RESEARCH ARTICLE

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Regeneration of safflower genotypes through callus mediated organogenesis using cotyledonary node explants

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Abstract

In this study, the cotyledon nodes of five safflower genotypes (Balcı, Linas, inbred lines 24, 25, and 55) were cultured for shoot regeneration via organogenesis in MS medium involving different TDZ (0.1, 0.5, and 1 mg L^{-1}) and NAA (0, 0.2, and 0.5 mg L^{-1}) doses. The highest rate of shoot forming calli was obtained from genotype 25 in all NAA and TDZ combinations, and there was no statistical difference between genotypes 24 and 25. The number of shoots per callus was found to be low in genotypes with a high rate of shoot forming calli. The maximum shoot number was obtained from the cultivar Linas on medium containing 1 mg L^{-1} TDZ, with 9.6 shoots/per callus and this value was followed by cultivar Balcı cultured at the same dose with 6.7 shoots/per callus. The rooting of safflower genotypes differed depending on the NAA content of the medium. Better rooting was achieved on medium with 2 mg L^{-1} NAA for Balcı, 1 mg L^{-1} NAA for Linas, and 0.1 mg L^{-1} NAA for genotypes 25 and 55. On the other hand, genotype 24 indicated rooting only on medium with 2 mg L^{-1} NAA, but it was very low. As a result; regeneration of safflower genotypes via callus-mediated organogenesis from cotyledonary explants was varied depending on TDZ and NAA doses, and many shoots were induced in Linas cultivar at 1 mg L^{-1} TDZ. However, the rooting of the regenerated shoots was quite low at different NAA doses.

Keywords: Safflower, Cotyledonary node, In vitro regeneration, TDZ, NAA

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INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is a mostly self-pollinated annual plant and is used both as a nutrition and energy crop (Knowles, 1969; Bérvillé et al., 2005). Safflower oil is important for human nutrition due to its rich unsaturated fatty acids such as oleic and linoleic acid (Liu et al., 2016; Katkade et al., 2018). On the other hand, increasing energy demand worldwide and concerns about environmental protection rise the interest in bioenergy crops such as safflower. It has a production potential in very different climates and soil conditions (Abd El-Lattief, 2012; La Bella et al., 2019).

The main goal of safflower breeding programs is to develop new varieties with high seed yield, oil content and quality, wide adaptability, and resistance to diseases and insects (Babaoglu and Guzel, 2015). Plant tissue culture applications are widely used as contributor methods in conventional breeding programs and gene transfer manipulations, however, the efficient use of these techniques in the improvement of plants requires a high-frequency shoot regeneration from tissues and cells. On the other hand, plant regeneration is specific for

each genotype and is affected by the nutrient media, hormones and environmental factors.

Safflower regeneration is quite difficult and there are specific effects on safflower regeneration of genotype, seedling age, explant type, media components, plant growth regulators and other additives (Fan and Guo, 2013). A higher regeneration rate was obtained in studies using varieties originating from India (Vijaya Kumar et al., 2008; Sri Shilpa et al., 2010) and Australia (Belide et al., 2011) compared to studies using varieties originating from China (Yang et al., 2009), Turkey (Başalma et al., 2008) and Iran (Motamedi et al., 2011). In addition, mostly cotyledon and hypocotyl segments were preferred as explant in these studies and callus-based shoot bud regeneration was achieved (Mandal and Gupta, 2001; Walia et al., 2005 Yang et al., 2009). On the orher hand, the use of cotyledonary nodes for *Agrobacterim*-mediated transformation resulted in genotype-independent regeneration in MS medium supplemented with BAP (6-Benzylaminopurine), NAA (Naphthalene acetic acid), and ascorbic acid in safflower (Patial et al., 2016).

In this study, there are 2 registered safflower cultivars (Balcı and Linas) and 3 hopeful inbred lines (24, 25, and 55) selected for winter hardiness, and their regeneration potential has not been determined by using cotyledonary nodes. The aim of this study was to reveal the effect of TDZ and NAA combinations on direct shoot regeneration from cotyledonary nodes in these safflower genotypes.

MATERIALS AND METHODS

Seeds of five safflower genotypes, 2 commercial cultivars (Balcı and Linas) and 3 inbred lines (24, 25 and 55) selected for winter hardiness, were used to establish callus mediated shoot regeneration and their names, flower colors and spininess are indicated in Table 1. Two plant growth regulators, 1-Naphthaleneacetic acid [(NAA), (Sigma-Aldrich product no: N0640)] and Thidiazuron [(TDZ), (Sigma-Aldrich product no: 45686)] were used for shoot bud regeneration of safflower genotypes. NAA was also used in induction of rooting. Seed surface sterilization was performed with mercuric chloride (PubChem CID: 24085) as described below.

The design of experiment was a completely randomized involving three factors with three replications. First of these factors was safflower genotypes, second was doses of NAA (0, 0.2, and 0.5 mg L^{-1}) and third was doses of TDZ (0.1, 0.5 and 1 mg L^{-1}).

Table 1. Flower colors and spininess of safflower genotypes.

Genotypes	Flower colors	Spininess
Balcı	Yellow	Spiny
Linas	Red	Spiny
Line 25	Yellow	Very few spines
Line 55	Orange/Red	Spiny
Line 24	Yellow	Few spines

First of all, the seeds were washed with tap water for 10 minutes and then were treated with 70% alcohol for 2 minutes and then with 0.1% of mercuric chloride ($HgCl_2$) for 5 minutes. Finally, these seeds were rinsed 5 times with sterile distilled water and were planted on 60 × 15 mm petri dishes including 10 ml of ¼ MS media (Murashige and Skoog, 1962) with 0.75 % sucrose.

These seeds were cultured at 25 °C in the dark condition and 5-6 days after germination (Fig. 1a) , the cotyledon nodes without primary leaves were moved to the full-strength MS medium including 3 % sucrose in different combinations of TDZ and NAA by cutting half of the cotyledon leaves (Fig. 1b) to induce shoot bud regeneration.

These cultures were initially maintained for 2 weeks at 25 °C in darkness and then incubated at the same temperature under 16/8 hours (day/night) at 50-60 % humidity. After 4-6 weeks from the beginning of the culture, the rates of shoot-forming callus (RSFC) were determined and these calli were moved to MS medium comprising 0.5 mg L^{-1} of kinetin with 3 % sucrose to induce shoot elongation. The number of shoots per callus (NSPC) was determined in these cultures two weeks later. Developed shoots were planted to half-strength MS medium involving 0.1, 0.5, 1, and 2 mg L^{-1} of NAA for root induction.

The data were analyzed by using JMP 14 statistical package program. Arcsin √x transformation was performed to values of RSFC. Significant mean values were compared with LSD test.

RESULTS AND DISCUSSION

Rates of shoot-forming callus

In general, hard calli with shoots were induced in all combinations. Analysis of variance indicated that RSFC was significantly influenced by the safflower genotypes, doses of NAA and doses of TDZ and their interactions (Table 2). The response of safflower genotypes to callus induction varied and the highest and lowest RSFC among safflower genotypes were in Line 25 and Line 55, respectively. These results confirmed the findings of Rajendra Prasad et al. (1991) and Mandal and Gupta (2001) on callus induction and regeneration of safflower.

While the effects of increasing NAA doses on RSFC were positive, the lowest dose of TDZ resulted in the highest RSFC (Table 2). No callus induction was obtained on the medium including 0.5 mg L^{-1} of TDZ in Line 55 (Table 3). On the other hand, Line 25 produced the highest RSFC as averaged value over all NAA and TDZ combinations. Although NAA was not required for shoot forming callus induction in this study (Table 3), other research indicated that the media enriched with combinations of auxin and cytokinin is widely used in tissue cultures to increase

callus induction (Baskaran et al., 2006; Soheilikhah et al., 2013; Ghasempour et al., 2014; Ali and Afrasiab, 2014).

Shoots were produced from calli after 4-6 weeks of the beginning of culture (Fig. 1c and Fig. 1 d). The NSPC varied with the safflower genotypes, and also a high RSFC did not produce high shoot regeneration (Table 2 and Table 4). Radhika et al. (2006) and Nikhil et al. (2014) reported that there were significant differences in shoot induction from calli in different safflower genotypes. In many studies, it has been determined that safflower genotypes with Indian and Australian origins (Mandal

Table 2. The results of variance analysis and differences between mean values of RSFC and NSPC resulting from cultured cotyledonary node segments of safflower genotypes at various TDZ and NAA doses. (Mean ± standard error).

Factors	Ratios of shoot-forming callus [(%), (RSFC)]	Number of shoots per callus (NSPC)
Safflower genotypes		
Balcı	88.5±3.30^{b†}	2.11±0.35^{b}
Linas	87.7±3.70^{b}	2.42±0.51^{a}
Line 24	92.7±2.89^{ab}	1.13±0.05^{c}
Line 25	98.1±1.28^{a}	1.17±0.06^{c}
Line 55	72.2±6.85^{c}	1.17±0.15^{c}
NAA doses (mg L^{-1})		
0	81.7±4.16^{b}	2.44±0.36^{a}
0.2	89.1±3.24^{a}	1.26±0.05^{b}
0.5	92.7±2.19^{a}	1.12±0.07^{b}
TDZ doses (mg L^{-1})		
0.1	94.5±1.90^{a}	1.21±0.07^{b}
0.5	81.2±4.23^{c}	1.31±0.10^{b}
1	87.8±3.23^{b}	2.29±0.36^{a}
Analysis of variance		
Genotypes (A)	**	**
NAA doses (B)	*	**
TDZ doses (C)	*	**
A × B	*	**
A × C	**	**
B × C	*	**
A × B × C	**	**

*, **: Significant level of 5% and 1%, respectively †: Different letters indicate different groups at the 5% level.

Table 3. The effect of different NAA and TDZ doses on shoot-forming callus ratio (%) in different safflower genotypes. (Mean ± standard error).

		TDZ doses (mg L^{-1})		
Safflower genotypes	**NAA doses (mg L^{-1})**	**0.1**	**0.5**	**1**
Balcı	0	83±8.34^{abc*}	100±0^{a}	75±14.45^{bcd}
	0.2	100±0^{a}	87±6.78^{abc}	93±6.78^{ab}
	0.5	83±16.97^{abc}	83±16.97^{abc}	92±8.48^{abc}
Linas	0	100±0^{a}	50±0de	100±0^{a}
	0.2	100±0^{a}	73±6.78^{cd}	100±0^{a}
	0.5	83±16.97^{abc}	92±8.48^{abc}	92±8.48^{abc}
Line 24	0	85±7.77^{abc}	75±14.75^{bcd}	100±0^{a}
	0.2	100±0^{a}	92±8.48^{abc}	83±16.97^{abc}
	0.5	100±0^{a}	100±0^{a}	100±0^{a}
Line 25	0	92±8.48^{abc}	100±0^{a}	100±0^{a}
	0.2	100±0^{a}	100±0^{a}	100±0^{a}
	0.5	100±0^{a}	100±0^{a}	92±8.48^{abc}
Line 55	0	92±8.48^{abc}	0±0^{f}	75±0^{bbd}
	0.2	100±0^{a}	83±16.97^{abc}	25±0^{e}
	0.5	100±0^{a}	83±8.48^{abc}	92±8.48^{abc}

* Different letters indicate different groups at the 5% level for genotype × NAA × TDZ interactions.

and Gupta, 2001; Vijaya Kumar et al., 2008; Sri Shilpa et al., 2010; Belide et al., 2011) showed higher regeneration than those with Turkish, Chinese and Iranian origins (Başalma et al., 2008; Yang et al., 2009; Motamedi et al., 2011).

In general, increasing NAA doses with combinations of 0.5 and 1 mg/l TDZ negatively affected the NSPC except for Line 55 as indicated in Table 4. The highest NSPC was found in Linas at 1 mg L^{-1} of TDZ without NAA and this genotype was followed by the Balcı at the same dose (Table 4). A wide range of TDZ + NAA combinations was used for high shoot regeneration of safflower. Nikhil et al. (2014) supported the finding that the high shoot regeneration of safflower in a medium comprising of low NAA and high TDZ combination.

Rooting

Elongated shoots without vitrification were transferred to the rooting medium (Fig. 1e) and it was clear in the first week whether there would be rooting or not of these shoots. (Fig. 1f). When rooting was delayed, the shoots completely died off, and regeneration of the green shoot did not occur over again even if roots were induced from the callus. All safflower genotypes were rooted at different NAA doses, even at low frequency (Table 5). Better rooting in Balcı was achieved at 2 mg L^{-1} of NAA, 1 mg L^{-1} in Linas, and 0.1 mg L^{-1} in Line 25 and Line 55 and also poor rooting was obtained in Line 24 at only 2 mg L^{-1} of NAA dose.

Rooting and subsequent acclimatization are among the most challenging issues for safflower regeneration (Sujatha, 2007). Rooting studies on safflower genotypes have reported successful rooting at different doses of NAA such as 0.1 mg L^{-1} (Mandal and Gupta, 2003; Walia et al., 2007), 0.5 mg L^{-1} (Radhika et al., 2006), 1 mg L^{-1} (Mandal and Gupta, 2001). Also, Yang et al. (2009) determined that a combination of 2 mg L^{-1} of NAA and 0.5 mg L^{-1} of IAA (Indole-3-acetic acid) promoted the rooting of safflower.

Table 4. The effect of various NAA and TDZ doses on the number of shoots per callus in different safflower genotypes. (Mean ± standard error).

		TDZ doses (mg L^{-1})		
Safflower genotypes	**NAA doses (mg L^{-1})**	**0.1**	**0.5**	**1**
Balcı	0	$1.0\pm0^{i*}$	2.2 ± 0.27^{de}	6.7 ± 0.4^{b}
	0.2	1.2 ± 0.11^{hı}	1.2 ± 0.11^{hı}	2.1 ± 0.48^{ef}
	0.5	2.0 ± 0.98^{efg}	1.0 ± 0^{i}	1.4 ± 0.44^{ghı}
Linas	0	$1.8\pm0.14^{e-h}$	3.0 ± 0^{c}	9.6 ± 0.08^{a}
	0.2	$1.5\pm0.05^{f-ı}$	1.4 ± 0.18^{hı}	$1.7\pm0.24^{e-ı}$
	0.5	1.0 ± 0^{i}	1.0 ± 0^{i}	1.0 ± 0^{i}
Line 24	0	1.0 ± 0^{i}	$1.8\pm0.14^{e-h}$	1.0 ± 0^{i}
	0.2	1.0 ± 0^{i}	1.4 ± 0.22^{ghı}	1.0 ± 0.06^{i}
	0.5	1.0±0	1.0 ± 0^{i}	1.0 ± 0^{i}
Line 25	0	1.1 ± 0.06^{hı}	1.3 ± 0.22^{hı}	$1.8\pm0.44^{e-h}$
	0.2	1.0 ± 0^{i}	1.0 ± 0^{i}	1.0 ± 0.06^{i}
	0.5	1.0 ± 0^{i}	1.2 ± 0.16^{hı}	1.1 ± 0.06^{hı}
Line 55	0	1.4 ± 0.44^{ghı}	0.0 ± 0^{j}	2.9 ± 0.6^{cd}
	0.2	1.0 ± 0.06^{i}	1.2 ± 0.14^{hı}	1.0 ± 0^{i}
	0.5	1.0 ± 0^{i}	1.0 ± 0^{i}	1.0 ± 0^{i}

* Different letters indicate different groups at the 5% level for genotype × NAA × TDZ interactions

Table 5. The observation of NAA doses on rooting in different safflower genotypes.

Safflower genotypes	NAA doses (mg L^{-1})			
	0.1	**0.5**	**1**	**2**
Balcı	+	-	-	++
Linas	+	-	++	-
Line 24	-	-	-	+
Line 25	++	-	-	+
Line 55	++	-	-	-

+low, +++++high

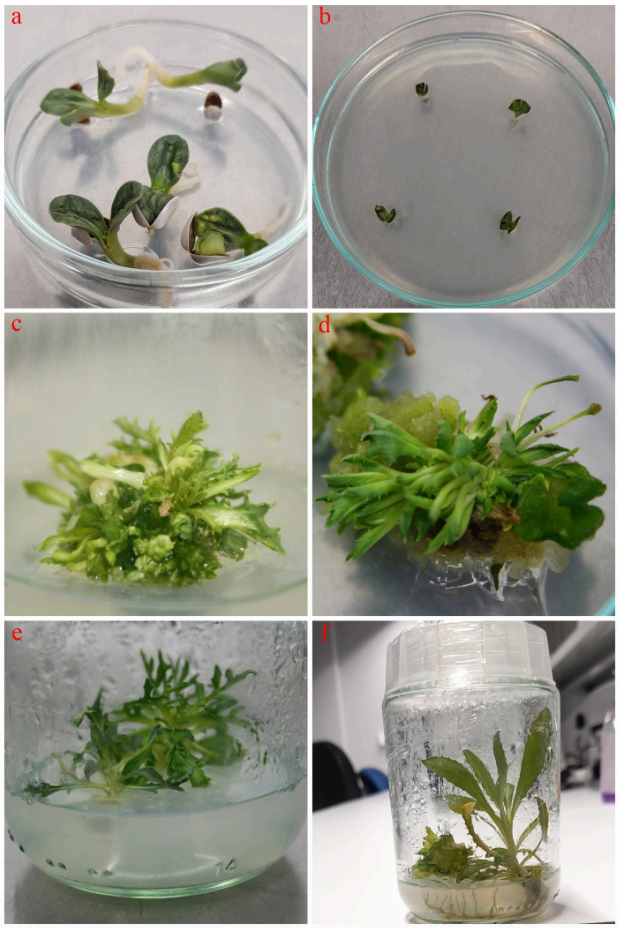

Figure 1. Callus-based shoot bud regeneration and rooting in different safflower genotypes. 1a: Stage of cotyledon node explants after germination from safflower seeds, 1b: Cotyledon node explants with half-cut cotyledon leaves, 1c and 1d: Shoots developing on callus in Linas and Balcı cultivars, 1e: Transferring of the elongated shoots into the rooting medium, and 1f: rooted safflower shoots.

CONCLUSION

In this study, callus-mediated organogenic shoot regeneration was obtained in different TDZ and NAA combinations by using cotyledon nodes in 2 cultivars and 3 inbred lines of safflowers. The NSPC was found to be low in genotypes with a high RSFC. Linas and Balcı cultivars produced high NSPC than inbred lines of safflower. Increasing doses of NAA and TDZ affected positively on RSFC and NSPC, respectively. It is possible to conclude that NAA has generally not any significant effect on the callus induction and shoot regeneration from cotyledonary segments of safflower. Rooting differed according to genotypes and occurred at a low frequency. In sum, Linas and Balcı indicated high number of shoots per callus in media enriched with 1 mg L^{-1} of TDZ without NAA.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

All authors declare that they have no conflicts of interest

Author contribution

The contribution of the authors to the present study is equal.

All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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