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Effects of NH_4^+ and Total Nitrogen Content in Culture Medium on Shoot Regeneration from Calli in Saffron (*Crocus sativus* L.)

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Saffron proliferates vegetatively through corm, and its stigmas and styles are used as a spice and as a raw material in medicine. The practical purposes of tissue culture of saffron are the development of a micropropagation method and the establishment of a new method for mass propagation of pistils through *in vitro* organogenesis of stigma- and style-like structures from pistils[1-3]. Concerning the micropropagation method, shoot regeneration from calli has not yet been extensively studied, except for reports describing shoot regeneration from the calli derived from corms[4,5] and from protoplasts isolated from the calli derived from corms[6]. Extensive examination of explant sources and culture conditions has not yet been done. In this paper we show efficient culture conditions for regenerating shoots from calli which were induced from young ovaries, and particularly the inhibitory effect of NH_4^+ on shoot regeneration.

The explants, 5-mm-long young ovaries, were cultured on MS media[7] supplemented with 30 g/*l* sucrose, 2 g/*l* Gellan Gum, auxin (NAA or 2, 4-D) and cytokinin (BAP, zeatin or kinetin) at 0, 0.5, 5, 50 μM. After primary culturing the explants for 60 days and subsequent two or three times subculturing for 20 days in the dark at 25°C, two types of calli, a nodular one and a friable one, were induced. The nodular calli had lustrous with rugged surfaces (**Fig. 1-A**) and high regenerating ability, as mentioned below, while the friable ones were spongy and had low regenerating ability. Nodular calli were induced in combinations of NAA and cytokinin, especially BAP. Appropriate concentrations for the induction of nodular calli were 0.5-50 μM NAA and 50 μM BAP in combination, where the frequency of callus induction was 10-50%. Poor callus induction and necrosis were always observed in 2, 4-D containing medium with or without cytokinin. 2, 4-D has been reported to be effective for callus induction in many monocotyledonous plants[8], including the culture of saffron corm[4,5]. However, in this study, 2, 4-D was inhibitory for callus induction. We are not able to explain definitely what caused the discrepancy between our results and others', although a different kind of explant was used.

The induced nodular calli resembled the so-called embryogenic callus reported in Gramineae[8], which had a lustrous and rugged surface and regenerating ability. Isa and Ogasawara induced calli from corms by treatment with 2, 4-D and zeatin, then transplanted the calli to a medium containing NAA and BAP and succeeded in the regeneration of shoots after the formation of enlarged nodular tissues[5]. We suppose the enlarged nodular tissues they described are similar to the nodular calli we induced.

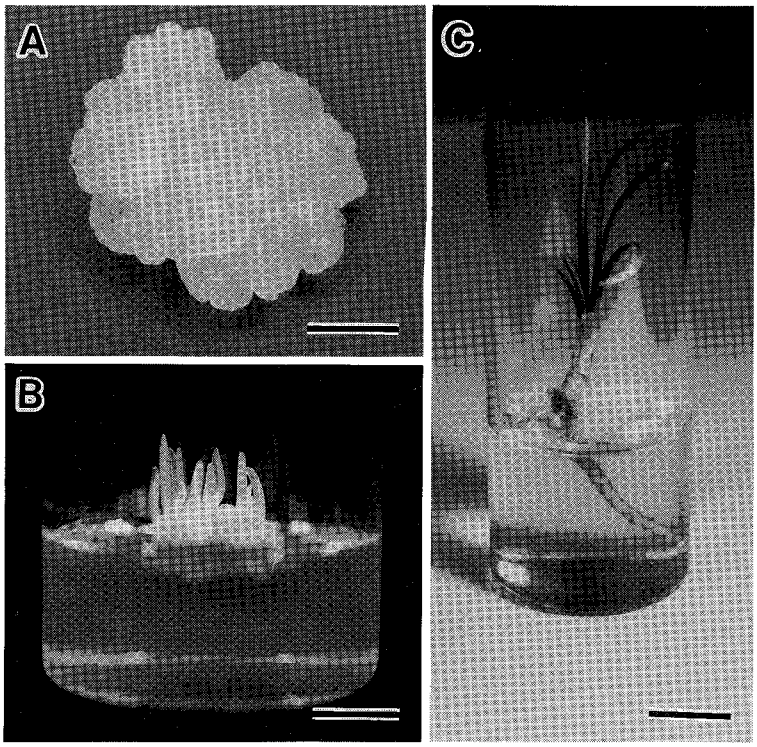

Fig. 1 Shoot regeneration from the nodular callus derived from the ovary.
A, a nodular callus 20 days after subculturing on the subculture medium, MS medium containing 5 μM NAA and 50 μM BAP; bar=2 mm; B, a shoot clump formed on the nodular callus 42 days after transplantation on NH_4^+-free MS medium containing 20 mM KNO_3, 0.5 μM NAA and 0.5 μM BAP; bar=1 cm; C, a plantlet 60 days after transplantation on to the growth medium, MS medium containing 0.5 μM NAA and 0.5 μM BAP; bar=1 cm.

The effect of NAA and BAP on shoot regeneration was examined. The nodular calli that were subcultured every 20 days for about a year were transplanted on MS media supplemented with NAA at 0, 0.5, 5 μM and BAP at 0, 0.5, 5, 50 μM in combination. Vegetative shoots were formed 20-40 days after transplantation at the regeneration frequency of 20-50% in a wide range of NAA and BAP concentrations. There was no distinct specific effect of hormone concentration on regeneration frequency. The maximal regeneration frequency was 50% in the combination of 0.5 μM NAA and 0.5-5 μM BAP; the average number of the regenerated shoots per callus was 2.6.

The effects of the total amount and kinds of nitrogen sources on shoot regeneration in the optimal concentrations of NAA and BAP (0.5 μM NAA and 0.5 μM BAP) were then investigated. The regeneration frequency was improved by reducing the total amount of nitrogen source in the media and by reducing the ratio of NH_4^+ to NO_3^- in the same total nitrogen concentration (**Table 1**). Especially in the NH_4^+-free medium with 20 mM total nitrogen (KNO_3 alone), both the regeneration frequency (78%) and the average number of regenerated shoots per callus (5.0) showed the maximum values, and vigorous shoot regeneration was observed (**Fig. 1-B**). In other words, shoot regeneration from calli was inhibited by NH_4^+ in the media, as reported previously[9,10].

When the obtained shoots were transplanted onto the growth medium and cultured at 25°C under 4000 lux illumination for a 15 hour daylength, the shoots grew into plantlets, of which more than two thirds rooted (**Fig. 1-C**). On further subculture of the plantlets with or without roots, small corms

Table 1. Effects of nitrogen sources on shoot regeneration from the nodullar calli on the medium containing 0.5 μM NAA and 0.5 μM BAP.

Total nitrogen (mM)	NH_4^+ (mM)	NO_3^- (mM)	Regeneration frequency (%)*1 (n=50)	Average number of shoots*2
60	30	30	17*3	2.2
	20	40	38	2.1
	0	60	38	2.6
40	20	20	27*3	2.9
	13.3	26.7	49	3.2
	0	40	38	3.3
20	10	10	34*3	2.4
	6.7	13.3	44	3.1
	0	20	78	5.0

*1 Regeneration frequency (%) = number of calli with shoots/total number of calli. Shoots were counted 42 days after transplantation.

*2 Number of shoots per callus which formed shoots.

*3 Sample number n=30 in the conditions of 30 mM NH_4^++30 mM NO_3^-, 20 mM NH_4^++20 mM NO_3^-, 10 mM NH_4^++10 mM NO_3^-.

(5-15 mm in diameter) were formed on the basal portion of the shoots (data not shown).

Furthermore, we studied shoot regeneration from calli which were obtained from young leaves and shoot apices. Both the positive effect of NAA and BAP and the negative effect of 2, 4-D on callus induction and the inhibitory effect of NH_4^+ on shoot regeneration were also observed as in the case of young ovaries (data not shown).

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《和文要約》

サフランのシュート再分化における培地中 NH_4^+ と総窒素量の影響

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カルス誘導，シュート再分化に対するホルモンの種類と濃度，培地中窒素源の影響を検討した．子房からのカルス誘導にはNAAとBAPが有効であり，球塊状のカルスが形成された．0.5 μMのNAAとBAPの最適条件下において，培地中の NH_4^+ を除き総窒素量を下げることでカルスから高効率にシュートを再分化させることができた．