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SELECTION OF HIGH NICOTINE-PRODUCING CELL LINES OF TOBACCO CALLUS BY SINGLE-CELL CLONING

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; plant tissue culture; selection; variation; single-cell cloning; synthesis of nicotine.

Abstract—Single-cell clones of *Nicotiana tabacum* callus showed wide variation in the production of nicotine. An efficient screening of numerous clones was made possible by the adoption of the 'cell squash method' devised for estimating the approximate alkaloid content of a small piece of callus. From these clones, several cell lines with higher yield of nicotine (1.0–3.4% of dry wt) have been isolated by repeated clonal selection.

INTRODUCTION

Since Dawson [1] described the presence of nicotine in the tobacco callus in 1960, several workers have reported the synthesis of nicotine by various callus cultures of *Nicotiana* species [2–7]. However, the nicotine contents of these callus cultures were strikingly low compared with whole plants, except for a culture reported by Ohta *et al.* [7] which showed a content as high as 1.7% of the dry wt of tissue. Although the nicotine production by tobacco callus cultures is regulated by certain environmental factors such as plant growth regulators [4–6], the important role of genetic factors has also been suggested by Tabata and Hiraoka [8], who found wide variation in nicotine-producing capability among single-cell clones isolated from a callus tissue of *Nicotiana rustica*. The present experiments with *N. tabacum* callus cultures were conducted to examine not only the effect of clonal selection in the improvement of the nicotine productivity, but also the stability of the selected cell lines during a long-term subculturing. A simple device for detecting clones with higher alkaloid yield is also described in this paper.

RESULTS

The nicotine content of the original callus strain BY-12H used for the present experiments had fluctuated between 0.004 and 0.05% of dry wt during subculturing since 1967 before it was found to be as high as 0.89% in November, 1975. Although the reason for this sudden increase was not clear, the finding suggested the high biosynthetic potential of this strain and a selection program was initiated in June, 1976 to improve the nicotine-producing capability of the callus culture by repeated cloning and screening of single-cell clones.

Screening of clones by the cell squash method

A primary screening test for single-cell clones with higher yield of alkaloid was carried out by utilizing the 'cell squash method' described in the Experimental, which was devised to facilitate a rough but quick estimation of the relative alkaloidal quantity for a large number of minute tissue samples. The clones assayed by this method were scored from 0 to 4 according to the intensity of orange-colored spots that appeared on the filter paper underneath the squashed tissues after the application of Dragendorff's reagent. The correlation between the scores recorded by the squash method for 121 clonal samples and the actual nicotine contents determined by UV for the steam distillates from the corresponding tissue is illustrated in Fig. 1. Although the actual nicotine content varied to a considerable extent even among the tissues with the same score, a high correlation coefficient ($r = 0.80$) was obtained between the values measured by the two different methods. These results suggest that the cell squash method is of practical value for an efficient screening of high-yielding clones. The amount of callus tissue necessary for the detection of alkaloid by this

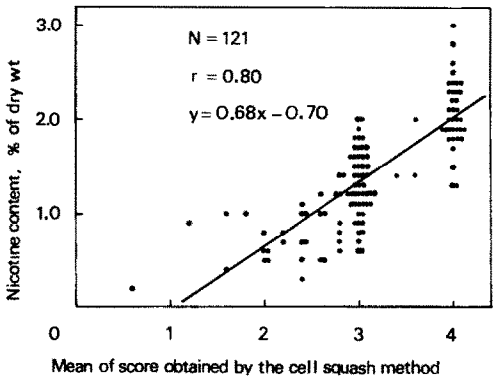

Fig. 1. Correlation between the nicotine content of single-cell clones and the score obtained by the cell squash method. The score of each point represents the mean of 5 tissue samples taken from each clone. For the explanation of scores (abscissa), see the Experimental.

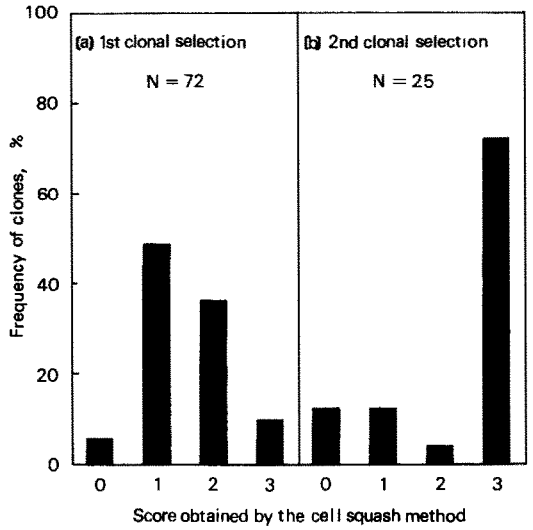

Fig. 2. Frequency distribution of single-cell clones with different nicotine contents. (a) Clones isolated by the first cloning of the original callus culture. (b) Clones developed from the single cells of a parent clone which showed the highest nicotine content in the first clonal selection. For the explanation of scores (abscissa), see the Experimental.

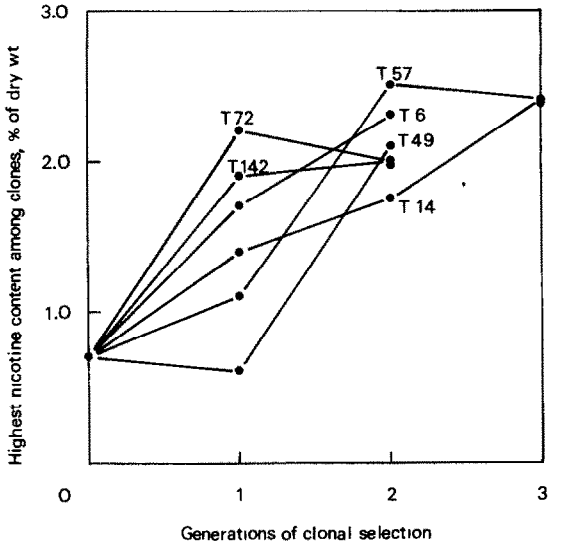

Fig. 3. Effect of repeated selection on the nicotine content in various cell clones.

method may be as small as 5 mg when the nicotine content is more than 0.5%. In the process of selection, only those clones with higher scores were subcultured to provide sufficient amounts of callus tissues necessary for confirming their actual nicotine contents by UV.

Isolation of high nicotine-producing cell lines

Single-cell clones from the original strain, whose nicotine content was 0.7%, showed wide variation in the nicotine content (for example see Fig. 2). The score obtained by the cell squash method varies from 0 to 3, the majority of clones scoring 1 and 2. Such variation in the nicotine content was observed also in the single-cell clones of the second cycle which were isolated from a callus culture showing the highest nicotine content in the first clonal selection. However, a great number of clones of the second cycle contained more nicotine than those of the first cycle.

Fig. 3 shows the highest nicotine contents found in *ca* 100 clones for each of 6 replicated experiments at consecutive cycles of clonal selection. It is seen that the nicotine content was remarkably increased by the first cycle of selection. In the second cycle, some of the cell lines gave rise to single-cell clones showing even higher nicotine contents. By the two cycles of cloning, 5 high nicotine-producing clones were selected from *ca* 1000 single-cell clones tested, i.e. clones T72 and T142 by the first clonal selection and clones T14, T57 and T49 by the second clonal selection. The highest nicotine content

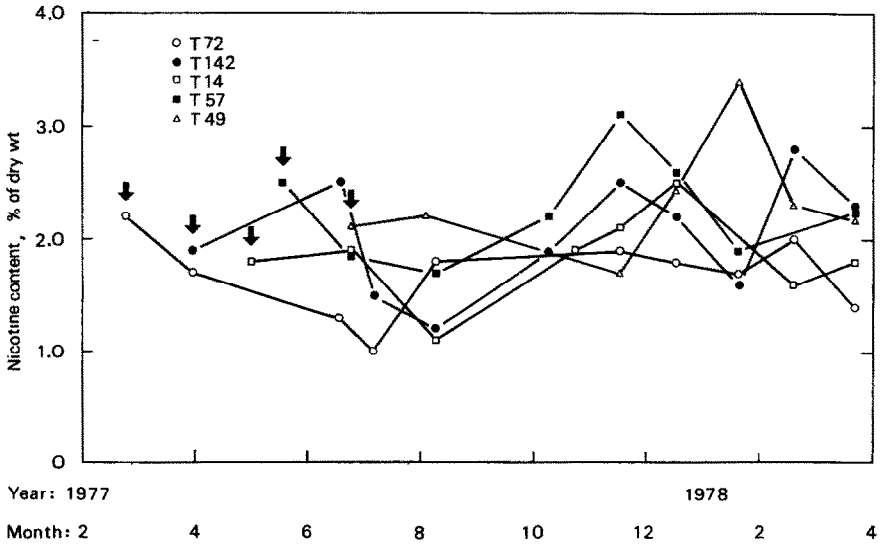

Fig. 4. Fluctuation in the nicotine contents of 5 selected cell lines during successive generations in the absence of artificial selection for nicotine content. Each arrow indicates the date of isolation of the high nicotine-producing cell line.

observed was 2.5% in clone T57. In contrast to the foregoing clonal selection, the third cycle of cloning was no longer very effective in improving the yield of nicotine.

The alkaloidal components of the 5 high nicotine-producing lines mentioned above were compared with those of the original strain BY-12H by TLC analysis. The results showed that the original strain contained nicotine as the major alkaloid and anatabine, anabasine and nornicotine as minor alkaloids. Nicotine was also the major alkaloid in all of the high nicotine-producing lines, but only nornicotine was detectable as the minor alkaloid in these lines.

Stability of cell lines

To examine the stability of the selected cell lines (T14, T49, T57, T72 and T142) during a long-term subculturing, their nicotine contents were measured at consecutive transfer generations. As shown in Fig. 4, the nicotine contents of the tested lines more or less fluctuated through a year of subculturing without selection. For example, the nicotine content of T72 once decreased from 2.2 to 1.0% and then increased up to 2.0%. In the case of T49, the nicotine content increased from 2.1 to 3.4% after 7 months.

Time course of nicotine production

Fig. 5 shows the time course of growth and nicotine production in the original strain and the selected line T14. Both cultures show a similar growth pattern without significant difference in the dry wt of tissue. The nicotine synthesis is initiated at the exponential growth phase (the 2nd week) in both cultures, but the selected line shows a higher rate of nicotine production during the growth period. At the 6th week, the nicotine content as well as the dry wt reach the maximum in both cultures, followed by a gradual decrease as the tissue begins to deteriorate. It is concluded therefore that the high nicotine content of T14 is not due to a shortening of the lag period prior to nicotine synthesis but to a higher production rate.

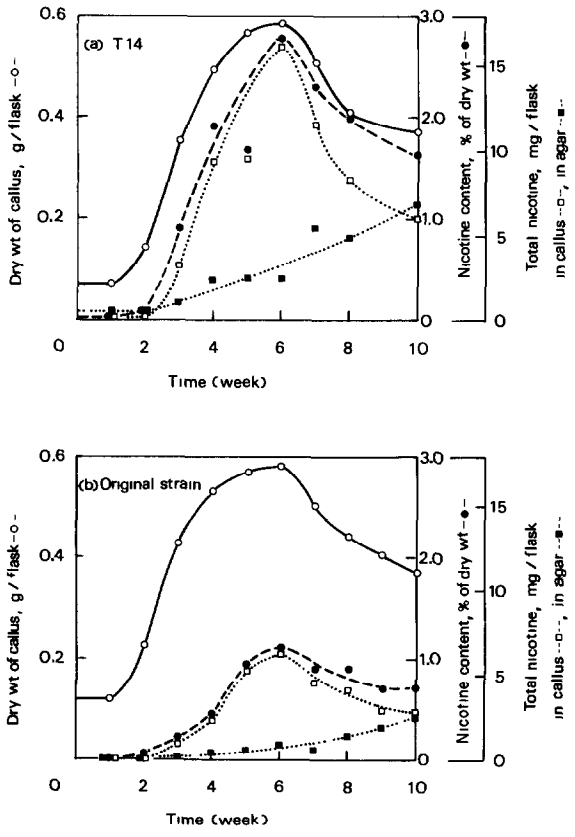

Fig. 5. Time course of growth and nicotine production in the high nicotine-producing line T14(a) and the original strain (b).

DISCUSSION

One of the major difficulties in attempting to improve the biosynthetic capability of cultured cells by artificial selection lies in the necessity of quantitative estimation of each specific secondary metabolite for a large number of clonal cultures isolated from the parent cultures. Therefore, most of the selection experiments hitherto reported have dealt with easily detectable variations in pigments such as anthocyanins [9], carotenoids [10] and naphthoquinones [11], except for a few works on non-pigmented products [8, 12]. Zenk *et al.* [12] applied radioimmunoassay for the quantitative determination of serpentine and ajmalicine contained in the cell clones of *Catharanthus roseus*. Although radioimmunoassay is an ideal method for the efficient analysis of minute amounts of secondary products, it requires the preparation of adequate haptens and specific antibodies, which are often difficult for particular compounds. Hence, we have devised the 'cell squash method' for a simple estimation of the alkaloid content of the tobacco cell clones. This method gives only a rough estimate of the alkaloid content, but it proved to be practical when used for primary screening of high alkaloid-producing clones. It is expected that this economical method could be applied to the detection of not only Dragendorff-positive alkaloids but also a variety of other compounds which react with specific reagents to give colored spots.

It has been suggested by the studies of single-cell clones derived from *Nicotiana rustica* [8] and *Catharanthus roseus* [12] that the callus cultures consist of heterogeneous cells which differ in their production of alkaloids and that selections of single cells are highly effective in increasing the alkaloid contents of cell lines. Zenk *et al.* [12] have succeeded in increasing the content of serpentine and ajmalicine up to 1.3% of dry wt in a selected cell line, a 1.5-fold increase over the original plant. Also, the present experiments on tobacco callus cultures have clearly demonstrated the effectiveness of cell selection and the selected cell line attained a nicotine content as high as 3.4%.

It has been shown that the nicotine content of the selected lines is more or less variable during successive transfer generations, even though they still maintain a nicotine content higher than 1% after a long period of subculturing over 12 months. This fact seems to suggest the necessity of making a continual selection to establish a more stable cell line.

Although the genetic basis of cellular variation regarding the biosynthetic capability in plant tissue cultures has not yet been clarified, the present study indicates the importance of the isolation and selection of variant cells for establishing a superior cell line with higher yield of useful secondary metabolite.

EXPERIMENTAL

Culture stock and culture method. The callus culture stock BY-12H, originally derived from the stem pith of *Nicotiana tabacum* L. cv 'Bright Yellow' in 1965, has been maintained on the Linsmaier–Skoog basal agar medium [13] supplemented with 10^{-5} M IAA and 10^{-6} M kinetin (standard medium) at 25° in the dark for 11 yr by successive transfers carried out with 1 month interval.

Single-cell cloning. Small pieces of the callus tissue were inoculated in 30 ml of the Linsmaier–Skoog liquid medium supplemented with 10^{-5} M kinetin in a 100 ml Erlenmeyer flask and cultured for 1 week on a reciprocating shaker at a speed of 95 rpm. IAA was omitted from this medium, because dividing cells showed a tendency to form a multicellular chain in the presence of auxin. The cell suspension obtained was filtered through a nylon sieve (mesh size: 148 μm). The filtrate contained single cells and a small number of linearly dividing cells consisting of 2–8 cells which were too small to be removed by filtering. This filtrate was centrifuged at 80 *g* to concentrate the cells. Since the single cells of BY-12H failed to grow when plated directly on any nutrient agar medium, they were grown to form colonies by the application of the nurse culture technique [14]: a suspension (0.5 ml) containing 1000–5000 cells was poured uniformly from a pipette onto filter paper, which had been placed on the previous day on a callus aggregate of the parental stock cultures on standard medium and after 2–3 weeks, each of small clones (*ca* 1 mm in dia) developing from single cells were isolated individually using a needle and transferred to standard medium (10 ml) to continue culture for 1–2 months. Single cell clones thus established were subjected to screening tests for alkaloid content and subcultured to provide the materials for a further single-cell cloning.

Cell squash method. The primary screening of high alkaloid-producing clones was carried out by the 'cell squash method' as follows. A small piece of callus (*ca* 2.5 mm in dia) was sampled aseptically from each clone. 5 pieces were arranged linearly on a strip (2 × 20 cm) of filter paper (Toyo No. 51) with an interval of 3 cm between 2 glass plates and then squashed thoroughly by the downward pressure applied evenly from the top of the glass plate so that the cell sap squeezed out of the cells was absorbed into the filter paper. Then a modified Dragendorff's reagent [15] was sprayed on the reverse of the filter paper to visualise the alkaloids, which were scored according to intensity of the reddish orange color: 0 = uncolored; 1 = faint; 2 = moderate; 3 = deep; and 4 = very deep. Those clones scoring 3 or 4 were selected as high-alkaloid producers. The minimum amount of nicotine detectable by this method was *ca* 0.1 μg/callus piece.

Analysis of alkaloids. The analysis of alkaloids by TLC and the estimation of the nicotine content of callus tissues by UV were carried out according to ref. [8].

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