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Isolation of Protoplasts from Various Tissues of *Acacia mangium* Cultured *in vitro*

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Introduction

Acacia mangium Will. is widely planted in the tropics as a fast-growing tree species. This species has tolerance against acidic soils and is able to grow on waste lands (YONEKAWA and MIYAWAKI, 1983). However, since many highly acidic soils are distributed in the tropics, breeding of tolerant clones is of much importance from the point of silvicultural view.

Recent method of plant biotechnology using protoplasts is one of efficient ways to breed tolerant clones. Protoplasts have been isolated from various tree species and cultured in order to create new plants by cell fusion (OHGAWARA *et al.*, 1985) or transfection of a foreign gene (KAWAZU *et al.*, 1988). As for *A. mangium*, however, no efficient protocols of protoplast isolation have been established yet. In the present report, as the first step toward breeding of tolerant clones of *A. mangium*, we examined the optimal condition for protoplast isolation from tissues produced *in vitro*.

Materials and Methods

Five different kinds of tissues were used for the protoplast isolation. Two of the tissues were compound leaves and phyllodes of shoots induced *in vitro* (SAITO *et al.*, 1993). Similarly, compound leaves and cotyledons of aseptically germinated 12-day-old seedlings were also tested. The other tissue was callus induced on a hypocotyl of a seedling (AKAMATSU *et al.*, 1991) cultured on MURASHIGE and SKOOG (MS) medium (MURASHIGE and SKOOG, 1962) containing 5 μM 6-benzylaminopurine (BAP) and 5 μM α-naphthaleneacetic acid (NAA).

Tissues were cut into small pieces in a 0.6 M mannitol solution and transferred into about 20 m*l* of an enzyme solution in 50 m*l* Erlenmeyer flasks. The basic enzyme solution contained 0.6 M mannitol, 2 mM dithiothreitol, 0.001% potassium dextran sulfate, 1 mM 2-[N-morpholino] ethanesulfonic acid (MES) (pH 6), in addition to various combinations of Cellulase 'ONOZUKA' RS (Yakult Pharmaceutical Co., Tokyo), Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo) and Driselase (Kyowa Hakko Kogyo Co., Tokyo). The enzyme solutions were centrifuged at 100×*g* for 3 min. Then the supernatants were sterilized by passing through 0.22 μm membrane filters. The flask was shaken with 70 rounds per min for 2–3 hr in a water bath at 30℃ to digest cell walls.

After the incubation, debris were removed by a 32 μm nylon mesh filter, and then the filtrate was centrifuged at 100×*g* for 5 min to collect protoplasts. The pellet of protoplasts was resuspended in 10 m*l* of a washing solution containing 0.6 M mannitol and 5.3 mM $CaCl_2$. The number of protoplasts was counted in a haemacytometer.

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Table 1. Effects of enzyme components in enzyme solution on the numbers of isolated protoplasts from compound leaves of cultured shoots*

Enzyme component (%)			Number of isolated protoplasts per 1 g of tissue ($\times 10^4$)
Cellulase 'ONOZUKA' RS	Pectolyase Y-23	Driserase	
1	0.1	0	4.3
1	0.1	1	10.7
1	0.1	2	15.6
1	0.2	0	9.3
1	0.2	1	3.8
1	0.2	2	34.8
0.5	0.1	0	4.5
0.5	0.1	1	0
0.5	0.1	2	20.0
0.5	0.2	0	7.1
0.5	0.2	1	4.7
0.5	0.2	2	9.3

* The shoots were induced from seedlings on MS medium containing 5 μM BAP and 5 μM IBA.

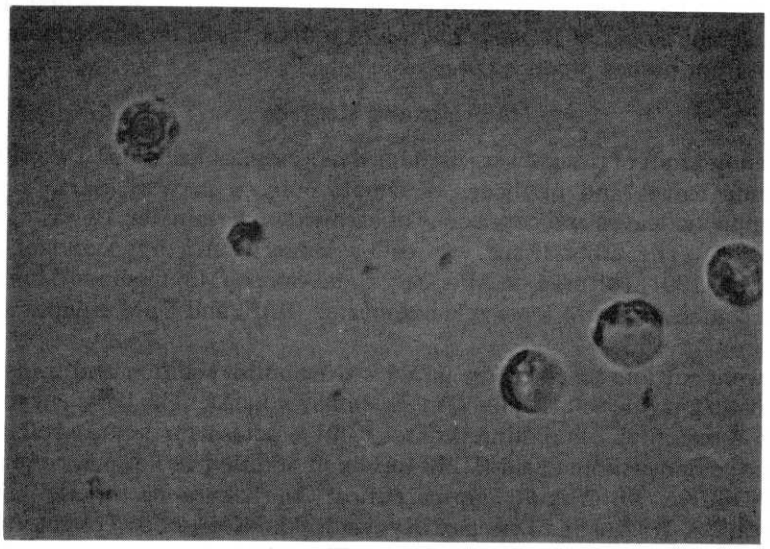

Fig. 1. Isolated protoplasts from the compound leaves of cultured shoot.

Results and Discussion

Enzyme composition for protoplast isolation

Twelve combinations of enzymes (Table 1) were tested to find the optimal combination for protoplast isolation from compound leaves of cultured shoots. Protoplasts were isolated in almost all enzyme combinations (Fig. 1). Yields of protoplasts are shown in Table 1. The enzyme solutions with Driselase were more effective for the protoplast isolation than those without Driselase. Especially the combinations with 2% Driselase were effective. Among the enzyme solutions that contained Driselase, the yields were higher in the solutions

Table 2. Difference in yields of protoplasts* from compound leaves, cotyledons and callus

Tissue	Number of isolated protoplasts per 1 g of tissue ($\times 10^4$)
Compound leaves**	3.8, 6.6
Cotyledons**	3.6, 8.7
Callus***	81.3

* The enzyme solution contained 1% Cellulaze 'ONOZUKA' RS, 0.2% Pectolyase Y-23 and 1% Driserase.

** Compound leaves and cotyledons of 12-day-old seedlings.

*** Callus were originated from hypocotyls of seedlings.

containing higher concentrations of Pectolyase Y-23 and Cellulase 'ONOZUKA' RS. The best yield of protoplasts was 3.5×10^5 per 1 g of tissue in the enzyme solution containing 1% Cellulase 'ONOZUKA' RS, 0.2% Pectolyase Y-23 and 2% Driselase.

SHIBATA *et al.* (1985) have already isolated protoplasts from seedlings of *A. mangium*, and the yields were at most 0.9×10^4 per 1 g of tissue. The yield of present experiment was about 40-fold higher than that. NEWELL and LUU (1985) reported that protoplasts were obtained from hypocotyls of *Glycine canescens*, a leguminous herb, and the yield averaged 1.4×10^6 per 1 g of tissue. In the case of trees, protoplasts were isolated from *Betula platyphylla* var. *japonica*, *Betula grossa* (IDE *et al.*, 1990), *Quercus acutissima* (IDE *et al.*, 1991) and *Populus alba* (SASAMOTO *et al.*, 1989), and the yields were between 1×10^5 and 1×10^7 per 1 g of tissue. In the present experiment, as the yield was equivalent to those from leguminous herb and other tree species, the method of protoplast isolation from *A. mangium* was improved.

Protoplast isolation from various kinds of tissues

HARAGUCHI *et al.* (1991) reported that the age and the firmness of tissue influence the efficiency of protoplast isolation. Therefore, the efficiency of protoplast isolation was examined for various kinds of tissues, phyllodes of cultured shoots, compound leaves and cotyledons of 12-day-old seedlings and callus. In the case of phyllodes, protoplasts were not isolated in the same enzyme solution used in the previous experiment. Probably, higher concentrations of enzymes or different combinations may be required for digesting the hard tissue of phyllodes.

The other tissues were treated with an enzyme solution containing 1% Cellulase 'ONOZUKA' RS, 0.2% Pectolyase Y-23, 1% Driselase. To avoid excessive digesting of young and soft tissues, the concentration of Driselase in the enzyme solution was decreased to 1%. The yields of protoplasts isolated from compound leaves and cotyledons were $3.6\text{–}8.7\times10^4$ (Table 2). In contrast, callus was more effective to isolate protoplasts. The yield from callus was about 8×10^5 per 1 g of tissue, being 10- to 20-fold higher than those from compound leaves and cotyledons. Callus is the best material for protoplast isolation in respect of yield.

In the present study, we established a procedure of protoplast isolation from *A. mangium* cultured *in vitro*. The isolated protoplasts formed no colony during the subsequent culture in MS medium. Our next step is to define a culture condition for successful proliferation of protoplasts of *A. mangium*.

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Summary

Various enzyme combinations were tested for protoplast isolation from compound leaves of *in vitro* cultured shoots of *Acacia mangium*. The combination of 1% Cellulase 'ONOZUKA' RS, 0.2% Pectolyase Y-23 and 2% Driselase was optimal to obtain a high yields of protoplasts. The protoplasts were also successfully isolated from compound leaves and cotyledons of young seedlings and callus, but not from phyllode.

Key words: *Acacia mangium*, Protoplast, Isolation

References

AKAMASTU, N., KOJIMA, K., IDE, Y. and SASAKI, S.: The growth regulator conditions for callus formation from seedlings of *Acacia mangium*. Abst. 102th Mtg. Jpn. For. Soc., 92, 1991.*

HARAGUCHI, M., YAMAMOTO, S. and IDE, Y.: Protoplast isolation from adventitious embryos of *Quercus acutissima*. Trans. 102th Mtg. Jpn. For. Soc., 383–389, 1991.**

IDE, Y., YAMAMOTO, S. and KONDO, A.: Isolation of mesophyll protoplasts from *in vitro* subcultured plantlets of *Betula platyphylla* var. *japonica* and *Betula grossa*. Trans. 101th Mtg. Jpn. For. Soc., 491–492, 1990.**

IDE, Y., YAMAMOTO, S. and KONDO, A.: Isolation and culture of mesophyll protoplasts from *in vitro* subcultured Japanese white birch. Bull. Tokyo Univ. For., **84**, 53–58, 1991.

KAWAZU, T., DOI, K. and SHIBATA, M.: Approaches to gene transfer into poplar protoplasts by electroporation. Trans. 36th Mtg. Chubu Br. Jpn. For. Soc., 13–14, 1988.*

MURASHIGE, T. and SKOOG, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., **15**, 473–497, 1962.

NEWELL, C. A. and LUU, H. T.: Protoplast culture and plant regeneration in *Glycine canescens* F. J. Herm. Plant Cell Tis. Org. Cult., **4**, 145–149, 1985.

OHGAWARA, T., KOBAYASHI, S., OHGAWARA, E., UCHIMIYA, H. and ISHII, S.: Somatic hybrid plants obtained by protoplast fusion between *Citrus sinensis* and *Poncirus trifoliata*. Theor. Appl. Genet., **71**, 1–4, 1985.

SAITO, Y., KOJIMA, K., IDE, Y. and SASAKI, S.: *In vitro* propagation from axillary buds of *Acaia mangium*, a legume tree in the tropics. Plant Tissue Cult. Lett., **10**, 163–168, 1993.

SASAMOTO, H., HOSHOI, Y., ISHII, K., SATO, T. and SAITO, A.: Factors affecting the formation of callus from leaf protoplasts of *Populus alba*. J. Jpn. For. Soc., **71**, 449–455, 1989.

SHIBATA, M., ITO, K., DOI, K. and TACHIMICHI, Y.: Basic study on protoplast isolations from woody plants (I) the most optimal condition for protoplast isolations from leaf cells. Trans. 33th Mtg. Chubu Br. Jpn. For. Soc., 137–140, 1985.*

YONEKAWA, S. and MIYAWAKI, S.: *Acacia mangium* in Brunei. The Tropical Forestry Quarterly Journal, **68**, 12–18, 1983.**

* Only in Japanese; the title was tentatively translated by the authors of this paper.

** Only in Japanese.

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アカシアマンギウムの種々の試験管内培養組織からのプロトプラストの単離

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要　　旨

アカシアマンギウムの培養シュートの本葉からプロトプラストを単離する酵素組成を検索した。酵素液は，セルラーゼ“オノズカ”RS 1%，ペクトリアーゼ Y-23, 0.2%，ドリセラーゼ 2%の添加が適当と判断された。また，芽生えの本葉，子葉およびカルスからプロトプラストを単離できたが，仮葉からは単離できなかった。

キーワード：　アカシアマンギウム，プロトプラスト，単離