

IN VITRO SHOOT REGENERATION AND PLANTLET DEVELOPMENT IN SAFFLOWER (*CARTHAMUS TINCTORIUS* L.)

MOHITE NIKHIL*, M. S. DUDHARE, P. V. JADHAV, M. P. MOHARIL AND A. G. DESHMUKH

Biotechnology Centre, Dr. Punjabrao Deshmukh Krishi Vidyapeeth,

Akola - 444 104 (M.S.), INDIA

e-mail: nikhilmohite17@gmail.com

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*Corresponding author

ABSTRACT

In vitro plant regeneration using cotyledonary leaf segments as explants was studied in two varieties of safflower (*Carthamus tinctorius* L.) namely AKS-207 and PKV-pink. Best result of callusing in both varieties was observed after 21 days of culture initiation on medium supplemented with TDZ (2.0mg/L) + NAA (0.5mg/L). Maximum percentage of shoot initiation (63.33%) in AKS-207 was observed on a medium fortified with TDZ (2.0mg/L) + NAA (0.1mg/L). While in PKV-pink it was observed on a medium fortified with TDZ (2.0mg/L) + NAA (0.5mg/L). Subsequent culturing of these responded explants resulted into maximum percentage of shoot multiplication on a medium fortified with TDZ (2.0mg/L) + NAA (0.1mg/L) for AKS-207 and TDZ (2.0mg/L) + NAA (0.5mg/L) for PKV-pink. Proliferated and well developed shoots were then rooted in medium having different concentrations of NAA. Rooted shoots were successfully acclimatized and established in greenhouse.

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) comes under family Asteraceae, is an important oilseed crop of semi-arid regions. Safflower occupies a unique position among oil seed crops due to the high linoleic content of its seed oil which has therapeutic value. Safflower has recently emerged as a broad-acre platform for production of transgenic products, including modified oils such as gamma-linolenic acid and pharmaceutically active proteins including human insulin and apolipoprotein (Belide *et al.*, 2011). It is very important to increase shoot multiplication frequency for genetic transformation studies in safflower. The major hurdle during *in vitro* regeneration of safflower is the phenolic secretions by explant which subsequently affects its growth. For avoiding such conditions, sub-culturing of explant and its rapid multiplication have to be considered. Rapid multiplication can be done by using TDZ along with different concentrations of auxins in the case of safflower.

Previous studies on Safflower reported that, regeneration frequency and rooting capacities in it were low (Basalma *et al.*, 2008). Organogenesis from various explants such as leaf explants and axillary buds (Sujatha and Dinesh Kumar., 2007), root, hypocotyl, cotyledon, primary leaf (Radhika *et al.*, 2006), leaf explants (Nikam and Shitole, 1999) and meristem tips, cotyledonary node (Basalma *et al.*, 2008) has been reported. Studies on plant regeneration using cotyledonary explants in safflower are known (Nikam and Shitole, 1999; Radhika *et al.*, 2006; Basalma *et al.*, 2008). *In vitro* regeneration ability in safflower is basically determined by the explant and genotype (Radhika *et al.*, 2006).

For genetic transformation, it is prerequisite to develop an efficient *in vitro* regeneration protocol for particular genotype. Plant growth regulators affect morphogenic responses in tissue culture. In this report of safflower regeneration a cytokinin, TDZ have been used with varying concentrations of different auxins. Since, regeneration is genotype dependent, two varieties used were differs in their responses to specific plant growth regulator requirement.

Therefore, in the present study, a protocol has been established for an efficient, rapid and high frequency plant regeneration in both AKS-207 and PKV-pink varieties of safflower.

MATERIALS AND METHODS

The seeds of two safflower (*Carthamus tinctorius* L.) varieties AKS-207 and PKV-pink were obtained from oilseed research unit, Dr. Punjabrao Deshmukh Krishi vidyapeeth, Akola (Maharashtra). These seeds were washed thoroughly in running tap water for 10 min, followed by washing with 1% Tween-20 solution for 5 min. and subsequently with sterile distilled water for 3-4 times. For surface sterilization, seeds were treated with 70% ethanol for 1 min. then these seeds were treated with 0.1% HgCl₂ for 5 min. followed by treatment with 0.1% Bavistin (fungicide) for 10 min. Finally, seeds were thoroughly washed with sterile double distilled water to remove traces of HgCl₂. Completely sterilized seeds were then kept on a blotting paper under LAF cabinet for complete drying.

Surface sterilized seeds were then inoculated on Murashige and Skoog's medium (1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar, and incubated at 25° temp. With 16 Hrs. of light period and 8 Hrs. of dark period. After 10-12

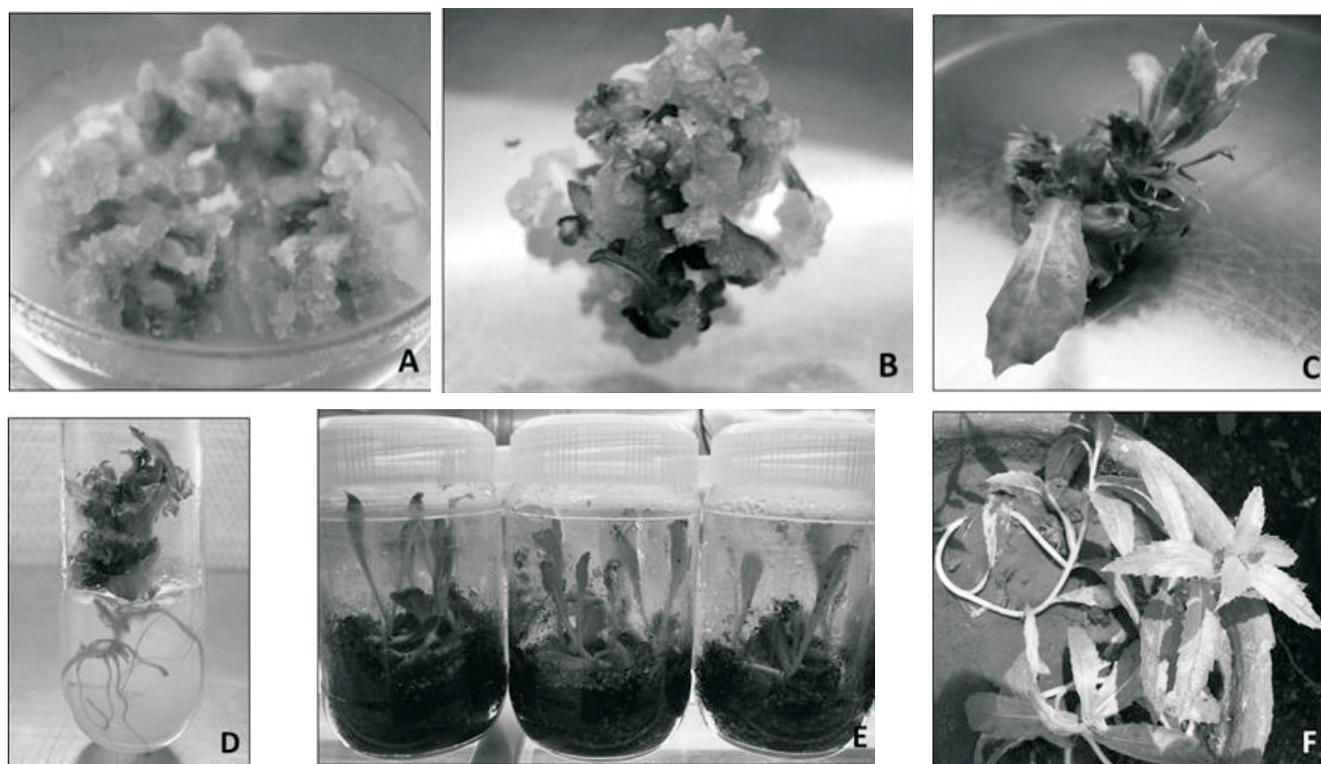


Figure 1 (A-F): *In-vitro* regeneration and plantlet development in safflower:

Fig A: Best callusing response from cotyledonary leaf segments on a medium containing TDZ (2.0 mg/l) + NAA (0.5mg/L), **Fig B:** Shoot induction response on a medium containing TDZ (2.0 mg/l) + NAA (0.1mg/L), **Fig C:** Maximum shoot multiplication response on a medium containing TDZ (2.0 mg/l) + NAA (0.1mg/L), **Fig D:** Best rooting response in regenerated shoots on a medium containing NAA (3 mg/L), **Fig E:** Primary hardening under controlled condition, **Fig F:** Completely developed hardened safflower plant in earthen pot

days cotyledonary leaves were obtained from germinating seeds and then these were sectioned transversely ($1 \times 1 \text{ cm}^2$) and taken as explants, as used earlier during *in vitro* propagation studies of safflower by Motamedi *et al.*, (2011). Different auxins as well as Ethanol diluted TDZ was incorporated into the medium which shows its activity even after autoclaving as described by Khawar *et al.* (2004). Cotyledonary leaf segments were then transferred aseptically on MS medium fortified with different concentrations of either TDZ alone or in combination with NAA, IAA and IBA. After 21-days of inoculation, completely differentiated, dense mass of callus showing further regeneration ability were taken as a standard measure to calculate percentage(%) of callusing. Each regeneration step was then further carried out for the period of 21-days. After 3 weeks of culture, responded explants were further transferred on fresh medium containing same concentrations of TDZ and auxins. Explants having minimum 5 shoots were taken as a standard measure to calculate percentage of shoot multiplication. Further for rooting, multiplied shoots which attended maximum length were excised and cultured on MS medium without any growth regulator and with either of 1mg/L of IAA, NAA and IBA or in combination with high percentage of sucrose. The rooting response was calculated by observing responded shoots to rooting out of 10 transferred on each medium.

Plantlets with well-developed roots were transferred to culture bottles containing soilrite: sterilized soil (2:1). These bottles were then tighten with caps to maintain humidity and were

initially allowed to grow under growth room conditions for 10 days before they were transferred to greenhouse. Such type of primary hardening in soilrite under controlled conditions has been attempted in earlier studies of safflower by Rohini and Rao (2000) to allow growth of germlings. Plants were irrigated with half MS solution during this period and finally, transferred to earthen pots containing soil: soilrite: FYM (2:1:1) and irrigated with water at regular intervals.

Statistical Analysis

All treatments of regeneration experiments repeated 3 times with 20 explants for each replication. Analysis of variance (ANOVA) of obtained data was calculated by using F-test at 1% probability level. Data given in percentages were subjected to Arc sin transformation prior to statistical analysis.

RESULTS AND DISCUSSION

The type and concentration of different phytohormones in the shooting medium was found to be crucial factor for controlling the multiple shoot induction in safflower. Pawar *et al.* (2012) also observed that the type and varying phytohormones plays a major role in determining multiple shoot induction in case of tomato. Multiple shoot formation with varying percentage was observed in almost all treatments. There exists a significant difference among the treatments for days required to callus initiation, structure of callus, days to shoot initiation as well as multiplication and overall percentage

response of shoot regeneration. Shoot initiation was observed from explants of both genotypes, while it was observed that PKV-pink gives slow and poor response for shoot initiation as compared to AKS-207. Earlier studies on safflower tissue culture pointed out the crucial role played by genotype to *in vitro* culture conditions. (George and Rao, 1982, Prasad et al., 1991, Ying et al., 1992, Radhika et al., 2006).

Several studies have been established that cotyledon was the best explant for promoting shoot organogenesis in safflower (Motamedi et al., 2011; Nikam and Shitole 1999; Basalma et al., 2008). All cotyledonary leaf segments used as explants showed symptoms of swelling after 48-72 Hrs. of culture initiation followed by callus initiation at their cut edges which were in contact with the medium. A completely grown, dense,

compact mass of callus was obtained after 21 days of culture initiation. Similar results were observed by Radhika et al. (2006) in several explants of safflower cultivars. Motamedi et al. (2011) also observed a clearly visible callus formation on the margins of cotyledon segments after 21 days of culture initiation. The intermediate callus obtained was observed as fragile and white to green in colour. The maximum callusing response was observed in concentration of TDZ+NAA (2.0mg/L+0.5mg/L) in both varieties that is, in AKS-207 it is 66.67% and in PKV-pink it is 61.67% (Table 1). In contrast, Srikanti Kumari and Pandey (2010) reported the highest mass of callus by using auxins (2, 4-D) alone from different parts of explants in case of A-1 variety of safflower. Regardless of the media and the genotype, formation of callus and subsequent regeneration of shoots through the induced callus was observed. Radhika et al. (2006) previously reported formation of callus in cotyledonary explants and subsequent regeneration of shoots from it. Basalma et al. (2008) also reported that the formation of compact callus after 22-25 days of culture followed by development of adventitious shoots after 45-50 days in case of cotyledonary leaves. Similarly, Motamedi et al. (2011) also observed compact mass of callus followed by shooting in case of cotyledonary leaf segments while, supplemented with different concentrations of NAA and BAP in the medium. Maximum shoot induction response that is 63.53% was observed on a medium fortified with TDZ (2.0mg/L) + NAA (0.1mg/L) in AKS-207 followed by PKV-pink that is 53.33% on a medium containing TDZ (2.0mg/L) + NAA (0.5mg/L) (Table 2). When explants which were responded to shoot initiation were transferred on fresh MS medium with TDZ (2.0mg/L) + NAA (0.1mg/L), maximum shoot multiplication efficiency was observed in AKS-207 (61.67%) while, in PKV-pink (51.67%) it was observed on medium supplemented with TDZ (2.0mg/L) + NAA (0.5mg/L). Sujatha and Dinesh Kumar (2007) observed that leaf segments of *Carthamus arborescens* shows high frequency of shoot regeneration on TDZ (1.0mg/L) + NAA (0.2mg/L) as compared to other *Carthamus* species. This indicates that, shooting responses in safflower are purely genotype as well as species specific.

Table 1: Callusing response in safflower genotypes on mediums supplemented with different concentrations of TDZ alone or in combination with different auxins for 21 days

Treatments	% of callusing	
	AKS-207	PKV-pink
TDZ (1 mg/L)	43.33	40
TDZ (2 mg/L)	43.33	41.67
TDZ (3 mg/L)	48.33	51.67
TDZ (4 mg/L)	51.67	50
TDZ (1.0 mg/L) + NAA (0.1mg/L)	46.67	43.33
TDZ (1.0 mg/L) + NAA (0.5mg/L)	40	43.33
TDZ (2.0 mg/L) + NAA (0.1mg/L)	61.67	55
TDZ (2.0 mg/L) + NAA (0.5mg/L)	66.67*	61.67*
TDZ (1.0 mg/L) + IBA (0.1mg/L)	45	41.67
TDZ (1.0 mg/L) + IBA (0.5mg/L)	53.33	50
TDZ (2.0 mg/L) + IBA (0.1mg/L)	46.67	53.33
TDZ (2.0 mg/L) + IBA (0.5mg/L)	55	51.67
TDZ (1.0 mg/L) + IAA (0.1mg/L)	43.33	38.33
TDZ (1.0 mg/L) + IAA (0.5mg/L)	36.67	40
TDZ (2.0 mg/L) + IAA (0.1mg/L)	53.33	46.67
TDZ (2.0 mg/L) + IAA (0.5mg/L)	36.67	33.33
SE (m)±	0.085	0.064
CD (0.01%)	0.332	0.250

*indicates maximum percentage of callusing response on adjacent treatment.

Table 2: Shoot induction and shoot multiplication responses in safflower after sub-culturing explants after 21 days respectively

Treatments	% shoot induction		% of shoot multiplication	
	AKS-207	PKV-pink	AKS-207	PKV-pink
TDZ (1 mg/L)	41.67	40	40	36.67
TDZ (2 mg/L)	40	36.67	40	30
TDZ (3 mg/L)	45	50	33.33	48.33
TDZ (4 mg/L)	46.67	43.33	31.67	36.67
TDZ (1.0 mg/L) + NAA (0.1mg/L)	30	36.67	28.33	35
TDZ (1.0 mg/L) + NAA (0.5mg/L)	35	43.33	33.33	33.33
TDZ (2.0 mg/L) + NAA (0.1mg/L)	63.33*	46.67	61.67*	45
TDZ (2.0 mg/L) + NAA (0.5mg/L)	58.33	53.33*	58.33	51.67*
TDZ (1.0 mg/L) + IBA (0.1mg/L)	43.33	41.67	41.67	36.67
TDZ (1.0 mg/L) + IBA (0.5mg/L)	51.67	46.67	48.33	41.67
TDZ (2.0 mg/L) + IBA (0.1mg/L)	46.67	48.33	45	40
TDZ (2.0 mg/L) + IBA (0.5mg/L)	56.67	46.67	50	36.67
TDZ (1.0 mg/L) + IAA (0.1mg/L)	41.67	33.33	36.67	25
TDZ (1.0 mg/L) + IAA (0.5mg/L)	23.33	31.67	18.33	30
TDZ (2.0 mg/L) + IAA (0.1mg/L)	46.67	41.67	35	36.67
TDZ (2.0 mg/L) + IAA (0.5mg/L)	28.33	28.33	26.67	18.33
SE (m)±	0.089	0.078	0.077	0.083
CD (0.01%)	0.349	0.302**	0.299	0.324**

*indicates maximum percentage of shoot induction and multiplication response on adjacent treatment. ** indicates non-significant values.

Table 3: Percentage of rooting from *in vitro* raised shoots of safflower genotypes

Treatments	% of rooting	
	AKS-207	PKV-pink
MS	-	-
MS + NAA (1 mg/L)	13.33	6.67
MS + NAA (2 mg/L)	13.33	16.67
MS + NAA (3 mg/L)	36.67*	23.33*
MS + Sucrose (3%)	-	-
MS + Sucrose (6%)	-	-
MS + Sucrose (9%)	-	-

- indicates no response on adjacent treatment. *indicates maximum percentage of rooting response on adjacent treatment.

Basalma *et al.* (2008) reported that with increase in TDZ concentration (upto 0.5 mg/L) there was corresponding increase in the frequency of shoot induction and multiplication. In present study, increase in TDZ concentration alone or in combination with NAA resulted into good shoot regeneration response upto certain extent. In contrast, as the concentration of TDZ increased (>2.0mg/L), it gave poor response for shoot regeneration. Medium supplemented with TDZ + NAA exhibited better results than medium supplemented with TDZ alone. It has been already reported by Radhika *et al.* (2006) that, increasing TDZ concentration (>2.0mg/L) along with auxins subsequently decreases percentage of shooting in cotyledon and other explants of safflower. However, when TDZ was used alone in culture medium it resulted in less percentage of callus formation, required more days for shoot initiation and also showed low shoot regeneration as compared to medium supplemented with combination of TDZ and NAA. Previous reports on safflower research shows that TDZ in combination with NAA or IBA improves the efficiency of shoot regeneration in safflower. (Radhika *et al.* 2006; Sujatha and Dinesh Kumar. 2007; Basalma *et al.*, 2008).

In the present investigation, both the varieties of safflower showed poor percentage of rooting. Similar reports by Nikam and Shitole (1999) also indicated that frequency of shoot rooting was low in safflower. Among different concentrations used, MS medium with NAA 3mg/l showed satisfactory results for rooting, it was found to be 36.67% in AKS-207 and 23.33% in PKV-pink (Table 3). After transferring on a rooting medium shoot explants showed clearly visible, brown, stalk like structure, which ultimately resulted into rooting response. It was found that, rooting takes much more time (35-40 days) and subsequent sub-culturing in both safflower genotypes. MS medium fortified with higher percentage of sucrose shows no rooting response in safflower. While, previous reports of George and Rao (1982) as well as Tejavathi and Anwar (1987) indicates certain percentage (less than 10%) of rooting on a hormone free medium supplemented with sucrose. Most of the earlier safflower reports define the use of medium supplemented with NAA for rhizogenesis as well as non-supplemented MS medium but the frequency of rooting was not upto desired extent (Singh 1991, Sujatha and Suganya 1996). Radhika *et al.* (2006) were obtained rooting in safflower on half strength MS medium fortified with 0.5 mg/L of NAA. While, Sujatha and Dinesh Kumar observed that, elongated shoots were rooted on half-strength MS medium supplemented with 1 mg/L each of IBA and phloroglucinol. However, the response varies from with the cultivar and regeneration. All

the rooted plantlets were hardened primarily in capped culture bottle containing soilrite:sterilized soil in 2:1 proportion and kept in growth room to prevent moisture. This resulted into early acclimatization of plantlet. Rohini and Rao (2000) also used such type of technique for 5-6 days old germlings before they were transferred to greenhouse. Similarly, reports of Sujatha and Dinesh Kumar (2007) also indicated the use of sterile *Vermiculite* for initial hardening of rooted shoots in safflower which were maintained under high humidity for a week. Then, these plantlets were hardened in green house which survived and exhibit normal morphological growth.

In present research work, we report a highly improved, efficient and reproducible protocol for *in vitro* plant regeneration in safflower. Among two varieties used, AKS-207 was found to be more responsive to *in vitro* regeneration as compared to PKV-pink. Complete plant regeneration which is desired for genetic transformation was achieved by using different concentrations of TDZ and NAA. This optimized regeneration protocol can be efficiently used for *Agrobacterium* mediated genetic transformation in safflower.

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