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REVIEW

Recent Progress in Plant Cell Culture

Research on the Production of Useful Plant Metabolites in Japan

MASANARU MISAWA* AND TAKEO SUZUKI

Tokyo Research Laboratory, Kyowa Hakko Kogyo Co. Ltd., Machida-shi, Tokyo, Japan

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Introduction

The membership list of the International Association for Plant Tissue Culture (IAPTC) in 1979 included more than 300 including substaining members in Japan, the second largest contingent in the world. The activities of Japanese scientists are directed not only to fundamental research, but also to applications such as breeding and propagation through plant tissue cultures. Because of the numerous advantages to be expected, research on the production of useful plant metabolites is one of the most interesting fields, as described in many reviews *(1–7)*.

Since industrial fermentation technology in Japan has become highly developed, it is quite reasonable to expect that there would be production of a variety of plant metabolites using suspensions of cultured cells in large fermentors, much as microbial cultivation techniques are used to produce antibiotics, and so on. However,

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studies on such systems at industrial and government research facilities and in the universities remain at the laboratory scale, indicating that industrial applications have not yet been achieved. The main reasons are the slow growth rate of plant cells, the low concentrations of products, and unstable productivity, all of which make production expensive.

In spite of these disadvantages, certain recent advances in West Germany, the United States, Canada, Japan, and other countries hold promise of overcoming present difficulties hindering the industrial application of plant tissue cultures.

Approaches to Efficient Production

In the studies noted above, attempts to increase the productive ability of plant cells were effective and seem applicable to various cultures. The steps toward efficient production accomplished by several Japanese groups are summarized here.

1. Optimizing Environmental Conditions

A variety of chemical and physical factors affecting cultivation have been examined extensively in many kinds of plant cells and of their products. These factors consist of components of the medium, phytohormones, pH, temperature, aeration, agitation, light, etc.

In *Symphytum officinale* suspension cultures, use of enhanced levels of inorganic nitrogen compounds markedly increased the amount of L-glutamine produced in the cells, up to 20% of dry cell weight *(8)*.

The nature and concentration of phytohormones present in the medium sometimes has a dramatic effect on the production of metabolites and cell growth. As with microbial fermentations, optimizing the culture conditions is undoubtedly very important to the production of metabolites by plant cell cultures.

2. Addition of Precursors

To increase the level of a product, addition of its precursors may be effective. For example, Tabata et al. at Kyoto University *(9)* observed an increase in the level of alkaloids by addition of 500 μM tropic acid to *Scopolia japonica* cultures, which was 14 times as much as that produced without the precursors.

Furuya and his group at Kitasato University *(10)* have examined the biotransformation of steroids and alkaloids by plant cells. As seen in Reinhard's success with β-methyldigoxin formation from β-methyldigitoxin produced in *Digitalis lanata* cell culture *(11)*, the biotransformation of plant products is capable of contributing to the synthesis of various kinds of useful substances.

3. Selection of Cell Lines

Since excellent results were first achieved by Zenk et al. *(12)* in West Germany, the clonal selection of higher producing strains has been carried out by several groups in Japan.

From a number of single cells or small cell aggregates, cell lines having high producing ability for nicotine *(13)*, shikonin derivatives *(14)*, ubiquinones *(15)*, and so on have been isolated, as will be described later. The levels of these compounds produced by selected cells were much higher than those found in their parent strains.

4. Mutation

Although the 5-methyltryptophan-resistant mutants of *Catharanthus roseus* derived by Scott et al. *(16)* and Schallenberg et al. *(17)* were not able to accumulate higher levels of tryptamine and ajmalicin, the derivation of suitable auxotrophic or drug-resistant mutants should become one of the most effective methods of increasing producing capability. Unfortunately, except for high saponin-producing strains of *Panax ginseng* derived by means of nitrosoguanidine treatments or UV irradiation, as described in a patent by Furuya et al. *(18)*, no other such mutants have been obtained.

5. Differentiation

There is an idea that the expression of secondary metabolism is based on a differentiation process. Accordingly, dedifferentiated cells should hardly accumulate secondary products.

Hiraoka et al. *(19)* at Kyoto University has observed that development of shoots and roots from the callus of *Datura innoxia* promoted the accumulation of alkaloids.

The amount of rotenoids accumulated in the root-differentiated tissues of *Derris elliptica* was 160 μg/g of cells dry weight, but that produced in the callus was only 2.9 μg/g *(20)*.

As the author and his colleagues have reported regarding the rapid propagation of ornamental plants such as begonias and lilies *(21, 22)*, suspension cultures of partially differentiated plants should be considered if dedifferentiated cells do not produce the desired metabolites.

6. Immobilized Cells

Brodelius et al. in Sweden *(23)*, using alginate-immobilized plant cells, were successful in producing anthraquinones, indole alkaloids, and digoxin. The advantages of using immobilized cells are the possibility of reusing the cells and easier separation of the products from the cells; product costs are therefore reduced. Reports of similar studies have not yet been published in Japan.

Products

In this review, recent (mostly since 1975) Japanese studies of the production of useful substances by plant tissue culture will be described from an economical point of view. This necessitates the omission of discussion of much excellent fundamental research whose economic value is not yet apparent.

Alkaloids

Many alkaloids have a variety of biological activities, and most of them are products of higher plants; therefore, there are many papers and patents on the production of alkaloids.

Although nicotine is not an interesting alkaloid from the viewpoint of manufacture by tissue culture because of its low price, there are several interesting reports on its production.

In the study reported by Tabata et al. (1976) *(24)*, nicotine content in the callus tissues of *Nicotiana rustica* L *var. brasilia* rapidly decreased to trace amounts on succeeding subculture, but a cell line selected from these clones could produce nicotine at a high rate, 0.29% dry wt., even in the 54th passage. Ogino et al. (1978) *(13)* in the same group also obtained a high nicotine-producing cell line of *N. tabacum* by repeated clonal selection whose yield was 3.4% dry wt. Its production was inhibited by light, but was restored completely when the tissue was transferred to the dark (1978) *(25)*. An increse of the NAA level in the medium also inhibited production (1978) *(26)*. Comparing a nicotine-producing with a nonproducing strain, Ohta et al. at Nagoya University (1980) *(27)* indicated that the latter was lacking in putrescine *N*-methyltransferrase.

Callus tissues of *Coptis japonica* were found to contain all the main alkaloids, such as berberine and jatrorrhizine, found in the rhizome of the mother plant, although their contents were much lower (1975) *(28)*. Restoration of alkaloid content was observed in the rhizome of the plant regenerated from the callus. On the other hand, 13.2 mg of berberine HCl and 0.8 mg of palmatine HCl were isolated from 535 g of *Phellodendron amurense* callus tissues in a patent by Kanebo Ltd. (1975) *(29)*.

A production method for *Rauwolfia* alkaloids was patented in 1977 by Sanyo Kasei Co. Ltd. *(30)* in which plantlets redifferentiated from the callus of *Rauwolfia serpentina* accumulated 0.034% dry wt. of the alkaloids after cultivation for 39 days at 25°C. The plantlets were also cultivated in suspension. Accumulation of 0.01–0.02% ajmalicine in the callus of the same plant was reported by another group (1979) *(31)*.

The production of morphine alkaloids is one of the interesting targets for plant tissue cultures. Kamimura et al. at Sankyo Co. Ltd. found that *Papaver bracteatum* cells cultivated in suspension formed L-stylopine and protopine, which had not been isolated from the mother plants, even after long periods of subculturing (1976) *(32)*. The levels of the alkaloids were approximately 0.2 and 0.15 μg per g of plant cells. Both compounds were also accumulated in the culture filtrate.

When the cells were cultured successively in a hormone-free medium, bud-like protrusions were formed in some of the cell aggregates. The increase in the number of the aggregates having the protrusions correlated with that in the concentration of thebaine accumulated. The highest amount of thebaine, 0.13 mg/g of cells, dry wt., was obtained when the cells were transferred to the medium containing 1 mg/L kinetin and 10% coconut milk from the hormone-free medium *(33)*.

Using suspension-cultured cells of *Papaver somniferum*, Furuya et al. (1978) *(34)* recognized that *(RS)*-reticuline was stereospecifically converted to

(−)-(*s*)-scoulerine and (−)-(*s*)-cheilanthifoline. (−)-(*R*)-reticuline was also obtained from *(RS)*-reticuline. The biotransformation of (−)-condeinone to (−)-condeine with *P. somniferum* cells occurred in a high yield, 60.8%, at 3 days.

Akasu et al. at the Tokyo College of Pharmacy (1976) *(35)* reported the production of several kinds of alkaloids, such as norcepharadine, liriodenine, lysicamine, bermamine, and aromorine, by the callus of *Stephenia capharantha*. Among them, aromorine has not yet been found in the mother plant. The maximum content of total alkaloids in the callus was more than three times higher than that of the mother plant.

Because of high product costs, tissue culture methods for the production of metabolites should be applied only to high cost compounds. Thus, antitumor substances from higher plants are another suitable target. A patent of the production method of camptothecine, an antitumor alkaloid in *Camptotheca acuminata,* by Misawa et al. at Kyowa Hakko Kogyo Co. Ltd, was published in 1978 *(36)*. The same group recently established a radioimmunoassay of cephalotaxine, which is the mother compound of certain antitumor alkaloids (homoharringtonine, harringtonine, etc.) *(37)*. This might be a useful technique for the production of these valuable alkaloids by a tissue culture.

Steroids

Nabeta et al. at Kagawa University (1976) *(38)* isolated 0.3 mg of stigmasterol from 32 g dry wt. of *Stevia rebaudiana* callus tissues. Other groups also recognized the existence of various phytosterols in the callus tissues of *Mentha spicata* (1980) *(39)* and of *Solanum laciniatum* (1979) *(40)*.

Although the amounts were small, phytoecdysones such as inokosterone and ecdysterone were identified in the callus of *Achyranthes japonica* (1975) *(41)*.

The root of *Panax ginseng* has been widely used as a natural medicine and a production method using the dried plant material was patented by Furuya et al. (1978) *(18)*. The crown-gall cells, callus tissues, and redifferentiated roots of *P. ginseng* were cultivated on both agar and liquid media for several weeks. The R_f values of the saponins in cell culture extracts on thin-layer chromatography corresponded to those of ginsenoside Rb and Rg. The amounts of crude saponins in the callus (21%), in the crown gall (19.3%), and in the redifferentiated root (27.4%) were much higher than those in natural roots (4.1%). The yield of crystalline panaxatriol was 0.09% of the dried callus. The inventors obtained variants of *P. ginseng* that had higher saponin-producing ability by nitrosoguanidine treatment and UV irradiation.

The dried root of *Bupleurum falcatum* has been applied in oriental countries as an antipyretic, a tonic, and an anodyne. A patent filed by Shionogi and Co. Ltd. (1976) *(42)* showed a "Bupleuri radix" production method by tissue cultures. After incubation for 12 days, the callus of *B. falcatum* was transferred to the liquid medium containing a lower level of IAA. A number of plantlets with roots and primordia were formed by cultivation successively on a shaker. The level of saponins in the roots thus obtained was 19.4 mg/g on a dry wt. basis, which was the same as had accumulated in the mother plants (18.5 mg/g).

Furuya and his group have extensively investigated the biotransformation of steroids by plant cell cultures (1980) *(10)*. Among eight compounds detected in the biotransformation products from digitoxigenin using cultured cells of *Digitalis purpurea*, three compounds, digitoxigenone, epidigitoxigenine, and epidigitoxigenin-β-D-glucoside were isolated (1980) *(43)*.

The conversion of digitoxin to digoxin, gitoxin, and other compounds by *D. purpurea*, and that of pregnenolone to pregesterone and other steroids, had already been reported before 1973 by the same group. Furthermore, they recognized the formation of products including a 3-β-hydroxy-5-β-pregna-20-one from 5-β-pregnane-3,20-dione by *D. purpurea* cultures *(44)*.

The biotransformation of compounds by plant cells should contribute to the synthesis of many substances useful in industry.

Quinones

The callus tissues of *Cassia tora*, harvested after 55 days of cultivation in the dark, were found to accumulate chrysophanol, emodin, physion, etc., all of which are contained in the seeds of the mother plant (1975) *(45)*. The maximum content of anthraquinones on a fresh weight basis was 0.334%, which is higher than that in the seeds.

Accumulation of shikonin derivatives having high antibacterial activity was recognized in the callus of *Lithospermum erythrorhizon* by the same group (1975) *(46)*. As a result of experiments on nutritional factors, a higher level of sucrose and a lower level of nitrogen in the medium were found to stimulate production of the quinone compounds. The addition of streptomycin, ascorbic acid, or phenylalanine to the cultures stimulated the biosynthesis of shikonin derivatives, while that of Ca^{2+} and Fe^{2+} inhibited their production (1977) *(47)*.

Mizukami et al. (1978) *(48)* reported that different strains of *L. erythrorhizon* callus tissues showed wide variation in the production of shikonin derivatives, ranging from almost 0 to 1000 μg/g fresh wt. of the tissues. From these cultures, they obtained two high and stable pigment-producing strains by repeated selection, whose level in the cells was similar to that of the mother plant root. Using cultured cells, a biosynthetic pathway for shikonin has been investigated by Inouye et al. (1979) *(49)*.

Studies on ubiquinone production have been actively carried out by the Japan Tobacco and Salt Public Corp. According to Ikeda (1976) *(50)*, the highest level of ubiquinone-10 in the suspension cultured cells of *Nicotiana tabacum* BY-2 was 360 μg/g dry wt. at the 10th day of cultivation, which was about 10 times higher than that in the leaves. The ratio of ammonium nitrogen to nitrate nitrogen in the medium significantly affected production. At higher ratios, the level of ubiquinone in the cells was increased. A higher concentration of 2,4-D and a higher temperature also stimulated ubiquinone production (1977) *(51)*. In the presence of 5 mg/L of 2,4-D, the level of ubiquinone was 580 μg/g dry wt., but the level decreased slowly during subculture (1978) *(52)*. Matsumoto et al. (1980) *(15)* isolated a high-producing strain by cell cloning techniques that accumulated 1848 μg/g dry wt.

Plant cells also accumulate ubiquinone-9. From 470 g dry wt. of suspension cultured cells of *Cathmus tinctorius*, 19.6 mg of ubiquinone-9 was isolated by

Hagimori et al. (1978) *(53)*. Ikeda et al. at Eisai Ltd. obtained 2.4 and 2.5 mg of ubiquionone-9 from 36 g dry wt. of cultured rice and 43 g dry wt of cultured wheat cells, respectively (1979) *(54)*.

The suspension-cultured cells of *Datura innoxia* have glucosylating activity of dihydroxybenzene isomers (1976) *(55)*. Administration of hydroquinone ($10^{-3}M$) to cell suspensions yielded its mono-β-D-glucoside, arbutin. Similarly, resorcinol and cathechol were converted to *m*- or *o*-hydroxyphenyl-β-glucoside, respectively. By the controlled feeding of hydroquinone to the culture, 70% of the substrate was converted to arbutin.

α- *and* γ-*Pyrones*

α-*Pyrones:* Hydrangenol and unbelliferone were isolated from the callus of *Hydrangea macrophylla* in 1977 *(56)* and skimmin was prepared by cultivating the same callus tissue (1978) *(57)*. In the patent literature, 150 mg of skimmin was obtained from cells suspended in 10 L of the medium after 3 weeks of cultivation. The same group was issued a patent in which 110 mg of crystalline phyllodulcin was obtained from 1.5 kg (fresh wt) of the cells of *H. macrophylla* cultivated for 3 weeks in a jar fermentor (1978) *(58)*.

The contents of scopoletin and its glycoside were 0.07 μmol/g dry wt. and 0.391 μmol/g dry wt., respectively, in the callus of *Swertia japonica (1978) (59)*.

γ-*Pyrones:* A yellow pigment produced by cultured cells of *Stevia rebaudiana* was identified as rutin, quercetin-3-β-rhamnoglucoside, by Suzuki et al. (1976) *(60)*; its yield was 22.4 mg from 3 kg of the callus harvested.

Rotenoids (2.9 μg/g dry wt.) were detected in the callus of *Derris elliptica* by Kodama et al. at the University of Tokyo (1980) *(20)*, but the amount decreased on subculturing. The root-like organs differentiated from the callus in a medium containing 10–50 mg/L IAA were observed to contain rotenone and deguelin (160 μg/g dry wt as rotenone).

The same group investigated the production of anthocyanin pigments, and two patents concerning their studies were issued to Ajinomoto Co. Inc. *(61)* and Meito Sangyo Co. Ltd. *(62)* (1978, 1980). In the former, the callus of *Polygonum tinctorium* was cultivated for 2 months on an agar medium and the pigments were extracted from the cells with 0.1*N* HCl. In the latter, from 180 g (fresh wt.) of *Vitis* sp. cells cultivated in a jar fermentor for 17 days, 161 mg of anthocyanin pigments were obtained. Light was found to stimulate pigment production. A patent for producing anthocyanins was also obtained by Mitsui Petrochem. Ind. Ltd. using the callus of *Derris* sp. (1979) *(63)*.

In the course of studies on the constitutents of cultured cells, Ayabe et al. isolated echinatin (300 mg), biosynthetically unique retrochalcone, and licodione, a dibenzoylmethane derivative (54 mg), from 290 g dry wt. of *Glycyrrhiza echinata* cells (1980) *(64)*. Formonetin and 7,4′-dihydroxyflavone were also isolated.

Terpenes

The amount of essential oil from the cultured cells of *Perilla frutescens var. crispa* was found to be quite similar to that obtained from the intact plant (0.1 % of fresh

cells) by Sugisawa et al. at Kagawa University (1976) *(65)*. The major component was identified as limonene, whose concentration was 21% of the oil, followed by α-pinene and linalool.

To prepare glycylrrhizin, a triterpenoid saponin, Fujita et al. used the callus tissues with roots, stems, and leaves of *Glycyrrhiza urarensis* (1978) *(66)*. The callus was transferred to the basal medium containing $10^{-7}M$ 2,4-D and $10^{-5}M$ kinetin and cultured for 30 days at 25°C. From the differentiating cultures thus obtained, glycylrrhizin was isolated in a yield of 7% (dry wt.)

Karasawa et al. (1980) *(67)* observed that the concentration and ratio of phytohormones affect the ratio of triterpenes, such as oleanolic, ursolic, and pomolic acids, in *Mentha arvensis* var. *piperascens* callus tissues.

The Earth Chemical Co. Ltd. (1978) *(68)* obtained a patent that described a method for producing pyrethrin using a callus, green callus, or redifferentiated plant of *Chrysanthemum cinerariaefolium*.

Suga et al. at Hiroshima University (1976) *(69)* investigated the biotransformation of monoterpenes by suspension cultured cells of *N. tabacum*. To the cultures, cyclic monoterpenes were added and incubated for 7 days at 25°C on a rotary shaker. Linalool and its derivatives, such as dihydrolinalool, linalyl acetate, etc., were hydroxylated at the transmethyl group in the isopropyridene group. They also showed that the cells had the ability to hydroxylate the carbon–carbon double bond itself as well as its allylic positions using α-terpineol, trans-β-terpineol, and trans-β-terpinyl acetate. In their patent *(70)*, α-terpineol (20 mg in 100 mL of *N. tabacum* suspension culture) was converted to 7-hydroxy-α-terpineol (15.0%), trans-6-hydroxy-α-terpineol (5.9%), and *cis*-6-hydroxy-α-terpineol (3.9%) after 7 days incubation.

The author and his group found that cultured cells of *Tripterygium wilfordii* accumulated larger amounts of the antineoplastic diterpenes, triptolide and tripdiolide, than those in the intact plant *(71)*. According to a patent issued, stevioside was formed from steviol in a yield of 60% when the callus of *Stevia rebaudiana* (20 g) was cultured on a medium containing steviol (5 mg) (1980) *(72)*.

Proteins and Peptides

Several papers describing the production of biologically active proteins and polypeptides were published by the author and his group. In the course of screening for new biologically active substances, we found that the *Scopolia japonica* callus had potent inhibitory activity against the proteinase, plasmin *(73, 74)*. Since the overproduction of plasmin causes many disorders in human beings, the suspension culture of *S. japonica* was investigated as a producer of inhibitors. The amounts in the cells cultivated for a week was approximately the same as those in the roots. The inhibitors were isolated as four low-molecular weight protein fractions *(75)*. The inhibition of blood fibrinolysis by a clot lysis mechanism was found to be potent for rats and rabbits.

We reported accumulation of anti-plant viral substances in a suspension culture of *Phytolacca americana* in 1975 *(76)*. The active principles were five proteins

whose molecular weights were from 1.1×10^4 to 3.1×10^4. The highest molecular weight component contained sugars in the molecule. The inhibitory activity against tobacco mosaic virus in a cultured broth reached a maximum after 9 days incubation on a rotary shaker. In further studies to obtain a more potent cell line, we selected *Agrostemma githago* cultured cells from a vareity of cell lines *(77)*. The highest levels of the cell mass and the antiviral substances were obtained at the 5th or 6th day of cultivation at 28°C on a rotary shaker operated at 180 rpm. The cells were disrupted by a homogenizer for a few minutes and centrifuged. Application of the supernatant or of a crude preparation of the inhibitor to the leaves of tobacco, tomato, etc. markedly inhibited plant virus infection as well as infection of *P. americana*. The active component in *A. githago* was also a protein whose molecular weight was 2.5×10^4. About 80% of TMV infection was inhibited by 1 μg/mL of the purified inhibitor, which was rather stronger than those of other inhibitors from microorganisms *(78)*.

There are many reports describing the production of enzymes. For example, peroxidase, phosphodiesterase, and phosphatase were purified from the cultured *N. tabacum* cells and their characteristics were determined by Shinshi et al. (1977) *(79)*. Two patents on phosphodiesterase production were issued in 1979, one to Kyowa Hakko Kogyo Co. Ltd. *(80)* and another to the Japan Tobacco and Salt Pub. Corp. *(81)*.

Cell Mass

The Japan Tobacco and Salt Public Corp. has been studying the biomass production of *N. tabacum* by cell cultures to prepare tobacco raw materials. They have studied the effects on cell growth of aeration conditions, medium composition and so on in large-scale fermentors. To obtain cells of a suitable quality for cigarets, they developed a two-stage continuous cultural method with 60-L fermentors. According to their results *(82)*, the cell concentrations were 16.1 g/L and 16.1 g/L at dilution rates of 0.41 and 0.78 day^{-1} in each tank using different concentrations of nitrogen sources, and the growth rates were, therefore, 6.6 and 12.6 g/L/day.

To prepare artificial silkworm food, *Morus bombycis* (mulberry) cells were cultured in a flask or a jar fermentor for 2 weeks. Yamada et al *(83)* at Kyoto University (1977) concluded that the cultured cells grown in the light provided an adequate silkworm food, and could be used as a substitute for mulberry leaves.

Miscellaneous

Nishi et al. at Nihon Sinyaku Co. Ltd. in their patent *(84)* described the production of crude anti-peptic ulcer substances by cell cultures of *Isodon japonicus*. Administration of the water extracts of the cells to rats showed the same level of activity as extracts of the natural plant.

The present author has also described *(85)* the inhibition of a protozoan (coccidium) infection in chickens by the administration of more than 250 ppm of dried cells of *Vinca rosea* in the feed. In neither case was the active principle isolated.

Water extracts of suspension-cultured *Glycyrrhiza globra* were shown to have flavor-enhancing ability for cigarets *(86)*. Extracts of *Hydrangea macrophylla* and related plant callus tissues were also prepared for the same purpose *(87)*.

Conclusions

Commercial application of plant cell cultures for the production of useful metabolites on a large scale has not yet been realized. However, some recently published papers and patents seem very attractive for industrial use because their results and techniques are apparently applicable to a large-scale production of metabolites. The author believes that commercial success using plant tissue cultures will be realized in the near future with advanced techniques if product selection is determined by economic and political principles.

In 1982, the 5th International Congress of Plant Tissue and Cell Culture will be held in Japan and this undoubtedly will encourage research activity in our country.

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