

シラカンバ茎切片由来カルスにおける不定芽分化に対する α -aminooxy- β -phenylpropionic acidと α - aminooxyacetic acidの影響

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論 文

Effects of α -Aminoxy- β -phenylpropionic Acid and α -Aminoxyacetic Acid on Differentiation of Adventitious Buds in the Callus Derived from Stem Segments of *Betula platyphylla* SUKATCHEV var. *japonica* HARA

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FU, Lu-Min, HOGETSU, Taizo, IDE, Yuji and SUZUKI, Kazuo: **Effects of α -aminoxy- β -phenylpropionic acid and α -aminoxyacetic acid on differentiation of adventitious buds in the callus derived from stem segments of *Betula platyphylla* SUKATCHEV var. *japonica* HARA.** J. Jpn. For. Soc. 75: 331~337, 1993 Adventitious bud differentiation was studied in stem explants of *Betula platyphylla*. Examinations of sectioned materials by conventional light and epifluorescence microscopy showed that calli formed on the cut ends are derived mainly from phloem cells and the differentiation of adventitious buds occurs in the surface area of calli where the suberized periderm is lacking. Additions of α -aminoxy- β -phenylpropionic acid (AOPP) or α -aminoxyacetic acid (AOA), competitive inhibitors of phenylalanine ammonia-lyase (PAL), to the culture media retard the formation of the suberized periderm and accelerate adventitious bud differentiation in the surface area of calli at both ends of stem segments. These results suggested that adventitious bud formation is inhibited by the suberized periderm in the surface area of the callus and accelerated by the prevention of periderm formation. Thus, inhibitors of PAL like AOPP and AOA might become powerful tools to accelerate adventitious bud differentiation in the callus of various woody plants.

傳 禄敏・寶月岱造・井出雄二・鈴木和夫：シラカンバ茎切片由来カルスにおける不定芽分化に対する α -aminoxy- β -phenylpropionic acid と α -aminoxyacetic acid の影響 日林誌 75: 331~337, 1993 シラカンバの茎切片を組織培養すると切口にカルスが生じ、やがてカルスの上に不定芽が形成される。この不定芽形成に対する、phenylalanine ammonia-lyase の拮抗阻害剤である α -aminoxy- β -phenylpropionic acid (AOPP) と α -aminoxyacetic acid (AOA) の影響を調べた。はじめに、解剖学的にカルスを調べたところ、カルス表面近くにスベリン化した周皮が形成された。また、不定芽の所で周皮が途切れるが、その付近の細胞配列の観察から、不定芽は周皮ができていない部位に生じることが推定された。一方、培地に 30 μ M の AOPP あるいは 0.3 μ M の AOA を添加しておくことで不定芽形成が促進された。また、カルス表面近くの周皮形成が AOPP と AOA によって遅れた。これらの結果から、AOPP と AOA はカルス表面近くの周皮形成を遅らせることにより、不定芽形成を促進するものと推論した。これらの薬剤は、木本植物の分化を促進するものとして今後さらに他の樹種でも検討する価値があると考えられる。

I. Introduction

One of the most promising methods for rapid, clonal multiplication of plants is regeneration of plant individuals from tissue culture. Many attempts have been made to promote shoot redifferentiation from woody plant callus by changing the ingredients of the culture medium such as inorganic salts and growth regulators or by removing phenolic substances from the medium (LLOYD, 1981; ANDERSON, 1984; DUHEM *et al.*, 1988; DA CAMARA MACHADO *et al.*, 1991). At the present time, adventitious buds are able to be produced efficiently on explants of a considerable number of woody species (BAJAJ, 1986) as well as herbaceous ones (THORPE, 1990) and grown into individual plants. Nevertheless, redifferentiation of adventitious buds still

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remains unsuccessful in explants of many more woody species than herbaceous ones, suggesting that there are difficulties specific to woody species in differentiating shoots.

The ability of callus tissue to regenerate adventitious buds might depend on structural characteristics within the callus. Generally speaking, the periderm usually develops at the surface of the woody callus, but seldom on the herbaceous one. In the healing process of mechanical wounds in various tissues of woody plants, vigorous callus formation occurs first, and periderm formation in the surface region of the callus follows (YAMAMOTO *et al.*, 1983; RITTINGER *et al.*, 1987). Many anatomical studies have been undertaken involving tissue culture in herbaceous plants (MOTT and CURE, 1978; REYNOLDS, 1989; REDWAY, 1991), but microphotographs in these studies usually show no periderm in the callus. Since the periderm, whose cell wall is suberized, is rigid and impervious to water and air, the presence of the suberized periderm at the surface of the woody callus might be inhibitory for adventitious bud formation. Therefore, it could be assumed that the difficulty in differentiation of shoots from woody calli is derived mainly from being covered by the rigid periderm. Comparison of the periderm in the callus of woody species between sites at which adventitious buds are formed and not formed, is of much interest.

Suberin is a composite of polymeric phenylpropanoids and ester-linked long chain fatty acids and alcohols. The phenylpropanoid portion of suberin is considered to have a lignin-like structure to which both aliphatic domains and hydroxycinnamic acids are esterified (YAMAMOTO *et al.*, 1989). Both the pathway of biosynthesis of phenylpropanoids and enzymes involved in it are well established (VANCE *et al.*, 1980). Phenylalanine ammonia-lyase (PAL), which catalyzes the formation of *trans*-cinnamic acid, a precursor of a large number of phenolic substances including suberin, from L-phenylalanine, occupies a central position in plant secondary metabolism.

Several analogues of phenylalanine are known as competitive inhibitors of PAL, and they prevent lignification of tissue indirectly (MOERSCHBACHER *et al.*, 1990). Thus, it may be of interest to examine whether or not such analogues inhibit suberization of the periderm and promote shoot differentiation in woody callus. An artificial modification of suberization of the periderm over woody callus would provide evidence for or against the above assumption.

In this paper, we describe structural characteristics of the suberized periderm in the callus of *Betula platyphylla* SUKATCHEV var. *japonica* HARA and effects of two analogues of phenylalanine, α -aminooxy- β -phenylpropionic acid (AOPP) and α -aminooxyacetic acid (AOA), on suberization of the periderm and adventitious bud differentiation in the callus.

II. Materials and Methods

1. Culture of stem segments

Stem segments (4~5 mm in length, 1 mm in diameter) were excised from plantlets grown aseptically *in vitro*, put on a IS agar medium in 9 cm petri plates (SAITO and IDE, 1985) supplemented with 0.8 mg/l of 6-benzylaminopurine, 30.5 mg/l of gibberellin A₃ and 0.02 mg/l of 1-naphthaleneacetic acid, and incubated at 24°C in a cycle of 16 h of light (3,000 lx) and 8 h of darkness.

2. Anatomical and histochemical examinations

Segments were fixed in FAA (formalin-acetic acid-ethyl alcohol; 1:1:18, v/v/v). Sections were cut at thicknesses of 20~30 μ m by a sliding microtome equipped with a freezing stage, stained with saturated phloroglucinol in 20% HCl and observed under epifluorescence to detect suberin (BIGGS, 1985). The counter staining of sections with phloroglucinol canceled the intensive fluorescence of lignin and made fluorescence of suberin much more identifiable.

3. Chemicals

Two analogues of phenylalanine were used in this study. AOA hemihydrochloride was purchased from Sigma. AOPP was provided by Hodogaya Chemical Co., Tokyo, Japan.

III. Results

1. *Periderm formation during the adventitious bud formation in stem segments of B. platyphylla var. japonica*

A slight swelling of the cut ends appeared in about 85% of the segments (36 out of 42) after a 5-d incubation. Cell divisions of phloem cells near the swellings were prominent. The swellings grew later to be the callus, and the segments took a dumb-bell shape (Fig. 1a). After 30 d, the callus became 3 to 4 times larger in diameter than the stem.

In the earlier stage of growth, the calli were compact and green or yellowish green. Phloem cells continued to increase in number, and a few cells, walls of which were thick and lignified, appeared in the tissue on the inner side of the phloem and developed into a mass of tracheary elements. The suberized periderm was found in the surface area of 43% of the calli (6 out of 14) on the 20 d. The surface of the callus was covered partly by 2~3 layers of cells which accumulated suberin in their walls (Fig. 2b).

Some of calli then formed red spots, protuberances and then dark green spots on their surfaces. Near the surface of each callus, meristemoids, cells of which were small, globular, thin-walled and less vacuolated and had more discriminate nuclei, sometimes were found (Fig. 2a). The periderm also was found in the surface area of the callus, being interrupted on the peripheries of the meristemoids. The edges of the periderm were not warped, suggesting that the meristemoids do not break through the pre-existing periderm but are formed in gaps of the surface periderm.

The dark green spots became shoot primordia. Adventitious buds appeared on some calli after 20 to 25 d of incubation, and calli which bore adventitious buds increased in number afterward. Adventitious buds were differentiated on 14% of calli (10 out of 72 calli) after 35 d of culture. After 30 d, the surface area of the callus was covered mostly by the tough suberized periderm except for regions under the shoot primordium in 73% of calli (8 out of 11). Suberized cells in the periderm formed five to six layers. The periderm was interrupted beneath the shoot primordia, but suberized cells were not broken (Fig. 2c). Cell arrangement of the surface of the basal, narrow part of shoot primordia, where the suberized periderm terminated, did not seem to be disordered (Fig. 2d), suggesting that shoot primordia did not break through the completed periderm but developed where the periderm has not been completed.

2. *Effects of AOPP and AOA on callus formation*

In the presence of 30 μM AOPP or 0.3 μM AOA, calli also were formed on the cut ends of stem segments. The calli were dark green. There were many more protuberances on the surface of the calli than on non-treated calli (Fig. 1c, d).

AOPP and AOA at concentrations of more than 100 μM and 10 μM , respectively, inhibited growth of calli. All calli cultured at these concentrations of AOPP and AOA became yellowish-brown or brown after 10 d of culture.

3. *Effects of AOPP and AOA treatments on periderm formation*

Suberization of the periderm in calli was affected by the PAL inhibitors. The suberized periderm was found only in 17% of calli (1 out of 6) treated by 30 μM AOPP after 20 d of culture, whereas suberized periderm was formed in 43% of the non-treated calli (6 out of 14), and only 10% of the AOPP-treated calli (1 out of 10) formed suberized periderm with few gaps after 30 d, whereas in 73% of the non-treated calli (8 out of 11) such formation occurred. These periderms in AOPP-treated calli were composed of only two to three layers of suberized cells.

No suberized periderm was observed in the calli treated with 0.3 μM AOA (10 calli) after 20 d of culture, though in 70% of the AOA-treated calli (7 out of 10), tough periderm with few gaps was formed after 30 d. There was no obvious difference in the appearance of the periderm between non-treated and AOA-treated calli. These observations indicated that AOPP and AOA delay formation of suberized periderm in the surface area of the calli.

4. *Effects of AOPP and AOA on differentiation of adventitious buds on the callus*

AOPP was most effective in promoting adventitious bud differentiation at 30 μM , and the percentage of

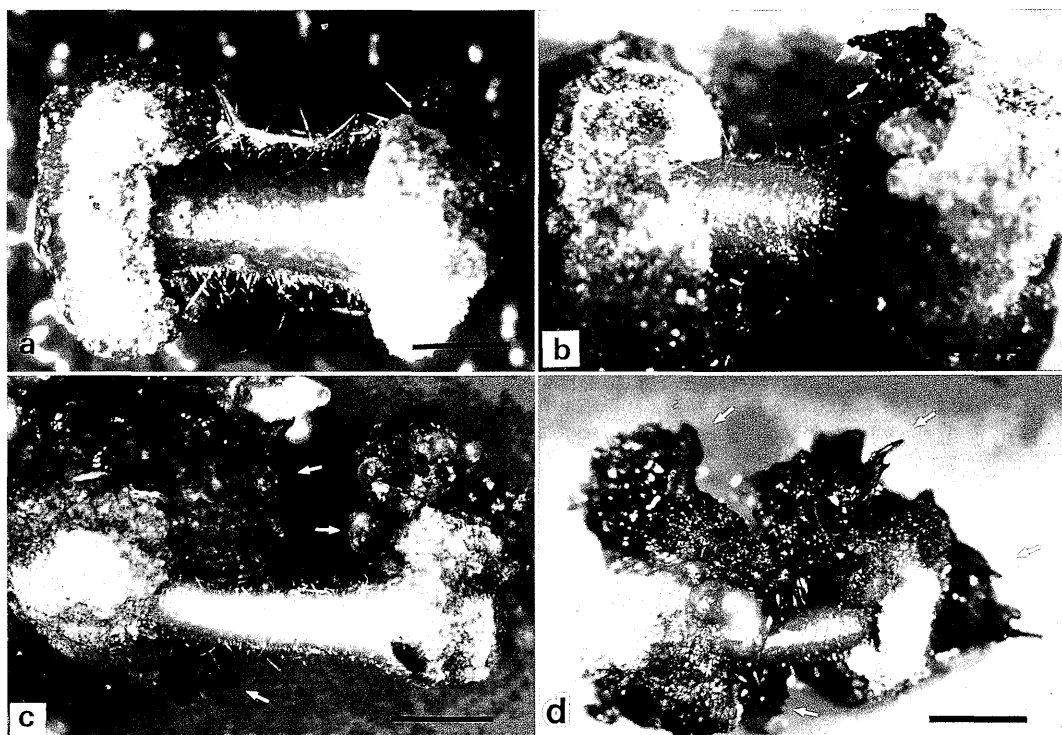


Fig. 1. Stem segments of *B. platyphylla* cultured on agar media with or without PAL inhibitors

a. Without inhibitors, after 10-d of culture; b. Without inhibitors, after 35 d of culture; c. With $30\ \mu\text{M}$ AOPP, after 35 d of culture; d. With $0.3\ \mu\text{M}$ AOA, after 35 d of culture. $\times 28$, bar= $50\ \mu\text{m}$. Arrows indicate adventitious buds. Note that many more adventitious buds were formed on the calli cultured with PAL inhibitors than those cultured without them.

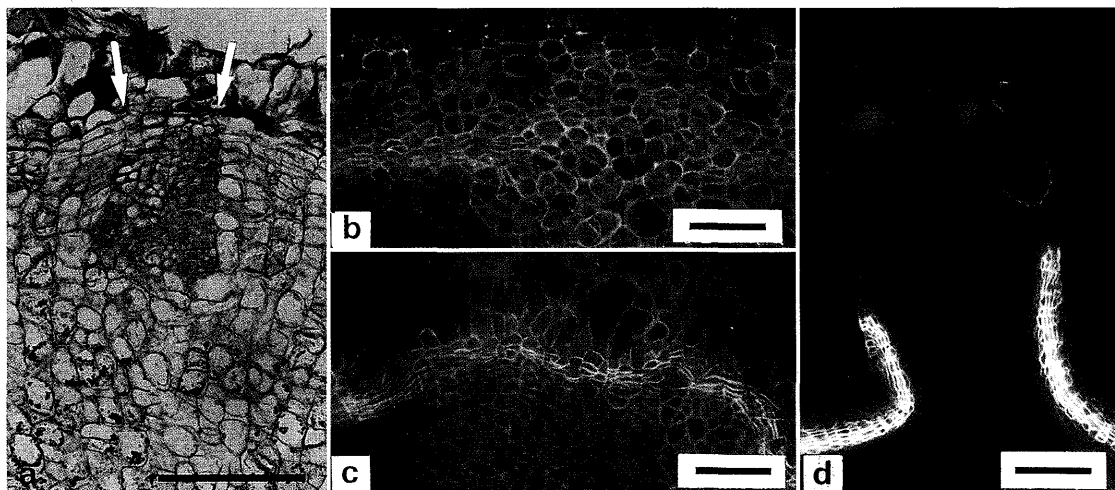


Fig. 2. Inner structure of the callus during adventitious bud differentiation

a. Meristematic region in a callus, after 15 d of culture. The periderm is terminated at the periphery (arrows) of a meristemoid; $\times 190$, bar= $10\ \mu\text{m}$. b. Suberin deposition (blue fluorescence) in a callus, after 15 d of culture. The outermost cells were suberized faintly, but the suberization proceeded no longer; $\times 100$, bar= $10\ \mu\text{m}$. c. Suberin deposition in a callus, after 25 d of culture; $\times 100$, bar= $10\ \mu\text{m}$. d. Suberin deposition in a differentiated adventitious bud, after 35 d of culture. $\times 100$, bar= $10\ \mu\text{m}$.

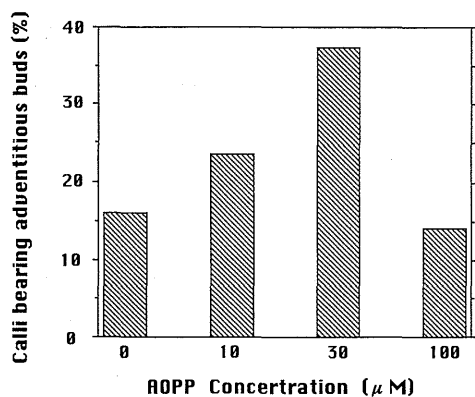


Fig. 3. Effect of AOPP treatment at various concentrations for 35 d on adventitious bud differentiation

Thirty six segments were examined for each treatment.

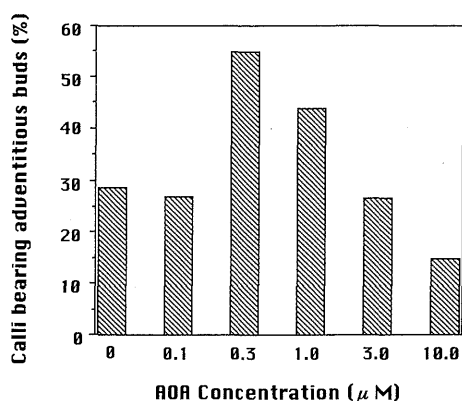


Fig. 5. Effect of the AOA treatment at various concentrations for 35 d on adventitious bud differentiation

Forty-two segments were examined for each treatment.

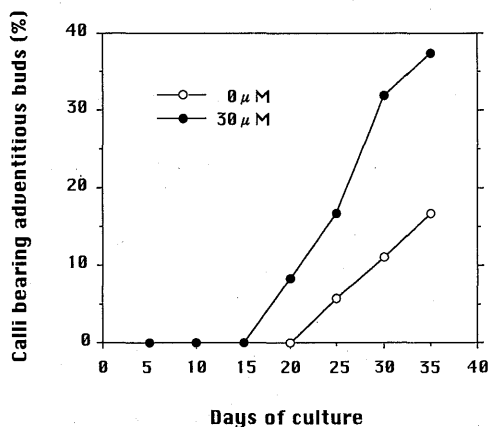


Fig. 4. Time course changes of adventitious bud differentiation in the absence and presence of 30 μM AOPP

Thirty six segments were examined for each treatment.

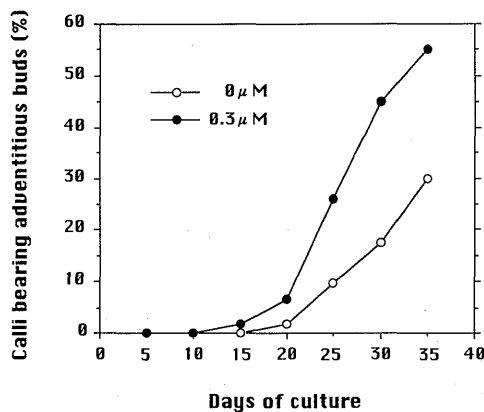


Fig. 6. Time course changes of adventitious bud differentiation in the absence and presence of 0.3 μM AOA

Forty-two segments were examined for each treatment.

shoot differentiation increased to about 38% (27 out of 72 calli) after 35 d of culture, being 2.3 times more than that without AOPP, *i.e.*, 17% (12 out of 72) (Fig. 3). This effect of AOPP was confirmed in another experiment of the same kind; 13% without AOPP and 37% with 30 μM AOPP. In stems treated with 30 μM AOPP, *de novo* formation of adventitious buds began after 15 d of culture, while formation in non-treated, control stems did not begin until the 20 d (Fig. 4).

AOA also accelerated adventitious bud differentiation on calli. The promotion by the treatment with AOA peaked at 0.3 μM, approximately doubling bud differentiation; 29% (24 out of 84) without AOA and 55% (46 out of 84) with it (Fig. 5). This effect of AOA was also confirmed in another experiment of the same kind; 33% without AOA and 54% with it. In the presence of 0.3 μM AOA, adventitious buds began to differentiate within 15 d, whereas in the control run adventitious buds began to form after 20 d (Fig. 6).

IV. Discussion

In our present study, the periderm was formed within the callus tissue of *B. platyphylla*, but not beneath

adventitious buds. There was no discontinuity of the outline and cell arrangements in the surface area where the periderm was interrupted. If adventitious buds developed by breaking through the pre-existing periderm, the outline and cell arrangements in the surface area of the regions where the periderm was terminated would be greatly disordered. Thus, this observation suggests that the adventitious buds were not formed at the sites where the suberized periderm had been already formed but at the sites where the suberized periderm had not been formed yet. The fact that meristemoids formed in the surface regions, which may develop to adventitious buds, seemed to be derived from gaps in the periderm coverage of callus surfaces also supports this suggestion. Although the causal relationship between shoot differentiation and periderm formation is not thoroughly clear, we tentatively assume that the ability of woody calli to form adventitious buds depends on the degree of coverage of callus surfaces by the suberized periderm.

We also found that the treatment of *B. platyphylla* stem segments with AOPP or AOA approximately doubled the rate of redifferentiation, and that AOPP and AOA inhibited partially, although not completely, suberization of the periderm in calli which were formed at both ends of stem segments. The inhibition of suberization of cell walls in the periderm by these inhibitors may reduce the rigidity of surface of the callus and make it easy to form meristematic regions, which would differentiate later adventitious buds, in the surface area of the woody callus.

A correlation between suberization and shoot formation also has been reported using tobacco explants. WILMS *et al.* (1990) reported that explants of tobacco stems on a medium with a large agar concentration form a complete layer of cells with suberized walls and lose their ability to form adventitious floral shoots, suggesting that the differentiation of floral buds in tobacco explants is inhibited by suberized periderm cells. It may be of interest to examine the effects of AOPP and AOA on shoot formation in the above system of tobacco explants.

AMRHEIN and GODEKE (1977) and AMRHEIN and GERHARDT (1979) reported that AOPP is a powerful inhibitor of PAL in higher plants and fungi both *in vitro* and *in vivo*. MASSALA *et al.* (1980) also reported that AOA is a competitive inhibitor of deamination and transamination of phenylalanine *in vitro*. Hydroxycinnamic acids, precursors of suberin, are formed from phenylalanine by several enzymes, including PAL as a key enzyme (VANCE *et al.*, 1980). Therefore, both AOPP and AOA may have prevented the surface of the callus from being suberized by inhibiting the PAL activity.

The fact that the treatments with large concentrations of AOPP and AOA prevent callus growth might, however, indicate the possibility of side effects other than PAL inhibition. MASSALA *et al.* (1980) pointed out that AOA inhibits some metabolic steps other than the phenylalanine metabolism. Some investigators also indicated that AOA is an inhibitor of transaminase reactions of alanine metabolism (BRUNK and RHODES, 1988) and of the photorespiratory metabolism (KUMAR and ABROL, 1989), suggesting that AOA is not so specific to PAL. More detailed examinations would be necessary for an understanding of the action mechanisms of AOPP and AOA.

In conclusion, we found that adventitious buds seem to be formed in the regions of calli of *B. platyphylla* where the suberized periderm does not cover, and that the additions of AOPP and AOA to the culture media retard suberization of the periderm and accelerate differentiation of the adventitious buds. If PAL inhibitors generally increase the adventitious bud differentiation in woody tissues, they would become powerful tools in the applied field of woody plant biotechnology.

Acknowledgement

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Literature cited

- AMRHEIN, N. and GODEKE, K.-H. (1977) α -Aminooxy- β -phenylpropionic acid-a potent inhibitor of L-phenylalanine ammonia-lyase *in vitro* and *in vivo*. Plant Sci. Lett. 8: 313~317.
- AMRHEIN, N. and GERHARDT, J. (1979) Superinduction of phenylalanine ammonia-lyase in gherkin hypocotyls caused by the inhibitor, L- α -aminooxy- β -phenylpropionic acid. Biochim. Biophys. Acta 583: 434~442.

- ANDERSON, W. C. (1984) A revised tissue culture medium for shoot multiplication of *Rhododendron*. J. Am. Soc. Hortic. Sci. **109**: 343~347.
- BAJAJ, Y. P. S. (1986) Biotechnology of tree improvement for rapid propagation and biomass energy production. In Biotechnology in agriculture and forestry, Vol. 1. (BAJAJ, Y. P. S., ed.). 1~23, Springer, Berlin, Heidelberg, New York, Tokyo.
- BIGGS, A. R. (1985) Detection of impervious tissue in tree bark with selective histochemistry and fluorescence microscopy. Stain Technol. **60**: 299~304.
- BRUNK, D. G. and RHODES, D. (1988) Amino acid metabolism of *Lemna minor* L. Plant Physiol. **87**: 447~453.
- DA CAMARA MACHADO, M. L., DA CAMARA MACHADO, A., HANZER, V., KALTHOFF, B., WEISS, H., MATTANOVICH, D., REGNER, F. and KATINGER, H. (1991) A new, efficient method using 8-hydroxy-quinolinol-sulfate for the initiation and establishment of tissue cultures of apple from adult material. Plant Cell, Tissue Organ Cult. **27**: 155~160.
- DUHEM, K., LE MERCIER, N. and BOXUS, P. (1988) Difficulties in the establishment of axenic *in vitro* cultures of field collected coffee and cacao germplasm. Acta Hortic. **225**: 67~75.
- KUMAR, P. A. and ABROL, Y. P. (1989) Effect of photorespiratory inhibitors on *in vivo* nitrate reduction in the leaves of barley and maize. Can. J. Bot. **67**: 3426~3427.
- LLOYD, G. (1981) Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip culture. Int. Plant Prop. Soc. **30**: 421~427.
- MASSALA, R., LEGRAND, M. and FRITIG, B. (1980) Effect of α -aminooxy-acetate, a competitive inhibitor of phenylalanine ammonia-lyase, on the hypersensitive resistance of tobacco to tobacco mosaic virus. Physiol. Plant. Pathol. **16**: 213~226.
- MOERSCHBACHER, B. M., NOLL, U., GORRICHON, L. and RESSENER, H.-J. (1990) Specific inhibition of lignification breaks hypersensitive resistance of wheat to stem rust. Plant Physiol. **93**: 465~470.
- MOTT, R. L. and CURE, W. W. (1978) Anatomy of maize tissue cultures. Physiol. Plant. **42**: 139~145.
- REDWAY, F. A. (1991) Histology and serological analysis of shoot formation in leaf callus of *Saintpaulia ionantha* WENDL (*African violet*). Plant Sci. **73**: 243~251.
- REYNOLDS, T. L. (1989) Adventive organogenesis from somatic tissue cultures of *Solanum carolinense*: origin and development of regenerated plants. Am. J. Bot. **76**: 609~613.
- RITTINGER, K. A., BIGGS, A. R. and PEIRSON, D. R. (1987) Histochemistry of lignin and suberin deposition in boundary layers formed after wounding in various plant species and organs. Can. J. Bot. **65**: 1886~1892.
- SAITO, A. and IDE, Y. (1985) *In vitro* plantlet regeneration from adventitious buds induced by petiole culture in Japanese white birch. J. Jpn. For. Soc. **67**: 373~375.
- THORPE, T. A. (1990) The current status of plant tissue culture. In Plant tissue culture: Applications and limitations (BHOJANI, S. S., ed.). 1~17, Elsevier, Amsterdam, Oxford, New York, Tokyo.
- VANCE, C. P., KIRK, T. K. and SHERWOOD, R. T. (1980) Lignification as a mechanism of disease resistance. Ann. Rev. Phytopathol. **18**: 259~288.
- WILMS, F. H. A., NEELISSEN, J. A. M. and KROH, M. (1990) Suberization and bud formation in tobacco explants. New Phytol. **114**: 563~570.
- YAMAMOTO, F., NAKAYAMA, T. and SUZAKI, T. (1983) Vascular differentiation in callus of *Cryptomeria japonica* D. DON *in vitro*. IAWA Bull. New Ser. **4**: 32~38.
- YAMAMOTO, E., BOKELMAN, G. H. and LEVIS, N. G. (1989) Phenylpropanoid metabolism in cell walls. An overview. In ACS symposium series 399 (Plant cell wall polymers), (LEVIS, N. G., ed.). 68~88, American Chemical Society, Washington, DC.

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