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Original article

Regeneration of *Carthamus tinctorius* from Jimsar

Li-jiao Fan, Mei-li Guo*

Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai 200433, China

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ABSTRACT

Objective To investigate the factors significantly affecting the shoot buds regeneration of *Carthamus tinctorius* from Jimsar (CTJ). **Methods** Through tissue culture experiments, the influences of culture temperature, illuminance, relative humidity, explants type, seedlings age, and plant growth regulators added in culture media on *in vitro* regeneration of CTJ were investigated. **Results** The culture temperature was set at 24 °C in daytime and 16 °C at night, illuminance at 9000 lx, and 60% relative humidity were suitable for CTJ regeneration. Cotyledons excised from 6-8 d old seedlings were more responsive than even-aged euphyll, hypocotyl, and root explants because of inducing adventitious buds. The highest percentage of regenerated shoots (79.1%) with about five adventitious buds per responding explant was obtained from MS basal medium containing 12.0 mg/L TDZ, 2.5 mg/L IBA, and 1.5 mg/L 2-ip (No. 14 medium). Regenerated shoot buds (80%) could elongate successfully after 1-2 weeks transferred to shoot elongation medium. **Conclusion** Suitable factors of shoot buds regeneration for CTJ were determined. It may also make a useful reference for regeneration researches of other *C. tinctorius* varieties in China.

Key words

Carthamus tinctorius; Compositae; regeneration; safflower; tissue culture

1. Introduction

Safflower (*Carthamus tinctorius* L.) is an annual herbaceous oilseed crop and medicinal plant. It belongs to the family Compositae, which is well adapted to semiarid conditions in the tropics and subtropics (Kumar et al, 2008a). India, America, Mexico, Argentina, and China are the main production countries of this crop. Safflower was mainly cultivated for its flowers and seeds that both have profitable industrial value. Its flowers have long been used to colour fibers, flavour foods, and treat diseases (Mandal et al, 1995). Especially for Chinese safflower varieties, they were mainly cultivated for pharmaceutical purpose. Safflower florets were used as a Chinese materia medica originally recorded in *Kaibao Bencao* (Guo et al, 1996), and its major function was to promote menstruation by activating blood, eliminating stasis, and analgesia (Pharmacopeia Committee of P. R. China, 2010). Safflower seed oil was widely used in food, medicine, paint, and lubricant industry. The levels of oleic and linoleic acids in safflower seed oil were the highest (75%−90%) among all plant oils. It is very desirable for human nutrition due to the high degree of unsaturation and notable level of α-tocopherol (Furuya et al, 1987), which has the ability to reduce blood cholesterol level and delay senility.

* **Corresponding author: Guo ML** Address: 325 Guohe Road, Yangpu District, Shanghai 200433, China E-mail: mlguo@126.com Tel/Fax: +86-21-8187 1302

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An efficient and reproducible plant regeneration system is the prerequisite for successful genetic transformation and germplasm improvement. Protocols for safflower regeneration have been reported by Indian (George and Rao, 1982), American (Ying et al, 1992), Turkish (Basalma et al, 2008), Iranian (Motamedi et al, 2011), Chinese (Yang et al, 2009), and Australian (Belide et al, 2011) cultivars. Regrettably, the reported safflower regeneration systems have low regeneration frequency and fail in efficient protocol suitable for this wide range of safflower cultivars. Chinese safflower was cultured mainly in Xinjiang Uygur Autonomous Region, Henan, Sichuan, and Yunnan provinces, and the representative varieties were *C. tinctorius* in Xinjiang Jimsar, Henan HX, Sichuan JY, and Yunnan WS. *C. tinctorius* in Jimsar (CTJ) was the major safflower variety in China, with large habitating areas and high content of flavonoids. However, there were abundant pigments in CTJ, which often made the explants get brown and hard to regenerate adventitious buds (Yi et al, 2005; Liu, 2011). This study took CTJ as an example to investigate the factors significantly affecting *in vitro* regeneration of safflower, which would make a reference for other Chinese safflower varieties.

2. Materials and methods

2.1 Materials

The seeds of *Carthamus tinctorius* L. in Jimsar (CTJ) used in this study were stored in our laboratory, numbered ZHH0019 in Chinese safflower germplasm resource library.

MS (Murashige and Skoog, 1962) medium with 0.4% phytagel (Sigma-Aldrich, Co., USA) and 3% sucrose was used as basal medium in all the experiments for plant materials growth. Plant growth regulators (PGRs) including 6-benzylaminopurine (6-BA), α-naphthaleneacetic acid (NAA), thidiazuron (TDZ), indole-3-butytric acid (IBA), and 2-isopentenyladenine (2-ip) were dispensed to the concentration of 1 mg/mL as stock solutions and sterilized by filtration, then stored at 4 ºC away from light. All the phytohormones used in this study were bought from Sigma-Aldrich, Ltd., USA.

2.2 Preparing media, sterilizing seeds, regeneration induction, and data collection

MS basal media were prepared with the pH adjusted to 5.8−6.2 by 1 mol/L HCl or NaOH, then autoclaved at 121 ºC for 25 min. Filtration-sterilized stock solutions of PGRs were added in MS basal medium in super clean bench according to the prescriptions of different inducing media (Table 1), when the sterilized media were cooled to about 50 ºC.

Safflower seeds were surfacely sterilized by 75% ethanol solution for 30 s and rinsed for three times with sterile distilled water for 3 min each time. Then the seeds were deep sterilized with 0.1% mercuric chloride ($HgCl_2$) for 25 min, followed by six washes for 5 min each time in sterile distilled water. Sterilized safflower seeds were dried with sterile filter papers and sowed on MS basal medium for germinating aseptic seedlings. Cotyledons, euphylls, hypocotyls, and roots excised from 6, 8, 10, 12, and 14 d old aseptic seedlings were used as primary explants and placed with the abaxial side on the surface of adventitious bud induction medium. This study was arranged in completely randomized design, each treatment had 20 replications (6 cm diameter culture bottle) and each replication consisted of four explants. The experiment was repeated for four times in all. To investigate the influences of culture conditions on shoot buds regeneration, cultures were maintained at 24 ºC in daytime and 16 ºC at night or at 24 ºC all the day, under cool white light with the photoperiod of 16 h light and 8 h dark, the illuminance was set at 4600, 7800, 9000, and 12 000 lx separately, and relative humidity was set at 40%, 60%, and 80%. After a period of induction, regenerated adventitious buds were transferred into elongation medium to grow up. The growth status of explants was recorded through visual observation every week after culture initiation. Data were subjected to ANOVA ($P \leq 0.05$) and the efficiencies of different inducing media were assessed on the basis of regeneration frequency, number of regenerated shoots per responding explant and bud appearance.

Table 1 Media used in this study

No.	Media / ($mg \cdot L^{-1}$)
1	MS + 6-BA (0.5) + NAA (0.1)
2	MS + 6-BA (0.5) + NAA (2.0)
3	MS + 6-BA (1.0) + NAA (0.2)
4	MS + 6-BA (1.0) + NAA (1.0)
5	MS + 6-BA (1.5) + NAA (0.5)
6	MS + 6-BA (2.0) + NAA (0.2)
7	MS + 6-BA (2.0) + NAA (0.5)
8	MS + 6-BA (2.0) + NAA(1.0)
9	MS + TDZ (0.2) + NAA (0.2)
10	MS + TDZ (0.5) + NAA (0.5)
11	MS + TDZ (2.0) + NAA (0.5)
12	MS + TDZ (6.0) + IBA (2.5) + 2-ip (1.5)
13	MS + TDZ(9.0) + IBA (2.5) + 2-ip (1.5)
14	MS + TDZ (12.0) + IBA (2.5) + 2-ip (1.5)
15	MS + TDZ (15.0) + IBA (2.5) + 2-ip (1.5)
16	MS + 6-BA (1.0) + NAA (1.0)
17	MS + GA3(0.2)
18	MS + myoinositol (400) + casamino acid (200) + *L*-glutamine (500) + thiamine HCl (250) + pyridoxine HCl (250) + nicotinic acid (300) + *L*-asparagine (50)
19	MS basal medium

1−15: adventitious bud induction media; 16−19: shoot elongation media

3. Results

All explants on adventitious bud induction medium swelled within 2 d of culture initiation, followed by callus formation at the cut ends. The calli induced on MS basal medium in combination with 6-BA + NAA were loose and friable with sporadic green spots. The calli formed on MS

contained TDZ + NAA were loose, friable, and occasionally translucent, while calli formed on MS with TDZ + 2-ip + IBA were in dark green, compacted and excrescent (Figure 1A). About two weeks after culture initiation, adventitious buds began to present at the adaxial cut ends of cotyledons explants on some media (Figure 1B). After another 2−4 weeks of induction, the number of cotyledons explants that regenerated adventitious buds increased gradually and reached the maximum (Table 2 and Figure 2). However, there were plenty of watery calli generated from hypocotyls and roots explants but no any adventitious bud. Euphylls explants could generate adventitious buds occasionally, but the number of responding euphylls explants was too small and hardly reproducible. In the all, cotyledons were the most responsive explants among even-aged euphyll, hypocotyl, and root explants for the adventitious buds regeneration of CTJ. In addition, cotyledons excised from 6−8 d old seedlings were more responsive than those excised from 10, 12, and 14 d old seedlings. In terms of culture temperature, we found that the calli or regenerated adventitious buds originated from explants cultured at continual 24 ºC were comparatively loose and watery, while those at 24−16 ºC rhythmic condition were compacted and viable. In the four levels of illuminance, explants at 9000 lx were more regenerative than those at other levels (data not presented). Most explants got terribly watery and brown at the illuminance level of 12 000 lx. At 40% relative humidity, explants were a little dry and regenerated less adventitious buds than those at higher relative humidity level. However, 80% relative humidity was too high to regenerate loose and watery adventitious buds. In the all, the optimal culture conditions were at 24 ºC in daytime and 16 ºC at night, illuminance at 9000 lx and 60% relative humidity. Adventitious buds induction experiment showed No. 1, 2, 4, 5, 7, 8, 12−14, and 15 media had different competence of inducing safflower adventitious buds from cotyledons explants (14.7%−79.1%), while No. 3, 6, and 9−11 media did not induce the adventitious bud at all (Table 3). As we raised the concentration of TDZ in No. 12−14 media, the regeneration frequency and numbers of adventitious buds per responding explant increased simultaneously (Table 3 and Figure 3). Nevertheless, regeneration frequency decreased with the level of TDZ increased further (No. 15 medium), while the number of adventitious buds per responding explant increased continuously. Thus, TDZ (12.0 mg/L) in combination with IBA (2.5 mg/L) and 2-ip (1.5 mg/L) (No.14 medium, Table 1) in MS was the most responsive adventitious buds induction medium composition for CTJ, with about five adventitious buds per responding explant. When compared the effectual regeneration frequencies of different inducing media between any two media, the effectual regeneration frequency (79.1%) on No. 14 medium had significant statistical differences to others (P = 0.00). The adventitious buds originated on No. 14 medium were fresh green and vigorous (Figure 1C). No. 15 medium also had a fairly higher regeneration frequency (65.9%) with about seven adventitious buds per responding explant. For shoot elongation, explants with regenerated shoot buds were transferred to No. 16−19 media separately. Well-developed shoots (4−5 cm tall; Figure 1D) were observed on No. 19 medium after 1−2 weeks induction. However, those explants with shoots cultured on No. 18 medium were infected with fungi acutely and those on No. 16 and 17 media turned brown gradually. Subsequently,

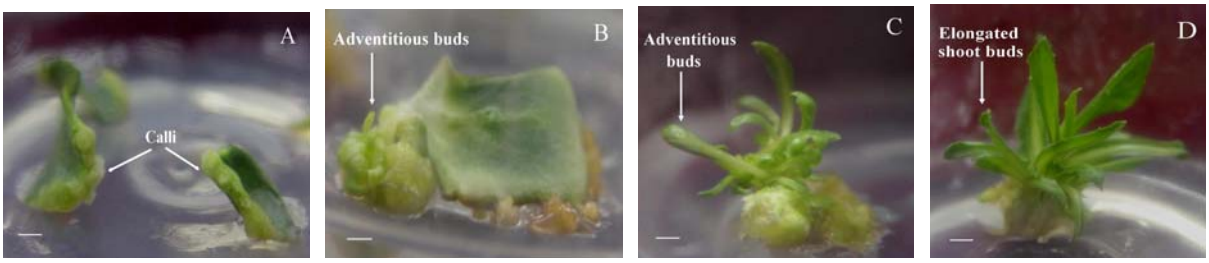

Figure 1 Callus formed from cotyledons explants after 8 d induction (A), adventitious buds began to present at adaxial cut ends of cotyledons explants after 15 d induction (B), adventitious buds with calli obtained from cotyledons explants after 6 weeks induction (C), and well developed shoots observed after 2 weeks elongation (D)

Table 2 Number of cotyledons explants regenerated adventitious buds on different adventitious bud induction media ($\bar{x} \pm s$)

Adventitious bud induction medium No.	Number of cotyledons explants regenerated adventitious buds				
	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
1	3.8 ± 2.1	8.2 ± 2.6	12.2 ± 2.1	14.0 ± 1.0	15.8 ± 1.6
2	5.2 ± 1.5	10.2 ± 2.3	14.0 ± 1.7	15.8 ± 0.5	17.6 ± 1.5
4	3.2 ± 0.9	4.2 ± 0.9	5.8 ± 0.8	7.2 ± 0.8	8.2 ± 1.3
5	6.2 ± 1.6	9.2 ± 4.8	12.6 ± 5.9	17.2 ± 1.3	18.0 ± 1.6
7	5.6 ± 2.4	10.6 ± 3.5	13.8 ± 1.9	15.6 ± 1.1	16.2 ± 1.1
8	5.2 ± 2.2	9.6 ± 2.7	14.4 ± 1.7	16.4 ± 1.3	17.0 ± 1.2
12	13.2 ± 1.3	19.8 ± 2.8	24.2 ± 2.2	26.8 ± 2.2	27.6 ± 1.5
13	12.2 ± 3.0	19.8 ± 3.9	24.0 ± 2.9	26.4 ± 2.4	27.8 ± 1.8
14	29.0 ± 1.6	39.6 ± 2.0	45.4 ± 1.3	48.6 ± 1.5	49.6 ± 2.0
15	26.4 ± 1.5	35.6 ± 2.0	40.4 ± 1.3	41.6 ± 1.1	42.2 ± 0.5

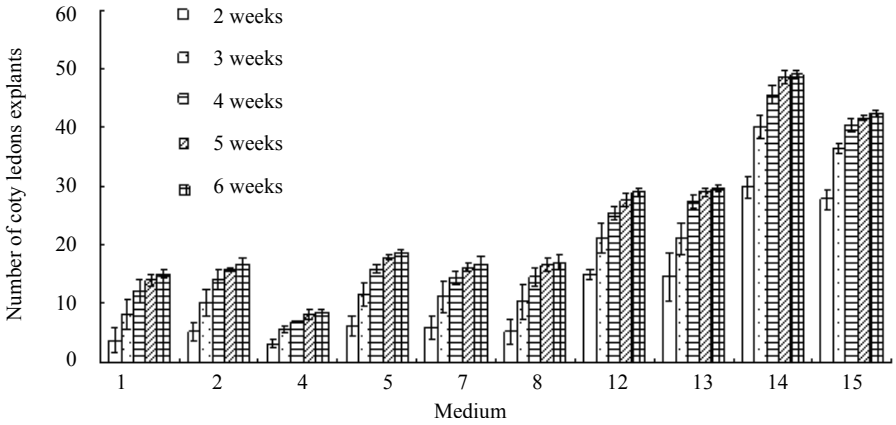

Figure 2 Number of cotyledons explants regenerated adventitious buds from different adventitious bud induction media after 2−6 weeks induction

Table 3 Regeneration responses of cotyledons explants on different adventitious buds induction media, assessed on basis of regeneration frequency, number of regenerated shoots per responding explant, and bud appearance after 6 weeks induction ($\bar{x} \pm s$)

Adventitious bud induction medium No.	Effectual explants number* / %	Effectual regeneration frequency** / %	Number of regenerated shoots per responded explant	Bud appearance
1	62.1 ± 3.2a	25.5 ± 3.4a	12.3 ± 0.1	normal
2	56.4 ± 2.1b	31.2 ± 3.4a	3.0 ± 0.0a	+++
4	55.9 ± 1.8b	14.7 ± 2.7	7.6 ± 0.2b	++
5	59.7 ± 4.3	30.3 ± 4.0a	10.4 ± 0.2	+
7	61.8 ± 3.3a	26.3 ± 2.6a	22.6 ± 0.4	++
8	58.6 ± 2.7	29.1 ± 2.8a	1.0 ± 0.0	+++
12	64.3 ± 1.6c	43.0 ± 3.0b	2.7 ± 0.2a	++
13	63.2 ± 3.5d	44.1 ± 4.3b	3.3 ± 0.9a	+
14	62.8 ± 1.9d	79.1 ± 4.6	5.3 ± 0.8	normal
15	64.1 ± 2.6c	65.9 ± 3.2	7.0 ± 0.2b	normal

*: number of explants without microbiological contamination or brown; **: number of cotyledons explants regenerated adventitious buds / effectual explants number; +: slight vitrification; ++: medium vitrification; +++: deep vitrification; Values followed by the same letter in each column are not significantly different at $P = 0.05$ by LSD

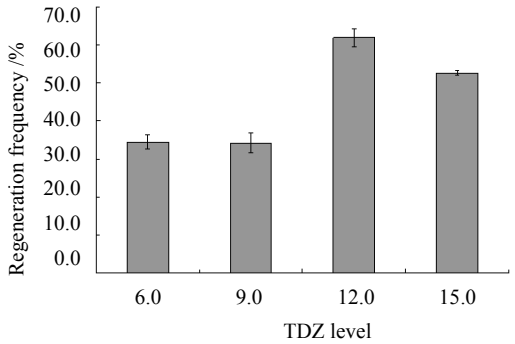

Figure 3 Adventitious buds regeneration frequencies of cotyledons explants at different TDZ levels in No. 12−15 media after 6 weeks induction

they all died at last. As a result, about 80% shoot buds elongated successfully on elongation No. 19 medium.

4. Discussion

Shoot buds regeneration was considered as one of the most difficult procedures in the whole regeneration system for safflower tissue culture. George and Rao (1982) demonstrated that the successful degree of safflower *in vitro* regeneration depended upon the genotype selected and the media used for culture.

This opinion has been agreed by subsequent reports of safflower regeneration. Furthermore, the kind of explants, seedling age, the combination and concentration of PGRs used and culture conditions all had great influences on safflower regeneration (Prasad et al, 1991; Nikam and Shitole, 1999; Mandal et al, 2001; Kumar et al, 2008b; Yang et al, 2009). Our adventitious buds induction experiment validated these viewpoints as well.

Orlikowska and Dyer (1993) first used TDZ in safflower regeneration study for two American varieties (Centennial and Montola). They added TDZ (0.1 mg/L) combined with NAA (0.1 mg/L) in MS as shoot regeneration medium and got a high frequency of shoot regeneration associated with the formation of large number of leafy shoot (data not given). Radhika et al (2006) used the same growth regulator combination at various concentration for three Indian safflower cultivars (A-1, Manjira, and HUS-305) and got a

very high induction rate (up to 98.5%). Similarly, Sujatha and Kumar (2007) reported 100% regeneration frequency for *C. arborescens*. We tried those high-frequency inducing media (No. 9-11) in our adventitious bud induction experiment, but did not get any regenerated shoot buds on them. It suggested that safflower explants of different genotypes might have different sensibility to the same inducing medium. In 2008, Kumar induced 94.3% embryogenic callus using MS + TDZ (6.0 mg/L) + 2-ip (1.5 mg/L) + IBA (2.5 mg/L), while 38.7% embryo germinated after transferred into generation medium (Kumari et al, 2008a; 2008b). We tried the same medium (No. 12) to induce adventitious bud, finally got 100% callus and a little higher shoot buds regeneration frequency (43.0%) without transferred into generation medium. As we raised the concentration of TDZ, into regeneration frequency of adventitious buds per responding explant increased during a range and decreased when beyond the range, while the numbers of adventitious buds per responding explant increased continuously. It suggested that TDZ might have a crucial regulative effect on safflower adventitious bud regeneration.

Such a high frequency regeneration protocol for CTJ made it as a platform for producing useful pharmaceutical secondary metabolites through genetic transformation and germplasm improvement possible. It also might be referenced by other safflower varieties regeneration.

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