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Tropane Alkaloids in Adventitious Root Cultures of *Physochlaina physaloides*

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Adventitious root cultures of *Physochlaina physaloides* were established and their growth and tropane alkaloid production were investigated. Although the roots cultured in Murashige-skoog liquid medium containing 1.0-3.0 mg/l IBA grew well, the addition of lower concentrations of auxins was more suitable for alkaloid production. The best alkaloid yield was obtained with 0.01 mg/l NAA in 1/2 MS liquid medium. In addition, the alkaloid differences between the cultured roots and the regenerated plant cultivated in the soil was studied. Cultured roots in this condition showed higher level of alkaloids when compared to leaf, stem and roots from the regenerated plant (3 months in field conditions).

Keywords: *Physochlaina physaloides*, Solanaceae, root culture, auxin, tropane alkaloid

Introduction

Physochlaina physaloides G. Don, a small solanaceous plant, grows mainly in the north and northeast parts of China, Inner Mongolia and Russian countries. The plant, containing tropane alkaloids such as scopolamine (**1**) and hyoscyamine (**2**) (Fig. 1), has been well-known in China as a medicinal herb for its antitussive and sedative properties[1]. The biochemical research on tropane alkaloid metabolism using plant tissue culture techniques has so far been intensively carried out in various solanaceous plants[2], however, there is no report on the tissue culture work of *Physochlaina physaloides* as far as we know. In this study, the adventitious root cultures of this plant were established and the growth and tropane alkaloid production in several different conditions were investigated.

Materials and Methods

Plant material

The seeds of *Physochlaina physaloides* were sterilized [2%

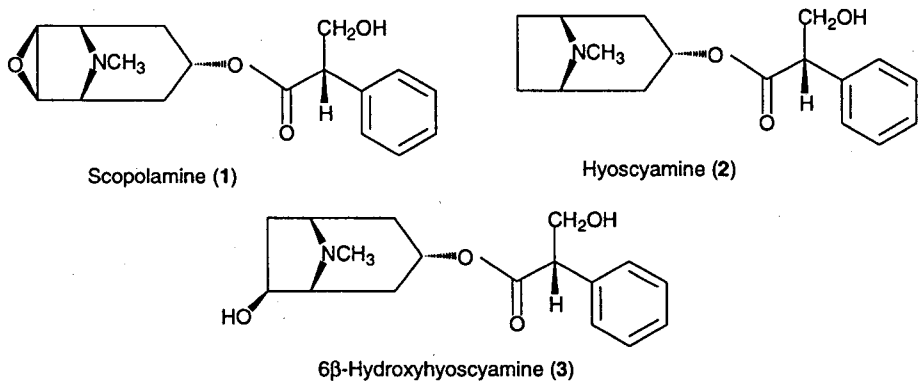

Fig. 1 Tropane alkaloids analyzed by HPLC in this study

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NaOCl with Tween 20 (1 drop in 50 ml)] for 10 min and germinated aseptically on 0.5% agar medium containing 0.5% sucrose under 16 h light/day (70 $\mu E \cdot m^{-2} \cdot S^{-1}$) at 25 ℃. The axenic plants were transferred to 1/2 Murashige-Skoog (MS)[3] solid medium containing 2% sucrose and subcultured monthly.

Induction of adventitious roots

The adventitious roots were induced with some calli from leaf segments (5 x 5 mm) of the axenic plants cultured on MS solid medium containing 1 mg/l indole-3-acetic acid (IAA) in the dark for 3 weeks. The roots were cut off and maintained in MS liquid medium with 1 mg/l IAA. Only one clone was selected by its rapid growth and high production of tropane alkaloids [**1**, **2** and related alkaloid 6β-hydroxyhyoscyamine (**3**)] (Fig. 1) and used for this experiment.

Analysis of alkaloids by HPLC

The sample preparation and the method of HPLC analysis for tropane alkaloids (**1-3**) were done by the same method as mentioned in our previous paper[4].

Effects of auxins

The adventitious roots [*ca.* 100 mg, fresh weight (fw)] were inoculated in MS liquid medium containing various concentrations of IAA (0.1, 0.5, 1 or 3 mg/l), indole-3-butylic acid (IBA) (0.1, 1 or 3 mg/l) or 1-naphthaleneacetic acid (NAA) (0.01, 0.1 or 1 mg/l). After 3 weeks of culture, the roots were harvested and their alkaloid contents were measured by HPLC.

Effects of media

The adventitious roots (*ca.* 150 mg, fw) were inoculated in four basal media [MS, 1/2 MS, Gamborg B5 (B5)[5] and Woody Plant (WP)[6]] supplemented with 0.01 mg/l NAA. After 3 weeks of culture, the growth and alkaloid production were determined.

Time course of growth and alkaloid production

The adventitious roots (*ca.* 150 mg, fw) were inoculated in 1/2 MS liquid medium containing 0.01 mg/l NAA. The growth and alkaloid production were measured periodically (once a week, from 1 to 6 weeks).

Alkaloid content in the regenerated plant cultivated in the soil

The axenic plant *in vitro* was transplanted to a pot and acclimatized in a phytotron at 25 ℃ in 70 % humidity under 16 h/day light (150 $\mu E \cdot m^{-2} \cdot S^{-1}$). After 3 weeks of culture, the plant was transferred to a field and cultivated for 3 months. The grown plant was separated to four portions (leaf, stem, root and rootlet) and their alkaloid contents were determined.

General experimental procedures

The volume of the liquid media (3 % sucrose) for the adventitious root cultures was 50 ml per 100 ml Erlenmeyer flask. The liquid cultures were maintained on a rotary shaker at 100 rpm in the dark at 25 ℃. All the media were adjusted to pH 5.7 before autoclaving at 121 ℃ for 15 min. Solidified medium was obtained by adding 0.2 % Gelrite. The data for all experiments are shown as the mean of three replicates.

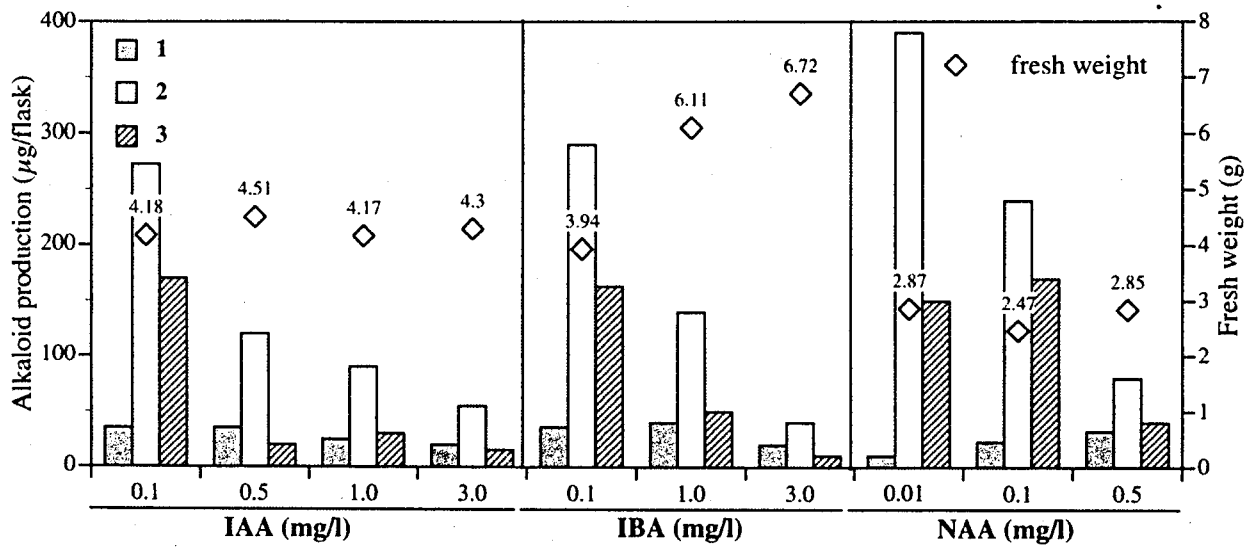

Fig. 2 Effects of auxins on the growth and alkaloid production in adventitious root cultures of *Physochlaina physaloides*

The roots (*ca.* 100 mg fw) were cultured in MS liquid medium (50 ml/100 ml flask) for 3 weeks at 25℃ in the dark on a rotary shaker (100 rpm). The value above symbol (◇) shows fresh weight (g). **1** (▨), scopolamine; **2** (□), hyosyamine; **3** (▨), 6β-hydroxyhyoscyamine.

Results and Discussion

The effects of three auxins, IAA, IBA and NAA, on the growth and alkaloid production (**1-3**) of *P. physaloides* adventitious roots were determined using MS liquid medium (Fig. 2). The roots (inoculum: *ca.* 100 mg fw) cultured with 1 mg/l or 3 mg/l IBA for 3 weeks showed the rapid growth (6.11 g and 6.72 g, fw, respectively) where the level was more than 2-fold compared to those cultured with NAA. In the media containing IAA the roots also showed satisfactory growth (4.17-4.51 g, fw). In this experiment, the roots accumulated **2** as the main alkaloid and lower concentrations of auxins were more effective for the tropane alkaloid production. Particularly, the highest alkaloid production was obtained with the addition of 0.01 mg/l NAA with yielding 390 μg/flask of **2** and 150 μg/flask of **3**. Although the tropane alkaloids in the culture medium were also determined by HPLC, they were at extremely low levels.

As the culture with 0.01 mg/l NAA was the most effective for alkaloid production, the effects of various basal media (MS, 1/2 MS, B5 and WP) containing 0.01 mg/l NAA on the growth and alkaloid production were tested (Fig. 3). The roots grew the best in 1/2 MS liquid medium of the four media tested. The roots cultured either in 1/2 MS or MS medium accumulated high amounts of **2**, however, B5 or WP media were not suitable for alkaloid production. The highest yield of total three alkaloids was obtained in the roots cultured in 1/2 MS liquid medium (397 μg/flask). Therefore contents of **1-3** and the growth of the adventitious root cultures in 1/2 MS liquid medium containing 0.01 mg/l NAA were periodically investigated (Fig 4).

Fresh weight of the roots increased from the beginning of the culture and it reached a plateau level after 5 weeks. The content of **2** showed a transient increment at week 2, and then after week 3, its level was constant. The contents of **1** and **3** gradually increased from the beginning until the end of the culture. The level of **3** was approximately 2-3 times larger than that of **1** in most part of the culture period.

To compare the alkaloid contents of the adventitious roots with that of the regenerated plant cultivated in the field, the axenic plants *in vitro* were transplanted to soil. After 3 weeks of acclimatization in a phytotron, the plants were transferred to a field and cultivated for 3 months. The grown plants were harvested and their alkaloid contents in four separated parts (leaf, stem, root and rootlet) were examined (Table 1). The levels of **1-3** were high in the underground part (especially in the rootlet), whereas they were remarkably low in the leaf and stem portions. The alkaloid production in the cultured adventitious roots was superior to those in the roots of the field-grown plant. This result indicates the usefulness of root cultures of this plant for the production of tropane alkaloids because the cultured roots grow much faster than field-grown roots and rootlets.

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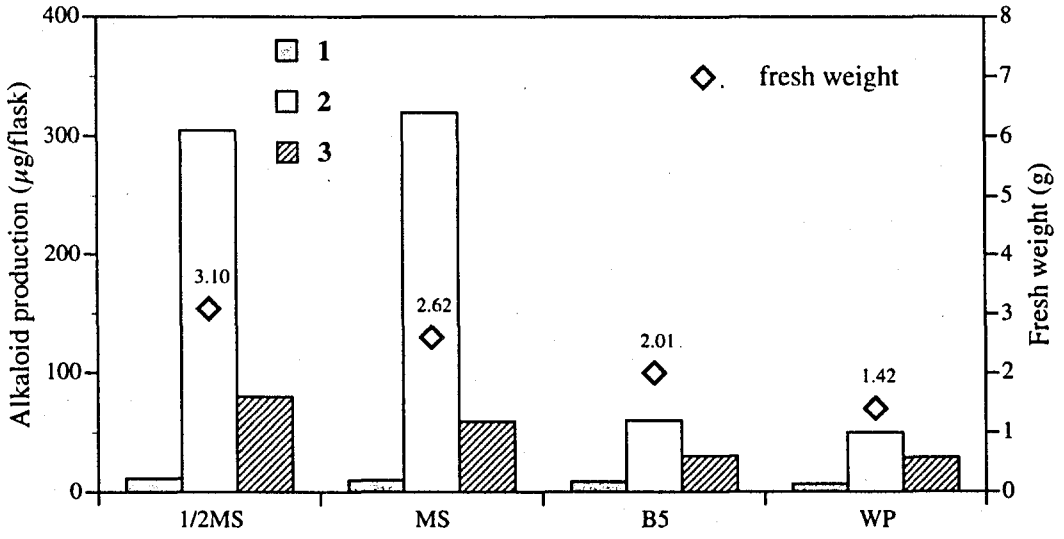

Fig. 3 Effects of various basal media on the growth and alkaloid production in adventitious root cultures of *P. physaloides*

The roots (*ca.* 150 mg fw) were cultured in various liquid medium containing 0.01 mg/l NAA (50 ml/100 ml flask) for 3 weeks at 25 ℃ in the dark on a rotary shaker (100 rpm). The value above symbol (◇) shows fresh weight (g). **1** (▨), scopolamine; **2** (□), hyosyamine; **3** (▨), 6β-hydroxyhyoscyamine.

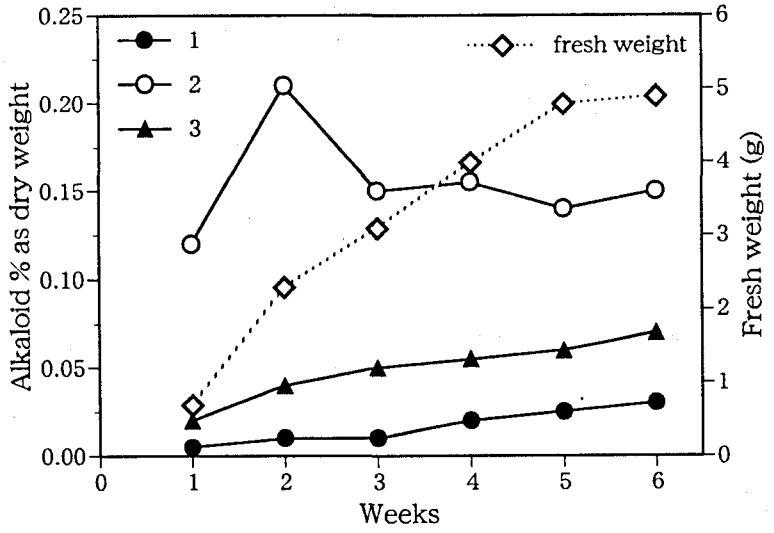

Fig. 4 Growth and alkaloid production in adventitious root culture of *P. physaloides*

The roots (*ca.* 150 mg fw) were cultured in 1/2 MS liquid medium containing 0.01 mg/l NAA (50 ml/100 ml flask) at 25 ℃ in the dark on a rotary shaker (100 rpm). ◇, fresh weight (g); **1** (●), scopolamine; **2** (○), hyosyamine; **3** (▲), 6β-hydroxyhyoscyamine.

Table 1 Tropane alkaloid contents in adventitious roots and regenerated plant

plant material		alkaloid % as dry weight		
		1	**2**	**3**
Adventitious roots		0.022	0.154	0.068
Plant	Leaf	trace	trace	0.004
	Stem	0.004	0.009	0.009
	Root	0.005	0.089	0.011
	Rootlet	0.052	0.341	0.230

Adventitious roots were cultured in 1/2 MS liquid medium (50 ml/100 ml flask) containing 0.01 mg/l NAA at 25 °C in the dark for 6 weeks. Axenic plantlets *in vitro* were transplanted to soil, acclimatized in a phytotron (25 °C, 70 % relative humidity, 16 h/day light) for 3 weeks, and then cultivated in a field for 3 months. **1**, scopolamine; **2**, hyosyamine; **3**, 6β-hydroxyhyoscyamine.

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