African Journal of Biotechnology Vol. 5 (2), pp. 143-148, 16 January 2006
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

Full Length Research Paper

A reproducible protocol for regeneration and transformation in canola (*Brassica napus* L.)

Reda E. A. Moghaieb, Mohamed A. El-Awady, Rabab G. El Mergawy, Sawsan S. Youssef and Ahmed M. El-Sharkawy*

Department of Genetics and Genetic Engineering Research Center (GERC), Faculty of Agriculture, Cairo University, Giza, Egypt.

Accepted 5 October, 2005

The objective of the present study is to develop an efficient protocol for shoot and plant regeneration using five commercial canola cultivars grown under the Egyptian agricultural conditions. The regeneration efficiency from hypocotyl explants was examined. The data indicated that embryonic calli were formed within two weeks in the presence of 1 mgl^{-1} 2,4-D. Adventitious shoots emerged from the embryonic callus in the presence of 4.5 mgl^{-1} BA. The cultivars showed a varied response to shoot regeneration. Regeneration frequency was high in the cultivar Sarow-4 (68%) followed by Masrri L-16 (64%) compared with the other cultivars tested. Hypocotyl explants from the cultivars Sarow-4 and Semu-249 were inoculated and co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pBI-121 containing the neomycin phosphotransferase-II gene (*NPT-II*). The resulted putative transgenic plantlets were able to grow under knanamycin containing medium. The stable integration of the *NPT-II* gene into the plant genomes was tested by PCR using *NPT-II* -specific primers. The GUS gene expression can be detected only in the transgenic plants. The reported protocol in the present study is repeatable and can be used to regenerate transgenic canola plants expressing the genes present in *A. tumifaciens* binary vectors.

Key words: *Agrobacterium*, canola, GUS assay, regeneration, fransformation, *NPT II* gene.

INTRODUCTION

Canol (*Brassica napus* L.) is considered as the most important source of vegetable oil and protein-rich meal worldwide. It ranks the third among the oil crops, following palm oil and Soya oil and the fifth among economically important crops, following rice, wheat, maize and cotton (Sovero, 1993; Cardoza and Stewart, 2003). There are increased domestic and export market opportunities for canola oil that can be realized through the development of high-oleic acid canola to replace saturated palm oil in food service applications (Spector, 1999; Stoujesdijk et al., 2000). Additionally, high-oleic acid oils are more nutritionally beneficial because oleic acid had cholesterol-lowering properties, whereas saturated fatty acids tend to raise blood cholesterol levels (Stoujesdijk et al., 2000). On the other hand, in Egypt there are agricultural opportunities to increase canola production by expanding into the new reclaimed regions. Therefore, *B. napus* has become an object of extensive tissue culture studies and breeding.

Cell and tissue culture relating to variability and selection efficiency are two essential components of molecular breeding (Lichtenstein and Draper, 1985). Genetic variation in canola is required to breed cultivars that are high yielding, and resistant to several biotic and abiotic stress conditions. It is well known that improvement of plant through conventional breeding methods is slow, time-consuming and labor-intensive. Non-conventional genetic improvement programs based on tissue culture and molecular genetics is essential as a complement to standard breeding (Lichtenstein and Draper, 1985).

*Corresponding author. E-mail: gecjica@link.net.

Since the early observation by Skoog and Miller (1957) that the balance of auxin and cytokinin in the culture medium regulates organogenesis, much progress has been made in identifying factors that control plant morphogenesis. These regulatory factors include both naturally occurring and synthetic plant growth substances as well as various environmental stimuli (Lakshmanan et al., 1997). In earlier studies, attention had been focused on determining the requirements of various plant growth substances and mineral nutrients for different organogenic processes (Murashige, 1974; Gamborg et al., 1977). More recently a number of investigations on organogenesis have been conducted from a physiological perspective to analyze various cellular processes associated with organogenesis (Thorpe ,1993).

Regeneration in *B. napus* is highly variable and genotype specific. Several papers have reported regeneration of shoots from seedlings or mature plant derived explants of *B. napus* (Dunwell, 1981). To date organogenesis has been achieved in a variety of explants such as stem sections (Pua et al., 1991), stem thin-cell layer (Klimaszewska and Keller, 1985), leaf discs (Dunwell, 1981), roots (Sharma and Thorpe, 1989), cotyledons (Moloney et al., 1989) and hypocotyls (Phogat et al., 2000).

It is well documented that efficient *Agrobacterium*-mediated transformation methods require a reliable and efficient callus induction and plantlet regeneration procedures (Riemenschiender et al., 1988). The aim of this study was to develop a procedure for the transformation and regeneration of viable shoots from hypocotyl explants of commercial Egyptian canola varieties. Five commercial cultivars of canola were successfully regenerated. Differences in the regeneration capacity among the cultivars were observed. The system described here can be used as a basis for the future development of commercial scale production of regenerated canola plants. In addition, the co-transformation of a foreign gene with T-DNA in the Ti-plasmid of *Agrobacterium tumifaciens* leads to an improvement of plant properties.

MATERIALS AND METHODS

Plant material

Three Egyptian commercial canola cultivars namely, Sarow-4, Masrri L-11, Masrri L-16, and two German cultivars, Semu-304 and Semu-249, were used. Canola seeds were surface sterilized by immersion in 70% ethanol followed by immersion in 3% (v/v) sodium hypochlorite, and rinsed in sterile distilled water. The sterilized seeds were germinated in flasks on 0.8% agar (w/v). The cultures were incubated at 25°C under a 16/8 h day/night photoperiod (1000-Lux).

Shoot induction medium

Hypocotyl segments (0.5 cm in length) were excised from 6-day-old canola seedlings. The explants were transferred to the callus induction medium containing MS salt, 3% (w/v) sucrose, B5 vitamins, 1% (w/v) agar in addition to 1 mg/l 2,4-D. Two weeks later the explants were transferred onto shoot induction (MS) medium (Murashige and Skoog, 1962), supplemented with BA at graded levels (0.0, 2.25, 4.50, 7.25 and 9 mg/l), pH 5.8. Each plate contained 10 segments and all the treatments were performed with 5 replications. The plates were sealed with parafilm and incubated at 25°C under a 16/8 h light/dark photoperiodic regime (1000-Lux). The explants were sub-cultured weekly on corresponding medium freshly prepared. Data was collected on the following traits: callus induction and the shoot induction frequencies (CIF and SIF) were calculated as follows:

$$CIF = \frac{\text{number calli-producing explants}}{\text{total number explants in the culture}} \times 100$$

$$SIF = \frac{\text{number shoots-producing explants}}{\text{total number explants in the culture}} \times 100$$

The LSD analysis was performed using the Analyse-it software LTD (PO box 77, Leeds, LS125XA, UK) according to Maxwell and Delaney (1989).

Elongation and acclimatization

Mature embryos were selected for germination based on their size. Plantlets (3.0 cm in height) were transferred to half strength MS medium in 200 ml flasks. The plantlets which showed a well-developed root system were transferred to sterilized soil in 15 cm plastic pots and irrigated with one tenth MS solution in a humid chamber at 25°C, under a 16/8 h day/night cycle. After acclimatization the plants were grown under greenhouse conditions. The plant regeneration frequency (PRF) was calculated based on:

$$PRF = \frac{\text{number plants-producing shoots} \times 100}{\text{total number of shoots initiated in the culture}}$$

Agrobacterium mediated gene transfer in canola

The *A. tumifaciens* strain LBA4404 harboring the binary vector pBI-121 (Jefferson et al., 1987) was grown overnight in 30 ml of LB medium containing 50 mgl/l kanamycin sulfate (Sigma-Aldrich, Japan) at 28°C, and then collected by centrifugation at 1120 x g for 5 min. The pellet was re-suspended in MS medium containing 100 µM acetosyringon. The hypocotyl segments (0.5 cm in length) were prepared from 6-day-old canola seedlings (cultivars: Serow-4 and Semu-249) and then immersed in the bacterial suspension for 5 min. Thereafter, the segments were blotted on sterilized filter paper, placed onto a co-cultivation medium, which consisted of growth regulators free MS medium (Murashige and Skoog, 1962) containing 100 µM acetosyringon, and then were incubated under dark conditions.

After co-cultivation for three days, the explants were transferred to the callus induction medium containing 1 mg/l 2,4-D, 500 mg/l cefotaxime and 50 mg/l kanamycin sulfate. Two weeks later the explants were transferred onto shoot induction medium supplemented with 4.5 mg/l BA and 500 mg/l cefotaxime in addition to 50 mg/l kanamycin sulfate. The plates were sealed with parafilm and incubated at 25°C under a 16/8-h light/dark photoperiodic

Table 1. Shoot regeneration frequencies on the meristematic end of the hypocotyl of five canola cultivars.

Genotype	No. of cultured explants	Callus inducing explants	Callus induction %	No. of shoot initiations	No. of regenerated Plants	Regeneration %
Sarow-4	600	599	99.8	467	320	68
Masrri L 11	600	600	100	396	191	48
Masrri L-16	600	600	100	180	116	64
Semu-304	600	600	100	363	161	44
Semu –249	600	599	99.8	288	152	52
$LSD_{0.05}$		-		14.76	11.93	

regime (1000-Lux). The explants were sub-cultured weekly on corresponding freshly prepared medium.

PCR analysis

In order to confirm the stable integration of the T-DNA into the plant genome, the putative transgenic plantlets were analyzed by PCR using *NPT-II* gene and 35 S-promoter specific primers. DNA samples were isolated from both of the transformed and non-transformed (control) plantlets according to the method described by Rogers and Bendich (1985). The reaction mixture (20 µl) contained 10 ng DNA, 200 µM dNTPs, 1 µM of each primer, 0.5 units of Red Hot Taq polymerase (ABgene Housse, UK) and 10-X Taq polymerase buffer (ABgene Housse, UK). Samples were heated to 94°C for 5 min and then subjected to 35 cycles of 1 min at 94°C; 1 min at 56°C and 1 min at 72°C. PCR products were separated by (2%) agarose gel electrophoresis and visualized with ethidium bromide. The forward and reverse primers for the *NPT-II* gene were 5`-CGCAGGTTCTCCGGCCGCTTGGG TGG-3` (position: 24-49) and 3`CTGAAGCGGAAGGGACTGGCTGCT-5` (position: 254-277). The sequences of the primers for the 35S-promoter detection were; 5`AAAGGAAGGTGGCTCCTACAAAT-3`and 3`CCT CTCCAAAT GAA ATGATCC-5`, respectively.

Histochemical *GUS* assay

The histochemical assay to screen for the expression of β-glucurodinase (*GUS*) activity in transgenic canola plants was carried out according to the method of Jefferson et al. (1987). For analysis, leaf tissue was incubated in a reaction buffer containing 12.5 mM $K_3Fe(CN)_6$, 12.5 mM $K_4Fe(CN)_6$, 20% methanol, 1% Triton X-100 and 38.3 mM 5-bromo-4-chloro-3-indolyl glucuronide as a substrate for the enzyme. The tissue was incubated in staining solution at 37°C for 24 h and the developed blue spots were recorded.

RESULTS

Hypocotyl explants exhibited an initial swelling followed by callus formation within two weeks of incubation. It was noted that callus proliferation started from cut ends of the hypocotyls on MS medium supplemented with 1.0 mg/l 2,4-D. In general, a high percentage of explants formed callus (99-100%, Table 1). No significant differences in callus induction between the cultivars were observed (Table 1).

Hypocotyl explants-derived calli were placed on regenerating media containing various concentrations of growth regulators. The nodular structures developed into shoot buds when the embryogenic calli were sub-cultured in the medium supplemented with BA within two weeks. The somatic embryos directly emerged from the body of the explants or indirectly germinated from the embryonic callus (Figure 1). Figure 1B depicts the different developmental stages of the plantlets.

The data in Figure 2 indicate that exogenous BA promoted shoot induction in all the cultivars, and the cultivars Sarow-4 and Semu-304 showed higher capability for production of somatic embryos at 4.5 mg/l BA compared with the other cultivars used.

The initiated shoots were matured and germinated on half strength MS medium. The seedlings were acclimatized and transferred to the greenhouse. Table 1 shows that the cultivars differed in the number of regenerated plants. Cultivar Sarow-4 showed higher regeneration percentage (68%) followed by Masrri L-16 (64%) while the cultivar Semu-304 showed the least regeneration frequency (44%).

Hypocotyl explants from the cultivars Sarow-4 and Semu -249 were co-cultivated with *A. tumefaciens* for 3 days. After co-cultivation, the infected hypocotyls were placed on MS medium with low selection pressure. Under these conditions, the ends of the hypocotyls gradually initiated the formation of callus. During the process of selection, the successfully transformed hypocotyls continued to grow vigorously to produce calli, whereas the untransformed ones failed to form callus and eventually bleached and became necrotic within 3 weeks. Shoots were usually regenerated within 4 to 6 weeks on the MS medium after co-cultivation. During the selection culture, sub-culturing the explants with a change of fresh medium containing 50 mg/l kanamycin greatly reduced the number of escapes. The average shoot regeneration percentages for the two tested canola cultivars after *Agrobacterium* infection and selection were 31% and 27%, with a total of 22 and 3 shoots produced for the cultivars Sarow-4 and Semu-249, respectively (Table 2). It was greatly reduced when compared to the 68% and 52% shoot regeneration from culture without the infection of *Agrobacterium* and selection with kanamycin (Table 1).

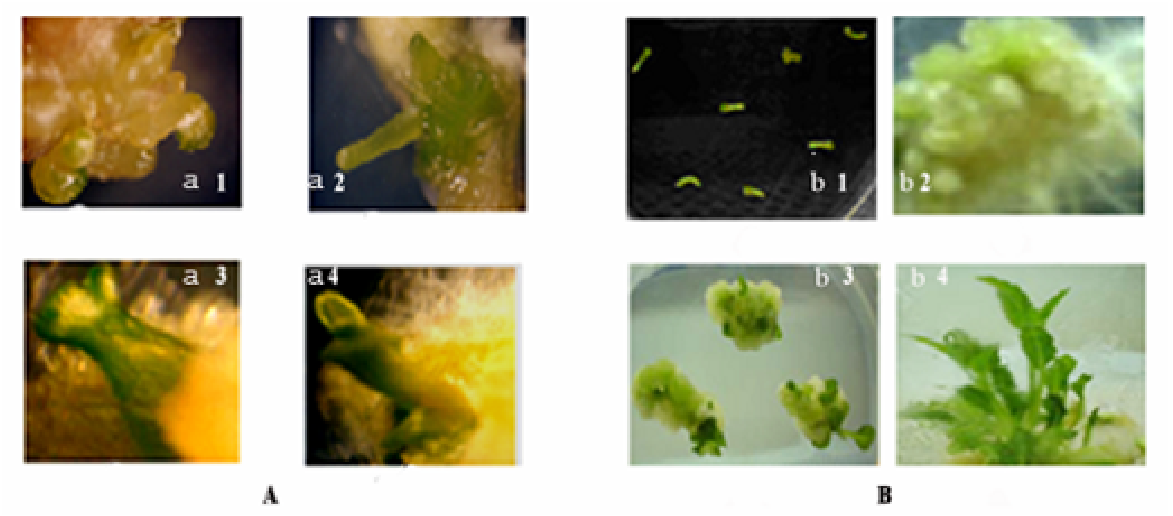

Figure 1. The organogenesis process in canola. (**A**) Different developmental stages of somatic embryos in canola (a1: globular stage, a2: torpedo stage, a3: heart stage and a4: cotyledonary stage. (**B**) Shoot induction in canola (b1: hypocotyl explants, b2: embryonic callus, b3: shoot buds and b4: regenerated plants).

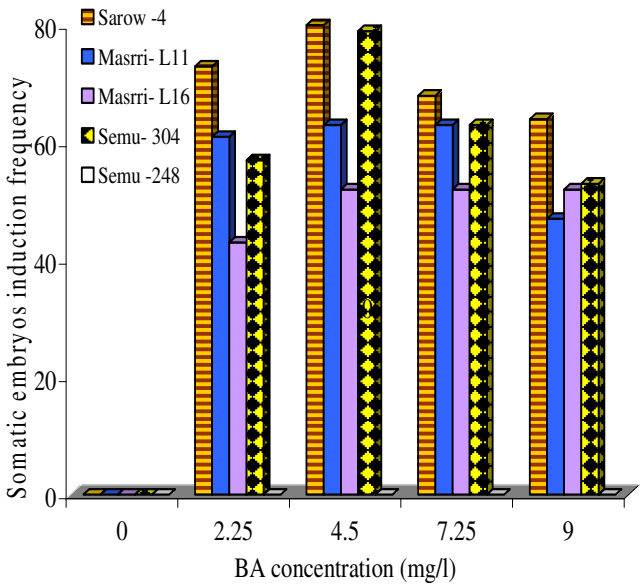

Figure 2. Somatic embryo induction frequency of five canola cultivars under different BA concentrations.

To confirm the presence of the *T-DNA* in the regenerated plants, all R0 plants were subjected to PCR analysis with the primers specific for the *NPT-II* gene and also for the 35 S-promoter. The PCR analysis indicated that all the regenerated plants examined showed a clear band corresponding to the relevant sequence of both of the *NPT-II* gene and the 35-S-promoter (Figures 3A and B). In the present study the kanamycin resistant plants showed GUS expression while the non-transgenic did not show any GUS activity (Figure 4).

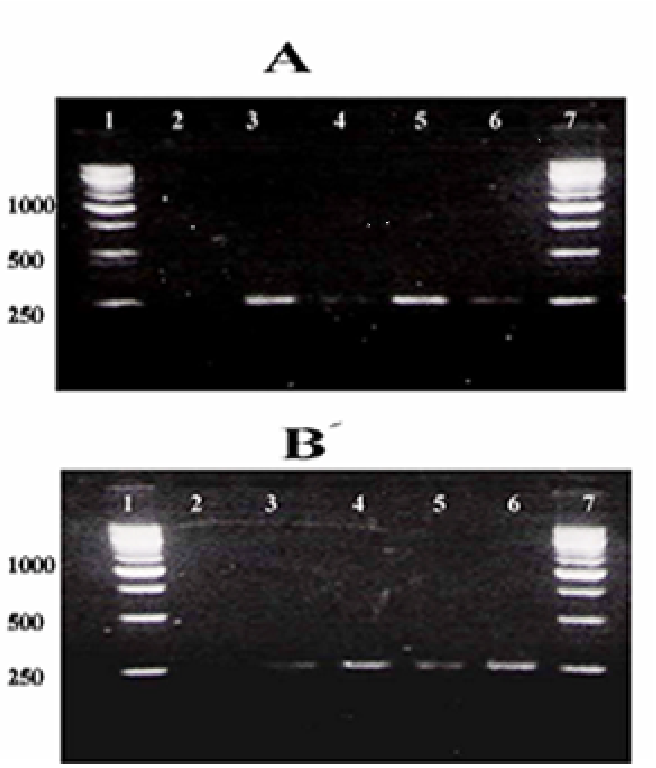

Figure 3. PCR analysis confirming the transformation of *T-DNA* into canola genome. **A.** The stable integration of the *NPT-II* gene into the genome of the regenerated plants. **B.** The detection of the 35-S-promoter in the transformed plants. Lanes 1 and 7: MW (XVI-250 bp ladder, Roche), lane 2: non-transgenic plant, lane 3-6: transgenic plants.

Table 2. Regeneration frequency of *GUS*-expressing plants derived from the hypocotyl explants of two canola cultivars infected with *A. tumifaciens* LBA4404.

Genotype	No. of explants	No of shoots	No. of regenerated plants	Regeneration %	GUS positive plants
Sarow-4	200	70	22	31	19
Semu –249	200	11	3	27	3

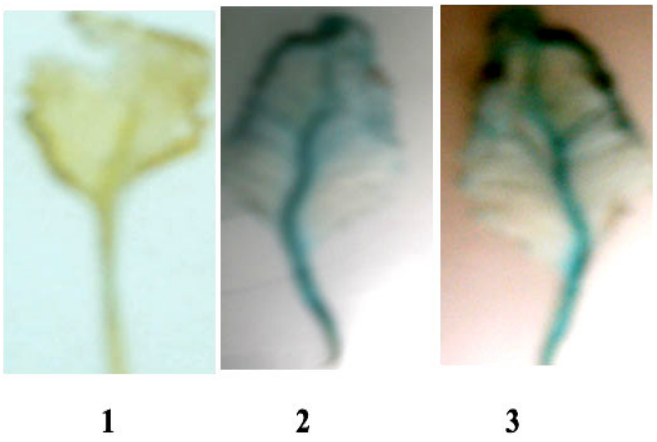

Figure 4. Histochemical GUS assay showing gene expression in transgenic plant leaves (2 and 3) while no expression can be detected in non-transgenic plant (1).

DISCUSSION

The response of explants to culture was observed within one week on callus inducing medium. In general, a high percentage of explants formed callus. A similar result was obtained by Muhammad et al. (2002a). These findings are also consistent with Turget et al. (1998). Narasimhulu and Chopra (1988) reported that shoot production efficiency became higher when the medium was supplemented with higher concentration of BA (2-4 mg/l). Muhammad et al. (2002a) reported that the highest frequency of shoot regeneration was achieved on the medium with 2.0 mg/l BA and 0.5 mg/l IAA using hypocotyl-derived calli. The data indicate that exogenous BA promoted shoot induction and the cultivars Sarow-4 and Semu-304 showed higher capability for production of somatic embryos at 4.5 mg/l BA compared with the other cultivars used. This finding supports the assumption that regeneration in canola is genotype-specific. These data are consistent with the findings reported by Szulc and Drozdowska (1997).

The most common method used for the transformation of *B. napus* is via *Agrobacterium* mediated gene transfer. Both *A. tumefaciens* and *A. rhizogenes* have been used for genetic transformation. *A. tumefaciens* mediated transformation has been attempted using a variety of explants such as hypocotyl (Muhammad et al., 2002b; Cardoza and Stewart 2003), and thin cell layer (Ovesna et al., 1993).

The efficiency of *A. tumefaciens*-mediated transformation technique in oilseed rape is influenced by cultivar specificity, donor plant age and explant type (Poulsen, 1996). Regeneration is also markedly genotype-dependent. The number of *Brassica* cultivars which have so far been used in transformation experiments is relatively limited (Poulsen, 1996). The regeneration protocol of the present study was efficiently used to produce transgenic canola plants expressing the *NPT-II* gene. The average shoot regeneration percentages for the two tested canola cultivars after *Agrobacterium* infection and selection were 31% and 27%, with a total of 22 and 3 shoots produced for the cultivars Sarow-4 and Semu-249, respectively (Table 2). It was greatly reduced when compared to the 68% and 52% shoot regeneration from culture without the infection of *Agrobacterium* and selection with kanamycin (Table 1). These results are in agreement with those reported by Xiang et al. (2000).

To confirm the presence of the *T-DNA* in the regenerated plants, we subjected all R0 plants to PCR analysis with the primers specific for the *NPT-II* gene and also for the 35 S-promoter. The PCR analysis indicated that all the regenerated plants examined showed a clear band corresponding to the relevant sequence of both of the *NPT-II* gene and the 35-S-promoter (Figure 3A and B). Kalfhill et al. (2001) used the green fluorescent gene (*GFP*) as a selectable marker to select the *Bt*-transformed canola plants.

Based on the data of the present study we can conclude that the reported regeneration system is repeatable and can be easily used to regenerate transgenic canola plants expressing the genes present in the *Agrobacterium* binary vector T-DNA. Using this regeneration and transformation protocol we can achieve our main goal which is the production of salt and drought tolerant canola plants.

ACKNOWLEDGMENTS

This research is fully supported by the National Strategy for Biotechnology and Genetic Engineering, Science and Technology Center at the Academy of Scientific Research and Technology, Ministry of Scientific Research. All the technical approaches of the research active-

ties are carried out at the Genetic Engineering Research Center (GERC), Faculty of Agriculture, Cairo University, Giza-Egypt.

REFERENCES

Cardoza V, Stewart CN (2003). Increased Agrobacterium-mediated transformation and rooting efficiencies in canola (*Brassica napus L.*) from hypocotyl segment explants. Plant Cell Rep. 21: 599.604.

Dunwell JM (1981). *In vitro* regeneration from excised leaf discs of *Brassica* species. J. Expt. Bot. 32: 789-799.

Gamborg OLF, Shyluk, JP, Brar, DS, Consble, F (1977). Morphogenesis and plant regeneration from callus of immature embryos of sorghum. Plant Sci. Lett. 10: 67 - 74.

Halfhill MD, Richards HA, Mabon SA, Stewart CN (2001). Expression of GFP and *Bt*-transgenes in *Brassica napus* and hybridization with *Brassica rapa*. Theor. Appl. Genet. 103: 659.667.

Jefferson RA, Kavanagh TA, Bevan MW (1987). GUS fusion. β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907.

Klimaszewska K, Keller WA (1985). High frequency plant regeneration from thin cell layer explants of *Brassica napus*. Plant Cell Tiss. Org. Cult. 4: 183-197.

Lakshmanan P, Siew KN, Loh SC, Goh JC (1997). Auxin, cytokinin and ethylene differentially regulate specific developmental states associated with shoot bud morphogenesis in leaf tissues of mangos teen (*Garcinia mangostana* L.) cultured in vitro. Plant Cell Physiol. 1: 59 - 64.

Lichtenstein C, Draper J (1985). Genetic engineering of plants, In: Glover D.M (ed.), DNA cloning. IRL Press, Oxford. pp. 67-l 19.

Maxwell SE, Delaney HD (1989). Designing Experiments and Analyzing Data. Wadsworth, Belmont, CA. pp. 241-260.

Moloney MM, Walker JM, Sharma KK (1989). High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Rep. 8: 238-242.

Muhammad RK, Rashid H, Quraishi A (2002-a). Effects of various growth regulators on callus formation and regeneration in *Brassica napus* Cv. Oscar. Pakistan J. Biol. Sci. 5: 693-695.

Muhammad RK, Rashid H, Quraishi A (2002-b). High frequency shoot regeneration from hypocotyl of canola (*Brassica napus* L.) cv. Dunkled. Plant Tissue Cult. 12: 131-138.

Murashige T (1974). Plant propagation through tissue culture. Annu. Rev. Plant Physiol. 25: 135 - 166.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.*, 15, 473 - 379.

Narasimhulu SB, Chopra VL (1988). Species specific shoot regeneration response of cotyledonary explants of Brassicas. Plant Cell Rep. 7: 104-106.

Ovesna J, Ptacek L, Opartny Z (1993). Factors influencing the regeneration capacity of oilseed rape and cauliflower in transformation experiments. Biol. Plant 35: 107.112.

Phogat SK, Burma PK, Pental D (2000). High frequency regeneration of *Brassica napus* varieties and genetic transformation of stocks containing fertility restorer genes for two cytoplasmic male sterility systems. J. Plant Biochem. Biotechol. 9: 73-79.

Poulsen GB (1996). Genetic transformation of *Brassica*. Plant Breed. 115: 209-225.

Pua EC, Mehra-Palta A, Nagy F, Chua NH (1991). Transgenic plants of *Brassica napus* L. Bio/Technology 5: 815-817.

Riemenschiender DF, Haissig BE, Bingham ET (1988). Genetic manipulations of woody plants. In: JW Hanover, DE Keathoey (Eds) Plenum Press, New York. pp: 433-494.

Rogers SO, Bendich AJ (1985). Extraction of DNA from milligram amounts of fresh herbarium and mummified plant tissues. Plant Mol. Biol. 5: 69 – 76.

Sharma KK, Thorpe TA (1989). *In vitro* regeneration of shoot buds and plantlets from seedling root segments of *Brassica napus* L. Plant Cell Tiss. Org. Cult. 18: 129-141.

Skoog F, Miller CO (1957). Chemical regulation of growth and organ formation in plant tissue cultivated *in vitro*. Symp. Soc. Exp. Biol. 11: 118- 131.

Sovero M (1993) Rapeseed, a new oilseed crop for theUnited States. In: Janick J, Simon JE (eds). New crops. Wiley, New York. pp. 302-307.

Spector AA (1999). Essentiality of fatty acids. J. Am. Oil Chem. Soc. 34: 51- 53.

Stoutjesdijk PA, Hurlestone C, Singh SP, Green AG (2000). High-oleic acid Australian *Brassica napus* and *B. juncea* varieties produced by co-suppression of endogenous Δ12-desaturases. Biochem. Soc. Trans. 28: 938 -940.

Szulc P, Drozdowska L (1997). The effect of kind of explant and growth regulators on plant regeneration of Polish winter oilseed genotypes. Rosliny Oleiste.

Thrope TA (1993). *In vitro* oganogenesis and somatic embryogenesis : physiological and biochemical aspects. In: (Morphogenesis in Plants). Edited By Roubelakis –Angelakis, K A and Tran Thaan Van K, Plenum Press, New York. pp. 19 –38.

Turget K, Barghchi M, Scott R (1998). Efficient shoot regeneration and somatic embryogenesis from immature cotyledons of *Brassica napus* L. Plant Breeding 117: 503 - 504.

Xiang Y, Wong W KR, Ma MC, Wong RSC (2000). *Agrobacterium*-mediated transformation of *Brassica campestris* ssp. Parachinensis with synthetic *Bacillus thuringiensis cry1Ab* and *cry1Ac* genes. Plant Cell Rep. 19: 251–256.