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Effect of methyl jasmonate on production of ariltetralin lignans in hairy root cultures of *Linum tauricum*

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ABSTRACT
Methyl jasmonate (MeJA) treatment increases the levels of plant secondary metabolites, including ariltetraline lignans, which are considered to be the main active compounds in *Linum tauricum*. This study was concentrated on the induction of production of ariltetralin lignans in hairy roots cultures of *L. tauricum,* transformed by *Agrobacterium rhyzogenes,* ATCC 15834 by exposing them to different concentrations (50-200 microM) of methyl jasmonate (MeJA) during the culture period. The content of 4'-demethyl-6-methoxypodo-phylotoxin (4'-DM-6MPTOX) and 6-methoxypodophyllotoxin (MPTOX), the main constituents in hairy roots of *L. tauricum* increased about 1.2 folds by elicitation of MeJA, however, the fresh weight, dry weight and growth ratio was inhibited by increasing MeJA concentrations. The highest total lignans yield was obtained with 150 microM MeJA treatment. These results suggest that MeJA elicitation is beneficial for lignan accumulation in the hairy roots cultures of *L. tauricum*.
Keywords: Lignans, *Linum tauricum*, hairy roots, Methyl jasmonate

INTRODUCTION

The aryltetralin lignan podophyllotoxin (PTOX) is currently being used as a lead compound for the semi-synthesis of anticancer drugs etoposide, teniposide, etopophos, which are used for the treatment of lung and testicular cancers and certain leukemias (1). Podophyllotoxin and related compounds are not only present in *Podophyllaceae*, but also in *Linaceae*. A detailed analysis of the lignans in the *Linaceae* is reviewed (2). *Linum tauricum* (Willd.) Petrova, endemic to the Balkan region, belongs to the Section Syllinum of the genus *Linum* (Linaceae). The podophyllotoxin derivatives 4'-demethyl-6-methoxypodophylotoxin (4'-DM-6MPTOX) and 6-methoxypodophyllotoxin (6MPTOX) have been isolated and identified by NMR and UV as the two main lignans in the aerial parts of *Linum tauricum* ssp. *tauricum* (3). The compound 4'DM-6MPTOX was for the first time isolated from wild medicinal plant from us and is interesting with its pharmacological activity. The *in vitro* investigation of its cytostatic properties has demonstrated 2 to 3.5 times higher activity than that of the referent antineoplastic drug etoposide. The data indicate that 4'-DM-6-Mptox acts as an apoptosis inductor. The 4'-DM-6-Mptox was found to induce concentration-dependent NF-kB inhibition in HeLa cells as assessed by the IL-6 luciferase gene reporter assay, which though not quite prominent, at least partly contributes to the cytotoxic potential of the tested lignan (4). Hairy-roots of *Linum tauricum* demonstrated high biosynthetic capacity (5). The major active constituent 4'-DM-6MPTOX can be easily isolated in reasonable amounts from the genetically transformed root cultures. Therefore optimization of the production in *L. tauricum* hairy roots is worthy of further consideration as an alternative production system of 4'-DM-6MPTOX, which is used as a template for the development of potential new therapeutic agents.

The accumulation of secondary metabolites in plants is part of the defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses (6). Therefore, the treatment of plant cells with biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures. The most frequently used elicitors in previous

studies were fungal carbohydrates, yeast extract, MeJA and chitosan. MeJA, a proven signal compound, is the most effective elicitor.

This study was concentrated on the optimization of production of ariltetralin lignans in hairy roots cultures of *L. tauricum* by exposing them to different concentrations (50-200 microM) of MeJA during the culture period.

MATERIALS AND METHODS

Plant material.

The plant material from *L. tauricum* was collected near Varna (Bulgaria) in July 2004. A plant specimen was deposited in the Herbarium of the Faculty of Pharmacy, Medical University of Sofia, Bulgaria (No FAF 0001, collected by N. Vasilev). The seeds were germinated on modified MS medium (7). Leaves excised from 4 and 5 weeks old in vitro plants were directly used as explants.

Bacterial strains and Hairy root induction

Agrobacterium rhizogenes strain ATCC 15834 was used in the present study. The induction was performed as described in (5).

Extraction and isolation of lignan aglycons.

A fine powder (0.2 g) of the lyophilised plant material was extracted with methanol (2 ml) in an ultrasonic bath (two times for 30 s). Distilled water (6 ml) was added, and the pH was adjusted to 5.0 by *o*-phosphoric acid. After adding of β-glucosidase (1 mg), the sample was incubated at 35 °C for 1 h. Methanol (12 ml) was added and the mixture incubated for another 10 min at 70 °C in an ultrasonic bath. After centrifugation, the supernatant was used directly or storage at –18 °C.

HPLC.

HPLC with a Thermo Quest HPLC system (Egelsbach, Germany) equipped with a photodiode array detector was used. Separation was performed using a GROM-SIL 120 ODS-5ST column with guard column (250 mm long, 4.6 mm i.d. and 40 mm long, 4.6 mm i.d., respectively; Grom Company, Herrenberg, Germany) and a gradient program with water (A) and acetonitrile (B) as eluents as follows: 0 to 17 min from 40 to 67% B, from 17 to 18 min to 40% B, and until 24 min back to 40% B. The flow rate increased from 0.8 mL/min at 0 min to 1.0 mL/min at 17.0 min and decreased again to 0.8 mL/min between 18 and 24 min.

Effect of Elicitor

In an elicitation experiment different concentrations of MJ (0, 50, 100, 150, 200 microM) was added to the cultures on the 21 day (3 weeks of growth) after inoculation. The fresh and dry weights were recorded after 4 weeks of culture.

Statistical analyses

Elicitors were added to the third generation of *L. tauricum* hairy roots in triplicate at the day 21 of the cultivation period. Cultures without elicitors were also included as control groups. Sampling was performed at the day 28, after addition of the elicitors in triplicate. All statistical analyses were performed by one way ANOVA test, $p<0.05$ and $n=3$.

RESULTS AND DISCUSSION

In the past years, numerous strategies such as cell line selection, medium optimization, cell immobilization, the use differentiated cells, elicitation and more recently metabolic engineering have been developed to improve the productivity of plant cell culture (2, 9). The accumulation of secondary metabolites in plants is part of the defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses (6). Elicitation has been proved to be effective way to increase secondary metabolite production. A number of elicitors and precursors such as methyl jasmonate (MeJA) have been used successfully for enhancing production of secondary metabolites such as taxoids, baccatins, ginsenosides during cell cultures of many plant species. Therefore, the treatment of plant cells with biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures (2). The process of elicitation makes use of the capacity of plants and plant cell cultivated in-vitro to react to various stress stimuli by a number of protective reactions leading to increased accumulation of secondary metabolites. In recent years, we have been searching for a strategy that could significantly affect the accumulation of lignans by focusing on commercially valuable compounds as a research target. Chemical elicitors like MeJA are known to enhance accumulation of secondary metabolites in *in vitro* cultures. In the present study, MeJA was used as an elicitor, because there are numbers of positive investigation for its influence on lignan biosynthesis (2).

Recently from above ground parts, cell and HR cultures of *Linum tauricum* we have isolated ariltetralin lignans, including two important compounds - 4'DM-6MPTOX and 6MPTOX (5). The concentration 100microM of the elicitor methyl jasmonate in the nutrition medium of suspension of *Linum tauricum*, leads to substantial increase of the levels of lignans 4'DM-6MPTOX and 6MPTOX from traces, reaching a maximum of 0.1180 mg/g dw and 0.1250 mg/g dw respectively. The results (10) indicate that addition of extracellular

Table 1: Effects of Methyl jasmonate, on the production of Lignans in L. tauricum-Hairy roots (ATCC 15835), clone Lt-A12

MJ concentration in medium (microM)	4'-DM-MPTOX (mg/g dry weight)	6-MPTOX (mg/g dry weight)
0	25.92±0.31	35.06±0.03
50	26.09±0.17	35.11±0.28
100	29.19±0.11	35.42±0.19
150	31.92±0.15	36.19±0.08
200	20.30±0.33	28.12±0.21

**Data was taken after 4 weeks of culture*

methyl jasmonate can not only increase the biosynthesis of both 4'DM-6MPTOX and 6MPTOX in a *L. tauricum* grown suspension culture, but also change the ratio of both compounds, in comparison with the intact plant and callus cultures, towards the more pharmacologically valuable 4'-DM-6MPTOX (4).

Effects of elicitor

Methyl jasmonate is known to influence production of secondary metabolites, particularly defense related polyphenolics. In the present study, we have examined the possibility of generating high lignan-yielding hairy root cultures of *Linum tauricum* using ATCC 15834 strains of *Agrobacterium rhizogenes*. Rapidly growing root lines (Lt-A12) were selected to increase the efficiency of the production of lignans. As the impact of elicitors on *L. tauricum* hairy roots has not been studied, the possibility of increase in the production of ariltetralin lignans by addition of MeJA to the culture medium was explored in this study.

Elicitor MeJA at different concentrations - 0, 50, 100, 150, 200 microM (1.0µl of ethanol per 1.0 ml medium) was added to the cultures after 21 day of inoculation (3 weeks of growth). The elicitors were filter sterilized by passing through 0.22 Mm filters (Millipore). Tissues were harvested on the 7th day, after addition of elicitors. Results of the addition of different concentrations of elicitors to the third generation of the fast growing hairy roots cultures are shown in Table 1. Amount of total lignans increased with increasing MeJA concentration, and reached a maximum at 150 microM MeJA. The accumulation of 4'-DM-6MPTOX was enhanced about 1.2 fold over controls in the hairy roots cultures of *L. tauricum* following the addition of MeJA to the cultivation medium, reaching 31.92±0.15±0.15 mg/g dry weight. There was slight increase in 6MPTOX accumulation following the addition of MeJA - up to 36.19±0.08 mg/g dry weight. These results showed that methyl jasmonate had significant effect on the accumulation of target compound 4'-DM-MPTOX in hairy rots cultures of *L. tauricum* and production reached up to 31.92 mg g/1 dry weight (DW). However, the fresh weight, dry weight and growth ratio were decreased with increasing MeJA concentration (the data not presenting in this article). Addition of MeJA at higher concentration (above 150 microM) was detrimental for biomass accumulation. Similar to the present results, MeJA inhibited the cell growth and promoted the secondary metabolite production cell cultures of *L. tauricum* (10). The results from this study demonstrate that MeJA elicitation strategy was quite useful to improve the yield of lignans in hairy roots cultures of *L. tauricum*.

CONCLUSION

The effect of MeJA on the accumulation and biosynthesis of lignans by hairy root cultures of *Linum tauricum* (Linaceae) was investigated. Elicitation resulted in about 1.2 times higher amounts of 4'-DM-MPTOX (31.92 mg g^{-1} dry weight (DW) than non elicitated (non treated with MeJA) hairy roots (25.92 mg g^{-1} DW) and slight increase of MPTOX (36.19 mg g^{-1} DW) than non elicitated (control). The highest yield was achieved by administering 150 µM of the synthetic elicitors on the 21 day and extracting the products on the 28 day of the culture period. Growth was affected by the addition of the high concentration of elicitor. MeJA exerted a moderate stimulating effect on the production of pharmacologically valuable 4'-DM-6MPTOX in *L. tauricum* hairy roots. The results from this study demonstrate that MJ elicitation strategy was quite useful to improve the yield of lignans in *in vitro* hairy root cultures of *L. tauricum*.

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