Improved shoot regeneration protocol for canola explants and pre-assessment of salinity tolerance in canola transgenic plants

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Abstract
Regeneration of explants plays a significant role in plant transformation. Explant type, hormonal concentration, and pre-culturing period are important in transformation efficiency. To get an efficient transformation of canola and optimize regeneration conditions, different explants along with different culture media were studied. Four canola varieties were used to evaluate regeneration ability of hypocotyledonary and cotyledonary leaf explants. In addition, cotyledonary leaf explants were evaluated on the MS medium containing five different concentrations of BAP. Suitability of cotyledonary explants in transformation experiment was assayed by an exogenous gene, coding for *P5CS*, the key enzyme in proline biosynthesis. The transformation of cotyledonary explants through *Agrobacterium tumefacience*-mediated gene transformation was used in a stepwise increased selection marker manner. PCR and proline content analysis confirmed the success of transformation. Cotyledonary explants displayed a higher regeneration efficiency than hypocotyledonary explants. In addition, BAP at 5 mg/l in the MS medium increased the rate of regeneration. Results showed that preculturing explants for 48 h increased the rate of transformation. Assessing the proline concentration further verified the expression and activity of the transformed gene. The *P5CS* transformed plants were more resistant to salinity compared to the non-transgenic control plants.

***Keywords*:** *Agrobacterium*, *Brassica napus* L., Proline, Salt tolerance, Δ^1-Pyrroline-5-carboxylate synthetase (*P5CS*).

INTRODUCTION

Canola (*Brassica napus* L.) is one of the top ranking oilseed crops of Iran and the third most important edible oil source in the world, after soybean and palm. Saline soils and saline irrigation waters are among the common problems in irrigated areas of Iran that, together with low rainfall, present potential hazards to canola production (Bybordi, 2010). Developing salinity-tolerant crops is essential for maintaining agricultural production. To achieve salt tolerance, plants have evolved different mechanisms to prevent damages associated with salt stress (Flowers, 2004). Among such mechanisms is the accumulation of low molecular weight metabolites such as proline (Sakamoto and Murata, 2000). Many researchers have focused on proline content as a proved evidence of salinity tolerance (Ashraf and Foolad, 2007; Cha-um and Kirdmanee, 2009; Goudarzi and Pakniyat, 2009; Ashrafijou *et al.*, 2010).

Crop productivity may be improved by introducing

various new traits such as salinity tolerance (Ashrafijou *et al.*, 2010), herbicide resistance (Kawahigashi *et al.*, 2007), protein quality (El-Shemy *et al.*, 2007), male-sterility (Zhang *et al.*, 2005) and insect resistance (Wu *et al.*, 2008). It is well known that the improvement of plants through conventional breeding methods is slow, time-consuming, and labor-intensive while non-conventional genetic improvement programs based on tissue culture and molecular genetics is far from these limitations (Moghaieb *et al.*, 2006). Gene transformation methods into plants offer a rapid way to salt tolerance in plants. Most studies in the field of regeneration and transformation of canola have focused on various sources of explants such as cotyledonary petioles (Moloney *et al.*, 1989), hypocotyl segments (De Block *et al.*, 1989; Radchuk *et al.*, 2000) thin cell layers (Ovesnaek *et al.*, 1993), stem segments (Cegielska-Taras *et al.*, 2008), protoplasts (Thomzik and Hain, 1990) and microspores (Dormann *et al.*, 1997). The best explants for efficient canola regeneration was found to be cotyledons and hypocotyls isolated from 4-6 day-old seedlings (De Block *et al.*, 1989).

The aim of this study was to develop an efficient transformation of canola explants and optimization of the regeneration condition.

MATERIALS AND METHODS

Plant material and tissue culture

Seeds of four canola varieties including PF, Modena, RGS003 and Hayola308 were used to evaluate regeneration rate of different explants, hypocotyledonary and cotyledonary leaves. Seeds were surface sterilized with 70% (v/v) ethanol (1 minute), 50% (v/v) sodium hypochlorite (15 minutes) and rinsed three times by sterile distilled water and germinated on the RM1 medium (Table 1), under a 16/8 hours (light/dark) photoperiod at 25±2°C. Cotyledonary leaves with small petioles and hypocotyls were separated from 4 to 6 day-old seedlings and used as explants. In addition, cotyledonary leaf explants were placed on the MS medium containing five different concentrations of BAP (0, 2.5, 5, 7.5 and 9 mg/l).

Transformation vector and *Agrobacterium* strains

The plasmid (PBI-*P5CS*) containing *Arabidopsis thaliana P5CS* gene, under transcriptional regulation of cauliflower mosaic virus 35S (CaMV 35S) promoter was used for canola transformation. *Agrobacterium tumefaciens* strain pGV3101 was used (Figure 1). The selective marker and reporter gene were kanamycin (*npt II*) and *uidA,* respectively.

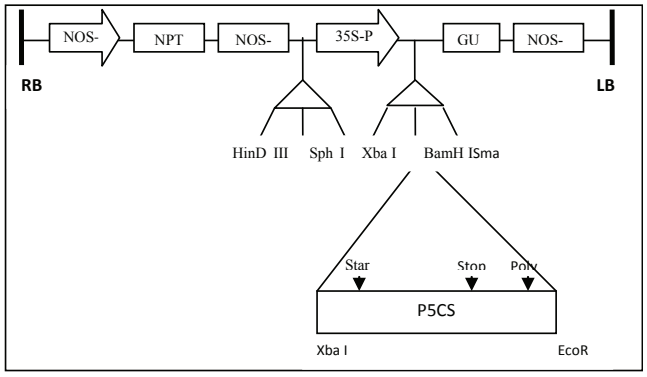

Figure 1. The plant expression vector of PBI-*P5CS*.

Regeneration and transformation protocols of canola

To obtain successful regeneration from transformed explants, a protocol involving the transfer of transformed cotyledonary petiole explants in a stepwise increased selection marker and reduced antibacterial cefatoxime was followed. The first medium was RM1 which was then replaced stepwise by the RM5 medium (Table 1) containing 2 mg/l IBA for inducing root formation. Explants were incubated primarily in dark for 48 h on the MSII medium containing 5 mg/l BAP. *Agrobacterium* cells were grown overnight in a 20 ml liquid LB medium and cotyledonary petioles were inoculated by the bacterial suspension at OD_{600} 0.5 for 30-60 seconds and dried by a sterile filter paper to remove the excess bacterial suspension. Inoculated explants were cultivated on RM2 and after 2 days, selected transformed explants were transferred to the RM3 medium. After 2 weeks, regenerated explants were transferred to RM4 and, after about 4 weeks, the elongated shoots were transferred to RM5 medium (Table 1 and Figure 2). The plantlets were washed and transferred to the autoclaved soil in 1 liter pots and grown in a plant growth chamber. They were covered by plastic domes to keep humidity. After 1 week, the plastic cover was gradually removed and plants were transferred to 3 liter pots and grown in the greenhouse. All cultures were maintained at 25±2°C, under a 16/8-h (light/dark) photoperiod, using cool white fluorescent bulbs.

DNA extraction and PCR analysis

Genomic DNA was extracted from the control and the

Table 1. Transformation media and protocols used for canola cotyledonary petioles regeneration.

Media	Composition	Sucrose (gr/l)	Hormone (mg/l)		Antibiotic (mg/l)	
			BAP	IBA	Kanamycine	Cefatoxime
RM1	MS	30	-	-	-	-
RM2	MS	30	5	-	-	-
RM3	MS	30	5	-	10	300
RM4	MS 1.2	20	-	-	25	200
RM5	MS 1.2	20	-	2	25	100

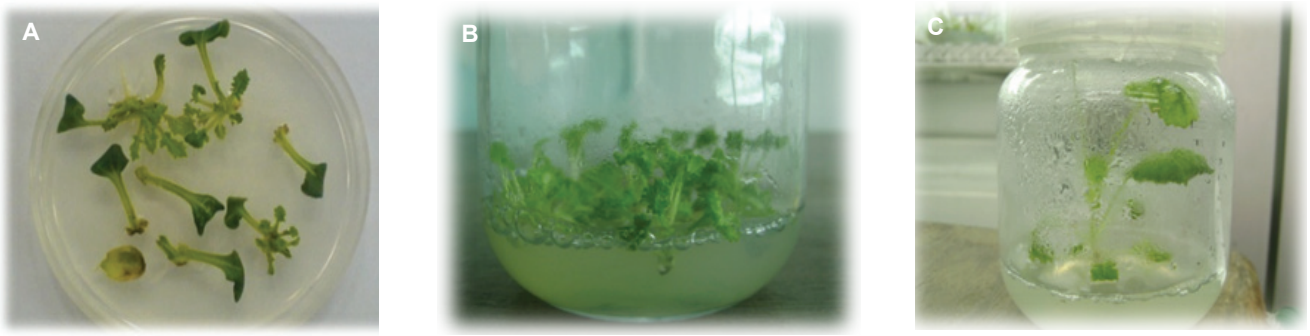

Figure 2. Transfer of explants into the root induction medium.

transformed leaves applying the CTAB (Murray and Thompson, 1980) protocol. PCR reactions were performed using specific primers to detect *Agrobacterium P5CS* gene (forward R-6-F: 5'-GGA TTG TGT GAT ATT CTC CAC TGA CG-3' and reverse R-2-R: 5'-CCT TCA ACA TCG CTC AGA AGA ATC AG-3'). The expected amplicon length was 765 bp. PCR reactions were carried out in 25 µl volume with the following conditions: pre-denaturing at 95°C for 10min, 35 cycles of denaturation at 94°C for 1min, annealing at 47.5°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. Amplification products were analyzed on a 1% agarose gel and visualized by ethidium bromide (1 µg/ml).

Osmotic stress and proline content analysis

Transgenic and non-transgenic canola regenerated explants were grown in the MS medium containing various concentrations of NaCl (0, 50, 100, 150, 200, 250, 300 mM) for 21 days. One gram of leaf tissue was collected and extracted with 3% sulfosalicylic acid and centrifuged at 3000 gr for 10 min. Determination of free proline content was performed according to the method of Bates *et al.*, (1973). Since proline content may vary from leaf to leaf and is dependent on the age of the plant, precaution was taken to select leaves from plants with a similar age and size (Figure 3). Factorial analysis was used based on the completely randomized design layout, with three replications. Factors including transgenic or non-transgenic samples and salinity treatment levels were applied. All data were subjected to analysis of variance (ANOVA), using the general linear model of SAS (version 9.1). Mean of the treatments were tested by Duncan's multiple range test (DMRT) and significance levels of sources ($p < 0.05$) were determined.

RESULTS

Tissue culture, regeneration and transformation

Comparative analysis of regeneration of the two different explants of canola (cotyledon petioles and hypocotyls) in the MS medium containing 5 mg/l of BAP showed that cotyledon petioles isolated from 4 to 6

Table 2. Regeneration of explants (cotyledon, petiole, and hypocotyls) of the four canola varieties studied.

Explant	Genotype	Number of explants used	Number of regenerated shoots	Regeneration percentage
Cotyledon petiole	PF	150	129	86.00
	RGS003	150	98	65.33
	Modena	150	117	78.00
	Hayola308	150	107	71.33
Hypocotyl	PF	150	114	76.00
	RGS003	150	90	60.00
	Modena	150	109	72.66
	Hayola308	150	94	62.00

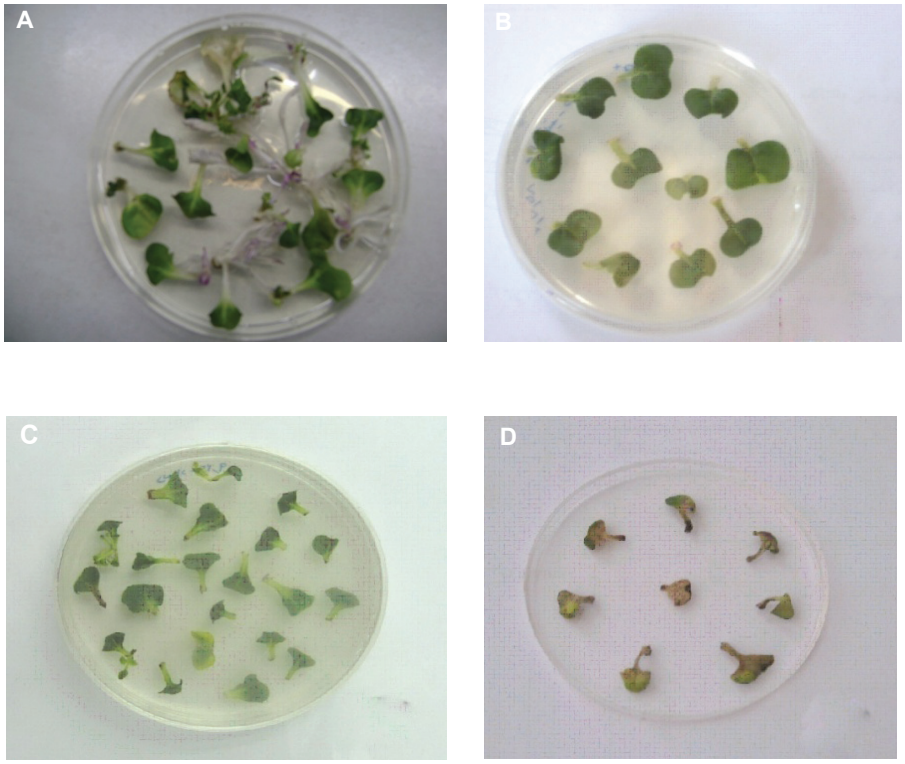

Figure 3. Development of plantlets on the selection media. **A**: Non-transformed plantlets were white and purple because of Kanamycin; **B**: Control plants with no regeneration; **C**: Transformed plantlets remained green and survived; **D**: Necrotic control plantlets.

days-old seedlings give better results than hypocotyl explants (Table 2). Furthermore, among different cultivars used, 'PF' cultivar reflected a higher regeneration efficiency (Table 2). Therefore, this cultivar was selected for the transformation of explants. The cotyledon petioles of this cultivar gave a higher regeneration efficiency. Our data indicate that cotyledon explants reflects almost 10% higher regeneration efficiency compared to hypocotyls. Among different concentrations of BAP (0, 2.5, 5, 7.5 and 9 mg/l), 5 mg/l of BAP caused the highest regeneration efficiency (Table 3). Compared to 'Modema' cultivar, 'PF' cultivar showed a higher regeneration frequency in all concentrations of BAP. Data showed that culturing cotyledon petiole explants

Table 3. The influence of different concentrations of BAP used in the MS medium on the regeneration efficiency of the two canola cultivars.

Concentration BAP (mg/l)	Genotype	Number of explants used	Number of regenerated shoots	Regeneration percentage
0	PF	150	12	0.08
	Modena	150	8	0.05
2.5	PF	150	114	76.00
	Modena	150	103	68.66
5	PF	150	129	86.00
	Modena	150	117	78.00
7.5	PF	150	105	70.00
	Modena	150	98	65.33
9	PF	150	97	64.66
	Modena	150	88	58.66

Table 4. The mean free proline content (mg/l) of non-transgenic and transgenic samples at different salinity levels.

NaCl (mM)	0	50	100	150	200	250	300
Non-Transgenic	33.53^{n}	47.98^{m}	70.70^{l}	112.3^{k}	158.86^{j}	209.82^{h}	231.39^{g}
Transgenic	190.86^{i}	272.65^{f}	328.03^{e}	427.81^{d}	747.74^{c}	1025.96^{b}	1791.5^{a}

play an important role in the efficiency of transformation. Pre-culturing explants for 2 days prior to co-cultivation with *Agrobacterium* gave a higher number of transformation frequency in comparison to the explants that were pre-cultured 1 or 3 days (Data not shown).

Transformation, proline concentration assessment, and the analysis of salt tolerance

Cotyledonary explants showed a higher regeneration efficiency, but the question that still remains is that whether they are suitable for transformation experiments or not. To explore their usefulness in gene transformation, *P5CS* gene was used as a model whose effects on plants cause resistance to osmotic stress. This gene has been the subject of many former studies (Yamchi *et al.*, 2007) and is well known. Following the *Agrobacterium*-mediated gene transformation, genomic DNA was extracted and PCR analysis was carried out on both the control and the transgenic plants. Observing the amplified fragment with 765 bp length from the transformed plants verified the successful transformation (Figure 4). Biochemical assays were carried out on both transgenic and the control plants to check proline production as a key product of the inserted gene. Plants were grown for

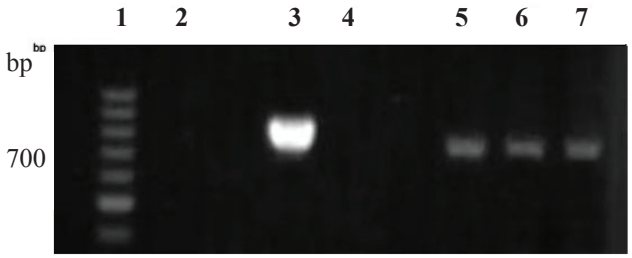

Figure 4. PCR analysis of the control and transgenic canola plants. Lane 1: molecular size marker (100 bp); Lane 2: negative control (master mix and primers without DNA); Lane 3: PBI121-*P5CS* vector plasmid extracted from *Agrobacterium*; Lane 4: non-transgenic control canola plant; Lanes 5, 6, 7: transgenic canola plants.

21 days in the MS medium supplemented with different concentrations of salt (0, 50, 100, 150, 200, 250, 300 mM of NaCl) and their proline content was measured (Figure 5). A higher proline content was observed in transgenic plants compared to the controls. Exposure of plants to the same salt concentrations in the MS medium during 21 days showed that the transgenic plants tolerated all salt concentrations while the control plants

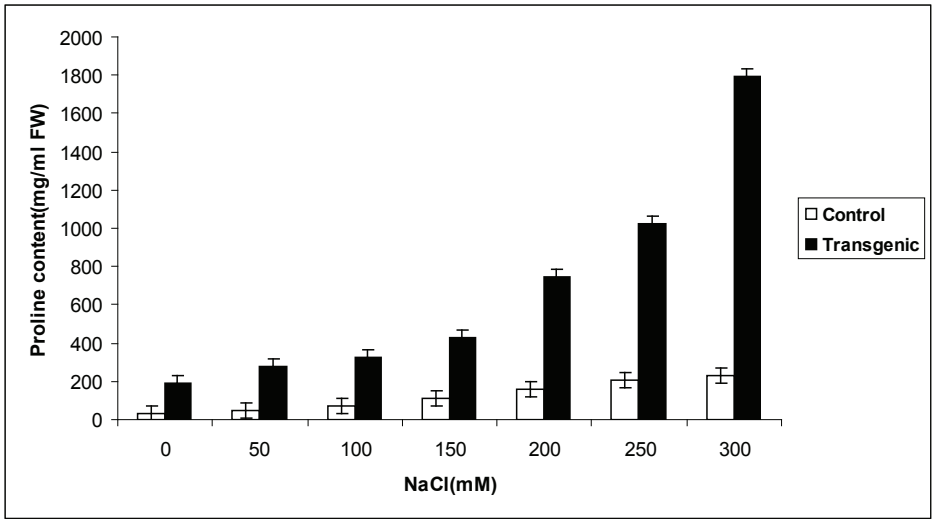

Figure 5. Free proline content of the control versus *P5CS* transgenic canola plants grown in a medium without NaCl as well as growth media containing various concentrations of the applied salt.

were only able to tolerate up to 200 mM of NaCl, the concentration at which plants showed wilting. This result confirmed primarily the success of transformation (Figure 3, Table 4).

DISCUSSION

Regeneration efficiency is among the most important factors influencing transformation efficiency. Factors suggested to be important in regeneration efficiency are genotype, explants type, age, hormonal concentration and pre-cultivation (Jonoubi *et al.*, 2005; Moghaieb *et al.*, 2006). To find the best source of explant and optimize the regeneration and transformation of canola plants, two formerly suggested sources of explant, *i.e.* cotyledonary petioles and hypocotyls, were compared. Formerly, DeBlock *et al*., (1989) have indicated that efficient regeneration can be obtained from cotyledons and hypocotyls isolated from 4 to 6 day-old seedlings, but they did not establish which one of these two sources had a higher regeneration efficiency and can be used for the successful transformation. Both the rate of regeneration as well as transformability of the regenerated explants play a significant role in the overall transformation. In this study, cotyledonary petioles showed a higher regeneration frequency in comparison to hypocotyls. Our results are in agreement with the results of a study conducted by Kamal *et al.,* (2007) on regeneration frequency of various canola explants (cotyledon, hypocotyls, and root). They reported that regeneration frequency of cotyledonary explants was higher in comparison to hypocotyl and root explants. While in contrast, Lingling *et al.,* (2005) found a higher regeneration frequency for hypocotyl explants. An important consideration in canola regeneration is finding the optimal concentration of hormonal supplements (Khan *et al.*, 2002; Moghaieb *et al.*, 2006). Using various concentrations of BAP in nutrient medium increases the regeneration frequency. Several attempts have been made to find this condition (Khan *et al.*, 2002; Lingling *et al.*, 2005; Kamal *et al.*, 2007). The results reported by Lingling *et al.,* (2005) showed that 2.0 mg/l BAP and 0.2 mg/l NAA in MS medium gave the highest regeneration frequency and Khan *et al.,* (2002) showed that the highest regeneration frequency was obtained in MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l IAA. Our results indicated that a higher concentration of BAP (5 mg/l) in the medium is required to obtain a higher regeneration frequency. A support to our results comes from the recent study conducted by Moghaieb *et al.,* (2006).

Necrosis is among the problems encountered in transformation experiments. This phenomenon was also observed in our study when cotyledonary petiole explants were used in the transformation experiments by co-cultivating with *Agrobacterium* harboring *P5CS* gene. In order to solve this problem, pre-culturing of explants was used before transformation. Previous experience of Ovesna *et al.,* (1993) on *Brassica napus*

has also marked that for improving transformation, pre-cultivation of explants could be helpful. Our results on canola supports this idea as we found pre-cultivation of cotyledonary explants for 2 days improved the rate of transformation by inhibiting necrosis. This result is in agreement with those of Pandian *et al.,* (2006) as well. In summary, our results indicated that cotyledonary petioles could be the appropriate source of explants for the transformation of canola by an exogenous gene, such as *P5CS* through *Agrobacterium tumefacience.* Our findings also demonstrated that optimized hormonal concentrations as well as pre-culturing of explants have a profound effect on the overall transformation efficiency. To monitor the efficiency of transformation, it is necessary to use a gene whose effect is known. For this purpose, *P5CS*, the regulatory gene of proline biosynthesis, could be an appropriate candidate. Proline accumulation in response to high salinity has been well documented (Pandian *et al.*, 2006). Producing proline in response to the osmotic stress and improvement of resistance has been noted in other plants such as potato (Hmida-Sayari *et al.*, 2005), wheat (Vendruscolo *et al.*, 2007), rice (Zhu *et al.*, 1998), Arabidopsis and tobacco (Yamchi *et al.*, 2007). Our results showed that free proline content was higher in transgenic plants compared to the controls and the transgenic plants showed more resistance to osmotic stress. The protocol described here can be used as a basis for the future development of commercial scale production. In addition, efficient transformation of a foreign gene, such as *P5CS* (Δ^1-pyrroline-5-carboxylate synthetase), improves the salinity tolerance of canola plantlets.

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REFERENCES

Ashraf M., and Foolad M. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany,* 59: 206-216.

Ashrafijou M., Noori S. A. S., Darbandi A. I., and Saghafi S. (2010). Effect of salinity and radiation on proline accumulation in seeds of canola (*Brassica napus* L.). *Plant Soil Environmental,* 56: 312-317.

Bates L., Waldren R., and Teare I. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil*, 39: 205-207.

Bybordi A. (2010). Effects of salinity on yield and component characters in canola (*Brassica napus* L.) cultivars. *Notulae Scientia Biologicae* 2: 81-83.

Cegielska-Taras T., Pniewski T., and Sza A. L. (2008). Transformation of microspore-derived embryos of winter oilseed rape (*Brassica napus* L.) by using *Agrobacterium tumefaciens*. *Journal of Applied Genetics,* 49: 343-347.

Cha-um S., and Kirdmanee C. (2009). Effect of salt stress on proline accumulation, photosynthetic ability and growth characters in two maize cultivars. *Pakistan Journal of Botany,* 41: 87-98.

De Block M., De Brouwer D., and Tenning P. (1989). Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the bar and neo genes in the transgenic plants. *Plant Physiology,* 91: 694-701.

Dormann M., Datla N., Hayden A., Puttick D., and Quandt J. (1997). Non-destructive screening of haploid embryos for glufosinate ammonium resistance four weeks after microspore transformation in *Brassica*. *Acta Horticult,* 459: 191-198.

El-Shemy H., Khalafalla M., Fujita K., and Ishimoto M. (2007). Improvement of protein quality in transgenic soybean plants. *Biologia Plantarum,* 51: 277-284.

Flowers, T. (2004). Improving crop salt tolerance. *Journal of Experimental Botany,* 55: 307-319.

Goudarzi M., and Pakniyat H. (2009). Salinity causes increase in proline and protein contents and peroxidase activity in wheat cultivars. *Journal of Applied Science,* 9: 348-353.

Hmida-Sayari A., Gargouri-Bouzid R., Bidani A., Jaoua L., Savouré A., and Jaoua S. (2005). Overexpression of [Delta] 1-pyrroline-5-carboxylate synthetase increases proline production and confers salt tolerance in transgenic potato plants. *Plant Science,* 169: 746-752.

Jonoubi P., Mousavi A., Majd A., Salmanian A., Jalali Javaran M., and Daneshian J. (2005). Efficient regeneration of *Brassica napus* L. hypocotyls and genetic transformation by A*grobacterium tumefaciens*. *Biologia Plantarum* , 49: 175-180.

Kamal G., Illich K. and Asadollah A. (2007). Effects of genotype, explant type and nutrient medium components on canola (*Brassica napus* L.) shoot *in vitro* organogenesis. *African Journal Biotechnology,* 6: 861-867.

Kawahigashi H., Hirose S., Ohkawa H., and Ohkawa Y. (2007). Herbicide resistance of transgenic rice plants expressing human CYP1A1. *Biotechnology Advances,* 25: 75-84.

Khan M., Rashid H., and Quraishi A. (2002). Effects of various growth regulators on callus formation and regeneration in *Brassica napus* Cv. oscar. *Pakistan Journal of Biology Science,* 5: 693-695.

Lingling L., Jianjun L., Ming S., Liyun L., and Bihao C. (2005). Study on transformation of cowpea trypsin inhibitor gene into cauliflower (*Brassica oleracea* L. var. botrytis). *African Journal of Biotechnology,* 4: 45-49.

Moghaieb R., El-Awady M., El Mergawy R., Youssef S., and El-Sharkawy A. (2006). A reproducible protocol for regeneration and transformation in canola (*Brassica napus* L.). *African Journal of Biotechnology,* 5: 143-148.

Moloney M., Walker J., and Sharma K. (1989). High efficiency transformation of *Brassica napus* using *Agrobac-*

terium vectors. *Plant Cell Reports,* 8: 238-242.

Murray M., and Thompson W. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research,* 8: 4321-4325.

Ovesna J., Ptá ek L., and Opatrný Z. (1993). Factors influencing the regeneration capacity of oilseed rape and cauliflower in transformation experiments. *Biologia Plantarum,* 35: 107-112.

Pandian A., Hurlstone C., Liu Q., Singh S., Salisbury P., and Green A. (2006). *Agrobacterium*-mediated transformation protocol to overcome necrosis in elite Australian *Brassica juncea* lines. *Plant Molecular Biology Reporter,* 24: 103-112.

Radchuk V., Klocke E., Radchuk R., Neumann M., and Blume Y. (2000). Production of transgenic rapeseed *Brassica napus* L. by transformation with *Agrobacterium tumefaciens*. *Genetika,* 36: 932-41.

Sakamoto A., and Murata N. (2000). Genetic engineering of glycinebetaine synthesis in plants: current status and implications for enhancement of stress tolerance. *Journal of Experimental Botany,* 51: 81-88.

Thomzik J., and Hain R. (1990). Transgenic *Brassica napus* plants obtained by cocultivation of protoplasts with *Agrobacterium tumefaciens*. *Plant Cell Reports,* 9: 233-236.

Vendruscolo E. C. G., Schuster I., Pileggi M., Scapim C. A., Molinari H. B. C., Marur C. J., and Vieira L. G. E. (2007). Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat. *Journal of Plant Physiology,* 164: 1367-1376.

Wu J., Wu, Q., Gai J., and Yu D. (2008). Constitutive overexpression of AOS-like gene from soybean enhanced tolerance to insect attack in transgenic tobacco. *Biotechnology Letters,* 30: 1693-1698.

Yamchi A., Jazii F., Mousavi A., and Karkhane A. (2007). Proline accumulation in transgenic tobacco as a result of expression of Arabidopsis Delta^ 1-Pyrroline-5-carboxylate synthetase (*P5CS*) during osmotic stress. *Journal of Plant Biochemistry and Biotechnology,* 16: 9-15.

Zhang Y., Singh M., Swoboda I., and Bhalla P. (2005). *Agrobacterium*-mediated transformation and generation of male sterile lines of Australian canola. *Australian Journal of Agriculture Research,* 56: 353-362.

Zhu B., Su J., Chang M., Verma D. P. S., Fan Y.L., and Wu R. (1998). Overexpression of a [Delta]1-pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water- and salt-stress in transgenic rice. *Plant Science,* 139: 41-48.