BIOLOGIA PLANTARUM 47 (1): 117-120, 2003/4

BRIEF COMMUNICATION

In vitro micropropagation of a medicinal plant species *Sophora flavescens*

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Abstract

A micro-propagating system based on the young stem node segments of *Sophora flavescens* Ait. (*Fabaceae*) was established. Murashige and Skoog (MS) basal medium supplemented with 8.88 μM 6-benzyladenine (BA) plus 2.69 μM α-naphthalene acetic acid (NAA) and that with only 5.37 μM NAA were found the best in promoting proliferation of shoots and induction of root, respectively. Percentages of shoot induction and number of shoot per explant were up to 93.4 % and 4.2 and rooting rate to 82.4 %, respectively. The segments of the regenerated shoots could be continuously induced to reproduce new shoots through subculture on the same medium in 30-d intervals and still kept this activity after being subcultured for 6 generations. After the regenerated plantlets were transplanted to field, they grew well, showing no any visible abnormalities.

Additional key words: auxins, multiple shoot formation, rooting

Sophora flavescens, a perennial shrub of family *Fabaceae* has been used in herbal medicine for centuries in China and Japan. It has been proved that alkaloids and flavonoids in its roots have many pharmacological functions, such as diuresis, anti-microbe, anti-insect, anti-ulceration, anti-arrhythmia, anti-tumour and curing hepatitis (Ryu *et al.* 1997). However, a long-term over-gathering of its roots has made this plant facing with the risk of being exhausted and therefore some researchers have tried the possibility of large-scale artificial cultivation of this plant species (Hu *et al.* 2001). Thus, a quantity of seeds or seedling will be in demand, but the germination rate of seeds is low, less than 50 % according to previous report (Hu *et al.* 2001) and our own tests, which limits the sexual propagation to some extent. Thereby asexual propagation is necessary. The present work was performed with the purpose of micro-propagation of the species. So far, there has been no person who had such an experience in *S. flavescens* in our knowledge.

The seeds collected from open country near Dalian city in China were disinfected first in 70 % ethanol for 3 min and then in 0.1 % $HgCl_2$ for 10 min. After thoroughly rinsed with sterile distilled water, they were transferred onto a half-strength agar Murashige and Skoog (MS) medium and allowed to germinate at temperature of 26 ± 2 °C. When the seedling grew up to 3 cm in height, their young stems were excised and cut into about 1-cm long segments with a node and then inoculated onto MS media supplemented with 3 % sucrose, 0.7 % agar and various combinations of TDZ, BA, NAA and 2,4-D (Table 1). The cultures were incubated under cool white fluorescent tubes (irradiance of 24 μmol m^{-2} s^{-1}, a 16-h photoperiod) and at temperature 26 ± 2 °C. After 30 d, the percentage of the explants regenerating shoots and the number of shoots formed on each explant were counted. Meanwhile shoots 2 - 3 cm in length were excised from the clustered shoots and inserted into the MS agar media supplemented with various concentrations of IAA, IBA or NAA (Table 2) for rooting. Plantlets were grown under continuous irradiance (0.48 μmol m^{-2} s^{-1}) and the same temperature. Rooting

Received 11 January 2002, *accepted* 24 July 2002.
Abbreviations: BA - 6-benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS medium - Murashige and Skoog medium; NAA - α-naphthaleneacetic acid; TDZ - thidiazuron.
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rates of the shoots were counted after culturing 30 d. All the media used were adjusted to pH 5.8 and autoclaved at 121 °C for 15 min.

In the 5 of 8 different media tested (Table 1), shoots could be induced. New shoots usually started to produce at node site of the stem explants after being cultured about one week and at the same time calli were formed at their bases (Fig. 1*A*). Media with 4.54 μM TDZ, 4.54 μM TDZ + 1.07 μM NAA, and 8.88 μM BA + 2.69 μM NAA were more effective than other ones in shoot induction rate (over 90 %). 8.4 and 6.2 of shoots per explant were formed on media with TDZ or TDZ plus NAA, but only 4.2 on medium with BA and NAA. However, the media with TDZ could not be regarded as the best shoot proliferation media since the shoots newly formed on them showed very short internodes (less than 1 cm) and densely grew together, which made it more difficult to further separate and excise for next subculture (Fig. 1*A*). Moreover they were hard to root (the rooting rate less than 8 %, Table 2). But those on the last medium usually grew higher (2 - 4 cm) and straighter with longer internodes (Fig. 1*A,B*), easier to be separated and cut into segments with node in the later subculture. Also the rooting rate was higher (over 82 %). Apparently the last medium was more suitable for the micropropagation of *Sophora flavescens* in the present study. On the medium without any regulator, neither shoot nor callus was formed, but on those containing 2,4-D, only callus was produced, which suggests that 2,4-D promoted the explants callusing and inhibited shoot organgenesis.

Table 1. Effect of different hormone combinations on shoot induction of stem explants of *S. flavescens*. Means ± SE of three repeated experiments with about 60 explants used in each treatment. Means followed by the same letter are not significantly different at the 5 % level according to Duncan's Multiple Range Test. n - no callus formation, p - callus only being formed at the base of stem explant, w - the whole explant changed into callus.

BA [μM]	TDZ [μM]	NAA [μM]	2,4-D [μM]	Shoot production [%]	Number of shoots [explant^{-1}]	Callus
0	0	0	0	0^{d}	0^{a}	n
4.44	0	2.69	0	71.1 ± 3.8^{c}	2.1 ± 0.2^{b}	p
8.88	0	2.69	0	93.4 ± 0.7^{ab}	4.2 ± 0.2^{d}	p
4.44	0	0	4.52	0^{e}	0^{a}	w
0	2.27	0	0	87.6 ± 5.8^{b}	3.2 ± 0.4^{c}	p
0	4.54	0	0	95.7 ± 3.7^{a}	8.4 ± 0.5^{f}	p
0	4.54	1.07	0	96.0 ± 3.5^{a}	6.2 ± 0.4^{e}	p
0	4.54	0	2.26	0^{d}	0^{a}	w

For further multiplication, the regenerated shoots from medium with 8.88 μM BA + 2.69 μM NAA were divided into segments with node and then subcultured on the same medium. The process could be repeated at 30-d intervals for not less than 6 cycles. Usually one such shoot might be cut into 2 - 3 pieces of segments with node, and if each segment reproduce 4 shoots, you will obtain from $4 \times (2 \times 4)^5$ to $4 \times (3 \times 4)^5$ (131072 - 995328) new shoots from one initial stem node within 6 months. This amount is extremely significant for the rapid propagation of the plant.

The new shoots from the 3 media showing more effective proliferation of shoots, were excised and transferred onto MS media with IAA, IBA or NAA of varied concentrations (Table 2). 5.37 μM NAA gave the highest rate of root induction for the shoots from medium with BA + NAA, up to 82.4 %, and 3 to 4 roots in average were formed per shoot after being cultured a month (Fig. 1*C*), but only 3.33 and 7.90 %, respectively, for the shoots from media with TDZ and TDZ + NAA. The significant difference of rooting rates between the shoots from media with and without TDZ implies that the

Table 2. Effect of various auxins on percentage of rooted shoots on media with BA + NAA. TDZ and TDZ + NAA. Means ± SE of three repeated experiments with about 60 explants used in each treatment. Means followed by the same letter are not significantly different at the 5 % level according to Duncan's Multiple Range Test. In statistics the rooted shoots with callusing at their bases were not included.

Auxin	[μM]	BA + NAA	TDZ	TDZ + NAA
Control		0^{a}	0^{a}	0^{a}
NAA	5.37	82.4 ± 3.4^{b}	3.33 ± 2.9^{a}	7.9 ± 3.4^{b}
	10.74	12.4 ± 3.7^{c}	0^{a}	0^{a}
IAA	5.71	0^{a}	0^{a}	0^{a}
	11.42	0^{a}	0^{a}	0^{a}
IBA	4.92	0^{a}	0^{a}	0^{a}
	9.84	0^{a}	0^{a}	0^{a}

remnant TDZ in the regenerated shoots possibly inhibited root organogenesis. When the concentration of NAA was raised up to 10.74 μM, the rooting rate of the shoots from the medium with BA + NAA was decreased to 12.4 %, instead calli were formed at their bases in most cases, but for those from media with TDZ and TDZ + NAA, only calli were produced at their bases without root formation. These results indicated that NAA at a higher concentration has obvious inhibitory impact on root production, whereas promoted callus formation. The similar result was also reported in *Robinia pseudoacacia* (Wang *et al.* 1999).

Both IBA and IAA failed to induce rooting of *Sophora flavescens* shoots, although IBA was successful in root induction of *Sophora japonica* shoots (Wang *et al.*

1992) and some other plants (Srivastava *et al.* 2001, Saritha *et al.* 2002).

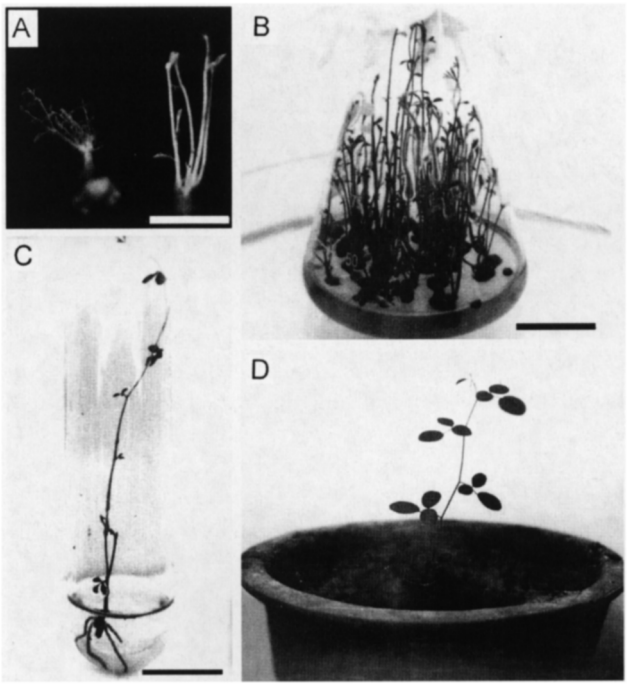

Fig. 1. Shoots and plantlets regenerated from young stem node explants of *S. flavenscens*: *A* - multiple shoots formed on MS medium with 4.54 μM TDZ + 1.07 μM NAA, gathering closely with shorter internodes (*left*), and those on medium with 8.88 μM BA + 2.69 μM NAA, being higher and straighter with longer internodes (*right*); a piece of tuberculiform extensive callus formed at the bases of stem explants (*bar* = 1.42 cm); *B* - multiple shoots formed on MS medium with 8.88 μM BA + 2.69 μM NAA (*bar* = 2.14 cm); *C* - a plantlet with roots on MS medium with 5.37 μM NAA (*bar* = 2.3 cm); *D* - a plantlet growing in pot.

In addition, it was also found that the frequency of rooting was apparently affected by irradiance: under 16-h photoperiod and irradiance 24 μmol m^{-2} s^{-1} only 37.1 % of shoots from medium with BA + NAA rooted on medium with 5.37 μM NAA, which was much lower than that under continuous irradiance of 0.48 μmol m^{-2} s^{-1} (82.4 %), suggesting that higher irradiance possibly inhibited root formation.

The regenerated plantlets were transplanted to an artificial soil mixture in pot and maintained in greenhouse with a 16-h photoperiod (24 μmol m^{-2} s^{-1}) at 26 ± 2 °C. In initial 10 d they were covered with a polyethylene membrane and irrigated with half-strength MS solution free of sucrose every 3 d. After 10 d they were gradually exposed to atmosphere and irrigated with tap water. One month later, 73.6 % (67 out of total 91) of the transplanted plantlets survived (Fig. 1*D*). The survival plantlets were transplanted to field in May. They grew flourishing and could re-germinate in the spring of next year, without visible abnormalities.

TDZ is among the most potent cytokinins and has been successful in inducing shoot proliferation in many plant species (Xu *et al.* 1996, Jain and Rashid 2001), including some species from *Fabaceae* family, such as *Swainsona salsula* (Yang *et al.* 2001), *Swainsona formosa* (Jusaitis 1997), *Glycine max* (Kaneda *et al.* 1997), *etc.* But in present study, apparently it was less appropriate than combination of BA and NAA for the proliferation of *S. flavescens* because of the difficulties in subculture and rooting of regenerated shoots. It was reported that combination of BA and NAA was also useful for the regeneration of *Sophora toromiro* (Iturriaga *et al.* 1994) and *Spilanthes acmella in vitro* (Saritha *et al.* 2002).

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