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GROWTH AND ALKALOID PRODUCTION OF CALLUS CULTURE INDUCED FROM *SECURINEGA SUFFRUTICOSA*

AKIO IDE, KENICHI NAGANO, NORIKO TANAKA, KOHJI IWASAKI, YOSHIO YAMANE, DAIZO KOGA, KAZUYOSHI YAGISHITA,* KAYOKO NAKAO,† YOSHIKO KURISU,† NAOMI FUJIOKA† HIROSHI KOHDA,† HIDEKI MIYAGAWA† and KAZUO YAMASAKI†

Department of Agricultural Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, Japan; †Institute of Pharmaceutical Sciences, School of Medicine, Hiroshima University, Hiroshima 734, Japan

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Key Word Index *Securinega suffruticosa*; Euphorbiaceae; callus; alkaloids; securinine; allosecurinine.

Abstract The growth of, and production of alkaloids by, callus derived from budding stem explants of the germinated seeds of *Securinega suffruticosa* has been studied. The major alkaloids produced were securinine and allosecurinine with the latter being present in the greatest amount. The effects of pH, growth hormones, sucrose concentration and light and dark on callus growth and alkaloid production have been examined in detail. The pattern of alkaloid production in the callus culture appeared to be similar to that in the root of the securinega plant.

INTRODUCTION

Securinine (**1**), one of the main alkaloids of *Securinega suffruticosa* Rehd., is a CNS stimulant which is useful in the treatment of paresis, paralysis following infectious disease, and psychical disorders [1]. Recently, it has been shown that **1** is a new class of GABA receptor antagonist [2]. Compound **1** and its congeners, including allosecurinine (**2**), have been isolated from *Securinega* and *Phyllanthus*: they all possess a tetracyclic structure related to **1** or its lower homologue [1], norsecurinine (**3**). The biosynthesis of **1** has been studied by three groups [3–5]. They found that **1** is formed from L-lysine and L-tyrosine. The former amino acid gives rise to the piperidine ring of **1** via cadaverine and Δ^2-piperidine, while the latter provides the remaining rings (Scheme 1).

The one study which has been carried out on the formation of **2**, which usually co-exists with **1** [4], showed that cadaverine is incorporated into the same positions in both alkaloids. This established that the biogenesis of both alkaloids is similar, although the branch point of the two routes may be at an early stage (Scheme 1).

With the aims of producing useful securinega alkaloids in a controlled manner and clarifying the mechanism which controls their biosynthesis, tissue culture of *S. suffruticosa* was attempted.

In this paper, we report on the production of **1** and **2** by callus induced from *S. suffruticosa*.

RESULTS AND DISCUSSION

Callus induction

This was examined on Murashige and Skoog's (MS) agar (1.3%) medium [6] supplemented with 0.1 μM kinetin (K) and with 2,4-dichlorophenoxyacetic acid (2,4-D) or 1-naphthalene acetic acid (NAA) in the dark. NAA at 10 μM and 2,4-D at 1–10 μM gave good callus formation (Fig. 1), but in the former case callus production was less active and root-like tissues were produced. The callus induced in the medium containing 0.1 μM K and 1 μM 2,4-D was used for further experiments.

Characterization of alkaloids

Two alkaloids were isolated from the callus. They were identified as **1** and **2** by comparison of IR spectra and mixed melting points with authentic samples isolated from the original plant. The amount of **2** obtained from the callus was more than that of **1**. Other alkaloids were detected in the callus by TLC but were not isolated.

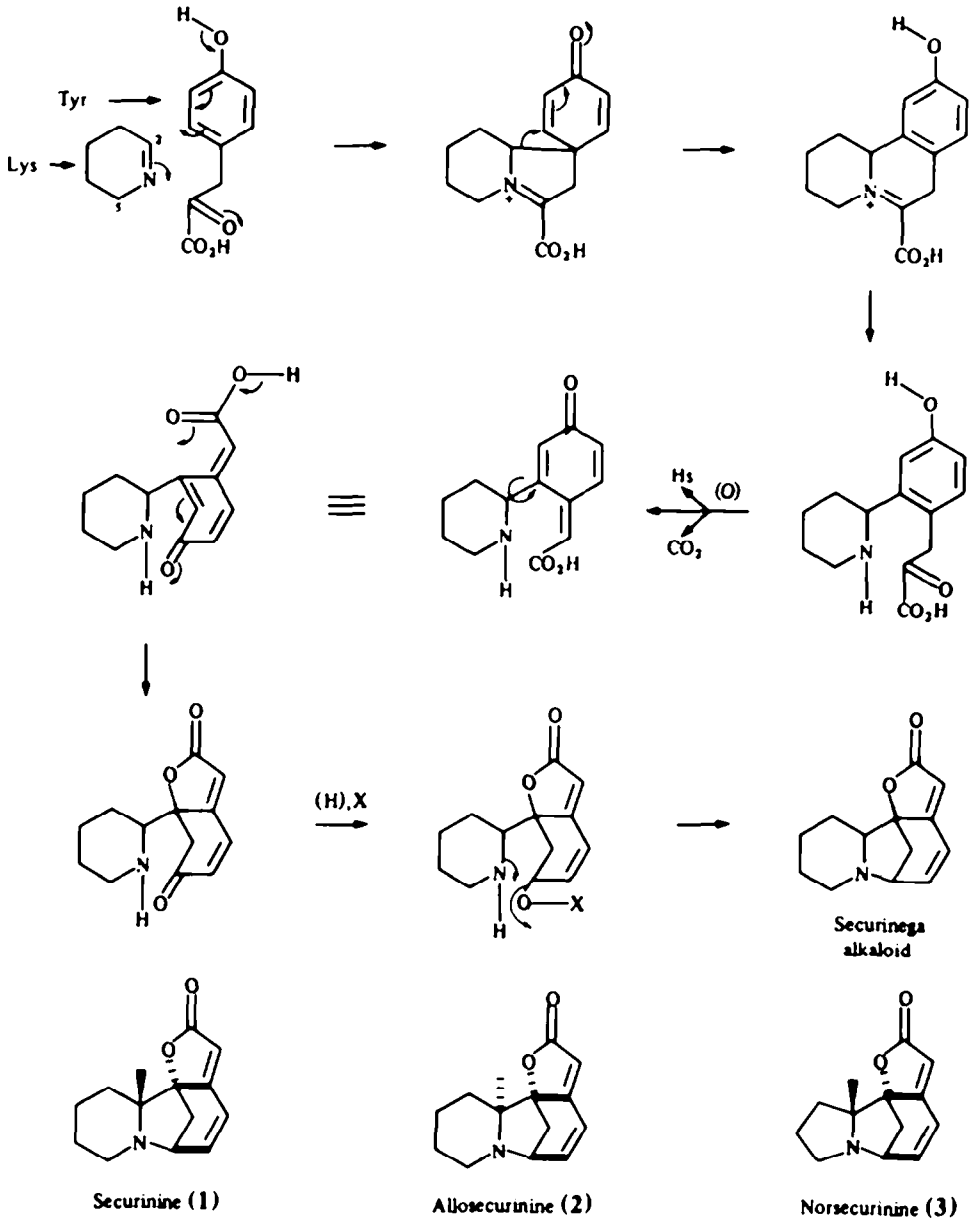
Time courses of callus growth and alkaloid production

Figure 2 presents the results obtained with calli which were grown on a medium supplemented with 2,4-D in the light or in the dark. Similar time courses for calli grown in a medium containing NAA were also determined (data not shown). Whatever the conditions, the callus showed a significant lag phase after inoculation. They reached the stationary state after 10–12 weeks via a logarithmic phase. The alkaloid content of the callus tissue increased slowly in step with callus growth. The maximum content of alkaloid was *ca* 1.6 mg/g dry wt (Fig. 2). However, callus growth in the medium containing NAA in the dark was slow compared with that under other conditions. Production of alkaloids, especially **2**, was more than that of **1** under any conditions, and was suppressed by 2,4-D and by light. Ten-week-old callus was used for determination of the optimum culture conditions.

Optimum culture condition

Callus growth and alkaloid production showed little change over the pH range 4–6. The optimum pH values

*Present address: Shoukei Junior College, 6-78, Kyuhinji-2, Kumamoto 862, Japan.



Scheme 1.

were pH 5.0 in the dark and pH 5.5 in the light (Fig. 3). Alkaloid production in the light was susceptible to changes in the pH more so than in the dark. Therefore, pH is an important factor in the control of alkaloid production in the light.

Optimum sugar concentration for callus growth was 5% in the dark and 3–5% in the light. In the dark, alkaloid production was more dependent on callus growth than in the light (Fig. 4). A medium containing 5% sugar was suitable for alkaloid production regardless of irradiation.

The callus grew better in a medium supplemented with 2,4-D than with NAA. The effect of the growth regulator on alkaloid production showed an opposite trend to that of callus growth in the dark. The optimum concentrations of the growth regulators for the callus growth were 1 μM 2,4-D for 0.1 and 0.01 μM K, and were not influenced by light (Figs. 5 and 6).

When the callus was grown in 2,4-D medium, compared with NAA it produced slightly more of **2** (1.3 mg/g dry wt) although the callus growth was not so good.

Summary of results

The callus grew better in the light than in the dark, and assumed a pale green colour. Its growth was very slow in the medium supplemented with NAA in the dark compared with the callus grown under the other conditions. In contrast, the production of alkaloids, especially **2**, is proportional to the growth of the callus. This pattern resembles that of the root of the securinega plant, which has a higher alkaloid content than the leaves, and contains more **2** than **1** than the leaves [7].

This suggests that the NAA-supplemented medium mimics the conditions in the root concerning the bio-

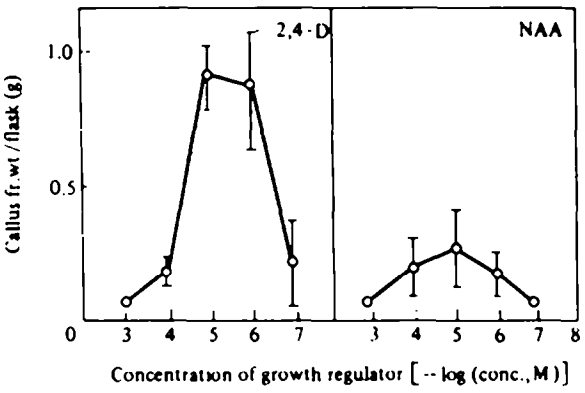


Fig. 1. Effect of growth regulators on callus induction from the leaf bud of *S. suffruticosa*. The tissues were incubated on MS agar medium supplemented with 2,4-D or NAA and with 0.1 μM kinetin at 27° for 6 weeks in the dark.

synthesis of the alkaloids. In other words, the presence of light and the addition of 2,4-D might change the biosynthetic mode into a system corresponding to the aerial part of the plant, where the total alkaloid synthesis is less, and the ratio of **1** to **2** is low. Concerning this, the requirement of sugar for callus growth was also influenced

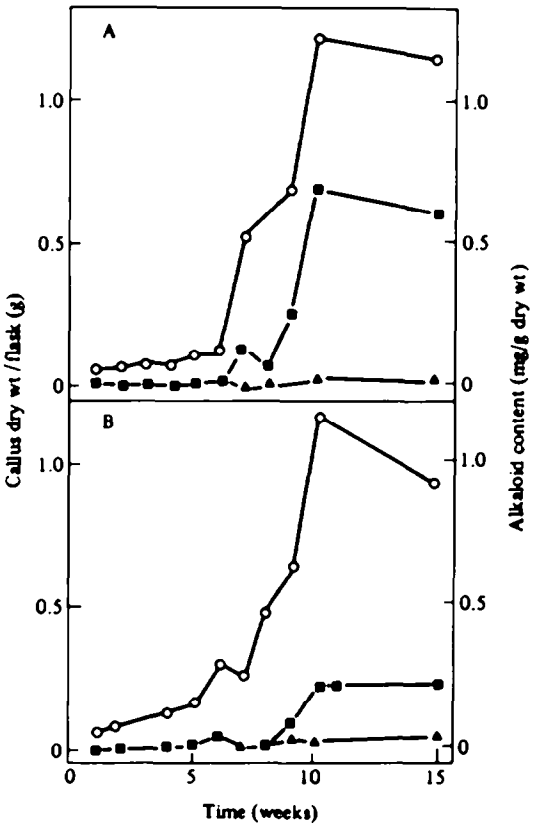


Fig. 2. Growth as measured by dry weight (O) and alkaloid contents (▲——▲, **1**; □——□, **2**) in callus of *S. suffruticosa*. The callus was grown on MS agar medium supplemented with 0.1 μM kinetin and 1 μM 2,4-D at 27° in the dark (A) or in the light (B). The same symbols have been used in all the figures.

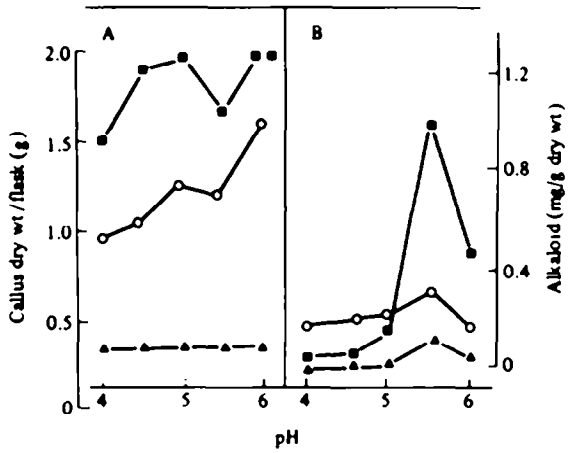


Fig. 3. Effect of pH on growth and production of alkaloids. In this and the following figures, calli were grown for 10 weeks on MS agar medium supplemented with 2,4-D (1 μM) and kinetin (0.1 μM) containing 5% sugar in the dark (A) or in the light (B).

by light (Fig. 4). It must be that consumption of sugar slowed down as the callus adapted to its surroundings and synthesized *de novo* the sugar for itself.

EXPERIMENTAL

Plant materials. *S. suffruticosa*, collected from Ujima Island, Hikari City, Japan, was grown in the Yamaguchi University campus. The seeds were collected in the autumn, air-dried and stored at 0°.

Callus induction. Seeds were dipped in 70% EtOH for 10 sec and then in 0.1% $HgCl_2$ soln for 1 min. They were then placed on sterilized agar medium (1.0%). After 3 weeks, sliced segments of the leaf buds from the germinated seeds were transferred to MS agar medium (1.3% agar, pH 5.8–6.0) supplemented with NAA or 2,4-D and 0.1 μM K, and incubated for 6 weeks in the dark.

Tissue culture. Newly induced callus tissue was maintained on MS agar medium (1.3% agar, pH 5.8–6.0) supplemented with 2,4-D (10 μM) and K (0.1 μM) at 27° for 4 weeks in the dark. The tissue was then cut into pieces weighing 0.5 g, each of which was inoculated into a milk bottle (180 ml capacity) containing 30 ml of MS medium supplemented with additives when required, and grown in the dark or in continuous light (2000–2500 lx). The

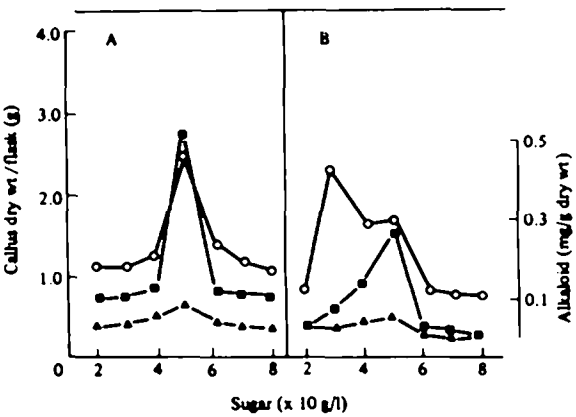


Fig. 4. Effect of sugar concentration on growth and alkaloid production in the dark (A) and in the light (B).

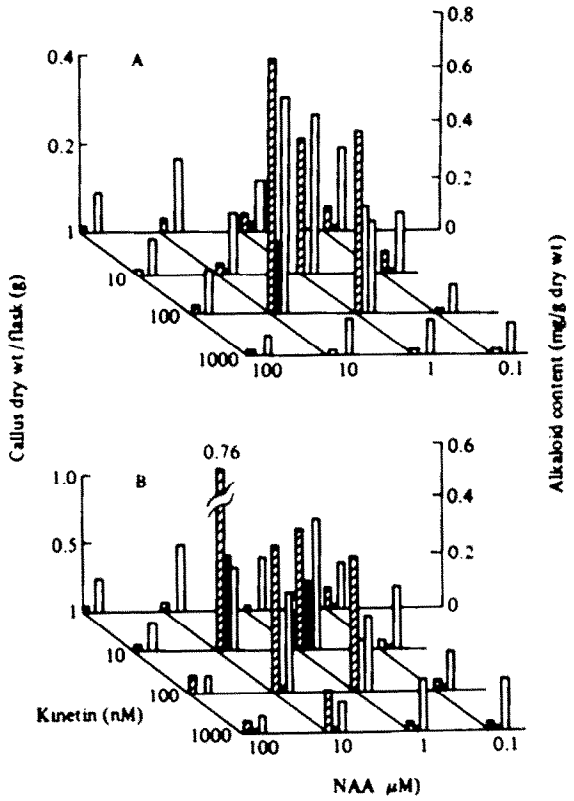


Fig. 5. Effects of NAA on growth and alkaloid production of callus of *S. suffruticosa*. In this and Fig. 6, callus was incubated on MS agar medium supplemented with kinetin at 27° for 10 weeks in the dark (A) or in the light (B). The media were supplemented with the indicated concentrations of NAA and 5% sugar, and the pH was adjusted to 5.5. □, Callus dry wt; ■, 1; ▨, 2. The same symbols are used in Fig. 6.

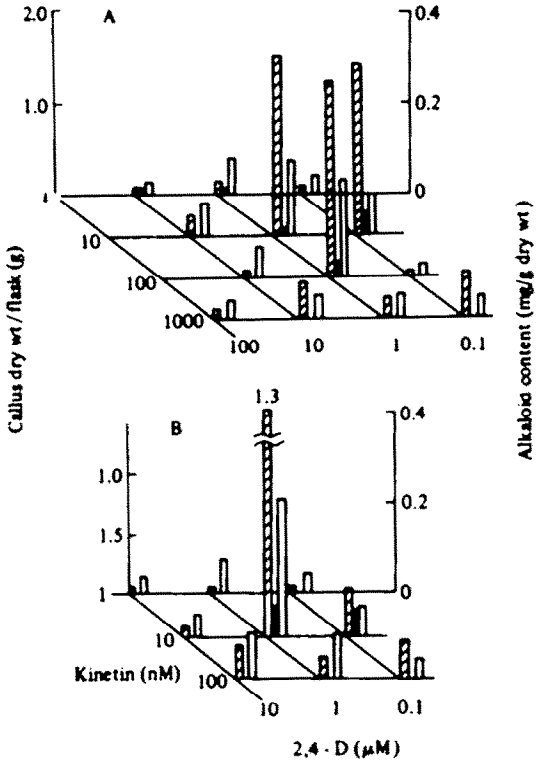


Fig. 6. Effects of 2,4-D on growth and alkaloid contents of callus of *S. suffruticosa*.

tissues were harvested at the end of 6 weeks' growth, except where stated otherwise.

Isolation of 1 *and* 2 *from callus.* 15-Week-old calli (365 g as fr. wt) were homogenized (Polytron PT 10-35, Kinematica) in a soln of 2% NH_4OH (1 l.) and $ClCH_2CH_2Cl$ (2 l.) for 30 sec in an ice bath. The homogenate was centrifuged for 10 min at 1500 rpm and the organic layer washed (× 3) with H_2O and extracted with 1 M H_2SO_4 (200 ml). The aq. layer was made alkaline with 10% NH_4OH and stirred with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried (Na_2SO_4) and the solvent removed. The viscous residue (26.4 mg) was dissolved in a small amount of Et_2O, subjected to CC on silica gel (1.2 × 40 cm) and eluted with C_6H_6–Et_2O (2:1). The fractions having the same R_f value as 1 on TLC were collected and evaporated to dryness. The residue (3.2 mg) was purified as its *d*-camphor sulphonate, according to the method of ref. [7], to afford 1.8 mg 1 as its free base (yellow needles, mp 139°). Mmp 141° (lit. [8] mp 142–143°).

Elution of the column with C_6H_6–Me_2CO (1:9) gave crude yellow needles which were purified via their oxalate, as described in ref. [7], to give 15.6 mg 2 as its free base (yellow needles). Mp 126° (lit. [9] 128°).

Determination of alkaloids. Calli were freeze-dried and weighed. Fresh calli from each treatment were treated as follows. A mixture of the calli (0.5–5 g), 10% NH_3 (1 ml), H_2O (14 ml), and $CHCl_3$ (15 ml) was homogenized (Polytron) in an ice bath and centrifuged. After collection of the $CHCl_3$ layer, the residue was extracted (× 2) with $CHCl_3$ (15 ml) as above. The combined $CHCl_3$ layer was washed with H_2O, dried (Na_2SO_4) and evaporated to dryness. The residue was dissolved in a suitable vol. of $CHCl_3$ and subjected to TLC on 0.2 mm silica gel 60 F 254 (precoated on Al sheet, Merck, Art. 5554) developed with Me_2CO–C_6H_6 (10:1) at 20°. The air-dried plates were analysed by means of a TLC scanner (CS-920, Shimadzu, UV mode, 254 nm). The R_f values of 1 and 2 were 0.56 and 0.22, respectively. There were other spots (R_fs 0.64, 0.76 and 0.79) on the plate. For quantitative analysis, a suitable amount of the alkaloid on the plate was 0.05–1 μg. All of the results are the average of triplicate expts.

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