011) who used the same combination of PGRs to regenerate transplastomic lettuce plants in selective media.

Table 1. Experimental treatments, their levels and corresponding coding numbers. Each treatment encoded by a 3-digit number which the first, the second and the third digits determine the codes of genotype, NAA and BAP concentrations respectively.

Level	Code	Level	Code
Genotype		NAA (mg l^{-1})	
Lorestani	1	0.1	1
American	2	0.2	2
Zirei	3		
Ahwazi	4	BAP (mg l^{-1})	
Orumia	5	0.1	1
Suri	6	0.2	2
Farangi	7	0.3	3

Table 2. Variance analysis of three different factors' effects on directly regenerated shoots in lettuce.

Source of variations	Degree of freedom	Mean square	
		Regenerated shoots per explant	% explants producing shoots
Genotype	6	14.563**	0.180**
NAA	1	19.824**	0.239**
BAP	2	13.777**	0.893**
Genotype × NAA	6	4.214**	0.040**
Genotype × BAP	12	2.140**	0.062**
NAA × BAP	2	2.254**	0.054**
Genotype × NAA × BAP	12	1.935**	0.122**
Error	84	14.563	0.007
% CV		21.5	16.96

** Statistically significant at the 0.01 level.

The effects of genotype and PGRs combinations on frequency of explants producing shoots are shown in Fig. 2. Direct shoot regeneration was observed in all genotypes within 14 days of culture. According to the results, Farangi genotype (Treatment 723) showed the least percentage of explants producing shoots. Additionally, two combinations of plant hormones, including 0.1 mg l^{-1} NAA with either 0.1 mg l^{-1} or 0.2 mg l^{-1} BAP resulted in a higher frequency of direct shoot regeneration in almost all genotypes. Our results are consistent with those previously reported by (Davoodi-Semiromi *et al.*, 2010; Ruhlman *et al.*, 2010; Boyhan and Daniell, 2011; Kanagaraj *et al.*, 2011) who used these combinations of PGRs to regenerate lettuce shoots directly. These results suggested that higher concentrations of PGRs did not increase the number of direct regeneration of shoots, which was similar to the results reported by (Hunter and Burritt, 2004; Mohebodini *et al.*, 2011) who obtained the greatest number of regenerated shoots in low concentrations of NAA and BAP.

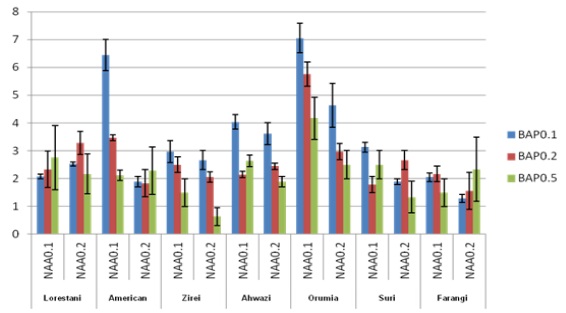

Fig. 1. Comparison of average number of directly regenerated shoots per leafy explant in different combinations of treatments.

In this study, genotype had a significant influence on direct shoot regeneration. Orumia genotype produced a large number of directly regenerated shoots (up to 7 shoots per each leafy explant) in all media containing different growth regulators concentrations. However, with respect to the percentage of lettuce explants producing shoots, different genotypes showed different responses. In general, the combination of 0.1 mg l^{-1} NAA with either 0.1 mg l^{-1} or 0.2 mg l^{-1} BAP in MS media induced the highest level of direct shoot regeneration from lettuce explants.

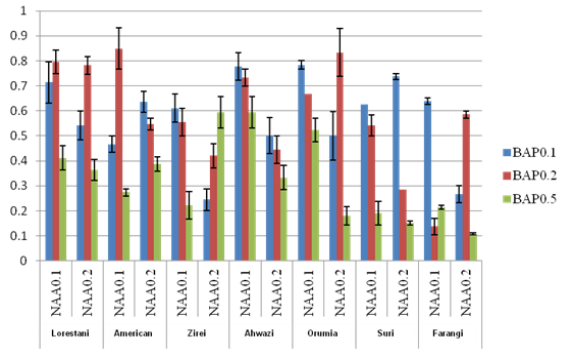

Fig. 2. Comparison of percentage of lettuce explants producing shoots in different combinations of treatments.

Evaluation of treatments' effects on qualitative features

The amount of callus induction and also the status of regenerated shoots are shown in Fig. 3. Treatments 411, 412, and 511 regarding to the Ahwazi and Orumia genotypes cultured in MS medium containing 0.1 mg l^{-1} NAA with either 0.1 mg l^{-1} or 0.2 mg l^{-1} BAP had the lowest amounts of produced calli (level 1) among all tested lettuce genotypes. Although other lettuce genotypes produced relative high amounts of calli in the replicated combinations of PGRs. Kruskal-Wallis test revealed that at least two out of 42 treatments were significantly different ($p < 0.01$) for the amount of callus induction ($\chi^2 = 100.353$). Different lettuce genotypes showed different responses to the PGRs combinations regarding to the status of regenerated shoots. Genotype was an important factor in almost all tested combinations of PGRs, but in overall, Orumia genotype had the best status of regenerated shoots in the tested combinations. In contrast, the weakest shoots were regenerated from explants of Farangi and Suri genotypes in all combinations of PGRs. In addition, results of Kruskal-Wallis test showed that at least two treatments among experimental treatments were significantly different ($p < 0.01$) for the status of regenerated shoots ($\chi^2 = 96.985$).

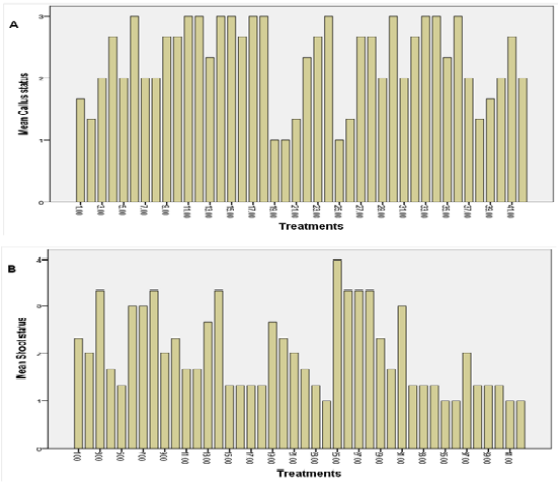

Fig. 3. Comparison of qualitative features in each combination of treatment. A) Amount of produced callus, B) Status of regenerated shoots.

To identify the relationship between the amount of produced callus and status of regenerated shoots, Spearman's correlation coefficient was computed. Spearman's correlation coefficient identified that the amount of produced callus and the status of regenerated shoots were negatively correlated (-0.303), and the correlation was statistically significant at the 0.01 level. Our results were in contrast to those obtained by (Mohebodini *et al.*, 2011) who reported positive correlation between callus induction and direct shoot regeneration in lettuce. Ampomah-Dwamena *et al.* (1997) declared that there was no correlation between callus production and shoot regeneration.

In conclusion, our study showed that direct regeneration of shoots in lettuce affecting by genotype and combinations of plant growth regulators. The highest number of regenerated shoots per explant was obtained using 0.1 mg l^{-1} NAA with either 0.1 mg l^{-1} or 0.2 mg l^{-1} BAP from Orumia and American genotypes. Also, the mentioned combinations of PGRs resulted in a high percentage of explants producing shoots in the studied lettuce genotypes. Xinrun and Conner (1992) previously reported that callus induction is strongly genotype-dependent. In this study, the minimum amount of callus induction was observed in Ahwazi and Orumia genotypes cultured in media containing 0.1 mg l^{-1} NAA with either 0.1 mg l^{-1} or 0.2 mg l^{-1} BAP. These results revealed that low concentrations of NAA and BAP induced low callus formation and increased direct shoot regeneration from lettuce explants. Furthermore, potent shoots were regenerated directly from explants of Orumia genotype in all media. The results showed that there was a negative correlation between callus induction and direct shoot regeneration. The outcomes of our study will be useful not only for lettuce genetic transformation using either *Agrobacterium*-mediated or biolistic methods, but also for commercial *in vitro* propagation of lettuce to produce virus-free plants.

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