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THE SELECTION OF CULTURED PLANT CELL LINES PRODUCING HIGH LEVELS OF BIOTIN

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Key Word Index—*Lavandula vera*; Labiatae; cultured plant cells; biotin content; selection; variation; gamma ray irradiation; pimelic acid.

Abstract—We have found that biotin is synthesized in many species of cultured plant cells, e.g. *Lavandula vera* (Labiatae), *Nicotiana tabacum* (Solanaceae) and *Glycine max* (Leguminosae). Cultured green *L. vera* cells grown under light contained the greatest amounts of free biotin of the cells studied although the specific amounts varied among the cell lines. Cell lines were selected after their free biotin contents had been analysed. Cells containing large amounts of free biotin were cultured repeatedly, analysed and reselected. Lines with high levels of free biotin were obtained from cells which survived on a medium containing pimelic acid and L-alanine or from gamma irradiated cells. One *L. vera* cell line obtained from irradiated cells contained seven times the amount of free biotin found in the original unselected cultured cells and four and a half times that found in the leaves.

INTRODUCTION

Cultured plant cells are heterogeneous and it is possible, therefore, to select for cells with a particular desired property. Photoautotrophic cells [1–3], resistant cells [4–6] and cells which contain higher concentrations of metabolites than the intact parent plant [7–11] have all been obtained. Research on the mass production of vitamins has produced cultured tobacco cells which contain more ubiquinone-10 than normal tobacco leaves [9] and cultured *Cytisus scoparius* cells which contain more vitamin B_6 than the leaves of the same plant [10].

Studies of the biosynthesis of biotin by microorganisms have been made in which pimelic acid and L-alanine are reported to be precursors of biotin [12, 13]. However, high concentrations of pimelic acid actually showed a toxic effect on cultured plant cells, but biotin did not have any such effect even though large amounts of it were taken into the cells. We, therefore, used pimelic acid as a means of selecting those cells which could change the acid into biotin.

One mutant of *E. coli* which produces excess biotin has been isolated by mutagenesis [14]. Based on this result, we used gamma ray irradiation to obtain those mutants which produce excess biotin.

Bound biotin which exists as a coenzyme is only present in trace amounts and cells with a high free biotin level are believed to contain a high total biotin level. Therefore, only the amounts of free biotin were used as indicators of selection. Cell lines with high levels of free biotin were obtained from cells resistant to pimelic acid. These lines could also be induced by gamma ray irradiation and were isolated by cell cloning.

RESULTS AND DISCUSSION

Variations in the free biotin content of cultured plant cells

When the free biotin content in cultured plant cells was measured (using the paper disc plate method with *Lactobacillus plantarum*), it was found to maximize during the stationary growth phase (Fig. 1). This increase was similar to those found for ubiquinone [15] and vitamin B_6 [10] in cultured plant cells.

The free biotin contents of various cultured plant cells were measured during the stationary growth phase. Some cultured plant cells contained free biotin. Cultured green *L. vera* cells grown under light contained the greatest amounts (0.061 μg/g fr. wt) of all the cells studied (Table 1). In some of the remaining cultures, no free biotin was detected. We confirmed that these cells did not contain any specific inhibitors against *L. plantarum* in the biotin bioassay system, since the bacteria grew when the corresponding cell extracts were added to the assay

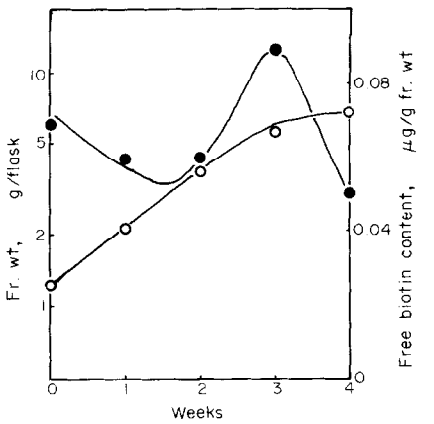

Fig. 1. Time courses showing growth (○) and free biotin content (●) in cultured green *L. vera* cells. Doses consisting of 1.3 g fr. wt of cells were inoculated on Linsmaier and Skoog agar medium (25 ml) with 10^{-5} M IBA and 10^{-6} M 6-benzyladenine and incubated at 27° under light (4 klx).

Table 1. Free biotin content in cultured plant cells

Cells from	Culture condition	Free biotin content (μg/g fr. wt)
Nicotiana tabacum var. Samsun	light	0.019
	dark	0.008
Nicotiana tabacum var. Bright Yellow	dark	0.011
Lavandula vera	light	0.061
	dark	0.028
Glycine max	light	0.015
Cytisus scoparius	light	N.D.
	dark	0.009
Bouvardia ternifolia	light	N.D.
	dark	N.D.
Jasminum officinale	dark	0.008
Beta vulgaris	light	N.D.
Rauwolfia serpentina	dark	N.D.
*Hyoscyamus niger**	light	0.011
	dark	0.011

N.D. = not detected.
*Liquid culture.

medium containing biotin (0.1 μg/ml). Thus, free biotin levels vary in the cell cultures from species to species. However, the higher levels were found only in cultured green cells grown under light (e.g. *L. vera*, *N. tabacum* and *G. max*). This indicates that specific green cells containing large amounts of free biotin must be chosen if biotin is to be produced.

Selection of cell lines which produce high levels of biotin and the effects of pimelic acid or gamma rays on the selection

Cultured green *L. vera* cells containing the highest amounts of free biotin were used for the selection assay (Table 1). After 3 weeks plate culture, 30% of the inoculated cell aggregates formed clones on the basal medium. The addition of pimelic acid (100 μg/ml) and L-alanine (100 μg/ml) to the medium decreased the rate of clone formation by one-fifth due to the toxicity of pimelic acid. Those cells able to resist pimelic acid and survive seem to have a greater capability of changing pimelic acid into non-toxic biotin.

To test the effect of gamma rays, cells were irradiated at 10 kR. This dosage decreased the rate of clone formation by one-tenth. Figure 2 shows changes in the average and maximum values of free biotin for each cell line selected. By selecting repeatedly, both values increased, particularly, the latter which was highest for those lines irradiated with gamma rays. The repeated selection using the high concentrations of pimelic acid demonstrated the effects of pimelic acid on the selection of cell lines containing high free biotin levels because lines on medium containing pimelic acid and L-alanine had higher average and maximum values than control cell lines grown only on the basal medium.

Figure 3 shows the distribution of free biotin content for each *L. vera* cell line based on the method of selection. By repeatedly selecting those using the high concentrations of pimelic acid, the maximum value of free biotin increased. At the time of the first selection of cells on the medium containing pimelic acid (100 μg/ml), only 6% of all inoculated cell aggregates survived. However, the second to the fifth generations of these surviving cells grew vigorously on the

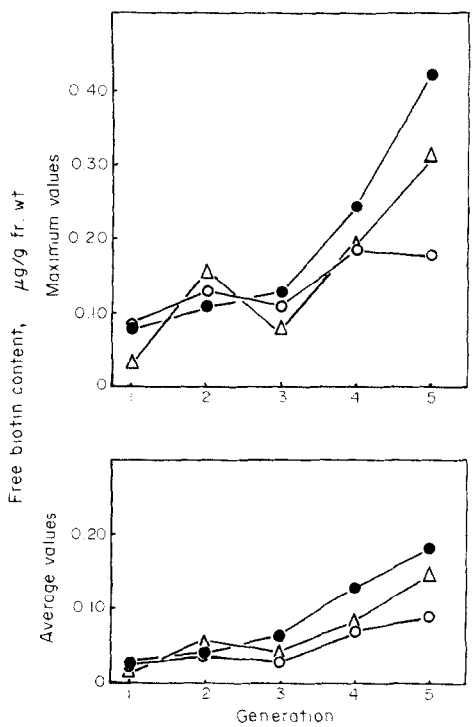

Fig. 2. Changes in the average and maximum values of free biotin contents from the first to the fifth generations in green cell lines of *L. vera* selected on each base. ○, control cell lines; △, cell lines with the high concentrations of pimelic acid (100 μg/ml); ●, cell lines selected by gamma ray irradiation.

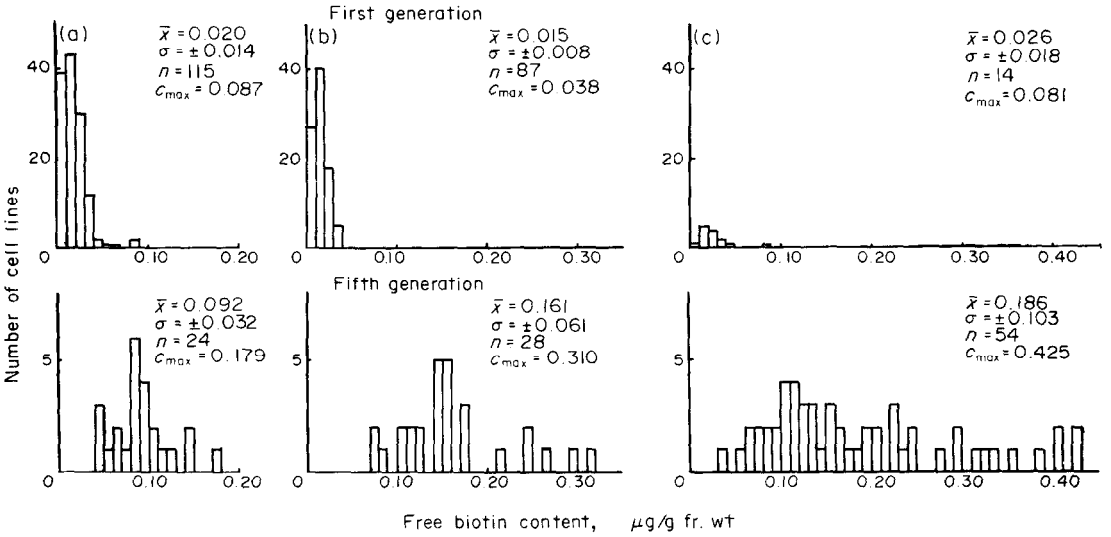

Fig. 3. Distribution of free biotin content of the first and the fifth generations in green cell lines of *L. vera* selected on each base. (a) Control cell lines. (b) Cell lines with the high concentrations of pimelic acid (100 μg/ml). (c) Cell lines selected by gamma ray irradiation. $\bar{x}$ = average value; σ = standard deviation; n = number of cell lines; c_{max} = maximum value.

same medium. The maximum value of free biotin of all these cell lines at the fifth generation was five times that of the original unselected *L. vera* cells (Table 1). At the fifth generation, the distribution of free biotin was from 0.073 to 0.310 μg/g fr. wt. This proves that variants with greater amounts of free biotin continuously appeared on culture medium which contained the high concentration of pimelic acid.

Free biotin in gamma irradiated cell lines showed a wide distribution from 0.031 to 0.425 μg/g fr. wt, at the fifth generation. After irradiation, the maximum value of free biotin at the fifth generation was seven times that of the original unselected cells (Table 1).

Thus, cell lines with significantly higher free biotin levels could be produced by repeated selection using either method. By selection using a high concentration of pimelic acid which is toxic, cell lines capable of changing pimelic acid into biotin were obtained. The increased biotin, however, was not equivalent to the amount of pimelic acid added and the cells seem to have other pathways by which they can detoxicate it.

Gamma ray irradiation increased the variation in the amounts of free biotin among the cell lines, and induced certain variants which contained very large amounts of free biotin. For cultured carrot cells, Nishi *et al.* have reported that various pigmented clones were produced after treatment with *N* - methyl - *N'* - nitro - *N* - nitrosoguanidine [16].

These experiments have demonstrated clearly the effects of pimelic acid and gamma rays on the selection of cell lines with high free biotin contents. Pimelic acid acted here as the toxic reagent and cells resistant to it survived. Gamma ray irradiation induced variants.

EXPERIMENTAL

Subculture of cell lines. The cultured plant cells used were *Nicotiana tabacum* var. Samsun, *N. tabacum* var. Bright Yellow, *Lavandula vera*, *Glycine max* var. Acme, *Cytisus scoparius*, *Bouvardia ternifolia*, *Rauwolfia serpentina* var. Bentham, *Jasminum officinale*, *Beta vulgaris* and *Hyoscyamus niger*. *G. max* cells were maintained on Miller's medium [17] with 3×10^{-5} M IAA and 2.5×10^{-6} M kinetin. Linsmaier and Skoog medium [18] was used as the basal medium for the other cells. It was then supplemented with 10^{-5} M NAA and 10^{-6} M 6-benzyladenine in the cases of *N. tabacum* var. Samsun, *C. scoparius*, *J. officinale* and *B. vulgaris* cells; with 10^{-5} M NAA and 10^{-8} M 6-benzyladenine in the cases of *B. ternifolia* and *H. niger* cells; with 10^{-5} M IBA and 10^{-6} M 6-benzyladenine in the case of *L. vera* cells; and 10^{-5} M IBA in the case of *N. tabacum* var. Bright Yellow cells. In the case of *R. serpentina* cells, ammonium nitrate in the basal medium was substituted for the double concentration of potassium nitrate, and 10^{-6} M 2,4-D and kinetin were added. Cells of *N. tabacum* var. Bright Yellow, *R. serpentina* and *J. officinale* were grown in the dark at 27°. Cells of *G. max* and *B. vulgaris* were grown under light at 27°. Some cells of each of *N. tabacum* var. Samsun, *L. vera*, *C. scoparius*, *B. ternifolia* and *H. niger* were grown in the dark, and some under light at 27°. All cells were transferred to fresh medium every 3 weeks.

Selection method. Cultured green *L. vera* cells were cultured on Linsmaier and Skoog agar medium [18] containing 10^{-5} M IBA and 10^{-6} M 6-benzyladenine. Cell aggregates (*ca* 10 cells/aggregate) were suspended in fresh liquid medium. The suspension (4×10^3 cells/ml) was then pipetted onto a fresh agar medium, and the plated Petri dishes were sealed and incubated. The clone cells formed were cultured until cell aggregates were sufficiently large as to allow analysis of the free biotin contents. Several cell lines which had high levels of free biotin were individually divided into small clusters (10 mg fr. wt/cluster) and these clusters were incubated on agar medium of the same composition. The free biotin contents of the cell lines which developed from these clusters were measured after 6 weeks. The several cell lines with the larger amounts of free biotin were consecutively cultured and selection was repeated five times.

In the selection using a high concentration of pimelic acid, both pimelic acid (100 μg/ml) and L-alanine (100 μg/ml) were added into the basal medium and the cells were cultured on this medium.

Gamma ray irradiation. Cultured green *L. vera* cells were put in a glass tube and irradiated for 1 hr with ^{60}Co (dose 10 kR/hr). After irradiation, the cells were washed with sterilized H_2O, plated on the basal medium and cultured. The selection method for cell lines was same as above except that the basal medium without pimelic acid and L-alanine was used.

Determination of free biotin content. Biotin contents were determined by microbiological assay using the paper disc plate method with *Lactobacillus plantarum* ATCC 8014 [19]. Sampled cells were frozen and homogenized. The homogenates were then centrifuged and the free biotin contents in the supernatants were measured.

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