

## Tannin Production in Hairy Root Cultures of *Sanguisorba officinalis* L.

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(Received April 22, 1991)

(Accepted May 30, 1991)

The hairy roots of *Sanguisorba officinalis* L. were induced by *Agrobacterium rhizogenes* A4 strain and analysed for the production of several tannins. Among the six clones of the hairy roots cultured in hormone free 1/2 Murashige-Skoog liquid medium, five clones produced mainly sanguin H-6 (0.217-0.569% fw) whereas the other one clone produced an especially high level of 1, 2, 3, 6-tetra-O-galloyl- $\beta$ -D-glucose (0.322% fw) and sanguin H-11 (0.221% fw).

### Introduction

The roots of *Sanguisorba officinalis* L. (Rosaceae), rich sources of phenolic compounds such as tannins<sup>1-4)</sup>, have been one of the important Chinese medicines ('ziyu' in Japanese) used as a hemostatic, antiphlogistic and astringent. Recently we have reported the production of eleven phenolic compounds in the adventitious root cultures of this plant<sup>5)</sup>. In the course of our chemical investigation on the tissue culture products of several medicinal plants<sup>6-12)</sup>, we succeeded in establishing the hairy root culture of this plant and production of five phenolic compounds, gallic acid (1), 1, 2, 3, 6-tetra-O-galloyl- $\beta$ -D-glucose (2)<sup>13)</sup>, 1, 2, 3, 4, 6-penta-O-galloyl- $\beta$ -D-glucose (3)<sup>14)</sup>, sanguins H-6 (4)<sup>4)</sup> and H-11 (5)<sup>4)</sup> in this culture.

### Material and Methods

*Plant Material and Induction of the Hairy Roots.* The shoot culture of *S. officinalis* was established and maintained as previously reported<sup>5)</sup>. The leaf segments (5 mm  $\times$  5 mm) cut from the axenic shoots were used for the explants for transformation. For the induction of the hairy roots we used *A. rhizogenes* A4 strain. *A. rhizogenes* A4 subcultured on YEB agar medium<sup>15)</sup> was transferred to YEB liquid medium (30 ml per 100 ml flask) and precultured for one day in the dark at 25°C on a rotary shaker (100 rpm). The solution of this *A. rhizogenes* A4 (200  $\mu$ l) and the leaf segments of *S. officinalis* were inoculated to 1/2 Murashige-Skoog (1/2 MS)<sup>16)</sup> liquid medium (30 ml per 100 ml flask) and co-cultured for 2 days in the same conditions mentioned above. The infected

leaf segments, after being rinsed with sterile water, were transferred to 1/2 MS agar medium (solidified with 0.2% gelrite containing 0.5 g/l Claforan) and incubated at 25°C in the dark. After 3 to 4 weeks, twelve hairy roots appeared on the segments. The tips of the hairy roots were cut off and cultured on the same medium to eliminate the bacteria. The axenic hairy roots thus obtained were maintained in hormone free 1/2 MS liquid media (50 ml per 100 ml flask) on a rotary shaker. Three clones (So-1~3) which showed sufficient growth were selected and used for this experiment. We also tried to induce the hairy roots by the infection with *A. rhizogenes* A4 strain precultured in YEB medium containing 100 µM acetosyringone. Procedures for the infection with the bacteria and the maintenance of the hairy roots were the same as mentioned above. In this case ten hairy roots were obtained, three of which (So-4~6) were selected for the experiment. Opines (agropine and mannopine) of the hairy roots were extracted and detected using high voltage paper electrophoresis<sup>17)</sup>(data not shown).

*Culture method of So-1~6.* So-1~6 (ca 50 mg, fw) were inoculated into hormone free 1/2 MS liquid medium (50 ml in 100 ml flask) and cultured at 25°C on a rotary shaker at 100 rpm in the dark. The hairy roots were harvested after 4 weeks culture (fw and dw are shown in Table 1 and 2).

*Analysis of the production of 1~5 in So-1~6.* The methods of preparation of the samples and the conditions for hplc were done the same as described in our previous paper<sup>5)</sup>. The standard compounds 1~5 used here were prepared from the mother plant<sup>5)</sup>. The contents of 1~5 in So-1~6 are shown in Table 2.

Culture media used throughout were 1/2 MS medium consisting of one half of the macro salt formulation of MS medium, containing 2% sucrose for co-culture with *A. rhizogenes* and 3% sucrose for the subculture of hairy roots. The media were adjusted to pH 5.7 before autoclaving at 121°C for 15 min. The data for this experiment are shown as the mean of three replicates.

## Results and Discussion

The induction of the hairy roots from the plant of *S. officinalis* using *Agrobacterium rhizogenes* A4 strain was fairly difficult due to the strong antibiosis of its tannin constituents. The direct infection method with *A. rhizogenes* A4 to the explants of this plant did not succeed because the explants, exposed to the air, easily began to show brownish coloration at the cut ends and infected sites and then died. Therefore, we selected the co-culture method for the induction of the hairy root. In 1985, Stachel *et al* reported that acetosyringone was the signal molecule which activated the virulence gene expression of *Agrobacterium*<sup>18)</sup>. Thereupon for this infection we also used *A. rhizogenes* strain precultured in the medium containing acetosyringone. But in this experiment, acetosyringone did not show a remarkable effect on the induction of hairy roots. Among the twenty two hairy roots obtained in this experiment, six clones (So-1~6) were selected for their sufficient growth. The growth of So-1~6 cultured in hormone free 1/2 MS liquid medium for 4 weeks were fairly superior to those of the adventitious (not transformed) roots cultured in MS, 1/2 MS, Gamborg B5 (B5)<sup>19)</sup>, Woody Plant (WP)<sup>20)</sup> and Root Culture (RC)<sup>21)</sup> liquid media containing 1 mg/l IAA (Table 1)<sup>5)</sup>. Especially, So-3 showed prominent growth which was almost three to five times larger than those of the other hairy roots.

In five clones (So-1, 2 and 4~6) the major constituent was 4 (0.217-0.569% fw) (Table 2) whose levels were higher than that of the mother plant (0.206% fw)<sup>4)</sup>. So-5, in spite of its poor growth, showed the highest level of 4. On the other hand, So-3 which showed the fastest growth indicated a high production of 5 (0.221%, fw) and 2 (0.322%, fw). Tanaka *et al* reported that in the mother

**Table 1.** Growth of the hairy roots (So-1~6) and the adventitious roots (So-N) cultured in different media for 4 weeks

material	medium	dw <sup>a)</sup>
So-1	1/2 MS	130.7
-2	1/2 MS	101.8
-3	1/2 MS	624.6
-4	1/2 MS	220.1
-5	1/2 MS	83.3
-6	1/2 MS	123.7
-N <sup>b)</sup>	MS <sup>c)</sup>	99.4
-N <sup>b)</sup>	1/2 MS <sup>c)</sup>	66.9
-N <sup>b)</sup>	B5 <sup>c)</sup>	70.9
-N <sup>b)</sup>	WP <sup>c)</sup>	82.0
-N <sup>b)</sup>	RC <sup>c)</sup>	18.2

a) dw (mg) of roots per flask

b) adventitious (not transformed) root

c) supplemented with 1 mg l<sup>-1</sup> IAA

**Table 2.** Production of 1~5 in So-1~6 cultured in 1/2 MS liquid medium for 4 weeks

material	fw <sup>a)</sup>	% as fw; (%) as dw <sup>b)</sup>				
		1	2	3	4	5
So-1	2.21	0.003 (0.05)	0.008 (0.14)	0.001 (0.01)	0.228 (3.84)	0.085 (1.44)
-2	1.71	0.002 (0.04)	0.003 (0.05)	0.001 (0.01)	0.264 (4.43)	0.048 (0.80)
-3	8.92	0.113 (1.62)	0.322 (4.60)	0.040 (0.57)	0.089 (1.28)	0.221 (3.16)
-4	3.95	0.005 (0.08)	0.011 (0.19)	0.001 (0.02)	0.217 (3.89)	0.124 (2.21)
-5	0.81	0.005 (0.05)	0.011 (0.11)	0.012 (0.12)	0.569 (5.53)	0.074 (0.72)
-6	1.45	0.007 (0.08)	0.021 (0.25)	0.007 (0.08)	0.264 (3.09)	0.166 (1.94)

a) fw(g) of roots per flask.

b) dw is shown in Table 1.

plant the content of **5** was not so high (0.119%, fw) and **2** was not produced<sup>4)</sup>. Therefore So-3 might have obtained the capability to produce specifically the secondary metabolites which had not biosynthesized as much in the mother plant. The hairy root cultures of *S. officinalis* seem to be useful for biosynthetic study as well as the production of high mol wt hydrolysable tannins such as **4** (mol wt, 1870) and **5** (mol wt, 3738). The tannin content in these hairy roots were almost the same as those of the adventitious roots<sup>5)</sup>. Taking into account the rapid growth, the hairy root cultures of this plant are more valuable than its normal root cultures for the production of these tannins.

*Acknowledgements*..... We are grateful to Prof. I. Nishioka (Faculty of Pharmaceutical Sciences, Kyushu University) and Prof. S. Natori (Meiji College of Pharmacy) for their generous support and encouragement of this work. This work was supported by Special Cooperation Funds for Promoting Science and Technology (Basic Research Core System) from Science and Technology Agency, Japan.

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## 《和文要約》

ワレモコウ (*Sanguisorba officinalis* L.) の毛状根培養によるタンニン類生産

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*Agrobacterium rhizogenes* A4 菌との共存培養法により, ワレモコウ (*Sanguisorba officinalis* L.) の毛状根を誘導し, その液体培養系におけるタンニン類の生産について検討した. ホルモン無添加 1/2 MS 液体培地で培養した毛状根 6 クローンのうち 5 クローンにおいては sanguin H-6 (0.217-0.569% fw) が主タンニンであり, 残りの 1 クローンにおいては, 1, 2, 3, 6-tetra-O-galloyl- $\beta$ -D-glucose (0.322% fw) と sanguin H-11 (0.221% fw) が高含量で生産されていた.