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ESTABLISHMENT OF REGENERATION AND TRANSFORMATION OF *Brassica napus*

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Canola (*Brassica napus* L.) ranked as the fifth amongst economically essential crops, following rice, wheat, maize and cotton (Sovero, 1993, Cardoza and Stewart, 2003). Canola is an essential source of vegetable oil and protein-rich meal worldwide, it ranks 3rd after palm oil and soybean and is considered as the second largest protein meal produced in the world after soybeans (economic report, USDA, ERS, 2012). Canola oil has many desirable qualities (high-oleic acid content) making it a desirable substitute for saturated palm oil in food industries (Spector, 1999, Stoutjesdijk *et al*., 2000). Moreover, high-oleic acid oils have extra nutritional benefits because oleic acid contains cholesterol-lowering properties, whereas saturated fatty acids tend to raise blood cholesterol levels (Stoutjesdijk *et al*., 2000). Genetic engineering techniques along with classic breeding methods have made it likely to produce new varieties of canola. There are information of gene transformation in canola that can create plants with innovative characteristics such as oil composition, tolerance to herbicides (De Block *et al*., 1989), protein composition and resistance to insects (Stewart *et al.*, 1996). *In vitro* regeneration and transgenic plant selection competence are two essential elements in molecular breeding (Cardoza and Stewart, 2003). Regeneration and gene transformation velocity to canola varieties are very changeable and is extremely dependable on genotype

(Khehra and Mathias, 1992, Poulsen, 1996, Cardoza and Stewart, 2004). Even though diverse transformation methods were applied in canola, *Agrobacterium* method is the main method of choice. Variety of plant tissues such as stem internodes (Fry *et al.*, 1987), stem segments (Pua *et al*., 1987), cotyledons (Moloney *et al*., 1989, Sharma *et al*., 1990, Hachey *et al*., 1991, Ono *et al*., 1994,), hypocotyls segments (Dunwell, 1981, Radke *et al*., 1988, De Block *et al*., 1989, Yang *et al*., 1991, Stewart *et al*., 1996), peduncle segments (Eapen and George, 1997), epithermal and sub-epidermal thin layer cells (Klimaszewska and Keller, 1985), roots (Sharma and Thorpe, 1989, Chi *et al*., 1990) and protoplast (Hu *et al*., 1999), have been applied as explants for gene transformation in canola plant. Cotyledon and hypocotyls have been recommended as the finest explants in regeneration and transformation experiments (Zhang and Bhalla, 2004). In a research implemented 100 varieties, the regeneration rate was 0 to 91% (Ono *et al*., 1994). *Agrobacterium*-mediated transformation requires high frequency regeneration technique (Zhang and Bhalla, 2004).

In Egypt, there is a serious gap in oil crop production (exceeding 90%, according to FAOSTAT, 2010), therefore, increasing oil production is a necessity, yet due to limitation in land and suitable irrigation water forcing strategic planners to expand plantation into new reclaimed desert area. In the current work, we anticipated on establishing a regeneration and transformation system for two varieties of canola that are well adapted for Egyptian condition as a first step to introduce canola cultivation into new reclaimed area.

MATERIALS AND METHODS

Seeds of two canola cultivars (Sarow-4 and Bactool) were kindly provided from Oil Crops Research Department, Field Crops Research Institute, Agriculture Research Center (ARC), Giza, Egypt. Seed sterilization was conducted by treating with 70% (v/v) alcohol for 1 min, followed by treatment with 20 % sodium hypochlorite (v/v) for 20 min. Seeds were rinsed several times with sterile distilled water and then placed on germination medium containing inorganic basal salt MS mixture (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose and 10 g/l agar. The pH was adjusted at 5.8 before autoclaving. All cultures within here were maintained in a controlled growth chamber at 26°C±2 under 8/16 h (dark/light) fluorescent lights.

Canola regeneration

Hypocotyls segment, were excised from 6-day-old canola seedlings and cultured on MS basal salt mixture supplemented with 0.5 mg/l BAP, 0.5 mg/l $AgNO_3$ and different concentrations of 2,4-D (0.0, 0.5, 1, 1.5 and 2 mg/l). Each experiment was conducted at least 3 times and each experiment had a total number of 100 explants. Calli pieces were then transferred to MS basal salt mixture supplemented with 4 mg/l BAP, 5 mg/l $AgNO_3$ along with different concentrations of

NAA (0.0, 0.1, 0.2, or 0.3 mg/l). Cultures were maintained on a freshly prepared medium every 4 weeks. Well- elongated shoots (5-4 cm) were cultured on rooting media supplemented with different concentrations of IBA, (0, 0.1, 0.2, 0.3, 0.4, or 0.5 mg/l IBA). Successfully rooted shoots were acclimatized in pots filled with equal volumes of peat moss, sand and clay, pots were covered with plastic bags for further 7-10 days under a photoperiod of 16/8 h light/dark in controlled greenhouse conditions.

Statistical analysis

Data for regeneration was statistically analyzed according to Silva (2011). The differences between means were compared using Duncan multiple test (1955).

Canola transformation

Plasmid and bacterial strain: The plasmid pISV2678 which is harboring the gus-intron under CaMV 35S promoter and *nos* terminator and the *bar* gene under *nos* promoter, AMV leader and pAg7 terminator, was cloned in pPin19 plasmid. This vector was kindly provided by Dr. P. Ratet, (ISV, CNRS, Gif-Sur-Yvette, France). The *Agrobacterium tumefaciens* strain LBA4404 was used for canola transformation.

Transformation conditions

Agrobacterium tumefaciens strain LBA4404 containing the binary vector pISV2678 was used throughout the transformation experiments. Transformation was carried out by placing hypocotyls explants on callus-induction medium (CIM) for three days prior of *Agrobacterium* inoculation. After three days, hypocotyls segment were merged in overnight growing bacterial culture for 10 min, explants were allowed to dry on sterile filter paper before being cultured on shoot induction medium. Three days later explants were transferred to a fresh shoot induction medium supplemented with 3 mg/l bialaphos and 300 mg/l carbenicillin. Well-developed shoots were excised and placed on suitable elongation medium until it reached 4-5 cm. Subsequently, shoots were transferred to suitable rooting medium and plantlets were acclimatized in the greenhouse. Each treatment was performed with 10 plates and each plate contains 10 explants with a total number of 100 explants, with three replicates for each treatment, so the total number of explants was 300 explants for each cultivar.

Survival curve

Explants of canola *cvs*. (Sarow-4 and Bactool) were cultured on regeneration medium supplemented with different concentrations of bialaphos, i.e., 0.0, 1.0, 2.0, 3.0, and 4.0 mg/l (Dovzhenko and Koop, 2003). Incubation conditions were the same as mentioned before. Three weeks further, the concentration of bialaphos which kill all explants was used in selection medium in the routine work of the plant transformation.

Histochemical gus assay

GUS-activity detection was applied

according to Jefferson *et al*. (1987). Two days post *Agrobacterium* inoculation explants were immersed in 1 ml GUS buffer and rapped with aluminum foil. The samples were then incubated on a rotary shaker (150 rpm) at 37°C for color development. Twelve hours later, buffer was removed and 70% ethanol was added for a few min and color development was scored.

Leaf painting

Recommended dose (2 g/l Basta) of Basta herbicide was used for leaf painting of canola. Non-transformed canola leaves were also treated to serve as a control. After a week the leaves showed colorless and others turned brown (necrosis) were considered as positive and negative results, respectively.

PCR analysis

Genomic DNA was isolated from young leaves of canola plants and using CTAB method (Lassner *et al*., 1989). PCR test was conducted using two sets of primers, the 1st set designated to amplify a 540 bp partial sequence of *bar* gene, while the second set were designated to amplify 2070 full length of *gus* gene. (BAR-F: 5'-AAA AGC TTC CAC CAT GAG CCC AGA ACG ACG-3', BAR-R: 5'-AAG GAT CCT CAG ATC TCG GTG ACG G-3', and GUS-F: 5'-CCA GAT CTA ACA ATG CGC GGT GGT CAG TCC C-3' and GUS-R: 5'-CCA GAT CTA TTC ATT GTT TGC CTC CCT GCT GC-3'). PCR reaction was carried in a 50 µl reaction as follow. Initial denaturation of DNA at 94°C for 5 min, 35 cycles comprised of 1 min denaturation at 94°C, 1 min. annealing at 55°C in GUS gene or 60°C in BAR gene, 1 min elongation step at 72°C followed by a final extension step at 72°C for 7 min. Amplicons were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Total RNA isolation and RT-PCR analysis

Total RNA was isolated from canola leaves (100 mg) using the Rneasy Plant Mini Kit (QiIAGEN, USA). The total RNA was converted into first strand cDNA using the commercially available kit (RevertAidTM First Strand cDNA Synthesis Kit, Fermentas, EU). The first strand cDNA was used for PCR reaction as a template. The reaction was conducted at the same conditions as mentioned above.

RESULTS AND DISCUSSION

Canola Regeneration

Regeneration system of canola *Brassica napus* L. *cv*s. Sarow-4 and Bactool was established using hypocotyls segment as explants. Different explants have been previously used for canola regeneration. Cardoza and Stewart (2004) and Ali *et al.* (2007) illustrated that hypocotyls stayed the most attractive explants for regeneration and transformation of canola due to their high regeneration ability. Additionally, Khan *et al.* (2010) showed that the percentage of regenerated shoots from explants varied greatly be-

tween the *B. napus* L. cultivars, Cyclone, Star and Westar, the shoot induction rate for hypocotyls in the three cultivars is higher than the cotoledenary explants, while there was no major difference between the different explants and among the canola cultivars for callus induction. In this study, hypocotyls segment, as explants, and different concentration of plant growth regulators were used for canola regeneration system establishment. For callus induction, results herein showed that hypocotyls explants exhibited a primary growth followed by callus development starting from cut ends of the hypocotyls within two weeks on media with different concentrations of 2,4-D. High percentage of friable calli with different size was produced and ranged from (97-100%) on callus induction medium (CIM) containing 1 mg/l 2, 4-D which revealed the best medium for callus formation for Sarow-4 and Bactool, respectively. This results is in agreement with Moghaieb *et al*. (2006) and Khan *et al*. (2010) which obtained high callus formation percentage 92-100 % of canola cultivars Sarow-4, Star, Westar and Cyclone using 1mg/l 2,4-D. In addition there was no major difference observed between both cultivars Sarow-4 and Bactool in callus induction, while in the case of shoot formation, Bactool was superior in shoot formation comparing with Sarow-4 cultivar.

Data in Table (1) illustrate that shoots were successfully differentiated and elongated for both cultivars on media supplemented with BAP (4 mg/l) and $AgNo_3$ (5 mg/l) and NAA with different concentrations, i.e., 0.1, 0.2 and 0.3 mg/l. In addition, medium RG3 with 0.2 mg/l NAA gave the highest percentage of shoot development (63.6% and 75.2%) for Sarow-4 and Bactool, respectively, followed by medium RG2 containing 0.1 mg/l NAA for Bactool giving percentage for about 70.5% and RG4 contains 0.3 mg/l for Sarow-4, with percentage of 57.5%. The lowest percentage of shoot formation was obtained in the absence of NAA in the two cultivars as it gives a percentage of 20% and 21.2% for Bactool and Sarow-4, respectively. On the other hand, statistically analysis showed significant differences among the treatment except the media RG2 and RG3 in the case of cultivar Bactool and with media RG3 and RG4 in Sarow-4.

Our regeneration frequency is located between the regeneration frequencies that had been previously obtained by Ali *et al*. (2007) who reached a regeneration frequency between (67-82%). It is an important to mention that shoot differentiation was obtained in the absence of 2,4-D as it is support callus formation but was not useful for shoot differentiation due to high rate of vitrification that it promoted. It was also noticed that callus size did not associate with shoot formation efficiency. These results agree with the results of Zhang *et al*. (2006) who reported that bigger calli did not generate more shoots. The influence of hormone regime is one of the most important factors for shoot regeneration (Khehra and Mathias, 1992). BAP is the major cytokinin efficiently used for canola shoot differentiation. Ali *et al*.

(2007) reported the combined BAP with NAA in addition to $AgNo_3$ reached canola regeneration frequency up to 67-82%. However, Al-Naggar *et al*. (2008) obtained a regeneration frequency 34%-40% when using only 2,4-D and BAP. Jain *et al*. (1988) show that BAP in combination with NAA inhibited shoot regeneration. Moghaieb *et al*. (2006) reached a regeneration frequency of about 68% in the canola cultivar Sarow-4 using shoot induction medium at high concentration (4.5 mg/l BAP). MS medium with a combination of 2 mg/l BAP and 0.2 mg/l IAA was recommended by Sharma *et al*. (2004) and Singh *et al*. (2009) as the best medium for canola regeneration. In the current study, silver nitrate was used at concentration of 5 mg/l in both canola cultivars Sarow-4 and Bactool. It's clearly know that $AgNo_3$ plays an important role of increase regeneration efficiency as Ag^+ ions can prevent ethylene-induction which is a potential inhibitor of many plant regeneration systems (Vain *et al*., 1989). Silver nitrate was also shown to be helpful in improving somatic embryogenesis and plant regeneration in a number of crop species including *Brassica* species (Zhang and Ling, 1995, Eapen and George 1997, Kuvshinov *et al*., 1999). Ali *et al*. (2007) was the first study the establishing regeneration system in three *Brassica napus* L. cultivars (Star, Cyclon and Westar) using silver nitrate at concentration 0.5mg/l resulting an efficient regenable callus formation.

Regenerated shoots were rooted on root inducing medium containing different concentration of IBA. Results in Table (2) shows that medium R4 containing 0.3 mg/l IBA revealed high significant variation among the other media under study of both cultivars. On the other hand, there are no significant differences observed among the other treatments. In addition, medium R4 gave the highest percentage (62.9% and 45% for Bactool and Sarow-4, respectively), followed by R5 medium containing 0.4 IBA (51.8% and 30%, for Bactool and Sarow-4, respectively). Therefore, R4 was the best medium using for root formation in both cultivars in this study. These results indicate that IBA is preferring auxine for producing root formation in canola.

Rooted canola cultivars (Sarow-4 and Bactool) plantlets were transferred after 3-4 weeks from rooting stage to pots containing sterile peat-moss for acclimatization under greenhouse conditions for two months. The main principle of acclimatization of canola from *in vitro* culture is to put it under conditions, where air humidity can be gradually lowered (Hazarika, 2003). Figure (1) illustrates the steps of regeneration system.

Canola Transformation

Due to the increase demands of canola oil, there are more research studies being pursued in improving cano1a breeding. Maheshwari *et al*. (2011) reported that canola is among the first crops to be genetically transformed, and genetically modified cultivars are in commercial production at very significant levels. Conventional breeding technique is time consum-

ing and it takes at least 8-10 generations to develop a new variety. Genetic engineering is an alternative method which reduces the time needed to develop new varieties. A significant amount of effort has been reported before trying to develop *A. tumefaciens*-mediated transformation effectiveness of canola, including choose the best plant material for the transformation and optimization of the infection and regeneration protocols (Zhang *et al*., 1999, Cardoza & Stewart, 2003, Zhang and Bhalla, 2004, Zhang *et al.*, 2005, Bhalla and Singh, 2008). *A. tumefaciens*-mediated transformation is conceder the pest option for transgenic plant production in many species because of its coast value, simplicity in handling, and high efficiency (Cardoza and Stewart, 2006, Qamarunnisa *et al*., 2008, Hao *et al*., 2010).

In this study, transformation system was established for both canola cultivars i.e., Sarow-4 and Bactool using hypocotyls segment as an explants and *Agrobacterium* containing plasmid plSV2678 as a source of *gus* and *bar* genes for selecting the proper concentration of bialaphos as a selective agent. Hypocotyls segment were cultured on different concentrations of bialaphos (0.0, 1.0, 2.0, 3.0 and 4.0 mg/l). Results showed that no explants survive on the medium with 3 mg/l bialaphos.

To improve transformation efficiency, pre-treatment of explants was performed in this study by applying the explants on CIM medium before the co-cultivation step for 3 days. Under these conditions, the hypocotyls cut ends gradually formed callus within 2-3 weeks. 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 on bialaphos medium. Trying to improve the factors affecting *Agrobacterium*-mediated transformation and regeneration of sweet orange and citrage. Yu *et al*., (2002) recommended the addition of 2,4-D in the explants pretreatment medium as it improved the transformation efficiency by decreasing the escape frequency.

Four weeks later, calli were transferred on RG3 medium with 3 mg/l bialaphos and 500 mg cefotaxime until the resistant callus produced the whole plantlets. Transformation rate was varied between the two cultivars as it reached 10.6% in Sarow-4 while, Bactool was only 8% (Table 3). Different rate for transformation were preview only in different canola genotype plants by different researchers. This variation in regeneration rate between the regenerated and transformed materials is generally due to the effect of *Agrobacterium*. Mathews *et al.* (1990) concluded that regeneration of transformed explants decrease the regeneration frequency after co-cultivation with *Agrobacterium*. Mashayekhi *et al*., (2008) reported that it cannot compare gene transformation rates obtained in different research due to different genotypes and

explants type used. Trying to establish a regeneration and transformation system in *Brassica juncea*, Sharma *et al*. (2004) illustrate that the regeneration frequency obtained using cotoledenary petiole or hypocotyls as explants was not high enough for *Agrobacterium* mediated transformation. Also Moghaieb *et al*. (2006) reported 9.5 and 1.5% transgenic plants creation in Sarow-4 and Semu-249 cultivars, correspondingly. Mashayekhi *et al*. (2008) demonstrated that transformation rate in canola plant ranged between 0.2% to 15.26% depending on genotypes and type of explants. Using $AgNO_3$ in a regeneration medium benefit for *Agrobacterium* enhancement, as ethylene produced by plants is one that inhibits *A. tumefaciens*-midiated transformation efficiency. For example, it has been reported that reducing the ethylene level increased the expression of the *vir* genes of *A. tumefaciens*, thereby increasing gene delivery efficiency (Nonaka *et al*., 2008). Moreover, application of ethylene inhibitors such as amino ethoxyvinylglycine or silver ions in the tissue culture medium has been reported to improve the transformation efficiency of many plant species, such as bottle gourd, cauliflower, apricot and apple trees (Chakrabarty *et al*., 2002, Burgos and Alburquerque, 2003, Han *et al*., 2005, Petri *et al*., 2005, Seong *et al*., 2005). Obtained putatively transgenic plants were transferred to the greenhouse for acclimatization. Two months later all plants (24 Bactool and 32 Sarow-4) survived under the greenhouse conditions

Molecular analysis

It is universal for population of putatively transgenic plants to be primary screen by PCR for integration of transgene(s) and then by RT-PCR for the integration of that gene(s) (Andrew and Minocha, 2004).

Polymerase Chain Reaction (PCR)

In vitro regenerated green shoots were utilized to isolate genomic DNA for molecular studies. All tested plants, 24 Bactool and 32 Sarow-4, were positive for *gus* and *bar*. The PCR amplification was carried out on isolated genomic DNA using *bar* and *gus* primers. Fragments of 540 bp and 2070 bp, were amplified representing *bar* and *gus* genes respectively, however, the control plants did not show any amplification product confirming the successful genes integration.

Histochemical assay

To detect *gus* expression, histochemical GUS assay was performed using x-gluc substrate on plant materials. All tested plant materials developed blue color with differed intensity which could be explained by the position effect of DNA integration (Fig. 2).

Leaf painting assay

Putative transgenic plants were treated with the recommended dose of Basta herbicide (2 g/l Basta) to prove the expression of the *bar* gene. The transgenic plant leaves were resistant to the herbicide

(stay green), while non transgenic as well as control plant leaves became sensitive to the herbicide and they turned yellow then dark brown (necrosis) within two days (Fig. 3).

RT-PCR analysis

RT-PCR is a quick and reliable technique for transgene expression analysis as an additional method to prove the expression of the gene. All canola plants that showed both bar positive results and gave the expected amplified fragment PCR analysis, were further subjected to RT-PCR using the specific oligonucleotides as primers. RT-PCR patterns presented in Fig. (4) showed that the tested plants give the expected fragment (540 bp). In the same time, control non-transformed clones gave no amplification products. All herbicide resistant canola plants gave positive results in PCR and RT-PCR, which means that the integration and expression of the *bar* gene was stable, the same results were obtained by Saker (2003).

Finally we can conclude that a reliable regeneration and transformation system were established for two of canola cultivars (Sarow-4 and Bactool) that are adapted to the Egyptian environment. In this study, a regeneration and transformation system was established for a new Egyptian canola cultivar (Bactool), results showed that this cultivar was superior on Sarow-4 in regeneration efficiency but less than Sarow-4 in transformation rate, so we tried to rise the efficiency of transformation for canola cultivar Sarow-4 that obtained 10.6% comparing with Moghaieb *et al*. (2006) that attained 9.5% only. This efficient regeneration and transformation system is being used to transfer the agronomically important genes, which are not possible to transfer *via* the conventional breeding methods.

SUMMARY

Production of genetically enhanced canola varieties with desirable new traits requires the establishment of efficient regeneration and transformation protocols. In the current work, experiments were conducted using two commercial cultivars Sarow-4 and Bactool, hypocotyl explants and *Agrobacterium* strain LBA4404 (harboring pISV2678 plasmid, which contains both *gus* and *bar* genes as reporter and selectable marker, respectively). Six days old hypocotyls segment were co-cultivated on callus induction medium (CIM) containing MS basal salt mixture and supplemented with 1mg/l 2,4-D for 3 days, *Agrobacterium* inoculation was conducted and cultures were incubated for 3 days before being placed on selection medium for a further 11 days. Two weeks old cultures were transferred to shoot induction RG3 medium supplemented with 4 mg/l BAP, 0.2 mg/l NAA, 5 mg/l $AgNo_3$, 500 mg cefotaxime and 3 mg/l bialaphos. Shoots were rooted on rooting R4 medium containing 0.3 mg/l IBA. Regenerated plantlets were successfully established in soil and plants were allowed to produce seeds. T_1 seeds were used for evaluation of transformation frequency using

histochemical GUS assay, leaf painting, PCR and RT-PCR. Our results indicated higher transformation frequency in Sarow-4 cultivar when compared to Bactool.

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Table (1): Effects of different concentrations of NAA on shoot formation for canola cvs. Bactool and Sarow-4.

Media	Bactool			Sarow-4		
	No. of calli	No. of shoots	%	No. of calli	No. of shoot	%
RG1: MS free hormones (control)	85	17c	20	66	14c	21.2
RG2: MS medium + 0.1mg/l NAA	85	60a	70.5	66	29b	43.9
RG3: MS medium + 0.2 mg/l NAA	85	64a	75.2	66	42a	63.6
RG4: MS medium + 0.3 mg/l NAA	85	43b	50.5	66	38a	57.5

Different letters indicate differ significantly at $p \leq 0.05$ according to Duncan Test at a level of 5% of probability.

Table (2): Effects of different concentrations of IBA growth regulators on root formation.

Media	Bactool			Sarow-4		
	No. of shoots	No. of pro-duced roots	% of pro-duced roots	No. of shoots	No. of pro-duced roots	% of pro-duced roots
R1: MS free hormones (control)	27	3d	11.1	20	1b	5
R2: MS medium + 0.1mg/l IBA	27	7cd	25.9	20	3b	15
R3: MS medium + 0.2 mg/l IBA	27	11bc	40.7	20	5ab	25
R4: MS medium + 0.3 mg/l IBA	27	17a	62.9	20	9a	45
R5: MS medium + 0.4 mg/l IBA	27	14ab	51.8	20	6ab	30
R6: MS medium + 0.5 mg/l IBA	27	12b	44.4	20	5ab	25

Different letters indicate differ significantly at $p \leq 0.05$ according to Duncan Test at a level of 5% of probability.

Table (3): Total number of callus, shoot and root regenerated after transformation by *Agrobacterium tumefaciens* LBA4404 strain carrying plasmid plSV2678.

Replicates		No. of Callus		No. of shoot		No. of root	
B	S	B	S	B	S	B	S
300	300	190	162	29	38	24	32
% trans.		63.3	54	9.6	13	8	10.6

B: Bactool, S: Sarow-4, % trans.: transformation percentage.

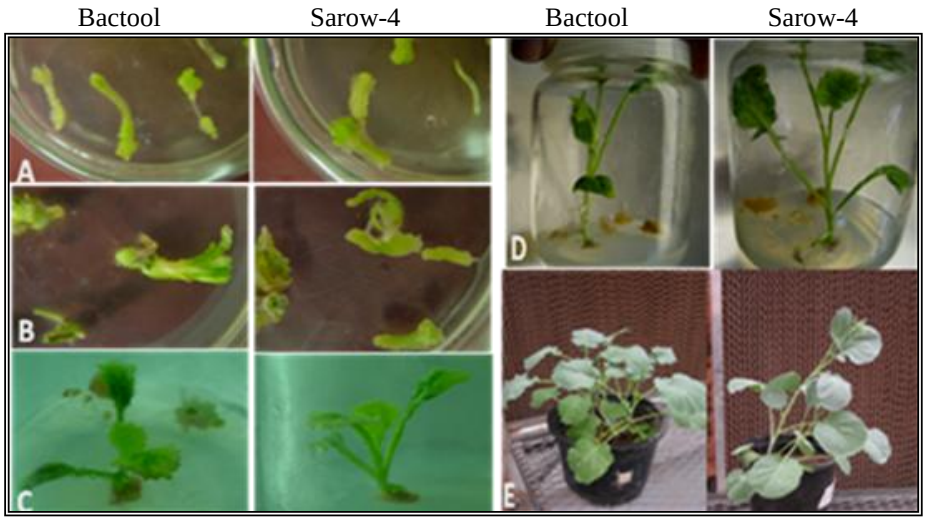

Fig. (1): Regeneration steps of canola cultivars Bactool (left), Sarow-4 (right) using hypocotyls explant. A: Callus induction stage after 2 weeks, B: Shoot differentiation stage, C: Elongation stage, D: Root formation stage and E: Acclimatization stage after 1-2 months.

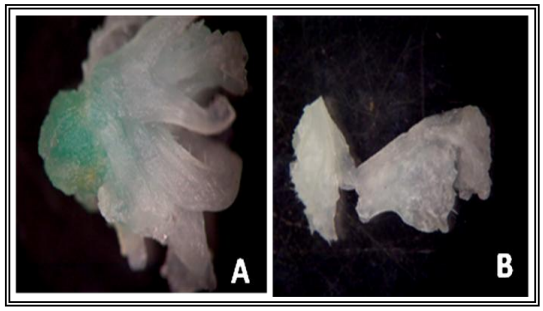

Fig. (2): GUS assay of leaves explants of canola cvs. Transformed with *Agrobacterium tumefaciens* LBA4404 strain carrying plasmid plSV2678. A: positive sample, B: control sample

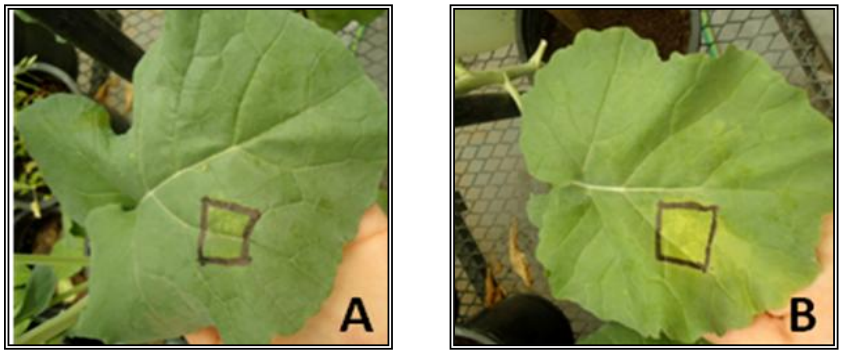

Fig. (3): Leaf painting assays by using Basta herbicide after one week. (A: positive sample, B: control sample).

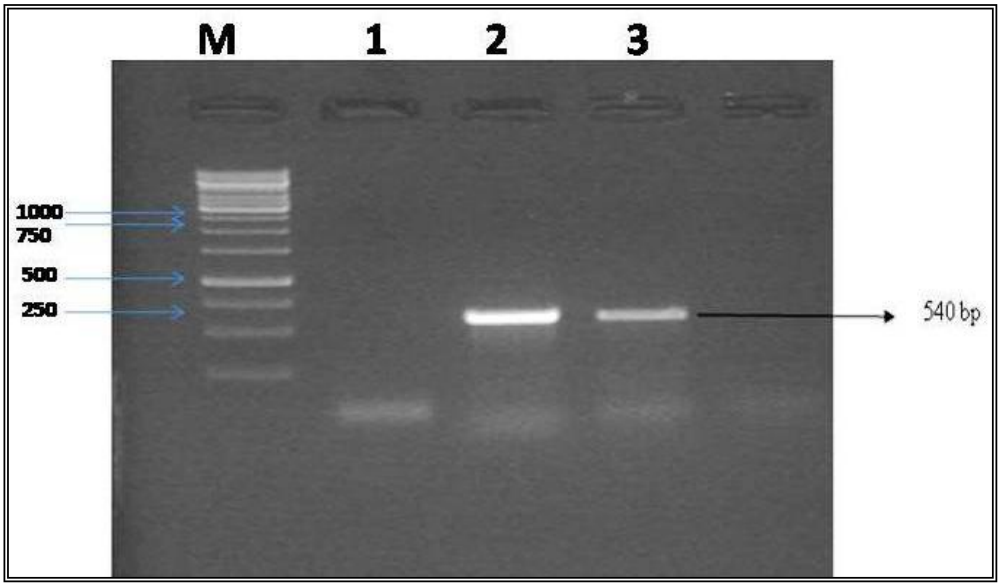

Fig. (4): RT- PCR confirmation for *bar* gene from cDNA using bar-specific primers to amplify 540bp of the gene. M: 1 kb DNA ladder, 1: non-transformed plant, 2: Positive control and 3: transformed plants