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Selection of high rosmarinic acid producing *Lavandula vera* MM cell lines

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Abstract

Selection of stable *Lavandula vera* MM cell lines producing high amounts of rosmarinic acid (RA) was performed using *m*-F-D,L-phenylalanine and *p*-F-D,L-phenylalanine. As a result, two callus lines *L. vera* MF and *L. vera* PF were obtained, which produced 1.95 and 1.71 times more rosmarinic acid in comparison with the parent culture. After adaptation of the selected callus lines in liquid media the achieved yields of rosmarinic acid were 2808.4 mg L^{-1} (in *L. vera* MF cell suspension) and 2594.6 mg L^{-1} (in *L. vera* PF cell suspension).

Keywords: Rosmarinic acid; *Lavandula vera* MM; Line selection; *m*-F-D,L-phenylalanine; *p*-F-D,L-phenylalanine

1. Introduction

Optimization of secondary metabolite production in different plant *in vitro* systems is a difficult and complicated process. Frequently, the stage of the selection of high-producing cell lines is underestimated. However, the selection of high-producing cell lines using selective agents was found to be an effective strategy for the enhancement of the accumulation of aromatic compounds (phenolics and shikonin derivates) in cultured plant cells [1,2].

Rosmarinic acid (RA) is a secondary metabolite which possesses antiviral, antibacterial, anti-inflammatory, antiallergic activities [3–5] and prevents the proliferation of human cancer cells [6]. The interesting biological activities of RA and its low content in the intact plants impose the development of alternative ways for the production of RA. Plant cell and tissue cultures are considered prominent producers of RA [7] and, therefore, a number of strategies for yield improvement have been developed, such as nutrient medium optimization [8], elicitation [9], optimization of culture conditions [10], etc. However, there is no information available about the selection of high-producing rosmarinic acid cell lines of lavender.

In this paper we report the data on the selection of *Lavandula vera* (hereafter simplified term *L. vera* MM) MM cell lines resistant to analogues of phenylalanine (*m*- and *p*-F-D,L-phenylalanine), their rosmarinic acid content, as well as on the adaptation of the obtained lines to the submerged cultivations.

2. Materials and methods

2.1. Plant cell culture and media

L. vera MM callus culture was obtained from stems of the oil-bearing sort Lavender ''Drujba'' and maintained as it has been previously described [11].

It was cultivated on standard Linsmayer–Skoog (LS) media [12], supplemented with 30 g L^{-1} sucrose, 0.2 mg L^{-1} 2,4-dichlorophenoxyacetic acid and 5.5 g L^{-1} plant agar. For experiments on selection, the modified LS nutrient media (added with 60 g L^{-1} sucrose and NO_3^-/NH_4^+ ratio of 40) was used [8].

Callus culture was grown in a thermostat at 26 °C, in darkness; suspension culture was cultivated in Erlenmayer flasks with total volume 500 mL (working volume 100 mL) on a shaker (110 rad s^{-1}), at 26 °C in darkness. For inoculation, 20% (v/v) cell suspension cultivated under the above mentioned conditions for 7 days was used.

2.2. Experimental settings

m-F-D,L-phenylalanine (MFP) and *p*-F-D,L-phenylalanine (PFP) (Sigma–Aldrich, USA) were dissolved in distilled water and sterilized by filtration (Stericup, GV Durapore Membrane, 0.2 μm). Selection was carried out using the method described by Gonzales and Widholm [13], which includes mixing of medium (containing the relevant amounts of selection agent and agar) with equal amounts of 7-day old suspension culture. After 42-days, the grown colonies were transferred to standard LS solid media and cultivated for another

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21 days. Their growth was checked by the scale 0 (without growth) to 5 (excellent growth). The rosmarinic acid content of the selected lines was checked as well.

2.3. Analysis

2.3.1. Growth of the L. vera lines

Growth of the culture lines was monitored by measurement of dry weight (60 °C to constant weight) [14].

2.3.2. Rosmarinic acid extraction and determination

Frozen cell biomasses were extracted with 50% ethanol at 70 °C for 1 h (3 × 20 min). Ethanolic extracts were combined and evaporated under reduced pressure (vacuum evaporator, Laborota 4002, Heidolph). The samples were dissolved in 70% ethanol and were stored for 24 h at −10 °C. The obtained precipitate was separated and the supernatant was used for analysis of rosmarinic acid. Quantitative determination of rosmarinic acid was performed spectrophotometrically (spectrophotometer UV/VIS Shimadzu 1240) at 327 nm [15]. As a standard, pure rosmarinic acid (Extrasynthese, Genay, France) was used.

The data presented are the averages from two independent experiments, each repeated twice (±S.D.). All determinations were performed in three replicates.

3. Results and discussion

3.1. Selection of L. vera MM callus lines with high and stable rosmarinic acid production

The advantage of the amino acid analogues as selection agents is that they kill the majority of the wild type cells, while the desired variants (high phenolics producing cell lines) survive the treatment [16]. This phenomenon is attributed to the fact that only cells with increased phenylalanine ammonia-lyase activity (PAL) (key enzyme in phenolics biosynthesis pathway) can be detoxified by them and survive. As far as PAL is one of the key enzymes included in the biosynthetic pathway of RA, it can be expected that the cell lines with enhanced activity of PAL will produce higher amounts of RA [7].

As selective agents, *m*- and *p*-F-D,L-phenylalanine were used in concentration from 0.2 – 0.1 mM and 0.2 – 0.8 mM, respectively. After a 42-day cultivation, the colonies were transferred to standard LS solid media and after another 21 days their growth was checked (Table 1). Callus lines demonstrated different growth, but the tendency is inhibition of growth with increase of MFP and PFP concentration. However, when MPF was used, weaker inhibition of growth of *L. vera* MM cell culture was observed as compared with PFP.

Isolated plant cell lines were analyzed for their rosmarinic acid content (Tables 2 and 3). It was established that in the variants with MFP concentrations between 0.2 and 0.4 mM, the

Table 1
Influence of selective agent's concentrations on growth of *Lavandula vera* MM cell culture

Selection agent	Isolated colonies	Growth
Without selection agent (control)	8	+++++
0.2 mM MFP	5	++++
0.4 mM MFP	5	++++
0.5 mM MFP	4	+++
0.6 mM MFP	3	++
0.8 mM MFP	3	+
1.0 mM MFP	–	–
0.2 mM PFP	4	++++
0.4 mM PFP	4	+++
0.5 mM PFP	3	++
0.6 mM PFP	3	+
0.8 mM PFP	–	–

+++++ excellent (5); ++++ very good (4); +++ good (3); ++ middle (2); + weak (1); – without growth (0).

Table 2
Rosmarinic acid content in *m*-F-D,L-phenylalanine selected *Lavandula vera* MM callus lines

MFP concentration (mM)	Line	Rosmarinic acid (mg g^{-1} dry weight)	Percentage from control
–	Control	5.08 ± 0.11	100
0.2 mM MFP	$MF1_1$	4.56 ± 0.19	90
	$MF1_2$	5.17 ± 0.18	102
	$MF1_3$	4.47 ± 0.06	88
	$MF1_4$	5.60 ± 0.11	110
	$MF1_5$	4.60 ± 0.09	91
0.4 mM MFP	$MF2_1$	4.82 ± 0.17	95
	$MF2_2$	5.51 ± 0.13	109
	$MF2_3$	5.17 ± 0.13	102
	$MF2_4$	5.73 ± 0.08	113
	$MF2_5$	6.12 ± 0.16	121
0.5 mM MFP	$MF3_1$	6.12 ± 0.19	121
	$MF3_2$	6.69 ± 0.21	132
	$MF3_3$	5.95 ± 0.12	117
	$MF3_4$	5.47 ± 0.08	108
0.6 mM MFP	$MF4_1$	8.94 ± 0.19	176
	$MF4_2$	7.11 ± 0.15	140
	$MF4_3$	6.40 ± 0.15	126
0.8 mM MFP	$MF5_1$	9.90 ± 0.17	195
	$MF5_2$	8.21 ± 0.26	162
	$MF5_3$	8.81 ± 0.11	173

Table 3
Rosmarinic acid content in *p*-F-D,L-phenylalanine selected *Lavandula vera* MM callus lines

PFP concentration (mM)	Line	Rosmarinic acid (mg g^{-1} dry weight)	Percentage from control
–	Control	5.08 ± 0.11	100
0.2 mM PFP	$PF1_1$	8.16 ± 0.18	161
	$PF1_2$	7.95 ± 0.22	156
	$PF1_3$	8.68 ± 0.16	171
	$PF1_4$	6.56 ± 0.19	129
0.4 mM PFP	$PF2_1$	7.16 ± 0.25	141
	$PF2_2$	5.47 ± 0.13	108
	$PF2_3$	5.43 ± 0.15	107
	$PF2_4$	7.82 ± 0.26	154
0.5 mM PFP	$PF3_1$	5.25 ± 0.18	103
	$PF3_2$	3.65 ± 0.05	72
	$PF3_3$	4.43 ± 0.09	87
0.6 mM PFP	$PF4_1$	3.82 ± 0.11	75
	$PF4_2$	3.08 ± 0.07	61
	$PF4_3$	2.47 ± 0.03	49