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Induction of Callus from Pistils of *Crocus sativus* L. and Production of Color Compounds in the Callus

Hidetaka HORI, Katsuyoshi ENOMOTO, and Makoto NAKAYA

Gakken Institute of Plant Technology, 1-2-14 Honson, Chigasaki, Kanagawa 255, Japan

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The calli were induced from the pistils of saffron. MS, 1/2 MS, and NN medium with BA and NAA at 1 mg/*l* each were the most effective for the induction and growth of the calli. The calli produced crocin, crocetin-di (monoglucosyl-diglucosyl) ester and crocetin-di (monoglucosyl) ester. The calli producing those pigments have been maintained for more than two years under dark conditions.

Saffron, *Crocus sativus* L., was originated from south Europe and has been recognized for its medicinal properties for a long time. The serum cholesterol levels of rabbits were reduced by half after intra muscular injection of crocetin.[1] Its principle value, however, now is as a food spice and colorant.[2] The main pigments of saffron are mono- and diglycosyl esters of crocetin. Besides these crocetin derivatives, small amounts of α-carotene, β-carotene, lycopene and zeaxanthin have been isolated.[3] The cultivation of saffron and harvesting of pistils require intensive labor.

We have tried to produce color and medicinal compounds from callus derived from the pistil of saffron in vitro. Here we show the induction of callus from saffron, *Crocus sativus* L. and the pigmentation.

Materials and Methods

Bulb of saffron. The bulb of saffron was purchased from a local market and sprouted at room temperature under the natural sun beam. The sprouts sized 3-5 cm were cut with a blade and sterilized by dipping into 70% ethanol 2-3 times. The sheaths of the sprouts were removed and the pistils collected from the sprouts. The pistils were cut about 5 mm long from the top of the stigma and used as explants. Six explants were placed onto the 10 ml of agar medium in a disposable plastic petridish.

Culture of explants. The explants were cultured for three months under the continuous illumination of fluorescent lamp at 3,000 lx at 20℃. The callus induction was checked with a microscope at one month after the inoculation. The growth of the callus were checked with the naked eyes at three months after the inoculation.

The explants were also cultured in a flask containing 5 ml of liquid media listed in **Table 2** and gyrated at 100 rpm.

Maintenance of the callus. Three months after the initiation of culture, half of each callus was maintained by changing the agar medium every three months under light conditions. The rest of the half was kept in the dark. When the callus was placed onto the new medium a part with no color was removed by cutting with a blade. Since no difference in color was observed between the calli maintained under the dark and that in light conditions for one year, all calli have cur-

rently been maintained in the dark.

Medium. The basal media of Murashige and Skoog[4] (MS), White[5] (WH) and Nitsch and Nitsch[6] (NN) were used and they were supplemented with various minor components and vitamins as they described. The 1/2 MS medium was also used, which was the same as MS medium but it contained half amount of the major elements of MS medium. Sucrose was added as a carbon source at 3% (w/v) concentration in the all media used. Agar was added at 0.8% (w/v) to solidify those liquid media. For the liquid culture of the explants the agar was omitted from the media used.

High performance liquid chromatography. Five hundreds mg of the callus, the same amount of newly harvested saffron pistils, and dried pistils purchased from a local market were extracted with 70% ethanol using a glass/Teflon homogenizer. The extracts were centrifuged to precipitate insoluble materials and analyzed by HPLC equipped with Lichrosorb RP-18, 5 μm (Merck) (ID 0.4 $\times$ 25 cm) and Lichrosorb SI-60, 7 μm (Merck) (ID 0.4 $\times$ 25cm) according to the method of Pfander and Rychener.[7]

Results

Effects of basal medium and plant hormones on the induction and growth of calli from the explants of saffron

The media of MS, WH, NN, and 1/2 MS with various plant hormones were used for callus induction from the explants of saffron. Benzyladenine (BA) and kinetin (KI) were used as cytokinin. Indoleacetic acid (IAA), naphthalene acetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were used as auxin.

When BA and NAA were added into the media of MS, WH, NN, and 1/2 MS at 1 mg/*l*, 22-37% of the explants placed onto these media formed callus (**Table 1**). But the highest frequency of callus induction (50%) were observed in WH medium with a combination of BA and IAA at 1 mg/*l* each. The combination of BA and IAA, however, showed the very low frequency of the callus induction

Table 1. The effects of various plant hormones on callus induction of the explants from *Crocus sativus* L. in various media.

Components of plant hormone (mg/*l*)			Medium			
			1/2 SM	MS	WH	NN
BA (1.0)	NAA (1.0)		37[a]	22	34	25
	IAA (1.0)		16	0	50	10
	2, 4-D (1.0)		—	—	17	17
KI (1.0)	NAA (1.0)		13	0	0	7
	IAA (1.0)		24	4	0	3
	2, 4-D (1.0)		6	13	3	4
BA (0.1)	NAA (0.1)		13	2	13	0
	IAA (0.1)		0	3	0	0
	2, 4-D (0.1)		—	—	21	13
KI (0.1)	NAA (0.1)		0	0	3	0
	IAA (0.1)		10	0	3	10
	2, 4-D (0.1)		0	3	0	3
Hormone free			11	0	3	0

[a] Induction frequency (%) was calculated in the following equation

$$: \frac{\text{No. of calli induced}}{\text{No. of explants incubated}} \times 100$$

The value of percentage was obtained from 20-50 explants inoculated.

in all other media such as 1/2 MS, MS, and NN. The rate of callus formation significantly decreased in any hormonal combination when both auxin and cytokinin were added into the medium at 0.1 mg/*l* each.

Almost all calli induced in an early stage were yellow or reddish yellow (**Fig. 1**) and the color did not seem to be depend either on the basal medium nor plant hormones.

The calli were maintained for three months on the agar plates without changing the medium. The stable growth of the callus during this period was observed only in the explants placed on MS, 1/2 MS, and NN media containing BA/NAA or KI/2, 4-D at 1 mg/*l* each (**Table 2**). The calli maintained on these media did not lose the yellow or reddish yellow color. All the rest of the calli which had not grown lost the pigments or necrotized during three months. The size of the callus grown was about 5 mm in diameter. In NN medium with BA and NAA at 1 mg/*l* each, about 40% of the callus formed in an early stage had grown to that size. On the other hand, in MS

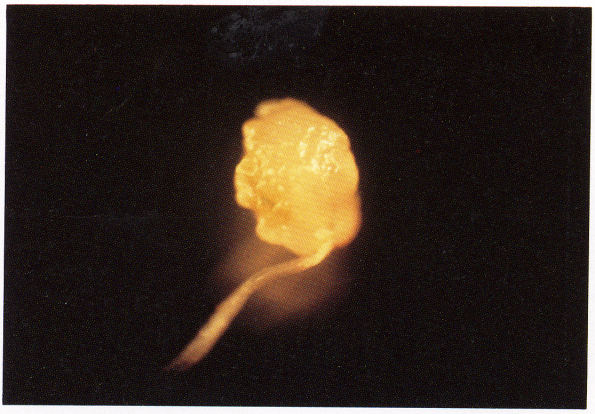

Fig. 1. The callus induced on the pistil. The pistil was placed on the medium of 1/2 MS with BA and NAA at 1.0 mg/*l* each. The photograph was taken at 2 months after the inoculation.

Fig. 2. The callus growing in the medium of 1/2 MS with BA, NAA at 1.0 mg/*l*. The photograph was taken at two years after the inoculation of the pistil into the agar medium.

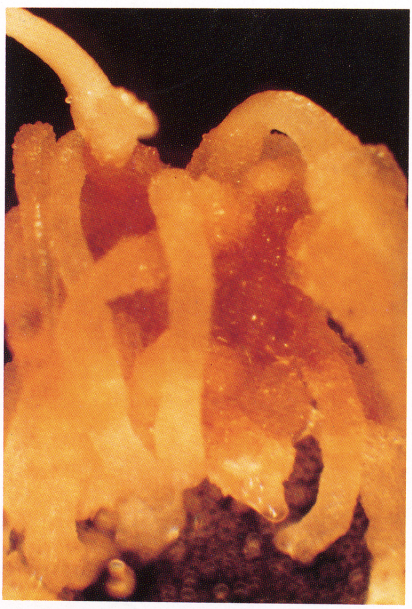

Fig. 3. The callus differentiating the organ in the medium of MS with BA, NAA at 1.0 mg/*l* each. The photograph was taken at the same time as in Fig. 2.

Table 2. Suitable media and plant hormones for the growth of the calli.

Medium	Hormones (mg/*l*)	No. of explants having callus induced	No. of explants having callus grown	Efficiency (%)
MS	BA (1.0) NAA (1.0)	49	7	14
1/2MS	BA (1.0) NAA (1.0)	52	8	15
1/2MS	KI (1.0) 2,4-D (1.0)	36	2	8
NN	BA (1.0) NAA (1.0)	24	10	42

and 1/2 MS, about 20% of the callus grew to that size. The calli have been maintained on the plate of 1/2 MS and NN medium for more than one year under dark conditions and they have shown slow but steady growth (**Fig. 2**).

In the media of MS and NN containing BA and NAA at 1 mg/*l* each, many calli differentiated the rod-shaped organ. This organ abounded reddish yellow pigment (**Fig. 3**).

We have also selected the callus which did not differentiate the organ for more than one year. The callus having low tendency to differentiate the organs has been obtained. In some cases the callus could be maintained without differentiation of any organs and the callus has produced yellow or reddish yellow pigment (**Fig. 2**).

Effects of the physiological age of the pistils on the callus formation

In order to check the effects of physiological age of the explants on the induction of callus, the pistils were grouped into three categories by their color, i.e., bright red, reddish yellow and yellow. The shorter was younger and yellow and the longer was older and bright red.

The grouped explants were cultured in the media which were the same as shown in **Table 1**. The induction frequency was calculated in each explant. We expected the tendency in which the younger explants showed the higher frequency of the callus induction. But no such a tendency was observed (data not shown).

Liquid culture of explants

The explants were cultured in the media of MS, 1/2 MS and NN for two months. The explants significantly swelled during the first one month but during the next month no callus induction was observed. During the two-months-culture the explants did not lose the color. The reddish pigment was accumulated in the medium during the culture and analyzed by HPLC.

High performance liquid chromatography of the extract from the calli, the pistils, and the liquid medium

Five hundred mg of pistils newly prepared from the sprout, dried pistils purchased from a local market, and the calli were extracted with 70% ethanol. The extracts were analyzed by HPLC. The liquid MS medium in which the pistils have been cultured was lyophilized, extracted with 70% ethanol, and analyzed by HPLC.

As shown in **Fig. 4**, although several unknown peaks were observed, the peaks of crocin, crocetin-di (monoglucosyl-diglucosyl) ester, and crocetin-di (monoglucosyl) ester were detected. The content-ratio of these three pigments in the callus was calculated from the chromatogram in ODS column as 20 : 10 : 1 and was the same as in the styles (**Fig. 4** panel B and C). The amount of these pigment in the callus, however, was only one tenth of that in the pistils.

Discussion

The callus induction was observed with about 20 to 40% of frequency in MS, 1/2 MS, WH, and NN media containing BA and NAA at 1.0 mg/*l* each as shown in **Table 1**. At present, it is still unclear that either both BA and NAA or at least one of them is necessary at 1.0 mg/*l* for callus

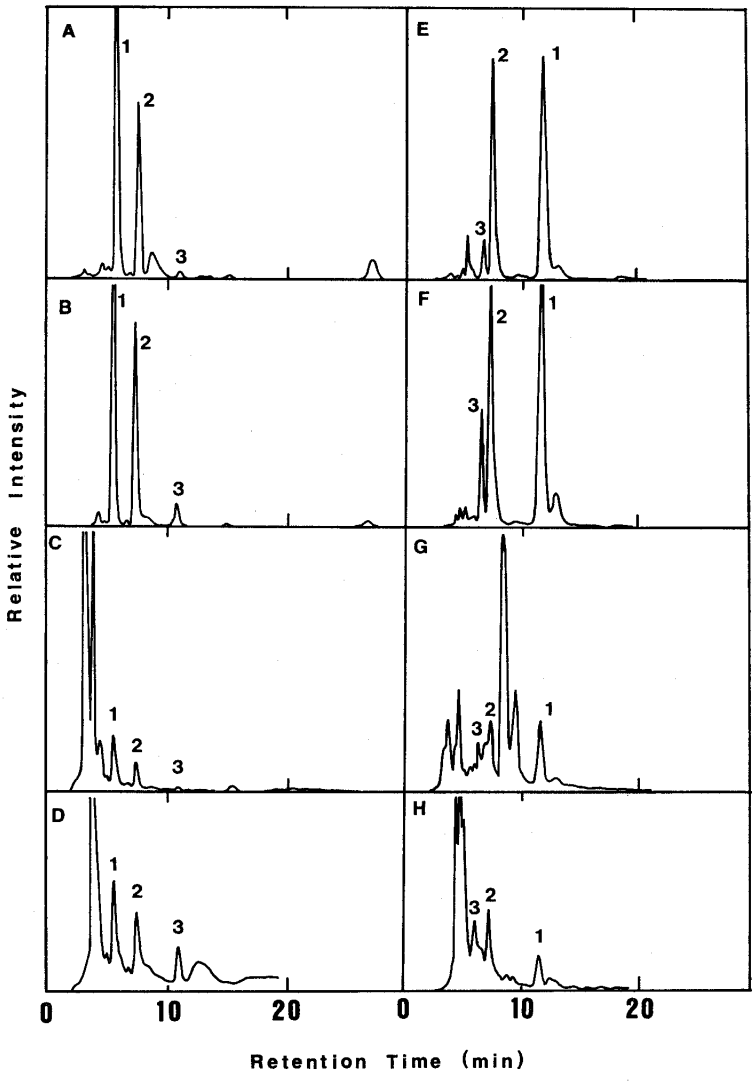

Fig. 4. The elution pattern of HPLC of the pigments from dried pistils (A, E), pistils (B, F), callus (C, G), and the medium (D, H). The chromatography was performed by a ODS column (left panel) and a silica column (right panel). The peaks numbered were estimated as 1: crocin [=crocetin-di (diglucosyl) ester], 2: crocetin-di (monoglucosyl-diglucosyl) ester, and 3: crocetin-di(monoglucosyl) ester.

induction. To address this problem the results of Ding[8] may be suggestive. They induced the callus from the corm of saffron with 83-93% induction frequency in MS medium containing only NAA and IAA or 2, 4-D alone at 1 mg/*l* each. This suggests that at least higher auxin level may be enough for the callus induction.

The growth rate of the callus obtained after one year of subculture on the optimum medium was steady but very slow. The growth rate of the rod-shaped organ (**Fig. 3**) seemed to be much faster than that of the callus. Therefore, it may be useful to establish the cultures having a tendency to differentiate the organ for the high production of the pigments.

The callus was maintained for more than one year without differentiation of any organs. Here we insisted that the callus produced the pigments. It is very difficult, however, to rule out completely the possibility that the callus may contain small differentiated primodia.

Sano and Himeno recently reported the in vitro proliferation of stigma from saffron and pigment production.[9,10] The differentiated organ we obtained (**Fig. 3**) a little bit looked different from their stigma-like structure. Because the organ we obtained was much thinner and longer than the stigma-like organ they obtained (**Fig. 3**). The morphological precise survey, however, is necessary to insist that the organ could be different from the stigma reported by Sano and Himeno.[9,10]

The HPLC analysis revealed that the callus produced the pigments such as crocin, crocetin-trisaccharides and crocetin-disaccharides derivatives. Although the contents of these pigments were lower than that of the pistils, it is worth to note that the content-ratio of these pigments in the callus was the same as in the styles (**Fig. 4** panel B, C). Beside these pigments, two major color components were detected in the callus. These unknown components with a higher polarity than crocin were not β-carotene.

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≪和文要約≫

サフランの雌しべからのカルス誘導と色素の生産

堀　秀隆，榎本克義，中谷　誠
学研(株)植物工学研究所

長さ 3～5 cm の蕾をサフラン球根から採集し，滅菌処理の後，雌ずいを寒天培地に置床してカルス誘導を試みた．用いた培地は，Murashige-Skoog (MS), White (WH), Nitsch-Nitsch (NN) および MS 培地の基本培地を半分に希釈した物 (1/2 MS) である．添加した植物ホルモンは，ベンジルアデニン，カイネチン，インドール酢酸，ナフタレン酢酸である．カルス誘導は MS, 1/2 MS, NN 培地で BA と NAA が 1 mg/l の時最も良好であった．一部カルスは柱頭状器官を分化した．器官分化しないカルスを選抜法により確立した．分化しないカルスの生長は分化するものに比べ著しく遅かった．カルスは赤色色素を生産した．カルスの色素から，クロシン，クロセチン三グルコース，クロセチン二グルコースの 3 種が検出された．三者の重量比は 20：10：1 で雌しべにおける比と同じであったが，生産量は雌しべに比べ 1/10 であった．