Lupin Alkaloids in Tissue Culture of *Sophora flavescens* var. *angustifolia*: Greening Induced Production of Matrine

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Green callus and multiple shoots were induced from the epicotyl segments of *Sophora flavescens* var. *angustifolia* (Leguminosae) on the Murashige and Skoog (MS) agar medium in the presence of 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) or 1-naphthaleneacetic acid (NAA). The green callus produced matrine as the only detectable alkaloid. The concentrations of matrine in the callus were positively correlated to the amounts of chlorophyll in the cells. No alkaloid was produced in the non-green callus. The multiple shoots accumulated not only matrine but also 5,6-dehydrolupanine and anagyrine. These results suggest that the production of lupin alkaloids in *S. flavescens* var. *angustifolia* is related to the tissue differentiation, in particular, to the formation of chloroplasts and that the biosyntheses of matrine-type and anagyrine-type alkaloids are differently regulated in different developmental stages of the cells.

Keywords *Sophora flavescens* var. *angustifolia*; Leguminosae; lupin alkaloid; quinolizidine alkaloid; matrine; 5,6-dehydrolupanine; anagyrine; green callus; multiple shoot; cell differentiation

A hundred years ago in 1889, Nagai[1] first reported the isolation of matrine from the dry roots of *Sophora flavescens* SOLANDER *ex* AITON var. *angustifolia* KITAGAWA (苦参). This crude drug has been used as a bitter medicine for centuries in China and Japan.[2] In 1936, the skeletal structure of matrine was proposed by Tsuda[3] and subsequently it was proved by synthetic studies.[4] The absolute structure of (+)-matrine was confirmed by Okuda *et al.*[5] in 1966. We have continued the phytochemical studies on lupin alkaloids in leguminous plants which grow mainly in Japan. In the course of these studies, several new alkaloids related to matrine were isolated and their structures were determined.[6] The biosynthesis of matrine was also investigated in intact plants of *S. flavescens* var. *angustifolia* by Shibata and Sankawa[7] and related species.[8] A number of interesting pharmacological activities were reported for matrine and the extracts of this plant, for example, a diuretic activity,[2] an antimicrobial activity,[2] an antiulcerogenic activity,[9] and an antiarrhythmic activity.[10]

Furuya and Ikuta[11] established callus culture of *S. flavescens* var. *angustifolia* and demonstrated that the callus produced the flavonoids, (−)-maackiain and pterocarpin. However, no report has been published on production of matrine and related lupin alkaloids in tissue culture of *S. flavescens* var. *angustifolia*. Recently we confirmed that the production of (+)-lupanine is induced by greening of the callus of *Thermopsis lupinoides* LINK,[12] which is also used as a source plant in Chinese traditional medicine.[13]

In the present communication, we report the production of matrine and other lupin alkaloids in the green callus and in the multiple shoots of *S. flavescens* var. *angustifolia*.

Results

Our previous study[12] indicated that the production of lupin alkaloids, in particular, of (+)-lupanine, was induced by the greening of the callus of *T. lupinoides*. Thus, we tried to obtain the alkaloid-producing green callus of *S. flavescens* var. *angustifolia*. The green callus was induced from explants of 10—14 d seedlings under illumination on Murashige and Skoog (MS) medium in the presence of phytohormones. We tried callus induction from epicotyls, roots and cotyledons of aseptic seedlings. Among these explant segments, epicotyls were best for the formation of green callus and multiple shoots. We studied the effects of 6-benzylaminopurine (BAP) and other phytohormones on the induction of green callus (Table I, Fig. 1), since BAP was shown to be essential for greening of the callus from the previous findings on *T. lupinoides*[12a] and our preliminary study on *S. flavescens* var. *angustifolia*.[12b] The effective auxin concentrations were 2.0 mg/l for 1-naphthaleneacetic acid (NAA) and 1.0 or 2.0 mg/l for 2,4-dichlorophenoxyacetic acid (2,4-D). BAP was also effective at 1.0 or 2.0 mg/l. The combinations of these concentrations of auxin and cytokinin gave up to 100% induction of green callus per epicotyl explant. However, kinetin has a very weak inducing effect on the formation of green callus. The green callus has been stably maintained at a specific growth rate of 1.5 to 2.0 per 3 weeks in sealed plastic plates. When the green callus was transferred onto the same medium in an Erlenmeyer flask with aluminum foil on the top, it tended to turn brown within a couple of days. This might be because of the lower concentration of oxygen in a sealed plate.

Multiple shoots were induced in the presence of 1.0 or 0.1 mg/l NAA and 1.0 or 2.0 mg/l BAP (Table II, Fig. 1). These are lower concentrations of NAA than those used for the formation of green callus. No multiple shoot was induced in the presence of 2,4-D, suggesting an inhibitory effect of 2,4-D for shoot differentiation. The multiple shoots were formed with small calli on the ends of excised epicotyls.

The total alkaloidal fractions were obtained from the green callus and the multiple shoots. The alkaloids were analyzed by gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC). The green callus produc-

We wish to dedicate this paper to the late Professor Nagai to commemorate the hundredth anniversary of his first report on the isolation of (+)-matrine.[1]

ed matrine as the only detectable alkaloid and no other lupin base was detected. Matrine was identified by its characteristic fragment ions at m/z 248 (M^+), 206, 205, 150, 98 and 96 in comparison with those of an authentic sample on GC-MS and also by HPLC as described previously.[14] The concentrations of matrine in the green callus, however, were two or three orders of magnitude lower than that of the differentiated plant.[6] The contents of chlorophyll were quantified fluorophotometrically in the green callus and in the multiple shoots. The concentrations of matrine in the callus were positively correlated to those of chlorophyll ($r=0.705$, $n=19$) (Fig. 2). The green calli which were originally derived from white calli also produced matrine, although they had accumulated no detectable alkaloid when they were still white. These results indicate that the production of matrine is inducible by the greening of the callus tissue.

The multiple shoots, in contrast, produced not only matrine but also 5,6-dehydrolupanine and anagyrine in some cell lines as shown in Table III. The concentrations of both matrine and chlorophyll in the multiple shoots were several fold higher than those of green callus. Among the cell lines, the cell line No. 1 contained the highest concentration of chlorophyll and was highly differentiated. This cell line also accumulated the greatest amount of alkaloids.

Table I. Effect of Phytohormones on the Induction of Green Callus on the Epicotyl Explants of *S. flavescens* var. *angustifolia*

Cytokinin (mg/l)	Auxin (mg/l)					
	NAA			2,4-D		
	2.0	1.0	0.1	2.0	1.0	0.1
BAP						
2.0	30/30[a] (100)[b]	8/30 (26)	0/21 (0)	12/15 (80)	17/17 (100)	8/14 (57)
1.0	13/15 (87)	4/14 (29)	0/14 (0)	4/15 (27)	15/15 (100)	10/15 (67)
0.1	6/14 (43)	0/14 (0)	0/14 (0)	5/16 (31)	9/14 (64)	1/15 (0.7)
Kinetin						
2.0	0/15 (0)	—	—	0/14 (0)	—	—
0.1	0/20 (0)	—	—	—	0/20 (0)	—

a) Number of epicotyl explants forming green callus/number of epicotyl explants examined. *b*) Percentage of green callus-forming explants with respect to total explants.

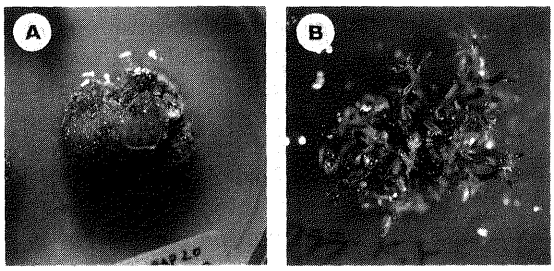

Fig. 1. Green Callus and Multiple Shoots of *S. flavescens* var. *angustifolia* on MS Agar Medium

(A) The green callus induced in the presence of 1.0 mg/l 2,4-D and 2.0 mg/l BAP. (B) The multiple shoots induced in the presence of 0.1 mg/l NAA and 2.0 mg/l BAP.

Table II. Effect of Phytohormones on the Induction of Multiple Shoots on the Epicotyl Explants of *S. flavescens* var. *angustifolia*

Cytokinin (mg/l)	Auxin (mg/l)					
	NAA			2,4-D		
	2.0	1.0	0.1	2.0	1.0	0.1
BAP						
2.0	0/30[a] (0)[b]	12/31 (39)	7/21 (33)	0/15 (0)	0/17 (0)	0/14 (0)
1.0	0/15 (0)	0/14 (0)	5/14 (36)	0/15 (0)	0/15 (0)	0/15 (0)
0.1	0/14 (0)	0/14 (0)	1/14 (0.7)	0/16 (0)	0/14 (0)	0/15 (0)
Kinetin						
2.0	0/15 (0)	—	—	0/14 (0)	—	—
0.1	0/20 (0)	—	—	—	0/20 (0)	—

a) Number of epicotyl explants forming multiple shoots/number of epicotyl explants examined. *b*) Percentage of multiple shoot-forming explants with respect to total explants.

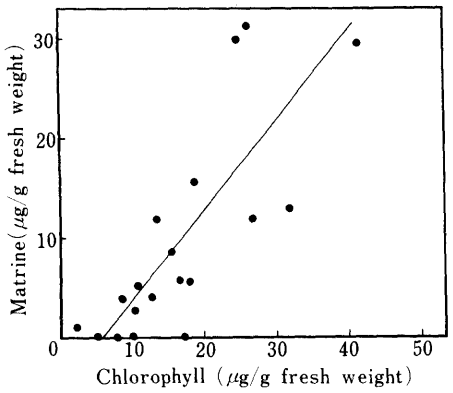

Fig. 2. Correlation between Matrine and Chlorophyll Concentrations in Green Callus of *S. flavescens* var. *angustifolia*

Table III. The Alkaloids in Multiple Shoots of *S. flavescens* var. *angustifolia*

Cell line	Phytohormones (mg/l)	Concentration in tissue (μg/g fresh weight)			
		Chlorophyll	Matrine	5,6-Dehydrolupanine	Anagyrine
1	BAP (2.0), NAA (1.0)	297	173	31.8	118
2	BAP (2.0), NAA (0.1)	107	29.2	14.8	16.5
3	BAP (2.0), NAA (1.0)	128	17.5	n.d.	n.d.
4	BAP (2.0), NAA (1.0)	151	31.4	Trace	11.8

n.d., not detected.

Discussion

In our previous paper,[12a] we demonstrated that the production of lupin alkaloids in *T. lupinoides* was related to the greening of the callus, and (+)-lupanine was the sole detectable alkaloid. Wink *et al.*[15] also reported that the production of lupanine was correlated to greening in *Lupinus* and other plants. They also established that the enzymatic activity of lysine decarboxylase, which is postulated as an initial enzyme for the biosynthesis of lupin alkaloids, was located in leaf chloroplasts of a differentiated plant of *L. polyphyllus*.[16] Our present results indicate that in *S. flavescens* var. *angustifolia* the biosynthesis of matrine is closely correlated to the formation of chloroplasts in the cells. Matrine and lupanine are assumed to be the initial metabolites in the biosynthetic pathways of matrine-type and anagyrine-type alkaloids, respectively. The finding that matrine or lupanine was the only alkaloid produced in undifferentiated callus tissues may suggest that the biotransformation activity responsible for matrine and lupanine is suppressed in the undifferentiated tissue.

The differentiated multiple shoots in which root organ is not developed synthesized 5,6-dehydrolupanine and anagyrine as well as matrine in higher concentrations than in green callus. These findings suggest that the expression of biosynthetic activities for matrine-type and anagyrine-type alkaloids, which have different modes of formation of their carbon skeletons, are differently controlled during the developmental stages of *S. flavescens* var. *angustifolia* cells. The occurrence of 5,6-dehydrolupanine, in particular, is intriguing from a biosynthetic point of view. 5,6-Dehydrolupanine is postulated to be a key intermediate for biosynthesis of anagyrine-type alkaloids.[17a] However, 5,6-dehydrolupanine is present in only trace amounts in differentiated plants, although the concentrations of matrine and anagyrine are, for example, 1—2 mg/g of fresh weight of seeds. The substantial accumulation of 5,6-dehydrolupanine, besides anagyrine, in the multiple shoots indicates that the biosynthetic activity to anagyrine *via* 5,6-dehydrolupanine is expressed in the multiple shoots.

The dry root of *S. flavescens* var. *angustifolia* is used as a crude drug. A high concentration of matrine N-oxide is normally detected in this dry root. However, in our cultured tissue matrine N-oxide could not be detected. Thus, it is possible that the root organ is responsible for storage and biotransformation of lupin alkaloids produced in leaves, suggesting cooperation for secondary metabolism in plants.[18]

Experimental

Induction of Green Callus and Multiple Shoots The epicotyls, roots and cotyledons were excised from the aseptic seedlings (10—14 d) of *S. flavescens* var. *angustifolia* grown on the half-strength MS agar medium without any phytohormones. The explant segments (1—1.5 cm, length) were put onto the MS agar (0.8%) medium supplemented with the appropriate phytohormones for callus induction as shown in Table I. The plastic plates (9 cm, diameter; 1.8 cm, depth) containing the agar medium (25 ml) were sealed with a commercial paraffin film (Novix-II, Iwaki Glass). The green callus was induced within 2 to 3 weeks at 25 °C under illumination (2000 lux, 16 h/d). The callus was excised from the original explants and transferred onto the same agar medium. The established callus was maintained in the sealed plastic plates with transfer every 3 to 4 weeks. The multiple shoots were obtained by using the same procedure described above, except for the combinations of phytohormones as indicated in Table II.

Extraction of Alkaloid Fraction The cultured tissue (0.5 to 1 g fresh weight) was homogenized with 2 ml of 80% EtOH containing 40 μg of benzoyllupinine as an internal standard. Benzoyllupinine is not found naturally and it was prepared from (−)-lupinine. The homogenate was centrifuged at 2000 rpm for 2 min, and an aliquot of the resulting supernatant was used for the determination of chlorophyll (*vide infra*). The remaining supernatant was concentrated *in vacuo* and acidified with 2 ml of 0.5 N HCl. The acidified solution was extracted with 2 ml of ethyl acetate. After discarding the organic layer, powdered potassium carbonate was added to the aqueous layer to saturation. The resulting alkaline solution was extracted twice with 2 ml of ethyl acetate. The combined organic layer was evaporated to dryness.

Determination of Lupin Alkaloids The standard lupin alkaloids were isolated in our previous studies.[6,17] The alkaloids in callus and multiple shoots were identified by GC-MS and HPLC. GC-MS was carried out using a Shimadzu QP-1000 system equipped with a glass column (2 m × 3 mm) containing 2% OV-17 on Gas Chrom Q. HPLC was performed as described previously.[14] The quantitative determination of the alkaloids was carried out by GC on a packed glass column with 2% OV-17 at 250 °C coupled with a chromato-integrator (Hitachi, D-2500).

Determination of Chlorophyll The contents of chlorophyll in the cells were determined as described elsewhere[19] with modifications. Tissue was homogenized and extracted with 80% EtOH. After centrifugation at 2000 rpm for 2 min, the chlorophyll in the supernatant was quantified fluorophotometrically at an excitation wavelength of 413 nm and an emission wavelength of 672 nm.

Acknowledgment The authors wish to thank Professor T. Furuya, Kitasato University, and Professor S. Okuda, The Kitasato Institute, for their interest and encouragement throughout this work.

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