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Biotransformation of Hydroquinone by Hairy Roots of *Brugmansia candida* and Effect of Sugars and Free-Radical Scavengers

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ABSTRACT

Hairy roots of *Brugmansia candida* were used to bioconvert hydroquinone into arbutin. The highest bioconversion, with the lowest damage to the cells, was attained when concentrations of 20–40 mg/L hydroquinone were used. Sugars (sucrose, glucose, mannitol, and sorbitol) at concentrations of 30–120 g/L enhanced bioconversion, and, of these, sucrose was the most effective. Two different free-radical scavengers were also tested: sodium benzoate and gallic acid. The first one diminished biotransformation efficiency; gallic acid did not affect biotransformation at all. Preliminary permeabilization treatments tested failed to liberate arbutin into the medium, and provoked a total loss in cell viability.

Index Entries: *Brugmansia candida*; Solanaceae; hydroquinone; arbutin; hairy roots; biotransformation.

INTRODUCTION

Hydroquinone (HQ) is employed in medicine and cosmetics as a depigmenting agent, because of its inhibitory activity on tyrosinase. However, the toxicity of this drug and its side effects *(1)* encourage the search for substitutes. Arbutin, the monoglucoside of HQ, which is used as a urethral disinfectant, has been shown to be a suppressor of melanin

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synthesis in human skin *(2)*, without apparent side effects *(3)*, therefore being an interesting candidate to replace HQ in cosmetics.

Plant cell cultures possess the ability to specifically transform exogenous substrates, thus constituting an interesting system for the transformation of cheap and plentiful substances into expensive compounds. Many researchers have reported on the use of plant cell cultures as bioreactors, with the object of transforming exogenous substrates *(4)*. In particular, it has been demonstrated that *Datura* suspension cultures are capable of glucosylating substrates such as HQ *(5,6)*. The bioconversion is catalyzed by the enzyme β-glucosyl transferase, being the glucose donor the high energy nucleotide UDP-glucose. In this system, exogenous HQ is converted almost completely into the monoglucoside arbutin within 24 h *(7)*. The use of cultured cells in this case represents two important advantages over chemical synthesis: The enzymatic reaction yields a position-specific glucosylation (β), and, in addition, it is a one-step glucosylation, therefore making it more efficient than the chemical synthesis, which involves a glucosylation and a deacetylation *(6)*.

A major drawback of suspension cultures is the phenomenon of somaclonal variation, which may lead to unstable biochemical behavior *(8)*. This problem can be circumvented by the use of organized tissues, such as root and shoot cultures. Hairy roots, obtained by transformation of plant cells with the bacterial soil pathogen *Agrobacterium rhizogenes*, grow at high rates comparable to unorganized plant cell suspensions, while retaining genetic and biochemical stability *(9,10)*. It has been reported that hairy roots are capable of biotransforming xenobiotics *(8,11)*.

In this research, hairy root cultures of *Brugmansia candida* were employed to biotransform exogenous HQ into its monoglucoside, arbutin. Initially, the culture stage during which the highest glucosylation capacity could be observed was analyzed. The addition of different concentrations of HQ and the sequent addition of this substrate were then studied. The effects of different types and concentrations of sugars and antioxidants on biotransformation efficiency were also tested. Finally, preliminary permeabilization treatments were carried out in order to explore the possibility of liberating arbutin into the medium.

MATERIALS AND METHODS

Plant Material and Culture Conditions

Hairy roots of *B. candida* were obtained through inoculation of sterile seedlings with *A. rhizogenes* LBA 9402, according to the method described by Pitta-Alvarez and Giulietti *(12)*. From the clones obtained (~50), clone No. 7.3.4. was selected because it showed the highest growth rate and capacity to

produce hyoscyamine and scopolamine. The transformed roots were cultured in liquid Gamborg (B5) medium *(13)* devoid of hormones. In order to avoid dedifferentiation, the medium used contained half concentration of mineral salts ($B5_{1/2}$) and 1.5% sucrose *(12)*. The roots were incubated on a rotary shaker at 100 rpm, a temperature of 25°C ± 2, and a 16 h photoperiod by using fluorescent lamps at a light intensity of approx 1.8 w/m^2 seg. In all experiments performed, the initial step consisted of the inoculation of approx 150 mg fresh weight (fw) of roots in 25 mL of $B5_{1/2}$ medium, supplemented with 1.5% sucrose, contained in 125-mL Erlenmeyer flasks. Whenever HQ was added, the entire biomass was transferred to $B5_{1/2}$ fresh medium. All the assays had three replicates, and each study was repeated once.

Analytical Methods

Extraction of Arbutin and HQ in Roots

Roots were separated from the medium by vacuum filtration, washed with dH_2O and then used for the determination of fw and the extraction of phenolic substances. The extraction was carried out according to the method described by Meravi *(14)*, with slight modifications: 200 mg (fw) of roots were extracted 3× (each time 15 min long) with 0.5 mL of boiling methanol under retro-cooler. The homogenate was then filtered and the solvent was evaporated at 55°C. The residue was dissolved in 1 mL of the mobile phase later used for HPLC analysis.

Extraction of Arbutin and HQ in the Medium

The medium was lyophilized and afterwards redissolved in the mobile phase.

Arbutin and HQ Analysis

Arbutin and HQ were analyzed on a Spherisorb ODS 2 column, using as mobile phase 5% methyl alcohol (adjusted to pH 3.0 with phosphoric acid) and monitoring at 285 nm *(15)*.

Fresh Weight (fw)

For fw determinations, root tissues were separated from the medium by filtration through glass fiber paper (Whatman GF/A) under vacuum, and weighed.

Biotransformation Studies

On hairy root cultures of 14, 19, and 22 d, HQ (dissolved in bidistilled water) was added aseptically to the medium to obtain different final concentrations (20, 40, 60, 80, and 100 mg/L). The cultures were then incubated in the dark on rotary shakers at 100 rpm and a temperature of 25°C ± 2.

Twenty-four hours after the addition of HQ, the hairy roots were separated from the medium by vacuum filtration. HQ and arbutin were determined in both the roots and the medium. The bioconversion was expressed as the percentage substrate (HQ) that was converted into the desired product (arbutin).

In order to study the effect of HQ concentration on biotransformation capacity, the following experiments were carried out: HQ was aseptically added to the cultures of 22 d to give final concentrations of 20, 200, 400, 800, and 1000 mg/L.

To study the effect of the sequential addition of HQ, 40 mg/LH Q were supplied according to one of the following strategies: 10 mg/L were administered every 2 h during a period of 8 h; 5 mg/L were administered every hour during a period of 8 h; the total amount was supplied initially. All the cultures were incubated during 48 h after the first incorporation of HQ. One-third of the total amount was added once a day during 3 d; one-fourth of the total amount was added once a day during 4 d; the total amount was supplied on d 22. All the cultures were incubated during 4 d after the last addition of HQ.

The incubation conditions and the subsequent determination of arbutin and HQ were as described previously.

In order to learn the effect of sugar addition on biotransformation capacity, the following experiments were carried out: After culturing the roots for 22 d, HQ was added to give a final concentration of 40 mg/L to different media. The media were basically constituted by the $B5_{1/2}$ medium already described, but supplemented with different types and amounts of sugars. The media were supplied with either sucrose, glucose, mannitol, or sorbitol in the following final concentrations: 30, 60, 90, and 120 mg/L. The medium, deprived of sugars, was used as control. The conditions used for incubation of cultures and for determination of HQ and arbutin after 24 h in the roots and the media were the same described above.

Concerning the addition of free-radical scavenger compounds, sodium benzoate and gallic acid were used according to the following experiment: After culturing the roots for 22 d, these were inoculated in 25 mL of $B5_{1/2}$ medium without sucrose, and HQ was added to give a final concentration of 40 mg/L. The media were supplied with either sodium benzoate (concentrations: 10, 20, and 30 m*M*) or gallic acid (concentrations: 40, 200, and 400 µg/L). The cultures were incubated for 24 h. The incubation conditions and the subsequent determination of arbutin and HQ were as described previously.

For the study of permeabilization procedures, Triton and dimethylsulfoxide (DMSO) were tested as follows: HQ was added in a final concentration of 40 mg/L to 22-d-old *B. candida* hairy root cultures in $B5_{1/2}$ supplemented with 3% sucrose. Twenty-four h later, 100 ppm of filter-

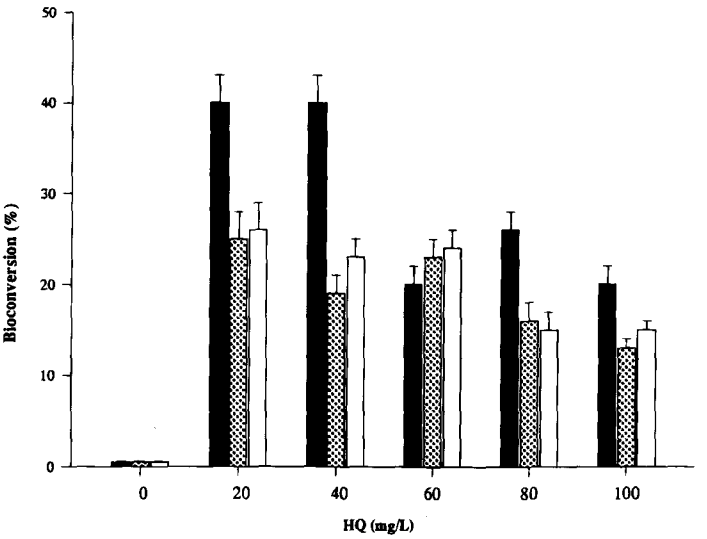

Fig. 1. Bioconversion of HQ into arbutin by different ages of *B. candida* hairy root cultures in different phases of growth ([■], d 22; [⊠], d 19; [□], d 14) and with different concentrations of HQ.

sterilized 1% Triton 100× *(16)* was incorporated to the medium. The flasks were incubated for 4 h under the conditions described above. Half of the flasks containing the treated tissue were subjected to the determination of fw, arbutin, and HQ. The remaining treated tissue was removed from the medium, rinsed 3× with sterile dH_2O sterilely blotted and weighed, and immersed in shake flasks containing $B5_{1/2}$ medium supplemented with 1.5% sucrose (without Triton). The roots were subsequently allowed to grow for 2 wk. In a second test, using DMSO as a permeabilizing agent, 24 h after HQ was added to the medium to give a final concentration of 40 mg/L, the hairy roots were permeabilized with DMSO according to Brodelius and Nilsson *(17)*. After shaking with DMSO for 30 min, the experimental procedure followed was the same as the one described for Triton.

RESULTS AND DISCUSSION

Figure 1 shows the bioconversion of HQ into arbutin carried out by *B. candida* hairy roots in three distinct phases of growth, corresponding to the exponential (14 d), progressive deceleration (19 d), and stationary phases (22 d). These phases had been previously determined by kinetic studies of the *B. candida* hairy root line employed *(12)*. The highest bioconversion was observed in hairy roots at d 22 of culture, corresponding to the stationary phase (Fig. 1). This behavior was observed at practically all the concentra-

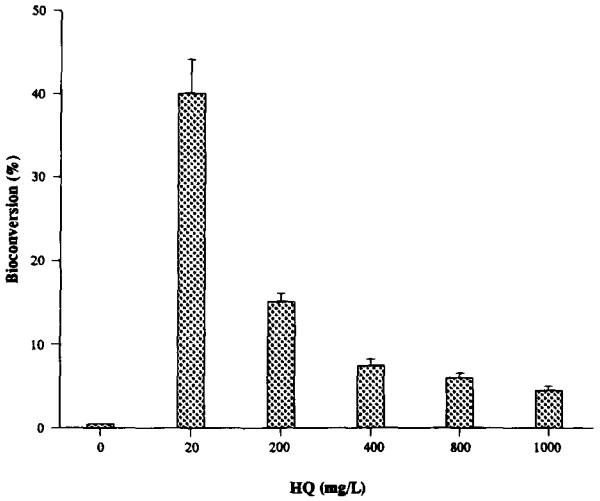

Fig. 2. Bioconversion of HQ into arbutin by 22-d-old *B. candida* hairy root cultures with different concentrations of HQ.

tions tested. The results seen on d 14 and 19 (exponential and deceleration phases respectively) were very similar for all the levels of HQ assayed.

It has been observed in previous reports that HQ damages the cell and thus inhibits the growth of young cultures in the exponential phase of growth *(19)*. Because this damage reduces the bioconversion capacity of the roots, it was suggested that HQ be added as late as stationary phase *(18)*.

The improved performance corresponding to the latest stage of growth was most noticeable when HQ was administered in concentrations between 20 and 40 mg/L. Since glycosylation in plant cells is a detoxifying mechanism *(19)*, these results would suggest that higher HQ concentrations would surpass this capacity, probably because of the damage provoked to the cells or to enzymatic limitations. However, it is noteworthy that, although levels of 80–120 mg/L HQ did not yield the high bioconversion obtained with lower concentrations, the bioconversion efficiency did not differ significantly among them, thus encouraging experiments with higher HQ concentrations.

Figure 2 shows that, in the range of 100–1000 mg/L, the bioconversion efficiency quickly diminished. In fact, within 24 h, these elevated levels of HQ induced a series of changes in the cultures, such as browning and the initiation of root disintegration, which implicated a loss in viability. These modifications increased with HQ concentration increments, and, finally, concentrations up to 800 mg/L HQ produced loss of root morphology, accompanied by total disintegration after 24 h of incubation. At any substrate concentration, neither arbutin nor HQ could be detected in the medium.

Table 1
Effect of Sequential Addition of HQ on Bioconversion into Arbutin by 22-d *B. candida* Hairy Root Cultures

	Arbutin concentration in roots (mg/g fw)	Bioconversion capacity (%)
A[a]	6,9 ± 0,5	36 ± 2
B[a]	6,3 ± 0,5	38 ± 2
C[a]	6,3 ± 0,5	34 ± 2
D[b]	6,5 ± 0,5	37 ± 2
E[b]	6,3 ± 0,5	39 ± 2
F[b]	6,6 ± 0,5	36 ± 2

[a]during 8 h.
[b]during 96 h.
In all experiments the total amount of HQ was 40 mg/L. A, 10 mg of HQ administered every 2 h; B, 5 mg of HQ administered every hour; C, 40 mg of HQ supplied initially; D, 13.3 mg of HQ administered every day for 3 d; E, 10 mg of HQ administered every day for 4 d; F, 40 mg of HQ supplied initially. HQ was not detected either in the medium or in the roots. Arbutin was not detected in the medium.

Table 1 shows the results of sequential addition of HQ on the bioconversion capacity of hairy root cultures. Since 40 mg/L HQ had yielded the highest bioconversion efficiency, with practically no visible damage to the roots, this was the concentration used. No significant changes were observed between the treatments, even when slower expositions to lower concentrations of HQ were used (D, E, and F treatments). Moreover, in cultures which had been exposed to the total amount of HQ, the results obtained were the same 24 and 96 h after the administration of the substrate, indicating that there was no increase in enzyme activity after the 3 d following the first determination. HQ was not detected either in the medium or in the roots. Arbutin was detected only in the roots.

The addition of sucrose or glucose at all the concentrations tested enhanced production of arbutin from 8- to 12-fold on the basis of sugar free medium (Fig. 3). Sorbitol and mannitol, in all the concentrations, also promoted the production of arbutin. The fact that sorbitol enhanced the production of arbutin in the same manner as did sucrose or glucose suggests that sugars were not utilized as a nutrient. In principle, this is in agreement with the hypothesis sustained by Yokoyama et al. *(20)*, who, working with *Catharanthus roseus* cell cultures, proposed that the effect of sugars on the production of arbutin may be, at least in part, caused by the scavenging of hydroxyl radicals. The free-radical scavenging would prevent injury to the cells resulting from the oxidation of hydroquinone and consequent pro-

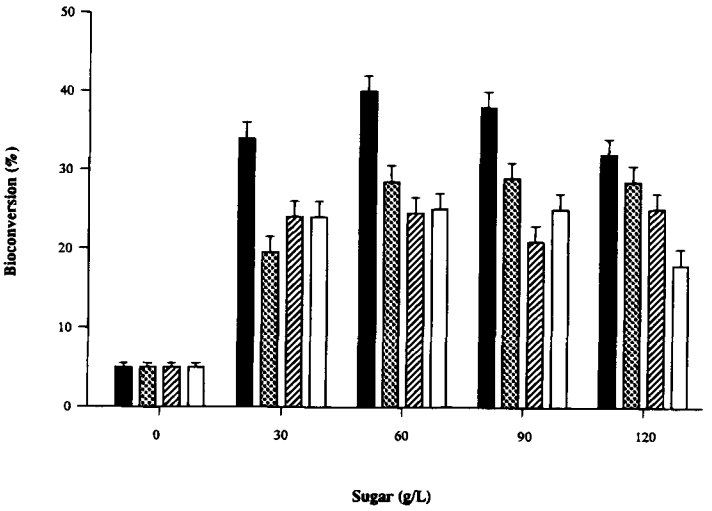

Fig. 3. Effect of several concentrations of different sugars ([■], sucrose; [▨], glucose; [▨], manitol; [□], sorbitol) on bioconversion of HQ into arbutin by 22-d-old *B. candida* hairy root and with 40 mg/L HQ.

duction of hydroxyl radical *(20)*. The improved cell viability would, thus, render the cells more capable of glucosylating the substrate.

When no sugars were added to the medium, the hairy roots turned a dark brown throughout their extension after the 24 h exposition to HQ. When sucrose or glucose were used, the roots appeared, after 24 h, uniformly pale brown. However, when mannitol or sorbitol were employed, the root tips presented an extremely dark brown color. This would suggest that, besides a common protective mechanism, such as that described by Yokohama et al. *(20)*, glucose and sucrose could also be used in part to generate UDP-glucose in the rapidly dividing meristematic region. Thus, glucose and sucrose would protect the cells by free-radical scavenging, and also by procuring the necessary substrate for the biotransformation to take place. Experiments designed to test this hypothesis are currently under way in our laboratory.

The hypothesis that sugars might act as scavengers of hydroxyl radicals prompted us to examine other free-radical scavengers that might increase bioconversion efficiency. With respect to sodium benzoate, levels of 10 m*M* did not affect bioconversion by the roots, but at high concentrations (20–30 m*M*) the bioconversion decreased. This could be attributed to the fact that this molecule can also act as substrate for glucosyltransferases *(21)*, thus probably competing for the same enzyme as HQ.

Phenoloxidases are enzymes directly involved in the oxidation of HQ and the subsequent production of free radicals. Gallic acid was

selected for this test not only for its properties as a hydroxyl scavenger, but also because it acts as substrate for phenoloxidases, and generally inhibits enzymes, of which phenoloxidases could be a target *(7)*. The three concentrations tested (20, 200, and 400 μg/L) increased fourfold the bioconversion rates.

Permeabilization Treatments

Neither one of the permeabilization treatments (Triton 100 ppm and DMSO 10%) were successful, and, in both cases, neither arbutin nor HQ release was detected. Glucosyl conjugation represents a phenomenon of detoxification *(18)* at the cellular level, by which the compounds become more soluble and can be stored in vacuoles. Since a prerequisite for the recovery of the arbutin retained in the vacuole of the cells is the opening of the tonoplast, we are conducting experiments employing lipases specific to plant membranes that could serve as permeabilizing agents while maintaining cell viability *(22)*. Finally, the roots that were subsequently transferred to fresh medium did not grow, indicating a loss of viability provoked by the treatment *(17)*.

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