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Artigo

ENHANCED TRITERPENE PRODUCTION IN *Tabernaemontana catharinensis* CELL SUSPENSION CULTURES IN RESPONSE TO BIOTIC ELICITORS

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Cell suspension cultures of *Tabernaemontana catharinensis* were treated with autoclaved homogenates of *Candida albicans*, *Fusarium oxysporum*, *Penicillium avelanium* and *Saccharomyces cerevisiae*. The effects caused by the concentration, exposure time and the type of elicitor on the accumulation of pentacyclic triterpenes were monitored. When exposed to biotic elicitors for longer periods, some cell lines redoubled the production of those triterpenes. *Saccharomyces cerevisiae* homogenate was the best elicitor of triterpenes in all cell lines investigated.

Keywords: *Tabernaemontana catharinensis*; plant cell culture; elicitation.

INTRODUCTION

Tabernaemontana catharinensis (A.DC.) Miers (Syn. *Peschiera catharinensis*, *T. hilariana*, *T. affinis*, *T. australis*, *T. acuminata*, *T. hibrida*, *P. albidiflora* and *T. salicifolia*) belongs to the Apocynaceae family[1]. Besides indole alkaloids several pentacyclic triterpenoids were isolated from *Tabernaemontana* species: lupeol, α- and β–amirin, baurenil acetate, sitosterol, and others[2,3]. Acid triterpenes are of great interest due to the diversity of pharmacological activities they display: anti-inflammatory, hepatoprotective, antitumoral, among others[4-7].

Fungal elicitation has been an effective tool to enhance the yield of secondary metabolites[8], also helping in the elucidation of mechanisms of plant responses to biotic stress agents[9]. Most fungal elicitation strategies utilize fairly undefined mixtures such as autoclaved fungal homogenates[10,11] or fungal culture filtrates[9].

Several reports have shown that the use of yeast and fungi as elicitors provoked an accumulation of triterpenoid phytoalexins[12-16]. In elicited cultures of *Catharanthus roseus* (L.), the qualitative profile of terpenoid products detected was similar to that found in control cultures[15], but different from the observation with *Tabernaemontana divaricata*[14] and *Uncaria tomentosa*[16].

In this study, 4 different types of biotic elicitors, yeast and fungi, were tested to stimulate triterpene production. We report here the effects of concentration and exposure time of those elicitors on *T. catharinensis* cell lines.

EXPERIMENTAL

Callus induction and maintenance

Callus cultures were induced from disinfested leaf explants and maintained on solid MS medium supplemented with 30 g sucrose l^{-1} in two different hormone combination: T43 (1.0 mg 2,4-dichlorophenoxyacetic acid l^{-1} and 1.0 mg kinetin l^{-1}) and T44 (1.0 mg 2,4-dichlorophenoxyacetic acid l^{-1} and 0.1 mg kinetin l^{-1}). Two different cell lines - chlorophyllated cells and non-chlorophyllated cells were selected from cultures carried out in T44 medium while only one cell line was obtained in T43 medium. Cells were subcultured every 30 days and maintained at 28 ± 2 ºC under 16-hour-day photoperiod.

Suspension cultures

Six-year-old callus cultures were inoculated into a 250 mL erlenmeyer flask containing 100 mL of MS medium with 30 g sucrose L^{-1}. The suspension cultures were established using different callus lines: calli previously cultured on T43 semisolid MS medium were inoculated into T43 liquid MS medium (Culture 2); non-chlorophyllated calli cultivated on T44 semisolid MS medium were inoculated into both T43 liquid MS medium (Culture 1) and T44 liquid MS medium (Culture 4); and chlorophyllated calli cultured on T44 solid MS medium were inoculated into T44 liquid MS medium (Culture 3). Cultures were subcultured every 30 days and maintained in a growth room, under agitation (110 rpm) on orbital shaker, at 28 ± 2 ºC exposed to a 16-hour-day photoperiod.

Preparation of biotic agents for experiments of elicitation

Candida albicans, *Fusarium oxysporum* and *Penicilium avelanium* were cultured in liquid potato/dextrose medium under agitation (110 rpm) on orbital shaker at 28 ºC. After 96 h of incubation the cultures were autoclaved and the micelia were collected by filtration and then dried. *C. albicans*, *F. oxysporum*, *P. avelanium* and *Saccharomyces cerevisiae* micelia (5, 10 and 50 mg mL^{-1}) were homogenized in deionized water and autoclaved. Fungal homogenates were added separately to the suspension cultures either at the early (14 days) or late exponential phase of growth (20 days). No elicitor was added to the control cultures. All experiments were conducted with four *T. catharinensis* suspension lines (Culture 1 to 4). For a time course study, untreated and elicited suspension cultures were harvested at different time intervals (24, 48 and 72 h) by vacuum filtration. Triplicate flasks were run for each treatment and controls.

Analytical procedures

Dry and powered material (200 mg) was extracted overnight, successively with chloroform and methanol (5 mL each) at room

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temperature. Chloroform and methanol extracts were grouped and the solvent evaporated at room temperature. Crude extract was then resuspended in water and partitioned three times with ethyl acetate. The combined organic extracts were evaporated under vacuum, residues were dissolved in 1 mL of methanol and analysed by HPLC.

HPLC analysis conditions: Shimadzu LC10AD*vp* system equipped with Supelco LC18 column (250 x 4.6 mm), coupled to a diode array detector. Samples were eluted with methanol:H_2O (Acetic acid, 0.1%), 85:15, at 1 mL min^{-1} under isocratic condition and monitored at 210 nm. 20 µL of each solution from oleanolic acid, ursolic acid and samples were used for quantitative analysis. The identification of oleanolic and ursolic acid was done by comparing their retention times and spectral data with those of standard compounds. All quantifications were performed in triplicate the independent experiments: elicited and control cultures. The quantification was evaluated using external calibration curves. Calibration curves with theirs respective standards were developed with diluted samples of standard compounds within the range of 1.0 to 0.01 mg/mL. Authentic ursolic acid and oleanolic acid were purchased from Aldrich Co.

RESULTS

Cell culture

Callus culture was initiated from axenic leaf explants of *T. catharinensis* inoculated on MS solid medium supplemented with two combinations of 2,4-D and kinetin (1:1 and 1:0.1). Calli cultured on T43 medium developed homogenous friable of yellow pigmented biomass and calli maintained on T44 medium developed heterogenous friable biomass with chlorophyllated and non-chlorophyllated cells. Chlorophyllated and non-chlorophyllated cells were subcultured in T44 liquid medium. Thereafter, all callus and suspension cultures were maintained and subcultured in T44 or T43 media.

Induction of ursolic and oleanolic acid production by elicitation

As a response to the biotic stress caused by microorganisms, the *T. catharinensis* cells was stimulated increasing the biosynthesis of triterpenoids (Table 1 to 4). The experiments were carried out with *T. catharinensis* cells either in the early or late exponential phase of growth (14 or 20-day-old). Cultures were homogenous in appearance, but when stressed with fungal homogenates there was a rapid change in their color.

The effects of various elicitors on triterpene accumulation in *T. catharinensis* suspension cultures are shown in Tables 1 to 4. Addition of cell wall homogenates, no matter the concentration or type of microorganism source tested, resulted in increased triterpene levels. Cultures 2 and 3 were more susceptible to fungal homogenate treatments showing higher yields of oleanolic and ursolic acids. Among the elicitors tested, *S. cerevisae* showed better results in terms of triterpene accumulation, producing 5 and 7 mg g^{-1} dw, of ursolic and oleanolic acids respectively, followed by *C. albicans,* which produced 2 and 3 mg g^{-1} dw, and *P. avelanium* 1 to 2 mg g^{-1}. Triterpene accumulation in cultured cells treated with *F. oxysporum* homogenates was slight stimulated. In most experiments, the maximum production of triterpenes was achieved around 72 h of culture in an elicitor dose related accumulation. When fungal homogenates were added to the suspension cultures at the early exponential phase, 14 days of culture, the production of triterpenes was not significant compared to control cells. This situation was verified twice in the third and sixth subcultures (Experiments 1 and 2).

Table 1. Accumulation of triterpenes in *T. catharinensis* suspension cultures after fungal elicitation (Culture 1)

Experiments		Triterpenes (mg g^{-1} dw)					
		Oleanolic acid			Ursolic acid		
		24 h	48 h	72 h	24 h	48 h	72 h
Exp. 2	Control	0.384 ± 0.086	0.351 ± 0.079	0.699 ± 0.269	nd	nd	nd
3rd subculture	*Ca*-5 mg	0.768 ± 0.131	0.418 ± 0.049	0.672 ± 0.172	0.581 ± 0.170	0.242 ± 0.128	nd
14-day-old	*Sc*-5 mg	0.683 ± 0.218	0.468 ± 0.099	0.754 ± 0.243	nd	nd	nd
	Pa-5 mg	0.919 ± 0.108	0.416 ± 0.107	0.434 ± 0.215	nd	nd	nd
Exp. 3	Control	0.085 ± 0.000	nd	nd	0.050 ± 0.000	nd	nd
3rd subculture	*Sc*-5 mg	0.503 ± 0.241	1.271 ± 0.383	0.582 ± 0.288	0.145 ± 0.052	nd	0.163 ± 0.074
20-day-old	*Sc*-10 mg	0.094 ± 0.039	nd	nd	0.064 ± 0.035	nd	nd
	Sc-50 mg	0.342 ± 0.186	0.310 ± 0.156	0.314 ± 0.080	nd	nd	nd
Exp. 4	Control	0.157 ± 0.000	nd	nd	0.070 ± 0.000	nd	nd
3rd subculture	*Pa*-5 mg	0.242 ± 0.084	0.158 ± 0.016	nd	0.110 ± 0.036	0.057 ± 0.000	nd
20-day-old	*Pa*-10 mg	0.379 ± 0.017	0.134 ± 0.070	nd	0.196 ± 0.000	0.106 ± 0.000	nd
	Pa-50 mg	0.369 ± 0.153	0.161 ± 0.027	0.243 ± 0.041	nd	nd	0.144 ± 0.010
Exp. 5	Control	0.104 ± 0.072	nd	nd	0.036 ± 0.015		
3rd subculture	*Ca*-5 mg	0.310 ± 0.043	0.143 ± 0.065	0.175 ± 0.047	0.124 ± 0.009	0.055 ± 0.014	0.087 ± 0.019
20-day-old	*Ca*-10 mg	1.153 ± 0.009	0.268 ± 0.059	0.028 ± 0.000	0.408 ± 0.020	0.087 ± 0.019	0.013 ± 0.000
	Ca-50 mg	0.109 ± 0.019	0.799 ± 0.044	0.043 ± 0.016	0.057 ± 0.011	0.307 ± 0.024	0.026 ± 0.014
Exp. 6	Control	nd	nd	nd	nd	nd	nd
3rd subculture	*Fo*-5 mg	0.179 ± 0.003	0.209 ± 0.177	nd	0.057 ± 0.000	0.073 ± 0.000	nd
20-day-old	*Fo*-10 mg	0.546 ± 0.287	0.553 ± 0.162	nd	0.211 ± 0.009	0.113 ± 0.038	nd
	Fo-50 mg	0.479 ± 0.000	nd	nd	nd	nd	nd

Ca = Candida albicans; *Sc= Sacharomyces cerevisiae*; *Pa = Penicillium avelanium*; *Fo = Fusarium oxysporum*; nd = not detected. The results represent the mean of triplicate with standard deviations.

Table 2. Accumulation of triterpenes in suspension cultures of *T. catharinensis* after fungal treatment (Culture 2)

Experiments		Triterpenes (mg g^{-1} dw)					
		Oleanolic acid			Ursolic acid		
		24 h	48 h	72 h	24 h	48 h	72 h
Exp. 2	Control	0.358 ± 0.040	0.457 ± 0.215	0.244 ± 0.019	nd	nd	0.430 ± 0.057
3rd subculture	*Ca*-5 mg	0.399 ± 0.019	0.663 ± 0.150	0.647 ± 0.074	0.517 ± 0.098	0.662 ± 0.199	0.783 ± 0.055
14-day-old	*Sc*-5 mg	0.256 ± 0.202	0.774 ± 0.143	0.480 ± 0.070	0.156 ± 0.036	0.808 ± 0.135	0.609 ± 0.133
	Pa-5 mg	0.360 ± 0.018	0.578 ± 0.173	0.498 ± 0.030	0.406 ± 0.030	0.688 ± 0.234	0.563 ± 0.013
Exp. 3	Control	0.950 ± 0.112	0.597 ± 0.019	0.641 ± 0.172	1.914 ± 0.231	1.137 ± 0.685	1.278 ± 0.438
3rd subculture	*Sc*-5 mg	1.541 ± 0.167	2.124 ± 0.260	4.601 ± 1.076	2.226 ± 0.298	2.888 ± 0.343	3.289 ± 0.410
20-day-old	*Sc*-10 mg	1.862 ± 0.195	2.408 ± 0.116	2.539 ± 0.373	3.509 ± 0.426	3.451 ± 0.340	3.911 ± 0.635
	Sc-50 mg	2.319 ± 0.550	2.604 ± 0.365	7.460 ± 2.632	4.632 ± 0.433	3.638 ± 0.629	4.674 ± 0.836
Exp. 4	Control	nd	nd	nd	nd	nd	nd
3rd subculture	*Pa*-5 mg	0.366 ± 0.038	0.516 ± 0.058	0.671 ± 0.055	0.547 ± 0.070	0.818 ± 0.106	1.095 ± 0.083
20-day-old	*Pa*-10 mg	0.496 ± 0.244	0.600 ± 0.079	0.609 ± 0.156	0.744 ± 0.383	0.955 ± 0.210	1.149 ± 0.338
	Pa-50 mg	0.981 ± 0.073	0.832 ± 0.152	1.212 ± 0.158	1.568 ± 0.136	1.244 ± 0.231	2.021 ± 0.174
Exp. 5	Control	nd	nd	nd	nd	nd	nd
3rd subculture	*Ca*-5 mg	0.507 ± 0.312	1.070 ± 0.082	1.236 ± 0.072	1.446 ± 0.000	1.905 ± 0.082	2.410 ± 0.130
20-day-old	*Ca*-10 mg	0.576 ± 0.025	1.118 ± 0.165	1.623 ± 0.074	0.835 ± 0.072	1.979 ± 0.135	2.759 ± 0.437
	Ca-50 mg	1.216 ± 0.329	1.845 ± 0.052	1.916 ± 0.133	1.553 ± 0.384	2.395 ± 0.045	2.961 ± 0.203
Exp. 6	Control	0.336 ± 0.089	nd	0.078 ± 0.028	0.208 ± 0.034	nd	0.069 ± 0.018
3rd subculture	*Fo*O-5 mg	nd	0.039 ± 0.012	0.037 ± 0.004	0.102 ± 0.051	0.080 ± 0.025	0.119 ± 0.017
20-day-old	*Fo*-10 mg	0.046 ± 0.008	0.069 ± 0.002	0.125 ± 0.035	0.145 ± 0.063	0.207 ± 0.021	0.267 ± 0.044
	Fo-50 mg	0.040 ± 0.004	0.054 ± 0.005	0.089 ± 0.032	0.139 ± 0.057	0.172 ± 0.001	0.202 ± 0.053

Ca = *Candida albicans*; *Sc*= *Sacharomyces cerevisiae*; *Pa* = *Penicillium avelanium*; *Fo* = *Fusarium oxysporum*; nd = not detected. The results represent the mean of triplicate with standard deviations.

Table 3. Accumulation of triterpenes in suspension cultures of *T. catharinensis* after fungal treatment (Culture 3)

Experiments		Triterpenes (mg g^{-1} DW)					
		Oleanolic acid			Ursolic acid		
		24 h	48 h	72 h	24 h	48 h	72 h
Exp. 1	Control	0.069 ± 0.028	0.014 ± 0.001	0.015 ± 0.001	0.057 ± 0.026	0.009 ± 0.001	0.019 ± 0.003
6th subculture	*Ca*-5 mg	0.212 ± 0.081	0.133 ± 0.065	0.166 ± 0.038	0.179 ± 0.036	0.118 ± 0.039	0.134 ± 0.020
14-day-old	*Sc*-5 mg	0.080 ± 0.009	0.097 ± 0.030	0.080 ± 0.017	0.056 ± 0.006	0.053 ± 0.006	0.048 ± 0.006
	Pa-5 mg	0.237 ± 0.028	0.077 ± 0.009	0.181 ± 0.040	0.165 ± 0.017	0.080 ± 0.027	0.164 ± 0.014
Exp. 3	Control	0.209 ± 0.027	0.263 ± 0.226	nd	0.025 ± 0.061	0.071 ± 0.018	nd
3rd subculture	*Sc*-5 mg	2.476 ± 0.176	3.418 ± 0.529	5.591 ± 1.765	1.579 ± 0.013	1.628 ± 0.518	2.695 ± 1.029
20-day-old	*Sc*-10 mg	2.821 ± 0.280	4.081 ± 0.167	5.572 ± 0.746	1.838 ± 0.391	2.621 ± 0.398	2.815 ± 0.599
	Sc-50 mg	3.310 ± 0.549	4.969 ± 1.037	6.764 ± 0.991	2.517 ± 0.742	2.779 ± 0.840	4.538 ± 0.579
Exp. 4	Control	0.250 ± 0.110	nd	nd	0.244 ± 0.157	nd	nd
3rd subculture	*Pa*-5 mg	1.383 ± 0.256	1.575 ± 0.006	3.435 ± 0.356	1.094 ± 0.524	1.043 ± 0.278	1.567 ± 0.345
20-day-old	*Pa*-10 mg	1.899 ± 0.114	2.164 ± 0.628	4.458 ± 0.560	0.852 ± 0.132	0.767 ± 0.239	1.410 ± 0.420
	Pa-50 mg	1.393 ± 0.177	2.749 ± 1.150	3.009 ± 0.594	0.723 ± 0.064	1.239 ± 0.182	1.758 ± 0.419
Exp. 5	Control	nd	nd	nd	nd	nd	nd
3rd subculture	*Ca*-5 mg	0.984 ± 0.338	2.037 ± 0.091	2.138 ± 0.070	0.800 ± 0.311	1.129 ± 0.376	1.918 ± 0.201
20-day-old	*Ca*-10 mg	1.359 ± 0.163	2.106 ± 0.118	2.514 ± 0.181	1.307 ± 0.151	1.722 ± 0.215	1.915 ± 0.189
	Ca-50 mg	1.166 ± 0.104	1.771 ± 0.145	1.884 ± 0.065	1.038 ± 0.123	1.422 ± 0.146	1.372 ± 0.106
Exp. 6	Control	nd	nd	nd	nd	nd	nd
3rd subculture	*Fo*-5 mg	2.295 ± 0.502	2.279 ± 0.040	3.026 ± 0.220	1.247 ± 0.129	1.030 ± 0.076	1.342 ± 0.115
20-day-old	*Fo*-10 mg	2.129 ± 0.157	2.474 ± 1.058	2.957 ± 0.218	1.543 ± 0.382	1.766 ± 0.592	1.560 ± 0.134
	Fo-50 mg	1.466 ± 0.179	1.730 ± 0.303	3.446 ± 0.467	0.679 ± 0.060	0.815 ± 0.045	2.090 ± 0.427

Ca = *Candida albicans*; *Sc* = *Sacharomyces cerevisiae*; *Pa* = *Penicillium avelanium*; *Fo* = *Fusarium oxysporum*; nd = not detected. The results represent the mean of triplicate with standard deviations.

Table 4. Accumulation of triterpenes in suspension cultures of *T. catharinensis* after fungal treatment (Culture 4)

Experiments		Triterpenes (mg g^{-1} DW)					
		Oleanolic acid			Ursolic acid		
		24 h	48 h	72 h	24 h	48 h	72 h
Exp. 1	Control	nd	nd	nd	0.010 ± 0.000	0.004 ± 0.000	nd
6th subculture	*Ca*-5 mg	0.038 ± 0.020	0.005 ± 0.001	0.004 ± 0.000	nd	nd	nd
14-day-old	*Sc*-5 mg	0.179 ± 0.063	0.007 ± 0.003	0.006 ± 0.001	0.103 ± 0.016	0.002 ± 0.000	0.001 ± 0.000
	Pa-5 mg	0.042 ± 0.010	0.006 ± 0.001	0.009 ± 0.001	nd	nd	nd
Exp. 2	Control	0.303 ± 0.013	0.323 ± 0.029	0.252 ± 0.190	nd	nd	nd
3rd subculture	*Ca*-5 mg	0.193 ± 0.071	0.229 ± 0.069	0.115 ± 0.023	0.042 ± 0.000	nd	nd
14-day-old	*Sc*-5 mg	0.462 ± 0.107	0.304 ± 0.088	0.128 ± 0.074	nd	nd	0.034 ± 0.000
	Pa-5 mg	0.233 ± 0.082	0.288 ± 0.035	0.238 ± 0.107	0.076 ± 0.000	nd	nd
Exp. 4	Control	nd	nd	nd	nd	nd	nd
3rd subculture	*Pa*-5 mg	0.066 ± 0.023	nd	nd	nd	nd	nd
20-day-old	*Pa*-10 mg	2.176 ± 0.034	nd	nd	0.210 ± 0.013	nd	nd
	Pa-50 mg	0.377 ± 0.161	nd	nd	0.185 ± 0.042	nd	nd
Exp. 5	Control	nd	nd	nd	nd	nd	0.254 ± 0.000
3rd subculture	*Ca*-5 mg	0.386 ± 0.115	0.676 ± 0.003	0.881 ± 0.532	nd	0.120 ± 0.030	0.261 ± 0.000
20-day-old	*Ca*-10 mg	0.829 ± 0.123	1.501 ± 0.183	1.133 ± 0.508	0.202 ± 0.018	0.186 ± 0.013	0.124 ± 0.000
	Ca-50 mg	1.670 ± 0.615	2.494 ± 0.253	1.607 ± 0.380	0.321 ± 0.124	nd	nd
Exp. 6	Control	nd	nd	nd	nd	nd	nd
3rd subculture	*Fo*-5 mg	0.539 ± 0.135	0.145 ± 0.086	0.204 ± 0.098	0.156 ± 0.024	0.066 ± 0.031	0.052 ± 0.017
20-day-old	*Fo*-10 mg	0.677 ± 0.182	0.297 ± 0.046	0.120 ± 0.005	0.170 ± 0.056	0.145 ± 0.026	0.071 ± 0.001
	Fo-50 mg	0.533 ± 0.155	0.297 ± 0.102	0.272 ± 0.135	0.204 ± 0.064	0.094 ± 0.031	0.059 ± 0.035

Ca = *Candida albicans*; *Sc* = *Sacharomyces cerevisiae*; *Pa* = *Penicillium avelanium*; *Fo* = *Fusarium oxysporum*; nd = not detected. The results represent the mean of triplicate with standard deviations.

DISCUSSION

Several reports have shown that secondary metabolism in plants cell culture were stimulated or inhibited with fungal homogenates[8,9,11]. In our study elicitors induced a rapid stimulation of the secondary metabolism pathway of *T. catharinensis* cells increasing the biosynthesis of triterpenoids. The rapid change in suspension cultures with fungal homogenates was also reported in elicitation experiments with other plant cells[12-16].

Obtained results corroborate the theory that components of the cell wall homogenates act as signaling molecules of triterpenoid biosynthetic pathway, triggering the production of oleanolic acid and ursolic acid by treated cells. When the extracts of *T. catharinensis* unelicited cells were quantified, triterpenes were almost not detected. The regulation and enzymology of pentacyclic triterpenoid phytoalexin biosynthesis in cell suspension cultures of *T. divaricata* has been investigated. In *T. divaricata*, *C. albicans* elicitor preparations resulted in the production of considerable amounts of ursolic acid type pentacyclic triterpenoids while alkaloid production was blocked under such conditions[13,14]. A similar regulatory mechanism would be expected in *T. catharinensis* cells. Neither TLC nor HPLC analysis were performed in this work for alkaloid production.

The procedures described in this work may be employed in strategies for enhancement in productivity of secondary metabolites and for investigating the complex secondary metabolite pathways in plant tissue cultures.

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