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Full Length Article

Effect of TIBA and other Plant Growth Regulators on Callogenic Response from Different Explants of Safflower (*Carthamus tinctorius*)

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Abstract

An efficient protocol for callogenesis of safflower (*Carthamus tinctorius* L.) using leaf, root and internode explants of 21-days old *in vitro* grown seedlings on Murashige and Skoog (MS) basal medium were optimized by studying the influence of different growth regulators (2, 4-D, TIBA, NAA, BAP, IBA) either alone or in different combinations. Moreover, the effect of light and dark conditions was also studied on callogenesis. The highest percentage of callogenic response (100%) was obtained in MS medium supplemented with 10.7 µM NAA and 2.22 µM BAP from leaf explants and 97% in MS medium containing 10.7 µM NAA and 2.22 µM BAP from internodal explants under light condition. Furthermore, MS medium supplemented with 6.0 µM TIBA proved to be the best for callus induction (95%) and proliferation from root explants under dark condition. Under light condition, leaf and internodal explants derived calli were best proliferated, however dark condition had more impact on callus induction as compared to light condition for root explants.

Keywords: Safflower; Callogenesis; 2, 4-D; TIBA; NAA; BAP; IBA

Introduction

Safflower *(Carthamus tinctorius* L.) is an annual medicinal plant, which grows well in semi-arid conditions particularly in the tropics and sub-tropics (Mandal *et al*., 1995). It belongs to family Asteraceae, subfamily Tubuliflоreae, and tribe Cynareae. It has 25 species. The centre of origin of this genus is the eastern part of the Mediterranean (Ashri and Knowles, 1960; Sehgal and Raina, 2005). There are many wild and weedy relatives of *Carthamus tinctorius* (Norov, 2005). Traditionally, its flowers were used for coloring foods; as spice for flavoring foods and in dyes making (Gao and Fan, 2000). Its seeds were used as bird seed and oil extracted from its seeds was used as edible oil (Keso, 1962). Safflower contains yellow and red quinochalcone pigments. From its florets two dyes, differing in color and solubility (carthamidin which is water soluble and yellow in color, while carthamin is alkali soluble and red in color), can be obtained (Dajue and Mundel, 1996). Safflower seeds contain high linoleic acid associated with the reduction of cholesterol level in the blood. It is also a source of important biochemicals like α–tocopherol and carthamin (Ramaswamy, 2001).

Safflower is an oil seed crop which is well adapted to low rainfall and stress conditions of cold dry lands. It is much more drought tolerant than other oilseed and most cultivated crops (Rashid *et al*., 2002). The area under safflower in India has significantly decreased from 8 lac hectares in 1986 to 3.63 lac hectares in 2006 (Anonymous, 2007). In Pakistan, safflower was introduced as oilseed crop in 1960s. It is mainly being cultivated in Sindh and Balochistan provinces. Being a drought tolerant crop, it is recommended for planting in rain-fed (barani) areas. Safflower seed contains 26-37% oil constituting oleic acid and linoleic acid. At the National Agricultural Research Centre, Islamabad, Pakistan, USDA World Collection has evaluated 1294 lines considering the factors like yield, plant height, days to flowering and number of flowers per plant (Aslam and Hazara, 1993).

Only a fews attempts have been made on tissue culture of safflower (Mandal *et al*., 1995; Mandal and Gupta, 2001; Dilek *et al.,* 2008), which suggest thorough studies to be carried out on the establishment of an optimum callus culture medium. Keeping in view this fact, the present study was conducted to evaluate the efficacy of various plant growth regulator combinations under different conditions (light, dark) for induction of callus from different explants obtained from *in vitro* grown seedlings of safflower.

Materials and Methods

Plant Material

Seeds of safflower were washed thoroughly under running tap water to remove all the adhering dust particles and microbes from the surface. They were then washed with house hold detergent followed by washing under tap water to remove the detergent. The seeds were then sterilized with 0.15% w/v of mercuric chloride ($HgCl_2$) for another 10-15 min with constant shaking to get thorough sterilization. Following four rinses of sterile distilled water, the seeds

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were transferred aseptically to full strength MS basal salt media containing 3% sucrose and 0.8% Bacto-agar. The pH of the medium was adjusted at 5.8 using 0.1 N NaOH or 0.1 N HCl.

Explant Culture Technique

Leaf, internode and root explants excised from 21-day-old *in vitro* seedlings grown on MS basal medium, were cut into 0.5-1.0 cm pieces and placed horizontally in culture tubes containing 20 mL MS basal salt medium supplemented with 2,4-D (4.52, 9.04, 13.56 and 18.04 µM) or TIBA (1.0, 2.0, 4.0 and 6.0 µM) or NAA (2.67, 5.35, 10.7 and 16.05 µM) with BAP (2.22 µM) or IBA (2.45, 4.90, 9.80 and 14.70 µM) with BAP (2.22 µM). Two sets of cultures were made for light and dark conditions.

Callus Maintenance Medium

All the cultures were maintained in an air conditioned culture room at a temperature of 25±2°C. One set of cultures was placed in dark and one set was subjected to light. The source of illumination consisted of 2.5 feet fluorescent tubes (40 W) and the intensity of illumination was 2,000-3,000 lux at the level of cultures. A 16 h light regime, followed by 8 h dark in heated culture room during winter season and in air conditioned culture room during summer season. Each treatment was repeated at least thrice.

Statistical Analysis

Treatments consisted of three explants (leaf, internode and root), four media and two photo-environments (light and dark). Each treatment had ten replicates consisting of test tubes and was arranged in a factorial experiment as a completely randomized design. All the experiments were performed in triplicates. The data were subjected to analysis of variance (ANOVA) and the difference between the means was compared by Duncan's multiple range test.

Results

The explants responded differently on MS media with different plant growth regulators (2,4-D, NAA+BAP, IBA+BAP and TIBA) either alone or in combination. Among different concentrations of 2,4-D (4.52, 9.04, 13.56 and 18.04 µM) tried for callus induction, all the three explants (leaf, internode and root) showed poor response. The highest rate of callus induction from leaf (33%) and root (23%) explants were observed in MS medium supplemented with 9.04 µM 2,4-D, while maximum callogenic response from internodal explant (26%) was recorded in MS medium containing 4.52 µM 2,4-D under light condition (Table 1). Callus produced was greenish, granular, and non-proliferated in appearance. Under dark condition, callogenic response from leaf and internodal explants was non-significant (6%) after 20 days of inoculation. However, no response was shown by root explants in all the concentrations of 2, 4-D tried (Table 2).

Table 1: Callogenic response of different explants of *Carthamus tinctorius* to different growth regulators under light condition

Growth Regulators	Concentration (µM)	Callogenic response from different explants (%)		
		Leaf	Internode	Root
2,4-D	4.52	6±6.67^{a}	26±12.01^{a}	6±3.33^{a}
	9.04	33±14.52^{a}	16±6.67^{a}	23±2.01^{a}
	13.56	10±10.0^{a}	20±5.27^{a}	20±1.0^{a}
	18.04	14±3.33^{a}	14±1.33^{a}	-
TIBA	1.0	50±5.77^{a}	77±3.33^{a}	76±1.20^{a}
	2.0	60±5.77^{a}	87±8.81^{ab}	83±1.20^{a}
	4.0	60±10.0^{a}	90±5.77^{a}	90±5.77^{a}
	6.0	66±3.33^{a}	93±6.67^{a}	93±3.33^{a}
NAA+BAP	2.67+2.22	96±3.33^{a}	90±5.77^{a}	80±10.00^{b}
	5.35+2.22	93±3.33^{a}	96±3.33^{a}	70±6.66^{a}
	10.7+2.22	100±0.00^{a}	97±3.33^{b}	90±3.33^{b}
	16.05+2.22	93±3.33^{a}	93±6.66^{b}	80±5.77^{b}
IBA+BAP	2.45+2.22	60±11.53^{a}	73±8.81^{a}	77±8.82^{a}
	4.90+2.22	76±3.33^{ab}	87±8.81^{a}	80±5.77^{a}
	9.80+2.22	73±8.81^{ab}	77±6.67^{a}	83±4.63^{a}
	14.70+2.22	90±5.77^{b}	93±6.67^{a}	70±5.27^{a}

Table 2: Callogenic response of different explants *Carthamus tinctorius* to different growth regulators under dark condition

Growth Regulators	Concentration (µM)	Callogenic response from different explants (%)		
		Leaf	Internode	Root
2,4-D	4.52	-	6±3.33^{a}	-
	9.04	6±3.33^{a}	3±14.52^{a}	-
	13.56	-	-	-
	18.04	-	2±3.33^{a}	-
TIBA	1.0	70±5.77^{a}	93±6.66^{a}	83±1.20^{a}
	2.0	90±0.00^{b}	93±3.33^{a}	96±1.15^{a}
	4.0	83±5.77^{b}	90±5.77^{a}	90±8.81^{a}
	6.0	96±3.33^{b}	93±6.66^{a}	96±3.33^{a}
NAA+BAP	2.67+2.22	83±3.33^{a}	80±10.00^{a}	93±6.67^{b}
	5.35+2.22	86±3.33^{a}	80±3.55^{a}	76±8.82^{b}
	10.7+2.22	86±3.33^{a}	83±6.67^{b}	93±6.67^{b}
	16.05+2.22	83±3.33^{a}	80±5.77^{b}	90±0.00^{b}
IBA+BAP	2.45+2.22	63±14.55^{a}	63±3.33^{a}	80±4.55^{a}
	4.90+2.22	80±5.77^{a}	76±5.57^{a}	56±2.77^{a}
	9.80+2.22	70±10.0^{a}	73±6.67^{a}	83±3.33^{a}
	14.70+2.22	73±8.81^{a}	85±3.33^{a}	80±5.77^{a}

Data followed by different letters show significant difference at P≤0.05
Each experiment was performed in triplicate

Among the different concentrations (1.0, 2.0, 4.0 and 6.0 µM) of TIBA used in MS medium, 6.0 µM proved to be the best concentration for callus induction from all the explants under both light and dark conditions. Under light condition, 93% callus induction response from internodes and roots, followed by 66% from leaf explants was observed (Table 1). Under dark condition, 96% callogenic response from leaves and roots, followed by 93% from internodal explants was recorded in the same medium (Table 2). In MS medium supplemented with 2.0 µM TIBA, maximum response to callus induction was shown by root (96%) followed by internode (93%) and leaf (90%) explants, respectively under dark condition. The calli produced from all the explants were mostly friable and

proliferated under both the conditions. However, color ranged from whitish to light green under dark condition while greenish under light condition with dark green nodules (Table 1, 2; Fig. 1).

Among the different combinations and concentrations of NAA with BAP in MS medium tried, MS medium containing 10.74 µM NAA with 2.22 µM BAP showed significant callogenic response from all the three explants under both light and dark conditions. Highest percentage (100%) was observed from leaf followed by internode (97%) and root (90%) explants under light condition (Table 1; Fig. 2). At the concentration of 2.67 µM NAA and 2.22 µM BAP callus along with shoots was observed from internodal explants (Fig. 3). Similarly significantly high callus formation was obtained in the same medium under dark condition from all explants (Table 2).

In the combination of IBA with BAP, maximum response (93%) to callus induction was obtained from internodes followed by leaf (90%) explants in MS medium supplemented with 14.70 µM IBA with 2.22 µM BAP under light condition (Table 1; Fig. 4). Similarly maximum response (85%) was obtained from internodal explants in the same medium under dark condition (Table 2; Fig. 4).

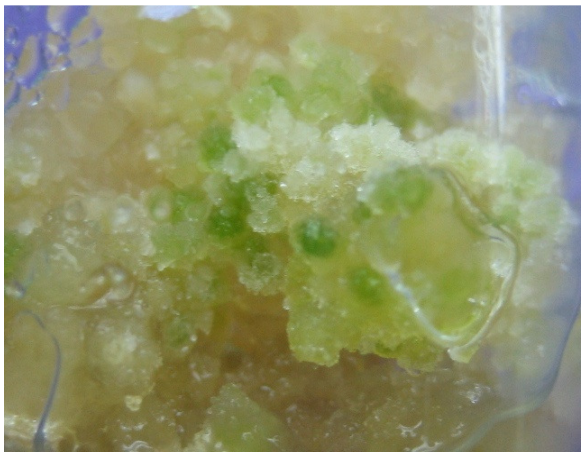

Fig. 1: Callus induction from inter-nodal explant on MS medium supplemented with 6.0 µM TIBA (bar=2 mm)

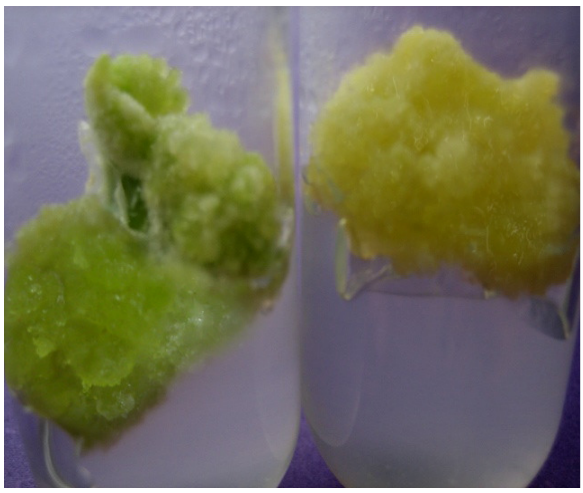

Fig. 2: Callus induction from leaf explants on MS medium supplemented with 10.7 µM NAA and 2.22 µM BAP (Left: callus from light, Right: callus from dark) (bar=5 mm)

Discussion

The effect of 2,4-D on callogenesis has been reported by many workers on different plants (Malik *et al*., 2003; Summart *et al*., 2008; Tahir *et al*., 2011). Our studies showed that at 9.04 µM concentration of 2, 4-D in MS medium under light condition, leaf and root explants gave better callogenic response when compared with internodal explant which gave better result at a lower concentration of 4.52 µM. However, under dark condition no significant response to callogenesis was observed (Tables 1 and 2). Kumari and Pandey (2010) also established callus cultures on 2,4-D alone and reported good callus within 15 days of inoculation from root, leaf and hypocotyls from *in vitro* grown safflower. However, in our results the callogenic response of all three explants was not very significant in any concentration of 2, 4-D under either light or dark condition which can be attributed to different genotype.

In the present work, the effect of polar auxin inhibitor, triiodo benzoic acid (TIBA) was also studied. Polar auxin transport inhibitors are reported to be important tools in assessing the role of polar auxin transport in plant development (Hadfi *et al*., 1998; Mattsson *et al.,* 1999). In tissue culture, many workers have reported the effect of TIBA in combination with other auxin like 2, 4-D on somatic embryogenesis (Chen and Chang, 2004; Venkatesh *et al.,* 2009; Habibi *et al*., 2009; Zarif *et al.,* 2013).

As compared to 2, 4-D, different concentrations of TIBA, gave significantly better response to callogenesis. Out of all the combinations tried, 6.0 µM concentration of TIBA in culture medium gave maximum response from all the explants tried under light condition. Under dark condition, all the concentrations of TIBA tried, showed significantly better results as compared to light condition (Tables 1 and 2). Bhau and Wakhlu (2001) reported that TIBA (0.1 mg/L) in combination with 1.0 mg/L BA in MS medium improved the long-term organogenic potential of the callus of *Morus alba*. However, Sambe *et al*. (2010) reported that the concentrations of TIBA (2.0, 3.0 and 5.0 mg/L) seem to be more effective in the reduction of the rate and the diameter of the callus in *Parkia biglobosa.* TIBA inhibits the formation of shoots and roots of *Nicotiana tabacum* while interfering with the endogenous auxins at the time of their implications in the organization of the cellular division (Dhaliwal *et al*., 2004). Our results showed promotive effect of TIBA for callus formation than 2, 4-D.

Most excised tissues require the addition of one or more growth regulators to initiate callus formation

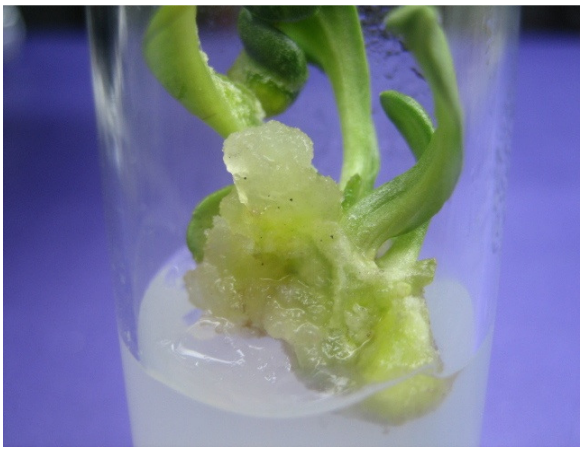

Fig. 3: Shoot formation along with callus from internode-derived callus on MS medium Supplemented with 2.67 µM NAA and 2.22 µM BAP (bar =7 mm)

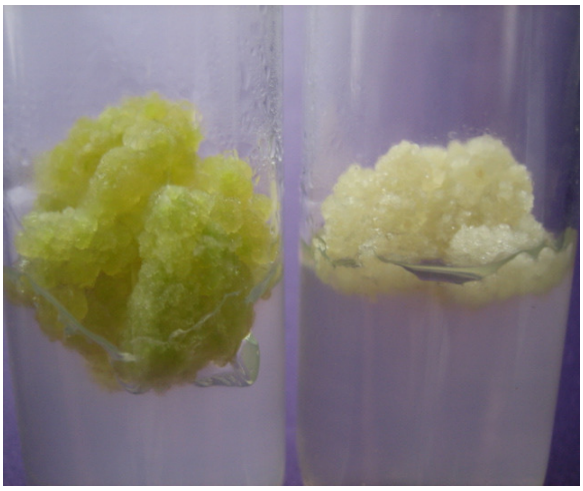

Fig. 4: Callus induction from inter-nodal explant on MS medium supplemented with 14.70 µM IBA and 2.22 µM BAP (Left: callus from light, Right: callus from dark) (bar =4.5 mm)

(Yeoman and Macleod, 1977). The promotive effect of auxin-cytokinin interaction on callus proliferation has been reported by many workers in different plants (Neibaur *et al*., 2008; Bohidar and Thirunavoukkrasu, 2008; Gopitha *et al.*, 2010). Generally a high concentration of auxin with a low concentration of cytokinin has been recommended for initiation and proliferation of callus.

In the present investigation, a combination of different concentrations of NAA with BAP were also used for callus formation from all three explants and 10.74 µM NAA with 2.22 µM BAP proved to be the best medium exhibiting highly proliferated and friable calli. Radhika *et al.* (2006) has also reported callus induction from different explants of safflower on different combination of BA (0.1-2.0 mg/L) and NAA (0-2.0 mg/L) growth regulatore.

Similarly the effect of different concentrations of IBA and BAP in MS medium was also studied on explants of safflower in both conditions (light and dark). All the explants exhibited good callus in all concentrations of this combination. The calli were granular and friable. Radhika *et al*. (2006) reported watery callus formation in safflower in MS medium supplemented with BA and IBA, while Aghaei *et al.* (2013) reported high rate of callus induction from stem explants of *Pistacia atlantica* in MS medium supplemented with BAP and low concentration of IBA.

Callus is an unorganized mass of parenchymatous cells, produced as a result of dedifferentiation from differentiated tissues and induced by exogenous growth regulators under *in vitro* conditions. Regeneration from callus can be achieved via organogenesis or somatic embryogenesis. Every differentiated plant tissue is totipotent but the conditions required for dedifferentiation varies from species to species and even tissue to tissue within the same plant (Ezhova, 2003). Callus cultures are useful in the amplification of limited plant material. In addition, plants regenerated from long term derived callus are also a source of somaclonal variation which results either from an existing genetic variability or induction of mutations as a result of application of plant growth regulators.

Light is known to influence the rate of cell division in different plants (Fraser *et al*., 1967; George and Sherrington, 1984). In our work, differences were observed in the callus morphology between the treatments of light and dark. Calli obtained from leaf and internodes were green and compact in light while pale yellow and loose in dark. Light seems to have less effect on callus induction from root explants as enhancement in callus induction from root explants have been observed in the dark. Although no significant difference in morphology of calli from roots was observed in light and dark. The reason for this enhancement may lay in the fact that natural auxin levels increases in the darkness and root growth is favorable in high auxin to cytokinin ratio (Skoog and Miller, 1957; Schmülling, 2004).

Among the three different explants used in this experiment, the leaves were shown to be the most totipotent with the highest percentage (100% callus at 10.74 µM NAA with 2.22 µM BAP) of callus formation. It has been reported by Twumasi *et al*. (2009) that mesophyll cells are easily reprogrammed through dedifferentiation into undifferentiated mass of cells with characteristics similar to meristematic tissue. These cells are relatively less recalcitrant to differentiation in culture medium due to their high responsiveness to growth regulators (San-José *et al*., 2010).

It is concluded from this study that a combination of NAA and BAP gave good result for callus induction for all three explants as compared to other hormonal combinations tried under light condition. The effect of TIBA on callogenesis from different explants has been reported and results show that TIBA alone gave good results under both light and dark conditions from all explants tried as compared to 2, 4-D. Irrespective of culture media, leaf tissue gave the best yield of callusing.

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