ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Hydrodynamic Stress Induces Monoterpenoid Oxindole Alkaloid Accumulation by *Uncaria tomentosa* (Willd) D. C. Cell Suspension Cultures via Oxidative Burst

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ABSTRACT: *Uncaria tomentosa* cell suspension cultures were grown in a 2-L stirred tank bioreactor operating at a shear rate $\dot{\gamma}_{avg} = 86\,s^{-1}$. The cultures showed an early monophasic oxidative burst measured as H_2O_2 production (2.15 μmol H_2O_2 g^{-1} dw). This response was followed by a transient production of monoterpenoid oxindole alkaloids (178 ± 40 μg L^{-1} at 24 h). At the stationary phase (144 h), the increase of the shear rate $\dot{\gamma}_{avg}$ up to 150 s^{-1} and/or oxygen tension up to 85% generated H_2O_2, restoring oxindole alkaloid production. *U. tomentosa* cells cultured in Erlenmeyer flasks also exhibited the monophasic oxidative burst but the H_2O_2 production was 16-fold lower and the alkaloids were not detected. These cells exposed to H_2O_2 generated in situ produced oxindole alkaloids reaching a maximum of 234 ± 40 μg L^{-1}. A positive correlation was observed between the oxindole alkaloid production and the endogenous H_2O_2 level. On the other hand, addition of 1 μM diphenyleneiodonium (NAD(P)H oxidase inhibitor) or 10 μM sodium azide (peroxidases inhibitor) reduced both H_2O_2 production and oxindole alkaloids build up, suggesting that these enzymes might play a role in the oxidative burst induced by the hydrodynamic stress.

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KEYWORDS: dissolved oxygen concentration; monoterpenoid oxindole alkaloid; oxidative burst; shear stress; stirred tank bioreactor; *Uncaria tomentosa*

Introduction

Uncaria tomentosa (Willd) D. C. (Rubiaceae) known as cat's claw is a plant widely used in traditional Peruvian medicine to treat cancer and chemotherapy side effects among a wide range of other diseases (Obregón-Vilches, 1995). Research on this matter led to the discovery of pentacyclic monoterpenoid oxindole alkaloids (MOA) with immunomodulatory, cytotoxic, anti-AIDS, and anti-leukemic activities (Laus, 2004; Winkler et al., 2004). The usual source of MOA is *U. tomentosa* powdered bark obtained

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from at least 8 years old native plants from the Amazon region (Obregón-Vilches, 1995). Since in the rainforest, there are only five specimens/hectare and the chemical synthesis of MOA is too complex, plant cell culture is a biotechnological alternative to produce these highly demanded and valuable compounds (Luna-Palencia et al., 2005; Trejo-Tapia et al., 2005).

The complete biosynthetic pathway of MOA in *U. tomentosa* still remains to be investigated but it has been proposed that in *Mitragyna*, these alkaloids are formed from monoterpenoid indole alkaloids through oxidation of the indole moiety (Shellard et al., 1969). Some oxidation experiments using indole derivatives, which are by far less complex substances than monoterpenoid indole alkaloids, have been carried out. Indeed, 5-fluoroindole-3-acetic is oxidized by horseradish peroxidase in the presence of H_2O_2 (Folkes et al., 2002), while indole derivatives are oxidized by adding H_2O_2 to suspension cultures of *Camellia sinensis*, *Nicotiana tabacum, Catharanthus roseus*, and *Daucus carota* (Takemoto et al., 2004). The effect of oxidative conditions on *U. tomentosa* alkaloids has not been studied.

The transient generation of reactive oxygen species (ROS), known as oxidative burst, is a hallmark of plant defense response to both biotic and abiotic stress (Apel and Hirt, 2004). The production of ROS has been established as one of the earliest signaling events involved in the response of plant to abiotic stress such as metals (Olmos et al., 2003), wounding (Orozco-Cárdenas and Ryan, 1999) and mechanical stress (Yahraus et al., 1995). ROS include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) (Apel and Hirt, 2004). Oxidases in mitochondria, chloroplasts, and peroxisomes generate ROS, but due to stress, the production of these active oxygen forms depends mainly on NAD(P)H oxidase located in plasmatic membrane (Auh and Murphy, 1995) and/or apoplastic peroxidases (Bolwell et al., 1998; Papadakis and Roubelakis-Angelakis, 1999).

On the other hand, abiotic stress has shown to induce first an oxidative burst and then accumulation of plant secondary metabolites in plant cell cultures (Zhao et al., 2005). For example, taxol production was induced by low-energy ultrasound in *Taxus chinensis* preceded by H_2O_2 generation (Wu and Ge, 2004), while shear stress in a viscosimeter stimulated the production of $O_2^{\bullet -}$ and taxol in *Taxus spp.* cell suspension cultures (Han and Yuan, 2004; Shi et al., 2003).

Plant cells cultivated in bioreactors are exposed to hydrodynamic stress (Doran, 1999). The increase in oxygen partial pressure induced H_2O_2 production in cells of *Panax notogingseng* cultured in an airlift bioreactor (Han and Zhong, 2003). *Glycine max* cell cultures subjected to mechanical stress or vigorous stirring in Erlenmeyer flasks experienced an oxidative burst (Legendre et al., 1993; Yahraus et al., 1995). No reports have shown specifically whether the shear stress and dissolved oxygen in a stirred tank bioreactor can induce an oxidative burst, which is required for induction of the alkaloid production in plant cell cultures.

In a previous work, we cultivated *U. tomentosa* cells (Uth-3 green line) in a 2-L stirred tank bioreactor. We found that MOA were accumulated in the culture medium and the alkaloid chemical profile was similar to that of the plant (Trejo-Tapia et al., 2005). MOA concentration was up to 10-fold higher than in Erlenmeyer flasks. Therefore, the aim of this work was to find out if shear stress or dissolved oxygen induced an oxidative burst in *U. tomentosa* cell suspension cultures and the link between this response and the alkaloid production.

Materials and Methods

Plant Cell cultures

U. tomentosa cultures (cell line green Uth-3b) were maintained in Murashige and Skoog (1962) (MS) medium supplemented with sucrose (20 g L^{-1}), 2,4-D (10 μM), and kinetin (10 μM) as described before (Trejo-Tapia et al., 2005). The pH of the medium was adjusted to 6.2 prior to sterilization. Cell suspension cultures were grown in 250-mL Erlenmeyer flasks (covered with aluminum foil) and subcultured every 14 days (10%, w/v). Cultures were grown in an orbital shaker at 110 rpm and $25 \pm 2^{\circ}C$ under a continuous illumination of 150 μmol $m^{-2}s^{-1}$.

Chemicals

Glucose, glucose oxidase (GO) from *Aspergillus niger*, H_2O_2, horseradish peroxidase (type II), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), diphenyleneiodonium (DPI) and Evan's blue were purchased from Sigma-Aldrich (St. Louis, MO) and sodium azide from Merck (Darmstadt, Germany).

Flask Cultures

Cell cultures were performed as described by Trejo-Tapia et al. (2005). Erlenmeyer flasks (125-mL) with 25 mL of MS culture medium were inoculated with 9-day-old suspension cultures (10%, w/v). Every 3 days, three flasks were harvested for analytical measurements.

Stirred Tank Bioreactor Cultures

Growth and MOA Production Kinetics

Culture of *U. tomentosa* in the stirred tank bioreactor was done as previously described (Trejo-Tapia et al., 2005). A 2-L stirred tank (Applikon, Schiedam, Netherlands) with a jacketed glass vessel and a multiport stainless head plate operating at a tip speed of 95 cm s^{-1} (average shear rate

$\dot{\gamma}_{avg} = 86\,s^{-1}$; agitation speed of 400 min^{-1}) was used. Dissolved oxygen tension (DOT) was automatically maintained above 20% by gassing oxygen when it was necessary. The fermenter (with an initial volume of 1.4 L) was inoculated with cells (10%, w/v) from 9-day-old suspension cultures. The pH was maintained over 5.0 ± 0.1 with NaOH (0.1 N) using a biocontroller ADI 1030 (Applikon, Schiedam, Netherlands). NaOH addition was only necessary for the first 96 h; about 250 mL of the solution were added during the experiment. Cells in the stirred tank were grown under continuous illumination of 150 μmol $m^{-2}s^{-1}$. Samples of 60 mL of cell culture were harvested from the vessel for its analysis every 3 days. In all cases, the results reported are the average of two independent experiments and the changes in the volume of the reactor were taken into account to calculate biomass and alkaloid concentrations.

Effect of Shear Stress and Dissolved Oxygen Concentration in Stationary Phase Cells

All experiments were performed with *U. tomentosa* cells of 144 h (early stationary phase) grown in the bioreactor as indicated before. *Control*: Constant impeller tip speed of 95 cm s^{-1} ($\dot{\gamma}_{avg} = 86\,s^{-1}$) without controlling DOT. *Increase in* $\dot{\gamma}_{avg}$: In this experiment, impeller tip speed was increased from 95 ($\dot{\gamma}_{avg} = 86\,s^{-1}$) to 167 cm s^{-1} ($\dot{\gamma}_{avg} = 150\,s^{-1}$) for 90 min without controlling DOT. *Increase in* $\dot{\gamma}_{avg}$ *with DOT constant*: One more set of experiments was done increasing $\dot{\gamma}_{avg}$ to 150 s^{-1} for 90 min and DOT was maintained *ca*. 25% by gassing air + nitrogen. *Increase in DOT*: Pure oxygen + air were gassed to increase DOT from 25 to *ca*. 85% for 90 min and $\dot{\gamma}_{avg}$ was constant as the control. For all experiments, the total gas flow was maintained at 0.1 vvm.

Estimation of Impeller Tip Speed and $\dot{\gamma}_{avg}$

The values of tip speed corresponding to the agitation speed of 400 and 700 min^{-1} were calculated as follows:

$$\text{tip speed} = \pi N Di \tag{1}$$

where tip speed is in cm s^{-1}, N = agitation speed (s^{-1}), and Di = turbine diameter (cm). The values of average shear rate ($\dot{\gamma}_{avg}$) corresponding to the agitation speed of 400 and 700 min^{-1} were calculated based on the equations proposed by Bowen (1986):

$$\dot{\gamma} = 4.2N\left(\frac{Di}{T}\right)^{0.3}\left(\frac{Di}{W}\right) \tag{2}$$

where $\dot{\gamma}_{avg}$ = average shear rate (s^{-1}), N = agitation speed (s^{-1}), Di = turbine diameter (cm), T = stirred tank diameter (cm), and W = turbine width (cm).

Biomass

Cell growth was measured as dry weight (dw) determined by filtration of aliquots (3 mL) through a filter paper of known weight that was dried to constant weight at 70°C.

Alkaloid Extraction and Quantification

MOA extraction and quantification were done as explained formerly (Trejo-Tapia et al., 2005). Briefly, liquid-nitrogen frozen cells (2 g of fresh weight) were macerated and sonically extracted with 5% hydrochloric acid (8 mL). Cells were vacuum filtered. Alkaloids were extracted from the cell extract or the culture medium, with chloroform (twofold) after adjustment of the pH to 8–9. The organic layer was evaporated under vacuum to dryness. The residue was dissolved in a 9:11 mixture of acetonitrile and 10 mM phosphate buffer at pH 7 (100 μL). The solutions were filtered through 0.45 μm nylon membranes and injected (20 μL) into the HPLC system (Varian Chromatograph Prostar 333 with a photodiode array detector; Varian, Walnut Creek, CA) equipped with a reversed-phase C18 column (Waters Spherisorb 5 μm 0DS2 of 250 mm length × 4.6 mm i. d.). Elution was carried out with the 9:11 mixture of acetonitrile and 10 mM phosphate buffer at a flow rate of 0.7 mL min^{-1}. Detection was done at 244 nm for MOA and at 215 for tryptamine alkaloids (TA). The alkaloid peaks were identified and quantified by employing the standard addition method and by comparison of the UV curves and retention times with those of the reference compounds which were obtained from the bark of *U. tomentosa* as described by Luna-Palencia et al. (2005).

Cell Viability

Cell membrane integrity was measured by the Evan's blue dye exclusion test reported by Rodríguez-Monroy and Galindo (1999). Cells were incubated with 0.25% Evans blue for 5 min and microscopically analyzed. A total of 700 cells were counted. The percent viability of a sample was determined based on the number of non-stained cells (viable) to that of total cells.

Measurement of Extracellular H_2O_2 Production

For H_2O_2 evaluation, the cell broth was centrifuged at 720 × g for 10 min. To 325 μL of the supernatant were added with 1,000 μL phosphate buffer (sodium phosphate 0.1 M, pH 7.0), later 150 μL luminol (2.5 mM in sodium phosphate buffer 1.0 M, pH 7.0) and finally 25 μL peroxidase (6 mU μL^{-1}). Luminescence was recorded immediately for 3 min at 30 s intervals in a luminometer (Betascout 2007, Perkin Erlmer Life Sciences, Turku, Finland) and the maximum level was registered (Baker et al., 1995). Phosphate buffer (1325 μL) was used as a blank and its luminescence value was subtracted from that of the

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sample. A standard curve was made between 0 and 50 μM H_2O_2 and hydrogen peroxide was expressed as μmol H_2O_2 per unit of dry weight.

Generation of H_2O_2 in situ

The oxidation of glucose with GO was used to generate H_2O_2 in situ in the cell cultures (Dorey et al., 1999) as follows: 10 mM glucose and 0.5, 5, and 50 U L^{-1} of GO (filtered through 0.22 μm nylon membranes) were added to stationary phase cells (8 to 9 days old, 12 g dw L^{-1}) cultivated in 250-mL Erlenmeyer flasks with 50 mL of MS medium. MOA and tryptamine production were evaluated 24 and 48 h later. Two flasks were used for each condition and the experiment was performed in three occasions.

Inhibition of NAD(P)H Oxidase and Peroxidases in Bioreactor Cultures

DPI was used as a specific NAD(P)H oxidase inhibitor (Auh and Murphy, 1995) and NaN_3 as peroxidases inhibitor. *U. tomentosa* cells were pretreated with 1 μM DPI (added as 10 mM stock in DMSO) or with 10 μM NaN_3 (added as a 100 mM stock in water) during 30 min before bioreactor inoculation. Culture media contained the same inhibitor concentration as treated cells. DMSO was assayed as a control to verify that it does not induce H_2O_2 production. Extracellular H_2O_2 production was followed for 24 h and then MOA accumulation was measured.

Results

Performance of *Uncaria Tomentosa* Cells in Stirred Tank Bioreactor and Shaken Flasks

U. tomentosa cells exhibited a similar growth profile in the stirred tank bioreactor and shaken flasks (Fig. 1A). In both experiments, cell viability was around 80% but the maximal biomass was 20% higher in the first case. A monophasic oxidative burst occurred during the first hour, although in stirred tank bioreactor the maximum H_2O_2 concentration was 2.15 μmol g^{-1} dw (Fig. 1B) which was 16-fold higher than in flasks. Throughout the remaining culture period, H_2O_2 was not detected in the medium. A transient pH increase—which coincided with the oxidative burst peak—followed by acidification, was observed in the stirred tank bioreactor. During the first 20 min, the pH increased from 5.2 to 5.5 and then decreased to 5.0 after 3.0 h (Fig. 1B), from this time until the culture ended the pH was controlled over 5.0.

In the stirred tank bioreactor, *U. tomentosa* cells produced a maximum MOA concentration of 178 ± 40 μg L^{-1} (21 ± 5 μg g^{-1} dw) 24 h after inoculation. Later MOA decreased to 44 ± 11 μg L^{-1} at 48 h and, from 100 to 340 h, the alkaloids were no longer detected (Fig. 2A). It was found that 95% of

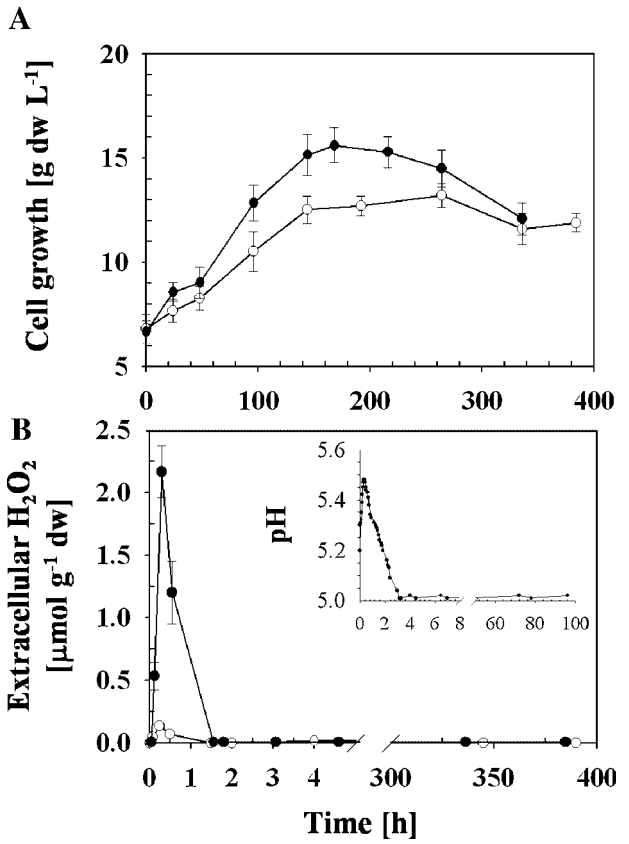

Figure 1. Cell growth kinetics (**A**) and H_2O_2 extracellular production (**B**) of *U. tomentosa* cell suspension cultures growing in a stirred tank bioreactor (•) and in Erlenmeyer flasks (○). Insert presents pH profile in the stirred tank during the first 100 h of culture. pH was controlled at 5.0 ± 0.1 after 3 h of culture. Error bars indicate standard deviation from the mean ($n = 4$).

the total MOA were excreted to the culture medium. The alkaloids were mainly pteropodine, isopteropodine, and mitraphylline, which are the three major MOA in *U. tomentosa* bark (Trejo-Tapia et al., 2005), but also tryptamine alkaloids (TA) in a range between 25 ± 5 and 50 ± 8 μg L^{-1} were produced (Fig. 2A). In contrast, the same cell line growing in shaken flasks did not produce MOA although it accumulated TA (between 25 ± 6 and 100 ± 12 μg L^{-1}) (Fig. 2B). These results suggest that MOA production could be linked to H_2O_2 production and thus to an oxidative burst.

Stimulation of MOA Production by H_2O_2 Generated in situ

With the aim of investigating the role of H_2O_2 in MOA production, H_2O_2 was generated in situ through oxidation of glucose with GO (Dorey et al., 1999) in cells growing in shaken flasks. First, the effect of 10 mM glucose and several amounts of the enzyme (0.5, 5.0 and 50.0 U GO L^{-1}) on cell viability was tested. When 50.0 U L^{-1} of GO

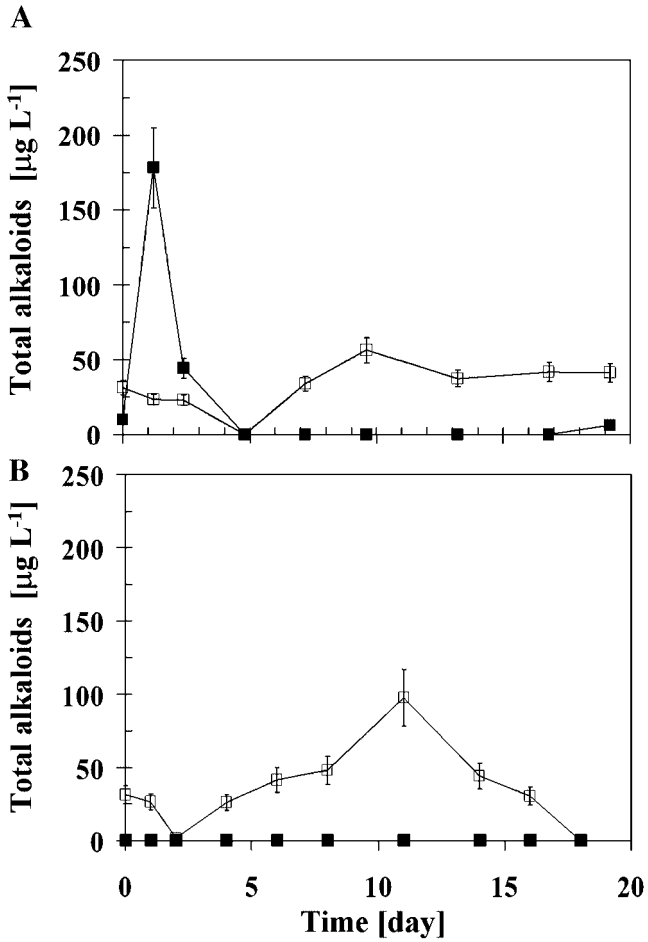

Figure 2. Production of MOA (■) and TA (□) in *U. tomentosa* cell suspension cultures growing in a stirred tank bioreactor (A) and in Erlenmeyer flasks (B). Error bars indicate standard deviation from the mean ($n = 4$).

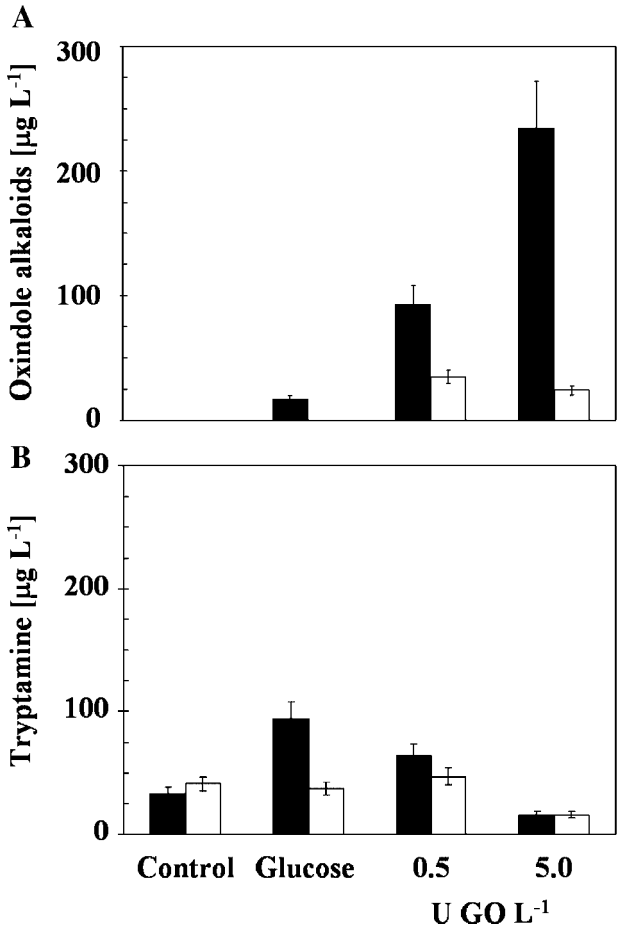

Figure 3. MOA (**A**) and tryptamine (**B**) production in *U. tomentosa* cell suspension cultures incubated in Erlenmeyer flasks with glucose (10 mM) and glucose oxidase (GO): (■) 24 (□) 48 h. Glucose/GO were added to 9 days-old cells (stationary phase). Error bars indicate standard deviation from the mean ($n = 9$).

(41.7 mmol H_2O_2 g^{-1} dw) were added, the cells turned orange-brown and the cell viability dropped drastically from 80 to 25% in the first 6 h, so the next experiments were performed with 0.5 or 5.0 U L^{-1} of GO (0.41 and 4.17 mmol H_2O_2 g^{-1} dw, respectively). Production of MOA was efficiently stimulated by in situ H_2O_2 generation; indeed when 5.0 U GO L^{-1} were added, the cells produced 2.5 times more MOA than with 0.5 U GO L^{-1} (Fig. 3A). Also, it was observed that these alkaloids were further metabolized. Additionally, 0.5 U GO L^{-1} increased tryptamine accumulation (Fig. 3B).

Effects of Shear Stress and Dissolved Oxygen Concentration on Stationary Phase Cells

During the stationary growth phase, *U. tomentosa* cells cultivated in the stirred tank bioreactor at a constant $\dot{\gamma}_{avg} = 86\,s^{-1}$ and DOT *ca.* 25% (Fig. 4A) did not accumulate H_2O_2 (Figs. 1 and 5A) neither MOA (Fig. 2A). In order to test if these cells could undergo oxidative burst, shear stress ($\dot{\gamma}_{avg}$), and DOT were increased together and individually. The increase in $\dot{\gamma}_{avg}$ from 86 to 150 s^{-1} provoke DOT enhancement from 25 to 57% (Fig. 4B). In another set of experiments, $\dot{\gamma}_{avg}$ was increased to 150 s^{-1} while DOT was maintained *ca.* 25% (Fig. 4C). To test the effect of dissolved oxygen, DOT was controlled to *ca.* 85% (Fig. 4D) and $\dot{\gamma}_{avg}$ was kept constant at 86 s^{-1}. In all cases, the cell viability was $\geq$75%. The increment in shear stress and DOT caused an oxidative burst in the cells and H_2O_2 production preceded MOA accumulation (Figs. 5 and 6). When DOT + $\dot{\gamma}_{avg}$ or DOT alone were increased, the cells exhibited a biphasic H_2O_2 production profile (Fig. 5B and D), while the increment in $\dot{\gamma}_{avg}$ alone produced a monophasic response (Fig. 5C). The increment in DOT + $\dot{\gamma}_{avg}$ induced the highest H_2O_2 (Fig. 5B) and MOA productions (Fig. 6). Actually, the alkaloid concentration was twofold higher than when DOT or $\dot{\gamma}_{avg}$ alone was increased. Indeed, all data obtained in stirred tank indicated a non-linear relationship between peroxide and oxindole alkaloid concentration

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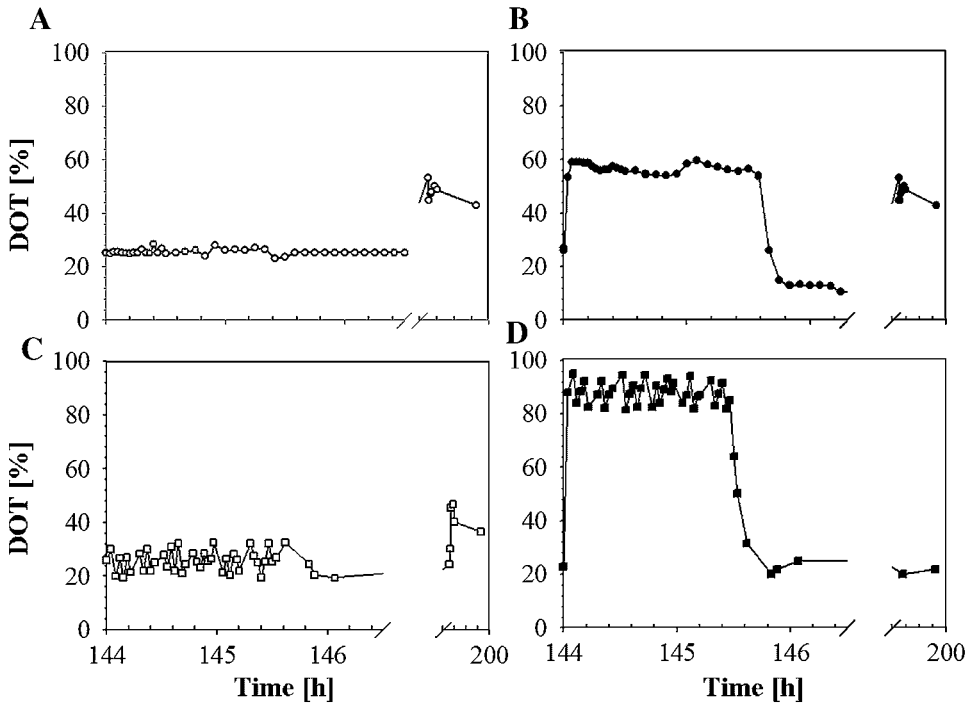

Figure 4. DOT profile of *U. tomentosa* cell cultures in the stirred tank bioreactor during the stationary phase. (**A**) $\dot{\gamma}_{avg}$ constant at 86 s^{-1} with DOT *ca.* 25% (control); (**B**) increase in $\dot{\gamma}_{avg}$ from 86 to 150 s^{-1} without DOT control; (**C**) increase in $\dot{\gamma}_{avg}$ from 86 to 150 s^{-1} with DOT *ca.* 25%; (**D**) increase in DOT from 25 to *ca.* 85% with $\dot{\gamma}_{avg}$ constant at 86 s^{-1}. Culture conditions were changed at 144 h and restored at 145.5 h. In all cases, air flow was constant at 0.1 vvm. Error bars indicate standard deviation from the mean ($n = 4$).

(Fig. 7) described by equation 3 ($R^2 = 0.848$).

$$\text{MOA} = 60 \times \ln H_2O_2 + 124.6 \qquad (3)$$

where MOA is the volumetric oxindole alkaloid concentration (in μg L^{-1}), and H_2O_2 is the hydrogen peroxide concentration (in μmol g^{-1} dw).

Effects of Inhibitors of Oxidases and Peroxidases on MOA Production

To confirm that ROS is involved in MOA biosynthesis, DPI a NAD(P)H oxidase inhibitor or NaN_3 a peroxidase inhibitor were added to *U. tomentosa* cell suspension cultures growing in the stirred tank bioreactor. At the concentrations used in the experiments, DPI and NaN_3 inhibited H_2O_2 production by 92 and 82%, respectively, and MOA biosynthesis by greater than 90% (Table I). Thus, MOA production strongly depends on the ROS generation and production of H_2O_2 is enzyme mediated.

Discussion

When *U. tomentosa* cells were inoculated into the stirred tank bioreactor the pH increased in a transient way, this increment coincided with the oxidative burst and was followed by the acidification of the broth (Fig. 1). Han and Yuan (2004) and Shi et al. (2003) also found a pH increase in *Taxus spp.* cell cultures exposed to shear stress in a viscosimeter. Moreover, in plant/pathogen interactions, a transient increase in the pH is characteristic of an oxidative burst and has been linked to the activation of peroxidases, which are involved in ROS generation (Bolwell et al., 2002). On the other hand, acidification is a common response of plant cells subjected to shear stress (Chen and Huang, 2000; Meijer et al., 1993).

U. tomentosa cells promptly produced MOA in the bioreactor as shown by the transient peak at 24 h of culture (Fig. 2A). Within 48–100 h, MOA accumulation quickly dropped suggesting a biosynthesis/degradation pattern, as reported for β-thujaplicin production by *Cupressuss lusitanica* cell suspension cultures (Zhao and Sakai, 2003). Most secondary metabolites, including alkaloids are toxic for the cells themselves, so plants have developed mechanisms for detoxification and compartmentalization. These mechanisms involve the metabolite transport to the vacuole or to the apoplast, where turnover reactions proceed. Once in the storage site, the metabolite can interact with chemicals and/or proteins to form longer-lasting structures, or can be degraded by catabolic enzymes and/or chemical reactions for recycling in other metabolic pathways (Roytrakul, 2004). In particular, indole alkaloids might be degraded either by

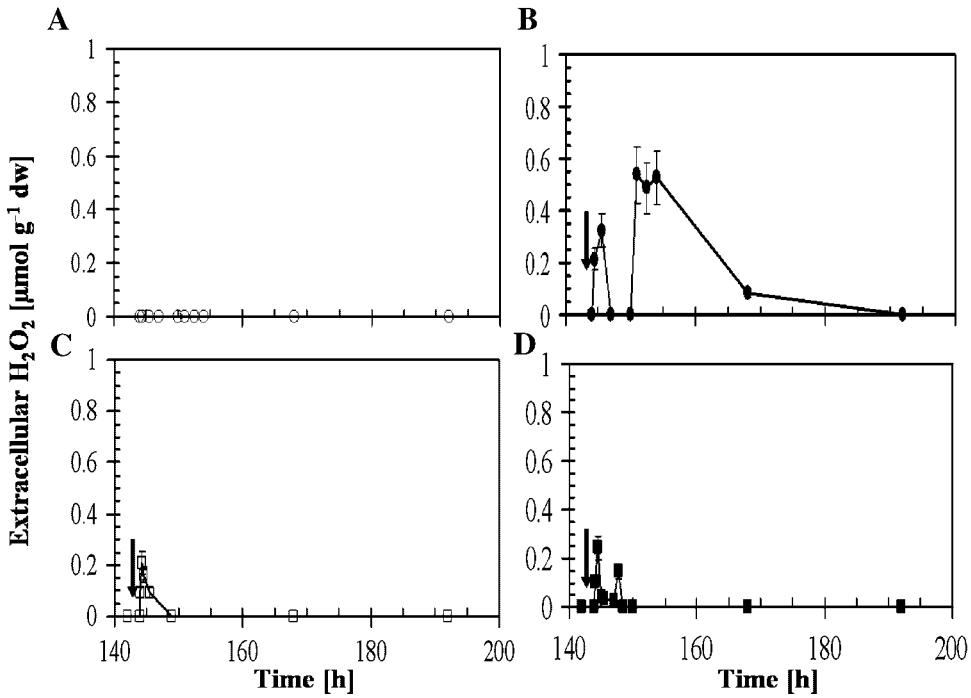

Figure 5. Extracellular H_2O_2 production in *U. tomentosa* cell cultures in the stirred tank bioreactor during the stationary phase. (**A**) $\dot{\gamma}_{avg}$ constant at 86 s^{-1} with DOT *ca.* 25% (control); (**B**) increase in $\dot{\gamma}_{avg}$ from 86 to 150 s^{-1} without DOT control; (**C**) increase in $\dot{\gamma}_{avg}$ from 86 to 150 s^{-1} with DOT *ca.* 25%; (**D**) increase in DOT from 25 to *ca.* 85% with $\dot{\gamma}_{avg}$ constant at 86 s^{-1}. (↓) Arrow indicates that culture conditions were changed at 144 h being restored at 145.5 h. In all cases, air flow was constant at 0.1 vvm. Error bars indicate standard deviation from the mean ($n = 4$).

catalytic enzymes or chemically, as in *Tabernaemontana divaricata* cell suspension cultures (Dagnino et al., 1993).

The maximum value of H_2O_2 obtained in this work was 2.15 μmol g^{-1} dw which is lower to that reported in *Panax notoginseng* cell cultures grown in an airlift bioreactor (1 mmol H_2O_2 g^{-1} dw) after 50 min of oxygen exposure (Han and Zhong, 2003). Our results show that certain levels of H_2O_2 are required to stimulate the production of MOA in non-producing *U. tomentosa* cells. It seems that a calculated threshold higher than 0.13 μmol g^{-1} dw is needed to stimulate MOA production. Also, it is observed a non-linear relationship between extracellular peroxide and oxindole alkaloid concentration (Fig. 7). These results agree with Mur et al. (2005) who reported that a minimum level of H_2O_2 is

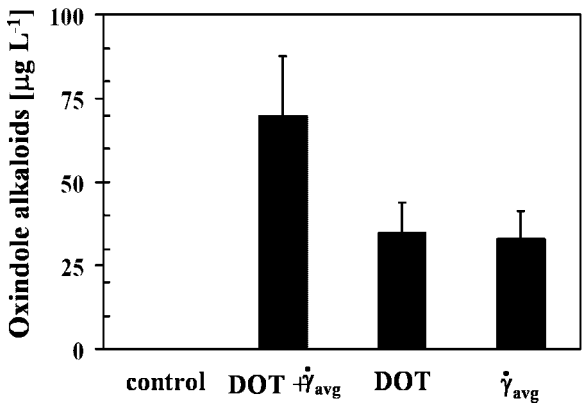

Figure 6. Influence of shear rate and DOT on MOA accumulation in *U. tomentosa* cell suspension cultures in a stirred tank bioreactor measured 24 h after the culture conditions were modified: (control) $\dot{\gamma}_{avg}$ constant at 86 s^{-1} with DOT *ca.* 25%; ($DOT + \dot{\gamma}_{avg}$) increase in $\dot{\gamma}_{avg}$ from 86 to 150 s^{-1} without DOT control; ($\dot{\gamma}_{avg}$) increase in $\dot{\gamma}_{avg}$ from 86 to 150 s^{-1} with DOT *ca.* 25%; (DOT) increase in DOT from 25 to *ca.* 85% with $\dot{\gamma}_{avg}$ constant at 86 s^{-1}. Error bars indicate standard deviation from the mean ($n = 4$).

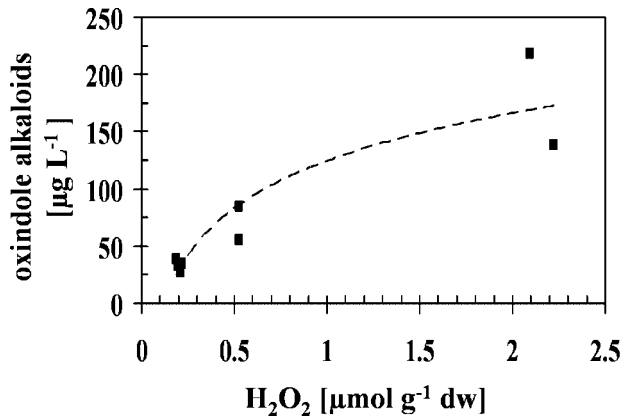

Figure 7. Relationship between H_2O_2 concentration and oxindole alkaloids in cell cultures in the stirred tank bioreactor.

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Table I. ROS inhibitors and their influence on H_2O_2 and MOA production in *U. tomentosa* cell suspension cultures growing in a stirred tank.

		Inhibition (%)	
Inhibitor (dose)	Targeted response	H_2O_2[a]	MOA[b]
Control (without inhibitors)		0	0
+DPI (1 μM)	NAD(P)H oxidase	92	95
+NaN_3 (10 μM)	Peroxidases	82	92

[a]H_2O_2 accumulation obtained after 30 min of stress in the bioreactor was set as the reference value (1.02×10^7 RLU g^{-1} dw).
[b]MOA accumulation observed after 24 h of culture in the bioreactor was set as the reference value (178 μg L^{-1}) (Standard error < 10%).

needed to induce the expression of transgenes AoPR10 and PR1a in the leaves of transformed *Nicotiana tabacum* plants. They also observed that although the minimum H_2O_2 level needed to induce each transgene was different, the activity of both transgenes was H_2O_2 dose-dependent. As a matter of fact both genes are related to the plant defense response against *Pseudomonas syringae*.

Even though the biochemical or molecular mechanisms in *U. tomentosa* are unknown, ROS should be implicated in secondary metabolism in several ways (Zhao et al., 2005). In *T. chinensis* cells exposed to low-energy ultrasound, ROS was involved as an upstream event for activation of jasmonic acid synthesis and both events were partially implicated in taxol biosynthesis regulation (Wu and Ge, 2004). Particularly, ROS is related to indole alkaloid biosynthesis, since the oxidation of the monoterpenoid indole alkaloids as ajmalicine to serpentine is catalyzed by peroxidases involving H_2O_2 (Blom et al., 1991). Our results suggest that in *U. tomentosa* cells a NAD(P)H oxidase and/or peroxidases could be implicated in H_2O_2 production. Although the biosynthetic pathway of MOA is not fully elucidated, it has been reported that indole moieties can be oxidized to oxindole by several classes of enzymes like cytochrome P_{450} (Gillam et al., 2000), chloroperoxidases (van de Velde et al., 2001) and peroxidases requiring H_2O_2 (Folkes et al., 2002; Takemoto et al., 2004). Taken together, these reports suggest that an oxidative condition may stimulate MOA production.

Plant cells are able to enter into a "refractory state" in which they are unable to respond to the same or even to a different stimulus for a second time (Legendre et al., 1993; Färber et al., 2003), thus desensitization avoids oxidative damage due to over stimulation. Therefore, the diminishing in the H_2O_2 level and the change in the ROS production profile in *U. tomentosa* cells observed between the early response (Fig. 1) and the stationary phase (Fig. 5) might be attributed to a desensitization phenomenon as a result of the first hydrodynamic stress contact. However, at the stationary phase, the cells developed in the bioreactor responded to an increment in the shear stress and DOT, producing H_2O_2 and later MOA. An increment of both DOT + $\dot{\gamma}_{avg}$ was more effective to stimulate MOA production than an increment on each of them.

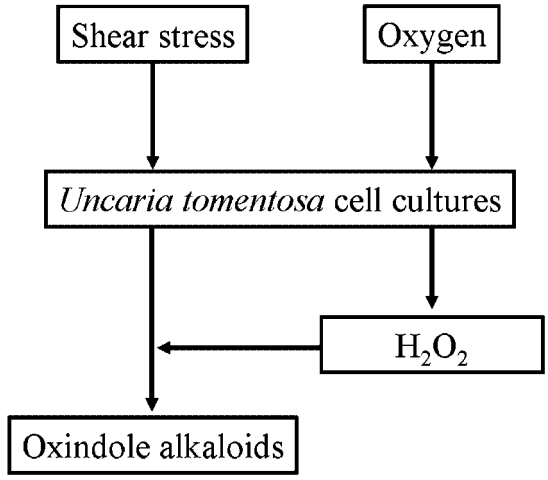

Figure 8. Proposed model for production of MOA in *U. tomentosa* cell suspension cultures induced by shear stress and oxygen.

We propose that shear stress and dissolved oxygen initiate an oxidative burst in *U. tomentosa* cell suspensions cultivated in a stirred tank bioreactor where the formed H_2O_2 stimulates MOA production. The suggested model, sketched in Figure 8, will be substantially improved when the studies about H_2O_2 acting either as a signal molecule or as a donor to oxidize MOA precursors are concluded.

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