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ORIGINAL ARTICLE

Growth retardants stimulate guggulsterone production in the presence of fungal elicitor in fed-batch cultures of *Commiphora wightii*

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Abstract Guggulsterone, a hypolipidemic natural agent, is produced in resin canals of the plant *Commiphora wightii*. In this study, the stimulatory effects of growth retardants [ALAR (*N*,*N*-dimethylaminosuccinamic acid) and CCC (chlormequat chloride)] and fungal elicitor on guggulsterone accumulation in cell cultures of *C. wightii* are reported. CCC at 1 mg l^{-1} enhanced guggulsterone content ($\sim$123 μg l^{-1}) when added on the fifth day after inoculation, while ALAR at 2.5 mg l^{-1} increased guggulsterone content ($\sim$116 μg l^{-1}) when added on the tenth day. In a two-stage fed-batch process, combined treatment with fungal elicitor and growth retardant caused a significant increase ($\sim$353 μg l^{-1}) in guggulsterone content in cell cultures after 17 days of growth. This represents an approximately fivefold increase over the guggulsterone contents in initial cultures of this plant.

Keywords *Commiphora wightii* · Fed-batch culture · Elicitor · Guggulsterone · Growth retardants

Abbreviations

2iP	6-(γ,γ-Dimethylallylamino)purine
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
ALAR	*N*,*N*-Dimethylaminosuccinamic acid
CCC	Chlormequat chloride
DM	Dry mass
MS	Murashige and Skoog medium

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Introduction

Guggulsterone (in the form of its *E*- and *Z*-isomers) is an effective antihyperlipidemic agent obtained from the gum resin of the guggul tree, *Commiphora wightii* (Arnott.) Bhandari (Ramawat et al. 2008). The overexploitation and slow growth of this tree, which is associated with poor seed set, have made this plant an endangered species (Kumar et al. 2003). The biotechnological production of guggulsterone has received attention as a promising alternative production source (Ramawat et al. 2008). Guggulsterone is an effective antagonist of the bile acid receptor (Wu et al. 2002), farnesoid X receptor (Urizar et al. 2002), and a ligand-dependent transcription factor that regulates the expression of the CYP 7A1 gene, which is involved in maintaining cholesterol/bile acid homoeostasis through the bile salt export pump (Owsley and Chiang 2003).

Optimum growth is negatively correlated with the production of secondary metabolites (Ramawat and Mathur 2007). Plant growth retardants play a crucial role in regulating plant growth and secondary metabolite production in aromatic and medicinal plants (Sangwan et al. 2001). Previous studies have indicated that CCC, triacontanol and ethrel are effective in stimulating the production of secondary metabolites such as essential oil components, artemisinin, ajmalicine, serpentine, vindoline and tabersonine (Haque et al. 2007).

We previously reported the accumulation of guggulsterone through the manipulation of medium constituents (Mathur et al. 2007), the marked influence of morphactin on guggulsterone accumulation in callus cultures (Tanwar et al. 2007), and guggulsterone accumulation in cell cultures grown in shake flasks and a bioreactor (Mathur and Ramawat 2007, 2008). In the present communication, we report improved guggulsterone accumulation through the

use of the growth retardants CCC and ALAR in combination with elicitor in a fed-batch culture.

Materials and methods

Cell culture

Cell suspension cultures were maintained in modified MS (Murashige and Skoog 1962) medium (NH_4NO_3 825 mg l^{-1}, KNO_3 950 mg l^{-1} and $CaCl_2{\cdot}6H_2O$ 220 mg l^{-1}) containing 2,4,5-trichlorophenoxyacetic acid (0.25 mg l^{-1}), 2iP (1 mg l^{-1}) and 3% w/v sucrose. The cultures were grown for 15 days in 250 ml Erlenmeyer flasks containing 100 ml medium. The culture conditions used and medium preparation were the same as described previously (Mathur and Ramawat 2007). To investigate the effects of plant growth retardants on the production of guggulsterones, three sets of experiments were devised, investigating: (1) the effect of CCC (1.0, 2.5, and 5.0 mg l^{-1}), (2) the effect of ALAR (1.0, 2.5, and 5.0 mg l^{-1}), and (3) the combined effect of growth retardant and the production medium in a two-stage fed-batch culture system.

Growth retardants were incorporated into the medium after different growth periods (0, fifth and tenth days of inoculation). In the two-stage culture system, the cells were first grown in suspension culture for 12 days in a 100 ml Erlenmeyer flask containing 100 ml of the growth medium as described above (Mathur and Ramawat 2007), and then the cells (∼400 mg dry weight) were transferred to a 100 ml production medium (PM) containing NH_4NO_3 1650 mg l^{-1}, KNO_3 475 mg l^{-1}, $CaCl_2{\cdot}6H_2O$ 220 mg l^{-1}, 2iP (1 mg l^{-1}), and sucrose:glucose (1:1, w/w) 4%. This medium was employed based on earlier results obtained with *C. wightii* (Mathur et al. 2007). In the second stage of culture (on the seventh day), CCC (1 mg l^{-1}) and dried powder of fungal cell wall obtained from autoclaved cultures of *Fusarium* species (500 mg l^{-1}) were added. As shown in Fig. 1, additional sugar was added to some cultures as 1 ml of an aqueous solution of sucrose:glucose (1:1 w/w) on the seventh and tenth days, so as to achieve final concentrations of 5 and 6%, respectively.

Guggulsterone extraction and HPLC analysis

The cell cultures were harvested, and the lyophilized cells (1 g DM) were finely ground and extracted overnight with 25 ml methanol, as described previously (Tanwar et al. 2007). In brief, the methanol was evaporated under vacuum; the residue was extracted with ethyl acetate, and a sample of this was injected into the HPLC after filtration through syringe filters (0.45 µm, 4 mm nylon filter). Separation was accomplished on a 250 × 4 mm C_{18} (5 µm) reversed-phase column protected by a guard column of the same material. The eluate was monitored at 245 nm. *E*- and *Z*-guggulsterones were obtained from Chromadex (Clearwater, FL, USA) and Natural Remedies (Bangalore, India), respectively.

Statistical analysis

All results were averaged over two separate analyses of two flasks to estimate guggulsterone, and two consecutive experiments with six replicate flasks in each treatment for growth value determination. The results were expressed as µg g^{-1} cell DM. For individual guggulsterone contents, the data were analyzed by two-way ANOVAs followed by mean separation using a post hoc least significant difference (LSD) test.

Results and discussion

There was a marked influence of growth retardants in combination with fungal elicitor and sugar on

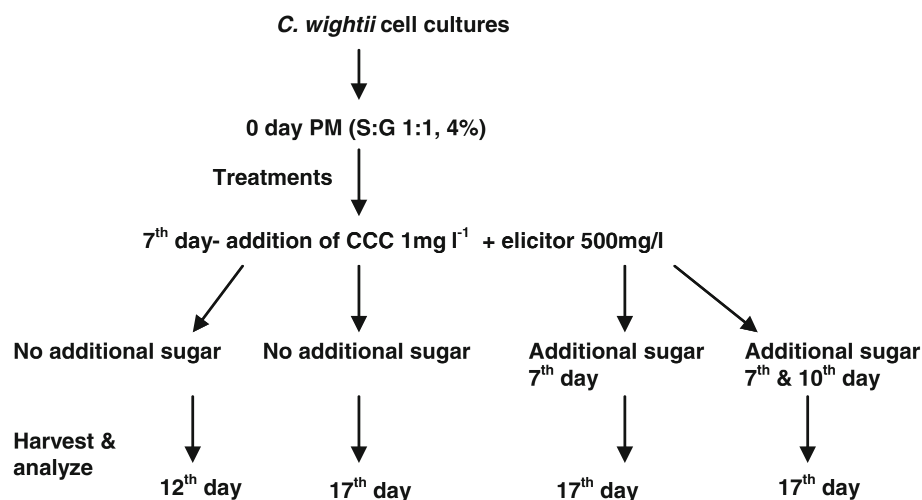

Fig. 1 Experimental scheme associated with the results presented in Table 3

guggulsterone accumulation in cell cultures of *C. wightii*. Guggulsterone induction in the cultures treated with CCC is presented in Table 1. The maximum guggulsterone content (123 μg l^{-1}) occurred for 1.0 mg l^{-1} CCC added on the fifth day of inoculation. At higher concentrations of CCC (2.5 and 5.0 mg l^{-1}), a decrease in guggulsterone content was recorded. When cells were treated with CCC on the day of inoculation, it was observed that both the growth and the guggulsterone content were lower than those of the control (Table 1). Maximum growth (7.6 g l^{-1} DM) was recorded for 1.0 mg l^{-1} CCC added on the tenth day. Though growth medium was used in this experiment, the growth was retarded when the retardant was added early on during the culture. The guggulsterone content was increased by about 3.4-fold compared to that obtained with growth medium and 2,4-D (Mathur and Ramawat 2007) by about twofold compared to growth medium with 2,4,5-T.

A stimulatory effect of ALAR on guggulsterone accumulation was only observed when added during the late growth phase (Table 2). The maximum production (116 μg l^{-1}) was recorded for 2.5 mg l^{-1} of ALAR added on the tenth day after inoculation. When ALAR was added during the early phase, the guggulsterone content and the growth were both inhibited.

Addition of CCC and fungal elicitor to the PM resulted in enhanced accumulation of guggulterone in the cell cultures (Table 3). A further increase in guggulsterone accumulation was recorded when the sugar level in the medium was increased. A marked ~3.5-fold increase in total guggulsterone yield (353 μg l^{-1}) was recorded in the cells grown in the medium fed twice with sugars on the seventh and tenth days. In this two-stage culture system, an increase in growth (10.8 g l^{-1} DM) was also observed (Table 3). Therefore, the fed-batch culture system was found to be more effective

Table 1 Effect of CCC on cell growth and guggulsterone production in cell cultures of *Commiphora wightii*

Day of addition	CCC (mg l^{-1})	DM (g l^{-1}) ± SD*	Guggulsterone content (μg g^{-1}) ± SD*			Yield (μg l^{-1})
			GE	GZ	Total content	
0	1.0	3.9 ± 0.3^{f}	5.6 ± 0.3^{e}	2.8 ± 0.1^{d,e}	8.4	33
	2.5	3.5 ± 0.2^{f,g}	4.4 ± 0.4^{e,f}	2.5 ± 0.2^{e,f}	6.9	24
	5.0	3.2 ± 0.2^{g}	4.2 ± 0.3^{f}	1.9 ± 0.1^{g}	6.1	20
5	1.0	5.9 ± 0.4^{c}	14.2 ± 0.9^{a}	6.7 ± 0.5^{a}	20.9	123
	2.5	5.4 ± 0.3^{d}	8.5 ± 0.7^{c}	4.1 ± 0.3^{b}	12.6	68
	5.0	4.8 ± 0.4^{e}	6.9 ± 0.5^{d}	3.2 ± 0.2^{c,d}	10.1	48
10	1.0	7.6 ± 0.6^{a}	9.3 ± 0.8^{b}	4.4 ± 0.3^{b}	13.7	104
	2.5	7.2 ± 0.3^{a}	7.1 ± 0.6^{d}	3.4 ± 0.2^{c}	10.5	76
	5.0	6.7 ± 0.5^{b}	4.9 ± 0.3^{e,f}	2.3 ± 0.2^{f,g}	7.2	48

Control values: dry mass, 8.7 g l^{-1}; total guggulsterone content, 8.1 μg g^{-1} DM; yield, 70.4 μg l^{-1}

* Mean values with common letters are not significantly different at $P \leq 0.01$, according to the least significant difference (*LSD*) test

Table 2 Effect of ALAR on cell growth and guggulsterone production in cell cultures of *Commiphora wightii*

Day of addition	ALAR (mg l^{-1})	DM (g l^{-1}) ± SD*	Guggulsterone content (μg g^{-1}) ± SD*			Yield (μg l^{-1})
			GE	GZ	Total content	
0	1.0	4.3 ± 0.2^{d}	4.3 ± 0.3^{d}	2.1 ± 0.2^{d}	6.4	27
	2.5	4.2 ± 0.3^{d}	4.7 ± 0.4^{d}	2.4 ± 0.1^{d}	7.1	30
	5.0	3.9 ± 0.2^{d}	3.8 ± 0.2^{d}	1.9 ± 0.1^{e}	5.7	22
5	1.0	6.9 ± 0.4^{b}	8.8 ± 0.6^{b}	4.1 ± 0.3^{b}	12.9	89
	2.5	6.3 ± 0.5^{c}	9.3 ± 0.8^{b}	4.5 ± 0.2^{a,b}	13.8	87
	5.0	5.8 ± 0.4^{c}	6.3 ± 0.6^{c}	2.9 ± 0.1^{c}	9.2	53
10	1.0	7.8 ± 0.6^{a}	9.7 ± 0.8^{b}	4.4 ± 0.3^{a,b}	14.1	110
	2.5	7.3 ± 0.4^{a,b}	11.1 ± 0.9^{a}	4.8 ± 0.3^{a}	15.9	116
	5.0	6.9 ± 0.7^{b}	7.1 ± 0.7^{c}	3.2 ± 0.2^{c}	10.3	71

Control values: dry mass, 8.7 g l^{-1}; total guggulsterone content, 8.1 μg g^{-1} DM; yield, 70.4 μg l^{-1}

* Mean values with common letters are not significantly different at $P \leq 0.01$, according to the least significant difference (*LSD*) test

Table 3 Guggulsterone production in cell cultures of *Commiphora wightii* grown in production medium containing 1 mg l^{-1} 2iP and sucrose:glucose (1:1, 4%) with a fed-batch process

Day of sugar addition	Day of harvest	DM (g l^{-1}) ± SD*	Guggulsterone content (μg g^{-1}) ± SD*			Yield (μg l^{-1})
			GE	GZ	Total content	
–	12th	8.1 ± 0.6[b]	17.8 ± 0.9[a]	9.4 ± 0.7[b]	27.2	220
–	17th	8.3 ± 0.8[b]	15.5 ± 1.1[a]	8.7 ± 0.6[b]	24.2	201
7th	17th	9.4 ± 0.7[a,b]	16.6 ± 1.2[a]	9.0 ± 0.9[b]	25.6	241
7th and 10th	17th	10.8 ± 0.9[a]	17.6 ± 1.4[a]	15.1 ± 1.3[a]	32.7	353

Experimental scheme is shown in Fig. 1

Control values in PM without CCC and fungal elicitor: dry mass, 9.1 g l^{-1}; total guggulsterone content, 11.2 μg g^{-1} DM; yield, 101 μg l^{-1}

* Mean values with common letters are not significantly different at $P \leq 0.01$, according to the least significant difference (*LSD*) test

than the addition of a high sugar concentration at the initial stage (Mathur and Ramawat 2008).

The dynamics of the inter-relationship between primary metabolism and secondary metabolite formation is well established. The reduced growth induced by the plant growth retardants may result in an enhanced flux of photoassimilates towards the biosynthetic pathway of secondary metabolites (Ramawat and Mathur 2007). The effect of plant growth regulators on guggulsterone biosynthesis and accumulation could also be due to their direct effect on the enzyme(s) of the biosynthetic pathway. CCC is known to inhibit the cyclization of geranylgeraniol, leading to the accumulation of gernaylgeraniol (Barnes et al. 1969). The physiological basis of growth retardation by chlormequat chloride involves its inhibitory effect on gibberellin biosynthesis. The growth retardants paclobutrazol and CCC have been found to affect anthocyanin production in cell suspension cultures of *Daucus carota* (Ilan and Dougall 1992). Since the mevalonate biosynthetic pathway is involved in the synthesis of guggulsterones, inhibiting the synthesis of some metabolites may have resulted in the enhanced accumulation of guggulsterones.

ALAR is considered one of the most systemic growth retardants, so it has various effects in plants, including reduction of growth and biomass (El-Sheibany et al. 2007; Basra 1994). It belongs to the succinic acid group; unlike other growth retardants, ALAR contains no benzene ring, quaternary ammonium or phosphonium cation, nor any substituent that is small, nucleophilic and nonionizable, with a C–C–C–N system (Weaver 1972; Wareing and Philips 1975). This study showed that combined treatment in a two-stage process with a high-density culture is a practical approach for producing guggulsterones.

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