Medicinal Plant Biotechnology[1]

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[1] Presented at the 37th Annual Congress of the Society for Medicinal Plant Research, Braunschweig, F.R.G., September 5-9, 1989
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Abstract

Biotechnology is described as methodology to enhance the formation and accumulation of desirable natural products and possible product modification in medicinal plants. Micropropagation, cell and hairy root culture as well as gene technology are being highlighted.

Key words

Biotechnology, cell cultures, gene technology, hairy root cultures, micropropagation.

Introduction

Medicinal plants have been the subject of man's curiosity and purpose since time immemorial. Pharmacognosy is the scientific discipline for the description and recognition of medicinal plants and plant products. Recently, its 200-year history has seen most significant advances. New and novel technologies improved the separation and analysis of plant products by several orders of magnitude, and immunochemical techniques are poised to extend our capabilities even further. As a result phytochemistry, biochemistry, and physiology have entered pharmacognosy and transformed it to pharmaceutical biology. Today, pharmacognosy is, again, at the threshold of a major expansion. Cell and gene technologies have changed our capabilities from the mode of plant description to control of development and design of products. This new technology, commonly referred to as biotechnology, may enhance the formation of desirable plant products and improve our health industry. It decidedly is meant also to broaden our understanding of the biology of product formation in medicinal plants. In a wider context, biotechnology will enable us to learn about one of biology's major concerns – differentiation. It may actually re-establish ties between pharmacognosy and biology.

Cell Technology

Micropropagation

The oldest root of medicinal plant biotechnology has grown from cell technology, specifically plant tissue culture. In fifty years since its inception as a tool in physiology (1–3) tissue culture has found most visible application in plant regeneration from *in vitro* cultured cells. One commercial spin-off of this technology is micropropagation (4). Regeneration of plants has been achieved with cells and tissues excised from various medicinal plants as well (5). The multiplication factor can be high. Recent work with *Cephaelis ipecacuanha* yielded 100 plantlets per shoot tip explant per year or 600 plantlets per axenic shoot (6).

Micropropagation would be of special interest when applied to species which require several years of development. Here, accelerated crop rotation can be expected. This concept showed prospects of success when Chang and Hsing (7) obtained whole plant regeneration by way of embryogenesis in root-derived callus of ginseng *(Panax ginseng)*. Commercial micropropagation of elite ginseng plants, however, appears to be afflicted by problems; transfer of plantlets from test tubes to soil is often affected by root rot (Kao, personal communication).

Slow in being accepted by industry, micropropagation may still develop into a major component in medicinal plant breeding. Its benefit derives from *in vitro* culture and multiplication of axenic shoots excluding callus formation and associated problems. This kind of vegetative propagation permits cloning and preservation of elite plants obtained by cross-pollination. With *Digitalis lanata*, for example, cultivars with high cardenolide content were obtained by inbreeding and subsequent crossing of selected genotypes. Isolation and *in vitro* culture of shoot tips led to the formation of adventitious shoots. After short- and long-term culture, rooting of such shoots on solid medium established plantlets which were transferred to soil. Cardenolide yields equalled those of the parents (8). In similar experimentation with *Digitalis lanata* (9) final adaptation of *in vitro* grown plants to field conditions was affected by transpiration problems and required a special protocol. Interestingly, *in vitro* cloned plants had to be vernalized, whether explants had been derived from 1- or 2-year old

source material. In a similar fashion valuable genotypes of chamomile *(Chamomilla recutita)* have been cloned (10).

Axenic shoot (tip) cultures can be employed not only for multiplication of stock, but also for storage. If kept at 4 °C on media with reduced mineral salt concentration chamomile and *D. lanata* shoot tips have been preserved for at least 12 months. Cryopreservation permitted the establishment of a gene bank for elite chamomile and *Digitalis* plants (9, 10).

Somaclonal variation (11), which may be expressed in the selfed progeny of regenerant plants from callus cultures, has been employed as a means to select for medicinal and industrial plants with altered levels of phytochemicals. Opium poppy cell cultures, for example, have been exposed to conditions for regeneration and have provided an ample supply of *in vitro* grown plants which subsequently set flowers and seed (12). At PBI, selfing regenerants and their offspring produced second and third generations for alkaloid analysis. While individual plants showed values of thebaine, codeine, and morphine which exceeded those of seed born control plants, segregants with heritable capacity for high or low concentrations of these alkaloids or "codeine plants" have not been obtained. It would appear that ten years of experimentation with somaclonal variation has not yielded stable chemovariant plants, the greatest handicap being the lack of adequate selection procedures for high volume samples.

Cell culture

The production of pharmaceuticals by *in vitro* culture of plant cells, tissues, and roots has been pursued for 30 years (13) and still, the results have not met expectations. In total only a handful of species are being used by industry (14). Difficulties with predicting the future of *in vitro* production of pharmaceuticals stem from problems with cell technology as well as from uncertainties in the markets, regulatory aspects concerning pharmaceuticals from tissue cultures, and research funding. A case history may best illustrate the extent to which plant cell culture can lead to producing pharmaceuticals.

Opium poppy (*Papaver somniferum* L. cv. Marianne) cell cultures were established with callus from hypocotyl segments of seedlings in 1977 (15). An initial report on the occurrence of codeine in these cultures (16) remained unconfirmed. Recent analyses using RIA technology (Abuscreen test kit, LaRoche Diagnostics) demonstrated the occurrence of morphinan alkaloids in young callus at a level of 0.4 ng/mg fresh weight over a period of 9 months from explantation or as long as calli could be induced to regenerate plantlets and retained/produced laticifer-like cells (17). The application of a two-step culture system as suggested and employed by Zenk et al. (18) which requires the increase in biomass using growth media followed by transfer of cells to production media and harvest for extraction of products failed to demonstrate morphinan alkaloids (15). Elicitation of these opium poppy cell cultures by homogenates of *Botrytis* spec. mycelium resulted in the formation not of morphinan alkaloids but exclusively of sanguinarine and up to 3 % of dry weight. Remarkably, sanguinarine accumulation was observed in cells and, to a lesser extent, their medium. It occurred while cells were suspended in growth medium, i.e. without transfer of cells to special production media, and within 3 days rather than after several weeks of culture (19). Today, at a pilot stage, the same cultures are producing 200–400 g of sanguinarine per week in one 300 l bioreactor and in 250 ml suspensions levels of 11 % of dry weight have been observed (K. Giles, personal communication). Sanguinarine has demonstrated antibiotic activity against bacteria causing plaque formation on teeth and, thus, has for some time been the active ingredient in a variety of dental care products (20). Natural sources of sanguinarine are bloodroot (*Sanguinaria canadensis* L.) and *Macleaya* species. The latter are being grown in plantations but may not satisfy the demand.

The process of elicitation as employed here remains under investigation for academic and practical reasons (21, 22). Apart from the observation that elicitation by biotic and abiotic agents of plant cells grown *in vitro* does not necessarily increase yields of desirable compounds, as shown for shikonin and berberine, or double-up yields when combined with transfer of cells to production media, its application leads to variation in yields. This variation in cell response may reside in the quality and quantity of the active principle of the elicitor. Experiments designed to define the *Botrytis* elicitor principle are on-going. Since chitin was found to simulate the effect of *Botrytis* homogenate, hydrolyzates of chitin are being tested for elicitor activity (Tyler, personal communication). The analyses follow procedures as employed by Conrath et al. (23) who investigated the synthesis of callose and coumarin derivatives in parsley cells exposed to chitosan. On the other hand, the exposure of poppy cells to fungal homogenate for 48 h leaves the cell structure intact (24, 25) and, upon a recovery by subculture in regular (1-B5) growth medium, permits a second and third treatment of the biomass for sanguinarine production. Increasing product yields have been observed as a result (26), and a semi-continuous process for the production of phytochemicals has subsequently been proposed (27).

Cell culture of medicinal plants will invariably involve a consideration of the interrelationship of structural and biochemical differentiation. The diversity of phytochemicals forbids general statements on this topic. Strong arguments for coregulation of structural and biochemical differentiation originated with attempts to use cell cultures for the production of essential oils. With mint cell cultures, for example, the lack of gland cells and subcuticular compartments has been seen as an impediment for mint oil synthesis and accumulation. A recent literature review demonstrated widespread, low level occurrence of essential oils, and more so of volatile compounds, in plant cell cultures (28). In pelargonium and mint cell cultures the level of isoprenoid compounds was affected by growth regulators, immobilization of cells, elicitation, polyploidization, and adsorption by Miglyol® (29). It would appear that in the absence of specialized structures in cultures the separation of essential oils containing cytotoxic compounds from cultured cells removes a/the limiting factor to measurable accumulation (30). Structural and biochemical differentiation, therefore, may be separated, must not be subject to coregulation (cf. 31).

Hairy root culture

Hairy roots are hormone autonomous roots obtained by transformation with *Agrobacterium rhizogenes*. Their biosynthetic potential is being explored in several laboratories. A massive increase of biomass over relatively short periods of culture and complete differentiation of root tissue warrant the production of root-specific phytochemicals at substantial levels. And, indeed, hairy root cultures may become raw material for industrial processes towards pharmaceuticals or flavors or pigments. A few examples may illustrate the potential of hairy root cultures. They permit us to obtain compounds which occur in native roots in traces only. Hairy root cultures of opium poppy produced sanguinarine at levels up to 2% of dry weight (32). Also, hairy root cultures may equal the productivity of native roots and exceed other *in vitro* systems as with red thiarubrines, strong antibacterial and antifungal compounds in *Chaenactis douglasii* (33, 34). Finally, a survey of the potential of 24 solanaceous species showed tropane levels in hairy roots similar to those in native roots (35). Analyses of hairy roots of a *Datura candida* hybrid furnished yields of 0.68% of dry weight which is 1.6 and 2.6 times the amount found in the aerial parts and in the roots of the parent plants, respectively. Scopolamine was the principal alkaloid and the scopolamine/hyoscyamine ratio of 5 : 1 makes these hairy root cultures subject to considerations as a source of scopolamine (36).

Cell cultures and hairy root cultures would gain in importance as source of pharmaceuticals if products including desirable compounds would be released into the medium and enable continuous, on-line, non-destructive harvest procedures. Forced release by organic solvents as applied once to immobilized cells (37) has not been found satisfactory. The poration of cells as generally practised with protoplasts for purposes of DNA incorporation (38) would appear to be impractical at this time. A better prospect would appear to result from observation of active excretion of berberine by cell cultures of *Thalictrum minus* (39). Subsequent experimentation admittedly showed that excretion of berberine differed with *Thalictrum* species and appeared to be largely under genetic control (40). While these observations and conclusions could prevent further studies today, they may in future become subject to genetic manipulation and widen the range of material designed to excrete desirable products.

Gene Technology

Enhancement of productivity

Transgenic plants have become reality. Their novelty is expressed as a change, i.e. enhancement, in plant performance (herbicide, insect resistance) and productivity (pigmentation, storage proteins) (41). Transgenic cell cultures of medicinal plants with modified or enhanced productivity and micro-organisms producing phytochemicals are conceivable and may further increase the feasibility of phytochemical production by *in vitro* methods. The first steps in this direction have been taken and include the purification and characterization of key enzymes of biosynthetic pathways, the isolation of cDNA clones and gene synthesis. The list of genes being characterized is growing steadily, those which code for phenylalanine ammonia-lyase, chalcone synthase (42), tryptophan decarboxylase (43), strictosidine synthase (44) or 6β-hydroxylase (45) are of interest. Enzymes of the isoquinoline monoterpene indole, tropane alkaloid, and flavonoid synthesis are receiving prime attention.

The application of gene technology to medicinal plant material can best be described by reference to experiments designed to overcome one particular problem. The occurrence of vinblastine has been reported for callus and hairy root cultures of *Catharanthus roseus* (periwinkle) (46, 47), but these observations lack persistent confirmation. Likewise, vindoline, one of the two moieties of vinblastine, has not been demonstrated for *in vitro* cell cultures with certainty (48). Its abundance in vinblastine-producing leaves of periwinkle has been well documented (49). How to force accumulation of vindoline and vinblastine in periwinkle cell cultures? Analyses of periwinkle cell cultures did not assist in the search for vindoline; elicitation by homogenates of *Pythium aphanidermatum* and other biotic and abiotic stress agents induced substantial increases of catharanthine, the second moiety of vinblastine, but not of vindoline and of enzymes catalyzing vindoline synthesis (50, 51). The ability of shoots regenerated from cell cultures to produce vindoline (52) had demonstrated that such biosynthetic potential is present but silent in cultures. Cell cultures, therefore, were replaced as experimental material by seedlings in order to elucidate the problems at hand. Vindoline was detected in 6-day-old material upon illumination. Also, over time, vindoline appeared in increasing amounts while the level of tabersonine decreased. This behavior prompted an elucidation of the biosynthetic pathway from tabersonine to vindoline and led to the description of a series of enzyme reactions, acetyl transfer being the sixth and last step, resulting in the formation of vindoline (53). Today, the acetyl coenzyme A :deacetylvindoline *O*-acetyl transferase has been purified and characterized. It will be employed in isolating its respective gene for reintroduction into cultured cells of periwinkle and test for regulation of gene expression (54). The question whether such experiments will bring about vindoline biosynthesis in cell cultures remains to be answered.

The studies performed with seedlings of periwinkle have demonstrated that the enzymes involved in vindoline synthesis are under strict developmental and tissue specific control (53, 55). The enzymes involved in the early stages of vindoline biosynthesis (tryptophan decarboxylase and strictosidine synthase) were induced and peaked by 4 days after germination, whereas enzymes involved in later stages (*N*-methyl transferase and deacetylvindoline acetyl transferase) peaked 6 days after germination. Only the latter enzyme required light for induction and showed a 10-fold increase in activity after light treatment of the seedlings.

It would appear from these observations that photoautotrophic cell cultures of periwinkle would constitute the most suitable host material for incorporation and expression of genes which code for enzymes of vindoline synthesis. Such cell cultures have been selected for and found to be incapable of vindoline formation under conditions tested so far (56). Also, enzymes catalyzing vindoline formation could not be detected.

Enhancement of morphinan accumulation in cell cultures of opium poppy is complicated by the factor of laticifer differentiation. Could expression of latex specific proteins in opium poppy cell cultures enhance conditions for morphinan accumulation? An answer to this question may have to wait for some time. Still, major, i.e. abundant latex specific proteins of opium poppy have recently been separated and characterized (57, 58). Also, a partial amino acid sequence of this protein has been obtained. The protein fragment was used to design a major latex protein primer. A specific DNA probe was obtained and used to hybridize with mRNA of latex, tissue cultures and roots and shoots of poppy. No hybridization signal was detected in mRNA extracted from poppy callus or cell suspension cultures. The major latex protein mRNA accumulates exclusively in laticifers (59).

Modification of products

The employment of DNA technology to genetically modify plants for products designed for special usages has begun already. Modification of *Brassica* plants to produce oil of specific chemical composition is the objective of several laboratories (60), as is the effort to reduce the level of glucosinolates in *Brassica* meal (60). The suggestion to reduce the level of cyanogenic glycosides in cassava plants to zero is part of a recommendation by an international advisory council for research on tropical agriculture (61). At present the only procedure which could be regarded as realistic is the interdiction of biosynthetic pathways by employing antisense mRNA. It would be the procedure of choice to eliminate glucosinolates or cyanogenic glycosides. A target for this kind of modification with medicinal plants has not been identified in the literature, but may well be at hand. While industrial application of such technology may materialize for agricultural and horticultural commodities, costs of investment may preclude application to medicinal plants.

The best known model for genetic modification of plants for designer products results from work on antisense mRNA activity, first detected with bacterial and mammalian systems, now shown for plants (62). Experimentation with petunia presented as molecular flower breeding (63, 64) can well be used as a model. The work required interdiction of a resident pathway in petunia by expressing the reverse orientation of a defined pigmentation gene. Upon transcription of the wrong DNA gene an antisense RNA strand is formed which can interact with the complementary RNA strand to form a duplex. The mRNA then cannot contribute to protein synthesis, here chalcone synthase, and the effect will be similar to a mutation. In petunia a pure white flower color is the result. Novel color patterns, indeed, have been demonstrated.

Outlook

The application of biotechnology to medicinal plants does include not only biological but also bio-engineering aspects. The design and development of reactors which respect the particular characteristics of plant cells suspended in liquid medium or immobilized in a matrix or on surfaces is an on-going concern in an attempt to bring input costs down and yields up. Important, also, is the development of legislation which covers products obtained by plant cell cultures as medicinals. Compounds obtained with transgenic plant materials may require additional legislation. Finally, funding of research projects focused on the synthesis, accumulation, and excretion of phytochemicals by *in vitro* culture systems is in need of continuance and must not be affected by temporarily volatile market conditions or policies. The advances made in medicinal plant biotechnology generally are of broad application and may show very significant spin-offs in the field of plant protection or further our understanding of the plant and its environment.

The immediate future of medicinal plant biotechnology may well be determined not by economic, but by socio-political factors. Work would be based on technology at hand, cryopreservation and micropropagation. It may gain increasing importance in preserving species threatened by extinction. This general concern includes medicinal plants. Since Douglas (65) described the propagation of the last specimen of a palm *(Hyophorbe amaricaulis)* by tissue culture methods the number of similar reports has increased and includes rare medicinal plants, *Saussurea lappa* (66) and *Coleus forskohlii* (67), for instance, both endangered species in the Himalayas.

References

[1] Gautheret, R. J. (1939) C. R. Hebd. Seances Acad. Sci. 208, 118–121.
[2] White, P. R. (1939) Am. J. Bot. 26, 59–64.
[3] Nobécourt, P. (1939) C. R. Seances Soc. Biol. 130, 1270–1271.
[4] Pierik, R. L. M. (1987) *In vitro* Culture of Higher Plants, 3rd ed., M. Nijhoff, Boston.
[5] Rao, P. S. (1987) Cell Culture and Somatic Cell Genetics of Plants, Vol. 4, (Constabel, F., Vasil, I. K., eds.), Academic Press, New York.
[6] Ikeda, K., Teshima, D., Aoyama, T., Satake, M., Shimomura, K. (1988) Plant Cell Rep. 7, 288, 291.
[7] Chang, W. C., Hsing, Y. (1978) Theor. Appl. Genet, 57, 133–135.
[8] Schöner, S., Reinhard, E. (1986) Planta Med. 478–481
[9] Diettrich, B., Mertinat, H., Luckner, M. (1990) Planta Med. 56, 53–58.
[10] Donath, P., Diettrich, B., Hannig, H. J., Luckner, M. (1989) Plant Cell Tissue Organ Cult., submitted.
[11] Good, A. R. (1986) Cell Culture and Somatic Cell Genetics of Plants, Vol. 3, (Vasil, I. K., ed.), Academic Press, New York.
[12] Yoshikawa, T., Furuya, T. (1983) Experientia 39, 1031–1033.
[13] Staba, E. J. (1963) Rev. Appl. Microbiol. 4, 193–123.
[14] Fontanel, A., Tabata, M. (1987) Nestle Research News 1986/87, Vevey.
[15] Constabel, F. (1985) The Chemistry and Biology of Isoquinoline Alkaloids, (Phillipson, J. D., Roberts, M. F., Zenk, M. H., eds.), Springer Verlag, Berlin.
[16] Tam, W. H. J., Constabel, F., Kurz, W. G. W. (1980) Phytochemistry 19, 486–487.
[17] Griffing, L. R., Fowke, L. C., Constabel, F. (1989) J. Plant Physiol. 134, 645–650.
[18] Zenk, M. H. El-Shagi, H., Arens, H., Stöckigt, J., Weiler, E. W., Deus, B. (1977) Plant Tissue Culture and its Bio-Technological Application, (Barz, W., Reinhard, E., Zenk, M. H., eds.), Springer Verlag, Berlin.
[19] Eilert, U., Kurz, W. G. W., Constabel, F. (1985) J. Plant Physiol. 119, 65–76.
[20] Southard, G. L., Boulware, R. T., Walborn, D. R., Groznik, W. J., Thorne, E. E., Yankell, S. L. (1984) J. Am. Dent. Assoc. 108, 338–341.
[21] Eilert, U. (1987) Cell Culture and Somatic Cell Genetics of Plants, Vol. 4, (Constabel, F., Vasil, I. K., eds.), Academic Press, New York.
[22] Ebel, J. (1989) Primary and Secondary Metabolism of Plant Cell Cultures II, (Kurz, W. G. W., ed.), Springer Verlag, Berlin.
[23] Conrath, U., Domard, A., Kauss, H. (1989) Plant Cell Rep. 8, 152–155.
[24] Eilert, U., Constabel, F. (1985) Protoplasma 128, 38–42.
[25] Cline, S. D., Coscia, C. J. (1989) Planta 178, 303–314.
[26] Tyler, R. T., Eilert, U., Rijnders, C. O. M., Roewer, I. A., Kurz, W. G. W. (1988) Plant Cell Rep. 7, 410–413.
[27] Constabel, F., Kurz, W. G. W., Eilert, U. F. K. "Repeated Elicitor Treatment, A Method for Semicontinuous Metabolite Production by Plant Cells Cultured *In Vitro*." Can. Pat. granted 1989.
[28] Koch-Heitzmann, I., Schultze, W. (1988) Bioflavour '87, (Schreier, P., ed.), W. de Gruyter Verlag, Berlin.
[29] Charlwood, B. V., Brown, J. T., Moustou, C., Morris, G. S., Charlwood, K. A. (1988) Bioflavour '87, (Schreier, P., ed.), W. de Gruyter Verlag, Berlin.
[30] Beiderbeck, R., Knoop, B. (1987) Cell Culture and Somatic Cell Genetics of Plants, Vol. 4, (Constabel, F., Vasil, I. K., eds.), Academic Press, New York.
[31] Dougall, D. K. (1977) Plant Cell and Tissue Culture, (Sharp, W. R., Larsen, P. O., Paddock, E. F., Raghavan, V., eds.), Ohio State University Press, Columbus/Ohio.
[32] Williams, R., Ellis, B. E. (1989) Annu. Meeting Can. Soc., Plant Physiol., Toronto (Abstract).
[33] Constabel, C. P., Towers, G. H. N. (1988) J. Plant Physiol. 133, 67–70.
[34] Constabel, C. P., Towers, G. H. N. (1989) Planta med. 55, 35–37.
[35] Knopp, B., Strauss, A., Wehrli, W. (1988) Plant Cell Rep. 7, 590–593.
[36] Christen, P., Roberts, M. F., Phillipson, J. D., Evans, W. C. (1989) Plant Cell Rep. 8, 75–77.
[37] Brodelius, P. E., Deus, B., Mosbach, K., Zenk, M. H. (1979) FEBS Lett. 103–107.
[38] Brodelius, P. E., Funk, C., Shillito, R. D. (1988) Plant Cell Rep. 7, 186–189.
[39] Yamamoto, H., Suzuki, M., Suga, Y., Fukui, H., Tabata, M. (1987) Plant Cell Rep. 6, 356–359.
[40] Suzuki, M., Nakagawa, K., Fukui, H., Tabata, M. (1988) Plant Cell Rep. 7, 26–29.
[41] Shell, J., Vasil, I. K., eds., (1989) Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Academic Press, New York.
[42] Kreuzaler, F., Ragg, H., Fautz, E., Kuhn, D. N., Hahlbrock, K. (1983) Proc. Natl. Acad. Sci., USA 80, 2591–2593.
[43] De Luca, V., Marineau, C., Brisson, N. (1989) Proc. Natl. Acad. Sci. USA 86, 2582–2586.
[44] Kutchan, T. M., Hampp, N., Lottspeich, F., Beyreuther, K., Zenk, M. H. (1988) FEBS Lett. 237, 40–44.
[45] Hashimoto, T., Yamada, Y. (1987) Eur. J. Biochem. 164, 277–285.
[46] Miura, Y., Okasaki, M. (1983) Japanese Patent # 8 31201982.
[47] Parr, A. J., Peerless, A. C. J., Hamill, J. D., Walton, N. J., Robins, R. J., Rhodes, M. J. C. (1988) Plant Cell Rep. 7, 309–312.
[48] Scott, A. I., Mizukami, H., Hirato, T., Lee, S. L. (1980) Phytochemistry 19, 488–489.
[49] Westkemper, P., Wieczorek, U., Gueritte, F., Langlois, Y., Potier, P., Zenk, M. H. (1980) Planta Med. 39, 24–37.
[50] Eilert, U., Constabel, F., Kurz, W. G. W. (1986) J. Plant Physiol. 126, 11–22.
[51] Smith, J. I., Smart, N. J., Misawa, M., Kurz, W. G. W., Tallevi, S. G., DiCosmo, F. (1987) Plant Cell Rep. 6, 142–145.
[52] Constabel, F., Gaudet-La Prairie, P., Kurz, W. G. W., Kutney, J. P. (1982) Plant Cell Rep. 1, 139–142.
[53] De Luca, V., Balsevich, J., Tyler, R. T., Eilert, U., Panchuk, B., Kurz, W. G. W. (1986) J. Plant Physiol. 125, 147–156.
[54] Power, R. (1989) M. Sc. Thesis, University of Saskatchewan, Saskatoon.
[55] De Luca, V. (1989) Primary and Secondary Metabolism of Plant Cell Cultures II, (Kurz, W. G. W., ed.), Springer Verlag, Berlin.
[56] Tyler, R. T., Kurz, W. G. W., Panchuk, B. D. (1986) Plant Cell Rep. 3, 195–198.
[57] Nessler, C. L. (1988) J. Plant Physiol. 132, 588–592.
[58] Griffing, L. R., Nessler, C. L. (1989) J. Plant Physiol. 134, 357–363.
[59] Nessler, C. L., von der Haar, R. A. (1989) Planta, submitted.
[60] Annual Report 1988/9, Plant Biotechnology Institute, National Research Council, Saskatoon (1989).
[61] Centro Internacional de Agricultura Tropical, Working document #5; 2, June, 1989.
[62] Inouye, M. (1988) Gene 72, 25–34.
[63] Van der Krol, A. R., Mol, J. N. M., Stuitje, A. R. (1988) BioTechniques 6, 958–976.
[64] Koes, R. E., Spelt, C. E., Mol, J. N. M. (1989) Plant Mol. Biol. 12, 213–225.
[65] Douglas, G. C. (1987) J. Plant Physiol. 130, 73–78.
[66] Arora, R., Bhojwani, S. S. (1989) Plant Cell Rep. 8, 44–47.
[67] Sharma, N., Chandel, S. (1989) personal communication.