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DEVELOPMENT OF AN EFFECTIVE TECHNIQUE FOR *IN VITRO* *Agrobacterium* - MEDIATED GENETIC TRANSFORMATION OF WINTER RAPE *Brassica napus* L.

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Abstract

The aim of the research was to improve the genetic transformation and adaptation conditions of commercial winter rapeseed line Brassica napus L. with subsequent production of transgenic seeds.It was optimized the technique of Agrobacterium-mediated genetic transformation of winter rapeseed Bn1 line (Brassica napus L.) using hypocotyls of 6-day-old seedlings as explants. GUS activity histochemical analysis showed a positive reaction in calli cultures and regenerant plant leaves obtained after Agrobacterium tumefaciens GV3101 transformation, which carried pCB203 plasmid with gus and bar genes. The optimal transformation conditions were determined as follows: the bacterial suspension optical density at a 600 nm wavelength - OD = 0.5, 10 min of inoculation and 48 h of co-cultivation. Stable integration of marker genes was confirmed by both histochemical and PCR analysis.

Key words*: Brassica napus, winter rapeseed, genetic transformation, bar gene, gus gene.*

INTRODUCTION

In many countries of the world, rapeseed is primarily cultivated as an oilseed. In the United States, canola's oil has an official safe status for human consumption since 1985. The improvement in the rapeseed oil quality has caused a sharp increase in demand for it throughout the world. Oilseed rape varieties – winter (*Brassica napus* var. *oleifera biennis*) and spring (*Brassica napus* var. *oleifera annua*) do not differ morphologically (U S Pharmacopeia, 1989). However, among the oilseed *Brassicaceae* family winter rape takes first place in oil content, which in its seeds contain 48-52%, 16-29% of proteins, 6.7% of fats and 17% carbohydrates. Rapeseed oil consumes in kind, and it is the best material for the production of sandwich butter, margarine, mayonnaise, dressings and confectionery. Rapeseed oil also produces plastics, paints, varnishes.

In order to improve the genetic characteristics of rapeseed, protocols have been developed for its *Agrobacterium*-mediated transformation since the late 1980s. (Fry et al., 1987; De Block et al., 1989; Moloney et al., 1989). Genetic transformation using agrobacteria has several advantages and remains one of the most common methods for modifying the plant genome. The efficiency of genetic transformation depends on many factors, including the genotype of the original plant, its susceptibility to transformation, explants type, transformation and cultivation conditions. The ability to regenerate plants from transformed cells is also essential for successful transformation (Bhalla & Singh, 2008).

Although some commercial rapeseed lines have already been successfully transformed (Bhalla & Singh, 2008; Mashayekhi et al., 2008; Rahnama & Sheykhhasan, 2016; Bates et al., 2017), to maximize the release of transgenic plants the *Agrobacterium*-mediated genetic transformation protocol should be optimized for each individual genotype. The urgency of the work is that the transformation conditions for the Ukrainian breeding winter rapeseed commercial line should be optimize. This protocol allows obtaining the first transgenic plants in 3-4 months after the beginning of the experiment, and the seeds in 10 months. Thus,

this technique can be use in further studies to create biotechnological rapeseed plants with improved genetic characteristics.

MATERIALS AND METHODS

Plant material and *in vitro* culture

Seeds of *Bn1* winter rape line were kindly provided by Ltd "Ukrainian Scientific Institute of Plant Breeding" (VNIS). Seeds were surface sterilized according to the following method: 70% ethyl alcohol treatment for 5 min, soaking in 1.5% sodium hypochlorite solution for 20-30 min followed by washing in sterile distilled water three times for 5 min. After sterilization, the seeds were planted on a hormone-free nutrient medium MS (Murashige & Skoog, 1962), supplemented with 400 mg/l of antibiotic Ceftriaxone (MSG) and cultivated for 24 h in the dark at 24°C. Further, the dishes were cultured for another 5 days in the culture room at 16 h photoperiod and 24°C.

Callus induction and regeneration

To induce callus formation and shoot regeneration a modified technique (Rahnama & Sheykhhasan, 2016) was used.

3-day old calli cultures obtained from hypocotyl segments (0.5-1 cm) of 6-day old rapeseed seedlings on MS nutrient medium supplemented with 1 mg/l 2.4-Dichlorophenoxyacetic acid (2.4-D) (MSC) were used as explants for genetic transformation.

The organogenesis induction in the transformed tissues was performed on MS nutrient medium supplemented with 4 mg/l 6-Benzylaminopurine (BAP), 2 mg/l N6-(Δ2-Isopentenyl) adenine (2-iP), 5 mg/l $AgNO_3$ and 5 mg/l DL-Phosphinothricin (ppt) as a selective agent (MSO). After 2-3 weeks of cultivation, when the adventitious buds were formed, the explants were transferred to regeneration MS medium containing 3 mg/l BAP, 2 mg/L 2-iP and 8 mg/l ppt (MSR). The shoots elongation was carried out on nutrient MS medium with half macro- and micro-salts content and supplemented with 0.1 mg/l BAP and 3 mg/l ppt (MSE). Rooting of plants was performed on a hormone-free MS medium with half macro- and micro-salts content and supplemented with 3 mg/l ppt (MSA). All media were supplemented with 400 mg/l Ceftriaxone (Ct); the composition of all media is shown in Table 1.

Adaptation and vernalization of plants

The regenerated plants were planted in a peat mixture and grown under greenhouse conditions (16 h photoperiod, 24°C) for 6-8 weeks. For vernalization, the plants were placed in a climate chamber with a temperature 4ºC and an 8 h photoperiod for 8 weeks. After the vernalization, the plants were grown under greenhouse conditions (24°C, 16 h photoperiod); the bud formation was observed after 4-5 weeks. To obtain seeds, all peduncles were covered with plastic insulators and self-pollinated.

Bacterial strains and vectors

Agrobacteria *Agrobacterium tumefaciens,* strain GV3101, containing plasmid pCB203, was used for *Agrobacterium*-mediated gene transfer. Bacterial strain GV3101 (Holsters et al., 1980) was obtained from the Genetic Engineering department Bank of Bacterial strains and Plasmids of the Institute of Cell Biology and Genetic Engineering (Kyiv, Ukraine). The construct contains a β-glucuronidase (*gus*) reporter gene and a selective phosphinothricin acetyltransferase (*bar*) gene that provides plant cells resistance to the Basta® herbicide (the active substance - L-phosphinothricin). The product of the *bar* gene activity is phosphinothricin acetyltransferase, an enzyme that neutralizes phosphinotricin, providing growth and rooting of transgenic plants on a selective medium. Polyubiquitin-1 (Ubi1) promoter and maize introns were used in the vector to achieve high expression levels of these genes (Christensen & Quail, 1996) (Figure 1).

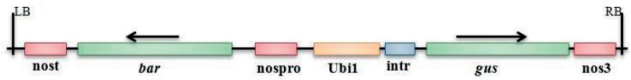

Figure 1. Schematic representation of the pCB203 vector T-DNA

Genetic transformation

A. tumefaciens GV3101 was grown overnight at 28ºC for 18 hours in LB medium (Bertani, 1951), which contained following antibiotics: Rifampicin (Rf) - 50 mg/l, Ampicillin (Amp) -

100 mg/l, Gentamicin (Gm) - 25 mg/l. The bacterial cells were centrifuged and resuspended in the inoculation medium MSI (Table 1). So that the optical density of the bacteria suspension was 0.2, 0.5 or 1.0, at a wavelength of light 600 nm. The inoculation of the pre-cultured hypocotyl explants was performed for 10, 30 or 60 minutes, followed by drying on sterile filter paper. Then the explants were placed on MSC medium (Table 1) and co-cultured in the dark at 24°C. After 48 hours, they were transplanted to MSC medium supplemented with 400 mg/l antibiotic Ceftriaxone to eliminate bacterial cells and cultured at the same conditions for another 10-12 days. Next, the explants were transplanted onto MSO medium (Table 1) supplemented with 5 mg/l ppt for selection of transformed cells followed with increasing of the amount of herbicide in the MSR medium (Table 1) up to 8 mg/l. All media at this stage contained 400 mg/l Ct. Every two weeks explants were transferred onto the fresh medium.

Total DNA extraction

The total DNA was extracted by CTAB method (Sambrook et al., 1989) from leaf material of regenerated canola plants, *B. napus* L., obtained after *in vitro* transformation using *A. tumefaciens* pCB203 genetic construct.

Polymerase chain reaction

The reaction mixtures included: specific primers, 2 µl of PCR buffer 10xDreamTaq™ GreenBuffer (Thermo Scientific) 0.2 mM of each deoxyribonucleoside triphosphate (Thermo Scientific), 2 units polymerase DreamTaq™ DNA Polymerase (Thermo Scientific), 100 ng of total DNA. The reaction mixture was adjusted to a final volume of 20 µl with deionized water Milli-Q.

GUS forward primer: 5'-ATG-GGT-CAG-TCC-CTT-ATG-TTA-3'

GUS reverse primer: 5'-ATA-AAG-ACT-TCG-CGC-TGA-T-3'

The expected band size - 239 bp.

The reactions were performed using the following profiles: initial denaturation 5 min at 95°C, 40 cycles - denaturation 40 sec at 95°C, annealing 40 sec at 50°C, elongation 45 sec at 72°C, final elongation 7 min at 72°C.

Histochemical GUS expression

To analyse the *gus* gene expression in transformed plants, histochemical analysis of GUS activity was performed using method (Jefferson, 1987). The organ and callus fragments were incubated overnight with 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) solution followed by washing in 70% ethyl alcohol.

Table 1. The composition of the nutrient media used in the study

Designation of nutrient medium	Basic salt composition	Sucrose, g/l	Agar-agar, g/l	pH	Growth regulators, mg/L			Other components		
					2.4-D, mg/l	BAP, mg/l	2-iP, mg/l	$AgNO_3$, mg/l	Ct, mg/l	ppt, mg/l
MSG	MS	20	7	5.7-5.8	-	-	-	-	400	-
MSC	MS	30	7	5.7-5.8	1	-	-	-	-	-
MSI	1/2 MS	20	-	5.2	-	-	-	5	-	-
MSO	MS	30	7	5.7-5.8	-	4	2	5	400	5
MSR	MS	30	7	5.7-5.8	-	3	2	-	400	8
MSE	1/2 MS	20	8	5.7-5.8	-	0.1	-	-	400	3
MSA	1/2 MS	20	8	5.7-5.8	-	-	-	-	400	3

Statistical data processing

The experiments were conducted in three replicates. The regeneration frequency of transformed tissues was calculated as the ratio of the explants number that formed organs on a selective medium to the total number of explants used in the experiment. The transformation frequency was calculated as the ratio of PCR-positive plants to the original

number of explants. The results were statistically processed using Microsoft Office Excel.

RESULTS AND DISCUSSIONS

The aim of the research was to improve the *Agrobacterium*-mediated genetic transformation conditions (the duration of inoculation with the agrobacterial suspension, the optical density of the suspension and the composition of the inoculation and regeneration media) of commercial winter rapeseed Ukrainian breeding line in order to develop a cost-effective and efficient transformation technique.

Callus induction and regeneration via hypocotyl explants

0.5-1.0 cm explants obtained from 6-day old rapeseed seedling hypocotyls have proven optimal for callus production (Cardoza & Stewart, 2003; Rahnama & Sheykhhasan, 2016). The cotyledons were separated to ensure the absence of meristem explants. The callus was formed at the cut sites on the third day of cultivation on MSC medium (Table 1). With increasing of pre-cultivation time on MSC medium up to 1 week, further regeneration occurred by rhizogenesis.

Search for transformation conditions for Ukrainian breeding *Bn1* line of winter rape

For transformation of commercial rapeseed hypocotyl explants agrobacterial suspension is used with an optical density at a wavelength of 600 nm (OD_{600}) from 0.2 (Maheshwari et al., 2011), 0.4 (Hussain et al., 2014), 0.5 (Liu et al., 2015) to 0.8 (Mashayekhi et al., 2008) and 1.0 (Hao et al., 2010). Currently the influence of inoculation time on the regeneration and transformation frequency of winter commercial rape lines is not studied yet. To determine the optimal bacterial cell density the following values OD_{600} were used: 0.2, 0.5 and 1. The inoculation time was 10, 30 and 60 min. The control was considered the regeneration frequency of winter rapeseed explants *Bn1* line without inoculation and selection. During the experiments, it was determined that the optical density of bacteria OD_{600} = 0.5 is optimal for inoculation of rapeseed explants, since the regeneration frequency after transformation is significantly reduced at OD_{600} = 0.2 and OD_{600} = 1.0. In addition, at OD_{600} = 1.0, the agrobacterial contamination and necrosis formation frequency increases after co-cultivation (Figure 2).

The maximum rate of explant regeneration (25.02% ± 4.59%) after transformation was observed with tissue inoculation time 10 min (Figure 3). Increasing of inoculation time contributed to the significant necrosis and contamination of tissues, which made the process of obtaining regenerated plants more complicated (Figure 3).

Often, to increase the *Agrobacterium*-mediated transformation frequency, acetosyringone (50-200 μM), a synthetic phenolic compound that activates *A. tumefaciens* virulence genes, is added into the inoculation medium (Boulter et al., 1990; Cardoza & Stewart, 2003; Mashayekhi et al., 2008; Hao et al., 2010; Maheshwari et al., 2011; Hussain et al., 2014; Liu et al., 2015; Rahnama & Sheykhhasan, 2016; Ravanfar et al., 2017). It was established, that the addition of acetosyringone to the inoculation medium is not appropriate because it causes necrosis of rapeseed tissues and reduces the winter rape *Bn1* line regeneration frequency by 20%.

Step-by-step selection with increasing of phosphinothricin concentration in the regeneration MSR medium up to 8 mg/l allowed to select the maximum number of transgenic plants and to reject false-positive variants. In order to form roots by the plants, the ppt concentration was reduced to 3 mg/l in MSE and MSA media.

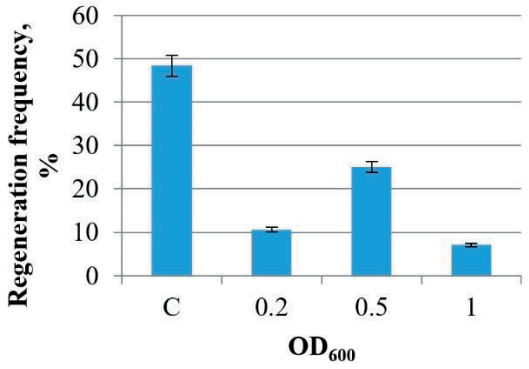

Figure 2. Dependence of the regeneration frequency of winter rapeseed *Bn1* line on the selective MSR medium on the optical density of the bacterial suspension OD at a wavelength 600 nm (with 10 min inoculation); C - Control

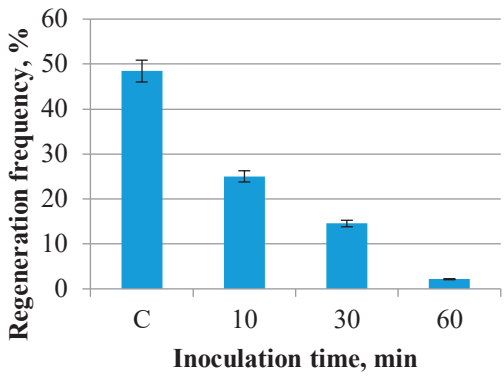

Figure 3. Dependence of the regeneration frequency of winter rapeseed *Bn1* line on the selective MSR medium on the inoculation time (optical density of bacterial suspension OD_{600} = 0.5); C - Control

Checking the marker gene stable integration

Histochemical analysis of GUS activity revealed the β-glucuronidase expression in regenerated plants and in all fertile winter rapeseed plants of *Bn1* line (Figure 4).

PCR by the *gus* gene confirmed the presence of the transgene in 20 of the 22 rapeseed plants.

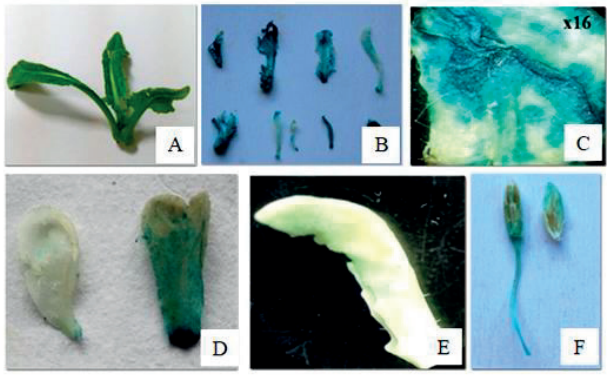

Figure 4. Histochemical analysis of β-glucuronidase expression in plant tissues:

A - winter rape *Bn1* line regenerated plant, obtained after genetic transformation using the pCB203 construct; *B, C, F* - the β-glucuronidase expression in leaves and buds of transformed rapeseed; *D* - negative and positive control (*Nicotiana tabacum* L.); *E* - negative control (*Brassica napus* L.)

Thus, the transformation rate of winter rapeseed *Bn1* line is 22.75% ± 2.28% (Figure 5).

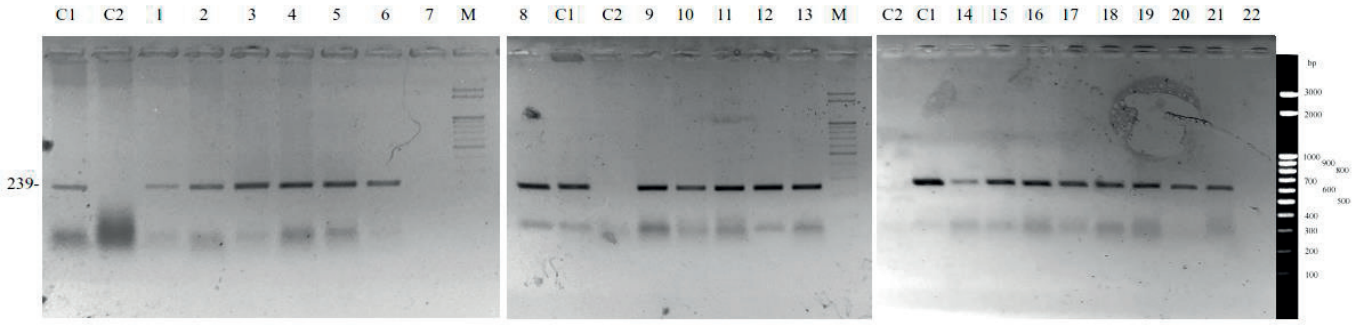

Figure 5. PCR analysis of transgenic rape plants using *gus* specific primers:

Lanes 1-22 - test samples; *C1* - positive control - *Agrobacterium tumefaciens* GV3101 pCB203 colony; *C2* - negative control - intact rapeseed *Bn1* line DNA; *M* - a molecular marker DNA LadderMix

Winter rapeseed *Bn1* line transgenic seeds obtaining

After rooting on MSA medium all regenerated winter rapeseed *Bn1* line plants were planted in peat mixture and vernalized by (Savelieva & Tarakanov 2014; Filek et al., 2007; Waalen et al., 2014). Seeds were obtained from vernalized winter plants (Figure 6).

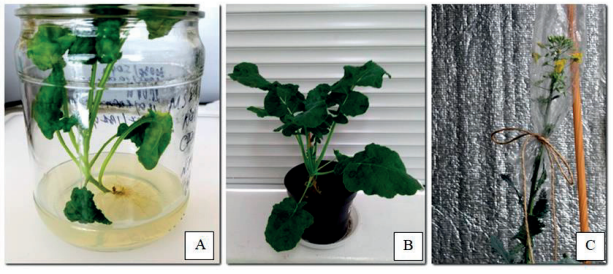

Figure 6. Transgenic seeds of winter rapeseed Bn1 line obtaining after transformation by pCB203 vector:
A - rooting of regenerated plants on the MSA medium;
B - adaptation of plants to the *in vivo* conditions;
C - isolation of peduncles after vernalization for seeds obtaining

CONCLUSIONS

This study presents an optimized protocol for *Agrobacterium*-mediated genetic transformation of Ukrainian breeding winter rape. The aim of transformation was to choose the optimal inoculation time with the agrobacterial suspension, suspension optical density and the composition of the inoculation and regeneration media. We selected following parameters: the optical density of the bacterial suspension for plant inoculation should be OD_{600} = 0.5, and the inoculation time - 10 min. The addition of acetosyringone caused a negative effect on the rapeseed regeneration rate in our experiments. Also in our work we used 2-iP as a cytokinin instead of Zeatin, which is 5 times more expensive than the first one. According to the PCR results, the transformation frequency of the *Bn1* line by the *gus* gene is 22.75% ± 2.28%. After vernalization of rapeseed plants seeds, that do not morphologically differ from native, were obtained. Thus, this technique can be used to obtain biotechnological winter rape plants and further research.

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REFERENCES

Bates, R., Craze, M., Wallington, E.J. (2017). *Agrobacterium*-mediated transformation of oilseed rape (*Brassica napus*). *Current Protocols in Plant Biology*, 2, 287-298.

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology*, 62, 293-300.

Bhalla, P.L., Singh, M.B. (2008). *Agrobacterium*-mediated transformation of *Brassica napus* and *Brassica oleracea*. *Nature Protocols*, 3, 181-189.

Boulter, M.E., Croy, E., Simpson, P., Shields, R., Croy, R.R.D., Shirsat, A.H. (1990). Transformation of *Brassica napus* L. (oilseed rape) using *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* - a comparison. Plant Science, 70, 91-99.

Cardoza, V., Stewart, C.N. (2003). Increased *Agrobacterium*-mediated transformation and rooting efficiencies in canola (*Brassica napus* L.) from hypocotyl segment explants. *Plant Cell Reports*, 21, 599-604.

Christensen A.H., Quail, P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research*, 5, 213–218.

De Block, M., De Brouwer, D., 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.

Filek, M., Koscielniak, J., Macháčková, I., Krekule, J. (2007). Generative development of winter rape (*Brassica napus* L.) - The role of vernalization. *International Journal of Plant Developmental Biology*, 1, 57-63.

Fry, J., Barnason, A., Horsch, R.B. (1987). Transformation of *Brassica napus* with *Agrobacterium tumefaciens* based vectors. *Plant Cell Reports,* 6, 321-325.

Hao, Y., Charles, C.T., Glick, R.B. (2010). ACC deaminase increases the *Agrobacterium tumefaciens*-mediated transformation frequency of commercial canola cultivars. *FEMS Microbiol Lett,* 307, 185-190.

Holsters, M., Silva, B., Van Vliet, F., Genetello, C., De Block, M., Dhaese, P., Depicker, A., Inze, D., Engler, G., Villarroel, R., Van Montagu, M. Schell, J. (1980) The functional organization of the nopaline A. tumefaciens plasmid pTiC58. *Plasmid*, 3, 212–230.

Hussain, S., Rasheed, A., Latif, M., Mahmood, T., Saqlan Naqvi, S.M. (2014). Canola (*Brassica napus* L.) regeneration and transformation via hypocotyl and hypocotyl derived calli. *Sarhad Journal of Agriculture*, 30, 165-172.

Jefferson, R. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Molecular Biology Reporter, 5. 387-405.

Liu, X.X., Lang, S.R., Su, L.Q., Liu, X., Wang, X.F. (2015). Improved *Agrobacterium*-mediated transformation and high efficiency of root formation from hypocotyl meristem of spring *Brassica napus* "Precocity" cultivar. *Genetics and Molecular Research*, 14, 16840-16855.

Maheshwari, P., Selvaraj, G., Kovalchuk, I. (2011). Optimization of *Brassica napus* (canola) explant regeneration for genetic transformation. *New Biotechnology,* 29, 144-155.

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

Moloney, M.M., Walker, J.M., Sharma, K.K. (1989). High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Reports*, 8, 238-242.

Murashige, T., Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15, 473-497.

Rahnama, H., Sheykhhasan, M. (2016). Transformation and Light Inducible Expression of *cry1Ab* Gene in Oilseed Rape (*Brassica napus* L.). *Journal of Sciences*, 27, 313-319.

Ravanfar, S.A., Orbovic, V., Moradpour, M., Abdul Aziz, M., Karan, R., Wallace, S., Parajuli, S. (2017). Improvement of tissue culture, genetic transformation, and applications of biotechnology to *Brassica. Biotechnology and Genetic Engineering Reviews*, 33, 1-25.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Savelieva, E.M., Tarakanov, I.G. (2014). Control of flowering in canola plants with various response to photoperiodic and low-temperature induction. *Izvestiya of Timiryazev Agricultural Academy*, 2, 57-68.

Waalen, W.M., Stavang, J.A., Olsen, J.E., Rognli, O.A. (2014). The relationship between vernalization saturation and the maintenance of freezing tolerance in winter rapeseed, *Environmental and Experimental Botany*, 106, 164-173.

***U S Pharmacopeia, United States Pharmacopedia Co (1989). *United States Pharmacopia Information*. Consumer Reports Books.