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GREENING INDUCED PRODUCTION OF (+)-LUPANINE IN TISSUE CULTURE OF *THERMOPSIS LUPINOIDES*

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Key Word Index—*Thermopsis lupinoides*; Leguminosae; green callus; lupin alkaloid; quinolizidine alkaloid; (+)-lupanine; absolute configuration.

Abstract—We have established three different types of tissue culture of *Thermopsis lupinoides* (Leguminosae): white callus, adventitious roots and green callus. Only the green callus accumulated lupin alkaloid, and its concentrations in the callus were correlated to the amounts of chlorophyll in the cells. (+)-Lupanine (6*R*,7*S*,9*S*,11*S*) was the sole alkaloid produced in green callus, although the differentiated plant contains not only (+)-lupanine but also α-pyridone-type alkaloids of the opposite absolute configuration (7*R*,9*R*,11*R*). These results suggest that the production of (+)-lupanine is related to the greening of the tissue but that of the enantiomeric alkaloids, e.g. (−)-lupanine, (−)-anagyrine, is not.

INTRODUCTION

Plant secondary metabolism is an interesting subject, not only because of the commercial value of some specific metabolites but also because of its relationship to plant cell differentiation. The different features of secondary metabolism, i.e. biosynthesis, transport, accumulation, transformation, degradation, release etc., depend on the state of cell differentiation [1]. For example, nicotine and tropane alkaloids in the Solanaceae are known to be synthesized in roots and transported to leaves [2]. It is also reported by Wink [3] that lupin alkaloids in *Lupinus* and several other species are produced in green parts of plants. However, no stereochemical study has been done on alkaloids produced by tissue culture of lupin plants, although lupin alkaloids provide us with intriguing stereochemical issues. Thus, it is interesting to investigate the biosynthetic capacity for lupin alkaloids in different types of cultured cells from a stereochemical point of view.

Thermopsis lupinoides Link. (Leguminosae) is used as a medicinal plant (ye jue ming) in China [4] and a rich source of lupin alkaloids [5]. We have isolated nine alkaloids from several organs of *T. lupinoides* [6–8]. This plant contains both (+)-lupanine-type and (−)-anagyrine-type alkaloids. These two type alkaloids have the opposite absolute configurations each other. Thus, it is suggested that *T. lupinoides* has the biosynthetic ability for alkaloids of both enantiomeric configurations [8].

In the present study, we have established three different kinds of tissue culture of *T. lupinoides*: white callus, adventitious roots and green callus. The production of alkaloids was induced by greening of the callus. (+)-Lupanine (6*R*,7*S*,9*S*,11*S*) (**1**) was the only alkaloid accumulated.

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RESULTS

Induction of white callus

The undifferentiated white callus was induced from the seed of *T. lupinoides* in the presence of 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l kinetin in Murashige and Skoog medium [9] (Table 1). The effects of plant hormones on the growth rate of the callus were investigated as shown in Fig. 1. The most rapid growth was obtained on medium D containing 1 mg/l 2,4-D and 0.1 mg/l kinetin. However, the white callus accumulated no detectable alkaloids.

Formation of adventitious roots

The white callus maintained on medium D was transferred to media A, B, C, D and E. After two months, the calli on media A, B and E spontaneously formed adventitious roots (Table 2). On the other hand, no roots were formed on media containing 2,4-D (media C and D), suggesting an inhibitory effect of 2,4-D on root formation of *T. lupinoides*. It has been reported that the frequency of root formation of seed legumes is quite high, despite the concentration of auxins and cytokinins [10]. Certainly, *T. lupinoides* also has a similar high propensity for root initiation. Again, alkaloids could not be detected in these adventitious roots, although the root tissue of differentiated plant is rich source of alkaloids [6, 8].

Formation of green callus

Green callus was obtained from white callus in the presence of 2 mg/ml 6-benzylaminopurine (BAP) (media G, H) under illumination (Table 2). However, every callus on medium A to F failed to turn green under illumination. 2,4-D and/or kinetin may have an inhibitory effect on greening of *T. lupinoides* callus.

Table 1. The media for tissue culture of *Thermopsis lupinoides**

Name	(mg/l)		Phytohormone additives
A	—		—
B	kinetin	0.1	—
C	2,4-D	1.0	—
D	2,4-D	1.0	—
	kinetin	0.1	
E	IAA	1.0	—
	kinetin	0.1	
F	2,4-D	1.0	coconut milk 1%
	kinetin	0.1	
G	BAP	2.0	—
H	BAP	2.0	$AgNO_3$ 10 mg/l.
I	2,4-D	1.0	$AgNO_3$ 10 mg/l.
	BAP	0.1	

*All media contained Murashige and Skoog medium [9], 3% sucrose and 0.8% agar (pH 5.7).

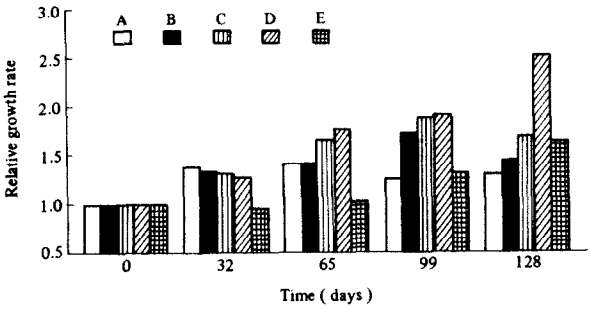

Fig. 1. Effects of plant hormones on the growth of white callus of *T. lupinoides*. The media A–E correspond to those in Table 1.

Medium H contained 10 mg/l silver nitrate, a compound reported to be a potent inhibitor of ethylene action [11] and which stimulates shoot formation in some callus culture [12]. In the first month, the callus grown on medium H was more green than that on medium G, but later the callus on medium H began to necrose. These suggest that silver nitrate is effective for initiation of green callus but is also toxic for *T. lupinoides* green callus.

We also induced green callus directly from seeds. During the first two weeks, callus was induced on medium I. At this stage the callus had a pale green colour. Then the callus was transferred to either medium G or H. Within one month, the callus had turned green.

Production of (+)-lupanine in green callus

Every green callus analysed accumulated lupanine as the only detectable alkaloid. This was confirmed by GC-MS and HPLC analysis. The amounts of lupanine in the callus were positively correlated to those of chlorophyll (Fig. 2). These results indicate that the production of lupin alkaloids, particularly that of lupanine, is closely correlated with the formation of chloroplasts.

It has already been shown by our recent investigations [6–8] that *T. lupinoides* and *T. chinensis* contain both (+)-(7*S*,9*S*,11*S*) lupanine-type and (−)-(7*R*,9*R*,11*R*) anagyrine-type alkaloids. It means that these plants have the biosynthetic ability for the alkaloids of opposite absolute configurations. Thus, we determined the absolute configuration of lupanine produced by green callus of *T. lupinoides*. Lupanine was extracted from the green callus and purified by HPLC. The purified lupanine gave a positive Cotton effect, $[\Theta]_{222}+12\,700$. This value was almost comparable to that of standard (+)-lupanine, $[\Theta]_{222}+13\,000$. These results indicated that (+)-lupanine (6*R*,7*S*,9*S*,11*S*) was produced by green callus, as it is in differentiated plants.

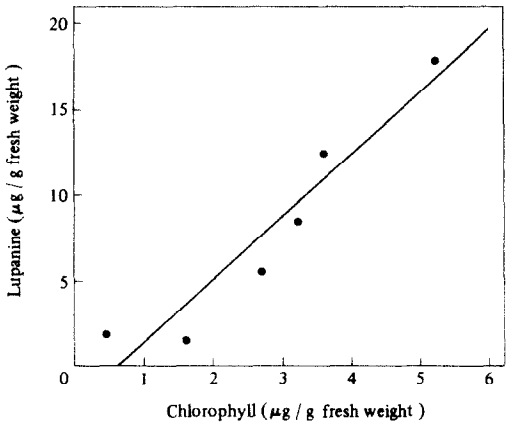

Fig. 2. Correlation between (+)-lupanine and chlorophyll concentrations in green callus on medium G.

Table 2. The formation of the adventitious roots and the green callus from the white callus

Medium	Illumination (2000 lx 16 hr/day)	Number of the callus forming adventitious roots	Number of the callus forming green callus	Production of (+)-lupanine
A	No	4/18 (22%)	—	—
B	No	5/19 (26%)	—	—
C	No	0/17 (0%)	—	—
D	No	0/16 (0%)	—	—
E	No	4/13 (31%)	—	—
F	Yes	—	0/12 (0%)	—
G	Yes	—	12/12 (100%)	++
H	Yes	—	12/12 (100%)	++

DISCUSSION

Our present results indicate that the biosynthesis of (+)-lupanine in *T. lupinoides* is closely related to the formation of chloroplasts and that the root tissue does not participate in biosynthesis but probably serves as a storage organ. Wink *et al.* [13–16] reported, in agreement with our findings, that lupanine production was correlated to the greening in some lupin plants. Recently it was shown that pyrrolizidine alkaloids in *Senecio vulgaris* are synthesized in root cultures [17], while *in vitro* root cultures of *Lupinus* explant failed to produce lupanine and sparteine [18]. These findings are also consistent with our present results that the adventitious roots do not produce any alkaloids.

We showed in this study that (+)-lupanine was the sole detectable alkaloid in the green callus, although no report has been published on the absolute configuration of the lupanine so far. Differentiated plants, on the contrary, contain not only (+)-lupanine (6*R*,7*S*,9*S*,11*S*) (**1**) but also (−)-anagyrine-type alkaloids (7*R*,9*R*,11*R*), e.g. (−)-anagyrine (**2**), (−)-cytisine and others, the alkaloids which have the enantiomeric absolute configuration compared with that of (+)-lupanine. Of the lupin alkaloids, lupanine is postulated as an important intermediate in the biosynthesis of tetracyclic sparteine-type alkaloids [19–21]. The pyridone-type alkaloids, i.e. anagyrine, cytisine etc., are derived from lupanine. In this context, it is interesting that (+)-lupanine was the only alkaloid detected in the callus culture and no (−)-lupanine- and (−)-anagyrine-type alkaloids were present. These results suggest either that the biosynthetic ability for (6*S*,7*R*,9*R*,11*R*) alkaloids, e.g. (−)-lupanine, (−)-anagyrine, (−)-cytisine, is suppressed in cultured cells, or that the degradation rate of the (6*S*,7*R*,9*R*,11*R*) alkaloids is so rapid that they are hardly accumulated compared with (+)-lupanine.

EXPERIMENTAL

Callus culture of T. lupinoides. Seeds were surface sterilized in 75% EtOH for 2 min and 3% NaOCl for 15 min. White callus was induced from these seeds and maintained at 25° in the dark on medium D (Table 1). The callus was transferred every 3 to 4 weeks to fresh medium. Green callus was induced from white callus on either medium G or H (Tables 1 and 2) under illumination (2000 lx, 16 hr/day). Green callus was also obtained directly from seeds. Sterile seeds were put onto medium I for 14 days under illumination and then transferred to either medium G or H.

Extraction of alkaloid fraction. The callus (1–3 g of the fr. wt) was homogenized with 2 to 3 ml of 0.5 M HCl containing 10 μg of lupinine benzoate as an internal standard. After the homogenate was centrifuged at 2500 rpm for 3 min, the clear supernatant was extracted with 2 ml of EtOAc. After removing the organic layer, powdered K_2CO_3 was added to the aq. layer to satn. The resulting alkali soln was extracted twice with 2 ml of EtOAc. The combined organic layer was evapd *in vacuo*.

Determination of alkaliods and chlorophyll. The lupin alkaloids in the calli were identified by GC-MS and HPLC [22]. The standard alkaloids were isolated by our previous studies [6–8, 23, 24]. The quantitative determination of the alkaloids was performed by GC (2% OV-17, 2 m, 250°). Lupinine benzoate was prepared from lupinine and used as an internal standard. The contents of chlorophyll were determined fluorophotometrically as described previously [25].

Determination of absolute configuration of lupanine produced by green callus. The alkaloid fraction obtained from 16.5 g of fr. wt. of the green callus was subjected to HPLC (equipped with a Lichrosorb Si60, 5 μm, 4.6 × 250 mm). A mobile phase of 25% MeOH in Et_2O–5% NH_4OH (25:1) was used at a flow-rate of 1.5 ml/min with monitoring at 220 nm as ref. [17]. The HPLC separation was repeated twice. The lupanine containing fractions were combined and concd to give 0.2 mg pure lupanine. CD $[\Theta]_{222}$ + 12 700 (MeOH). The standard (+)-lupanine was obtained as described in refs [5–7] and gave CD $[\Theta]_{222}$ + 13 000 (MeOH).

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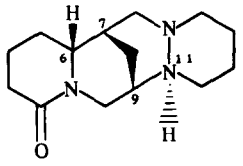
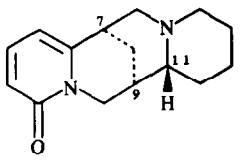


1 (6*R*,7*S*,9*S*,11*S*)



2 (7*R*,9*R*,11*R*)

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