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ABILITY OF A *NICOTIANA PLUMBAGINIFOLIA* CELL SUSPENSION TO DEMETHYLATE NICOTINE INTO NORNICOTINE

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Key Word Index—*Nicotiana plumbaginifolia*; Solanaceae; *in vitro* culture, plant cell, bioconversion, *N*-demethylation; nicotine; nornicotine

Abstract—A cell suspension of *Nicotiana plumbaginifolia* which does not accumulate tobacco alkaloids was found to keep the ability to demethylate nicotine into nornicotine. The highest bioconversion yield was 53.2%. The influence of some environmental factors upon the reaction has been studied. In particular, it appears that light enhances the catalytic activity of the cells which leads to the hypothesis that this metabolic step of tobacco alkaloids is bound to photodependent systems

INTRODUCTION

The ability of plant cell cultures to produce secondary metabolites depends on the nature of these. If some plant cell cultures are able to produce larger amounts of some metabolites than the whole plant [1], only small amounts or even none of some others (alkaloids) have been detected in plant cultures [2]. The reasons for such a low production are still unknown although several hypotheses have been suggested. One of them may be that the enzymatic systems required for the secondary metabolism are lacking or inhibited in plant cell cultures.

Nornicotine is the main alkaloid of *Nicotiana plumbaginifolia* Viv. It is formed by the *N*-demethylation of nicotine [3]. Neither nicotine nor nornicotine are accumulated in cell suspension cultures derived from this plant and cultured in our laboratory, so we investigated at which step of the metabolic pathway an alteration might occur and whether the lack of nornicotine in cultured cells came from the absence of nicotine or of the requisite enzymatic system or even the inhibition of the later. In this paper, we show that a *Nicotiana plumbaginifolia* cell suspension, unable to produce nicotine or nornicotine, is able to convert exogeneous nicotine into nornicotine, which proves that the required enzymatic system is present in these cells.

RESULTS AND DISCUSSION

Time-course of the bioconversion of nicotine into nornicotine

In order to ascertain whether or not the *N. plumbaginifolia* cell suspension (with a negative alkaloid test) could convert nicotine into nornicotine, the culture medium was supplemented with 1 g nicotine/l (MM1), i.e. 15.4 μmol/g cells (fr. wt), at the time of subculture. Simultaneously, cells were cultured without nicotine (medium MM). The time-course (14 days) of cell growth and the alkaloid accumulation, both in the medium and in the cells, are shown in Fig. 1a. Almost no growth of cells occurred on MM medium. When cells were fed on nicotine (MM1), growth was unchanged, thus nicotine had no toxic effect on the cells.

The amount of nicotine declined sharply in the medium during the first two days (from 15.4 to 2.2 μmol nicotine/g cells), then more slowly. No more nicotine was detected at day 10. Nicotine entered into cells; the concentration of the alkaloid increased until day four (0.68 μmol/g cells at day 2; 0.98 μmol/g cells at day 4) and then decreased until day 14 when only traces of nicotine were detected in cells (0.01 μmol/g cells). Simultaneously, nornicotine appeared both in the medium and in the cells from the second day. Its amount rapidly increased and reached the higher content on day 10 (2.4 μmol/g in the cells and 5.8 μmol/g in the medium). Then the nornicotine amount slightly decreased until day 14. Most of the nornicotine produced was found in the medium (70% on day 10).

In two control cultures (cells cultured without nicotine and nicotine added to culture medium without cells) no nornicotine was detected. Therefore, the *N. plumbaginifolia* cell suspension culture had kept the ability to demethylate exogenous nicotine into nornicotine; its inability to produce alkaloids was not due to a lack or an inhibition of the enzymatic activity required for this reaction. The non-production of alkaloids by the cells in the absence of nicotine might due to an alteration(s) in the biosynthetic pathway placed before the studied step, perhaps on the level of the enzymes diverting the primary metabolite into the secondary pathway as suggested by Berlin *et al.* [2].

The bioconversion yield (μmol of detected nornicotine/μmol of added nicotine × 100) fluctuated in the course of the cell culture cycle (Table 1A): it reached the highest value (53.2%) on day 10 and then slightly decreased until day 14 (42.7%). The demethylation rate (μmol of detected nornicotine/g fresh cells/day) also varied, reaching its maximum (1.3) between days two and four. This can have connection with the time, several hr in some cases [4], for alkaloids to be transferred from the culture medium to the cells and from one cell compartment to another one

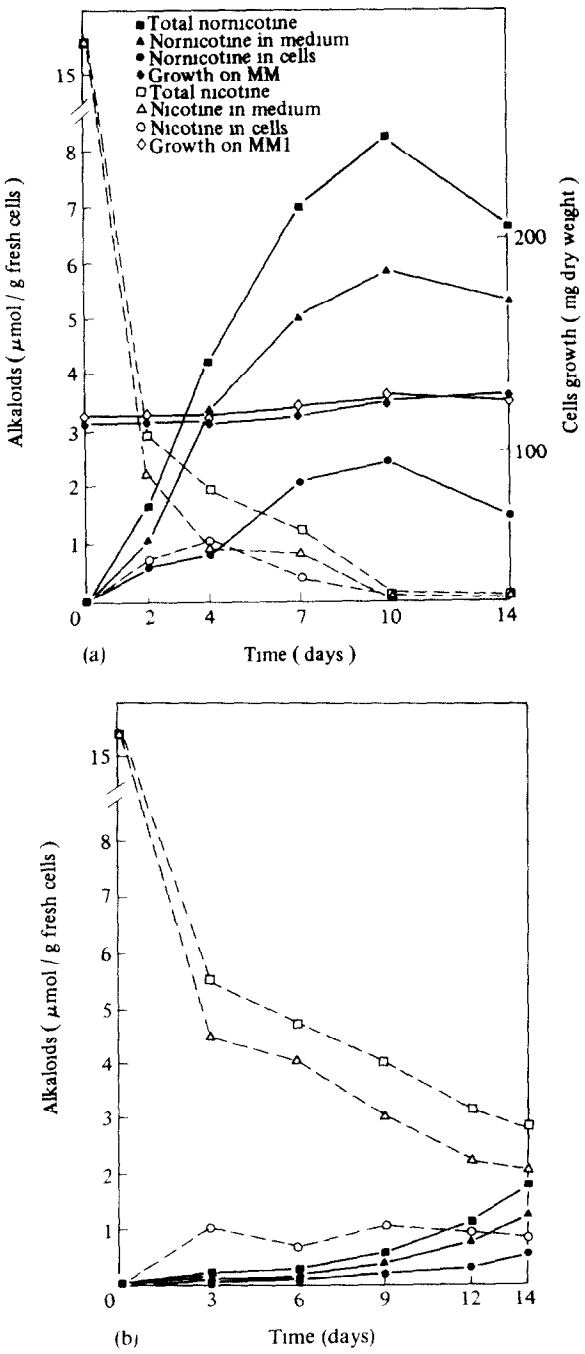

Fig 1 Time-course of growth and nornicotine production in *N plumbaginifolia* cell suspension (a) under neon illumination, (b) in the dark

and *vice versa* The bioconversion ratio (μmol of detected nornicotine/μmol of missing nicotine × 100) was the highest on day 10, only 53 2% of nicotine was transformed, while no nicotine could be detected in the culture Therefore, nicotine was not completely converted into nornicotine (Fig. 1a) The amount of detected nornicotine was smaller than the amount of missing nicotine all along the culture cycle Several authors, on the basis of a comparison between various analytical methods [5, 6] or to explain the absorption and accumulation mechanisms of exogeneous alkaloids by cell suspension cultures [7, 8], have suggested that alkaloids form a complex in plant cell cultures. In such a case, a part of alkaloids present in the cell suspension culture, both nicotine and nornicotine might be bound to cellular components and be unavailable to be evaluated because they are not released during the extraction phase of the analytical process Therefore, the apparent amounts of alkaloids are lower than the true ones Moreover, the bound part of nicotine should be unavailable to be converted into nornicotine Another hypothesis following from this is that the intermediates involved in the demethylation of nicotine are currently unknown [3] These may be alkaloid or non-alkaloid compounds not detected in our analytical conditions. Moreover, according to Poulton [9], demethylation probably initiates more extensive processes of degradation of the molecules To determine whether nornicotine is degraded or not, *N. plumbaginifolia* cell suspension was cultured on MM medium supplemented with nornicotine (1g/1). No degradation was observed, all the nornicotine being found unchanged in the medium at the end of the culture It was surprising that nornicotine was not taken up by the cells, whereas usually cell cultures or vacuoles from plant species which synthesize some alkaloids are able to specifically accumulate these [10, 11]

Effect of the added nicotine–cell density ratio on nornicotine production

In order to investigate the effect of nicotine concentration added to the culture medium on nornicotine formation, cells were cultured in media containing 7.7–154 μmol nicotine/g fresh cells for 10 days (i e 0 5–10 g/1). The highest concentration of nicotine did not have any toxic effect, according to growth and macroscopic aspect of cells. This high tolerance of nicotine by *N plumbaginifolia* cell suspension culture might be explained by the fact that nicotine is a normal constituent of *Nicotiana.*

The greater the amount of nicotine added to the cells, the more nicotine is missing (Table 2) Up to 15 4 μmol, the whole added nicotine disappeared, beyond these concentrations, nicotine remains in the medium. The highest amount of nornicotine formed was obtained for 53 μmol nicotine/g fresh cells In the other experiments, nornicotine production was lower, which probably means that cells reached saturation point. The best bioconversion ratio (53 2%) and bioconversion yield were achieved for 15 4 μmol nicotine/g fresh cells (1 g/1).

Effect of light on nornicotine production

In order to evaluate the influence of illumination conditions on the biotransformation of nicotine by *N plumbaginifolia* Viv cell suspension cultures, the results obtained with cells maintained in standard conditions were compared with those obtained with cells cultured in darkness (Fig 1a and b) It appeared that when cells were maintained in darkness, nicotine disappeared more slowly from the medium, some of it remaining on day 14 (1,9 μmol/g fresh cells). Nicotine entered into the cells and the concentration remained unchanged between days three and day 14. The nornicotine production was lower and the maximum content was obtained only on day 14

Table 1. Evolution of bioconversion yield (nornicotine formed/nicotine added × 100) demethylation rate (μmol nornicotine/g cell fr. wt/day) and bioconversion ratio (nornicotine formed/nicotine lacking × 100)

Table 1A: *Nicotiana plumbaginifolia* cell suspension cultured with exogeneous nicotine (MM1), in the light

Day	Bioconversion yield	Demethylation rate	Bioconversion ratio
2	10.4	0.8	13
4	27	1.3	30
7	45 4	0 9	50
10	53 2	0 4	53
14	42.7	—	42

Table 1B: *Nicotiana plumbaginifolia* cell suspension cultured with exogeneous nicotine (MM1), in the dark

Day	Bioconversion yield	Demethylation rate	Bioconversion ratio
3	1 02	0 05	15
6	1 4	0 02	2
9	3.6	0 12	5
12	7	0 16	8
14	11.6	0.23	14

Data are the mean value of five experiments

Table 2 Effect of the added nicotine/cell density ratio on the bioconversion of nicotine into nornicotine, at day 10

Nicotine μmol/gfresh cell	7 7	15.4	53	77	154
Nicotine lacking μmol/gcell fr wt	7.7	15.4	41	61	127
Nornicotine formed μmol/gcell fr wt	2.54	8 2	13 2	8 8	3.2
Bioconversion ratio nornicotine formed/nicotine lacking × 100	33	53.2	32	14 4	2.5
Bioconversion yield nornicotine formed/nicotine added × 100	33	53.2	24 9	11 4	2

Data are the mean value of five experiments

(1.8 μmol nornicotine/g fresh cells). The low values of transformation ratio, demethylation rate and transformation yield (Table 1B) showed the slowness and the weakness of the biotransformation process, when the cells were maintained in darkness. Illumination is an important factor for the optimization of the transformation yield. These results allow us to conclude that the *N*-demethylation of nicotine to form nornicotine is bound to photodependent systems inside the cells.

EXPERIMENTAL

Plant material and tissue culture. Cell suspension cultures were initiated from leaves of hypohaploid *Nicotiana plumbaginifolia* with wide chlorophyllous leaves, regenerated from thin cell layers of haploid plants [2]. They were cultured since 1985 in GM1 medium [3] with 0.2 mg/l 2,4-D and 0 1 mg/l BAP in 250 ml conical flasks filled with 125 ml medium and 20 g fresh cells. Flasks were agitated on a rotary shaker (orbital diameter 8 mm at 100 rpm) maintained at 24 ± 1° in fluorescent illumination 'warm white' (2 500 erg/cm^2; photoperiod: 16 hr). Unless otherwise mentioned, the standard protocol for the biotransformation experiments was as follows: cells were removed from GM1 medium during their exponential growth phase (day 8 of the culture cycle) and transferred into a mineral medium [macroelements of Linsmaier and Skoog medium (13)], supplemented with 1 g/l nicotine (MM1). The medium/cells ratio was 2.5 (ml/g fresh cells), i e. 15 4 μmol nicotine/g fresh cells.

Extraction and purification of tobacco alkaloids The cells were separated from the medium by filtration and samples of both were taken for analysis. Alkaloids were extracted by MeOH–NH_4OH = 9:1 (v/v) under reflux, for 3 hr. After filtration, crude extract was evapd to dryness under red. pres. The residue was treated with 3 × 10 ml 0.1 M H_2SO_4. The aq. solns

were collected, filtered and the filtrate was basified to pH 9 with NH_4OH, then extracted by shaking with 3×20 ml $CHCl_3$. Pooled extracts were dried (Na_2SO_4) and evapd to dryness under vacuum

GC analysis of tobacco alkaloids. Analysis were performed with a GC equipped with FID and fused silica capillary SE-30 basic column (25 m × 0 25 mm) Oven temp 150°, injector and detector temp. 250° He was used as carrier gas with an inlet pressure of 0 9 bars Tropine was used as int standard. In these conditions, R_ts times were 2 6 min for tropine, 3 8 min for nicotine and 4 6 min for nornicotine The system was calibrated with standard nicotine and nornicotine (Extrasynthese, France) MeOH, solns

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