Journal of Cell and Tissue Research Vol. 15(1) 4849-4854 (2015)
(Available online at www.tcrjournals.com)
ISSN: 0973-0028; E-ISSN: 0974-0910

Original Article

REFINEMENT OF *IN-VITRO* REGENERATION SYSTEM IN ELITE SAFFLOWER (*CARTHAMUS TINCTORIUS* L.) GENOTYPES

DHUMALE DIPTI, R.?, DUDHARE, M. S., MOHITE, N. R., SHINGOTE, P. R., JADHAV, P.V. AND MOHARIL, M. P.

Biotechnology centre, Department of Agricultural Botany, Dr. Panjabrao Deshmukh Agricultural University, Akola- 444104, Maharashtra, India.
E. mail: diptidhumale@gmail.com, Cell: 09657701118

Received: March 6, 2015; Accepted: March 30, 2015

Abstract: In-vitro regeneration system for two safflower genotypes, AKS-207 and PKV-Pink using cotyledonary leaf explant was established. Highest percentage of callusing response was observed on MS medium with equal concentration of 2,4-D and KIN (1 mg/l each) in both the genotypes. Shoot induction as well as multiple shoot formation recorded maximally on medium supplemented with 3 mg/l BAP in AKS-207 and 4 mg/l BAP in PKV-Pink. Highest percentage of shoot elongation was achieved by using GA_3 at 1 mg/l in both the genotypes. Rooting was achieved on half strength MS medium fortified with NAA (2 mg/l) in both the genotypes. In-vitro rooted plantlets were transferred to pre-hardening conditions but failed to respond ex-vitro. To overcome the major constrain of verification in the present regeneration system, repeated sub-culturing of explants on fresh shooting medium was practiced. Overall regeneration system indicated that, safflower genotype AKS-207 is more responsive than PKV-Pink for in-vitro study.

Key words: *Carthamus tinctorius* L., *In-vitro* regeneration.

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is a multipurpose oilseed crop cultivated mainly for production of high quality edible oil rich in poly-unsaturated fatty acids. It is widely grown in arid and semi-arid regions of the world. Safflower petals are important source of medicinal preparations, natural food colours, and dyes for colouring fabrics, curing several chronic diseases such as hypertension, coronary heart ailments, rheumatism as well as male and female fertility problems [1]. Spineless genotypes of safflower also used as cut flowers in Western Europe, Japan and America.

Tissue culture techniques are widely used in Safflower improvement programs [2,3]. Reliable regeneration methods are important for selection of Safflower *in-vitro* and for genetic transformation to introduce novel phenotypes that are impossible to introduce through conventional breeding. Somatic embryogenesis has become the method of choice for high volume propagation, pathogen free delivery systems in multiplying Safflower varieties [4,5]. Production of transgenic plants through transformation method requires an highly efficient and reliable plant regeneration system in case of safflower [6-8]. *In-vitro* regeneration frequency and rooting capacity of Safflower is very low [9,10]. Rooting was found to be totally depended on the genotype, type and age of the selected explants, media composition etc. [11-13]. Moreover, Rooting frequency is also strongly influenced by genotype, shoot quality, medium to which the shoots were habituated and the period of culture [14].

In Safflower, there are only few reports dealing with indirect mode of regeneration from different explants [9], while there are many available literature of somatic embryogenesis and adventitious shoot formation [11-13,15]. In the present study, attempts were made to optimise indirect regeneration system for two Safflower genotypes namelyAKS-207 and PKV-Pink.

MATERIALS AND METHODS

Plant material: Seeds of two safflower (*Carthamus tinctorius* L.) genotypes i.e. AKS-207 and PKV-Pink having oil content 27 % and 33 % respectively were obtained from Oilseed Research Unit Dr. Panjabrao Deshmukh Agricultural University, Akola, Maharashtra. The seeds were washed with 1% Tween-20 detergent for 5 min, sterilised in 0.1% mercuric chloride ($HgCl_2$) for 10 min and rinsed in sterile distilled water (3 times). Surface sterilised seeds were then transferred to half strength of MS medium for germination. After 8-10 days of germination cotyledonary leaves from germinated seedlings were isolated and its petiole, 1-2 mm lower region was trimmed off to obtain the explant size of about 0.5-1cm^2 each.

Culture conditions: The basal Murashige and Skoog [16] medium with 3% sucrose and solidifying agent i.e., bacteriological agar at 0.8% was used during media preparation. The pH of the medium was adjusted to 5.8 with 1N NaOH or 1N HCl before autoclaving at 121ºC for 15 min. *In-vitro* cultures were incubated in growth room under 16 h photoperiod with cool fluorescent light at 25±2ºC.

Regeneration system: For callus induction different concentrations of auxins and cytokinins singly or in combinations were used. Variable concentrations of 2,4-D , KIN singly and 2,4-D + KIN in combination were used for callusing (Table 1). Completely regenerated responsive calli were transferred to shooting medium containing different concentrations of BAP alone or in combination with NAA (Table 2). Shoots showing maximum outgrowth were further cultured on MS medium fortified with different concentrations of GA_3 (Table 2). Optimally elongated shoots were then transferred for rooting on half strength MS medium containing IAA, IBA, NAA (1 to 4 mg/L) each. Rooted plantlets were excised from the culture bottles and adhering medium was removed by washing with sterile distilled water. The Plantlets were transferred in a pro-tray containing 1:1 ratio of sterilised soil and soilrite. Plantlets were kept in green house under suitable conditions and were observed for *ex-vitro* response. All the experiments described here were repeated thrice using minimum 10 explants for each treatment. Numbers of responsive explants at each step were calculated in percentage and represented in tabular forms.

RESULTS AND DISCUSSION

Optimization of stable *in-vitro* regeneration system is indispensible to apply molecular approaches in Safflower crop. Due to its profound impact on genetic transformation studies, we established a reproducible and effectual *in-vitro* regeneration system in safflower genotypes AKS-207 and PKV-Pink.Use of 2,4-D alone in MS medium resulted in the proliferative callusing. Cotyledonary leaf was used as an explant because of high differentiation potential for regeneration system as well as it is reported as an ideal source for rapid callus formation [17,18].

The callus formation percentage increases with increase in the concentration of 2,4-D. Compact callus was observed on 2,4-D supplemented medium and increasing hardness of callus was directly proportionate to the concentration of 2,4-D. The callus formation was prominent on MS medium supplemented with 2, 4-D+KIN hormonal combinations for both the genotypes (Table 1). Auxin and cytokinin in combination reported to play an important role in callus induction and its proliferation [19]. Among the several concentrations used, 2, 4-D+KIN

Explanations of figures

Figs. 1 to 8 are *In-vitro* regeneration response in safflower genotypes. **Fig. 1:** Callusing response on MS medium with 2,4-D+KIN 1 mg/l each, **Fig. 2:** Shoot induction response from callus on MS medium with BAP 3 mg/l, **Fig. 3:** Vitrification (hyperhydrated shoots) on shooting medium, **Fig. 4:** Shooting response of AKS-207 on medium containing BAP 3 mg/l, **Fig. 5:** Shooting response of PKV-Pink on medium containing BAP 4 mg/l , **Fig. 6:** Shoot elongation response on MS medium with GA_3 1 mg/l, **G Fig. 7:** Rooting response on MS medium containing 2 mg/l NAA, **Fig. 8:** Hardening response of regenerated safflower plantlets.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Table 1: Optimization of auxin and cytokinine alone and in different combination for maximum callusing in Safflower genotypes. (Percentage data represents three replications with minimum 10 explants per replication. * indicates highest percentage of response on corresponding medium.)

Media composition (mg/L)	Growth of callus (%) after 3-4 weeks of culture	
	AKS-207	PKV-Pink
MS+ 2,4-D(1)	83.21	82.52
MS+ 2,4-D(2)	95.41	92.52
MS+ 2,4-D(3)	96.26	94.85
MS+ KIN(1)	51.20	50.23
MS+ KIN(2)	67.23	66.10
MS+ KIN(3)	73.56	70.23
MS+2,4-D(1)+ KIN(1)	100*	99.99*
MS+2,4-D(1)+ KIN(2)	99.85	98.52
MS+2,4-D(1)+ KIN(3)	98.00	97.23

at 1 mg/l each shown highest callusing response (100% in AKS-207). Callus obtained on equal ratio of 2, 4-D:KIN was fragile in nature and pale yellow to brownish color (Fig 1). Both the genotypes showed different responses on all callus formation media (Table 1) indicating genotypes based response, which supports the previous researcher's findings [13]. Inline results in A-1 variety of safflower also indicated maximum callus induction on 2, 4-D (3 to 5 mg/L) alone and in combination with KIN (0.1 to 0.5mg/L) [17].

After 4-5 weeks of callus formation, maximum shoot induction was observed when calli were cultured on BAP containing media. Cytokinin, BAP is widely used plant growth regulator to induce shooting in different crops under *in-vitro* condition. MS medium with BAP alone found effective than BAP and NAA in combination. With increasing concentration of BAP the shoot induction also increases upto 3mg/l (Fig. 2) however; increased concentrations of BAP beyond optimal level slightly declined responses. The maximum shoot induction was observed on MS medium containing BAP (3mg/l) for AKS-207 and PKV-Pink with 4mg/l BAP (Table 2). Induced shoots were sub-cultured after 15 days of interval on MS medium supplemented with different concentrations of BAP for multiple shoot formation (Figs. 4,5). For AKS-207 highest numbers of multiple shoots i.e., 15 were obtained on MS medium with 3 mg/l BAP. While, in PKV-Pink highest multiple shoots, approximately 13 were observed at 4 mg/l BAP in MS medium (Table 2) after 2-3 weeks of induced shoots. The frequency of shoot regeneration from calli (2-3 weeks old) of safflower reported in this study is in agreement to those reported earlier [20].

On BAP supplemented MS medium, vitrification (hyperhydrated shoots) was observed (Fig. 3). hyperhydricity or vitrification is clearly visible as a physiological malformation resulted into excessive hydration and reduced mechanical strength [21]. This observed to be a major problem of shoot regeneration in Safflower as reported by previous researchers [10,20]. To overcome the shoot hyperhydricity, we used fresh shooting medium (with same concentrations of BAP) for sub-culturing of multiple shoots. It was observed that these shoots were recovered somewhat when transferred to fresh medium with certain increase in agar concentrations

Table 2: Optimization of Shoot regeneration from callus derived from Safflower genotypes. (Percentage data represents three replications with minimum 10 explants per replication. * indicates highest percentage of response on corresponding medium.)

Media composition(mg/L)	Percentage of Shoot induction		No. Of Multiple Shoots formation		Percentage of Shoot Elongation	
	AKS-207	PKV- Pink	AKS-207	PKV-Pink	AKS-207	PKV- Pink
MS+BAP(1)	40.66	34.00	5.35	3.55	-	-
MS+BAP(2)	54.33	40.00	6.33	3.66	-	-
MS+BAP(3)	67.33*	55.35	15.66*	10.33	-	-
MS+BAP(4)	66.85	65.00*	11.66	13.33*	-	-
MS+BAP(5)	66.55	61.00	9.33	8.00	-	-
BAP(1)+NAA(0.5)	41.35	39.23	-	-	-	-
BAP(2)+NAA(0.5)	43.33	41.33	-	-	-	-
BAP(3)+NAA(0.5)	50.33	52.00	-	-	-	-
BAP(4)+NAA(0.5)	49.33	45.20	-	-	-	-
BAP(5)+NAA(0.5)	49.00	46.21	-	-	-	-
GA_3(0.5)	-	-	-	-	39.33	41.66
GA_3(1.0)	-	-	-	-	47.00*	37.33*
GA_3(2.0)	-	-	-	-	32.00	27.66
GA_3(3.0)	-	-	-	-	24.33.	21.66

(0.85-0.9%). A polysaccharide, iota-carrageena used in the available report of safflower to reduce this shoot hyperhydricity problem [10]. Hyperhydricity of shoots was also reduced by using TDZ in shooting medium during earlier safflower studies [20].

After 1-2 weeks of multiple shoots formation, maximum shoot elongation upto 47.00 % and 37.33 % was observed in AKS-207 and PKV-Pink respectively (Table 2), on medium supplied with GA_3 1mg/l (Fig. 6). Further increase in the concentration of GA_3 resulted in declining shoot elongation percentage accompanied with senescence of shoots. Gibberllic acid (GA_3), a phytohormone generally regulates cell elongation and usually employed for internodal elongation under *in-vitro* studies. In previous studies of this species [6], BAP (0.2 to 0.5 mg/L) was employed for shoot multiplication as well as elongation but we recorded responded GA_3 concentrations rather than BAP for elongation of regenerated safflower shoots.

Different hormonal levels tested, including those reported earlier as successful for rooting in safflower [7,9,12,20,22,23]. It was observed that, the use of IAA and IBA at different (1, 2, 3 and 4 mg/l) concentration resulted into zero response for root induction. After 3-4 weeks of transferring to rooting medium, more responsive shoots for rooting was recorded on half strength of MS media supplemented with NAA (2 mg/l) in both genotypes (Fig. 7). However, the rooting frequency recorded in both genotypes were less than 16% (data not shown). In Safflower, rooting frequency reported to be very low in different varieties tested [9,10,24]. It was identified that, rhizogenesis in safflower completely depends upon the genotype, type and age of explant selected, media composition etc. [11-13]. Inline reports of rooting in safflower with different concentration of NAA were optimised by many workers with different genotypes [11,13,20,24]. The rooted shoots when transferred to hardening conditions in pro-tray, showed wilting symptoms and were unable to survive after 7-8 days (Fig . 8). Previous findings on different genotypes of safflower also indicated low success rate when transferred to the soil [23,24].

The observations recorded during the present investigation clearly suggest that safflower regeneration is genotype specific and AKS-207 is found to be more responsive than PKV-Pink. In addition to this, use of specific growth regulator and its concentrations influence the organogenesis in safflower.

ACKNOWLEDGMENTS

The authors are greatly thankful to Biotechnology centre, Department of Agril. Botany, Dr. Panjabrao Deshmukh Agricultural University, Akola for providing laboratory facilities and financial support to carry out the research work.

REFERENCES

[1] Dajue, Li. and Hans-Henning, M.: International Plant Genetic Resources Institute, Rome, Italy, pp 1-74 (1996).
[2] Ying, M., Dyer, W.E., and Bergman, J.W.: Plant Cell Report, 11: 581-585 (1992).
[3] Motamedi, J., Zebarjadi, A., Kahrizi, D. and Salmanian, A.H.: Australian J. Crop Science, 5(4): 479-486 (2011).
[4] Vijaya Kumar, J., Ranjitha Kumari, B.D., Sujatha, G. and Castano, E.: Plant Cell Tiss. Organ Cult., 93: 85-96 (2008).
[5] Vijaya Kumar, J., Ranjitha Kumari, B.D. and Castano, E.: Biologia Plantarum, 52 (3): 429-436 (2008).
[6] Shilpa, K.S., Dinesh Kumar, V. and Sujatha, M.: Plant Cell Tiss. Organ Cult., 103: 387-401 (2010).
[7] George, L. and Rao, P.S.: Proc. Indian Nat. Sci. Acad. B., 48: 791-794 (1982).
[8] Tejovathi, G. and Anwar, S.Y.: Plant Sci. Letters, 36: 165–168 (1984).
[9] Nikam, T. D. and Shitole, M. G.: Plant Cell Tiss. Org Cult., 5: 15-22(1999).
[10] Belide, S.,Hac, L., Singh, S.P., Green, A.G. and Wood, C.C.: Plant methods, 7: 12 (2011).
[11] Mandal, A.K.A., Chatterji, A.K. and Dutta, G.S. : Plant Cell, Tiss. Organ Cult., 43(3):287-290 (1995).
[12] Mandal, A.K.A. and Gupta, S.D.: *In-vitro* Cell. Dev. Biol. Plant, 37: 50-54 (2001).
[13] Radhika, K., Sujatha, M. and Rao, N. T.: Biologia Plantarum, 50: 174-179 (2006).
[14] Sujatha, M.: Proc. 7th Int. Safflower Conference, WaggaWagga, Australia (2008).
[15] Sujatha, M. and Dinesh Kumar, V.: Biologia Plantarum, 51(4): 782-786 (2007).
[16] Murashige, T. and Skoog, F.A.: Physiologia Plantarum, 15: 473-497 (1962).
[17] Kumari, S. and Pandey, R.K.: The Bioscan, 5(2): 247-250 (2010).
[18] Mohite, N., Dudhare M.S., Jadhav P.V., Moharil M.P. and Deskmukh A.G: The Bioscan., 9(2): 551-555 (2014).
[19] Bhansali, R.R. and Singh, K.: Phytomorphol., 32: 167-170 (1984).
[20] Orlikowska, T.K. and Dyer, W.E.: Plant Sci., 93: 151-

157 (1993).
[21] Debergh, P., Aitken-Christie, D., Cohen, D., Grout, B., Von Arnold, S., Zimmerman, R. and Ziv, M.: Plant Cell Tiss. Organ Cult., 30: 135-140 (1992).
[22] Tejovathi, G. and Anwar, S.Y.: In: *Proceeding of Symposium on Plant Cell and Tissue Culture of Economically Important Plants*. Osmania University, Hyderabad, India, 347-353 (1987).
[23] Baker, C.M. and Dyer, W.E.: Plant Cell Reports, 16: 106-110 (1996).
[24] Walia, N., Kaur, A. and Babbar, S.B.: J. Plant Biochem. Biotech., 14: 65-68 (2005