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# EFFECTS OF CHEMICALS ON ALKALOID PRODUCTION BY TRANSFORMED ROOTS OF BELLADONNA

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**Key Word Index**—*Atropa belladonna*; Solanaceae; tropane alkaloids; transformed root culture; hydrogen peroxide.

**Abstract**—The growth and tropane content in transformed root cultures of *Atropa belladonna* (strain M8) grown in 300 ml flasks were studied. After one month in culture, the biomass of the transformed roots increased 15 times and reached 5 g dry wt $l^{-1}$; the alkaloid contents were 0.7% for (−)-hyoscyamine, 0.1% for 6β-hydroxyhyoscyamine, 0.02% for scopolamine and 0.02% for littorine. During the culture period, no alkaloid could be detected in the medium. Seven chemicals were individually added to 18-day-old cultures. Glutathione (5 mM), chitin (0.1%), chitosan (0.1%) or yeast extract (0.1%) had no effect on the release of alkaloids into the medium. However, treatment with 5 mM $H_2O_2$ induced a transient release of tropane alkaloids from transformed roots and the maximum amount of alkaloids in the medium was ca 0.35 mg per flask (10% of alkaloid contained in tissues); tissue viability was still preserved. $Cu^{2+}$ and $Cd^{2+}$ (5 mM) also improved the excretion of tropane alkaloids into the medium. However, their addition led to lysis of transformed roots.


## INTRODUCTION

Tropane alkaloids, such as (−)-hyoscyamine and scopolamine, are abundant in solanaceous plants, such as *Atropa*, *Datura*, *Hyoscyamus*, *Scopolia* and *Duboisia* [1]. High and stable productivity of tropane alkaloids in transformed roots of Solanaceae has led to many studies on the production of tropane derivatives, which include (−)-hyoscyamine, 6β-hydroxyhyoscyamine, scopolamine and littorine [2–4]. Some chemical substances, called elicitors, can enhance productivity of phytoalexins from plant cell/tissue cultures. Some sesquiterpenoids (7-epi-debneyol, 1-hydroxydebneyol, 8-hydroxydebneyol, capsidiol and debneyol) have been isolated from cellulase-treated cell suspension of *Nicotiana tabacum* [5]. When transformed root cultures of *Hyoscyamus muticus* were treated with a crude cell wall preparation of the root pathogenic fungus, *Rhizoctonia solani*, the sesquiterpenes (solavetivone and lubimin) were induced and secreted into the culture medium within 24–48 h [6]. The treatment of transformed root cultures of *Datura stramonium* with copper and cadmium salts at external concentrations of 1 mM has been found to induce the rapid accumulation of high amounts of sesquiterpenoid-defensive compounds, notably lubimin and 3-hydroxylubimin [7]. Some chemicals such as dimethyl sulfoxide (DMSO), Triton X-100 and Tween-20 also caused release of secondary metabolites from plant cell/tissue cultures [8–10]. In the present work, we examined the effect of heavy meal ions ($Cu^{2+}$ and $Cd^{2+}$) and some chemicals ($H_2O_2$, glutathione (GSH), chitin, chitosan and yeast extract) on tropane alkaloid production by transformed root cultures of *Atropa belladonna* strain M8.

## RESULTS AND DISCUSSION

Transformed root of *A. belladonna* M8 was established by co-culture of leaf discs with *Agrobacterium rhizogenes* MAFF 03-01724 [4]. Transformed root cultures were incubated in hormone-free half strength MS (1/2 MS) liquid medium containing 3% sucrose [4] on a gyratory shaker (100 rpm) in the dark. Figure 1 illustrates profiles of growth and alkaloid content as a function of cultivation time, during one month of culture in 300 ml conical flasks (200 ml medium).

From an initial inoculum of ca 1 g fresh wt, transformed roots grew 15 times in biomass and reached

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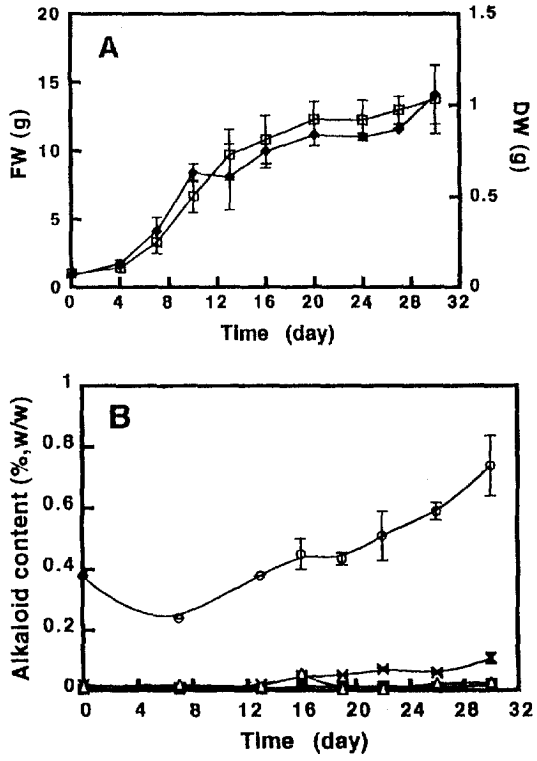


Fig. 1. Growth curve (A) and alkaloid content (B) of transformed root cultures in *Atropa belladonna* M8 grown in 300 ml conical flasks. Bars represent standard deviation ($n = 3$). □—□, FW (fr. wt); ◆—◆, DW (dry wt); ○—○, (−)-hyoscyamine; ×—×, 6β-hydroxyhyoscyamine; ■—■, scopolamine; △—△, littorine.

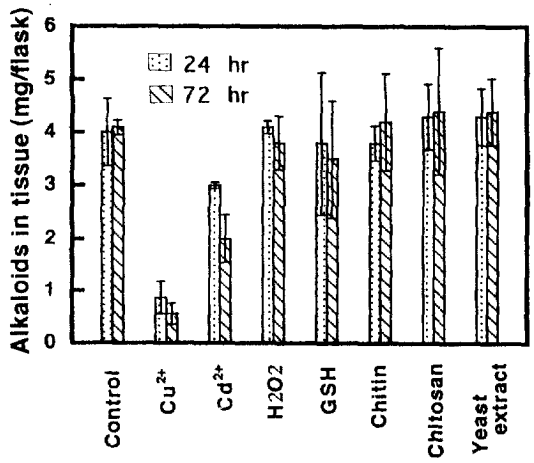


Fig. 2. Total alkaloid content in transformed root cultures of *Atropa belladonna* M8 after treatment with various chemicals for 24 and 72 h. Bars represent standard deviation ($n = 3$).

5 g dry wt $l^{-1}$ medium after one month of culture (Fig. 1A). Four major tropane alkaloid were found in the transformed roots: (−)-hyoscyamine, 6β-hydroxyhyoscyamine, scopolamine and littorine [4]. The (−)-hyoscyamine content began to increase after one week of inoculation and reached 0.7% (dry wt) in the roots after one month of culture (Fig. 1B). The content of the other alkaloids was 0.1% for 6β-hydroxyhyoscyamine, 0.02% for scopolamine and 0.02% for littorine. Compared with the previous paper [4], we further found that transformed roots of *A. belladonna* M8 had maintained a stable ability for tropane alkaloid production but no alkaloids could be detected in the medium during the culture period.

It is reported that some chemical substances called elicitors can enhance the production of phytoalexins from plants [6]. In this study, we have examined the effects of some of these, viz, 5 mM $H_2O_2$, 5 mM $CuCl_2$, 5 mM $CdCl_2$, 5 mM glutathione (GSH), 0.1% (w/v) chitin, 0.1% chitosan and 0.1% yeast extract, on tropane alkaloid production by transformed root cultures of *A. belladonna* strain M8. Sterile preparations of these chemicals were added to 18-day-old root cultures and the amounts of alkaloids in the tissue and culture medium were analyzed after incubation times of 24 and 72 h. After 24 h treatment with 5 mM $Cu^{2+}$, $Cd^{2+}$, a decrease of alkaloid in the roots was observed (Fig. 2) and 120%, 27.8% and 6% of the amount contained in untreated transformed root cultures was detected in the medium by 5 mM $Cu^{2+}$, $Cd^{2+}$ or $H_2O_2$ treatment, respectively (Table 1). No effect on the release of alkaloids from transformed roots into the medium was observed as a result of treatment in the 5 mM GSH, 0.1% chitin, chitosan or yeast extract. None of the chemicals at the concentration used increased tropane alkaloid accumulation in the tissues of transformed root cultures. However, release of alkaloids from the transformed roots into the medium was observed after $Cu^{2+}$, $Cd^{2+}$ or $H_2O_2$ treatment. This suggests that additional, more specific release process may also operate, induced by these treatments [7].

Plant secondary metabolites are often stored in vacuoles which makes the semi-continuous/continuous production of secondary metabolites by plant cell/tissue cultures difficult. Currently, chemicals, such as DMSO, Triton X-100, Tween-20, some monoterpenes and fatty acids, have been employed to release intracellularly stored products from plant cells or tissues. This is achieved by destroying the plasma membrane of the cells [8–11]. The chemicals that we investigated here, $Cu^{2+}$, $Cd^{2+}$ and $H_2O_2$, also showed a permeabilization effect for alkaloid production from transformed root cultures of *A. belladonna* strain M8. In order to evaluation the permeation effect on tissue viability, 18-day-old transformed root cultures were treated with $Cu^{2+}$ (1 and 5 mM), $Cd^{2+}$ (1 and 5 mM), and $H_2O_2$ (5, 15 and 50 mM). Tissue viability was assayed, 2, 6, 18, 24, 36, 48 and 72 h later by monitoring tissue respiratory efficiency using 2,3,5-triphenyl tetrazolium chloride (TTC). In this assay, dehydrogenase activity of living tissues reduces TTC to red formazan, which is then detected by measuring the $A$ at 485 nm [12–14]. Tissue viability of these

Table 1. Alkaloid released into the medium by *Atropa belladonna* M8 root cultures 24 and 72 after treatment with various chemicals

| | Alkaloid in medium | | | |
|---|---|---|---|---|
| | 24 h | | 72 h | |
| Treatment | (mg) | %* | (mg) | %* |
| Control | —† | — | — | — |
| 5 mM $CuCl_2$ | 4.82±0.24 | 120.5 | 2.71±0.95 | 66.1 |
| 5 mM $CdCl_2$ | 1.15±0.20 | 27.8 | 1.13±0.02 | 27.6 |
| 5 mM $H_2O_2$ | 0.24±0.08 | 6.0 | 0.13±0.06 | 3.2 |
| 0.1% Glutathione | — | — | — | — |
| 0.1% Chitin | — | — | — | — |
| 0.1% Chitosan | — | — | — | — |
| 0.1% Yeast extract | — | — | — | — |

* As percentage of mean control values in tissue.
† Less than detection limit.
± Standard deviation for triplication experiments for each treatment.

treatments were compared with control cultures treated with sterile water. $Cu^{2+}$, $Cd^{2+}$ damaged the root cultures seriously. After 2 h treatment with 1 mM $Cu^{2+}$, 5 mM $Cu^{2+}$, 1 mM $Cd^{2+}$ and 5 mM $Cd^{2+}$, tissue viability was 5, 33, 45 and 65% respectively, but after 72 h treatment, tissue viability had declined to 2, 2, 5 and 12%, respectively. $Cu^{2+}$ and $Cd^{2+}$ treatment also led to lysis of transformed roots. Tissue native viability of transformed root cultures decreased to 15% 2 h after treating with 5 mM $H_2O_2$, but recovered to 100% after 18 h and increased to 142% after 48 h (Fig. 3). When treated with 15 mM $H_2O_2$, the tissue viability decreased to 43% after 12 h, then recovered rapidly. After 50 mM $H_2O_2$ treatment tissue viability recovered to 89% after 72 h. Transformed root cultures also showed a transiently high (more than 100%) relative tissue viability in the $H_2O_2$ treatment (Fig. 3). Tissues treated with 50 mM $H_2O_2$ for up to 72 h resumed root growth following subculture to fresh 1/2 MS medium and attained a biomass of 75% of that of control cultures after 14 days. Furze *et al.* [7] reported that when *Datura stramonium* transformed root cultures were treated with 1 mM $Cu^{2+}$ for 64 h, the biomass of the subculture was reduced to 50% of that of the control culture [7]. The above data show that the viability of the belladonna roots was much better after $H_2O_2$ treatment than after $Cu^{2+}$ or $Cd^{2+}$ treatment.

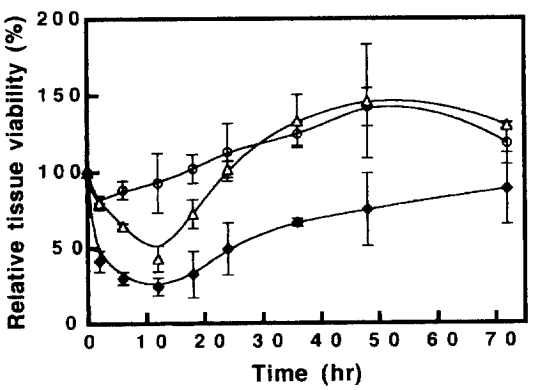


Fig. 3. Time-course of tissue viability transformed root cultures in *Atropa belladonna* M8 after treatment with 5, 15 and 50 mM $H_2O_2$. Bars represent standard deviation ($n = 3$). ○—○, 5 mM; △—△, 15 mM; ◆—◆, 50 mM.

In order to study the time-course of alkaloid release by $H_2O_2$, 5 mM $H_2O_2$ was selected as suitable concentration. The amounts of alkaloids in the medium were determined periodically for up to 48 h after $H_2O_2$ treatment. Alkaloids could be detected in the medium within 3 h of adding $H_2O_2$. The release of (−)-hyoscyamine reached a maximum after 12 h, whereas scopolamine reached a maximum after 18 h, showing a lag phase of ca 4 h (Fig. 4). During the 24 h treatment, both (−)-hyoscyamine and scopolamine decreased in the medium. 6β-hydroxyhyoscyamine was not detected in the medium during treatment. As $H_2O_2$ had no destructive effect on authentic tropane alkaloids in fresh medium (data not shown), the decrease of alkaloids in the medium suggests a reabsorption of alkaloids in the tissues or an enzymatic transformation/degradation. The maximum amounts of alkaloid in medium was ca 0.35 mg per flask (10% of alkaloid contained in the tissues) and it was detected at 12 h after treating with 5 mM $H_2O_2$.

Cell/tissue viability after permeabilization treatment is of fundamental importance for the development of a process involving intermittent release of intracellularly stored products by permeabilization. A variety of organic solvents and detergents have been tested on various plant cell/tissue cultures but have always shown a deleterious effect [8–11]. These permeabilization treatments caused an irreversible loss of

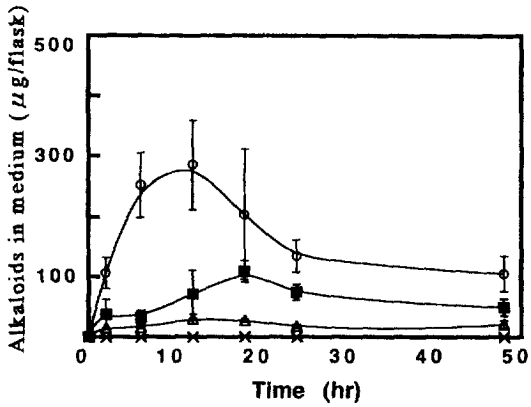


Fig. 4. Time-course of tropane alkaloid content in medium released from transformed root cultures in *Atropa belladonna* M8 after treatment with 5 mM $H_2O_2$. Bars represent standard deviation ($n = 5$). ○—○, (−)-hyoscyamine; ×—×, 6β-hydroxyhyoscyamine; ■—■, scopolamine; △—△, littorine

membrane integrity which led to a loss of cell/tissue viability or caused death, depending on the concentration of permeabilizing agent and incubation time of permeabilization used. In biological systems, $H_2O_2$ stress might cause a series of reactions involving catalase and peroxidase. These reactions would occur before $H_2O_2$ is actually metabolized. Thus, efficient use of $H_2O_2$ is possible as a physiologically-acceptable permeabilizing agent.

## EXPERIMENTAL

### *Plant material*

Transformed roots of *A. belladonna* M8 were established by co-culture of leaf discs with *Agrobacterium rhizogenes* MAFF 03-01724 [4]. Transformed root cultures were incubated in hormone-free half-strength MS (1/2 MS) liquid containing 3% (w/v) sucrose in 300 ml conical flasks (200 ml medium). Roots were subcultured every 12–14 days. All cultures were maintained on a gyratory shaker at 100 rpm in the dark at 30°. For culture and time-course expts, ca 1 g fr. wt per flask of roots were inoculated. Three flasks were used for each expt. At 3 to 4-day intervals, roots were harvested and both biomass (fr. wt and dry wt) and tropane alkaloid content were measured.

### *Sample preparation and HPLC analysis*

About 50 mg of each sample was extracted with 5 ml $CHCl_3$–MeOH–$NH_4OH$ (15:5:1) using sonication (20 min) [3, 4]. Further sample prepn was carried out as previously described [2–4]. Alkaloid extracts were dissolved in 1 ml MeOH, and 10 μl were analysed by HPLC [3, 4]. Alkaloid contents in culture media were determined as follows: $NH_4OH$ (28%) (5 ml) was added to 50 ml of culture medium and alkaloids extracted × 2 with 20 ml of $CHCl_3$. The combined frs were then evaporated at 35°. The dried residue was redissolved in 500 μl MeOH and analysed by HPLC.

### *Preparation and addition of chemicals*

Chemicals were prepared as concd solns and autoclaved ($Cu^{2+}$, $Cd^{2+}$, chitin, chitosan and yeast extract), or filter-sterilized (GSH) [8, 15, 16], except for $H_2O_2$, before being added to 18-day-old root cultures. Bacto-yeast extract (DIFCO) was used [17].

### *Tissue viability*

Tissue viability was assayed using the 2,3,5-triphenyltetrazolium chloride (TTC) assay [12–14]. Harvested root cultures were placed on tissue paper to remove culture medium, and 250 mg roots (fr. wt) were then treated with 3 ml of 0.6% TTC soln (pH 7.4), and incubated for 14 h.

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