Enhanced Rosmarinic Acid Production by *Lavandula vera* MM Cell Suspension Culture through Elicitation with Vanadyl Sulfate

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The influence of elicitation on rosmarinic acid biosynthesis by *Lavandula vera* MM cell suspension culture was investigated using vanadyl sulfate as an abiotic elicitor. It was established that 12 h after treatment with 25 mg/l vanadyl sulfate the rosmarinic acid production was increased up to 3.92 g/l (2.8 times higher compared to the control cultivation). No significant amounts of rosmarinic acid were detected in the culture medium in comparison with its intracellular content. However, it was observed that the extracellular content of rosmarinic acid is 3.3 times higher compared to the control variant (4 h after treatment at elicitor concentration 25 mg/l).

Key words: Elicitation, *Lavandula vera* MM, Rosmarinic Acid, Vanadyl Sulfate

Introduction

Rosmarinic acid (RA) possesses various biological activities (antimicrobial, anti-inflammatory, antimutagenic) and also shows strong radical scavenging activities (Nakamura *et al.*, 1998; Petersen and Simmonds, 2003). These features outline it as an interesting product for both pharmaceutical and cosmetic industries.

For enhancement of RA yields in plant cell and tissue cultures different strategies such as feeding with phenylalanine as a precursor (Hippolyte *et al.*, 1992) and optimization of nutrient media composition and cultivation conditions in a bioreactor (Pavlov *et al.*, 2000; Pavlov *et al.*, 2005) were used. The elicitation with biotic (Szabo *et al.*, 1999) and abiotic (Mizukami *et al.*, 1993; Kim *et al.*, 2001) elicitors was recognized as one of the most promising strategies for enhancement of RA yields, regarding to its role in the plant defense system against pathogens and herbivores (Szabo *et al.*, 1999; Petersen and Simmonds, 2003).

According to literature data vanadyl sulfate was found as an effective elicitor for enhancement of paclitaxel, 10-deacetylbaccatin and thiarubrine A accumulation in different plant cell cultures (Bhagwath and Hjortso, 2000; Furmanowa *et al.*, 2000). Currently, there is no information available about the influence of vanadyl sulfate on rosmarinic acid production**.**

In this paper the influence of vanadyl sulfate, an abiotic elicitor, on the growth of *Lavandula vera* MM cell suspension culture and biosynthesis of RA are covered.

Materials and Methods

Cell culture

Lavandula vera MM cell suspension culture was used for the experiments. The culture was grown in a liquid LS nutrient medium (Linsmayer and Skoog, 1965), supplemented with 0.2 mg/l 2,4-dichlorophenoxyacetic acid and 30 g/l sucrose. For the elicitation experiments optimized liquid LS medium for RA production by *L. vera* MM was used (Pavlov *et al.*, 2000).

Elicitor

Vanadyl sulfate (Sigma-Aldrich) was dissolved in distilled water and then the solution was sterilized using a Millipore Stericup™ disposable filter (0.22 μm, Sigma-Aldrich, USA).

Experimental design

The experiments on the elicitation were performed in 100 ml Erlenmeyer flasks, containing 16 ml optimized LS medium, in the dark at 26 °C on a shaker (11.6 rad/s). For inoculation 20% (v/v) 7-day-old cell suspension culture was used. Vanadyl sulfate was added to the flasks on day 11 from the beginning of the cultivation at five different final concentrations: 6.25 mg/l, 12.5 mg/l, 25 mg/l, 50 mg/l and 75 mg/l. The samples were taken and the rosmarinic acid content in the biomass and culture medium were checked at 0, 4, 8, 12, 24, 36 and 48 h after vanadyl sulfate addition. As a control samples without added elicitor were taken and analyzed as well.

Analysis

Growth

The growth of the cell suspension was monitored by measurement of dry biomass (at 60 °C to constant weight) (Dixon, 1985).

Rosmarinic acid extraction and determination

Rosmarinic acid was extracted from cell biomass with 50% ethanol at 70 °C for 1 h (Georgiev *et al.*, 2004). The extract was evaporated to dryness and the dry residue was dissolved in 70% ethanol. For detection of extracellular RA in the culture medium, it was evaporated to dryness and then the dry residue was dissolved in 70% ethanol. The solutions were stored at – 10 °C for 24 h and the obtained precipitate was filtered off. The filtrate was used for determination of RA.

Rosmarinic acid in each sample was determined spectrophotometrically at 327 nm (spectrophotometer: Shimadzu UV/vis – 1240) (Lopez-Arnaldos *et al.*, 1995).

The data presented are averages from two independent experiments; each repeated twice ± standard deviation (SD).

Results and Discussion

In our previous experiments it was found that *L. vera* MM cell suspension culture is a promising producer of RA, as the achieved yields are several times higher in comparison with field plants from this species (Pavlov *et al.*, 2000, 2005). However the elicitation of *L. vera* MM with abiotic elicitors was not investigated.

Based on our preliminary experiments it was found that the most appropriate time for addition of vanadyl sulfate is the 11th day, which coincide with the beginning of the stationary phase of growth of *L. vera* MM cell culture. Further it was observed that the plant cell suspension of *L. vera* MM was sensitive to the elicitor, so the proper dosages of vanadyl sulfate were determined as well.

Influence of vanadyl sulfate on growth of L. vera MM cell suspension

After 4 h a decrease in the accumulated dry biomass was observed, as a response to all studied concentrations of the elicitor (Fig. 1), which probably was due to a defense response (Chen and Chen, 2000). Further, 8 h after the elicitor treatment this effect was overcome and the quantities of accumulated biomass were enhanced. This enhancement was most significant when the cells were treated with 6.25 mg/l and 12.5 mg/l vanadyl sulfate (about 2–6 % enhancement), while for the higher elicitor concentrations (50 mg/l and 75 mg/l) the amounts of accumulated biomass were lower compared to the non-elicited cells (about 4–12% decrease) (Fig. 1).

Influence of vanadyl sulfate on rosmarinic acid accumulation in the cells and on its secretion in the culture medium

For all concentrations under study the addition of vanadyl sulfate provoked significant enhancement of intracellular rosmarinic acid production, but the produced RA amounts and the time for

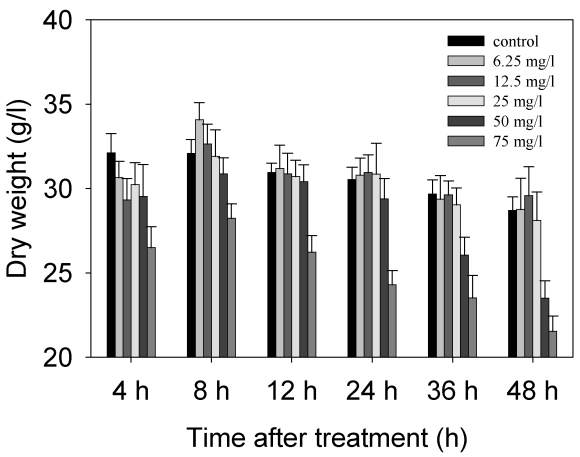

Fig. 1. Time course of growth of *L. vera* MM cell suspension after treatment with different concentrations of vanadyl sulfate (the elicitor was added on day 11 of cultivation). Bars represent standard deviation.

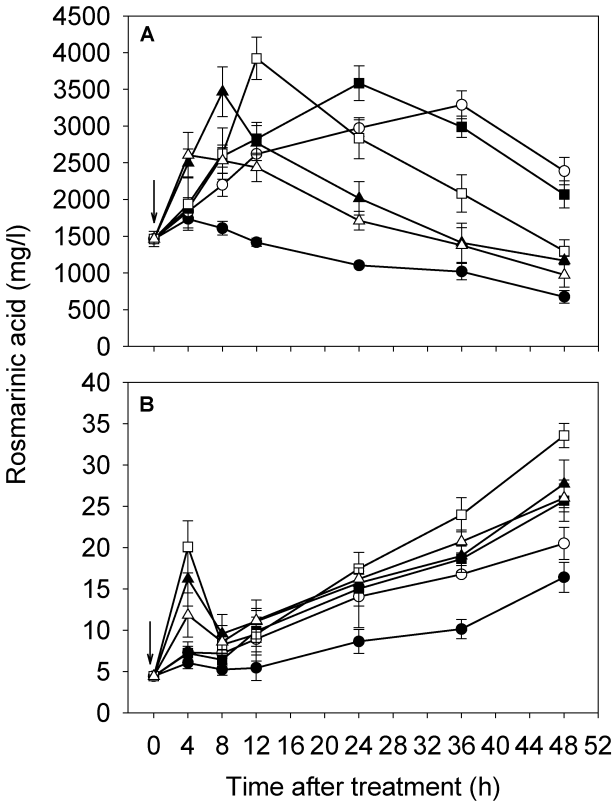

Fig. 2. Time course of rosmarinic acid content in cell biomass (A) and in culture media (B) of *L. vera* MM cell suspension culture after addition of different concentrations of vanadyl sulfate. Symbols: ↓ time of elicitor addition (on day 11 of cultivation); (●) control; (○) 6.25 mg/l; (■) 12.5 mg/l; (□) 25 mg/l; (▲) 50 mg/l; (△) 75 mg/l. Bars represent standard deviation.

their achievement were different (Fig. 2A). The maximum in RA production appeared when the cells were treated with 25 mg/l vanadyl sulfate (about 3.9 g/l). In comparison to the non-elicited cells (control cultivation) this yield is 2.8 times higher and the achieved productivity of 339 mg/(l·d) rosmarinic acid is one of the highest reported so far. No significant amounts of RA were detected in the culture medium in comparison with its intracellular content (Fig. 2B) and the possible reason for this is the presence of active peroxidases in it, which rapidly destroy RA (Szabo *et al.*, 1999). Four hours after the treatment with elicitor, the detected amounts of rosmarinic acid in the culture medium were between 7.3 mg/l and 20.1 mg/l (Fig. 2B), which is 1.2–3.3 times higher, compared to non-elicited cells. This fact revealed possibilities for further two phase cultivation of *L. vera* MM cells with the aim to "capture" these amounts of RA and to save it from destructive peroxidases (Pavlov *et al.*, 2001). Further from 12 h to 48 h after the addition of vanadyl sulfate the determined amounts of extracellular RA were about 34 mg/l; this enhancement is probably due to the lysis processes and connected with this leakage of RA in the culture medium.

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