Australian Journal of Crop Science AJCS
AJCS 5(4):479-486 (2011) ISSN:1835-2707

In vitro propagation and *Agrobacterium*-mediated transformation of safflower (*Carthamus tinctorius* L.) using a bacterial mutated *aro*A gene

Javad Motamedi [1, 2], Alireza Zebarjadi[* 1, 3], Danial Kahrizi[1, 3], Ali Hatef Salmanian[4]

[1]Agronomy and Plant Breeding Department, Faculty of Agriculture, Razi University, Kermanshah, Iran
[2]Young Researchers Club, Islamic Azad University, Branch of Kermanshah, Iran
[3]Biotechnology for Drought Tolerance Research Department, Razi University, Kermanshah, Iran
[4]National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

***Corresponding author: zebarjadiali@yahoo.com; zebarjadi@razi.ac.ir**

Abstract

Callus induction and *in vitro* plantlet regeneration systems following *Agrobacterium*-mediated transformation using a mutated bacterial *aro*A gene were obtained in two cultivars of safflower (*Carthamus tinctorius* L.). Mean comparison showed that the highest percentage of induced callus was occurred on MS medium containing (1 mg l^{-1} NAA + 1 mg l^{-1} BAP) in cv. Dincer (94.33%) and (0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BAP) in hypocotyl explant (97%), respectively. In addition, the highest percentage of shoot regeneration also was achieved on a range of media supplemented with 0.1 mg l^{-1} NAA + 2 mg l^{-1} BAP from cotyledon explant of Dincer cultivar (35.1%). In respect of transformation efficiency, the highest percentage of putative regenerated shoots on selective medium containing 50 mg l^{-1} kanamycin was achieved in cv. Dincer and LBA4404 strain (20.61%). PCR analysis of sixteen Dincer plantlets for both strains confirmed that mutated *aro*A gene was amplified using specific primers (EPS1F/EPS2R) and (EPS2R/35SF) yielded fragments of 1300 bp and 1800 bp, respectively whereas no plantlets contained this gene in Sina cultivar. This study revealed that cotyledon explant of cv. Dincer of safflower have a good potential for direct shoot regeneration, and *A. tumefaciens* LBA4404 can be established a beneficial method for the transformation of this oilseed crop by mutated *aro*A gene.

Keywords: Callus induction; Direct shoot regeneration; EPSPS; Plant transformation; Safflower
Abbreviations: *aro*A- Aromatic amino acids; BAP- 6-Benzylaminopurine; IBA- Indole-3-butyricacid; NAA- α-Naphthalene acetic-acid; PGR- Plant growth regulator(s)

Introduction

Safflower (*Carthamus tinctorius* L.) owes its importance to the demand for oil, which results from the highly polyunsaturated/saturated ratios of fatty acids coupled with elevated levels of α-tocopherol (Sujatha, 2002) as they help in reducing the cholesterol level in blood (Singh and Nimbkar 2006). Moreover, safflower is reported to be the best example of a crop with variability for fatty acid composition in seed oil (Knowles, 1989). Glyphosate is a broad-spectrum, post-emergence herbicide that blocks plant growth by inhibiting the production of aromatic amino acids, leading to arrest of protein production and prevention of secondary compound formation. The glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a nuclear-encoded chloroplast-localized enzyme in the shikimic acid pathway of plants and microorganisms (Ye et al., 2001). The EPSPS is of agronomic importance since it is the primary target of the nonselective herbicide glyphosate and weeds have evolved limited resistance to glyphosate (Baerson et al., 2002). Bacterial forms and mutants of EPSPS with decreased affinity for glyphosate have been discovered and used to confer resistance to glyphosate in plants (Wang et al., 2003). Reports of a mutation in the EPSPS of *E. coli* conferring glyphosate resistance in transgenic maize involved the double mutations T102I and P106S (Dill, 2005) and in transgenic rapeseed involved the G96A and A183T substitution (Kahrizi et al., 2007; Kahrizi and Salmanian, 2008). Genetically, engineering herbicide-resistant cultivars would be highly worthwhile from the producers' viewpoint, because the primary agronomic problem related with safflower production is the lack of effective herbicides (Ying et al., 1992). Tissue culture techniques have been developed for the Indian and American safflower cultivars. In general, the mode of regeneration was through direct or indirect organogenesis (Orlikowska and Dyer, 1993; Nikam and Shitole, 1999; Mandal and Gupta, 2001; Radhika et al., 2006; Basalma et al., 2008). Production of transgenic safflower via *Agrobacterium tumefaciens*-mediated transformations is only reported in Centennial, A-1 and A-300 cultivars (Ying et al., 1992; Orlikowska et al., 1995; Rohini and Sankara Rao, 2000). In the present work we optimized the callus culture protocol and to assess direct regeneration system for safflower in Dincer (a high yielding Turkish cultivar) and Sina (a landrace Iranian cultivar). In addition, we report the successful transformation of safflower by bacterial mutated *aro*A gene via *Agrobacterium tumefaciens*.

Materials and methods

Plant material

Seeds were surface-disinfected with 70% (v/v) ethanol for 1 min, treated with 0.1% (v/v) (g l^{-1}) mercuric chloride ($HgCl_2$) for 7 min, thoroughly washed with sterile distilled water four times, and were blot-dried inside a laminar hood. The sterile seeds were then placed on to MS medium (Murashige and

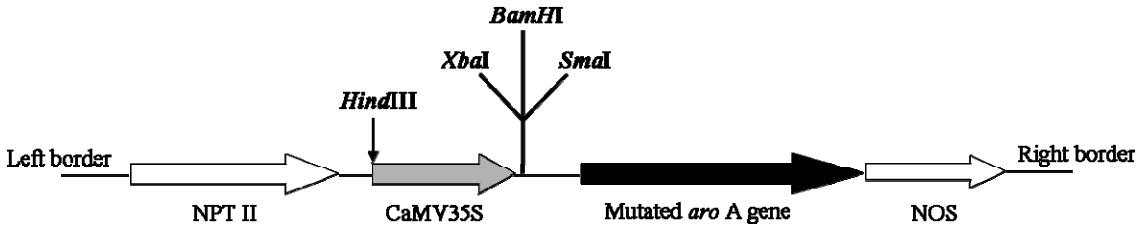

Fig 1. Schematic representation of T-DNA region of pBI121 expression vector containing mutated *aro* A gene (Kahrizi et al., 2007).

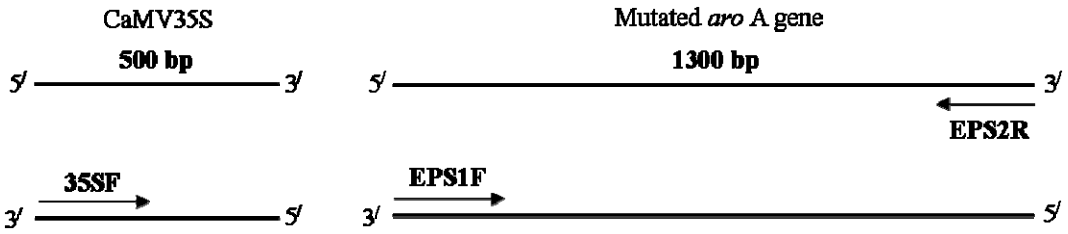

Fig 2. Schematic representation of placing specific primers and size of amplified fragments in PCR analysis.

Skoog, 1962) containing 0.7% (v/v) (g l^{-1}) agar and 3% (v/v) (g l^{-1}) sucrose without any plant growth regulator (PGR) and incubated at 25°C under the 16-h light/8-h dark photoperiod for germination. After 10 days the hypocotyls with one centimeter long were excised and cultured on the same media. The cotyledons were sectioned transversely (1×1 cm^2) and used as explants.

Plant culture Media and growth conditions

The MS basal salts and B_5 vitamins supplemented with various concentrations of α-naphthaleneacetic acid, NAA (0, 0.5 and 1 mg l^{-1}) either singly or in combination with 6-benzylaminopurine, BAP (0, 0.5 and 1 mg l^{-1}) for callus induction and NAA (0, 0.1 and 0.2 mg l^{-1}) in combination with BAP (0, 1 and 2 mg l^{-1}) for direct shoot organogenesis were used throughout the experiments. The cotyledon explants were placed on culture medium with abaxial surface. The percentage of hypocotyls and cotyledons producing calli and shoot regeneration were determined after 21 and 35 days, respectively. The efficiency of plant growth regulators and their concentrations were analyzed on the basis of visual observation and expressed as the percentage of callus formed and shoots produced per explant. All the cultures were incubated at 25±1°C under cool-white light with a 16-h photoperiod (40-60 µmol m^{-2} s^{-1}), relative humidity 35-40% and subculture on fresh media at 14 days interval. All the components were supplied by Duchefa (The Netherlands)

Root induction and acclimatization

Direct regenerated shoots fromhypocotyls and cotyledons were excised , rinsed with tap water and transferred to the rooting medium containing MS salts supplemented with IBA (2 mg l^{-1}). Rooted plantlets were transferred to a sterile pot mixture and acclimatized in a growth chamber with 14-h light and 10-h dark photo-period at 25±1°C for 14 days.

Statistical analysis and experimental design

The experiment was laid out as a factorial experiment based on completely randomized design with three replications and each replicate was made by using 3 Petri dishes per medium which contains nine explants. Therefore, each treatment comprised 27 explants and was repeated twice. In the first experiment, the rate of callus induction and shoot regeneration from cotyledon and hypocotyl explants were measured in two cultivars (Dincer and Sina) in front of different concentrations of NAA and BAP phytohormones. The transformation efficiency for two cultivars was evaluated with two *Agrobacterium tumefaciens* strains as a factorial based on completely randomized design. Each treatment consisted of 6 explants per Petri dish with 3 replicates. Analysis of variance was calculated, and significance of differences between means was conducted using Duncan's multiple range test at P=0.01. Data given in percentages were subjected to Arc sin (√X) transformation before statistical analysis.

Bacterial strains and plasmid construct

A. tumefaciens LBA4404 and GV3101 strains were used for plant transformation. Bacteria were grown in LB medium at appropriate temperatures (28°C) with shaking (200 rpm). Recombinant pBI121-*aro*A plasmid was used as a binary plant expression vector. The vector contains the bacterial mutated *aro*A gene (G96A and A183T) driven by the CaMV 35S promoter and the nopaline synthase terminator (Kahrizi et al., 2007) (Fig.1).

Plant transformation procedures

The cotyledon segments (1×1 cm^2) were immersed in a suspension of *A. tumefaciens* containing the recombinant plasmid pBI121-*aro*A for 1-2 min with constant shaking, blotted with sterile filter paper to remove excess bacteria, and co-cultivated on MS solidified medium containing 0.1 mg l^{-1} NAA and 2 mg l^{-1} BAP for 48 hours. Explants were then transferred onto selective medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin, and subcultured onto fresh selective medium every 15 days. Un-inoculated cotyledons were used as a negative control. After four weeks, the number of regenerated green shoots on selective medium to the number of cultured explants was determined. The

Table 1. Features of specific primers which used for transformation of safflower.

Primer name	Sequence	Site of restriction enzyme	Purpose	Forward/ Reverse	Paired with	Expected size (bp)
EPS1	5'-CGG,GAT,CCA,TGG,AAT, CCC,TGA,CGT,TAC, AA-3 (29mer)	GGATCC (*Bam*HI)	Amplify considered part of *aro* A gene	F	EPS2	1300
EPS2	3'-GCG,GAT,CC T,CAG,GCT,GC C,TGG,CTA,AT C-5'(27 mer)	GGATCC (*Bam*HI)	Amplify considered part of *aro* A gene	R	EPS1	1300
35S	5'-GGC,GAA,CAG,TTC, ATA, CAG,AGT,CT-3'(23 mer)	-	Amplify part of CaMV 35S promoter and *aro* A gene	F	EPS2	1800

Table 2. Analysis of variance for callus induction and shoot regeneration of safflower.

S. O. V	df	MS	
		Callus induction	Shoot regeneration
A (cultivars)	1	4231.26 **	291.39 **
B (explants)	1	3136.33 **	288.83 **
AB	1	4008.92 **	11.46 ns
C (NAA)	2	6334.75 **	65.71 **
AC	2	17.56 ns	1.37 ns
BC	2	5194.36 **	47.07 **
ABC	2	2669.28 **	3.52 ns
D (BAP)	2	4069.33 **	426.78 **
AD	2	404.03 *	27.72 **
BD	2	1204.11 **	93.80 **
ABD	2	167.14 ns	6.91 ns
CD	4	1035.91 **	92.58 **
ACD	4	383.42 *	11.04 *
BCD	4	6065.47 **	705.95 **
ABCD	4	175.42 ns	20.24 **
Error	72	112.67	3.44
CV (%)		15.51	14.12

ns: Non-significant, * and **: Significant at 0.05 and 0.01 probability levels, respectively. S.O.V: Source of variance, df: Degree of freedom, MS: Mean square CV: Coefficient of variance.

elongated shoots were transferred to rooting medium consisting of MS basal medium, 2 mg l^{-1} IBA and 500 mg l^{-1} cefotaxime. All cultures were incubated at 25±2°C under a 16/8 hours photoperiod.

Genomic surveys and PCR screening of transformants

Genomic DNA was extracted from young leaves of green putative transgenic and non-transgenic (control) plants, by the cetyl-trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). Recombinant pBI121-*aro*A plasmid was used as positive control. Integration of the desired gene into the plant genome was confirmed by amplification of CaMV 35S promoter/mutated *aro*A regions by PCR method and using specific primers (Fig 2 and Table 1). PCR analysis was performed to confirm the presence of a 1800 bp (CaMV-*aro*A) and a 1300 bp (*aro*A) gene fragments in the putative transformants. All the procedure carried out by the methods described previously (Kahrizi et al., 2007). The amplified fragments were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

Results

Callus induction and direct shoot regeneration

After 14 - 21 days, most of the hypocotyls and cotyledons became green and increased slightly in volume, and subsequently the compact callus was formed at the cut edges of the each explant (Fig.3). Significant and non-significant differences among main levels of NAA, BAP concentrations and their interactions have been represented in Table.2. There was a significant interaction among cultivar×explant×NAA concentrations on the frequency of callus induction ($P<0.01$). Induction of callus was observed in all media and there was no statistically difference between cultivars in responding to NAA concentrations (Table.2). In general, cv. Dincer and cotyledon explants were more responsive than cv. Sina and hypocotyl explants in callus formation (Fig.4 a). Considering both interaction among cultivar ×NAA×BAP and explant× NAA×BAP, the highest callus formation frequency in cv. Dincer (94.33%) was achieved on a medium supplemented with (NAA 1 mg l^{-1} + BAP1 mg l^{-1}). In hypocotyl explant of cv. Sina the highest callus formation (97%) was observed when the explant cultivated an media contain NAA (0.5mg l^{-1}) and BAP (0.5 mg l^{-1}) (Fig.4 b,c). According to Table.2, there was no statistically difference between interactions of cultivars × explant for shoot regeneration. A similar response was observed among BAP concentrations in respect of shoot regeneration as well as callus induction. Interestingly, interaction among cultivars× explants× NAA×BAP concentrations was significant for shoot regeneration (Table.2). The percentage of explants producing shoots were influenced by concentrations of NAA and BAP tested ($P<0.01$). The percentage of regenerated shoots was wary 7.94 – 35.1%. The highest percentage of shoots regenerated from cotyledon explant was obtained on a medium containing 0.1 mg l^{-1} NAA and 2 mg l^{-1} BAP (35.1%) in Dincer cultivar (Table.3). Cotyledon explants showed positive morphogenetic response and readily developed multiple shoots compared with

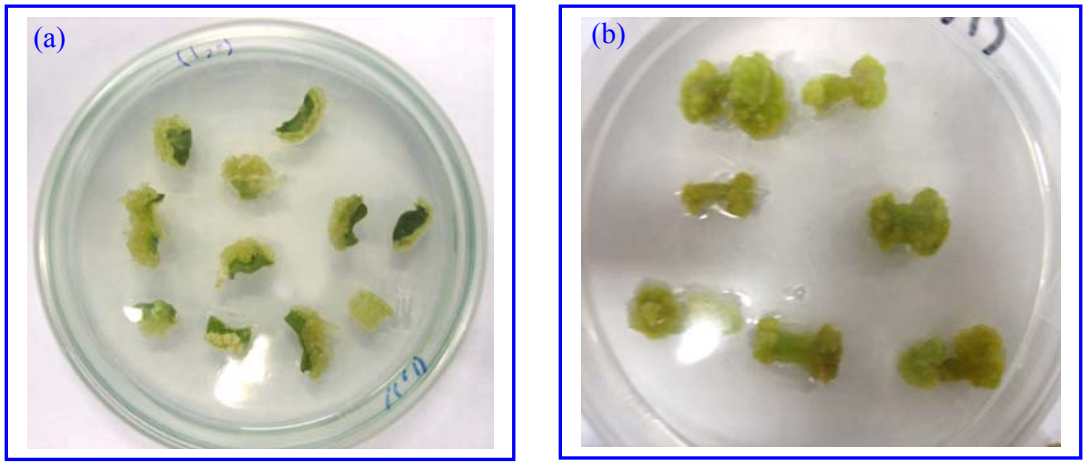

Fig 3. Callus formations after 21 d of culture of safflower cultivars on the medium supplemented with 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} BAP concentrations; *Hint*: callus formation was clearly visible (a) on the margins of the cotyledon and (b) cut surface of hypocotyl explants.

Table 3. Effect of different concentrations of NAA and BAP on direct shoot regeneration from hypocotyl and cotyledon explants of *C. tinctorius* cultivars after 28 - 35 d of culture.

Growth regulator concentrations (mg l^{-1})		% Explant producing shoots in cultivars			
NAA	BAP	Dincer		Sina	
		Hypocotyl	Cotyledon	Hypocotyl	Cotyledon
0	0	5.70 ^{n}	6.5 ^{mn}	5.74 ^{n}	5.70 ^{n}
	1	14.74 ^{efgh}	20.75 ^{cd}	5.74 ^{n}	12.93 ^{ghi}
	2	16.21 ^{efg}	13.18 ^{fghi}	12.45 ^{ghij}	9.88 ^{ijklmn}
0.1	0	11.42 ^{hijkl}	7.94 ^{jklmn}	11.02 ^{hijklm}	7.15 ^{lmn}
	1	17.83 ^{de}	6.53 ^{mn}	12.86 ^{ghi}	6.53 ^{mn}
	2	12.25 ^{ghij}	35.1 ^{a}	8.74 ^{ijklmn}	24.28 ^{c}
0.2	0	9.85 ^{ijklmn}	24.13 ^{c}	7.32 ^{klmn}	17.57 ^{def}
	1	6.50 ^{mn}	15.13 ^{efgh}	5.74 ^{n}	13.36 ^{efghi}
	2	29.23 ^{b}	11.84 ^{ghijk}	22.19 ^{c}	8.93 ^{ijklmn}

Values within a column followed by different letters are significantly different at the 0.01 probability level, analyzed by Duncan's multiple range test.

hypocotyl explants in response to BAP concentrations. This superiority of response to BAP concentrations was also observed in association with cultivars. Formation of initial primordia producing shoots were directly occurred from the explants within 21 d after culture in different concentrations of NAA and BAP and subsequently normal shoots were developed after 28 – 35 days. Moreover, shoot buds emerged directly from the adaxial surface and from the cutting end of the cotyledons and multiple shoots were formed after four weeks. (Fig.5). Regenerated shoots were transferred to medium without growth regulators and subsequently to MS medium supplemented with 2 mg l^{-1} IBA for root formation. Root was formed after 2-3 weeks. Rooted plantlets were transferred to soil pots (Fig.6).

Analysis of putative transformed plantlets

Transformation experiment with mutated *aro*A gene was carried out on the cultivars and *Agrobacterium* strains. Analysis of variance showed that there was a statistical difference between cultivars and *Agrobacterium* strains but their interaction was not significant (Table.4). The highest percentage (20.61%) of putative regenerated shoots on selective medium containing 50 mg l^{-1} kanamycin was achieved on cv. Dincer and LBA4404 strain; hence cv. Sina and GV3101 strain had the lowest percentage (8.6%) of shoot regeneration. Regeneration was not observed in non-transformed control plants, but the transgenic plants displayed a regeneration frequency of approximately 17% in the medium containing 50 mg l^{-1} kanamycin. After 4 weeks the regenerated (1.5 – 3 cm) were transferred to rooting medium.

Molecular analyses of transformed safflower

The PCR technique was used for molecular analyses of transgenic safflower plantlets. The presence of mutated *aro*A gene and the junction of CaMV 35 S promoter–*aro*A were checked by amplification of 1300 bp and 1800bp fragments, respectively. No amplification was observed in control DNA from non-transgenic plant (Fig.7 a and b). PCR analysis of sixteen Dincer cultivar showed that ten plantlets harboring the mutated *aro*A gene under the control of CaMV 35S promoter (Fig.7 a and b), whereas no amplification was observed for Sina cultivar. This data indicated that in safflower transformation of Dincer cultivar was more efficient than Sina. The frequency of transformation reached 10% in Dincer cv. (Table.5).

Discussion

Safflower has attracted very little attention as far as tissue culture and genetic transformation are concerned. Initial efforts in safflower were led to develop suitable culture conditions for whole plant propagation. Several biochemical

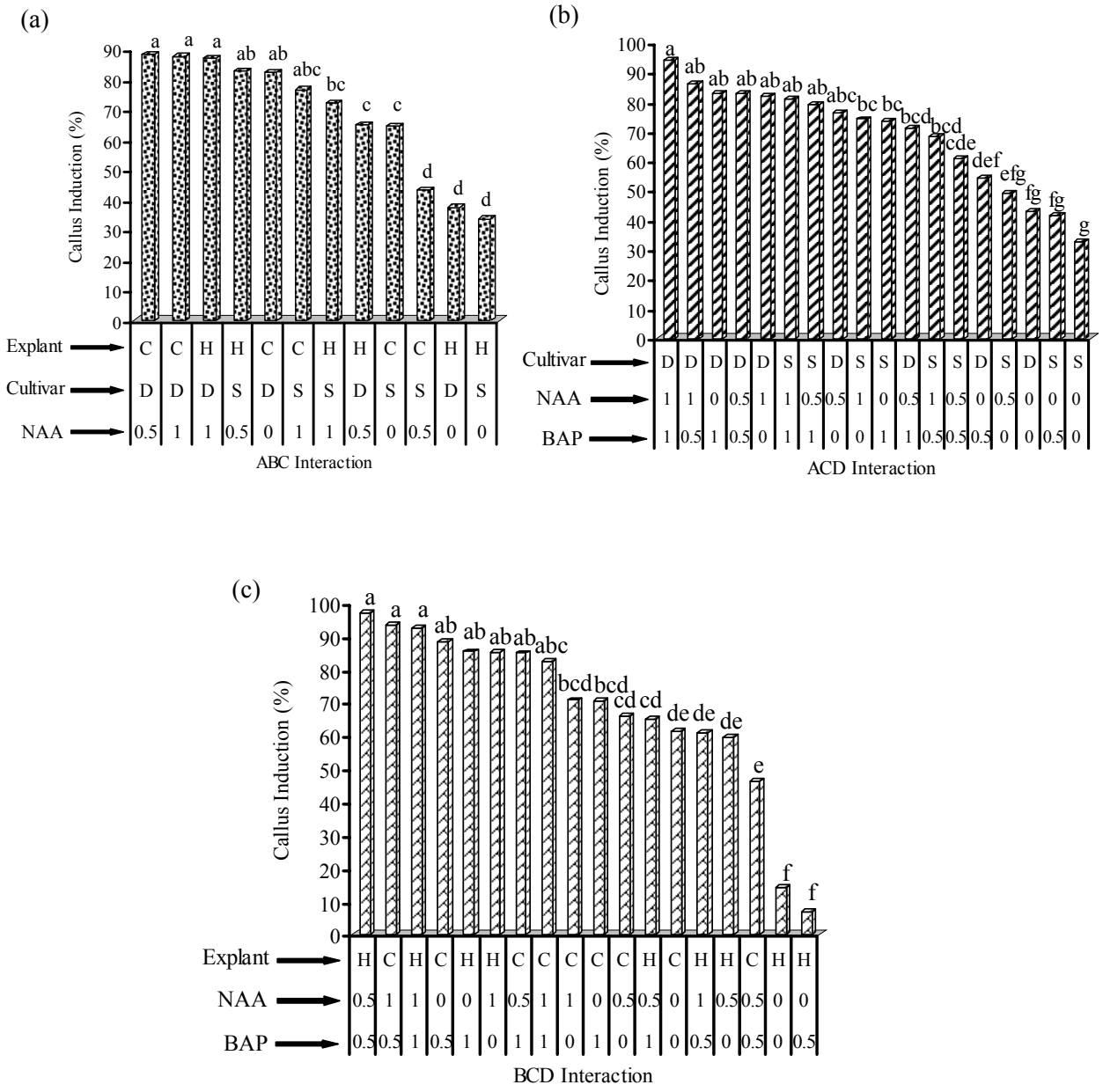

Fig 4. (a), (b) and (c) Mean comparison among cultivar and explant interactions with different levels of NAA and BAP concentrations on callus induction; values within a column followed by different letters are significantly different (P<0.01), analyzed by Duncan's multiple range test. Hint: D: Dincer, S: Sina, H: hypocotyl and C: cotyledon.

Table 4. Analysis of variance for putative transformed shoots of safflower in response to transformation with mutated *aro* A gene.

SOV	df	MS
		Putative transformed shoots
A (cultivars)	1	150.87 **
B (strains)	1	143.03 **
AB	1	32.70 ns
Error	8	11.21
CV (%)		15.67

ns: non-significant,**: Significant 0.01 probability level, S. O. V: Source of variance, df: Degree of freedom, MS: Mean square CV: Coefficient of variance.

processes are required for differentiation during shoot morphogenesis in plants. In general, the explant type, its orientation in the culture medium, and PGRs play a key role in regulating the differentiation process (Chawla, 2000). Selection of a suitable explant at correct development stage plays a key role in the successful establishment of culture under *in vitro* conditions. Morphological integrity of an explant along with the proper choice of plant growth regulators strongly influence induction of optimal callus and shoot regeneration (Khawar et al., 2005). Based on our finding, in different concentration of BAP concentrations the cotyledon explant in both cultivars (Dincer and Sina) had the better response to callus induction and shoot regeneration than hypocotyl explant. Many reports has been represented which

Table 5. Summary of transformation events in safflower cultivars Dincer and Sina.

Cultivars	No. of cotyledons infected	No. of putative shoots obtained		PCR analysis (mutated *aro* A gene)		Frequency (%)
		GV3101	LBA4404	GV3101	LBA4404	
Dincer	90	$6/_{45}$	$18/_{45}$	0	$9/_{45}$	10
Sina	90	$2/_{45}$	$2/_{45}$	0	0	0

supported accuracy of this method in other species or cultivars of safflower (Orlikowska and Dyer, 1993; Nikam and Shitole, 1999; Mandal and Gupta, 2001; Sujatha and Dinesh Kumar, 2007; Basalma et al., 2008). It seems, the cotyledon explant of safflower has a great organogenic potential for direct shoot formation from adaxial surface compared with other explants. Mandal and Gupta, (2001) stated that main cause of this superiority may be related to development of meristematic zones in subepidermal cells on the adaxial side of cotyledon explant. In fact, repeated divisions of subepidermal cells resulted in the formation of meristemoids, the progenitors of shoot buds. In other study, Basalma et al., (2008) confirmed that adventitious shoot buds were induced directly on the adaxial surface of cotyledon explants of safflower. Our result was in agreement with these reports and clearly underlines the importance of cotyledon explants in efficient organogenesis of safflower. Direct regeneration of shoots, another consequence that obtained from culture of cotyledon tissue is provided the possibility of multiple shoot regeneration which is very important in micropropagation and genetic engineering technique. In direct regeneration the somaclonal variation associated with callus culture could be avoided. The effect of cultivar is significant for callus induction, shoot regeneration and transformation efficiency. The key role of genotype was closely evident in the present study. Similarly, the genotypic effects have been reported for the Indian and American cultivars (see Introduction). In our experiments, the Dincer cultivar had better response than Sina cultivar. the tissue culture of the Dincer cultivar, was investigated by Baslama et al., (2008), also they reported that the range of callus induction was 93.33-100% and the highest percentage of regenerated shoots (33.33%) occurred on media supplemented with 0.5 mg l^{-1} TDZ + 0.25 mg l^{-1} IBA. Our result partially support this finding since that the best percentage of callus induction (97%) and the highest percentage of shoot regeneration (35.1%) on media supplemented with (0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BAP) and (0.1 mg l^{-1} NAA + 2 mg l^{-1} BAP) in cv. Dincer. In most studies, the best results in direct shoot regeneration (33 - 54%) was reported in in the presence of 0.2 - 5 mg l^{-1} of BAP and TDZ concentrations singly or in combination with 0.1 - 0.5 mg l^{-1} of NAA (Orlikowska and Dyer, 1993; Mandal and Gupta, 2001). However, maximum frequency of shoot regeneration in safflower (98.5%) achieved on media supplemented with 0.5 mg l^{-1} TDZ + 0.5mg l^{-1} NAA in HUS-305 cultivar (Radhika et al., 2006). These results show the crucial role of PGR types as exogenous hormones in morphogenic competence of organogenesis and their positive effects in changing of endogenous hormones levels for induction of adventitious shoots in safflower. Another noteworthy observation of this study was poor rooting of regenerated shoots of safflower under *in vitro* conditions especially after genetic transformation. The Dincer and Sina cultivars were found to be largely recalcitrant to rooting. Generally, regeneration frequency and rooting capacity of safflower were low Nikam and Shitole, (1999) and there are many reports on this plant which failed shoots to root induce formation in regenerated shoots. (Ying et al., 1992; Orlikowska and Dyer, 1993; Rohini and Sankara Rao, 2000). However, the investigations of Mandal and Gupta, (2001) and Radhika et al., (2006) show the amenability of safflower roots for organogenesis which is unlike our finding where root tissues had the lowest regenerability. This probably attributed to the discrepancies in the genetic determinants controlling organogenesis in Indian and American cultivars *vs*. other cultivars. The results of Baker and Dyer, (1997) show that, shoots infected with *A. rhizogenes* in cv. Centennial produced large numbers of fibrous roots, but shoots did not elongate or could not survive when transfer to soil. In disagreement our finding Basalma et al., (2008) reported that regenerated shoots of Dincer cultivar were rooted readily and all of the developing roots were physically vigorous and healthy. As previously described, we observed superiority of LBA4404 strain of Agrobacterium and Dincer cultivar for transformation efficiency compared with GV3101 strain and cv. Sina. Orlikowska et al., (1995) suggested that organogenetic potential of safflower is strongly influenced by the conditions of *A. tumefaciens*-mediated transformation such as *Agrobacterium* strain, selection agent and plant growth regulators. With regard to this, the frequency of putative transformed shoots was 17% when mutated *aro* A gene was used. Similar results were reported for *GUS* gene and LBA4404 strain in cv. Centennial (15%) (Ying et al., 1992). Sankara Rao and Rohini, (1999) proposed that frequency of putative transformed shoots for *UID A* gene using LBA4404 strain was fluctuated between 23-34% in A1 and A300 cultivars. As a result, genotype dependency of *A. tumefaciens* strains was clearly observed that has been previously reported in other oilseed crops such as rapeseed (Zhang and Bhalla, 1999) and sunflower (Mohamed et al., 2004). Furthermore our previous study, showed that frequency of transformation in rapeseed plant with *FAE* gene and using LBA4404 strain on selective medium containing 25 mg l^{-1} kanamycin was about 29% (Zebajadi et al., 2006). Wagiran et al, (2010) were used *A. tumefaciens* strain LBA4404 harbouring pCAMBIA1305.2 plasmid containing *ipt* gene and transformed rice suspension cell culture. The results shown that the highest percentage of transformation frequency based on GUS activity was 93% in the variety *Hayahishiki*, 77% in *Nippon Bare* and 30% in the variety *Fujisaka 5*. In another study, Saiful Islam et al, (2009) were reported use of *A. tumefaciens* strain LBA4404 strain for genetic transformation of Tossa Jute (*Corchorus olitorius* L.), according to the results the highest response to GUS assay was 86.6%. The current study is the first report of the use of mutated *aro*A gene for transformation of safflower via *A. tumefaciens* mediated method. In this respect, there were only two reports for transferring of the bacterial mutated *aro*A gene to rapeseed in order to make tolerance to glyphosate (Kahrizi et al., 2007; Kahrizi and Salmanian, 2008). To transform cotyledonary petioles of *B. napus*, they were also used *A. tumefaciens* LBA4404 and received to a regeneration frequency of 28% on the medium containing 25 mg l^{-1} kanamycin. In safflower transformation after PCR analysis of regenerated plantlets, the frequency of transformed shoots was reduced to 10% in Dincer cultivar. This could be due to escaping of many white, non-transformed shoots, which were formed in addition to the green putative transgenic shoots on the selective medium. Ying et al., (1992) realized that the main cause of reduction in regeneration efficiency is presumably

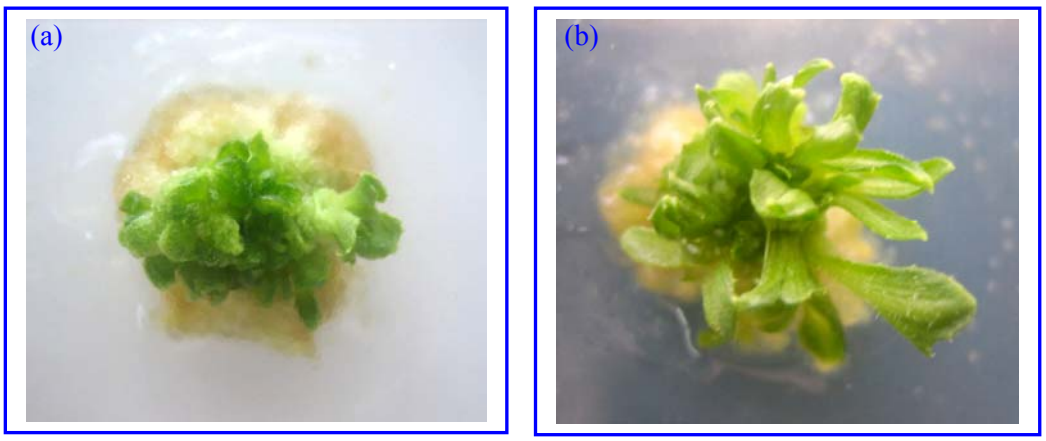

Fig 5. (a) Emerging of initial primordia producing shoots from the cotyledon explants within 21 d after culture in 0.1 mg l^{-1} NAA and 2 mg l^{-1} BAP concentrations and (b) direct regeneration of multiple shoots after 28 – 35 d on the same medium from adaxial surface of cotyledon explants in cv. Dincer.

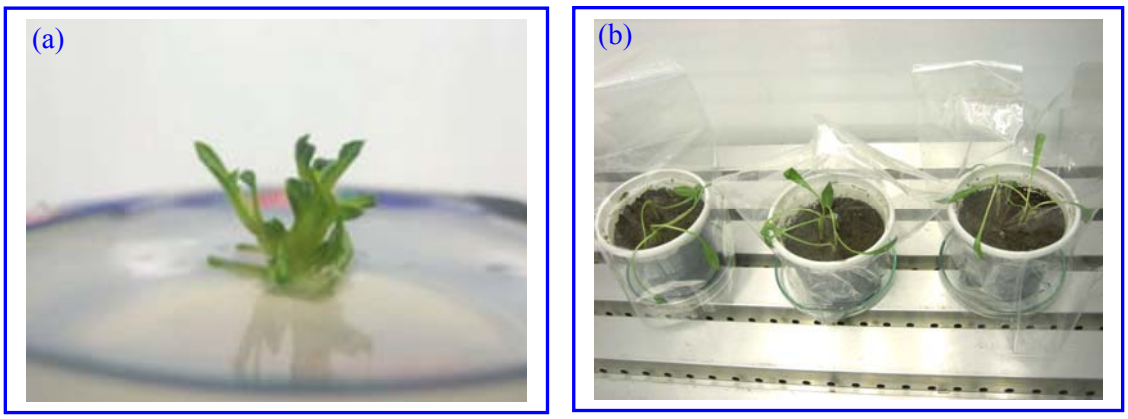

Fig 6. (a) Transferring of regenerated shoots to MS medium supplemented with 2 mg l^{-1} IBA for rooting and (b) transferring of rooted plantlets to soil pots and acclimatization conditions after 21 days.

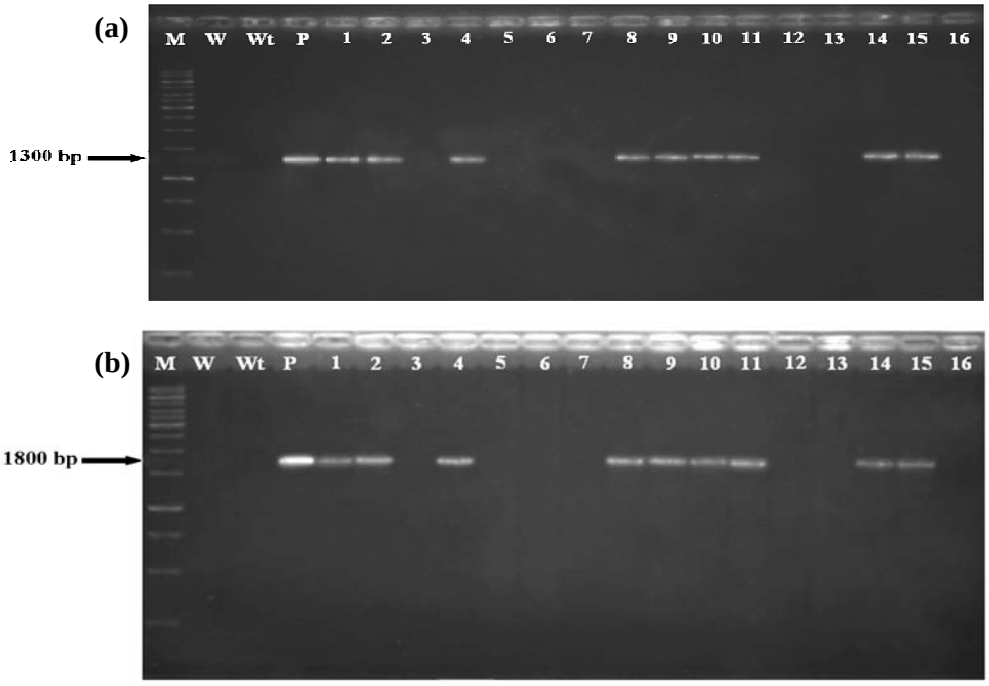

Fig 7. Determination of *aro* A gene for cv. Dincer of safflower on a 1% agarose gel (a) Amplification of a 1300 bp and (b) 1800 bp mutated *aro* A gene fragments. *Lane M*: marker (100 bp ladder), *Lane W*: negative control (Water), *Lane Wt*: negative control (DNA from non-transformed plant), *Lane P*: positive control (pBI121 plasmid containing mutated *aro* A gene), *Lanes 1-16*: DNA from the plants that survived infection, respectively.

inhibitory effects of kanamycin in the selective medium or related to adverse effects of the T-DNA insertion. The PCR analysis for confirm the putative transgenic plants were applied with some different scientific persons (Zebajadi et al., 2006; Wagiran et al, 2010).As a number of target genes conferring resistance/tolerance to a wide spectrum of biotic and abiotic stresses from diverse sources which are currently available. After this manner, we will try to perform additional analyses such as southern blot and RT-PCR and hope to obtain transgenic safflower genotypes that resistant to glyphosate using bioassay under *ex vitro* conditions.In conclusion, here we described a method for efficient transformation and regeneration of Dincer cultivar of safflower plant.

References

Baerson SR, Rodriguez DJ, Tran M, Feng Y, Biest NA, Dill GM (2002) Glyphosate-resistance goose grass: Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. Plant Physiol. 129: 1265–1275.

Baker CM, Dyer WE (1996) Improvements in rooting regenerated safflower (*Carthamus tinctorius* L.) shoots. Plant Cell Rep. 16: 106-110.

Basalma D, Uranbey S, Mirici S, Kolsarici Ö (2008) TDZ×IBA induced shoot regeneration from cotyledonary leaves and *in vitro* multiplication in safflower (*Carthamus tinctorius* L.). African J. Biotech. 7(8): 960-966.

Chawla HS (2000) Introduction to plant biotechnology. 2nd edn, Inc. Enfield, New Hampshire, USA, Science publisher, p 39–56.

Dill GM (2005) Glyphosate-resistant crops: history, status and future. Pest Manage Sci. 61: 219–224.

Kahrizi D, Salmanian AH (2008) Substitution of Ala183Thr in *aro* A product of *E. coli* (k12) and transformation of rapeseed (*Brassica napus* L.) with altered gene confers tolerance to Roundup. Transgenic Plant Journal 2(2): 170-175.

Kahrizi D, Salmanian AH, Afshari A, Moieni A, Mousavi A (2007) Simultaneous substitution of Gly96 to Ala and Ala183 to Thr in 5 enolpyruvylshikimate-3-phosphate synthase gene of *E. coli* (k12) and transformation of rapeseed (*Brassica napus* L.) in order to make tolerance to glyphosate. Plant Cell Rep. 26: 95-104.

Khawar KM, Sarihan E, Sevimay C, Çöçü S, Parmaksiz I, Uranbey S, Ipek A, Kaya MD, Sancak C, Özcan S (2005) Adventitious shoot regeneration and micropropagation of *Plantago lanceolata* L. Periodicum Biologorum 107(1):113-116.

Knowles PF (1989) Safflower: In Oil Crops of the World. In: Downey RK, Robellen G, Ashri A (ed), McGraw-Hill, New York, p 363–374.

Mandal AKA, Gupta SD (2001) Direct shoot organogenesis and plant regeneration in safflower. In vitro Cell Dev Biol. 37(1): 50-54.

Mohamed S, Boehm R, Binsfeld PC, Schnabl H (2004) *Agrobacterium*-mediated transformation of two high oleic sunflower *(Helianthus annuus* L.) genotypes: assessment and optimization of important parameters. Helia 27(40): 25-40.

Murashig ET, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15(3): 473-497.

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acid Res. 8(19): 4321–4325.

Nikam TD, Shitole MG (1999) *In vitro* culture of safflower L. cv. Bhima: initiation, growth optimization and organogenesis. Plant Cell Tiss Org Cult. 5: 15-22.

Orlikowska TK, Dyer WE (1993) *In vitro* regeneration and multiplication of safflower (*Carthamus tinctorius* L.). Plant Sci. 93: 151-157.

Orlikowska TK, Cranston HJ, Dyer WE (1995) Factors influencing *Agrobacterium tumefaciens* mediated transformation and regeneration of the safflower cultivar centennial. Plant Cell Tiss Org Cult. 40(1): 85–92.

Radhika K, Sujatha M, Rao NT (2006) Thidiazuron stimulates adventitious shoot regeneration in different safflower explants. Biologia Plantarum 50: 174-179.

Rohini VK, Sankara Rao K (2000) Embryo transformation, a practical approach for realizing transgenic plants of safflower (*Carthamus tinctorius* L.). Ann Bot. 86: 1043-1049.

Saiful Islam M, Kamrul Huda MM, Mahmud F, Akhter Banu S, Ang MH (2009). Regeneration and genetic transformation of Tassa Jute (*Corchorus olitorius* L.). Australian Journal of Crop Science. 3(5): 287-293.

Sambrook J, Russel DW (2001) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, New York, p 12.1–12.114.

Sankara Rao K, Rohini VK (1999) Gene transfer into Indian cultivars of safflower (*Carthamus tinctorius* L.) using *Agrobacterium tumefaciens*. Plant Biotech. 16(3): 201-206.

Singh V, Nimbkar N (2006) Safflower (*Carthamus tinctorius* L.). Chapter 6, p 167-194.

Sujatha M (2002) Current status and future prospects of *in vitro* techniques and biotechnology in safflower breeding. Sesame and safflower Newsletter 17: 92-97.

Sujatha M, Dinesh Kumar V (2007) *In vitro* bud regeneration of *Carthamus tinctorius* and wild *Carthamus* species from leaf explants and axillary buds. Biologia Plantarum 51(4): 782-786.

Wagiran A, Ismail I, Che Mohd Zain Ch, Abdullah R (2010) *Agrobacterium tumefaciens*-mediated transformation of the isopentenyltransferase gene in japonica rice suspension cell culture. Australian Journal of Crop Science. 4(6): 421-429.

Wang HY, Li YF, Xie LX, Xu P (2003) Expression of a bacterial *aro* A mutant, *aro*A-M1, encoding 5-enolpyruvylshikite-3-phosphate synthase for the production of glyphosate-resitant tobacco plants. J Plant Res. 116(6): 455–460.

Ye GN, Hajdukiewicz PTG, Broyles D, Rodriguez D, Xu CW, Nehra N, Staub JM (2001) Plastid-expressed 5-enolpyruvylshikimate-3-phosphate synthase genes provide high level glyphosate tolerance in tobacco. The Plant Journal 25(3): 261-270.

Ying M, Dyer WE, Bergman JW (1992) *Agrobacterium tumefaciens* mediated transformation of safflower (*Carthamus tinctorius* L.) cv. Centennial. Plant Cell Rep. 11(11): 581-585.

Zebarjadi AR, Jalali Javaran M, Karimzadeh G, Moeini A, Mousavi A, Salmanian AH (2006) Transformation of rapeseed (*Brassica napus* L.) plants with sense and antisense constructs of the fatty acid elongase gene. Iranian J Biotech. 4(2): 79-87.

Zhang Y, Bhalla PL (1999) Shoot regeneration potential from seedling explants of Australian cultivars of oil seed rape (*Brassica napus* L.). New Horizons for an old crop, Proceedings 10th International, Rapeseed Congress, Canberra, Australia