INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 6, No. 3, p. 319-329, 2015

RESEARCH PAPER OPEN ACCESS

Optimization of *in vitro* shoot regeneration and *Agrobacterium*-mediated transformation of *Brassica napus*

Amin Ravaei[1*], Parissa Jonoubi[1], Ali Hatef Salmanian[2]

[1]*Department of Plant Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran*

[2]*Department of Plant Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran*

Key words: Rapeseed, *Agrobacterium, GUS* gene, tissue culture.

http://dx.doi.org/10.12692/ijb/6.3.319-329 Article published on February 18, 2015

Abstract

Rapeseed (*Brassica napus* L.) is one of the most cultivated oil crops in the world. Its genetically engineered varieties harboring new characteristics rely significantly on establishment of an efficient in vitro tissue culture and transformation. In this study the potential of shoot regeneration of two *B.napus* cultivars, RGS003 and PF7045-91, and their *Agrobacterium*-mediated transformation using three methods were investigated. Hypocotyl and cotyledonary explants of 5, 10 and 15 day-old seedlings were used for indirect and direct shoot regeneration, respectively. The hypocotyl explants showed best callus formation in 0.5 mg/L 2,4-D. The calli and cotyledonary explants were regenerated in 3 to 4.5 mg/L BAP, successfully. The regenerated shoots were maturated and then rooted in 0.5 mg/L IBA. For genetic transformation, both explants were infected by *Agrobacterium* using immersing, incubation and well-based methods. None of hypocotyl explants get transformed. The transformation frequency of cotyledonary explants in well-based method was higher than the others, showing an increase of 15%. The obtained putative transgenic plantlets maintained green in presence of kanamycin. The transformation confirmed by PCR and histochemical GUS assay. The well-based inoculation method appeared promising to enhance the efficiency of cotyledon-based transformation.

* **Corresponding Author:** Amin Ravaei ✉ aminravaei@hotmail.com

Introduction

Molecular biotechnology has quickly broadened the horizons of plant breeding and crop improvement by offering modern technologies. Nowadays, genetic engineering by providing new facilities rectifies the disabilities of conventional breeding approaches, which depend on the unplanned rearrangement of existing gene between parent plants (Lemaux, 2008). Numerous studies have addressed the genetic modification of several plants such as soybean, maize, cotton, sugar beet, wheat, potato, rice and rapeseed (Stirn and Lorz, 2003, and references therein). Rapeseed (*Brassica napus* L.), that is one of the most genetically modified plants, by supplying the majority of Brassica-derived vegetable oil in the world is considered as the most economically important of *Brassica* genus (Bonnema, 2011; Rahman *et al.*, 2013). Although, crop improvement by providing desired properties caused the resurgence of rapeseed (Salisbury and Wratten, 1999; Burbulis *et al.*, 2009) going through the conventional crop improvement is low yield and takes more time to be completed (Moghaieb *et al.*, 2006; Moose and Mumm, 2008; Burbulis *et al.*, 2010). Fortunately, effective strategies have emerged and leaded to appearance of new traits in rapeseed (Tinland, 1996). These new characteristics can be classified in those involving in agronomic traits (Pan *et al.*, 2009), plant development (Denis *et al.*, 1993), quality traits (Venkatramesh *et al.*, 2003) and pharmaceutical production (Amani *et al.*, 2011).

The majority of new characteristics result from gene technology (Cardoza and Stewart, 2004). *Agrobacterium tumefaciens* which contributes to gene technology is considered popular for genetic manipulation because of transferring low copy number of genes and transgene stability (Travella *et al.*, 2005; Lee and Zhang, 2014). Considering genotype and explant influences, the *Agrobacterium*-mediated transformation may be low efficiency leading to low percent of transformation (Cardoza and Stewart, 2004; Burbulis *et al.*, 2010; Tang *et al.*, 2011; Lee and Zhang, 2014). Therefore, any potential approach able to take advantages of *Agrobacterium*-mediated transformation can serve plant biotechnology, significantly. The aims of this study were optimization of shoot regeneration and development of an efficient approach for *Agrobacterium*-mediated transformation of *Brassica napus* cv. RGS003 and PF7045-91.

Materials and methods

Plant material and sterilization

Seeds of commercial rapeseed cultivars PF 7045-91 and RGS003 were obtained from the Seed and Plant Improvement Research Institute of Iran. The seeds were surface sterilized using 30% sodium hypochlorite and 0.01% tween 20 mixture for 10 min. Then the seeds were rinsed by sterile distilled water for three times and germinated on half-strength MS medium (Murashige and Skoog, 1962) with 30 g/L sucrose and 8 g/L agar under condition of 16/8 h photoperiod at 40-50 $\mu Em^{-2}s^{-1}$ light intensity and 25 °C.

Tissue culture

The 5, 10 and 15 day-old seedlings were used for cotyledon and hypocotyl explants preparation. The cotyledonary explants were obtained by removing the shoot and the latter were prepared by cutting the hypocotyl in 1 cm segments.

The hypocotyl explants were initially cultured in Callus Induction Medium (CIM) including B5 (Gamborg *et al.*, 1968) medium supplemented with 0.5, 1 and 1.5 mg/L of 2,4-dichlorophenoxyacetic (2,4-D) growth regulator. Afterward the explants were transferred into Shoot Induction Medium (SIM) composing of B5 medium enriched with 1.5, 3 and 4.5 mg/L 6-benzyl amino purine (BAP), and every two weeks they were transferred in the same fresh media till appearance of new shoots. The regenerated shoots were carefully cut and transferred into Shoot Maturation Medium (SMM) consisting of MS medium without any growth regulators. The enlarged shoots then transferred in Root Induction Medium (RIM) supplemented with 0.5, 0.1 and 1.5 mg/L Indole-3-butyric acid (IBA). The cotyledonary explants were cultured in SIM, SMM and RIM,

respectively. The composition of these media is similar to hypocotyl's one with exception of B5 medium which replaced by MS medium. All CIM, SIM, SMM were supplemented by 30 g/L sucrose and 8 g/L agar at pH 5.8. The RIM had the same amount of sucrose and pH but 9 g/L agar for efficient rooting. The rooted plantlets were transferred in pots containing mixture of peat and perlite with a cover of plastic bag for supplying the humidity. After 2 weeks the cover removed.

Agrobacterium-mediated transformation

To transform the rapeseed explants *Agrobacterium tumefaciense* strain LBA4404 was used. It was cultured overnight in liquid Luria-Bertani (LB) medium containing 34 mg/L rifampicin and 50 mg/L kanamycin. The culture medium with OD_{600}: 0.8 was centrifuged and the cells were suspended in infection medium composed of MS salts plus 5% glucose at pH 5.2.

Immersing-based infection

The hypocotyl and petiole of cotyledonary explants were immersed in infection medium containing *Agrobacterium* for 5, 10 and 15 s and then were wiped with sterile filter paper and cultured in co-culture medium.

Explant Incubation-based infection

The hypocotyls and cotyledons were incubated for 5, 10 and 15 min in infection medium containing the *Agrobacterium* at room temperature. Then the explants were wiped with sterile filter paper and cultured in co-culture medium.

Well-based infection

In co-culture media 10 to 15 wells were made by means of a sterile Pasteur pipettes. In the wells 10, 20 and 30 µl of the infection medium were poured and 2 to 3 mm of cut end of the cotyledonary explants were embedded in the wells.

Co-cultivation

The co-cultivation was done for hypocotyl and cotyledon explants in B5 and MS media respectively. The culture was done without/with 0.5 and 1 mg/L of 2,4-D growth regulator at pH 5.6 in dark or light condition at 25 °C for 48 or 72 h. After co-cultivation the explants were transferred to above-mentioned culture media supplemented with 200 to 400 mg/L cefotaxime and 5, 10 and 15 mg/L kanamycin (in CIM/SIM, SMM and RIM, respectively) as selective agents.

Histochemical GUS assay

The leaves of putative transgenic plants were obtained and incubated overnight at 37 °C in 50 mM phosphate buffer (pH 7.0), 1 mM X-GLUC (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid), 1 mM EDTA (Ethylenediaminetetraacetic acid), 0.001% Triton and 10 mM β-mercaptoethanol. The chlorophyll was eliminated by soaking in 96% ethanol for easy detection of blue color.

Polymerase Chain Reaction analysis

The genomic DNA (deoxyribonucleic acid) of transformed and control plants was extracted by DNA extraction kit (BIONEER). PCR was performed by specific primers (5'-CCGGCATAGTTAAAGAAATCATG-3'; 5'-GGTGGTCAGTCCCTTATGTTACG-3') in TECHNE termocycler as follow: 30 cycles of denaturation at 94 °C for 1 min, annealing at 61 °C for 1 min, primer extension at 72 °C for 1 min, followed by final extension for 7 min at 72 °C, and initial denaturation was at 94 °C for 4 min. The PCR products were electrophoresed on 1% agarose gel.

Statistical analysis

All experiments were conducted randomly with three replicates per treatment using 60 explants for each replicate. The percentages of shoot regeneration and transformation were calculated as follow: [(total number of shoots produced by explants/total number of explants) × 100] and [(number of transformed plants/total number of *Agrobacterium*-infected explants) ×100].

Results

Shoot regeneration

The present study showed that the cotyledonary and hypocotyl explants regenerated on MS and B5 media containing different concentrations of growth regulators, respectively.

The hypocotyl explants formed callus in cut ends in CIM containing 0.5, 1 and 1.5 mg/L 2,4-D within 7 to 10 days. The calli were extremely tough and light green (Fig. 1a and 1b). Only 0.5 mg/L 2,4-D cultured explants regenerated successfully (Fig. 1c and 1d) and the others formed hair roots in SIM (Fig. 1e and 1f). Prolonged culture of hypocotyl explants in CIM prevented shoot initiation and induced hair root formation. The regeneration results of both RGS003 and PF7045-91 cultivars considering different conditions were presented in Table 1. The best results observed when 15 day-old explants of RGS003 and PF7045-91 cultivars were cultured in 3 and 4.5 mg/L BAP, respectively.

Table 1. Shoot regeneration frequencies of hypocotyl explants of two RGS003 and PF7045-91 cultivars cultured on different concentrations of 2,4-D and BAP growth regulators.

CIM 2,4-D (mg/L)	SIM BAP (mg/L)	Shoot Regeneration Frequency (%)					
		RGS003			PF7045-91		
		Age (day)			Age (day)		
		5	10	15	5	10	15
0.5	1.5	11.2	13	13.7	9	10.2	12
0.5	3	17.4	20	27	14	14.7	17.3
0.5	4.5	14	18.8	21.5	16.2	20.4	23.3

The cotyledon leaves initiated the regeneration at cut end of the petioles within 11-14 days and then were differentiated into shoots with normal growth and development. The shoot regeneration frequency was different considering the age, BAP concentration and cultivar variations (Table 2). The best results, as shown in Fig. 1g and 1h, were recorded when the medium supplemented with 3 mg/L BAP in 15 day-old cotyledon leaves for both PF7045-91 and RGS003 cultivars with 173% and 210%, respectively. The 1.5 mg/L BAP in SIM resulted in formation of callus at petioles. The calli were relatively tough, big and pale (Fig. 1i and 1j). When the calli cut and transferred to SIM containing 3 mg/L BAP could regenerate significantly. SIM supplemented with 4.5 mg/L BAP decreased the bud creation frequency and caused shoot vitrification.

Table 2. Shoot regeneration frequencies of cotyledonary explants of two RGS003 and PF7045-91 cultivars cultured on different concentrations of BAP growth regulator.

SIM BAP (mg/L)	Shoot Regeneration Frequency (%)					
	RGS003			PF7045-91		
	Age (day)			Age (day)		
	5	10	15	5	10	15
1.5	70.5	142	164.7	67	90	146
3	93	182.8	210	88.3	150	173
4.5	90	176.8	199	72	145.7	160.5

By increasing the age of hypocotyl and cotyledonary explants the regeneration frequency raised (Table 1 and 2). The regenerated shoots from both explants were enlarged in SMM within 3 weeks (Fig. 2a and 2b). Most of shoots rooted in SMM and the others were rooted in the presence of IBA (Fig. 2c and 2d). All concentrations of IBA caused shoots produce root. After 6 weeks of culture in RIM, the plants were transferred to pots for acclimatization (Fig. 2e and 2f).

Agrobacterium-mediated transformation

The hypocotyl and cotyledon explants which were immersed in infection medium showed different results as all hypocotyl explants became brownish and finally putrefied. The transformation percentages for cotyledonary explants in 5s and 10s immersing were 5% and 7% for PF7045-91, 5.8% and 11% for RGS003, respectively (Table 3). The 10s immersing showed some *Agrobacterium* contaminations and explant putrefaction. In 15s immersing explant putrefaction and contamination of SIM and SMM with *Agrobacterium* was high that was preventing the successful enlargement of shoots and rooting.

Table 3. Transformation frequencies of 15 day-old cotyledonary explants of two RGS003 and PF7045-91 cultivars inoculated with *Agrobacterium* using three different methods in growth regulator free MS medium in light condition for 48h.

Agrobacterium Inoculation Method / Cultivar	Transformation Frequency (%)								
	Immersing (s)			Incubation (min)			Well (µl)		
	5	10	15	5	10	15	10	20	30
RGS003	5.8	11	4	4	-	-	6.6	20	19
PF7045-91	5	7	4.3	2.3	-	-	7	18	15.6

Using the incubation approach all hypocotyl explants get putrefied leading to stopped procedure. The cotyledon leaves get transformed in 5 min incubation with 2.3% for PF7045-91 and 4% for RGS003, respectively, but in 10 min- and 15 min-incubation all explants became highly contaminated with *Agrobacterium*, as all measurements were not successful (Table 3).

The well-based co-cultivation approach resulted in transformation of cotyledonary explants with 7%, 18% and 15.6% rates for PF7045-91 and 6.6%, 20% and 19% for RGS003 using 10, 20 and 30 µl of infection medium, respectively (Table 3). The 30 µl volume of infection medium showed more *Agrobacterium* contamination within shoot and root induction media.

Table 4. Transformation frequencies of 15 day-old cotyledonary explants of two RGS003 and PF7045-91 cultivars inoculated with *Agrobacterium* using well-based method loaded with 20 µl of *Agrobacterium* suspension.

2,4-D (mg/L)	Cocultivation Time (h)	Lighting	Transformation Frequency (%)	
			RGS003	PF7045-91
0.5	48	L	22.5	21
0.5	48	D	24.7	23
0.5	72	L	23.2	19.2
0.5	72	D	27	21.8
1	48	L	7.3	3.4
1	48	D	R	R
1	72	L	5.8	R
1	72	D	R	R

D= Darkness, L= Light, R=Root formation.

The use of 0.5 mg/L 2,4-D in well-based co-cultivation medium showed an increase in transformation rate but absence of 2,4-D resulted in low transformation frequency and the 1 mg/L 2,4-D induced the formation of root in SIM. The dark condition increased the transformation frequency and 48h co-cultivation was best for PF7045-91 and 72h co-cultivation was best for RGS003 cultivar (Table 4).

The potential transformed shoots maintained green in SIM, SMM and RIM supplemented with 5, 10 and 15 mg/L of kanamycin as selective agent, respectively, while nontransformed shoots became white or purple (Fig. 3a).

Histochemical GUS assay

The histochemical GUS assay showed that 73% of the transformed plants had GUS activity by displaying blue color (Fig. 3b).

PCR analysis

PCR assay of plants confirmed the presence of *GUS* gene by exhibiting a 520 bp fragment in agarose gel, while the electrophoresis of PCR product of nontransgenic plants did not exhibit any amplified fragment (Fig. 3c).

Discussion

Development of genetically engineered plants harboring new traits is one of the major goals of plant biotechnology. Attempts to achieve this goal by applying *Agrobacterium* go through optimization of in vitro tissue culture and gene transformation of a putative plant. *Brassica napus* which serves as an amenable host for many new traits in regard to optimization of in vitro tissue culture and gene transformation is extremely genotype dependent (Jonoubi *et al.*, 2004; Mashayekhi *et al.*, 2008; Burbulis *et al.*, 2010). Therefore, measurements such as improving the efficiency of *Agrobacterium* introduction ways to the plant, concentrations of growth regulators, and co-culture conditions should be taken. This study aimed to optimize tissue culture conditions of *Brassica napus* cv. PF7045-91 and RGS003, and develop an efficient method for their transformation mediating by *Agrobacterium*.

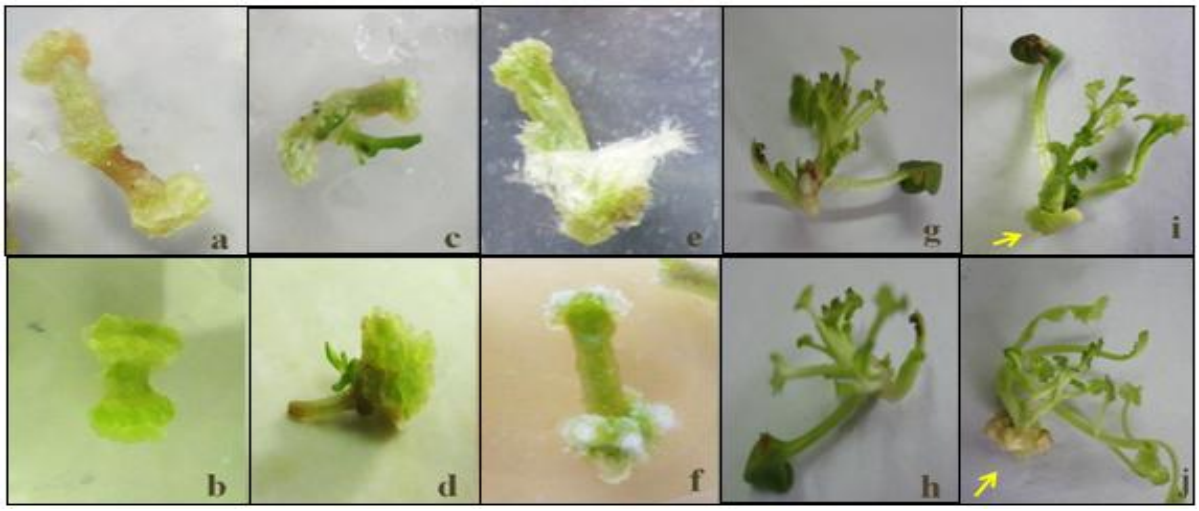

Fig. 1. Callusogenesis and regeneration of hypocotyl and cotyledonary explants of *B.napus*: (a) and (b) morphogenic callus formation of hypocotyl explants of PF7045-91 and RGS003 cultivars treated with 0.5 mg/L 2,4-D in CIM, (c) and (d) shoot initiation from hypocotyl explants of PF7045-91 and RGS003 cultivars treated with 3 mg/L BAP, (e) and (f) root produced hypocotyl explants of PF7045-91and RGS003 cultivars treated with 1 mg/L 2,4-D in CIM, (g) and (h) direct regeneration of cotyledonary explants of PF7045-91 and RGS003 cultivars treated with 3 mg/L BAP in SIM, (i) and (j) morphogenic callus formation and indirect shoot regeneration of cotyledonary explants of PF7045-91 and RGS003 cultivars treated with 1.5 mg/L BAP in SIM.

In rapeseed tissue culture, the cotyledonary and hypocotyl explants are often applied than other parts because of high regeneration frequency and transformation capability (Zhang and Bhalla, 2004; Burbulis *et al.*, 2008; Burbulis *et al.*, 2009). These explants can be obtained numerously over a short time by the seeds growing in sterile germination medium (Burbulis *et al.*, 2008). The regeneration and transformation frequencies depend significantly on explants type and genotype (Moghaieb *et al.*, 2006;

Zeynali *et al.*, 2010). As recorded by present study the best regeneration frequencies for cotyledonary explants of RGS003 and PF7045-91 cultivars were 210% and 173%, respectively. These frequencies for hypocotyl explants of respective cultivars were 27% and 23.3% indicating the role of genotype in differences between cultivars regeneration. There are various studies reporting different regeneration frequencies for distinctive genotypes (Cardoza and Stewart, 2004; Mashayekhi *et al.*, 2008). Burbulis *et al.*, (2009) investigated the regeneration frequencies of ten winter rapeseed cultivars and observed 0% to 34.67% regeneration frequencies among the cultivars. Burbulis *et al.*, (2010) reported a range between 0% to 26.0% for regeneration frequency for eleven spring rapeseed cultivars. Moghaieb *et al.*, (2006) studied the regeneration ability of five rapeseed cultivars and the most frequency was 68% for Sarow-4 cultivar and the least frequency was 44% for Semu-304 cultivar.

The cotyledonary explants of both cultivars showed more regeneration frequency than hypocotyl ones indicating the role of explant type in response to tissue culture conditions. This finding is in coincidence with results reported by Travella *et al.*, 2005; Kamal *et al.*, 2007; Burbulis *et al.*, 2008; Ghnaya *et al.*, 2008; Burbulis *et al.*, 2009; Burbulis *et al.*, 2010.

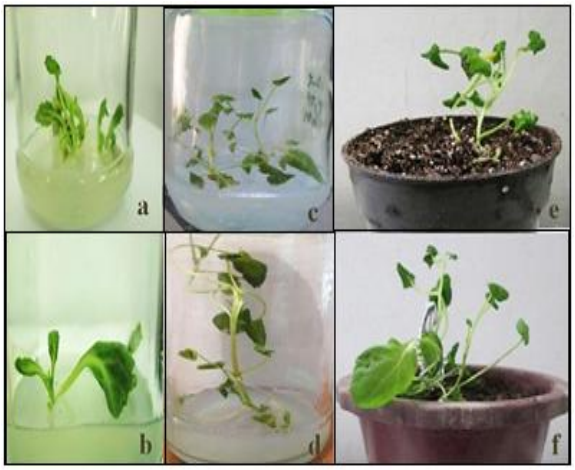

Fig. 2. In vitro maturated and rooted shoots of *B.napus* explants: (a) and (b) regenerated shoots of PF7045-91and RGS003 cultivars in SMM, (c) and (d) rooted shoots of PF7045-91 and RGS003 cultivars in RIM, (e) and (f) in vitro produced plantlets of PF7045-91 and RGS003 cultivars transferred to pots for acclimation.

The use of 2,4-D in CIM induced callus formation in hypocotyl explants efficiently. Different concentrations of 2,4-D resulted in different organogenesis as 0.5 mg/L concentration caused the calli to differentiate into shoots in SIM. Shameen-Alam *et al.*, (2013) reported that 0.5 mg/L 2,4-D is optimum concentration for rapeseed callus formation. Ali *et al.*, (2007) also observed that the best callusogenesis of three rapeseed cultivars is achieved in 0.5 mg/L 2,4-D. While 1 and 1.5 mg/L concentrations contributed to root formation in SIM implying its accumulation in epidermal cells (Eshel and Beeckman, 2013). Ramzan-khan *et al.*, (2002) reported that callus proliferation would be inhibited in higher concentrations of 2,4-D.

Utilization of BAP in shoot induction media led to bud formation and shoot initiation. Its different concentrations also can affect explant fate as lower concentration caused callus formation and higher concentration resulted in decreased shoot regeneration in cotyledonary explants. Shameen-Alam *et al.*, (2014) observed that higher concentrations of BAP decreased shoot initiation. Ramzan-khan *et al.*, (2002) documented that lower concentrations of BAP can affect morphogenesis adversely. These observations indicate the dependence of plant growth regulators effect on their concentrations and interaction with the endogenous growth regulators of plant (Roy and Banerjee, 2003).

By increasing the hypocotyl and cotyledonary explants age, the regeneration frequency raised. This result corresponds well with the finding of Cardoza and Stewart, 2004; and Jonoubi *et al.*, 2004. This is because of the level of indigenous growth regulators that is affected by plant growth. In the case of cotyledonary explants, by growing the seedlings the region of multipotent cells (Fig. 4a) expands which provides more suitable cells and also contributes to more convenient explant preparation which in turn avoids losing multipotent cells.

In this study the introduction of *Agrobacterium* to *B.napus* explants were conducted by three approaches. All hypocotyl explants of both cultivars

which infected by immersing or incubation approaches did not transform and finally get putrefied. This was partly due to *Agrobacterium* contamination. Thus, the explants washed with cefotaxime after co-cultivation; however, they did not transform. Preconditioning of hypocotyl explants in 2,4-D did not lead to transformation, likewise. Probably, the hypocotyl explants of both cultivars and their meristematic cells are not impressionable by *Agrobacterium* (Block *et al*., 1989).

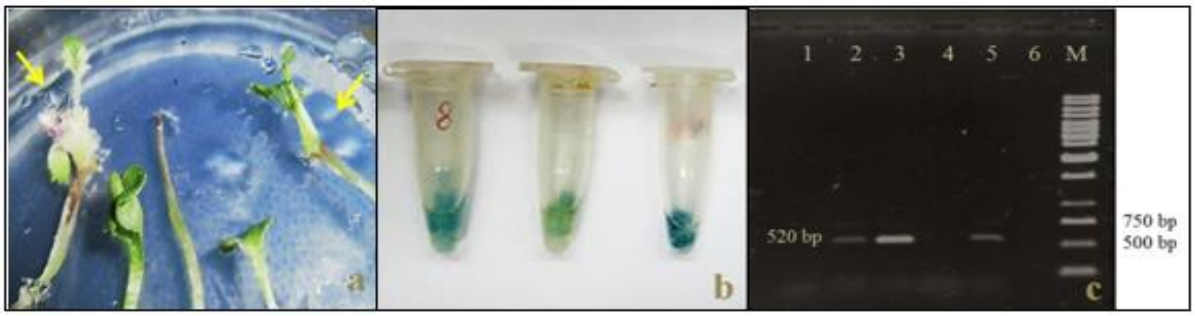

Fig. 3. Confirmation of *B.napus* transformation: (a) the left arrow shows the kanamycin susceptible regenerated shoot indicating unsuccessful transformation and the right arrow shows the green kanamycin resistant regenerated shoot capable to be transformed, (b) GUS-positive plants, (c) PCR amplification of *GUS* gene: 1 and 4= nontransgenic plant; 2 and 5= transgenic plants; 3= positive control; 6= negative control; M= 1 Kb DNA ladder.

By using cotyledonary explants all three methods resulted in rapeseed transformation with different frequencies. The well-based method showed more transformation rate and the incubation method had the least rate. The low transformation frequency of incubation method was due to explant waste. This method had more explant waste even when there was no *Agrobacterium* contamination. The immersing method, which can somehow be considered as incubation method, showed more transformation frequency than incubation method since during this method only the petiole of cotyledonary explants infected with *Agrobacterium* which prevents further contamination. However, this method is time consuming and laborious. The well-based method rectified these problems. By means of wells in co-cultivation medium the cotyledonary explants after preparation could be embedded in wells rapidly (Fig. 4b) which rescues the explant from wilting. The most responsive part of cotyledonary explants to transformation can be embedded in the well contacting directly with *Agrobacterium*. Since whole explant does not inoculate with bacterial suspension it prevents subsequent *Agrobacterium* contamination. The wells also provide appropriate space for adding certain materials which should not be inserted in medium composition. All of these outcomes result contributorily in higher transformation frequency of cotyledonary explants. Thus, for further optimization the well-based - *Agrobacterium* introduction were performed in dark/light, presence/absence of 2,4-D for 48/72h. Both cultivars showed more transformation in presence of 0.5 mg/L 2,4-D in dark conditions. This is due to modification of cell wall plasticity caused by darkness-induced endogenous auxin and exogenous 2,4-D levels which alter the cell permeability making explants able to be transformed (Tavakol-Afshari *et al*., 2011; Pitzschke, 2013).

Fig. 4. Multipotent cells of cotyledonary explants and their co-cultivation medium: (a) the arrow shows the region of dark green multipotent cells able to get regenerated and transformed, (b) the co-cultivation medium containing wells and cotyledonary explants.

The GUS and PCR assays were carried out on all green kanamycin resistant plantlets to confirm the *GUS* gene presence and expression. Appearance of blue color in leaves of putative plantlets and 520 bp fragment in agarose gel confirmed the genetically transformation of rapeseed.

In conclusion, the well-based method is recommended for *Agrobacterium* introduction to *B.napus* cv. RGS003 and PF7045-91 in presence of 0.5 mg/L 2,4-D and dark conditions. This method can be applicable for all plants capable to be transformed by cotyledonary explants. For other types of explants some modifications are necessary which require further investigations.

Acknowledgements

This work was supported by Faculty of Biological Sciences, Kharazmi University and National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.

References

Alam SS, Khaleda L, Al-Forkan M. 2014. In vitro plant regeneration from hypocotyl and cotyledon explants of BARI SARISHA-13 (*Brassica napus* L.). Asian Journal of Agriculture and Food Sciences **2,** 39-45.

Ali H, Ali Z, Ali H, Mehmood S, Ali W. 2007. In vitro regeneration of *Brassica napus* L., cultivars (Star, Cyclone and Westar) from hypocotyls and cotyledonary leaves. Pakistan Journal of Botany **39,** 1251-1256.

Amani J, Mousavi SL, Rafati S, Salmanian AH. 2011. Immunogenicity of a plant-derived edible chimeric EspA, Intimin and Tir of *Escherichia coli* O157:H7 in mice. Plant Science **180,** 620-627. http://dx.doi.org/10.1016/j.plantsci.2011.01.004

Block MD, Brouwer DD, 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.

Bonnema G. 2011. Diversity and taxonomy of *Brassica* oil crops. In: Kole C, Ed. Genetics, Genomics and Breeding of Oilseed Brassicas. FL, USA: CRC Press, 47-72 P.

Burbulis N, Blinstrubbiene A, Kupriene R, Jonytiene V, Rugienius R, Staniene. 2009. In vitro regeneration of *Brassica napus* L. shoots from hypocotyls and stem segments. Zemdirbyste-Agriculture **96,** 176-185.

Burbulis N, Blinstrubiene A, Kupriene R. 2010. Genotypic and growth regulator effects on shoot regeneration from hypocotyl and stem segment explants of spring rapeseed (*Brassica napus* L.). Journal of Food Agriculture and Environment **8,** 634-637.

Burbulis N, Kupriene R, Blinstrubiene A. 2008. Callus induction and plant regeneration from somatic tissue in spring rapeseed (*Brassica napus* L.). Biologija **54,** 258-263. http://dx.doi.org/10.2478/v10054-008-0053-1

Cardoza V, Stewart CN. 2004. *Agrobacterium*-mediated transformation of rapeseed. In: Curtis IS, Ed. Transgenic Crops of the World-Essential Protocols. Netherlands: Kluwer Academic Press, p. 379-387.

Cardoza V, Stewart CN. 2004. *Brassica* biotechnology: progress in cellular and molecular biology. In Vitro Cellular & Developmental Biology-Plant **40,** 542-551. http://dx.doi.org/10.1079/IVP2004568

Denis M, Delourme R, Gourret J, Mariani C, Renard M. 1993. Expression of engineered nuclear male sterility in *Brassica napus*. Plant Physiology **101,** 1295-1304.

Eshel A, Beeckman T. 2013. Plant Roots: The Hidden Half. 4th ed. Boca Raton, FL, USA: CRC Press.

Gamborg OL, Miller RA, Ojima K. 1968. Nutrient requirements of suspension culture of soybean cells. Experimental Cell Research **50,** 151-158.
http://dx.doi.org/10.1016/0014-4827(68)90403-5

Ghnaya AB, Charles G, Branchard M. 2008. Rapid shoot regeneration from thin cell layer explants excised from petioles and hypocotyls in four cultivars of *Brassica napus* L. Plant Cell Tissue and Organ Culture **92,** 25-30.
http://dx.doi.org/10.1007/s11240-007-9298-0

Jonoubi P, Mousavi A, Majd A, Daneshian J. 2004. Improved *Brassica napus* L. regeneration from hypocotyls using thidiazuron and benzyladenine as cytokinin sources. Pakistan Journal of Botany **36,** 321-329.

Kamal GB, Illich KG, Asadollah A. 2007. Effects of genotype, explant type and nutrient medium components on rapeseed (*Brassica napus* L.) shoot in viitro organogenesis. African Journal of Biotechnology **6,** 861-867.

Khan MR, Rashid H, Quraishi A. 2002. Effects of various growth regulators on callus formation and regeneration in *Brassica napus* cv. Oscar. Pakistan Journal of Biological Sciences **5,** 693-695.

Lee H, Zhang ZJ. 2014. *Agrobacterium*-mediated transformation of mize (Zea mays) immature embryos. Methods in Molecular Biology **1099,** 273-280.
http://dx.doi.org/10.1007/978-1-62703-715-0_22

Lemaux P. 2008. Genetically Engineered Plants and Foods: A Scientist's Analysis of the Issues (Part I). Annual Review of Plant Biology **59,** 771-812.
http://dx.doi.org/10.1146/annurev.arplant.58.032806.103840

Mashayekhi M, Shakib AM, Ahmad-Raji M, Ghasemi Bezdi K. 2008. Gene transformation potential of commercial rapeseed (*Brassica napus* L.) cultivars using cotyledon and hypocotyl explants. African Journal of Biotechnology **7,** 4459-4463.

Moghaieb REA, El-Awady MA, El Mergawy RG, Youssef SS, El-Sharkawy AM. 2006. A reproducible protocol for regeneration and transformation in rapeseed (*Brassica napus* L.). African Journal of Biotechnology **5,** 143-148.

Moose SP, Mumm RH. 2008. Molecular plant breeding as the foundation for 21st century crop improvement. Plant Physiology **147,** 969-977.
http://dx.doi.org/10.1104/pp.108.118232

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiologia Plantarum **15,** 473-497.
http://dx.doi.org/10.1111/j.13993054.1962.tb08052.x

Pan J, Wang J, Zhou Z, Yan Y, Zhang W, Lu W, Ping S, Dai Q, Yuan M. 2009. IrrE, a global regulator of extreme radiation resistance in *Deinococcus radiodurans,* enhance salt tolerance in *Escherichia coli* and *Brassica napus*. PloS One **4,** 1-9.
http://dx.doi.org/10.1371/journal.pone.0004422

Pitzschke A. 2013. *Agrobacterium* infection and plant defense-transformation success hangs by a thread. Frontiers in Plant Science **4,** 1-12.
http://dx.doi.org/10.3389/fpls.2013.00519

Rahman H, Harwood J, Weselake R. 2013. Increasing seed oil content in *Brassica* species through breeding and biotechnology. Lipid Technology **25,** 182-185.
http://dx.doi.org/10.1002/lite.201300291

Salisbury P, Wratten N. 1999. *Brassica napus* Breeding. In: Proceedings of the 10st International Rapeseed Congress. The Regional Institute, 07 P.

Shameem-Alam S, Khaleda L, Al-forkan M. 2013. Establishment of regeneration Protocol for rapeseed (*Brassica napus* L.). Global Journal of Science Frontier Research **13,** 7-11.

Stirn S, Lorz H. 2003. Genetically Modified Plants. In: Heller KJ, editor. Genetically Engineered Food: Methods and Detection. Weinheim, FRG: Wiley-VCH Verlag GmbH & Co. KGaA, 33-49 P.

Tang GX, Knecht K, Yang XF, Qin YB, Zhou WJ, Cai D. 2011. A two-step protocol for shoot regeneration from hypocotyl explants of oilseed rape and its application for *Agrobacterium*-mediated transformation. Biologia Plantarum **55,** 21-26.

Tavakol Afshari R, Angoshtari R, Kalantari S. 2011. Effects of light and different plant growth regulators on induction of callus growth in rapeseed (*Brassica napus* L.) genotypes. Plant OMICS **4,** 60-67.

Tinland B. 1996. The integration of T-DNA into plant genomes. Trends in Plant Science **1,** 178-184. http://dx.doi.org/10.1016/1360-1385(96)10020-0

Travella S, Ross SM, Harden J, Everett C, Snape JW, Harwood WA. 2005. A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium*-mediated techniques. Plant Cell Reports **23,** 780-789.

Venkatramesh M, Karunanandaa B, Sun B, Gunter CA, Boddupalli S, Kishore GM. 2003. Expression of a Streptomyces 3-hydroxysteroid oxidase gene in oilseeds for converting phytosterols to phytostanols. Phytochemistry **62,** 39-46. http://dx.doi.org/10.1016/S0031-9422(02)00431-4

Zeynali M, Zanjani BM, Amiri ME, Noruzian M, Aghajari SM. 2010. Influence of genotype and plant growth regulator on somatic embryogenesis in rapeseed (*Brassica napus* L.). African Journal of Biotechnology **9,** 4050-4055. http://dx.doi.org/10.5897/AJB09.1870

Zhang Y, Bhalla P. 2004. In vitro shoot regeneration from commercial cultivars of Australian rapeseed (*Brassica napus*). Australian Journal of Agricultural Research **55,** 753-756. http://dx.doi.org/10.1071/AR03209