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Research note

Micropropagation of *Cypripedium flavum* through multiple shoots of seedlings derived from mature seeds

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Abstract

Cypripedium flavum, known as the rare lady's slipper orchid, is one of the endemics with a yellow flower in China. Due to its conservation and commercial requirement, establishment of an efficient method for micropropogation is urgently needed. Multiple shoots were obtained by placing seedlings from seeds of *C. flavum* on Harvais media supplemented with two cytokinins (BAP or KIN) used alone or in addition to different concentration of potato homogenate. The effect of BAP was better than that of KIN on shoot multiplication. The Havais media supplemented with BAP (2.22 μM) and potato homogenate (20 g l^{-1}) was the most effective, providing high shoot multiplication frequencies (95%) associated with a high number of shoots per explant (2.55 shoots/plant). For root formation, high rooting and survival were achieved using 1/2 Harvais media supplemented with 0.6 g l^{-1}activated charcoals. High-level activated charcoal increased the number and the length of roots because the activated charcoal could absorb BAP in the media. This study demonstrated that *C. flavum* could be micropropagated by using multiple shoots of seedlings derived from mature seeds.

Abbreviations: ANOVA – analysis of variance; BAP – 6-benzyladenine; KIN – kinetin

Cypripedium flavum, known as the rare lady's slipper orchid, is the one of endangered endemics with a yellow flower in China. They grow under forests, at forest margins, among shrubs or in humid grasslands with many rocks at 1800–3450 m altitudes in China (Chen et al., 2005). *C. flavum* is typical non-rewarding orchid. The percentage of setting fruit was no more than 8% and few seeds could germinate in wild populations (our observation data) because the terrestrial orchid was restricted in utilizing the resources and in being pollinated in alpine ecological conditions. In recent years, ecological disturbance, tourism and increasing grazing pressure have resulted in considerable decline of *C. flavum* populations in China (Cribb, 1997; Cribb and Sandison, 1998). Constructing rapid and large-scale propagation of *C. flavum in vitro* will be beneficial to restoration of wild population in local environments.

Furthermore, *Cypripedium* is one of the most uncommon and conspicuous north temperate orchids. They grow so slowly that the wild population is the major source of material for horticulture (Cribb and Sandison, 1998). The collection of wild *Cypripedium* continues at levels ranging from hobbyist to large-scale illegal trade. The attractive nature of *Cypripedium* flowers combined with technical difficulties associated with their *ex situ* cultivation and propagation, have resulted in increased collection pressure and subsequent rarity of various *Cypripedium* species

(Case et al., 1998). To satisfy the interest of hobbyist through a large-scale micropropagation is one of the preferable options to prevent illegal collection.

Numerous studies on efficient micropropagation of many orchids through callus or protocorm-like bodies (PLBs) have been published (Arditti, 1977). But the propagation of the lady's slipper orchids was very difficult (De Pauw et al., 1995). Lee and Lee (2003), Shimura and Koda (2004) have successfully induced the protocorm-like bodies of *C. formosanum* and *C. macranthas* var. *rebunense* to regeneration. However, many seeds had to be constantly sowed because *Cypripedium* had to be induced the protocorm-like bodies for plantlet regeneration by these ways. We induced the multiple shoots of *C. flavum* to solve the problem that plantlet formation depends on the induction of protocorm-like bodies derived from seeds. As long as a small number of seeds were sowed (such as the seeds of one fruit) by this ways, many multiple shoots of seedlings were induced to form many plantlets.

Several capsules of *C. flavum* were harvested as soon as they turned green to yellowish green in the later September (about 100 days after pollination) in habitats. We sowed the seeds in ten 100-ml cultural flasks that contained 20 ml media supplemented with 2.22 μM BAP or 2.32 μM KIN (Huang and Hu, 2001). Each flask had about 100–200 seeds. The complete Harvais media (Harvais, 1973) formulation was used, which supplemented with 2% (w/v) sucrose and 0.7% (w/v) agar. The pH of the media was adjusted to 5.6 prior to autoclaving at 121 °C for 17 min. The seeds were incubated in darkness at 23 ± 2 °C. After 12 weeks, the percentage of seed germination reached $41.4 \pm 1.0\%$ and $38.6 \pm 3.2\%$ ($\pm$SE, $n = 5$) on the media supplemented with 2.22 μM (0.5 mg l^{-1}) BAP and 2.32 μM KIN (0.5 mg l^{-1}), respectively. The small protocorms (about 4 mm) were transferred to illuminated conditions in a 14-h photoperiod with a light intensity of 25–30 μmol m^{-2} s^{-1} from cool, white, fluorescent tubes (Philips LD 36W/54, SanXiong Electric Co., Guangdong, China) at the same temperature used for seed germination. We compared the effect of two cytokinins [KIN (2.32, 4.65 μM), BAP (2.22, 4.44 μM)] and various concentrations of potato homogenate (0, 20 and 40 g l^{-1}) on shoot multiplication. Potato were peeled and cut into 1 cm^3 section. The fresh materials were boiled for 15 min with 200 ml of distilled water and homogenized with a kitchen blender. Each experiment had five replicate cultures of 10 explants per flask and was conducted three times.

According to the number of shoots per explant, the percentage of explants with shoots, and the percentage of necrotic explants, the effective induction media of multiple shoots were selected after 6 weeks (Table 1). The results indicated that BAP (2.22–4.44 μM) was more effective for

Table 1. Shoot multiplication of *C. flavum* after 6 weeks of culture on Harvais media with different concentrations of cytokinins and potato homogenate

Cytokinins (μM)	Potato (g l^{-1})	*Shoots/explant	%Explants with shoots	% Necrotic explants
BAP2.22	0	1.54 ± 0.38^{abcd}	54^{ab}	22^{a}
2.22	20	2.55 ± 0.05^{e}	95^{c}	0^{a}
2.22	40	2.42 ± 0.20^{e}	78^{bc}	0^{a}
4.44	0	1.86 ± 0.25^{bcde}	76^{bc}	16^{a}
4.44	20	2.26 ± 0.16^{de}	86^{c}	2^{a}
4.44	40	1.56 ± 0.24^{abcd}	56^{ab}	22^{a}
KIN2.32	0	1.10 ± 0.30^{ab}	30^{a}	30^{a}
2.32	20	1.98 ± 0.26^{cde}	68^{ac}	6^{a}
2.32	40	1.80 ± 0.33^{bcde}	58^{ab}	4^{a}
4.65	0	1.56 ± 0.16^{abcd}	48^{ab}	14^{a}
4.65	20	1.46 ± 0.26^{abc}	48^{ab}	20^{a}
4.65	40	0.96 ± 0.29^{a}	24^{a}	26^{a}

* Values are means $\pm$ standard error.
Different letters show significant differences ($p \leq 0.05$).

Table 2. Analysis of variance of the effects of cytokinins and potato homogenate on shoot multiplication of *C. flavum*

Source of variation	df	Shoots/explants[a]	%Explants with shoots	%Necrotic explants[b]
BAP (B)	1	0.532^{NS}	0.036^{NS}	4.889^{NS}
KIN (K)	1	2.381^{NS}	1.972^{NS}	5.594^{NS}
Potato (P)	2	3.569*	3.614*	14.352^{NS}
B×P	5	2.494^{NS}	2.097^{NS}	20.578^{NS}
K×P	5	3.915**	2.547^{NS}	28.961^{NS}
B×K	3	4.265**	5.307**	18.209^{NS}

[a] F values from ANOVA.
[b] Chi square from Fisher exact test.
NS, *,** Nonsignificant or significant at $p \leq 0.05$ or 0.01, respectively.

induction of shoot multiplication than KIN (Table 2). Thus, the exogenous hormonal requirements for micropropagation of *Cypripedium* were species-specific (De Pauw, 1995). The Havais media supplemented with BAP (2.22 μM) and potato homogenate (20 g l^{-1}) was the most effective, providing high shoot multiplication frequencies (95%) associated with a high number of shoots per explant (2.55 shoots/plant) (Figure 1a). Many researchers suggested that the effect of organic supplements such as potato homogenate on micropropagation was complex (Tomita and Tomita, 1997; Lee and Lee, 2003). We observed that the number of induced shoots had no significant difference supplemented with 20 g l^{-1} or 40 g l^{-1} potato homogenate with 2.22 μM BAP, but the number of shoots supplemented with 40 g l^{-1} potato homogenate were significantly

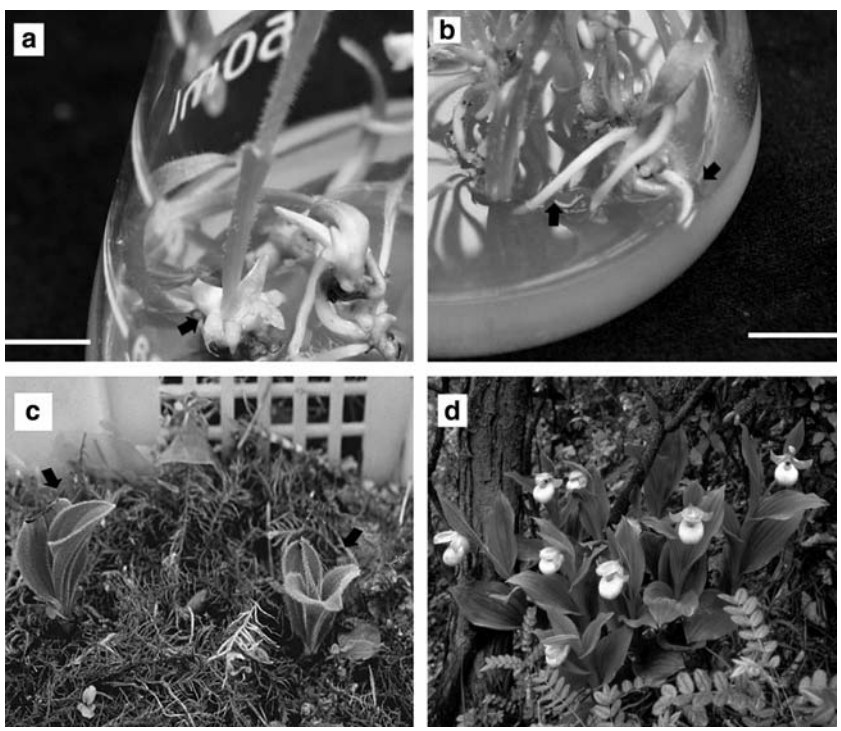

Figure 1. Established plants from multiple shoots of seedlings derived from seeds of *C. flavum*. (*a*) Shoot multiplication on Harvais media with 2.22 μM BAP and 20 g l^{-1} potato homogenate. (*b*) Root formation after 4 weeks in culture on 1/2 Harvais media with 0.6 g l^{-1} activated charcoal. (*c*) Seedlings from plantlets *in vitro* after 3 years. (*d*) *C. flavum* in habitats. Scale bar = 1 cm. Arrow in (*a*) indicate shoots. Arrows in (*b*) indicate roots. Arrows in (*c*) indicate seedlings in greenhouse.

Table 3. Rooting of *C. flavum* after 4 weeks of culture on Harvais and 1/2 Harvais media contained different proportion activated charcoal with or without BAP

Media	BAP (μM)	Activated charcoal (g l^{-1})	*No. roots per shoots	*Length/roots	%Explants with roots
Harvais	0	0	0.24 ± 0.05^{a}	0.473 ± 0.043^{bc}	20^{a}
Harvais	0	0.3	1.84 ± 0.20^{cd}	0.539 ± 0.031^{cde}	76^{bc}
Harvais	0	0.6	2.28 ± 0.09^{de}	0.675 ± 0.038^{ef}	92^{de}
Harvais	2.22	0	0^{a}	0^{a}	0^{a}
Harvais	2.22	0.3	0.94 ± 0.11^{b}	0.664 ± 0.052^{def}	58^{b}
Harvais	2.22	0.6	2.08 ± 0.22^{de}	0.772 ± 0.040^{f}	82^{cde}
½ Harvais	0	0	0.28 ± 0.11^{a}	0.340 ± 0.050^{b}	20^{a}
½ Harvais	0	0.3	2.04 ± 0.19^{de}	0.656 ± 0.036^{cdef}	90^{de}
½ Harvais	0	0.6	2.54 ± 0.14^{e}	0.738 ± 0.039^{f}	100^{e}
½ Harvais	2.22	0	1.35 ± 0.34^{bc}	0.484 ± 0.041^{bcd}	67.5^{bc}
½ Harvais	2.22	0.3	1.95 ± 0.10^{de}	0.750 ± 0.059^{f}	80^{cd}
½ Harvais	2.22	0.6	2.32 ± 0.38^{de}	0.828 ± 0.051^{f}	94^{de}

* Values are means ± standard error.
Different letters show significant differences ($p \leq 0.05$).

fewer than that in addition of 20 g l^{-1} with 4.44 μM BAP (Table 1).

To the multiple shoots, the induction of roots is an important procedure to form the plantlet. We studied the effect of activated charcoal (0, 0.3 and 0.6 g l^{-1}) on root initiation from micropropagated shoots with or without BAP (2.22 μM) on the Harvais and 1/2 Harvais media. The shoots (2–3 cm in length) were excised and transferred to root initiation media. After 4 weeks, maximum *in vitro* rooting was 100% for shoots treated without BAP and 0.6 g l^{-1} activated charcoals on 1/2 Harvais media (Table 3). There were significant differences in the mean number of roots and the mean length of roots between on Harvais media and on 1/2 Harvais media (Table 4). The results of root induction implied that the reduction of nutriment was beneficial for the rooting of *C. flavum*. We found the number of roots induced on the media without BAP was more than that of on the media with BAP (2.22 μM) in most treatments. The results suggested that BAP was not beneficial for the root induction of *C. flavum*. On the other hand, the highest level of activate charcoal (0.6 g l^{-1}) was more useful to induction of roots than the other two level (in addition to 0 or 0.3 g l^{-1} activated charcoal) (Table 4, Figure 1b). We considered that BAP absorbed by the activated charcoal was most likely the cause of the increased rooting. Activated charcoal has not only good adsorption properties but also creates partial darkness. The darkness is similar to the underground environment of *C. flavum* in habitats. The plantlets with one or more 1–2 cm roots were readily able to survive. The

Table 4. Analysis of variance of the effects of media, BAP and activated charcoal on root formation of *C. flavum*

Source of variation	df	No. roots[a]	Length/roots[a]	%Explants with roots[b]
BAP	1	0.193^{NS}	0.005^{NS}	14.309^{NS}
Activated carbon	2	58.206***	69.169***	49.969***
Media	1	4.579*	18.624***	13.827^{NS}
All treatments	11	23.579***	19.231***	120.287***

[a] F values from ANOVA.
[b] Chi square from Fisher exact test.
NS, *, *** Nonsignificant or significant at $p \leq 0.05$ or 0.001, respectively.

survival percentage of rooted *C. flavum* plants was about 60% in a greenhouse at 18–22 °C with 80% humidity. The seedlings grew slowly for the first 3 years (Figure 1c). We supposed that low percentage of seedling survival and slow growth were related to the lack of mycorrhizal fungi under greenhouse conditions. Thus, symbiotic germination of seeds and procedure of mycorrhizal fungi infecting *C. flavum* could be the subjects for further investigation.

In summary, The Harvais media supplemented with 2.22 μM BAP and 20 g l^{-1} potato homogenate had a significant effect on shoot multiplication of *C. flavum*. The shoots developed into plantlets after further incubation on 1/2 Harvais media without BAP plus 0.6 g l^{-1} activated charcoal. We were able to propagate many plants independent of wild populations by this technique. Efficiency for the production of plantlet from the multiple shoots was much higher than that through protocorm-like bodies derived from seeds.

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