*Original Papers* Plant Tissue Culture Letters, **9**(3), 196~201(1992)

# Tannin Production in *Liquidambar styraciflua* Callus Cultures

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(Received August 3, 1992)
(Accepted September 2, 1992)

The effects of $NH_4NO_3$, casein hydrolysate and some amino acids (glutamine, glycine and serine) on the growth and tannin production of *Liquidambar styraciflua* callus cultures were determined. The removal of $NH_4NO_3$ from the culture medium strongly increased the yield of tannins (both hydrolyzable and condensed ones) in the calli. The addition of casein hydrolysate also enhanced the growth and tannin production of the calli especially in the light whereas the treatment with amino acids was not as effective in either the light or dark conditions.

## Introduction

*Liquidambar styraciflua* is a plant in Hamamelidaceae family which is rich in phenolic constituents[1-3]. Recently we reported the production of several tannins (both hydrolyzable and

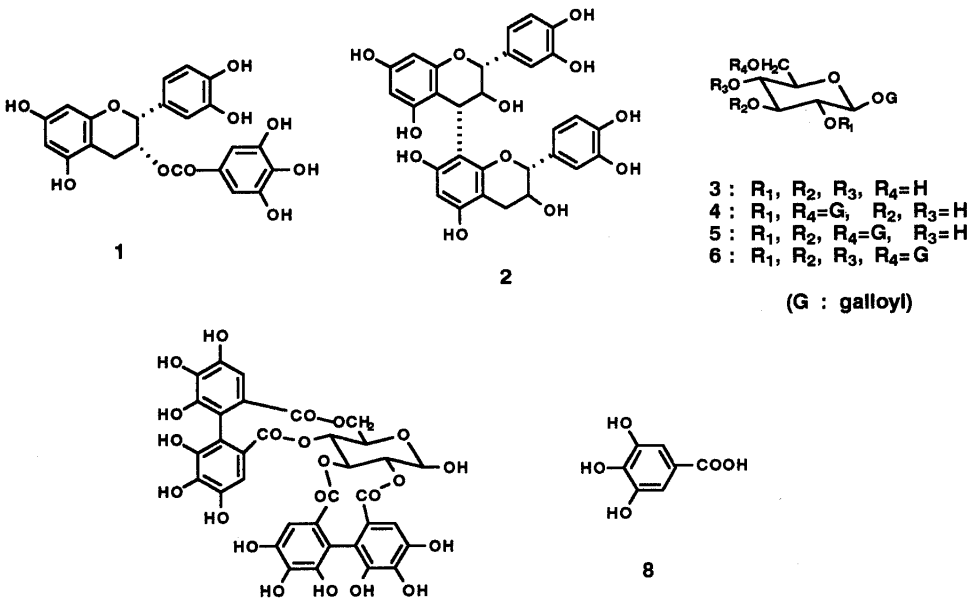


**Fig. 1** Structures of phenolics.

condensed ones) and related compounds in the callus and cell suspension cultures and in the intact plant (leaf) of *L. styraciflua*[4]. In this research, the effects of $NH_4NO_3$, casein hydrolysate and some amino acids (glutamine, glycine and serine) in the culture media on the production of several phenolics,: (−)-epicatechin 3-*O*-gallate (**1**)[5]; procyanidin B-3 (**2**)[6]; β-glucogallin (1-*O*-galloyl-β-D-glucose) (**3**)[7]; 1, 2, 6-tri-*O*-galloyl-β-D-glucose (**4**)[8]; 1, 2, 3, 6-tetra-*O*-galloyl-β-D-glucose (**5**)[8]; 1, 2, 3, 4, 6-penta-*O*-galloyl-β-D-glucose (**6**)[8]; pedunculagin (**7**)[9]; and gallic acid (**8**), in *L. styraciflua* calli were determined. This experiment was performed under light (16 hr photoperiod per a day) and dark conditions.

## Materials and Methods

***Callus induction*** : Calli of *L. styraciflua* were induced from leaf segments of the parent plant[4] and maintained on Murashige-Skoog (MS)[10] solid medium supplemented with 0.5 mg/*l* naphthaleneacetic acid (NAA) and 0.1 mg/*l* kinetin in the dark. For this experiment, calli subcultured for over one year were used.

***Callus cultures*** : A piece of callus (*ca.* 0.09 g, fw) was inoculated separately on MS solid media supplemented with nine combinations of NAA-kinetin (media A-I) (**Table 1**) and cultured for 5 weeks. The growth (g per test tube, fw) of the calli cultured on these media is shown in **Table 1**. The

**Table 1.** Growth (g, fw) of the calli cultured on MS solid media (A-I) for 5 weeks.

| medium | NAA (mg/*l*) | kinetin (mg/*l*) | control light | control dark | $-NH_4NO_3$ light | $-NH_4NO_3$ dark | +CH* light | +CH* dark | +AA** light | +AA** dark |
|---|---|---|---|---|---|---|---|---|---|---|
| A | 0.1 | 0.1 | 0.28 | 0.24 | 0.44 | 0.33 | 0.24 | 0.17 | 0.19 | 0.20 |
| B | 0.5 | 0.1 | 0.35 | 0.30 | 0.89 | 0.81 | 0.33 | 0.30 | 0.19 | 0.16 |
| C | 1.0 | 0.1 | 0.35 | 0.38 | 1.43 | 0.91 | 0.59 | 0.32 | 0.25 | 0.26 |
| D | 0.1 | 0.5 | 0.39 | 0.26 | 0.36 | 0.38 | 0.22 | 0.20 | 0.18 | 0.21 |
| E | 0.5 | 0.5 | 0.42 | 0.25 | 0.84 | 0.84 | 0.35 | 0.16 | 0.26 | 0.36 |
| F | 1.0 | 0.5 | 0.46 | 0.28 | 1.02 | 0.68 | 0.67 | 0.31 | 0.17 | 0.20 |
| G | 0.1 | 1.0 | 0.27 | 0.22 | 0.46 | 0.41 | 0.30 | 0.37 | 0.14 | 0.18 |
| H | 0.5 | 1.0 | 0.30 | 0.28 | 0.75 | 0.84 | 0.47 | 0.27 | 0.25 | 0.18 |
| I | 1.0 | 1.0 | 0.54 | 0.28 | 0.84 | 0.57 | 0.26 | 0.20 | 0.44 | 0.50 |

* casein hydrolysate, ** amino acids

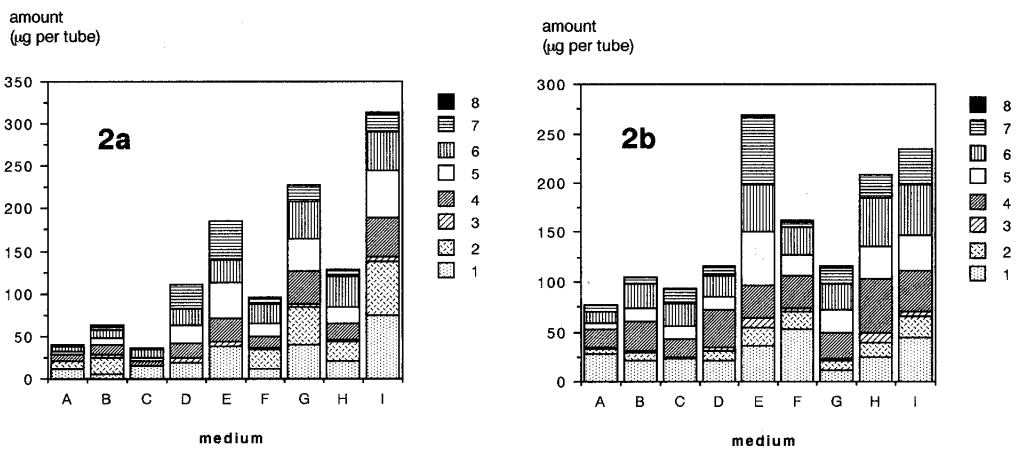


**Fig. 2** Tannin production in the calli cultured on MS solid media with NAA and kinetin for 5 weeks. 2a: in the light, 2b: in the dark

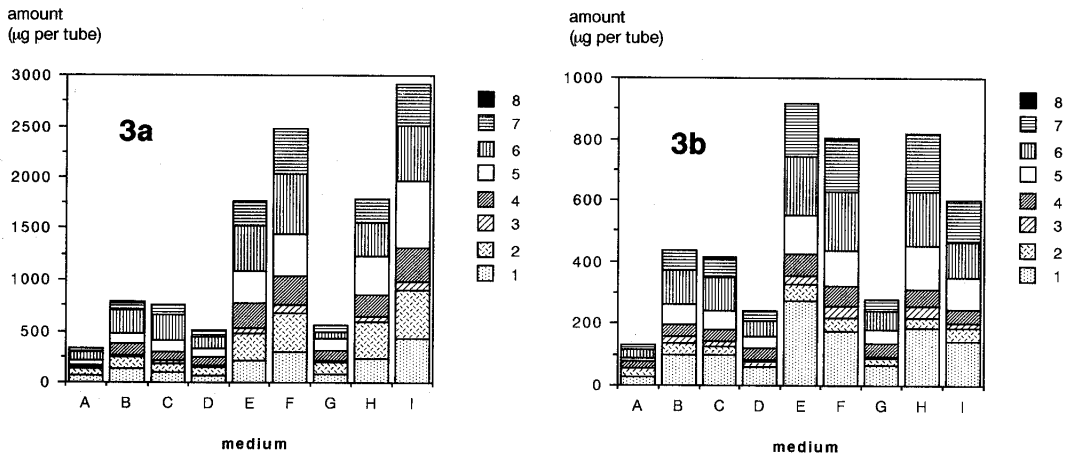


**Fig. 3** Tannin production in the calli cultured on MS solid media (minus $NH_4NO_3$) with NAA and kinetin for 5 weeks.
3a: in the light, 3b: in the dark

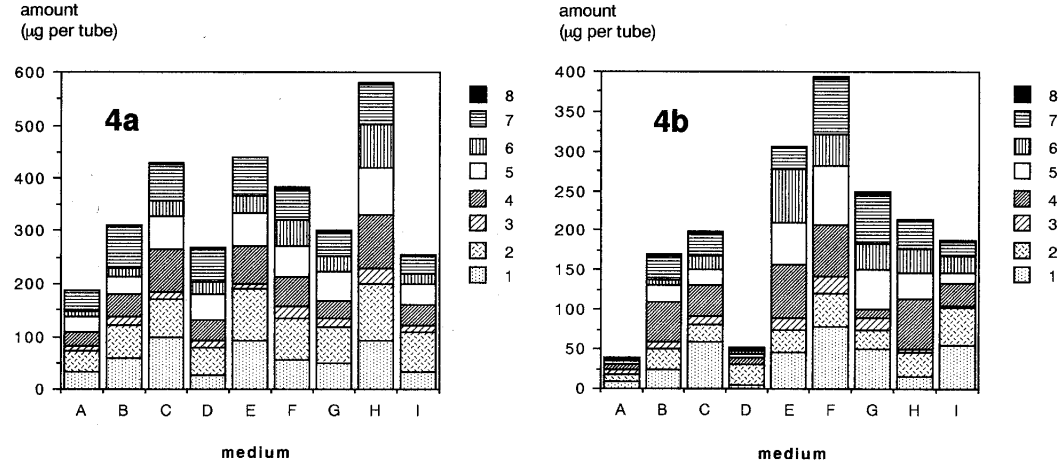


**Fig. 4** Tannin production in the calli cultured on MS solid media (plus casein hydrolysate) with NAA and kinetin for 5 weeks.
4a: in the light, 4b: in the dark

production of tannins in the calli was determined by HPLC (**Fig. 2**).

***Effects of the removal of $NH_4NO_3$*** : A piece of fresh callus (0.09 g) was inoculated separately on MS solid media (minus $NH_4NO_3$) containing various combinations of NAA-kinetin (media A-I) and cultured for 5 weeks. The growth (**Table 1**) and tannin production (**Fig. 3**) of the calli were determined.

***Effects of casein hydrolysate*** : A piece of fresh callus (0.09 g) was inoculated separately on MS solid media containing 100 mg/*l* casein hydrolysate with various combinations of NAA-kinetin (media A-I) and cultured for 5 weeks. The growth (**Table 1**) and tannin production (**Fig. 4**) of the calli were determined.

***Effects of some amino acids*** : A piece of fresh callus (0.09 g) was inoculated separately on MS solid media containing 1 g/*l* glutamine, 50 mg/*l* glycine and 50 mg/*l* serine with various combinations of NAA-kinetin (media A-I) and cultured for 5 weeks. The growth (**Table 1**) and tannin

production of the calli were determined.

***HPLC analysis*** : Lyophilized calli (*ca.* 20-60 mg) were mashed and extracted with 80% aq. acetone (2 m*l*) for 15 hr at room temperature. Each extract, after filtration with millipore filter (0.45 $\mu$m) was injected (2-4 $\mu l$) to HPLC. HPLC conditions were the same as described in our previous paper[4].

MS media used throughout contained 30 g/*l* sucrose. The volume of MS solid media used for all cultures was 10 m*l* for a piece of callus in a test tube (2.2 cm in the diameter). All media were adjusted to pH 5.7 before autoclaving at 121°C for 15 min. All cultures were placed in the light (3000 lux, 16 hr photoperiod per a day) or dark condition at 25°C. All data were shown as the mean of three replicates.

## Results and Discussion

The growth rates of the calli observed severally on media A-I were almost the same (0.22-0.46 g, fw per tube) except on one, medium I in the light condition (0.54 g, fw per tube) (**Table 1**). On medium I, the amount of the calli cultured in the light was 1.9 times larger than that in the dark. Except on medium C, the light condition seemed to be suitable for the growth of the calli.

The production of phenolic compounds (**1**-**8**) in the calli cultured on these media (A-I) under light or dark condition is shown in **Fig. 2**. In the light (**Fig. 2-a**) the calli produced significant amounts of phenolics on the media with a high concentration (over 0.5 mg/*l*) of kinetin (media D-I). Particularly, on three media, E, G and I, the total amount of **1**-**8** was observed over 160 $\mu$g per tube. In these three media hydrolyzable tannins such as gallotannin (**4**-**6**) and ellagitannin (**7**) were observed at high levels. The total amount of phenolics (**1**-**8**) showed the highest level (*ca.*320 $\mu$g per tube) on medium I which was supplemented with a high concentration (1 mg/*l*) of NAA and kinetin. On this medium the calli also produced a high amount of condensed tannin **2** (63.2 $\mu$g per tube) and flavan 3-ol **1** (74.8 $\mu$g per tube) which is one of the structural elements of condensed tannins. In the dark condition, the calli produced high amounts (over 210 $\mu$g per tube) of phenolics on media E, H and I (**Fig. 2-b**). Likewise in those observed in the light condition, galloylglucoses (**4**-**6**) appeared in a fairly good amount. The highest (*ca.* 270 $\mu$g per tube) level of phenolics was observed on medium E. The amount of **7** (69.2 $\mu$g per tube) observed on medium E was the largest quantity of any phenolic on any medium in the dark condition.

Nitrogen sources in culture medium are important factors for the production of tannins in plant tissue cultures[11,12]. In this experiment the effects of $NH_4NO_3$ on the growth and tannin production of *L. styraciflua* calli were determined. The growth of the calli cultured on media A-I without $NH_4NO_3$ is shown in **Table 1**. Both in the light and dark conditions the calli grew more satisfactorily in comparison with those cultured on the same media with $NH_4NO_3$. Especially, in the light condition, the callus growth was enhanced in proportion to the concentration of NAA. On medium C the calli showed the highest amount (1.43 g, fw per tube in the light). This level was almost 1.6 times that obtained on the same medium in the dark. Removal of $NH_4NO_3$ seemed to have a relatively positive effective on the growth of the calli when they were cultured with large amounts of NAA in the light.

The tannin production in the calli cultured on media A-I (minus $NH_4NO_3$) was also observed in high level (**Fig. 3**). Particularly, on four media E, F, H and I, the calli produced plenty of tannins (both hydrolyzable and condensed types) whose total amount (**1**-**8**) was almost 10 times that observed on the same media containing $NH_4NO_3$ (**Fig. 2**). On these media (E, F, H and I), the calli showed significant amounts of **2** (in the light), **1** and **7** (both in the light and dark). Particularly, on

medium I in the light condition, the calli produced the maximum level of phenolics (*ca.* 2900 μg per tube of total amount of **1**-**8**). With the results above, the removal of $NH_4NO_3$ was identified as being fairly effective in enhancing the production of tannins in *L. styraciflua* calli.

Casein hydrolysate, widely used in the medium of plant tissue cultures, has been reported to have some effect on the growth[13,14] and secondary metabolism[12] of the cultures. When *L. styraciflua* calli were cultured on MS media containing 100 mg/*l* casein hydrolysate with various combinations of NAA and kinetin (media A-I), the growth rate increased especially in the light condition (**Table 1**). On three media C, E and F, the callus growth in the light was observed as over twice the level of that in the dark. Addition of casein hydrolysate to the medium seemed to effect the growth of *L. styraciflua* calli only when they were cultured under light. This result was noteworthy when we considered the similar observation made in the callus cultures of *Sapium sebiferum*[12].

The calli cultured on media A-I containing casein hydrolysate also produced similar phenolics (**1**-**8**) which appeared on the same media without the additive (**Fig. 4**). In this culture, the light condition seemed to be suitable for both the production of phenolics and for callus growth. On the media with low content (0.1 mg/*l*) of kinetin (media A-C), the addition of casein hydrolysate seemed to be comparatively effective for tannin production in the light (**Fig. 4-a**).

Some amino acids (glutamine, glycine, serine *etc.*) are known to have several effects on growth[15-17] and secondary metabolism[15,18] in plant tissue cultures. The effects of three amino acids (1 g/*l* glutamine, 50 mg/*l* glycine and 50 mg/*l* serine) on *Quercus* plants (species of rich in tannins) cultured cells have been studied[19]. Therefore, we determined the effects of these three amino acids on the growth and tannin production of *L. styraciflua* callus. (With the exception of two conditions media E and I in the dark). When the calli were cultured on media A-I with these three amino acids, their growth level (**Table 1**) was similar to those observed on the same media without these additives. For the growth of the calli, the addition of these amino acids seemed to have little effect.

In this culture, in the light condition, the calli produced a significant level of tannins (over 350 μg/tube of total amount of **1**-**8**) only on two media, H and I, which contained high contents of NAA (over 0.5 mg/*l*) and kinetin (1 mg/*l*). Especially, the amount of **1** was relatively high (95.4 μg per tube on medium H and 111.7 μg per tube on medium I). For the other seven media (A-G), the addition of these amino acids seemed to have little effect on tannin production (below 150 μg/tube of total amount of **1**-**8**). In the dark condition, the calli showed good production of tannins only on one medium, I (over 250 μg/tube of total amount of **1**-**8**), and on the other media the production was fairy low (below 150 μg/tube of total amount of **1**-**8**). Therefore, the addition of these amino acids was identified as being not suitable for tannin production in *L. styraciflua* calli.

With the comparisons of the culture conditions above, callus cultures of this plant were determined to be usable for the production of tannins (both hydrolyzable and condensed), especially by removal of $NH_4NO_3$ from the culture medium (MS medium tested in this research) under light condition.

## References


1) Mayer, W., N. Kunz, F. Loebich, 1965. Liebigs Ann. Chem., **688**: 232-238.
2) Hatano, T., R. Kira, M. Yoshizaki, T. Okuda, 1986. Phytochemistry, **25**: 2787-2789.
3) Okuda, T., T. Hatano, T. Kaneda, M. Yoshizaki, T. Shingu, 1987. Phytochemistry, **26**: 2053-2055.
4) Neera, S., H. Arakawa, K. Ishimaru, Phytochemistry (in press).
5) Nonaka, G., O. Kawahara, I. Nishioka, 1983. Chem. Pharm. Bull.,**31**: 3906-3914.
6) Thompson, R. S., D. Jacques, E. Haslam, R. J. N. Tanner, 1972. J. Chem. Soc. Perkin Trans. I, 1387-1399.

7) Kashiwada, Y., G. Nonaka, I. Nishioka, 1984. Chem. Pharm. Bull., **32**: 3461-3470.
8) Elizabeth, A. H., Rai, K. Gupta, M. K. AI-Shafi Sabah, E. Haslam, D. Magnolato, 1982. J. Chem. Soc. Perkin Trans. I, 2515-2524.
9) Tanaka, T., G. Nonaka, I. Nishioka, 1985. J. Chem. Research (M), 2001-2029.
10) Murashige, T., F. Skoog, 1962. Physiol. Plant., **15**: 473-493.
11) Ishimaru, K., K. Shimomura, 1991. Phytochemistry, **30**: 825-828.
12) Neera, S., H. Arakawa, K. Ishimaru, 1992. Phytochemistry (in press).
13) Rai, V. R. S., K. S. J. Chandra, 1987. Plant Cell Tissue Organ Culture, **9**: 81-88.
14) Mridula, M. K., P. K.Gupta, A. F. Mascarenhas, 1983. Plant Cell Tissue Organ Culture, **2**: 133-139.
15) Jeorge, E.E., D. J. M.Pattodk, H. J.Jeorge, 1988. In "Plant Culture Media, Commentary and Analysis" Vol. 2, p. 338-369, Exegetics Limitted, England.
16) Kamada, H., H. Harada, 1979. J. Exp. Bot., **30**: 27-36.
17) Dodds, J. H., L. W. Roberts, 1985. In "Experiments in Plant Tissue Culture" p. 35-53, Cambridge University Press, England.
18) Luckner, M., L. Nover, H. Böhm, 1977. In "Molecular Biology Biochemistry and Biophysics 23" p. 104-119, Springer-Verlag, Berlin, Heidelberg, New York.
19) Romano, A., L. Gomes, M. A. Martins-Loucao (unpublished data).

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## 《和文要約》

### アメリカフウ(*Liquidambar styraciflua*)のカルス培養におけるタンニン類生産

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アメリカフウカルスの生育およびタンニン類生産に及ぼす $NH_4NO_3$，カゼイン分解物ならびにアミノ酸(グルタミン，グリシンおよびセリン)の効果について検討した．NAA および Kinetin 添加 MS 培地での培養において，$NH_4NO_3$ の除去はカルス中のタンニン生産量を大きく増加させた．カゼイン分解物の添加は光照射下においてカルスの生育とタンニン生産に効果的であったが，アミノ酸の添加は照明下また暗黒下においてともに大きな効果は示さなかった．