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Variability in tissue cultures of *Choisya ternata*. III Comparing alkaloid production in cell lines obtained by various strategies

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ABSTRACT

Callus cultures of **Choisya ternata** have been prepared by different strategies : aggregate clones, subclones and protoclones obtained from well-established strains ; protoclones obtained from mesophyll tissue ; cultures transformed by **Agrobacterium tumefaciens.** All of them show high variability in their dihydrofuroquinoline alkaloid production. As compared to the alkaloid content of the whole plant, one alkaloid (platydesminium) could be obtained in higher amounts in some lines, but it was impossible to get high-balfourodinium accumulating lines. Moreover balfourodinium-producing capacities were lower in transformed cells as compared to those of normal cell lines.

ABBREVIATIONS

MS : Murashige and Skoog (1962) ; B5 : Gamborg B5 (1968) medium ; 2,4-D : 2,4-dichlorophenoxyacetic acid ; NAA : 1-naphtaleneacetic acid ; BA : N^6-benzyladenine : EtOH : ethanol ; d.w. : dry weight ; sd : standard deviation.

INTRODUCTION

For several years we have been studying the dihydrofuroquinoline alkaloids occurring in some **Rutaceae** species [Rideau **et al.** 1979]. Some of them display cytotoxic and antimicrobial activities and currently we are using **in vitro** culture of **Choisya ternata** as an experimental model to evaluate the extent of variability in alkaloid accumulation according to the strategy adopted for the establishment of the cultures.

In previous studies, we first analyzed 33 **C. ternata** variant strains originating from a single plant [Crèche **et al.** 1985]. We further analyzed the alkaloid production of protoclones and aggregate clones obtained from five of these variants [Trémouillaux-Guiller **et al.** 1987]. In this study we have compared the alkaloid-accumulating capacities of cultures obtained by three other strategies : subclones prepared from a high-producing aggregate clone ; protoclones prepared from mesophyll cells ; cultures transformed with **Agrobacterium tumefaciens.**

MATERIALS AND METHODS

Plant material - The initiation and culture conditions of a population of 33 variant habituated strains of **Choisya ternata** Kunth (**Rutaceae**) originating from a single tree have been previously described [Crèche **et al.** 1985]. Five of these strains, chosen for their different dihydrofuroquinoline-accumulating properties, were used to prepare protoclones and aggregate clones [Trémouillaux-Guiller **et al.** 1985]. The aggregate clone Ac 28 (obtained from the strain V8AL) was used for obtaining subclones. It was maintained on Murashige and Skoog (1962) agar medium containing the following additives : 0.17 M glucose ; 0.55 M myo-inositol ; 48.6 µM pyridoxine-HCl ; 29.6 µM thiamine-HCl ; 40.6 µM nicotinic acid ; 26.64 µM glycine ; 0.8 % (w/v) agar . From the same tree we also obtained in 1982 a culture of foliated stems which was maintained on MS agar medium containing 41.6 mM glucose as sole organic substance [Crèche **et al.** 1985]. Subcultures were obtained by transferring stem explants with 2-3 internodes every 40 days to fresh medium.

Recloning of the Ac 28 aggregate clone - Ten g of 16 day-old tissue were dissociated at 25°C by rotary shaking (75 rpm) in 250 ml Erlenmeyer flasks containing 50 ml of liquid culture medium. After 5 days, the suspension was filtered through a nylon mesh (100 µm). The filtrate was diluted with 0.8 % (w/v) agar medium so that the final concentration was $5x10^4$ single cells and small aggregates (≤ 3 cells) per ml. Incubation was performed in Parafilm-sealed plastic Petri dishes (9 cm diameter ; 10 ml medium) under continuous white light (500 lx, Philips TLD 18 W/33 lamps) at 25°C. Aggregate-clones (1-2 mm diameter) were transferred onto agar culture medium (5.3 cm diameter Petri dishes ; 10 ml medium ; one clone per dish) and grown for 2 passages. One month-old tissues were lyophilized at the end of the second passage and their alkaloid contents were estimated.

Isolation and culture of mesophyll protoplasts - Protoplasts were isolated from leaves (0.2g) which had been removed from the upper part **in vitro** foliated stems described above. After peeling off the lower epidermis, the leaves were placed lower surface down onto 4 ml of a filter-sterilized solution (0.22 µm ; Millipore Ltd AH/A) containing 0.125 % (w/v) macerozyme R10, 0.25 % (w/v) cellulase R10, 0.34 M $CaCl_2 \cdot 2\ H_2O$ and 0.6 M mannitol in 5x diluted MS inorganic salts. Incubation was carried out at 27°C in the dark. After 14-16 hrs, the digested leaves were filtered through a nylon sieve (100 µm pore size) and the protoplasts were washed 3 times by gentle centrifugation (50xg, 3 min) in 4 ml fresh culture medium. Protoplast viability which was verified using the induction of fluorescence by a 23.5 µM fluorescein diacetate solution in acetone for 3 min, was about 90 %. The isolated protoplasts were initially cultured at a density of 10^5 per ml in a liquid medium containing the MS inorganic salts, 0.35 M mannitol, 0.25 M glucose, 4.52 µM 2,4-D, 5.37 µM NAA, 0.46 µM kinetin, 0.44 µM BA, and the organic additives mentioned above for Ac 28 cultures. Incubation was performed in Parafilm-sealed Corning Petri dishes (3.5 cm diameter) in the dark at 27° for one week (at this time about 20 % protoplasts have undergone at least one division). Then they were transferred to continuous light (500 lx). After one month the dishes were

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replenished every week with 100 µl of fresh medium (the same composition as above except that the glucose and mannitol concentrations were reduced to 0.1 M and 0.2 M respectively. After three months, visible protoclones (0.5 - 1 mm diameter) were transferred onto MS agar medium supplemented with the organic additives and growth substances mentioned above, 0.15 M glucose and 0.05 M mannitol. The next passages occurred on MS agar medium of the same composition except that the glucose concentration was 0.17 M and the mannitol was omitted. Protoclones were subcultured every 3 weeks.

Transformed cell lines - A nopaline strain of **Agrobacterium tumefaciens** (C58-587) was used. It was maintained on agar slopes containing 0.5 % (w/v) tryptone ; 0.25 % (w/v) yeast extract ; 0.27 M mannitol ; 680 µM L-glutamic acid ; 180 µM KH_2PO_4 ; 40 µM $MgSO_4 . 7H_2O$; 171 µM NaCl and 4.1 µM biotine. In March 1986, sterile foliated stems cultured **in vitro** were wounded between nodes and infected with a 36 hour-old bacterial suspension grown at 25° (about 5×10^9 bacteria per ml). Inoculated material was incubated at 25° in continuous white light (500 lx) for 6 weeks. Three galls which formed on the stems were aseptically excised, cut into small fragments and transferred to hormone-free B_5 [Gamborg **et al.** 1968] or MS media containing 0.6 mM cefotaxim. The media were solidified with 0.9 % (w/v) agar. The tumor tissues were maintained on cefotaxim-containing media for 3 passages (one month each). The antibiotic was then omitted and the tissues were repeatedly grown on B_5 or MS medium.

Nopaline assay - Samples of about 10 mg of callus (dry weight) were homogenized in 1.5 ml 85 % (v/v) EtOH. Nopaline dehydrogenase activity in extracts was detected by paper electrophoresis and phenanthrenequinone staining using the method of Otten and Schilperoort [1978].

Extraction and analysis of alkaloids - Alkaloid amounts were evaluated in 5 week-old tissues by a spectrofluorodensitometric method as previously described [Montagu **et al.** 1980]. The detection limits were 3 pmoles for platydesminium and 0.1 pmole for balfourodinium.

RESULTS

Variation of alkaloid in subclones - The strain V8AL belonging to a population of 33 variant strains [Crèche **et al.** 1985] was cloned using an aggregate cloning procedure. The frequency distributions of platydesminium and balfourodinium contents in clones are given in Fig. 2B-3B. One of the high platydesminium accumulating clone (named Ac 28) was selected, subcultured for one year and then recloned : we adopted the aggregate cloning procedure which was easier to carry out and which gave similar results to protoplast cloning [Trémouillaux-Guiller **et al.** 1987]. Fig 1C-D and 2C-D give the frequency distributions of alkaloid contents in subclones obtained from Ac28 in 2 separate experiments. The distribution patterns were similar in form to those obtained by cloning the strain V8AL (Fig. 1B-2B) or other strains of the population variant strains [Trémouillaux-Guiller **et al.** 1987]. The aggregate clone AC28 gave subclones accumulating very different levels of alkaloids : balfourodinium ranged from 0 to 300 $\mu g.g^{-1}$ d.w. and platydesminium ranged from 100 to about 3000 $\mu g.g^{-1}$ d.w. More than 60 % of subclones accumulated balfourodinium in amounts lower than 50 $\mu g.g^{-1}$ d.w. Morover, alkaloid mean values were lower than the corresponding values of the original cell line Ac28.

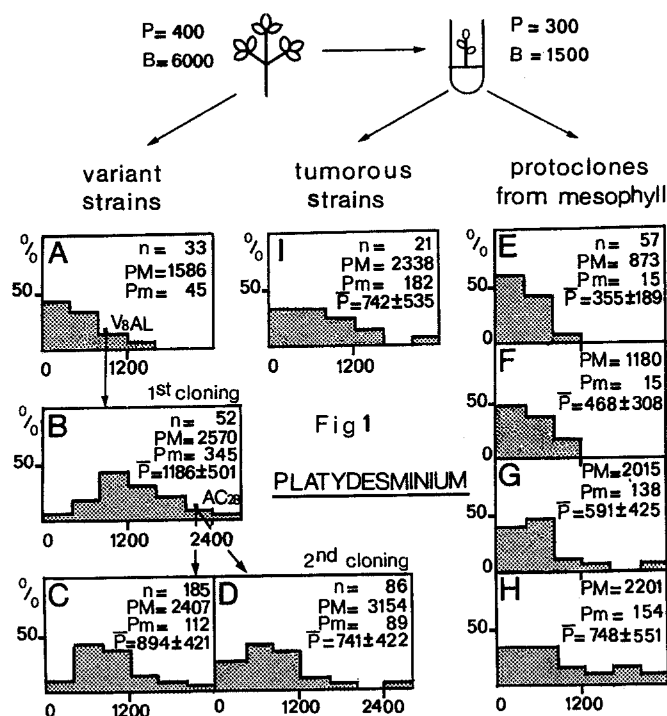
Alkaloid accumulation in protoclones derived from mesophyll tissue - As high balfourodinium production cannot be achieved by a cloning procedure applied to stabilized strains, we attempted to obtain protoclones directly from mesophyll cells in order to compare the extent of the variability of alkaloid contents with that observed in the populations of clones (and subclones) prepared from well-established strains. Mesophyll protoplasts were isolated from **in vitro** cultures of foliated stems originating from the same tree used for the establishment of the strains. Recently isolated protoclones were chlorophyllous and very hard. Their appearance was similar to primary callus developing on stem explants. Most of them however became more and more friable upon further subculturing. Analysis of 57 protoclones showed that all of them could accumulate platydesminium and balfourodinium but in different amounts. As for the aggregate clones and protoclones obtained from the established strains, the frequency distributions of alkaloid contents showed a Poisson's law pattern. The variability increased during several passages : for example, balfourodinium contents ranged from 12 to 192 $\mu g.g^{-1}$ (d. w.) at the first passage and from 9 to about 700 $\mu g.g^{-1}$ at the 5th passage (Fig. 2 E-H). During this period, the mean values in platydesminium and balfourodinium increased from 355 to 748 $\mu g.g^{-1}$ and from 68 to 165$\mu g.g^{-1}$ respectively.

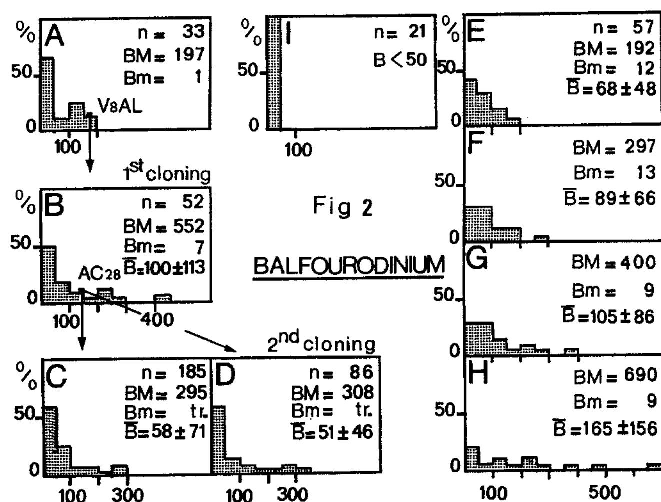
Alkaloid accumulation in transformed cell lines - Tumorous lines were established from 3 crown galls developed on sterile foliated stems cultured **in vitro,** after inoculation with **Agrobacterium tumefaciens.** Twenty one variant strains were obtained by plating small clumps of transformed tissues on solid medium, repeatedly growing callus tissues on various hormone-free media (MS or B_5) under various environmental conditions (light or dark), then checking visually for modified phenotypes. These variant strains showed different phenotypes and accumulated nopaline in various amounts. They showed a large variation in platydesminium content (from 180 to 2340 $\mu g.g^{-1}$: Fig. 1-I). In contrast, the balfourodinium content was very low (30$\mu g.g^{-1}$) (Fig. 2-1) in all strains studied.

DISCUSSION AND CONCLUSION

All the cultures studied here originated from a single tree. Results obtained by recloning an aggregate clone of **C. ternata** highly resemble those found by Dougall **et al.** (1980) on the production of anthocyanins in clones and subclones prepared from carrot tissue cultures. First, the alkaloid production of subclones was distributed widely as compared to that of the original aggregate clone. There is some evidence that the cloning process itself might have destabilizing effects on the cell phenotype [Pétiard **et al.** 1986]. This instability appears to be very high for balfourodinium accumulation. Second, if we compare maximum and mean values of alkaloid contents in the protoclones and aggregate clones derived from established strains (see results in Trémouillaux-Guiller **et al.** 1987) and in the subclones obtained from one of these previous clones, it seems that alkaloid accumulation capacities of all the clones reach an upper limit.

Mesophyll cells are considered to be more homogeneous than callus cells [Jullien 1980]. Despite this, protoclones derived from mesophyll cells of **C. ternata** differed from each other in their dihydrofuroquinoline alkaloid production : protoplasts from mesophyll can be considered as very small primary explants leading to different cell lines. Similarly, a great variability exists in indole alkaloid production of protoclones derived from one leaf of **Catharanthus roseus** [Constabel **et al.** 1981] or in scopolamine content of protoclones derived from mesophyll cells of **Hyoscyamus muticus** [Oksman-Caldentey and Strauss 1986]. Moreover, both the scopolamine and the dihydrofuroquinoline-accumulating capacities of the protoclones appear to be unstable : we found a progressive increase in the extent of variability during the first subcultures. We also established a population of **C. ternata** cultures transformed by **Agrobacterium tumefaciens.** These cultures were grown in phytohormone-free media and the transformation was confirmed by detection of nopaline. Although crown gall tumors and the transformed tissues derived from them





Figures 1-2 : Frequency distribution of platydesminium (Fig.1) and balfourodinium (Fig.2) yield in C. ternata cell lines.

(A) Variant strains obtained from two stem explants * ; (B) Aggregate clones prepared from the variant strain V8AL* ; (C,D) Subclones prepared from the aggregate clone AC28 ; (E-H) Protoclones prepared from mesophyll cells (E : 1st, F : 2nd, G : 3th, H : 5th passages) ; (I) Transformed cell lines. Each class of the distribution is characterized by its relative frequency. For each distribution, the following parameters are reported : n = total number of cell lines, XM = maximum yield, Xm = minimum yield, X = mean sd ; X = P for platydesminium, B for balfourodinium.
Alkaloid yields are given in µg g^{-1} dry weight. (*) : complete results in the previous work : Trémouillaux-Guiller **et al.** 1987.

have been studied for many years, there are relatively few works on secondary metabolite production by these tissues. Experiments with tumorous cultures of **Cinchona succirubra** [Payne **et al.** 1987] and **Catharanthus roseus** [Eilert **et al.** 1987] showed that they can accumulate secondary metabolites. Here we show that transformed cultures of **C. ternata** are able to synthesize dihydrofuroquinoline alkaloids. Regarding platydesminium, these cell lines behave like non-tumorous cultures and presented a wide variability in their capacities of accumulating this alkaloid. On the contrary, balfourodinium accumulating capacities are lower compared to those of non-tumorous cell lines.

If we compare the alkaloid-accumulating capacities of the different cell lines to those of the leaves (which accumulate the highest amounts of alkaloids), we can see that it is always possible to get lines which accumulate platydesminium in higher amounts than the mother plant. For balfourodinium, the best method was the cloning of protoplasts obtained from mesophyll cells : with this procedure we obtained the highest balfourodinium-accumulating lines (about 700 µg.g^{-1} d.w.). However it is necessary to monitor their production capacity during subculturing.

These results show that the use of inherent variability **in vitro** is an efficient procedure to get high-platydesminium accumulating cell lines whatever the strategy used. On the other hand we could not obtain high-balfourodinium accumulating lines : this 8-methoxy-derivative of platydesminium is always accumulated in much lower amounts than in the plant. Other strategies may be necessary for achieving high-balfourodinium production in cultures of **C. ternata.**

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