Section: Plant Cell Technology

New method to enhance ajmalicine production in *Catharanthus roseus* cell cultures based on the use of cyclodextrins.

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Abstract

The joint use of cyclodextrins and methyljasmonate, when accompanied by a short exposure to UV, enhanced extracellular ajmalicine accumulation to 1040 ± 26.6 mg/l in suspension cultured cells of *Catharanthus roseus*. The success of this strategy is due to the use of cyclodextrins, which not only induce ajmalicine biosynthesis but also promote adduct formation. This removes ajmalicine from the medium, reduces feedback inhibition and ajmalicine degradation, and allows its accumulation in the culture medium at elevated concentrations.

Keywords: Ajmalicine; *Catharanthus roseus*; cell culture; cyclodextrin; elicitation; methyl jasmonate.

Introduction

Catharanthus roseus synthesizes a range of indole alkaloids that have pharmaceutical applications. Serpentine and ajmalicine have anti-hypertensive activities and are used to combat heart arrhythmias and to improve blood circulation in the brain (van der Heijden et al. 2004). Global demand for these compounds is increasing and new methods are needed to enhance their production. Plant cell cultures could be considered an alternative for producing indole alkaloids rather than extracting them from the whole plant. One of the strategies to enhance metabolite production using suspension cultured cells (SCC) is to use elicitors.

Random methylated β -cyclodextrins (β -CDs) are modified cyclic oligosaccharides derived from starch with seven glucose residues linked by $\alpha(1\rightarrow4)$ glycosidic bonds. They can solubilize hydrophobic molecules by virtue of their hydrophobic interior. β -CDs are true elicitors since they act by inducing defence responses, including phytoalexin biosynthesis (Zamboni et al. 2009). Their action resides in their chemical structure, which is similar to that of the alkyl-derived pectic oligosaccharides released from the cell walls when a fungal infection occurs (Bru et al. 2006).

Plant cell cultures can also be elicited by jasmonates, resulting in increased accumulation of defense-related compounds (Xu & Dong 2008). Treatment of *C. roseus* SCC with methyl jasmonate (MJ) promoted ajmalicine extracellular accumulation (Lee-Parsons et al. 2004). In addition, UV-B light induces both the formation of indole alkaloids and signalling events which enhance the production of catharantine in SCC (Ramani & Chelliah 2008).

On the other hand, the production of high levels of indole alkaloids is dependent on both tissue and cellular compartmentation. Neumann et al. (1983) demonstrated that indole alkaloids were localized in the vacuoles of *C. roseus*. Therefore, the addition of sequestering agents, like β -CDs, to cell cultures might promote their secretion to the culture medium and improve their production by mimicking the effect of storage in the vacuoles or cell walls.

The objective of this study was to analyze the effect of these different inducing factors on ajmalicine production in *C. roseus* SCC.

Materials and Methods

Chemical reagents

β -CDs were from Wacker Chemie. Other chemicals were standard commercial products of analytical grade.

Plant Materials

C. roseus SCC cv. First Kiss Apricot were obtained from young leaf-derived callus by inoculating small friable callus pieces into 250 ml flasks containing 100 ml LS medium (Linsmaier & Skoog 1965) supplemented with 0.4 mg thiamine/l, 100 mg myo-inositol/l, 30 g sucrose/l, 0.19 mg naphthalene acetic acid/l and 0.22 mg 2,4-dichlorophenoxyacetic acid/l. The SCC were incubated in the dark at 25°C and shaken at 110 rpm.

Elicitor treatments

Elicitation experiments were performed in triplicate on 14 day old *C. roseus* SCC. Cells were transferred into 250 ml flasks and suspended in 100 ml of fresh medium supplemented with either β -CDs or MJ (in ethanol) or a combination of both. In other cases, elicitation was started with MJ at different concentrations in combination with 50 mM β -CDs and/or under UV-light at short and long exposure times in the presence or in the absence of the above chemical elicitors. Flasks were opened in a laminar flow hood and exposed to UV-C light (254 nm, 10 μ W/cm²) for 15 or 120 min.

Extraction, identification and quantification of ajmalicine

Ajmalicine was extracted three times from extracellular media by phase partitioning using ethyl acetate after adjusting pH to 10. The organic phase was collected and evaporated at 40 °C in vacuum. The solid residue was dissolved in 1ml of methanol, filtered and analyzed by HPLC-DAD (Jasco LC-NetII/ADC) as described Zhao et al. (2000) using a Teknokroma Mediterranea Sea 18 column (150 x 4.6 mm, 3 μ). Ajmalicine was identified and quantified by comparison with its commercial standard.

Results and discussion

Characterization of *C. roseus* cell growth

C. roseus growth was characterized by measuring g dry weight/l, pH and conductivity of the spent media and growth kinetic parameters. Fig. 1 shows a typical S-shaped growth curve with a maximum of 10 g dry wt/l at 14 days and depletion time, which represents the estimated time at which the scarcity of nutrients would cause stress and cell death, at 12 days.

Effect of cell density on ajmalicine production in *C. roseus* SCC elicited with β -CDs and MJ individually or in combination.

100 μ M MJ was used because this is considered as the optimal dose to induce metabolites in various plant species, including *C. roseus* (Lee-Pearson et al. 2004), while 50 mM β -CDs is considered sufficiently high not to limit metabolite production (Bru et al. 2006).

Ajmalicine production using low cell densities (100 g fresh wt/l or 5 g dry wt/l) was significantly lower than when high cell densities (200 g fresh wt/l) were used, in all conditions tested (Fig. 2). Our results represent an improvement over those of Lee & Shuler (2000), who studied the effect of cell-inoculum density on both fresh weight and ajmalicine production using *C. roseus* cells immobilized on alginate beads. They found that once the maximum fresh weight concentration was reached (450 g fresh wt/l) the maximum production of ajmalicine was over 120 mg/l. In our experiments, using 200 g fresh wt/l, the maximum value of extracellular ajmalicine reached in the presence of β -CDs (which act in a similar way to the amberlite XAD-7 resin used by the above authors) was 216 ± 18 mg/l. [Note that at a high cell density, the combined treatment of MJ and β -CDs led to much higher ajmalicine accumulation than the sum of the individual additions, so that the effect can be regarded as synergistic. This effect would be due to the presence of elicitors which both act as inducers of ajmalicine biosynthesis while the action of β -CDs also promotes the adsorption and recovery of ajmalicine from the medium and reduces feedback inhibition and degradation.]

A similar improvement was described by Zhao et al. (2001), who observed a synergistic effect on indole alkaloid accumulation in *C. roseus* SCC elicited with fungal preparations and chemicals. However, their highest ajmalicine levels were always below 100 mg/l.

Ajmalicine production is dependent on MJ dose, time of elicitation and scale-up

As shown in Fig. 3, the maximum ajmalicine production was reached when cell suspensions were incubated in the presence of 50 mM β -CDs and 100 μ M MJ. Extracellular ajmalicine increased linearly until 192 h ($= 930 \pm 16$ mg/l), and then decreased for the rest of the experiment (on day 14 = 740 ± 8 mg/l) – SEE Fig. 4). However, in the control culture medium, ajmalicine was hardly detected (data not shown).

Lee-Parsons et al. (2004) also studied the relationship between MJ dose and ajmalicine production using different MJ concentrations with resin (Amberlite XAD-7HP). Maximum ajmalicine accumulation (8.1 ± 0.8 mg/l) was obtained with 100 μ M MJ on day 15, a 240% increase over the controls. These values are well below those obtained using MJ and /or β -CDs in our experiments at a high cell density (Figs. 2 and 3) but are of the same order or above those observed when a lower cell density was used (Fig. 2: 0.7 ± 3.1 mg/l in the presence of MJ, and 11.9 ± 5 mg/l in the presence of MJ and β -CDs). In conclusion, the enhancement of extracellular ajmalicine production is not only dependent on the MJ dose but also on the cell density used and time of elicitation.

The effect of cell biomass and flask size on ajmalicine production was studied, using a cell suspension volume of 100, 200 and 400 ml in flasks of 250, 500 and 1000 ml of capacity, respectively (results not shown). Ajmalicine production slightly decreased as the flask size increased probably as a result of the amount of dissolved O₂ and CO₂, ethylene production, nutrient supply and cell suspension volume.

Effect of UV light exposure and elicitors on ajmalicine production

As shown in Fig. 5, ajmalicine production was dependent on UV-light exposure time, since long UV-light exposure (120 min) caused a reduction in ajmalicine accumulation in all treatments. Short UV-light had no stimulatory effect when used with MJ. However, with β -CDs, short exposure to UV-light led to a significant increase in ajmalicine production. At a similar short exposure time, the use of β -CDs and MJ led to a further increase in ajmalicine accumulation. Thus, in addition to the synergistic effect observed on ajmalicine accumulation provoked by the joint presence of β -CDs and MJ, there was also synergism when these elicitors were used in combination with short UV-light exposure. Similarly, Ramani & Chelliah (2008) observed an increase in the production of catharanthine and vindoline from *C. roseus* SCC when cells were irradiated with UV-B light for 5 min.

In conclusion, to achieve high ajmalicine production levels using a productive *C. roseus* cell line, the best operating conditions were elicitation using a combination of β -CDs, MJ and short UV-light exposure. β -CD was fixed at 50 mM and the optimal MJ dose for the production of ajmalicine was 100 μ M. Short exposure to UV-light led to a significant increase in ajmalicine while long UV-light irradiation was clearly detrimental to ajmalicine accumulation. Moreover, ajmalicine production increased as cell density increased. The enhancement of extracellular ajmalicine production was not only dependent on the culture growth stage and elicitor dose but also on cell density used and the duration of elicitation. The combined use of elicitors during a relatively short time could well be considered as a novel way for the industrial production of ajmalicine, since its accumulation is hardly affected as a result of scaling-up.

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Figure captions

Fig. 1 Time course of *C. roseus* cell growth measured as g dry weight/l. The initial cell density was 2 g dry weight/l.

Fig. 2 Effect of cell density on extracellular ajmalicine production in *C. roseus* SCC. Cell suspensions were treated with 50 mM β -CDs and 100 μ M MJ individually or in combination. Results were evaluated 96 h after treatment. Values are given as the mean $\pm$ SD of one experiment with three replicates.

Fig. 3 Effect of different MJ concentrations (25, 75, 100, 200 and 300 μ M) on extracellular ajmalicine production in *C. roseus* SCC treated with 50 mM β -CDs. Results were evaluated 96 h after treatment. Values are given as the mean $\pm$ SD of one experiment with three replicates.

Fig. 4 Time course of ajmalicine production in *C. roseus* SCC. Cell suspensions were treated with 50 mM β -CDs and 100 μ M MJ individually or in combination. Ajmalicine was extracted from the spent medium after different incubation periods. Values are given as the mean $\pm$ SD of one experiment with three replicates.

Fig. 5 Effect of UV-light exposure time (15 and 120 min) on extracellular ajmalicine production in *C. roseus* SCC elicited with 50 mM β -CDs and 100 μ M MJ individually or in combination. Results were evaluated 192 h after treatment. Values are given as the mean $\pm$ SD of three replicates

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