

# Somaclonal variation: a morphogenetic and biochemical analysis of *Mandevilla velutina* cultured cells

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## Abstract

Cell cultures of *Mandevilla velutina* have proved to be an interesting production system for biomass and secondary metabolites able to inhibit the hypotensive activity of bradykinin, a nonapeptide generated in plasma during tissue trauma. The crude ethyl acetate extract of cultured cells contains about 31- to 79-fold more potent anti-bradykinin compounds (e.g., velutinol A) than that obtained with equivalent extracts of tubers. Somaclonal variation may be an explanation for the wide range of inhibitor activity found in the cell cultures. The heterogeneity concerning morphology, differentiation, carbon dissimilation, and velutinol A production in *M. velutina* cell cultures is reported. Cell cultures showed an asynchronous growth and cells in distinct developmental stages. Meristematic cells were found as the major type, with several morphological variations. Cell aggregates consisting only of meristematic cells, differentiated cells containing specialized cell structures such as functional chloroplasts (cytodifferentiation) and cells with embryogenetic characteristics were observed. The time course for sucrose metabolism indicated cell populations with significant differences in growth and metabolic rates, with the highest biomass-producing cell line showing a cell cycle 60% shorter and a metabolic rate 33.6% higher than the control (F<sub>2</sub> cell population). MALDI-TOF mass spectrometric analysis of velutinol A in selected cell lines demonstrated the existence of velutinol A producing and nonproducing somaclones. These results point to a high genetic heterogeneity in general and also in terms of secondary metabolite content.

## Key words

- *Mandevilla velutina*
- Secondary metabolism
- Plant cell cultures
- Somaclonal variation
- Phytosteroid
- Anti-bradykinin compounds

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## Introduction

*Mandevilla velutina* (Apocynaceae) is a plant native to Brazil and in several central-western regions folk medicine prescribes the use of infusion or alcoholic extracts of its tuber for the treatment of inflammatory states, including those caused by *Bothrops jararaca* snake bites. The tubers are a source of bioactive compounds able to selectively antagonize the hypotensive activity of bradykinin (BK) and other kinins (1). These anti-BK compounds are secondary metabolites chemically characterized as triterpenoids, e.g., velutinol A (2). However, no significant amounts (~0.001 to 0.0001%, fresh weight) were found in crude plant extracts (3,4). Recently, a patent covering velutinol A and its glycosylated derivatives was issued in Canada (Canadian Patent No. 70857-228, September, 1998; 5), but large-scale biomass production by conventional methods seems not to be economically feasible.

As an alternative method to the destructive exploitation of native plant populations, *in vitro* cell culture of *M. velutina* was adopted (6). The extraction of the bioactive compounds with ethyl acetate from cultured cells yielded crude extracts with an anti-BK action about 31- to 79-fold more potent than that obtained with extract of tubers of the plant (3). This result indicated the potential of this method for obtaining the anti-BK compounds. Somaclonal variation, a common phenomenon in plant cell cultures, characterized by phenotypic variation of either genetic or epigenetic origin, may be an explanation for the wide range of inhibitor activity found. Accordingly, somaclonal variation can be either an advantage (variability increase) or disadvantage (loss of stability of cell lines), depending on the aim of the study (7-9). However, it has become apparent that, rather than being an unexplainable aberration in an otherwise uniform cell proliferation process, the appearance of variants in cell cultures may be a routine

occurrence for certain types of plants or specific explant sources. Since all cells of an organism are derived from a single cell, it has been assumed that plants derived from cells of a donor plant would yield identical individuals. Nonetheless, studies on somaclonal variation have indicated that clonal uniformity is now recognized as the exception rather than the rule (10,11). Recently, quantitative analyses of velutinol A and its glycosylated form (MV 8612) revealed the occurrence of higher content in cell culture extracts when compared to the native plant (12).

Since somaclonal variants result, by definition, from genetic changes ultimately caused by inheritance (13), several critical analyses of this subject have been conducted focusing on the phenotypic, genetic (e.g., aneuploidy, polyploidy, or mixoploidy), cytogenetic and molecular (DNA methylation) alterations found in plant cells and/or tissues in crop breeding programs (8). Several studies have shown that somaclonal variation can be assessed by analysis of phenotype, chromosome number and structure, proteins or direct DNA evaluation of plants (14). The types of variation that are frequently observed may differ from species to species, and it is often difficult to determine the genetic nature of the observed variation (15). However, one should keep in mind that the correspondence between changes at the phenotypic and cytological/molecular level should be considered, since good correlation might not be found between the extent of mutations (molecular level) and phenotypic changes (14). Thus, in the present state of knowledge, we are able to exploit somaclonal variation, but we are still unable to control it (16).

In contrast, analyses of the occurrence of variants in plant cell cultures concerning biochemical phenotype have been undertaken to a lesser extent. It has been recognized that this phenomenon may provide a source for useful variation, which can form the basis for the development of plant cell

lines with characteristics of interest even on an industrial scale. As an example, we may mention the production of shikonin from *Lithospermum erythrorhizon* (17), of berberine from *Coptis japonica* (18), and of ginsenosides from *Panax ginseng* (19). Thus, the information about somaclonal variability can be considered fundamental to studies concerning the production of secondary metabolites (7).

The *in vitro* screening and selection of *M. velutina* somaclones with a high potential for anti-BK production could be done on a chemical basis, taking into account the content of the triterpenoids of interest (13). However, the objective of the present study is to investigate the phenotypic heterogeneity of *M. velutina* cell cultures in a broader sense, taking into account their morphological and metabolic profiles, using light and scanning electron microscopy and growth analysis by the determination of the rate of metabolism of sugars by the cell populations. Furthermore, an investigation concerning secondary metabolites of interest in selected cell lines is reported. In fact, the improvement of the yield of velutinol A in *M. velutina* cell cultures is currently the bottleneck which remains to be overcome before the application of this biotechnological system on a commercial scale.

## Material and Methods

### Cell line cultures

Primary callus cultures were obtained using nodal segments (~8 mm long; 6.5-8.0 mg) from a single 4-month-old plantlet cultured *in vitro*, native to the Cerrado ecosystem (Coromandel, MG, Brazil), on semi-solid MS medium (20) supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid, 2 mg/l 6-benzylaminopurine, and 3 mg/l 6-furfurylaminopurine (MC<sub>24</sub> medium; 12). The cell lines were set up by a classical method (cell-aggregate cloning; 7), selecting high bio-

mass-producing clones from regular subcultures (28 days). Briefly, cell aggregates (~3 mm in diameter) were subcultured from a single cell colony 28 days after inoculation onto filter paper (21) in contact with liquid MC<sub>24</sub> medium. The cultures were kept at 24 ± 1°C, 16-h photoperiod, 31.5 μmol photons m<sup>2</sup> s<sup>-1</sup> (Philips TLF 33), and 85-90% relative humidity. Cell growth biomass was monitored daily by visual inspection for 28 days, followed by transferring ca. 0.1 g of cells of the higher biomass-producing clones selected to fresh semi-solid MC<sub>24</sub> culture medium. From each selected cell line, 0.5 g of 21-day-old cells were subcultured in MC<sub>24</sub> liquid medium to obtain cell suspension cultures as previously described (12). Cell suspensions were maintained in 250-ml Erlenmeyer flasks under continuous light (31.5 μmol photons m<sup>2</sup> s<sup>-1</sup>, Philips TLF 33) and shaking at 110 rpm at 24 ± 1°C. Stock cell line cultures have been cultured according to the above conditions for eight years in our laboratory.

### Determination of dissimilation curves

Growth analysis was performed by determination of dissimilation curves. This is a non-destructive analytical method previously described in detail (22). The dissimilation curves were determined using seven culture flasks (F<sub>1</sub>-F<sub>7</sub>), which were weighed at 24-h intervals on a Mettler analytical balance (range: 200 g, accuracy: 0.1 mg). Inoculated samples (initial cell density = 3 g cells/flask, accurately weighed) were aseptically collected from a single 21-day-old cell suspension stock-culture, and transferred to 250-ml Erlenmeyer flasks containing 50 ml MC<sub>24</sub> culture medium (accurately weighed). Serving as control in respect to water evaporation, three flasks containing only culture medium were also weighed. Silicosen T-32 plugs (Shin-Etsu Polymer Co., Japan, obtained from Simrit Holland B.V., Naarden, The Netherlands) were used to close the flasks in the experiments. The software for

the analysis of the data was kindly furnished by the Division of Pharmacognosy, Leiden University, Leiden, The Netherlands.

### **Synchronous cell cultures**

Synchronous cell cultures were obtained by collecting cell samples (10 ml) from a single 30-day-old cell suspension culture (3 g cells/50 ml culture medium - stationary phase), in order to minimize the effect of any error or variation source, followed by filtration (1.0 mm in diameter) and inoculation of 0.5 g cells on 20 ml MC<sub>24</sub> semi-solid medium. The growth analysis was carried out by determining the dissimilation curves for two independent cell populations.

### **Light microscopy**

Samples of 0.5 ml from 21-day-old *M. velutina* cell suspension cultures (2.1 x 10<sup>6</sup> cells/ml) were collected weekly, centrifuged at 251.5 g for 5 min and washed twice with sterile saline solution (0.85% NaCl). The cells were resuspended in saline solution and samples were stained with 0.2% Coomassie brilliant blue R-250 prior to visualization. Data were recorded by micrography under visible light (100X and 400X) using a microscope (Nikon Labophot, Tokyo, Japan) equipped with a Nikon FX-35 camera.

### **Scanning electron microscopy**

Cells resuspended in saline solution were centrifuged at 1062 g for 5 min and washed extensively with 0.1 M sodium cacodylate buffer, pH 7.2, followed by a 2% glutaraldehyde/2-h dark treatment. Samples were washed twice with sodium cacodylate buffer, resuspended in 1% OsO<sub>4</sub>/15 min in the dark and washed again (three times) in buffer. After centrifuging at 1062 g for 5 min, cells were sequentially treated with EtOH (50, 75, 90 and 100%, 15 min/concentration), gold coated. The material was exam-

ined with a Philips XL 30 scanning electron microscope operating at 15 kV and data were recorded with a Linhof camera.

### **Chromatographic analysis of velutinol A**

For the analysis of the steroid velutinol A in selected cell cultures, 150 g of cells (fresh weight) from 30-day-old cell cultures (inoculum density = 3 g cells/50 ml culture medium) were collected, lyophilized and stored at -20°C. The compound of interest was extracted from lyophilized cells with 2 volumes of ethyl acetate at 4°C/7 days. The extract was filtered, concentrated under reduced pressure (crude extract), followed by the isolation of velutinol A by column chromatography on silica gel, using methylene chloride with increasing amounts of ethyl acetate as eluent (23). The presence of the compound in the column effluent was monitored by TLC on silica gel (Kieselgel 60 F<sub>254</sub>, 0.25 mm, Merck, Darmstadt, Germany), using hexane:diisopropyl ether:acetone (4:4:3) as the solvent system. Detection was performed by first spraying the chromatoplates with anisaldehyde-sulfuric acid, followed by heating at 100°C (3).

### **MALDI-TOF mass spectrometry analysis of velutinol A**

For the detection of velutinol A in the organosolvent fraction of the cell lines we carried out matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, with a PerSeptive Biosystems (Framingham, MA, USA) Voyager MALDI-TOF spectrometer as previously described (24). Briefly, ethyl acetate samples concentrated under reduced pressure (crude extract, see above) were first resuspended in a minimum volume of acetone (~50 µl) and mixed (1:20) with the freshly prepared matrix  $\alpha$ -cyano-4-hydroxycinnamic acid (10 mg/ml), dissolved in H<sub>2</sub>O:trifluoroacetic acid:acetonitrile (4:1:5, v/v/v). A 1-µl aliquot was ap-

plied to the sample target and allowed to dry prior to mass spectrometry analysis. A nitrogen laser with a 337 nm output was used and the spectra were taken in the positive-ion mode using an accelerating voltage of 28 kV. The laser power output was set just above the threshold for ion production.

## Results and Discussion

Cell cultures of *M. velutina* showed an asynchronous growth pattern and somaclones in distinct development stages. Typical meristematic cells with a round shape and translucent cytoplasm were found as the major type, especially along the lag phase (Figure 1A,B,E,F). We also observed a tendency to an elongated shape as the cultures entered the exponential growth phase (Figure 1F). Plant cells are naturally asynchronous with respect to the mitotic cycle, and at any time a culture will contain a combination of cells in different cycle phases (25) as described here. The heterogeneity found might be attributed to the fact that the cell cultures did not originate from a single cell (26). In fact, to account for the lack of uniformity in multicellular explants as in this case, it has been suggested that explants derived from sources

other than protoplasts be called complex cultures due to their multicellular origin (27,28). Because it is unlikely that any two cell types have exactly the same proliferation rate, even after prolonged culture cell lines often remain highly heterogeneous in cellular constitution. As an example, a wide variation in berberine content of individual cells within a *Coptis japonica* cell suspension culture was detected by flow cytometric analysis (29). Indeed, it has been recognized that a cultured cell line seems better defined as a population formed by cells having varied characters at the genetic, biochemical, physiological, and morphological levels, with the resulting phenotype of the population being determined by the predominant cell type. Furthermore, the response of the cell types to external stimuli in terms of the biosynthesis of secondary metabolites, for instance, depends upon their morphological and/or physiological state (16). This is because the cell culture environment not only could induce variations in the cultured cells but could also select a particular cell type. So any cell carrying this trait has a selective advantage over the normal ones and the probability to obtain a mutant with a specific trait is highly increased (30).

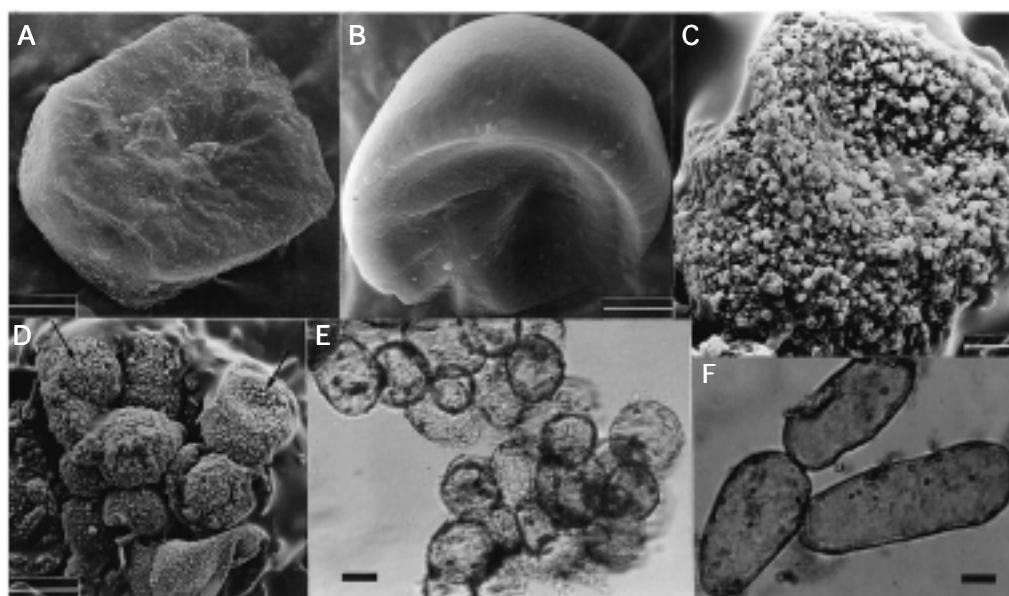


Figure 1. Scanning electron (A, B, C, and D) and light (E, F) micrographs of *Mandevilla velutina* cultured cells showing variations in size, morphology and cell aggregates. A, B, Typical meristematic 7- and 21-day-old cells, respectively (bar = 5 µm). C, Details of the cell surface rugosity of a single cell (bar = 10 µm). D, Cell aggregates with the predominance of globular cell types from a 21-day-old culture. Arrows show the cell surface rugosity (bar = 50 µm). E, Seven-day-old cells (bar = 60 µm). F, Protein staining with 0.2% Coomassie brilliant blue R-250 in 21-day-old cells showing an elongated shape (bar = 60 µm).

Clearly green cell aggregates were usually observed, being formed only of meristematic cells (Figure 1D,E), with a mean range of 16-22 cells/aggregate. We observed a strong tendency to form larger cell aggregates when the cells were cultured in medium containing growth regulators at concentrations ten times lower than used in this study (data not shown). The size of the cell aggregates (240-330  $\mu\text{m}$ ) is a determinant factor of culture behavior because of the different microenvironments to which the cells are exposed. It is also possible that such cells represent a variant phenotype with altered properties (26). Meaningful differences in cell surface rugosity were detected between single cells (Figures 1A-C and 2B) and cell aggregates (Figure 1D) along the culture stage. In fact, this trait seems to be useful for cell heterogeneity characterization in cultured plant cells, for clonal cell screening and for selection with polyclonal antibodies as biochemical markers (12). As a practical example, more recently the characterization of bacterial cells was performed

by analyzing surface molecules (biomarkers) by mass spectrometry (MALDI-TOF), resulting in a mass fingerprint typical of the particular species, and in some cases at the strain level, demonstrating the feasibility of this approach (31).

Cells with embryogenetic characteristics (Figure 2A,B) were also found, suggesting the existence of this morphogenetic pathway. To the best of our knowledge, the occurrence of embryogenesis in cultured cells of *M. velutina* has not been previously reported. As *M. velutina* cultured cells express such a development after a callus stage, this seems to be a case of indirect embryogenesis which is the most common embryogenetic pathway. Previous analyses of *M. velutina* calli showed the occurrence of embryogenetic cells only in cultures older than 21 days. It seems that frequent subculturing (time intervals <15 days) inhibits this morphogenetic pathway, since embryogenetic cells were not found in these populations (32). Again, the existence of any differentiation stage in *M. velutina* cell suspension cultures demonstrated in the present work is interesting, considering that the focus is on the secondary metabolite synthesis (33).

The cultures were able to synthesize chlorophyll (data not shown), thus exhibiting a specialized function, and further transmission electron microscopy analysis (Figure 2C) showed the presence of chloroplasts exhibiting a normal architecture. This aspect is worth mentioning since the absence of specialized cell structures in some cultures may be a further reason for the absence or at least for much reduced levels of accumulated secondary metabolites (16). Chlorophyll content has been used as a parameter to evaluate the occurrence of variations in plant cell and tissue cultures (30,34). In rye, as in other Gramineae, very frequent chlorophyll variations were observed in plants regenerated from immature embryo cultures (30). In *Picea mariana* and *P. glauca*, 0.1 and 0.3% of the plants regenerated by somatic em-

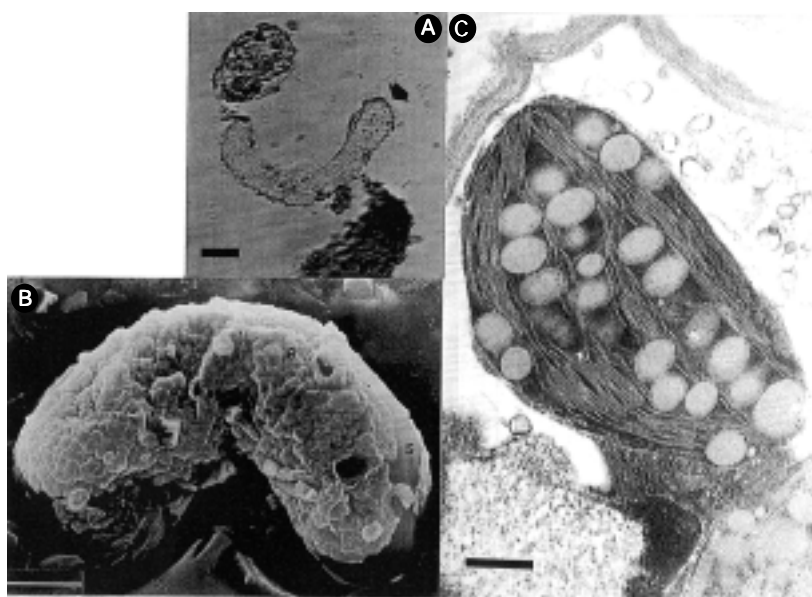


Figure 2. Light (A) and scanning electron (B) micrographs of 21-day-old *Mandevilla velutina* cells showing embryogenetic characteristics and specialized functions. A, Note the initial cell polarization (arrow). Bar = 15  $\mu\text{m}$ . B, Smooth (S) and rough (R) areas on the pro-embryogenetic cell surface. Bar = 3  $\mu\text{m}$ . C, Electron transmission micrograph showing a functional chloroplast containing starch-like granules. Bar = 400 nm.

bryogenesis were achlorophyllous and displayed different distributions and proportion of green and white tissues (*variegata* phenotype). However, chromosome number counts did not show differences between *variegata* and normal plants, emphasizing the fact that phenotypic evaluation seems to be a valuable tool to assess the genetic integrity of clonal propagation processes. Furthermore, in the absence of reliable genetic markers of somaclonal variation, phenotype still represents the easiest and fastest way to identify putative mutants (34).

The dissimilation curves observed are shown in Figure 3A,B. Cell cultures presented a variable growth index, suggesting the presence of somaclones with different metabolic rates in *M. velutina* cell suspension cultures. Similar results were found in semi-solid medium and in cell suspension culture kinetic experiments performed in the absence of light (12).

A detailed analysis of dissimilation curves for the cultures with the highest growth index (F<sub>2</sub>, F<sub>4</sub>, F<sub>6</sub>, and F<sub>7</sub>) revealed meaningful differences among them. The F<sub>2</sub> cell population showed a typical sigmoid growth ( $r^2 = 0.996$ ), with a lag and exponential phase duration of 4 and 14 days, respectively. This growth curve has been characteristic for *M. velutina* cell suspension cultures along the subcultures (>30), with small variations, and we have considered it as a standard (hereafter referred to as control) for the purpose of further comparative analysis. F<sub>6</sub> and F<sub>7</sub> cell populations differed from the control (F<sub>2</sub>) especially concerning lag phase duration (data not shown). A short lag phase (~24 h) was observed for both populations and their growth phase was longer, 16 and 10 days, respectively. In fact, a mathematical model based on linear regression seems to better represent the dissimilation curve for the F<sub>7</sub> cell line ( $r^2 = 0.956$ ), considering its lag and growth phase. High biomass-producing cell lines of *Tabernaemontana divaricata* cells displayed a similar profile for the growth

curve with a short lag phase and also a linear growth phase, but without alterations in the alkaloid content (35). Furthermore, a mathematical treatment by integrating the area of each dissimilation curve revealed highest metabolic rates for F<sub>6</sub> and F<sub>7</sub> cell lines, which were 22.4 and 33.5% greater than the control (F<sub>2</sub>), respectively, while F<sub>4</sub> showed a dissimilation 15.9% lower than the control. Despite the differences for the cell cycle observed here, the maximum dissimilation at the stationary phase did not differ for these

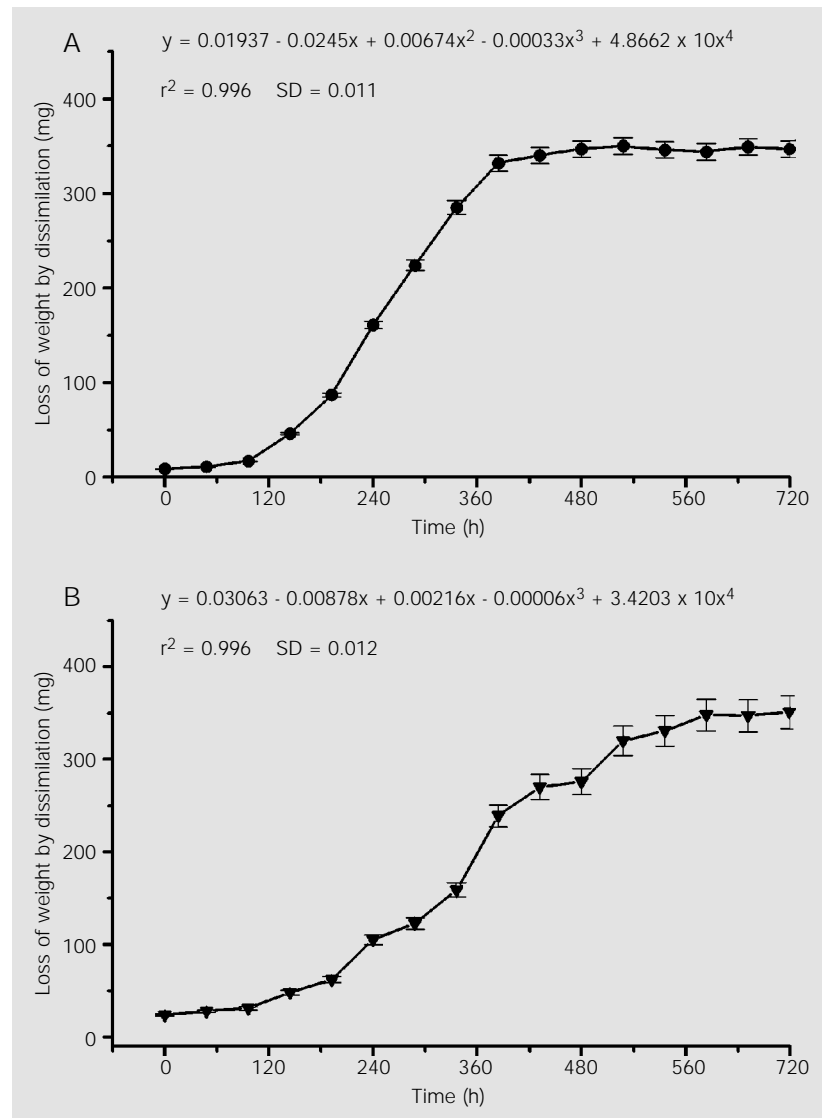


Figure 3. Representative dissimilation curve (A) for a *Mandevilla velutina* cell suspension culture showing a sigmoidal growth pattern - F<sub>2</sub> cell line. A distinct growth rate, with lower biomass accumulation, is shown in (B) for the F<sub>4</sub> cell line ( $P < 0.05$ ).

three populations compared to the control ( $F_2$ ), with a mean value of about 356 mg (25-day-old cultures), suggesting a similar potential for biomass production. The cell suspension cultures with a lower growth rate ( $F_1$ ,  $F_3$  and  $F_5$ ) did not show cells with morphological changes that indicate degeneration or death.

Growth rate has been suggested as a criterion in experiments designed to select cell lines with higher performance (33). Indeed, one might consider it an expression of the genetic variability in cell cultures, which could be correlated with the synthesis rate of the metabolites of interest. Distinct dissimilation rates and indole alkaloid content were

found in three *T. divaricata* cell strains along a culture period of nearly one year (36). The N strain accumulated a lot of intracellular glucose and fructose, with a relatively low dissimilation, whereas strain S behaved in the opposite manner and strain A showed an intermediate behavior. The highest indole alkaloid production content was found in strain N, while strain A showed a lower but stable alkaloid production. No indole alkaloid production was detected in strain S. Interestingly, no evidence for random genetic instability was found in the experiments. Following this approach, thin layer chromatography analysis of the organosolvent fraction (ethyl acetate) extracted from

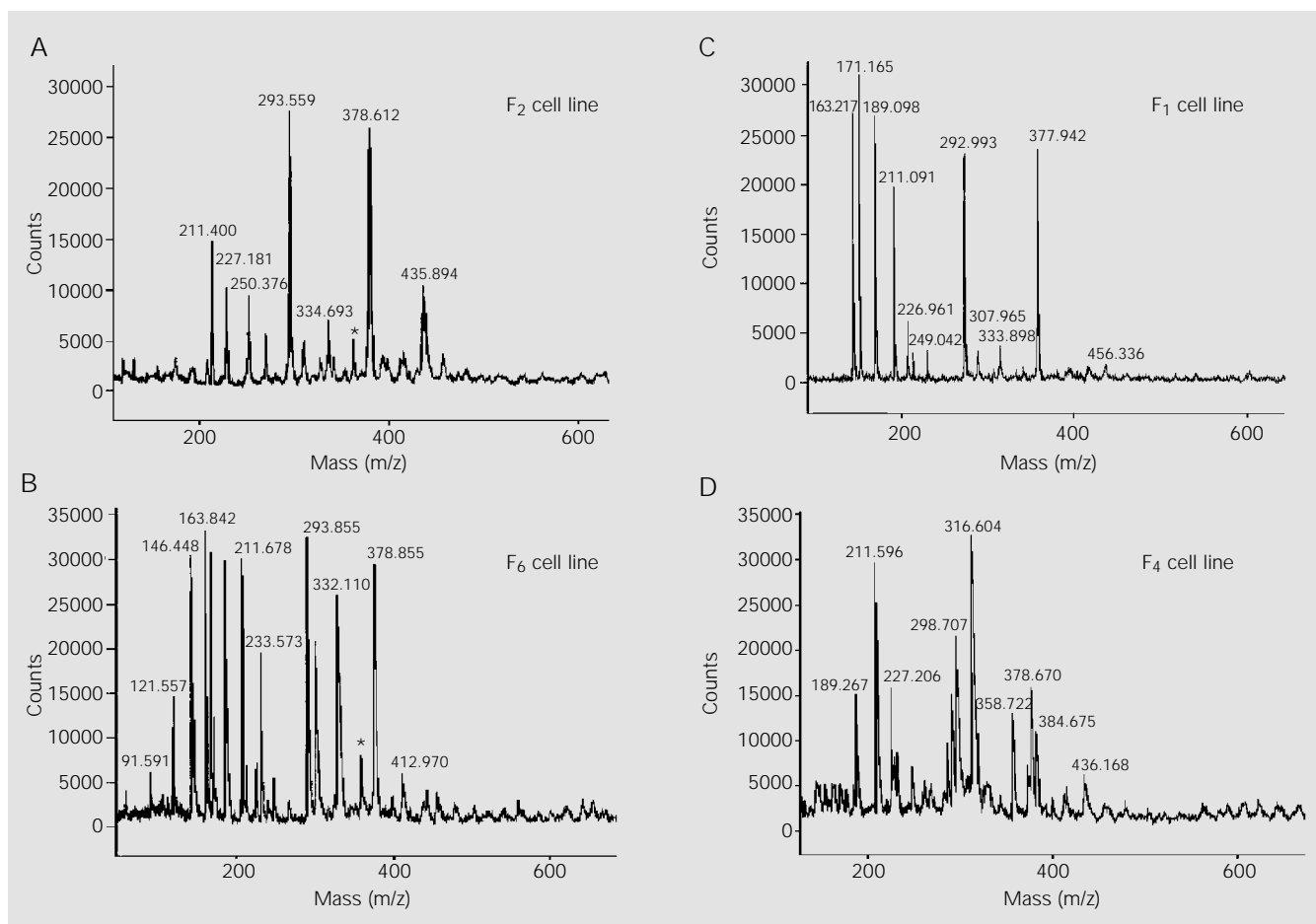


Figure 4. Partial, positive ion detection mode, MALDI-TOF mass spectrometry obtained from the ethyl acetate samples (crude extract) of producing and nonproducing cell lines of *Mandevilla velutina*. A, Producing  $F_2$  cell line and B, producing  $F_6$  cell line. The peak resulting from velutinol A ( $m/z$  362) is marked with an asterisk. C, Nonproducing  $F_1$  cell line and D, nonproducing  $F_4$  cell line. Notice the absence of the peak resulting from velutinol A ( $m/z$  = 362) in C and D.



*M. velutina* cell cultures revealed distinct chromatographic patterns for the compounds of interest (i.e., MV 8608, velutinol A and MV 8610, its glycosylated derivative - data not shown), indicating that some cell lines do not produce them. These findings prompted us to screen the cell lines selected according to their biomass yield in order to detect the compound of interest by using MALDI-TOF mass spectrometry with direct injection of the organosolvent samples into the mass spectrometer. For purposes of comparative analysis among cell lines with respect to velutinol A production, the F<sub>5</sub> cell line was used as control, as previously reported (24). Indeed, analysis of the MALDI-TOF spectra allowed us to confirm the chromatographic findings described above, pointing to the existence of velutinol A producing (Figure 4A,B) and nonproducing (Figure 4C,D) cell lines. Because of the soft ionization, MALDI mass spectrometry has been shown to be a robust technique for the analysis of compounds in the complex biological matrix of a crude plant extract, as shown for chlorinated bisbibenzyls in crude extracts of *Bazzania trilobata* (37). In a similar approach, MALDI-TOF mass spectrometry analysis of 3-deoxyanthocyanidins and anthocyanins was performed using crude extracts of *Sorghum bicolor* tissues, with sensitivities at 15 pmol/μl for 3-deoxyanthocyanidins and as low as 5 pmol/μl for pure samples of anthocyanidin (pelargonidin) and anthocyanin (malvin) (38). This approach would seem to be of interest in plant biotechnology programs for early identification and selection of high producing cell lines, reducing the duration of this usually time-consuming step, as well as for phytochemical studies, monitoring secondary metabolism in plant or animal cells and tissues, and for rapid and accurate characterization of microorganisms of interest (24,31,37). Generally, a primary metabolite is synthesized as a direct result of the metabolic processes that keep the cells alive and growing and it accu-

mulates in parallel with cell biomass. Conversely, a secondary metabolite is usually not formed as a direct result of metabolism that maintains cells in an actively dividing state and the accumulation of these metabolites tends to lag behind cell growth (16). Therefore, culture conditions favoring rapid growth are seldom considered as the best for the biosynthesis/accumulation of secondary metabolites. The present findings, especially considering the F<sub>6</sub> and F<sub>7</sub> cell lines, become interesting as one considers a two-stage strategy as a suitable way of increasing the productivity of plant cell cultures (39).

Since synchronous cell culture is an important tool for further studies of secondary metabolism, the results prompted us to pursue this goal. In preliminary attempts, growth analysis was carried out by determining the dissimilation curves for two independent cell populations and the results are shown in Figure 5A,B. A similar dissimilation profile

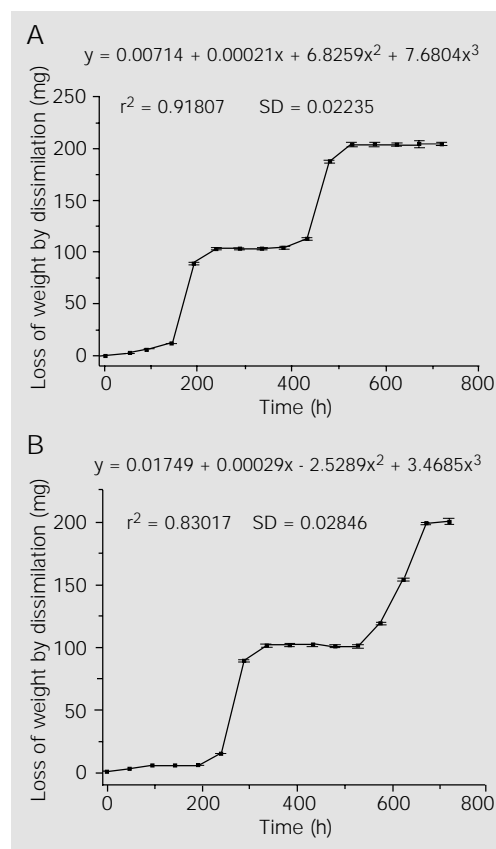


Figure 5. Loss of weight by dissimilation (mg) of two independent synchronous cell lines of *Mandevilla velutina* grown on MC<sub>24</sub> semi-solid medium. The duration of the whole cell cycle (lag-stationary phase) is shown, being 288 h for the S<sub>1</sub> population (A) and 384 h for the S<sub>2</sub> population (B).  $P < 0.05$  using the Student t-test.

was observed for the cell populations in the time course experiments, which showed a synchronous cell cycle. This might be due to the cell harvesting time (30 days after inoculation - stationary phase), since it has been reported that the induction of synchronization of the cell cycle in cultures along the stationary phase can be due to lack of any nutrient (limiting factor). The elapsed time for the growth cycle (lag → stationary phase) was about 288 and 384 h for S<sub>1</sub> and S<sub>2</sub> populations, respectively. Accordingly, the total amount of dissimilation of the former cell population was somewhat higher (35.3%) as calculated by integrating the area of its dissimilation curve.

The results point to a high cell variability present in the cultured cell populations with respect to morphogenetic and metabolic aspects. Furthermore, the findings are evidence that *M. velutina* cultured cells are heterogeneous in their ability to produce the compounds of interest, being mixtures of producing and nonproducing cells. Many plant cell cultures also exhibit variation in secondary product yield over successive subcultures. This phenomenon constitutes a poten-

tial problem for the commercialization of fine chemical production by plant cell cultures, particularly when the instability takes the form of a consistent reduction in yield. One should keep in mind that unpredictable yield fluctuations of 50% or more might make all the difference between an interesting observation and a commercial process. Thus, it seems clear that the variation generated via the plant cell culture process could be interpreted as a hindrance to progress, for example, in an industrial program aiming at drug production from plant cell cultures. In this situation, however, this process of yield deterioration can be arrested by making selections at regular intervals, coupled to techniques scaled up to meet industrial production levels (25,34). Since the somaclonal variation appears to provide an interesting approach to study secondary metabolism in order to select high producing cell lines, the performance of some producing velutinol A cell lines herein described was screened in a two-stage cell culture system (38) in order to optimize the yield of this compound and the results will be published elsewhere.

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