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Plant Regeneration and Microprojectile-Mediated Transient β-glucuronidase (*gus*) Gene Expression in Mature Embryos of Safflower (*Carthamus tinctorius* L.)

Badri Jyothi[1*] • Nisar Ahmed Ansari[1] • Mulpuri Sujatha[2]

[1] Department of Genetics and Plant Breeding, College of Agriculture, Rajendranagar, Hyderabad, Andhra Pradesh-500046, India
[2] Department of Biotechnology, Directorate of Oilseeds Research, Rajendranagar, Hyderabad, Andhra Pradesh-500046, India

Corresponding author: * jyothi_rishik@yahoo.com

ABSTRACT

An efficient *in-vitro* plant regeneration system is a basic necessity for any transgenic approach. *In-vitro* studies were carried out with the prime objective of developing a suitable protocol for high frequency *in-vitro* regeneration from mature embryos of safflower (*Carthamus tinctorius*). Attempts were made to determine the variability and regeneration abilities among selected genotypes *viz*., 'Bhima', 'CO-1', 'A-1' and 'JSI-7' and to identify suitable hormonal combination for morphogenesis. Kinetin + 2,4-D combinations resulted in callus induction while BA + NAA combinations yielded direct regeneration. MS medium supplemented with 2.0 mg l^{-1} BA + 0.5 mg l^{-1} NAA and 0.1 mg l^{-1} BA + 1.0 mg l^{-1} NAA induced maximum caulogenesis and rhizogenesis, respectively. Prolonged culture period without sub-culture or transfer to the rooting medium resulted in development of callus at the base. Transient *Gus* gene expression in mature embryos was studied in comparison with other seedling explants. Percentage transient *gus* gene expression was maximum in mature embryos followed by cotyledonary leaf, hypocotyls and root. Mature embryos can be used as an ideal target tissue for transformation as direct regeneration is possible without the involvement of a callus phase. Among the cultivars studied, 'Bhima' was better both for morphogenesis and transient *gus* expression percentage.

Keywords: morphogenesis, particle bombardment, root induction, shoot induction
Abbreviations: ANOVA, analysis of variance; **BA**, 6-benzyl adenine; **2,4-D**, 2,4-dichlorophenoxy acetic acid; **HSD**, highly significant difference; **MS**, Murashige and Skoog; **NAA**, α-naphthalene acetic acid; **PPFD**, photosynthetic photon flux density

INTRODUCTION

Carthamus tinctorius L. (safflower), Asteraceae, is a valuable oilseed crop of semi-arid regions. Safflower oil is desirable for human nutrition because of its high degree of poly unsaturation and elevated levels of α-tocopherol. The young plants are used as leaf vegetable (Anonymous 1950). India is one of the major safflower producers in the world contributing to as much as 52% of the world's acreage and 45% of the world's production (INDIA STAT 2007). However the average productivity of 694 kg/ha (INDIA STAT 2007) and oil content of 30-32% could be considered quite low as compared to the 1300 kg/ha seed yield and 40% seed oil obtained elsewhere (FAOSTAT 2008). Moreover, present safflower area of 0.377 Mha and production of 0.24 Mt (INDIA STAT 2007) have been reduced by 7 and 54%, respectively compared to 1992-93, due to lack of cultivars with high yield coupled with better adaptability to various biotic and abiotic stresses.

Genetic improvement of safflower is complicated as it involves simultaneous improvement of both seed yield and oil content. Genetic variability has allowed the successful breeding of cultivars with widely varying oil content and quality, ranging from high oleic varieties used for human consumption to high linoleic varieties used for industrial coatings and lubricants. Conventional breeding has helped in developing several elite cultivars, while *in-vitro* technology has served as an additional tool for further genetic improvement. The present day direct or vector mediated *in-vitro* gene transfer has not only obviated the limitations posed by conventional breeding approaches but also speeded and revolutionized gene transfer from any donor to any recipient across the taxonomic boundaries.

Microprojectile bombardment uses the acceleration of small DNA-coated particles to deliver foreign DNA into target cells able to regenerate whole plants, with the subsequent integration of the introduced DNA (Sanford *et al*. 1998). An efficient *in-vitro* plant regeneration system is a basic necessity for such approaches. Tissue culture and shoot regeneration protocols have been reported for Indian (George and Rao 1982; Tejovati and Anwar 1987; Prasad *et al*. 1991; Nikam and Shitole 1999; Mandal and Gupta 2001; Neetika *et al*. 2005; Radhika *et al*. 2006; Sujatha and Kumar 2007; Chavhan *et al*. 2008; Kumar *et al*. 2008) and other safflower cultivars (Ying *et al*. 1992; Orlikowska and Dyer 1993; Basalma *et al*. 2008). However the response varies with the cultivar and shoot regeneration and shoot rooting frequency are low. Attempts have been made to induce capitula (Tejovathi and Anwar 1984) and produce androgenic plants (Prasad *et al*. 1991). Shoot regeneration from immature zygotic embryos was studied by Yamini (2007). However, there are no reports on regeneration studies in safflower using mature embryos.

The first successful *Agrobacterium tumefaciens*-mediated transformation in safflower expressing the *gus* gene in transgenic shoot buds was reported by Ying *et al*. (1992). Rohini and Rao (2002) reported an *in planta* strategy for gene transfer into plants involving embryo transformation but without the involvement of a tissue culture-plant regeneration component. Tissue culture initiated from mature embryos may provide a good source of totipotent cells from which regenerable tissue culture can be initiated. The objective of the present study was to investigate the suitability of the mature embryos as an explant for regenerable tissue

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culture initiation from which whole plants can be regenerated and to study the transient *Gus* gene expression percentage in the mature embryos using particle bombardment.

MATERIALS AND METHODS

Seeds of four selected safflower genotypes viz., 'Bhima', 'CO-1', 'A-1' and 'JSI-7' were procured from the Directorate of Oilseeds Research, Rajendranagar, Hyderabad. Seeds were washed thoroughly with running tap water for 15 min and further surface sterilization was done using 0.1% (w/v) mercuric chloride and rinsed four times with sterile double distilled water. To obtain mature embryos, surface-sterilized seed was soaked in sterile double distilled water overnight and the seed coat was removed using forceps and scalpel blades and mature embryos were excised. Explants thus obtained were transferred to Murashige and Skoog (1962) (MS) medium fortified with various hormonal combinations and concentrations (mg l^{-1}, w/v) [Kinetin (Kin) + 2,4,-dichlorophenoxy acetic acid (2,4-D); benzyl adenine (BA) + α-naphthalene acetic acid (NAA) in the following combinations, all in MS: 1.0 Kin + 0.5 2,4-D; 1.0 Kin + 1.0 2,4-D; 2.0 Kin + 1.0 2,4-D; 1.0 Kin + 2.0 2,4-D; 2.0 Kin + 0.1 2,4-D; 0.1 Kin + 1.0 2,4-D; 1.0 BA + 1.0 NAA; 0.1 BA + 1.0 NAA; 0.5 BA + 1.0 NAA; 1.0 BA + 0.5 NAA; 2.0 BA + 0.5 NAA; 1.0 BA + 2.0 NAA] supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. The pH of the medium was adjusted to 5.6-5.8 before autoclaving at 121°C. These treatments were investigated to optimize the hormonal requirement for callus induction and regeneration from mature embryo explants. Seed surface sterilization, seed coat removal, embryo excision and mature embryo inoculation into the culture tubes were all carried out in the laminar air flow under aseptic conditions.

The cultures were maintained in a culture room at 22 ± 3°C under a 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 80 µE m^{-2} s^{-1} provided by white fluorescent tubes with 70% relative humidity. All the cultures were transferred every 2-3 weeks onto fresh medium with the same composition. After 3-4 weeks of culture period, once the shoot emergence was observed, individual shoots were transferred to the shooting medium. Multiple shoots regenerated from different explants were excised and individually transferred to MS medium fortified with various concentrations for root induction.

The data were scored in terms of percentage of mature embryo explants that responded and produced callus after four weeks, the number of shoots produced per shoot bud inoculated in the shooting phase and the number of roots formed in the rooting phase. Data were subjected to analysis of variance (ANOVA) to assess treatment differences and varietal response. Significance between means was tested by Tukey's Highly Significant Comparison (HSD) comparison at the 5% probability level using MSTAT-C (Michigan State University). Data given in percentages were subjected to arcsin ($\sqrt{X}$) transformation (Snedecor and Cochran 1967) before statistical analysis.

After assessing the potential for regeneration in mature embryos, mature embryos were used as the target material for transient *Gus* expression studies. In addition, transient *Gus* expression percentage (number of explants showing blue colour/total number of calli bombarded X 100) in seedling explants was also compared with that of mature embryos. To 50 µl of tungsten microprojectiles (0.1 g ml^{-1} of Sylvania M20 powder in 50% glycerol as previously described by Franks and Birch (1991) 10 µl of plasmid pBI 121 DNA (**Fig. 1**) harbouring the *UidA* gene encoding β-glucuronidase [obtained from Indian Agricultural Research Institute, New Delhi (plasmid multiplied in *E. coli* strain DH10B maintained in glycerol stocks)]. 50 µl of 2.5 M $CaCl_2$ and 20 µl of 100 mM spermidine (freshly prepared) were added sequentially with brief vortexing after adding each and placed on ice for 5 min to allow the tungsten/DNA preparation to precipitate at the bottom of the microcentrifuge tube. Then, 100 µl of supernatant was carefully removed. A gas control solenoid and syringe filter holder as described by Finer *et* al. (1992) were arranged to replace the barrel and stop plate in the CAP gun. The target material was placed on the osmoticum medium (MS + 0.2 M sorbitol + 0.2 M mannitol) for 4 h before bombardment (Vain *et al*. 1993). Embryos and their calli were bombarded with the DNA-coated microprojectiles using helium pressure of 12 kg cm^{-2}, at a particle travel distance of 15 cm and a partial vacuum of 550 mg (Livingstone and Birch 1995).

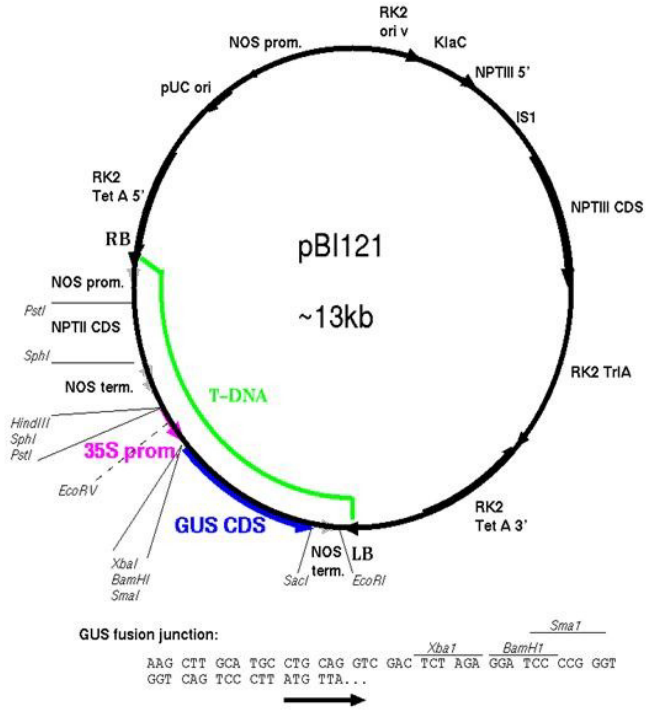

Fig. 1 pBI 121 construct.

A histochemical assay was done to detect the integration of the reporter gene (*Gus* gene) into the target tissue. After incubation in the osmoticum medium 4 h after bombardment (Vain *et al*. 1993) the plant tissue was incubated in the *Gus* histochemical assay according to Jefferson (1987) at 37°C for 24 h and GUS activity was determined microscopically.

RESULTS AND DISCUSSION

Regeneration

Callus induction alone was observed in MS medium supplemented with Kin + 2,4-D while no shoot/root induction was observed (**Fig. 2A**). In MS medium supplemented with BA + NAA, direct regeneration was observed and with prolonged culture period callus induction was also observed (**Fig. 2B, 2C**). After preliminary experiments with a total 12 different combinations and concentrations of hormones, the treatments Kin + 2,4-D, low NAA + high BA and high

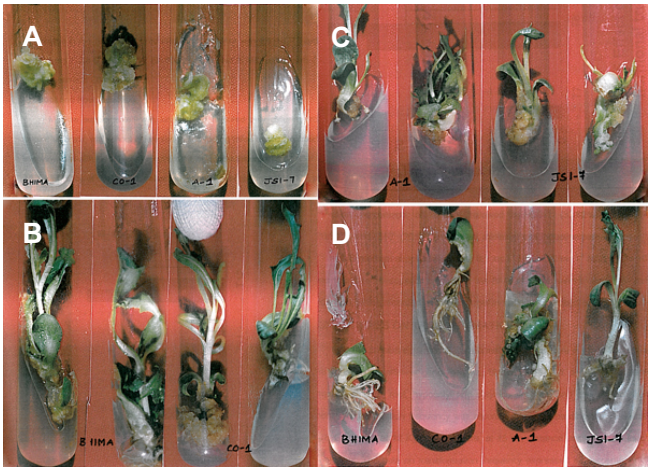

Fig. 2 Regeneration of safflower mature embryos. (**A**) Callus growth from mature embryos at 25 DAI from on MS medium with (in mg l^{-1}) 2.0 Kin + 0.1 2,4-D. (**B, C**) Shoot induction from mature embryos at 2.0 BA + 0.5 NAA. (**D**) Root of the *in-vitro* regenerated shoots on 0.1 BA + 2.0 NAA.

NAA + low BA were designated as callus induction, shooting and rooting media, respectively. The experimental results of the effect of the 12 treatments as designated were investigated on enhancing the percentage response of callus induction, number of shoots per callus and number of roots per shoot.

Mature embryos turned green 48 h after inoculation. Callus induction was observed after 2-3 weeks of culture and two-way ANOVA revealed that the treatments and varieties had a significant effect ($P < 0.05$) on percentage of callus induction response, shooting and rooting. A comparison between the hormonal combinations showed the maximum callus induction response from mature embryo explants in MS supplemented with Kin + 2,4-D and among the different Kin + 2,4-D concentrations, 2.0 Kin + 0.1 NAA was found better for callus induction (**Fig. 2A, 4**).

As the objective of the present study was to obtain direct regeneration from mature embryos without callus phase, shoots obtained on media with NAA + BA combinations were cultured in the shooting medium. Multiple shoots were visible after approximately two-three weeks of culture and the maximum number of shoot initials was observed in 4-5 week old cultures. A comparison of different BA + NAA concentrations showed that maximum response and shoot bud induction per shoot explant was observed in the MS medium containing 2.0 BA + 0.5 NAA (**Fig. 2B, 2C, 5**). The extensive branching pattern observed supports the suggestion that BA inhibits shoot growth and also promotes adventitious bud initiation. Maximum numbers of shoots were obtained after the 1st sub-culture. Retention of the explants with proliferated shoots in the initiation medium for more than 4 weeks resulted in a decline as evidenced from the formation of smaller new leaves and re-appearance of callus. Repeated sub-culturing in the shooting medium enabled continuous production of callus-free shoots. Prolonged delay in subculture resulted in accumulation of phenolics, resulting in the shoots turning brown with re-appearance of callus at the base. Ying *et al*. (1992) reported that direct regeneration of shoots from leaf tissue of safflower may be a useful alternative to regeneration from callus, because somaclonal variation associated with callus culture could be avoided. Basalma *et al*. (2008) developed a high frequency adventitious shoot regeneration protocol for safflower cv. 'Dincer' using cotyledonary leaves and achieved 100% shoot multiplication from cotyledonary node and meristem tip on a range of MS media supplemented with different concentrations of BAP and NAA. Radhika *et al*. (2006) studied adventitious shoot regeneration from different seedling explants and Yamini (2007) from immature zygotic embryos stimulated by thidiazuron. Regenerative potential of the zygotic embryos in several other crops was studied and has been found to give reproducible results in a wide range of genotypes (Finer 1987; Jeanin and Hahne 1991; Jeanin *et al*. 1995; Sujatha and Prabakaran 2001).

The regenerated shoots were excised and transferred to different rooting media treatments with NAA and BA. In most cases, root initiation started within two weeks of culture. The shoots that failed to form roots in this period were unable to produce roots even after prolonged periods of culture. MS medium supplemented with 0.1 mg l^{-1} BA + 1.0 mg l^{-1} NAA was found better for inducing maximum roots per shoot (**Fig. 2D, 5**). Though Baker and Dyer (1996) developed an optimal protocol for inducing root formation consisting of indole-3-butyric acid, survival rate after transfer to soil was quite low. Further research is required to develop a reliable method for rooting shoots and to improve the survival of rooted shoots when transferred to soil as it is a continuing obstacle for regenerating safflower plants.

Transient *gus* gene expression

Among the direct explants the percentage of transient *gus* expression was maximum in mature embryo explants followed by cotyledonary leaf, root and hypocotyls explants

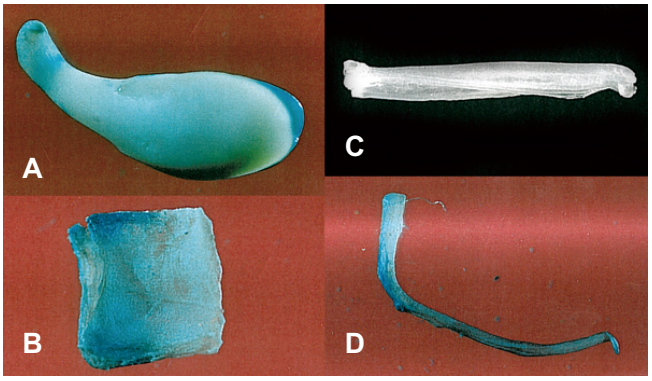

Fig. 3 Microphotographs of 'Bhima' explants showing transient *Gus* expression percentage. (**A**) Mature embryo, (**B**) cotyledonary leaf, (**C**) hypocotyl, (**B**) root.

irrespective of the genotype (**Fig. 3A-D, 7**). The suitability of zygotic embryos for particle bombardment was earlier reported by Lappara *et al*. (1995). However, Chen and Beversdorf (1994) reported that hypocotyls were more advantageous as target material than primary embryos. Hazel *et al*. (1998) reported differences in transformability of a cell line. Livingstone and Birch (1995) optimized the microprojectile bombardment parameters for high transient *gus* expression rates using peanut embryos and excised leaflets from mature embryos and reported higher transient *gus* expression percentage in embryos than in the leaflets. Rohini and Rao (2000) employed embryo axes of safflower cultivars A-1 and A-300 for *Agrobacterium*-mediated transformation and obtained a transformation frequency of 5.3% in A-1 and 1.3% in A-300 following histochemical assay and screening using southern blotting. In other oilseed crops embryos as target tissue for transformation were used by several groups (Burrus *et al*. 1992; Abenes *et al*. 1997; Sujatha and Sailaja 2005). Particle bombardment of zygotic embryos has been used as a transformation system in rice (Li *et al*. 1993), cotton (Banerjee *et al*. 2002) and maize (Kennedy *et al*. 2001). Zygotic embryos were used in *Agrobacterium*-mediated transformation of maize (Frame *et al*. 2002), *Carica papaya* (Azad and Rabbani 2005) and cotton (Banerjee *et al*. 2002). The suitability of zygotic embryos for particle bombardment was earlier reported by Lappara *et al*. (1995). However, Chen and Beversdorf (1994) reported that hypocotyls were more advantageous as target material than primary embryos. Shanjun *et al* (2005) showed the superiority of redifferentiated tissue from pollen embryoid-derived plantlets over leaf, root, stem, petiole, hypocotyls and zygotic embryos as well as pollen embryoids of *Hyoscyamus niger* for both efficient transformation and regeneration of transgenic plants in recalcitrant species. Aulinger *et al*. (2003) reported that in maize zygotic embryos are more efficient for transformation than gametic embryos though the latter can be more potentially transformed than the former.

Varietal response

Among the four safflower genotypes studied, callus induction and transient *Gus* expression percentage was high in 'Bhima' followed by 'CO-1', 'A-1' and 'JSI-7' irrespective of the hormonal concentrations and combinations. 'Bhima' and 'CO-1' showed no significant difference for shooting response and rooting and showed maximum response for the same when compared to 'JSI-1' and 'A-1' (**Figs. 5, 6**). Significant difference for rooting response was found between 'JSI-7 and 'A-1' but not for callus induction and shooting response. No significant difference was found between 'Bhima' and 'A-1' for transient *Gus* expression percentage which gave maximum response when compared to 'JSI-7' and 'CO-1' (**Fig. 7**). The individual effects of genotypes and various combinations and concentrations

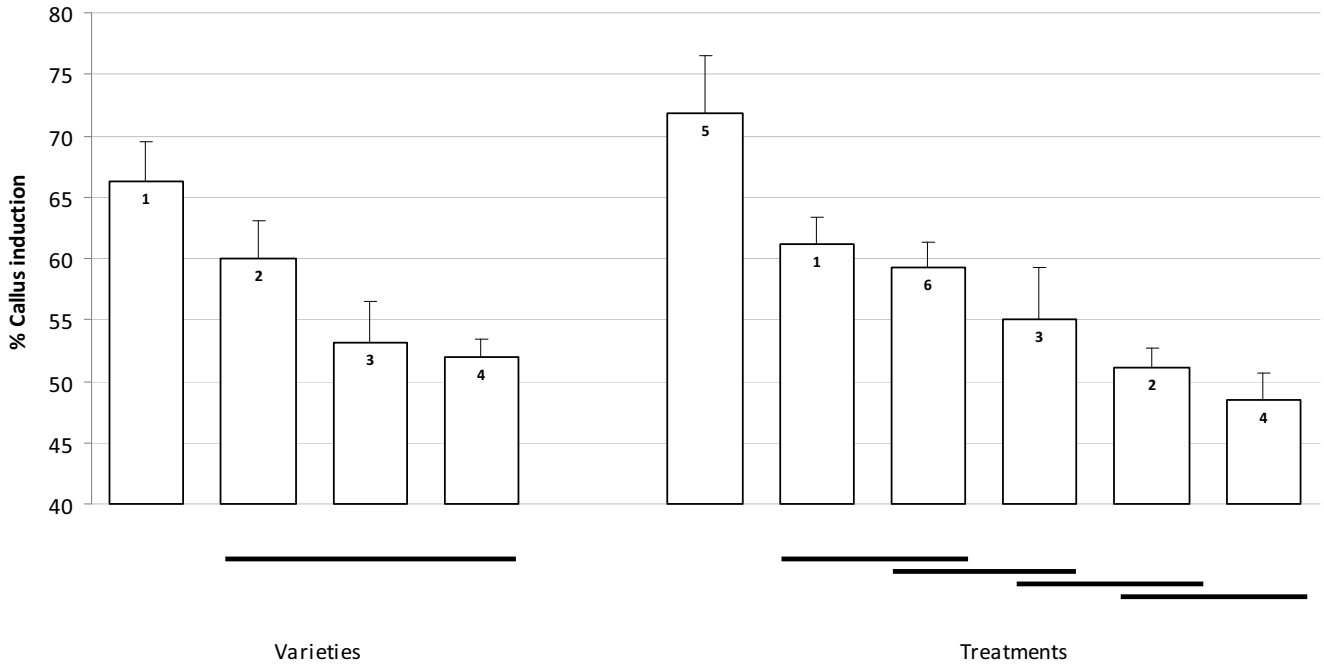

Fig. 4 Callus induction response (% callus induction) from mature embryos. Varieties: (**1**) **'**Bhima', (**2**) 'CO-1', (**3**) 'A-1', (**4**) 'JSI-7'; Treatments (values in mg l^{-1}): (**1**) 1.0 Kin + 0.5 2,4-D, (**2**) 1.0 Kin + 1.0 2,4-D, (**3**) 2.0 Kin + 1.0 2,4-D, (**4**) 1.0 Kin + 2.0 2,4-D, (**5**) 2.0 Kin + 0.1 2,4-D, (**6**) 0.1 Kin + 1.0 2,4-D. Horizontal lines below the columns indicate no significant differences between varieties or treatments.

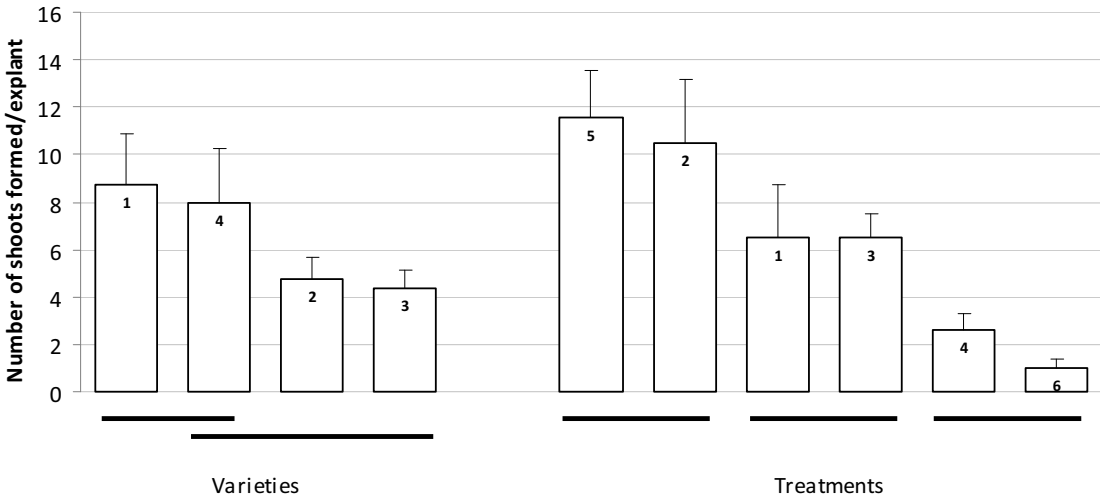

Fig. 5 Evaluation of varietal response (number of shoots formed/explant) in various NAA + BA combinations. Varieties: (**1**) **'**Bhima', (**2**) 'CO-1', (**3**) 'A-1', (**4**) 'JSI-7'; Treatments (values in mg l^{-1}): (**1**) 1.0 BA + 1.0 NAA, (**2**) 0.1 + 1.0 NAA, (**3**) 0.5 BA + 1.0 NAA, (**4**) 1.0 BA + 0.5 NAA, (**5**) 2.0 BA + 0.5 NAA, (**6**) 1.0 BA + 2.0 NAA. Horizontal lines below the columns indicate no significant differences between varieties or treatments.

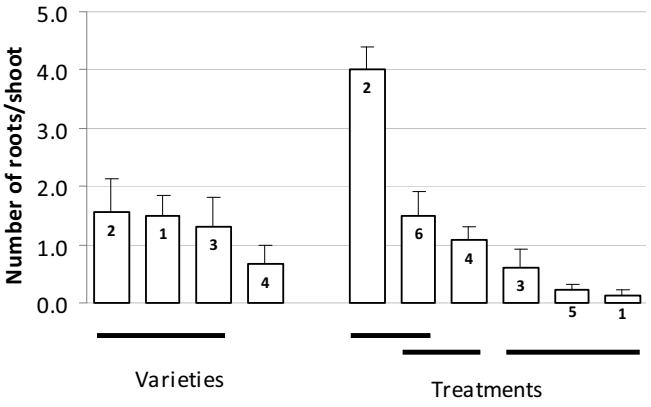

Fig. 6 Evaluation of varietal response in various NAA + BA concentration for rooting. Varieties: (**1**) **'**Bhima', (**2**) 'CO-1', (**3**) 'A-1', (**4**) 'JSI-7'; Treatments (values in mg l^{-1}): (**1**) 1.0 BA + 1.0 NAA, (**2**) 0.1 BA + 1.0 NAA, (**3**) 0.5 BA + 1.0 NAA, (**4**) 1.0 BA + 0.5 NAA, (**5**) 2.0 BA + 0.5 NAA, (**6**) 1.0 BA + 2.0 NAA. Horizontal lines below the columns indicate no significant differences between varieties or treatments.

were found to be highly significant with certain exceptions as mentioned. Many genotypic dependent effects were found to be caused by interactions between the genotype, the cultural environment and levels of endogenous growth substances that vary in different genotypes (George 1993). A relationship between genotype and *in-vitro* response, particularly regeneration was reported in several crop species (Bingham *et al*. 1975).

CONCLUDING REMARKS

The main objective of the study was to investigate upon the suitability of mature zygotic embryos as the target material for transient *Gus* expression studies in safflower and henceforth their ability to regenerate without callus phase. MS medium supplemented with 2.0 BA + 0.5 NAA is the best for direct regeneration from embryo explants. MS medium supplemented with 0.1 BA + 1.0 NAA is the best for inducing maximum roots per shoot. MS medium supplemented with 2,4-D + Kin favoured only callusing from mature embryos, while shoot differentiation and rooting failed to

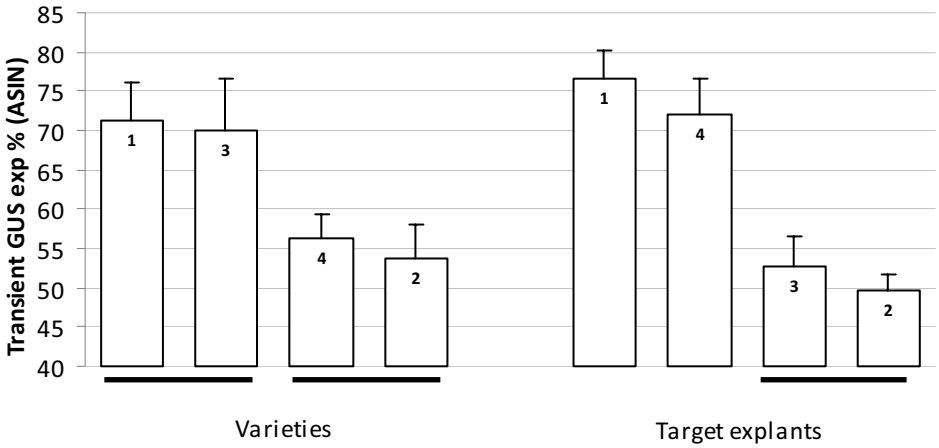

Fig. 7 Transient *Gus* gene expression percentage. Varieties: (**1**) **'**Bhima', (**2**) 'CO-1', (**3**) 'A-1', (**4**) 'JSI-7'; Target material: (**1**) Mature embryo, (**2**) root, (**3**) hypocotyl, (**4**) cotyledonary leaf. Horizontal lines below the columns indicate no significant differences between varieties or target explants.

occur on media supplemented with these growth regulators at different concentrations. Similar conclusions were made by Radhika *et al*. (2006) and Yamini (2007) about the failure of safflower explants to elicit an organogenic response on media containing 2,4-D alone or in combination with Kin. It is known that plant growth hormones play an important role during *in-vitro* culture of plants. The balance in the level of auxin and cytokinin determine the morphogenetic pattern. A relatively high level of auxin to cytokinin ratio stimulates rooting, whereas low auxin to cytokinin ratio produces shoots and almost equal proportion of each results in undifferentiated callus (Skoog and Miller 1957).

Every organ or tissue could be used as a source of explant (Evans *et al.* 1984). Nevertheless, the degree of success has been variable and hence in developing a protocol for commercial use, selection of explant should be carried out systematically and deliberately. Mathews (1987) stated that the highest rate of regeneration often depends not only on the selection of the most suitable explant, but also on the discovery of correct combination of growth regulators and/ or the best medium for that organ or tissue. The main advantage of direct morphogenesis over callus mediated regeneration is that the genetic and chromosomal variation, most of which is induced in callusing phase could be avoided by direct regeneration of shoots without callus formation. Directly regenerated transgenic shoots would retain the desirable agronomic traits of the starting material and thus eliminate or reduce the necessity of extensive backcrossing after introducing novel genes.

An ideal target for particle bombardment for production of transgenic plants should be capable of integrating introduced DNA and regenerating intact plants. The percentage transient *gus* gene expression is high in mature embryo explants than in other seeding explants. Among the four genotypes studied for regeneration ability and transient *gus* expression percentage, 'Bhima' was the best for regeneration and introduction of this transgene.

The present study indicates that genetic component of regeneration capacity exists in safflower both for caulogenesis and rhizogenesis. Overall, this study reveals that mature embryo explants are better for regeneration without callus induction which can be utilized *in-vitro* for production of transgenic plants of commercial importance without impairing genetic constitution. Genetic transformation in safflower is possible using particle inflow apparatus but further work needs to be done on the stable integration of the introduced gene and regeneration of transformed plants.

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