Hanguk Nonghwahak Hoechi
(J. Korean Agric. Chem. Soc.)
35(2), 99~105(1992)

Production of secondary metabolites by tissue culture of *Artemisia annua* L.

Nam-Cheol Kim, Jeong-Gu Kim, Hyung-Joon Lim*, Tae-Ryong Hahn* and Soo-Un Kim

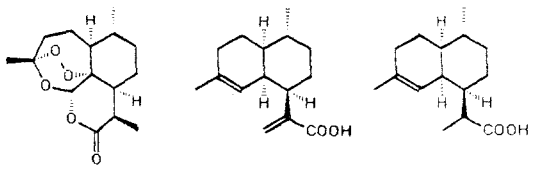
Department of Agricultural Chemistry and Reseach Center for New Bio-Materials in Agriculture, Seoul National University, Suwon 441-744, Korea
*Department of Genetic Engineering, Kyung Hee University, Yong-In 449-900, Korea

Abstract : *Artemisia annua* contains the antimalarial principle, artemisinin. The possibility of the production of this compound through tissue culture technique was studied. The optimum combinations of hormones for the induction of callus were *p*-chlorophenoxyacetic acid(pcPA) and 6-benzylaminopurine(BAP) or pcPA and *N*-isopentenylaminopurine(2*i*P) in 0.05 mg/*l* each. For the growth of callus, the same combination of pcPA and BAP was optimum in concentrations of 1.0 μM and 0.5 μM, respectively, and the optimal concentration of sucrose was also found to be 2%(w/v). Tissue culture from the crown gall grew faster than normal callus. In the suspension culture broth and the cells of normal callus or *Agrobacterium*-transformed tumors, arteannuic acid and 11,12-dihydroarteannuic acid were found together with common phytosterols, whereas artemisinin was not found(Received February 20, 1992, accepted March 31, 1922).

Although synthetic organic chemistry advanced considerably in the recent years, plants still serve as the prinicipal source of medicine, flavor, fragrance, and pigments.[1] The global search for useful secondary products of plants for such purposes is ongoing.[2] Artemisinin or qinghaosu(QHS, **1**), a sesquiterpene lactone containing a peroxide bridge from *Artemisia annua* L., is an exemplary result of the efforts.

The herb *A. annua* L., a member of the Compositae family, has been described in Chinese medical classics since 168 B.C.[3] In 1972, an antimalarial prinicple, subsequently named as artemisinin{**1**}, was isolated from the plant,[4] and its structure was determined in 1979.[5-7] The compound has been since used by the Chinese to treat patients infected with *Plasmodium vivax* and *P. falciparum*, resistant to quinine and its analogs. In spite of the several syntheses of this compound reported so far,[8,9] its complex structure makes economical synthesis impractical, thus the production of the sesquiterpenes by tissue culture technique has become a viable possibility.

Studies on the production of artemisinin{**1**} through plant cell and tissue culture have been recently conducted by a few groups.[10,11] Nair *et al.* found the concentration of artemisinin{**1**} in the suspension culture of *A. annua* to be as low as 8 μg/m*l*. However, the compound was not detected in calli



1 2 3

Key words : *Artemisia annua* L., tissue culture, secondary metabolites, arteannuic acid, 11,12-dihydroarteannuic acid artemisinin, *Agrobacterium tumefaciens, Agrobacterium rhizogens*
Corresponding author : S. U. Kim

or regenerated plantlet except in rooted ones. While these experiments did not verify the above method as being cost-effective, they did show the potential of the culture method for the production of the compound. In this report, the production of the secondary metabolites using cell and tissue cultures of *A. annua* was studied.

Materials and Methods

Chemicals

Chemicals for the cell and tissue cultures were the "plant cell culture tested grade" from Sigma. The solvents were GR grade of redistilled EP grade. Other chemicals were GR grade.

Induction of callus

A. annua was maintained in the garden of the College of Agriculture and Life Sciences, Seoul National University, Suwon. The leaves of the plant were severed and washed under tap water with a house detergent(Ponpon, Lucky Co.). They were sterilized for 15 min with 0.8% NaOCl solution containing 1% Triton X-100, and then washed with sterilized water. Explants were trimmed to 5 mm×5 mm segments and cultured on the Murashige and Skoog(MS) medium without glycine containing 2% of agar. Cultures were maintained at 27 ℃ at light regiments ranging from 1,500 to 2,000 Lux at a 16~8 hours light-dark cycle. Calli were subcultured every 4~5 weeks.

Concentration of each auxin was fixed at 0.05 mg/*l* and that of cytokinins were 0.05 and 0.5 mg/*l* for the initial callus induction. Media other than MS employed for the growth of the calli were Linsmaier-Skoog(LS)[12] and B5.[13]

Infection with *Agrobacterium*

Agrobacterium rhizogens(ATCC 15834) and *A. tumefaciens*(ATCC 15955) were cultured on Luria-Bertani(LB) medium[14] containing 2% of agar for about 2 days. Suspension of the *A. rhizogens* and *A. tumefaciens* was cultured on LB medium without agar until the absorbance reached at 0.5~1.0. *Agrobacterium* suspension was inoculated with a needle to the stem of the one-month-old plant grown aseptically and further grown for about 3 months to obtain tumor or hairy root.[15,16] The tumor-like crown gall or hairy root was removed from the plant and subcultured in the dark on MS medium stripped of phytohormones and with carbenicillin at the concentration of 500 μg/m*l*.

Suspension culture of *A. annua* Cell

Callus clumps, 30~40 days old, subcultured for several generations in the dark on a MS medium supplemented with 0.1% casein hydrolyzate, were used to initiate the suspension cultures. The culture were incubated in 500 m*l* Erlenmeyer flasks containing 125 m*l* of the liquid MS medium on a rotary shaker operating at 100 rpm and were subcultured every 2 to 3 weeks until harvest.

Extraction of compounds of from culture product

Suspension culture broth was filtered, and the filtrate was mixed with equal volume of 10% Na_2CO_3 solution. The filtrate was then extracted with petroleum ether or n-hexane. The organic phase was dried with $MgSO_4$ and concentrated *in vacuo*(Fraction I). The concentrate was then converted into Q260 as described by Zhao and Zeng[17] and subjected to the artemisinin{**1**} analysis. The remaining basic culture broth was acidified to pH 2~3 with 10% HCl and again extracted with petroleum ether or n-hexane. The organic acidic extract(Fraction II) was purified on TLC plate to obtain a crude acidic fraction with authentic arteannuic acid as a standard.

The cells from the suspension culture, callus, and tumor were homogenized with MeCN in a mortar and sonicated for 30 min. The mixture was shaken at 100 rpm overnight. The homogenate was filtered and evaporated. The residue was dissolved with alcohol, and the aliquot was used for the artemisinin{**1**} analysis. The remaining extraction procedure used to obtain the fractions was the same as that for the broth filtrate extraction procedure.

Instruments and Chromatography

GC/MS conditions were as follows; Hewlett-Packard 5980 II GC equipped with 25 m×0.33 μm×0.2 mm HP-5 capillary column, columm temperature programmed from 170 to 280 ℃(temperature program, 8 ℃/min; isothermal at 280 ℃), helium as the carrier gas at a flow rate of 1 ml/min, Hewlett-Packard 5988 EI-MS with an ionizing energy of 70 eV. For mass spectrometry, VG 70-SEQ operating at 70 eV was employed. The identification was established through computer-aided mass spectral library search.

HPLC conditions for artemisinin{**1**} analysis were as follows : mobile phase, 0.01 M phosphate buffer (pH 7.9), water : MeOH(55 : 45, v/v); flow rate, 0.5 ml/min; wavelength, 260 nm; detector sensitivity, 0.08 V; temperature, ambient temperature; quantitative data processing. The HPLC system consisted of a PU4010 pump, a PU4020 detector, and a 4 mm i.d.×25 cm stainless steel column packed with Li-Chrosorb C-18(5 μm) from E. Merck(Germany).

For thin layer chromatography, precoated TLC plate(Silica gel, 250 μm, E. Merck) was used. Developing solvent was cyclohexane : ether, 5 : 1(v/v).

Results and Discussion

Induction of callus

Ten days after the initiation of the calli, the calli were induced in all hormonal conditions on MS medium. The medium with indolacetic acid(IAA) and 1-naphthaleneacetic acid(NAA) were slow to induce the calli and could not maintained the calli growth further. The best hormonal conditions for calli induction were the combinations of *p*-chlorophenoxyacetic acid(pcPA) with 6-benzylaminopurine (BAP) and pcPA with 2-isopentenylaminopurine(2 *i*P). The optimal concentration of these hormones was 0.05 mg/*l*. When NAA or pcPA was used alone, the calli were also induced. Chlorophyll was generated in the light condition.

Organogenesis into shoot was observed in the condition of 0.035 mg/*l* of NAA with 0.3 mg/*l* of BAP. Root was generated when NAA was used

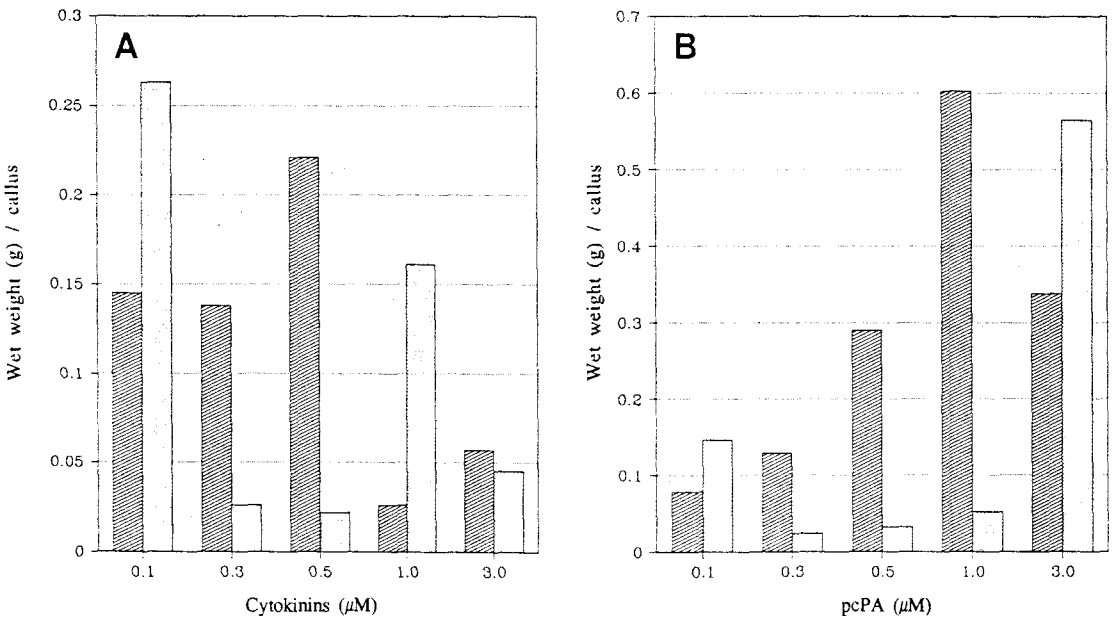


Fig. 1. Effect of varying concentrations of growth regulators, cytokinins(A) and pcPA(B) on the growth of *A. annua* calli.
(A) Concentration of pcPA was fixed at 0.3 μM, and the column with slashes denotes BAP and dots 2*i*P. (B) The column with slashed line represents 0.3 μM of BAP and dots 0.3 μM 2*i*P.

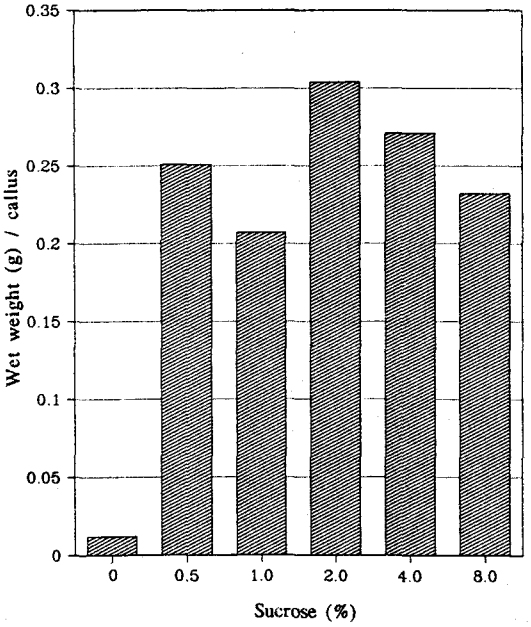


Fig. 2. Effect of varying concentrations of sucrose on the growth of *A. annua* calli.
Concentration of pcPA was at 1.0 μM and 2*i*P at 0.5 μM.

alone. Organogenesis did not occur with either pcPA, β-naphthoxyacetic acid(NOA), or indol-3-butyric acid(IBA) alone. In all conditions, calli were not induced from stems. From these results, the combination of pcPA and BAP was used in the ensuing induction and growth of calli.

Growth of calli

LS and B5 mediums were not suitable for the growth of the calli. The best hormonal conditions for the growth of callus were determined by systematically changing the concentration of the growth regulators. Auxins used in this experiment were pcPA and cytokinin BAP. The optimal concentrations of pcPA and BAP were determined as being 1.0 μM and 0.5 μM, respectively(Fig. 1).

The best growth of the calli were observed with 2% sucrose(w/v) as a carbon source(Fig. 2). Optimal phosphate(potassium phosphate monobasic) and inorganic nitrogen concentration(ammonium nitrate and potassium nitrate) were 0.344 mg/m*l*, and 1.652

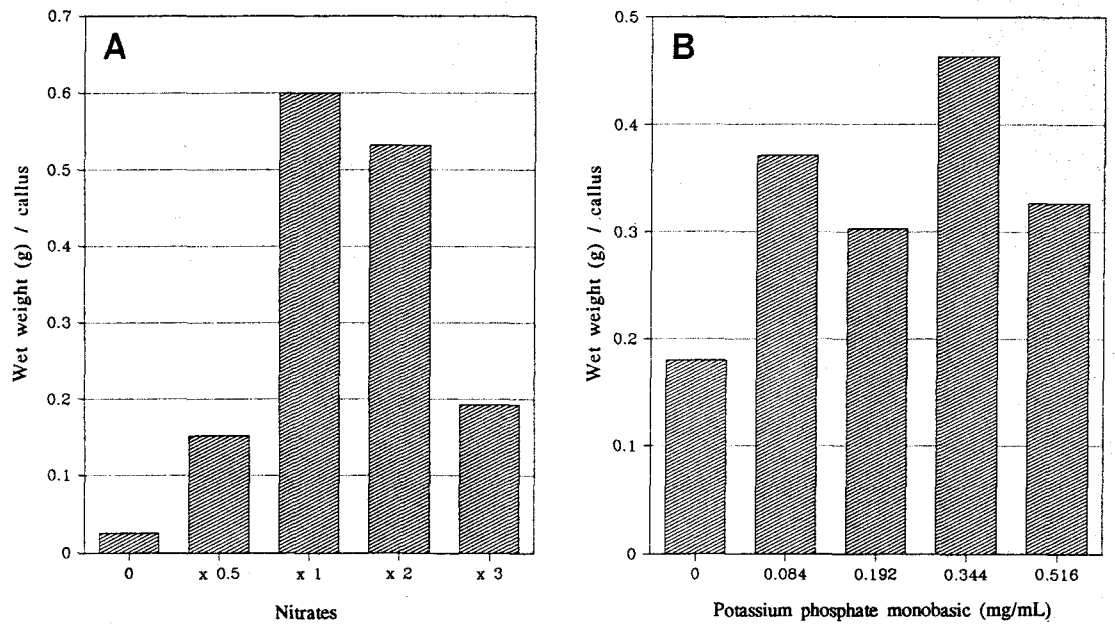


Fig. 3. Effect of varying concentrations of nitrogen(A) and phosphate(B) sources on the growth of *A. annua* calli.
In plate A, numbers denote multiples of concentration of original MS medium(1.652 mg/m*l* in ammonium nitrate and 1.900 mg/m*l* in potassium nitrate). Concentratiions of pcPA and BAP were 1 μM and 0.3 μM, respectively.

mg/ml and 1.900 mg/ml(Fig. 3). The optimal concentration of the inorganic phosphate found for *A. annua* was somewhat higher than the original MS medium. However, the nitrogen concentration was the same as the MS medium. Light rendered the calli non-friable. Calli became suitably soft and friable for the suspension culture when the casein hydrolysate at the concentration of 0.1% was supplemented and the calli were grown in the dark.

Transformation with *Agrobacteria*

Part of the stem of the plant inoculated with *A. tumefaciens* bulged in 2~3 months(Fig. 4A). When this enlarged stem was cut off and cultured on MS medium without phytohormones, the tissue grew rapidly, suggesting it to be a crown gall(Fig. 4B). Hairy root emerged from the plant inoculated with *A. rhizogens*, but the induced root did not maintain growth on the hormone-free MS medium and could not be used for further experiments. Tumor derived from crown gall grew more vigorously and was more friable than normal callus, which rendered the tumor suitable for the suspension culture.

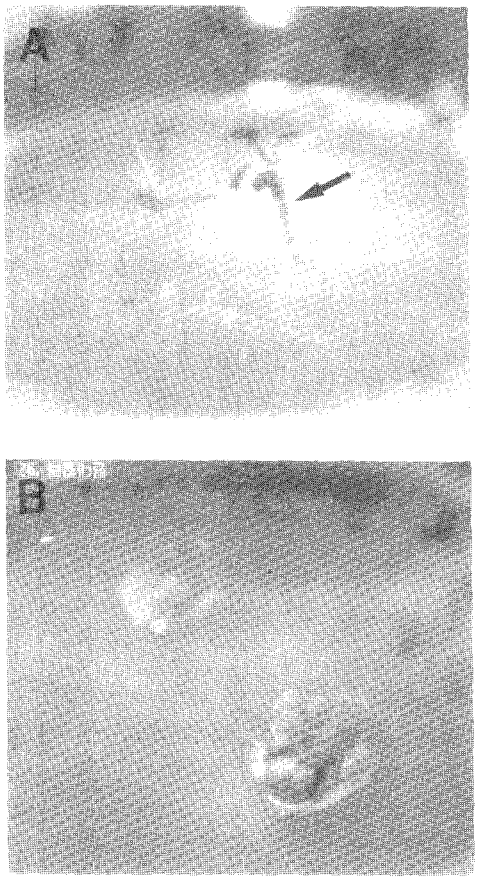


Fig. 4. The crown gall(A) emerged in parts of the stem of *Artemisia annua* inoculated with *Agrobacterium tumefaciens*(arrow), and tumor derived from the crown gall(B).

Identification of compounds from callus, tumor and culture broth

TLC analysis of the fractions from calli, tumors,

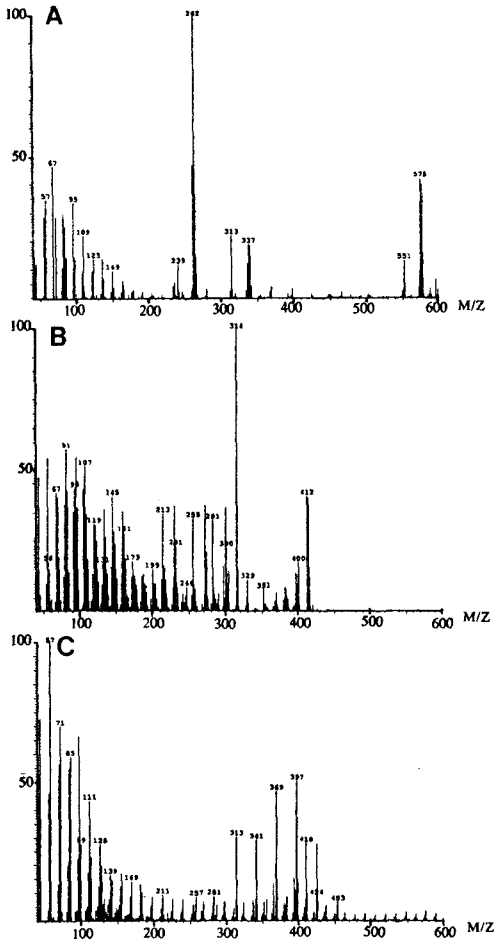


Fig. 5. Mass spectra(EI, 70 eV) of the compounds in organic fraction I obtained through the extraction step described in the experimental section, octadecenoic acid triglyceride(A), stigmasterol(B), and an unknown compound(C) with alkyl side chain.

and their suspension cultures showed no I_2-, bromocresol green- nor anisaldehyde-positive spots corresponding to the authentic artemisinin and arteannuic acid. However, several I_2-positive spots were apparent when fraction I were chromatographed on TLC plate. Thus, the organic fractions I from all the samples(calli, tumors, and the suspension culture broth and cells derived from normal calli and crown galls) were separated on a silica gel plate. The I_2-positive spots were scrapped, eluted from the plate, and subjected to mass spectrometry. The compounds were identified as stigmasterol with $M^+=412$, a common phytosterol found in plants, octadecenoic acid triglycerate($M^+=860$) and an unidentified compound with alkyl chain, presumably a wax(Fig. 5). The identification of the unknown compound was not vigorously established.

It is possible that artemisinin was not detected on TLC plate due to the low detection sensitivity of the visualization methods. However, HPLC analysis of the fractions I failed to detect artemisinin. The detection sensitivity of artemisinin through the pre-column modification was reported to be approximately 3 ng.[17] It is thus unlikely that the low sensitivity of the instrument precluded the detection of artemisinin.

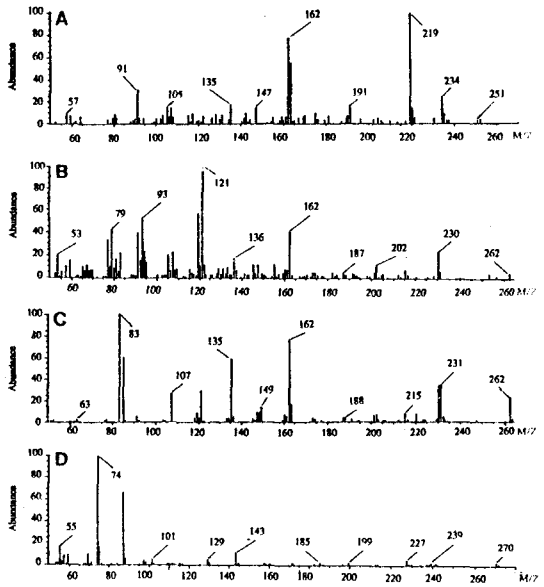


Fig. 6. GC/MS of the compounds separated from acidic fraction II through preparative TLC.
The isolated sample was methylated with diazomethane before analysis. The electron impact mass spectra of methyl 11,12-dihydroarteannuate(A), reaction products of arteannuic acid with diazomethane(B, C), and hexadecanoic acid(D).

Fraction II were separated by TLC into two spots. Through GC/MS analysis, arteannuic acid{**2**} and 11,12-dihydroarteannuic acid{**3**} were found in one of the spots only from the suspension culture broths(Fig. 6). Spectrum in Fig. 6A is almost identical to that of authentic methyl 11,12-dihydroarteannuate. $M.^+$ ion is absent. However, the peak at m/z 251, $[M+1].^+$, has arisen from the intermolecular reaction. The intermolecular reaction is probably due to the low amount of 11,12-dihydroarteannuic acid in the extract as judged from the total ion chromatogram(not shown here). The peak at m/z 234 is due to $[M\text{-}CH_3+1].^+$ and the base peak at m/z 219 to $[M\text{-}OCH_3].^+$. Spectral patterns shown in Fig. 6B and C have originated from the thermolysis products of the cycloaddition adduct formed between diazomethane and arteannuic acid.[18] The spectrum B is due to methyl 11-methylarteannuate ($M.^+=262$) and C is due to methyl 12-cyclopropylidenearteannuate. The total ion chromatogram suggests that arteannuic acid is the main components in the fractions II(not shown here). Also found was hexadecanoic acid(as methyl ester, $M.^+=270$).

This report is the first demonstration of the occurence of arteannuic acid{**2**} and 11,12-dihydroarteannuic aicd{**3**} in the tissue culture of *A. annua*. This result shows the potential of producing the acids via tissue culture technique. Furthermore, as the transformed calli grew more vigorously than normal calli, it would be possible to obtain the sesquiterpenes in higher amounts with the appropriate medium manipulation.

In light of the unpublished result from this laboratory, which demonstrated the formation of artemisinin{**1**} via nonenzymatic reaction from 11,12-dihydroarteannuic acid{**3**}, it is quite perplexing that artemisinin did not occur in suspension culture. The concentration of the dihydro acid was very low

compared to that of arteannuic acid, which could be explained by the inefficient conversion of the acid into the dihyro acid and/or the instability of the dihydro acid. Further tuning of the culture conditions and the analysis methods, thus, would be necessary for the demonstration of the artemisinin {**1**} production.

Acknowledgement

The authors wish to thank for support by the Ministry of Education(Genetic Engineering Research, 1989), Republic of Korea.

References

1. Balandrin, M. F., Klocke, J. A., Wurthele, E. S. and Bellinger, W. H. : Science, 288 : 1154(1985)
2. Booth, W. : Science, 237 : 963(1987)
3. Klayman, D. L. : Science, 228 : 1049(1985)
4. Qinghaosu Antimalaria Coordinating Research Group : Chin. Med. J., 92 : 811(1979)
5. China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials : J. Trad. Chin. Med., 2 : 17(1982)
6. Wang, Z., Nakashima, T. T., Kopecky, K. P. and Molina, J. : Can. J. Chem., 63 : 3070(1985)
7. Blasko, G., Cordell, G. A. and Lankin, D. C. : J. Nat. Prod., 51 : 1273(1988)
8. Schmidt, G. and Hofheinz, W. : J. Am. Chem. Soc., 105 : 624(1983)
9. Xu, X., Zhou, J., Huang, D. and Zhou, W. : Tetrahedron, 42 : 818(1986)
10. He, X., Zeng, M., Li, G. and Liang, Z. : Zhiwu Xuebao, 25 : 87(1983)
11. Nair, M. S. R., Acton, N., Klayman, D. L., Kendrick, K., Basile, D. V. and Mante, S. J. : J. Nat. Prod., 49 : 504(1986)
12. Linsmaier, E. M. and Skoog, F. : Physiol. Plant., 18 : 100(1965)
13. Gamborg, O. L., Miller, R. A. and Ojima, K. : Exp. Cell Res., 50 : 151(1968)
14. Maniatis, T., Fritsch, E. F. and Sambrook, J. : In 'Molecular Cloning. A Laboratory Manual', p. 440, Cold Spring Harbor Laboratory, New York(1982)
15. Kamada, H., Okamura, N., Satake, M., Harada, H. and Shimomura, K. : Plant Cell Rep., 5 : 239(1986)
16. Mugnier, J. : Plant Cell Rep., 7 : 9(1988)
17. Zhao, S. and Zeng, M. : Planta Med., 304 : 233 (1985)
18. Choi, J. -H. : Master's Thesis, Seoul National University (1992)

***Artemisia annua* L.의 조직배양을 이용한 이차대사 산물의 생산**

김남철 · 김정구 · 임형준* · 한태룡* · 김수언(서울대학교 농화학과 및 농업생물신소재연구센터, *경희대학교 유전공학과)

초록 : 국화과의 일종인 개똥쑥(*Artemisia annua* L., 青蒿)에는 항말라리아 물질인 artemisinin이 함유되어 이 물질을 조직배양 방법으로 얻을 수 있는 가능성을 연구하였다. 먼저 식물조직의 유기를 위해 생장조절 물질을 조사한 결과 pcPA와 BAP, 그리고 pcPA와 2*i*P의 가장 적절하였다. 식물조직의 생장도 역시 pcPA와 BAP가 각각 1.0 μM과 0.5 μM일 때, 그리고 sucrose가 2%일 때 가장 우수하였다. MS배지를 제외한 다른 배지들은 성장을 뒷바침하지 못하였다. 다른 생장조건들은 기존의 배지 조성을 능가하지 못하였다. 개똥쑥은 *Agrobacterium*으로 형질 전환되어 crown gall과 hairy root를 생성하였다. 그러나 hairy root는 MS 배지에서 배양하였을 때 더 이상의 생장을 보이지 않고 crown gall만이 정상 식물 조직보다 더욱 빠른 성장을 보였다. 배양된 조직과 형질 전환된 tumor의 현탁배양액에서 arteannuic acid와 dihydroarteannuic acid가 흔히 보이는 식물 steroid와 함께 발견되었으나 artemisinin은 발견되지 않았다.