7th International Safflower Conference
WAGGA WAGGA AUSTRALIA

Establishment of regeneration and transformation protocols in safflower (*Carthamus tinctorius* L.)

Narasimha Rao N[1], Sujatha M[1], Lakshmi Narasu[2], Dinesh Kumar V[1,3]
[1]Directorate of Oilseeds Research, Rajendranagar, Hyderabad 500 030, India
[2] Jawaharlal Nehru Technological University, Kukatpally, Hyderabad 500 072, India
[3] dineshkumar@dor-icar.org.in

Abstract
The present investigation has been undertaken to establish an efficient plant regeneration and genetic transformation in safflower for the Indian cultivars. In the first phase, we studied five genotypes with respect to regeneration frequency with different hormonal concentrations. Based on these studies, HUS-305 genotype that gave high regeneration with TDZ 1.0 + NAA 0.2mg/l when compared with other genotypes was selected for further experiments. In the second phase we standardized different parameters like bacterial culture O.D, *vir* gene inducers, vacuum infiltration, etc influencing the transformation frequency. Also, we studied different transformation methods for adoptability in safflower and kill curves of different selection agents for the cv HUS-305. Among all the parameters studied, bacterial concentration of 0.1O.D and vacuum infiltration for 30 minutes were identified as critical for getting good transformation frequency. Kill curve studies indicated that hygromycin @15 mg/l and phosphinothricin @1 mg/l were better selection regimes without allowing the escapes. With the standardized procedure, about forty percent of the hypocotyl explants gave transformed shoots.

Key words: Safflower – regeneration - *Agrobacterium* - phosphinothrcin - hygromycin

Introduction
Though there are several reports of plant regeneration and transformation in safflower, a consistent protocol which provides high frequency of transformation is not available. Safflower plantlets have been regenerated though somatic embryogenesis and organogenesis (Tejovathi and Anwar, 1987; Orlikowska and Dyer, 1993; Mandal and Dutta Gupta, 2001& 2003). The present investigation has been undertaken to establish efficient plant regeneration and genetic transformation system in safflower using the axenic seedling explants for the Indian cultivars.

Material and methods
In the initial experiments, an efficient regeneration system was established using seedling explants. In the preliminary experiment, sixty-three combinations of different growth regulators *viz.* TDZ+NAA, 2,4-D + kinetin, BA+NAA, TDZ+IBA, TDZ+IAA and 4, 2, 5-Cl_3-POP were tested for induction of organogenesis and embryogenesis. To induce elongation of the regenerated shoots MS medium supplemented with different concentrations (0.2 to 1.0 mg/l) of the cytokinins, kinetin and BAP were used. To study the effect of genotypes on regeneration, five genotypes *viz.* A-I, JSF-I, Manjira, Bhima, and HUS 305 were tested for their efficacy. Rooting of *in vitro* regenerated shoots is a major problem in safflower. Therefore, we studied different hormonal combinations such as phloroglucinol, phloroglucinol + activated charcoal, 2,4,5 phenoxy propionic acid, NAA, 2,4,5 phenoxy propionic acid + activated charcoal with full-strength MS and half-strength MS medium. The established regeneration protocol was employed for transformation studies. The *Agrobacterium* strain LBA4404 harbouring the binary vector pCAMBIA1305.2 was used for the standardization of transformation procedure. The PCAMBIA1305.2 vector contains hygromycin as selection agent and *Gus* as the reporter gene. To increase the transformation frequencies, several variables were tried. The tested variables included explants (root, hypocotyls and leaf), bacterial concentration (0.1, 0.2, 1.0, 1.5 and 2.0 OD), methods of infecting the explants (vacuum infiltration of the explant with the culture, sonication of explant with the bacterial culture, shaking the explants with the culture for 30 minutes), enzymatic pretreatment of explants with pectinase, hemicellulase and use of *vir* gene

inducers like acetosyringone. The kill curves were established for the two-selection agents hygromycin (0-100 mg/l) and phosphinothricin (0-10 mg/l).

Results

Regeneration response was best on the medium supplemented with TDZ 1.0mg/l + NAA 0.2mg/l. Shoots emerged as clusters within 2 weeks of culture from all the explants. Root explants gave shoots on medium containing TDZ 0.5mg/l + NAA 0.2 mg/l while the hypocotyls gave regeneration with TDZ 1.0mg/l + NAA 0.2mg/l. Shoots regenerated from explants were first transferred to 0.5mg/l or 1.0mg/l kinetin, and later transferred to 0.2mg/l BA medium for shoot elongation. Among the genotypes tried, better results were obtained with JSF-I followed by HUS 305. For inducing rooting, the elongated shoots were transferred to different rooting media. Best rooting was obtained on MS medium supplemented with phloroglucinol at1mg/l and IBA 0.2mg/l concentration. Kill curve studies indicated that hygromycin at15 mg/l and phosphinothricin at1 mg/l could act as effective selection agents for identifying the transformed shoots. *Agrobacterium* mediated transformation protocol was standardized using LBA4404 strain harboring pCAMBIA1305.2 vector that has *Gus* gene as the reporter and hygromycin phosphotrasferase (*hpt*) gene as the selectable marker. Explants used for co-cultivation included hypocotyls, roots and leaves. Vacuum infiltration method was used during co-cultivation. Co-cultivated explants produced multiple shoots within 10 to 15 days from the cut ends of the explants when transferred onto the medium containing cefotaxime (bacteriostat) as well as hygromycin (Fig.1). The shoots elongated on selection medium supplemented with BAP at 0.2 mg/l in the next 30-40 days. The elongated shoots rooted on the rooting medium and subsequently transferred to the greenhouse (Fig.2). Molecular analysis to confirm the presence of transferred genes was carried out (Fig.3).

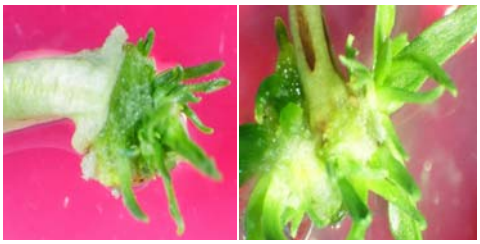

Fig.1 Multiple shoots from the cut ends of the explants

Fig.2 Acclimatized plants in the pots

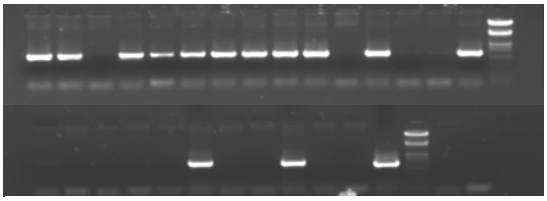

Fig.3 Determination of transgenicity of shoots using PCR positive plants

Discussion and conclusion

We have reported a simple and efficient protocol for regeneration and transformation of safflower. Though there are several protocols already reported in safflower, this protocol has advantages such as reduced time needed for getting the completely regenerated plants avoiding a prolonged callus phase. This should ensure very low frequency of somaclonal variability and will ensure less vitrified and healthy shoots. We have been able to get better rooting of the transformed shoots on medium containing phloroglucinol and thus this protocol is essentially an improvement over the existing methods of regeneration in safflower as in most of the cases it has been acknowledged that rooting of the regenerated shoots is a major problem.

Using this protocol, attempts are underway to develop transgenic male sterility system in safflower.

Acknowledgements
The funding given by Indian Council of Agricultural Research for carrying out this research through a grant under AP-Cess scheme to VDK is acknowledged. The contribution of all the associated research scholars and associates in the project is acknowledged.

References

Mandal, A.K. and Gupta, D.S. 2001 Direct shoot organogenesis formation and plant regeneration in safflower. *In Vitro Cell. Dev. Biol.* 37: 50–54.

Mandal, A.K. and Gupta, D.S. 2003 Somatic embryogenesis of safflower: influence of auxin and ontogeny of somatic embryos. *Plant Cell, Tissue and Organ Culture* 72: 27–31.

Orlikowska, T.K. and Dyer, W.E. 1993 *In vitro* regeneration and multiplication of safflower (*Carthamus tinctorius* L). *Plant Sciences*, 1993: 151-157.

Tejowathi, G. and Anwar, S.Y. 1987 Plantlet regeneration from cotyledonary cultures of safflower (*Carthamus tinctorius* L.). In: Reddy, G.M., ed. Plant cell and tissue culture of economically important plants. Osmania University, Hyderabad, India 1987; 347-353.

Ying, M., Dyer, W.E. and Bergman, J. 1992 *Agrobacterium tumefaciens* mediated transformation of safflower (*Carthamus tinctorius* L) cv 'Centennial'. *Plant Cell Reports*, 11: 581-585.