植物学通报 2004, **21** (4): 455~460
Chinese Bulletin of Botany

研 究 简 报

产藏红花素 1(crocin)愈伤组织的诱导及其细胞系的筛选①

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摘要 对藏红花(*Crocus sativus* L.) 愈伤组织的诱导条件进行了优化。结果表明：MS 是藏红花芽愈伤组织的最佳诱导培养基，而 B5是 叶子和花愈伤组织的最佳培养基。藏红花芽、叶和花愈伤组织的最佳诱导温度分别是18℃、25℃和21℃。光照是叶子愈伤组织诱导的有利因素，但不利于芽和花愈伤组织的诱导。1.5～2.0 mg·L^{-1} NAA和 0.25 mg·L^{-1} 6-BA是愈伤组织诱导的最佳激素组合。通过目视法和HPLC方法, 从229株细胞系中筛选出细胞系Corm1，其藏红花素1 的含量是1 677 mg·g^{-1}，生长较快，且不易褐化。为采用植物细胞工程法解决藏红花素1资源短缺问题打下了基础。

关键词 藏红花, 藏红花素1, 愈伤组织，筛选

Screening of *Crocus sativus* L. Callus Lines for Crocin Production

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Abstract Induction conditions of *Crocus sativus* L. callus were optimized. MS medium was optimal for shoot callus induction while B5 medium was optimal for leaf and flower callus induction. 18℃, 25℃ and 21℃ were the optimal temperature for callus induction of shoot, leaf and flower, respectively. Light was positive factor for leaf callus induction but negative for shoot and flower induction. Naphthaleneacetic acid (NAA, 1.5 - 2.0 mg·L^{-1}) and 6-benzyladenine (6-BA, 0.25 mg·L^{-1}) were the optimal hormones for callus induction. Under the optimized conditions, the induction rate reached 60% for shoot callus, 80% for leaf callus, and 35% for flower callus. Using eyesight and HPLC methods, one callus line which gave rise to 1 677 mg·g^{-1} crocin and would not brown was obtained from 229 callus lines.

Key words *Crocus sativus* L., Crocin, Callus, Screening

Crocin, the main pigment of saffron (*Crocus sativus* L.) stigma, is soluble in water and exhibits a high coloring capacity. A powerful quencher of free radicals and endowed with anticancer activity,

①Foundation item: 863 Program.

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Received: 2003-04-01 Accepted: 2003-04-08 Managing editor: SUN Dong-Hua

https://www.sciengine.com/doi/10.3724/CBB-t30-033

crocin could be a first choice of hydrosoluble natural yellow food colorant (Escribano and Alonso, 1996; Dufrene *et al.*, 1999). Commercial utilization of saffron pigment is restricted by a prohibitive price and limited availability. As a geophyte, saffron grows slowly and propagates only by vegetative production through daughter corm. It takes 150 000 - 200 000 flowers and over 400 h of hand-labor to produce 1 kg of saffron stigma (Plessner *et al.*, 1989). Plant tissue culture methods offer a great potential for crocin production.

One approach is to produce crocin by *in vitro* stigma-like-structure (SLS) culture; another is by the induction of crocin in callus tissues. Problems encountered in these approaches are the low frequency of SLS regeneration, slow growth, low crocin content and the browning of callus tissues (Loskutov *et al.*, 1999).

In order to get the quick growth, high crocin content and not easily browning callus tissues, induction and screening of saffron callus lines were systematically studied.

1 Materials and Methods

1.1 Plant material

Crocus sativus L. was collected from Zhejiang province of China.

1.2 Shoot, leaf and flower callus induction

Shoot, leaf and flower explants were rinsed by tap water for 1 - 2 h, washed with distilled water, dipped in 70% alcohol for 20 s, surface sterilized for 20 min in 1% sodium hypochlorite solution, and then rinsed five times with sterile distilled water. Afterward, explants were dissected as sided pieces of 0.5 - 1 cm and inoculated on MS medium (Murashige and Skoog, 1962), B5 medium (Gamborg *et al.*, 1968), White medium (White, 1963) and N6 medium (Nitsch and Nitsch, 1969), respectively. Dichlrophenoxyacetic acid (2,4-D, 2.5 mg·L^{-1}) and 6-BA (0.25 mg·L^{-1}) were added to all of the above mediums. The explants were cultured at 20℃ under darkness.

1.3 Optimization of the cultural conditions for induction of shoot, leaf and flower callus

The culture medium, light or temperature optimized in the last experiment was used to the subsequent experiments instead of basal condition.

1.4 Corm callus induction

Callus was developed from corm mainly as described by Piqueras *et al.* (1996). Briefly, surface sterilized and dissected corms were cultured on MS medium supplemented with 2,4-D (2.0 mg·L^{-1}) and 6-BA (0.25 mg·L^{-1}) at 21 ± 0.3℃ in dark. After 14 days, calli could be observed on the top of the explants.

1.5 Crocin extract

Calli were dried in an oven at 60℃ until their weight did not change and the dry weight was recorded. Each sample was ground, resuspended in ethanol:water (50:50, V/V), stirred for 1 h at 25℃ in dark. Later, the suspension was centrifuged at 3 500 r·min^{-1} for 20 min to separate callus residue and the supernatant was collected. The residue was further extracted by ethanol:water (50:50, V/V) and centrifuged again under the same conditions above. The residue was discarded and the combined

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extracts were passed through a filter (0.22 μm) for high performance liquid chromatograph (HPLC) analyses.

1.6 Crocin detection

HPLC was performed with a GB11A quad pump and with LCQ DECA XP Photodiode Array Detector (a UV-visible spectrophotometer: 190 - 800 nm). Reverse phase HPLC was done using a Nucleosil C18 column (3.9 mm × 150 mm, 5 μm particle size). Samples were eluted over 20 min linear of ethanol:water (53:47, V/V). Other conditions were as follows: detection meter, 440 nm; flow rate, 0.7 mL·min^{-1}; injection volume, 10 μL.

1.7 Standard crocin

Standard crocin was obtained from natural stigmas of *Crocus sativus* L. by thin layer chromatography (TLC) and purified three times through silica gel plate. Crocin showed a chromatographic (HPLC) purity of 97%, and its molecular weight was identified as 977.36 by Mass Spectrometry test (van Calsteren *et al.*, 1997).

1.8 The screening method of callus lines

Callus lines were firstly screened according to their color, growth, and browning characters. Either the yellow, red, quickly growing, or not easily browning lines were selected by eyesight method. The lines selected above were further selected according to crocin content analyzed by HPLC method.

2 Results and Discussion

2.1 *Crocus sativus* L. callus induction

Callus initiation time, induction rate and growth character varied with kind of medium, culture temperature, light and growth hormone used (Table 1 - 3). MS medium was the optimal for shoot callus induction while B5 was optimal for leaf and flower.

Temperature had a significant effect on callus initiation time, induction rate and growth character. 18℃, 25℃ and 21℃ were the optimal temperature for shoot, leaf and flower callus induction, respectively. Light was positive factor for leaf callus induction but was negative for shoot and flower calli.

Naphthaleneacetic acid (NAA, 1.5 - 2.0 mg·L^{-1}) and 6-benzyladenine (6-BA, 0.25 mg·L^{-1}) were the optimized hormones for callus induction. Under these optimized conditions, the induction rate reached 60% for shoot callus, 80% for leaf callus, and 35% for flower callus. 2, 4-D has been reported to be effective for callus induction in many monocotyledonous plant, including *Crocus sativus* L. (Vasil and Vasil, 1984), but it was not effective in this study. The discrepancy between this report and others may be due to different kind of explants used.

2.2 *Crocus sativus* L. callus lines screening by eyesight method

Table 4 indicated that the morphology, browning and growth characters of four kinds of callus lines were different, and the differences might be due to the different explants and mediums used.

In order to get appropriate callus lines for crocin production, calli were firstly screened by eyesight according to their color, growth rate and browning characters. Yellow or red callus lines were

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Table 1 Effects of different mediums on callus induction of *Crocus sativus* L. shoot, leaf and flower

Medium	Initiation time (d)			Induction rate (%)			Growth		
	Shoot	Leaf	Flower	Shoot	Leaf	Flower	Shoot	Leaf	Flower
B5	21	18	35	30	40	10	Slow; some brown	Quick; some brown	Slow; easily brown
MS	15	22	40	40	25	5	Quick; some brown	Slow; some brown	Slow; brown
N6	22	-	-	15	0	0	Slow; some brown		
White	16	25	-	35	10	0	Slow; brown	Slow; brown	

" - " indicates having no results and the callus induction rates were calculated within 60 d

Table 2 Effects of temperature and light on callus induction of *Crocus sativus* L. shoot, leaf and flower

Temperature (℃)		18±0.3	18±0.3	21±0.3	21±0.3	25±1	25±1
Light or dark		Dark	Light	Dark	Light	Dark	Light
Initiation time (d)	Shoot	15	20	15	20	16	21
	Leaf	25	19	26	20	23	18
	Flower	42	-	35	-	38	-
Induction rate (%)	Shoot	45	30	40	35	40	40
	Leaf	30	40	25	40	35	50
	Flower	5	0	20	0	10	0

" - " indicates having no results and the callus induction rates were calculated within 60 d. Light conditions were under 16 h cool-white fluorescent

Table 3 The effect of plant hormones on callus induction of *Crocus sativus* L. shoot, leaf and flower

Growth hormone		2,4-D	2,4-D	2,4-D	2,4-D	2,4-D	NAA	NAA	NAA	NAA	NAA
(mg L^{-1})		0.5	1.0	1.5	2.0	3.0	0.5	1.0	1.5	2.0	3.0
Initiation time (d)	Shoot	15	15	15	16	15	15	15	15	15	15
	Leaf	18	20	18	18	18	18	18	18	18	18
	Flower	-	-	45	-	-	35	40	40	35	38
Induction rate (%)	Shoot	35	35	45	40	35	45	50	60	25	35
	Leaf	40	40	40	35	35	40	70	80	50	50
	Flower	0	0	10	0	0	5	25	20	35	15

" - " indicates having no results. The callus induction rates of corm, shoot and leaf were calculated within 30 d while that of flower was within 45 d

Table 4 Screening of *Crocus sativus* L. calli by eyesight method

	Corm callus	Shoot callus	Leaf callus	Flower callus
Callus morphology	Granule or clump; loose or compact; white, yellow or red; opacity or translucence	Slice or clump; loose or compact; white or yellow; opacity or translucence	Granule or clump; loose or compact; white or yellow; opacity or translucence	Clump; loose; red or yellow; opacity or translucence
Brown	Some easily brown	Some easily brown	Some easily brown	Easily brown
Growth	A few grow rapidly	A few grow rapidly	A few grow rapidly	Grow slowly
Callus lines	102	55	50	22

selected since they might contain high concentration of crocin, and the quickly growing or not easily browning lines were selected as well. Thus, fifteen callus lines induced from corm, three from shoot, six from leaf and four from flower were obtained and used in the following experiments.

2.3 Screening of *Crocus sativus* L. callus lines by HPLC method

Table 5 shows the red, yellow and white callus lines contained a certain quantity of crocin.

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Table 5 Screening of *Crocus sativus* L. callus lines by HPLC method

Callus	Color	Brown	Growth	Crocin (mg·g^{-1})	Callus	Color	Brown	Growth	Crocin (mg·g^{-1})
Corm1	Yellow	Not	Rapidly	1667±58	Corm15	White	Slowly	Slowly	333±23
Corm2	White	Slowly	Rapidly	647±52	Shoot1	Yellow	Slowly	Slowly	1105±67
Corm3	Yellow	Easily	Slowly	2171±101	Shoot2	Yellow	Slowly	Rapidly	771±54
Corm4	Yellow	Easily	Slowly	1086±46	Shoot3	Yellow	Slowly	Slowly	1095±48
Corm5	White	Slowly	Slowly	419±28	Leaf1	Yellow	Easily	Slowly	2714±103
Corm6	White	Slowly	Slowly	187±16	Leaf2	Yellow	Easily	Slowly	2771±132
Corm7	Yellow	Slowly	Slowly	1229±72	Leaf3	White	Not	Rapidly	457±35
Corm8	Red	Easily	Slowly	6376±158	Leaf4	White	Slowly	Rapidly	495±38
Corm9	White	Slowly	Slowly	487±41	Leaf5	White	Not	Rapidly	343±42
Corm10	White	Not	Rapidly	343±25	Leaf6	Yellow	Slowly	Rapidly	695±50
Corm11	White	Not	Rapidly	209±38	Flower1	Yellow	Easily	Slowly	3171±153
Corm12	White	Not	Rapidly	505±40	Flower2	Yellow	Slowly	Slowly	1201±122
Corm13	Yellow	Slowly	Slowly	1124±72	Flower3	Red	Easily	Slowly	5213±184
Corm14	Yellow	Slowly	Slowly	1943±95	Flower4	Yellow	Slowly	Slowly	2281±102

The values are means of three separate experiments ± standard deviation

However, Visanath *et al.* (1990) reported that the combination of NAA plus kinetin or 2,4-D plus kinetin gave rise to yellow callus, but crocin was not detected in these yellow callus lines and was only detected in the red callus lines by TLC method. The differences may be due to the different mediums, plant hormones or crocin detection methods used as well as genotypic factors.

As shown in table 5, the colors of saffron callus were correlative to the crocin content and callus browning. Red and most of yellow callus lines had high crocin content (which confirms the applicability of eyesight method in screening lines of high crocin content), but most of them grew slowly and browned easily. On the contrary, white lines grew quickly and did not brown easily, but the crocin content was very low. In order to obtain a callus line with higher capability for crocin production, the crocin content and growth ability of lines should be considered in general. Therefore, Corm1 line was selected for further studies.

Acknowledgement This research was sponsored by 863 Program (the National High Technology Research and Development Program of China).

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