Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.8 No.3, Issue of December 15, 2005

Received May 5, 2005 / Accepted June 23, 2005
DOI: 10.2225/vol8-issue3-fulltext-6
RESEARCH ARTICLE

Effects of elicitor and copper sulfate on grindelic acid production in submerged cultures of *Grindelia pulchella*

Xenia E. Hernandez
Area de Química Orgánica (INTEQUI-CONICET)
Facultad de Química, Bioquímica y Farmacia
Universidad Nacional de San Luis
Chacabuco y Pedernera, 5700
San Luis, Argentina
Tel: 54 2652439909
Fax: 54 2652426711

Alejandro A. Orden
Area de Química Orgánica (INTEQUI-CONICET)
Facultad de Química, Bioquímica y Farmacia
Universidad Nacional de San Luis
Chacabuco y Pedernera, 5700
San Luis, Argentina
Tel: 54 2652439909
Fax: 54 2652426711
E-mail:aaorden@unsl.edu.ar

Oscar S. Giordano
Area de Química Orgánica (INTEQUI-CONICET)
Facultad de Química, Bioquímica y Farmacia
Universidad Nacional de San Luis
Chacabuco y Pedernera, 5700
San Luis, Argentina
Tel: 54 2652439909
Fax: 54 2652426711
E-mail:ogiord@unsl.edu.ar

Marcela Kurina*
Area de Química Orgánica (INTEQUI-CONICET)
Facultad de Química, Bioquímica y Farmacia
Universidad Nacional de San Luis
Chacabuco y Pedernera, 5700
San Luis, Argentina
Tel: 54 2652439909
Fax: 54 2652426711
E-mail: mkurina@unsl.edu.ar

Financial support: UNSL: PROIPRO 0201; CONICET: PID 2429; FONCyT; ANPCyT: PICT2002 Nº 06-10710.

Keywords: Cu^{2+}, dimethylsulfoxide permeabilization, grindelane diterpenes, jasmonic acid.

Abbreviations:
BA: N6-benzylaminopurine
CDCl3: chloroform-d
2,4-D: 2,4-dichlorophenoxyacetic acid
DMSO: dimethylsulfoxide
DW: dry weight
Et2O: Ethyl ether
GC: gas chromatography
GC-MS: gas chromatography-mass espectrometry
IBA: indole-3-butyric acid
JA: jasmonic acid
K: kinetin
MS: Murashige and Skoog (1962) medium containing 3% (w/v) sucrose
NAA: 1-naphthalene acetic acid
NMR: nuclear magnetic resonance
TLC: thin layer chromatography

*Corresponding author

Grindelia pulchella **callus and cell suspension cultures were established from seedling leaves. When several phytoregulator supplementations were assayed in solid Murashige and Skoog medium containing 3% (w/v) of sucrose (MS medium), combinations of indole-3-butyric acid (IBA) and N^6-benzylaminopurine (BA) resulted the most appropriate conditions to generate fast growing friable calli with detectable levels of grindelic acid. Moreover, the same basal media supplemented with 20.0 µM IBA/4.4 µM BA was found to be optimal for cell growth in submerged cultures (μ_{max} = 0.26 $days^{-1}$) while the addition of 20.0 µM IBA/18.0 µM BA resulted in a relative higher metabolite production (4.55 mg/gDW) when the inocula was 5% (v/v). Furthermore, three different stress factors and combinations of them were used to elicit cell suspensions. These experiments demonstrated that the combination of $CuSO_4$ and dimethylsulfoxide (DMSO) increase the grindelic acid production to 2.63 mg/gDW in the elicited essay versus 0.756 mg/gDW in the control, at expense of cell growth. In contrast, the addition of jasmonic acid (JA) alone and combined with DMSO neither affected cell growth nor grindelic acid accumulation.**

Grindelia species are widespread in South American semiarid regions. Most of these genuses are used in folk medicine as antispasmodic and diuretic, among other purposes. Grindelic acid (Figure 1) and minoritary hydroxylated metabolites were isolated from *G. pulchella* aerial parts (Guerreiro et al. 1981). Grindelic acid hydroxylated derivatives, the acids 6-β-hydroxygrindelic, 6-α-hydroxygrindelic and 3-β-hydroxygrindelic, were successfully obtained by fungal bioconvertion from grindelic acid (Hernandez et al. 1997; Hernandez et al. 2002; Orden et al. 2005) and showed bioactive properties towards *Tenebrio molitor* larvae species and phytopatogen fungi and bacteria. Furthermore, the resins of several species of the genus *Grindelia* such us *G. chiloensis, G. camporum* and *G. glutinosa*, particularly rich in diterpene acid derivatives, have been extensively studied due to their possible industrial applications like pine resin and raw material for the naval stores industry (Hoffmann and McLaughlin, 1986; Timmermann et al. 1987; Ravetta et al. 1996; Ravetta and Soriano, 1998; Zavala and Ravetta, 2001; Zavala and Ravetta, 2002; Wassner and Ravetta, 2005). Even some attempts have been made to generate protocols for vegetative propagation of *G. chiloensis* (Wassner and Ravetta, 2000).

There has been a long-standing interest in the exploration and utilization of plant cell cultures for the production of plant secondary metabolites. Searching for a biotechnological approach as an alternative for stable production of grindelic acid seems to be quite promising since a wide range of derivatives could be obtained by both chemical and biological transformations.

Elicitation by heavy metals is a procedure that has demonstrated to improve secondary metabolite accumulation in plant cell cultures (Rakwal et al. 1996; Oikawa et al. 2001; Mithöfer et al. 2004). Many authors have reported evidences that jasmonates are involved in the biosynthesis of a wide spectrum of secondary metabolites (Menke et al. 1999; Vom Endt et al. 2002) and we have previously demonstrated that DMSO helps to increase terpenoid metabolite accumulation in cell cultures (Kurina et al. 2000). In a preview personal communication, we have demonstrated that grindelic acid possesses antimicrobial activity toward several phytopatogenic fungal and bacterial strains. Taking into account that the biosynthesis and accumulation of this sort of secondary metabolites may be induced by biotic elicitation, JA, a proved mediator in plant-wounding and plant-microbe interactions, was chosen to try to induce the aforementioned metabolite accumulation *in vitro*.

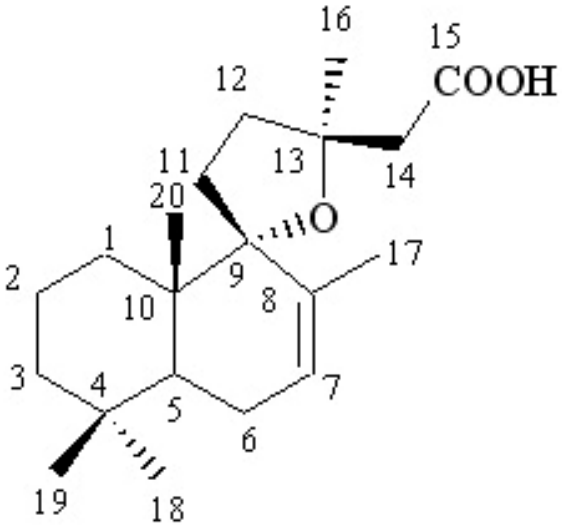


Figure 1. Grindelic acid structure.

The objective of this work was to establish submerged cultures of *G. pulchella* in order to study the possibility of producing stable amounts of grindelic acid. In this sense the effects of elicitors such as JA and copper salts were tested on DMSO-permeabilized cell suspension cultures.

MATERIALS AND METHODS

General

The ^1H NMR spectra were recorded in $CDCl_3$ at 200.13 MHz and ^{13}C NMR were obtained at 50.23 MHz on a Bruker AC-200. EIMS were collected at 70 eV on a Finnigan-Mat GCQ-plus instrument. Optical rotations were obtained on a Perkin-Elmer 341 polarimeter. CC were performed on Silica gel G 70-230 mesh and 60 H. TLC

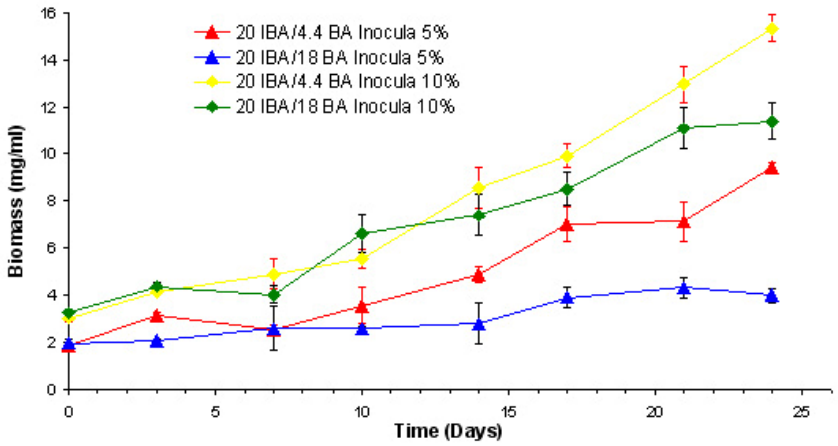


Figure 2. Phytoregulator ratios (µM) and inocula size (v/v) influence in biomass production in *G. pulchella* cell suspension cultures. Vertical bars denote standard deviation from the mean.

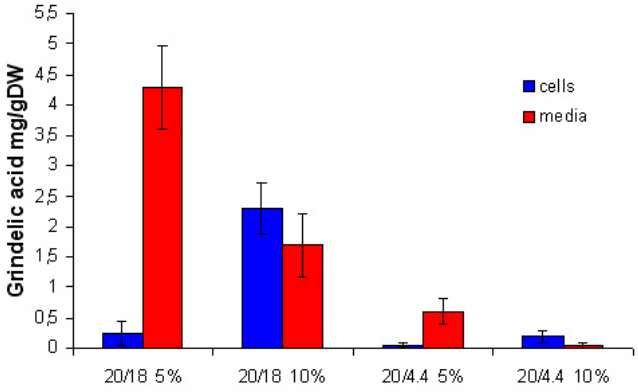


Figure 3. Phytoregulator and inocula size influence in grindelic acid accumulation rates in logarithmic phase of *G. pulchella* cell suspension cultures.

20/18 5%: 20.0 µM IBA /18.0 µM BA, inocula 5% (v/v); 20/18 10%: 20.0 µM IBA /18.0 µM BA, inocula 10% (v/v); 20/4.4 5%: 20.0 µM IBA /4.4 µM BA; inocula 5% (v/v); and 20/4.4 10%: 20.0 µM IBA /4.4 µM BA; inocula 10% (v/v). Vertical bars denote standard deviation from the mean.

were carried out on Silica gel 60 F_{254} (0.2 mm-thick plates). The chemicals used were JA and $CuSO_4$, purchased from Sigma-Aldrich**.**

Plant material

G. pulchella seeds and aerial parts were collected in Departamento Capital, San Luis, Argentina and identification was performed by Prof. L.A. Del Vitto. A voucher specimen Nº3616 (UNSL) is deposited in the Herbarium of the San Luis University.

Grindelic acid isolation and structure determination

Grindelic acid authentic samples were isolated from aerial parts of wild *G. pulchella* specimens as previously reported (Guerreiro et al. 1981), and structural identity was confirmed by ^{1}H NMR and ^{13}C NMR.

Grindelic acid

$[\alpha]_D^{25}$ = - 102.2° ($CHCl_3$; *c* 0.7). ^{1}H NMR spectral data ($CDCl_3$): d 5.6 (*brs*, H-7), 2.76 (*d*, *J* = 15 Hz, H-14a), 2.59 (*d*, *J* = 15 Hz, H-14b), 1.78 (*s*, H_3-17), 1.39 (*s*, H_3-16), 0.92 (*s*, H_3-18), 0.88 (*s*, H_3-19) y 0.81 (*s*, H_3-20). ^{13}C NMR spectral data ($CDCl_3$): d 173.5 (C-15), 133.4 (C-8), 127.9 (C-7), 91.8 (C-8), 81.0 (C-13), 47.4 (C-14), 42.6 (C-5), 41.7 (C-3), 40.5 (C-10), 38.9 (C-12), 33.0 (C-4), 32.7 (C-18), 32.5 (C-1), 27.6 (C-11), 26.7 (C-16), 24.0 (C-6), 21.9 (C-19), 21.0 (C-17), 18.5 (C-2), and 16.5 (C-20).

Culture conditions

G. pulchella seeds were surface disinfected and germinated in aseptic conditions. Seedling first true leaves were used to initiate callus cultures on MS medium. Different conditions were obtained by supplementing the above-mentioned salt basal medium with different auxin/cytokinin ratios (Table 1). Incubation was carried out at 22 ± 2ºC under a 16 h light 8 hrs dark cycle by fluorescent lamps at an irradiance of approximately 1.8 Wm^{-2}. Cells were subcultured for 25 or 30 days. Submerged cultures were initiated by transferring 4-5 g of calli into 100 ml liquid media with the same plant growth regulator supplements. After 4 weeks, when the cultures reached a suitable cell density for subculture, inocula of 5 and 10% v/v were transferred to fresh medium. Liquid cultures (50 ml) were grown in 125 ml flasks on a rotatory shaker (120 rpm) under the conditions described above.

Culture growth evaluation

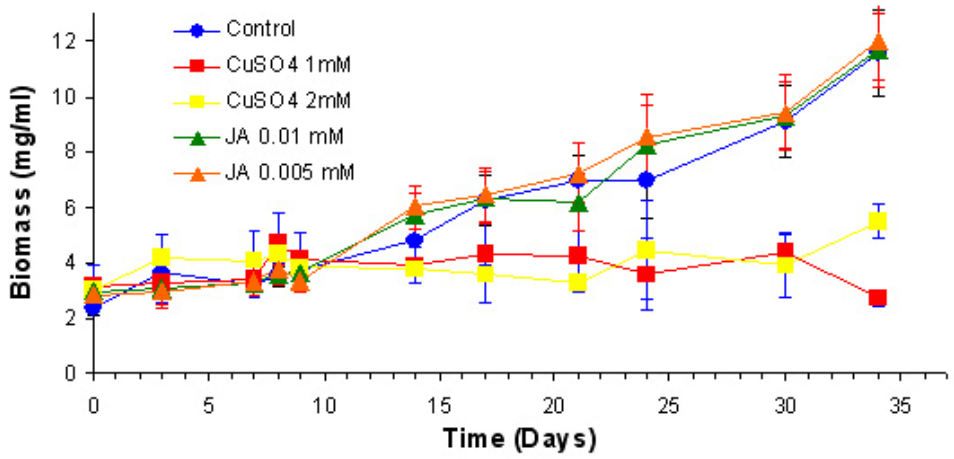


Figure 4. Elicitation influence in biomass production in *G. pulchella* cell suspension cultured in MS media supplemented with 20.0 µM IBA /18.0 µM BA, inocula size 10% (v/v). Vertical bars denote standard deviation from the mean.

Biomass was evaluated as dry weight (DW) of cells in vacuum at 40ºC.

Grindelic acid extraction and identification

The freeze-dried calli were extracted three times with methanol in reflux. Evaporation of solvent under reduced pressure gave the methanol extract, which was then partitioned between chloroform and water. The organic phase was evaporated and subjected to preparative TLC eluted with benzene: dioxane: acetic acid (30:5:1) to give grindelic acid, which identity was confirmed by ^{1}H NMR and ^{13}C NMR.

Grindelic acid quantification

Dried cells were homogenized and extracted in methanol by reflux (X3). Culture medium was acidified to pH 5/5.5 and extracted three times with Et_2O. Both methanolic and ethereal extracts were washed, concentrated, and methylated with diazomethane and redisolved in acetone. Quantification of methylgrindelate was carried out by GC using an OV 17 column, a N_2 flow rate of 30 ml min^{-1} and a temperature gradient from 200ºC to 270ºC. Grindelic acid methyl ester peaks were identified by co/chromatography with authentic grindelic acid samples derivatized in the same way. Methyl grindelate peak was confirmed also by GC-MS. MS *m/z* (rel. int.) 334 $[M]^+$ (0.45%) $[C_{20}H_{34}O_3]^+$, 319 (0.17%) $[C_{20}H_{31}O_3]^+$, 303 (0.27%) $[C_{20}H_{31}O_2]^+$, 261 (2.74%) $[C_{18}H_{19}O]^+$, 243 (2.24%) $[C_{18}H_{27}]^+$, 210 (100%) $[C_{12}H_{18}O_3]^+$, 136 (45.6%) $[C_9H_{12}O]^+$, 109 (26.2%) $[C_8H_{13}]^+$. Quantification was performed using a standard curve run under the same conditions. All experiments were repeated three times and statistical analyses were carried out using the ANOVA test.

Elicitation experiments

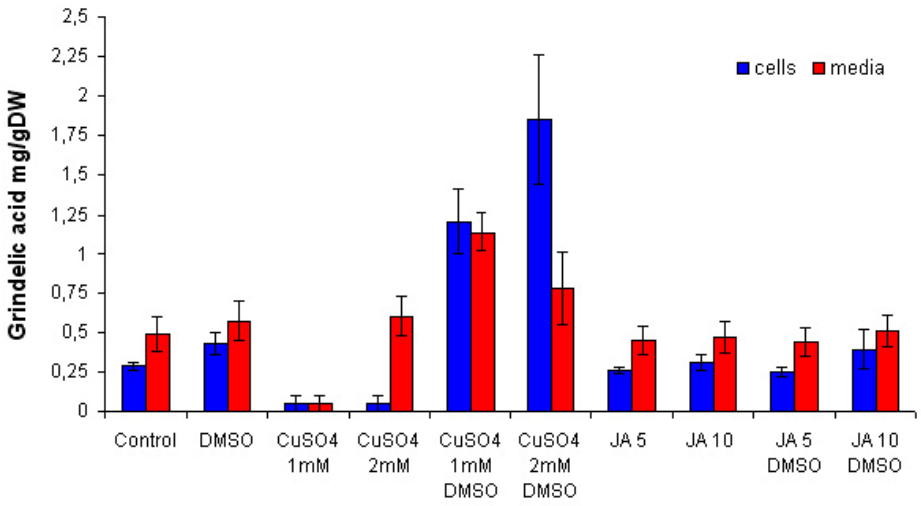
They were carried out on 7-day-old cultures in MS medium supplemented with 18.0 µM BA and 20.0 µM IBA. DMSO and $CuSO_4$ solutions were sterilized by filtration. DMSO was added to the cultures at final concentrations of 1.0 µl per ml of culture. Meanwhile $CuSO_4$ solutions were added to reach a final concentration of 1.0 and 2.0 mM. JA was dissolved in Et_2O, sterilized by filtration, and added to a final concentration of 0.005 or 0.01 mM. Grindelic acid accumulation in cells and media was evaluated over the growth cycle in elicited and non-elicited cultures and the maximal grindelic acid rates during logarithmic growth phase were registered. Three replicates of each experiment were performed.

RESULTS AND DISCUSSION

G. pulchella in vitro culture establishment

Calli of *G. pulchella* could be successfully induced from *in vitro* germinated seedlings when explants from different organs were cultured on MS media added with several plant growth regulator combinations and ratios (Table 1). The best results for callus production were obtained when explants were cultured in the presence of 20.0 µM of IBA and 18.0 µM of BA. Other auxin/cytokinin combinations and ratios were less effective in promoting callus development. Although several authors demonstrated that 2,4-D was necessary to initiate callus induction (Banthorpe, 1994), it was ineffective in our experiences and, even necrosis was evident after the first subculture. Microscopy revealed the presence of adult cells organized in differentiated tissues, particularly vascular ones. NAA tended to induce the regeneration of roots, independently of the origin of the explants.

The presence of grindelane diterpenes in the different callus lines was checked by extraction and further TLC analysis



Control: Blank essay; DMSO: elicited with 1 µl/ml DMSO; CuSO4 1mM: elicited with 1.0 mM CuSO4; CuSO4 2mM: elicited with 2.0 mM CuSO4; CuSO4 1mM DMSO: elicited with 1.0 mM CuSO4 and 1 µl/ml DMSO; CuSO4 2mM DMSO: elicited with 2.0 mM CuSO4 and 1 µl/ml DMSO; JA 5: elicited with 0.005 mM JA; JA 10: elicited with 0.010 mM JA; JA 5 DMSO: elicited with 0.005 mM JA and 1 µl/ml DMSO; and JA 10 DMSO: elicited with 0.010 mM JA and 1 µl/ml DMSO. Vertical bars denote standard deviation from the mean.

Figure 5. Elicitation influence in grindelic acid accumulation rates in logarithmic phase of *G. pulchella* cell suspension cultured in MS media supplemented with 20.0 µM IBA /18.0 µM BA, inocula size 10% (v/v).

compared with authentic samples. Grindelic acid was detected only in calli grown in MS media supplemented with different IBA/BA combinations. Its identity was confirmed by ^{1}HNMR and ^{13}CNMR spectroscopy. No other minor grindelane metabolites were observed by this methodology in the analyzed biomass samples.

Cell suspension cultures and grindelic acid accumulation

Suspension cultures were initiated from friable tissues using those callus lines developed in MS basal media supplemented with 20.0 µM IBA/18.0 µM BA and 20.0 µM IBA/4.4 µM BA that showed detectable grindelic acid accumulation as it was described above. Significant differences were observed when cell growth ratios of cultures grown in basal media supplemented with 20.0 µM IBA/18.0 µM BA were compared with those added with 20.0 µM IBA/4.4 µM BA using inocula sizes of 5 and 10 % v/v in both cases. In the former phytoregulator condition, a $\mu_{max} = 0.177$ days^{-1} was obtained when inocula was of 10%, and $\mu_{max} = 0.052$ days^{-1} with inocula of 5%. However, when MS media was supplemented with 20.0 µM IBA/4.4 µM BA, $\mu_{max} = 0.23$ days^{-1} and $\mu_{max} = 0.26$ days^{-1} for inocula of 10% and 5%, respectively, were observed (Figure 2).

Intra and extracellular grindelic acid accumulations were evaluated. The maximal grindelic acid levels during logarithmic growth phase were registered. Nevertheless, no other grindelane metabolites were detected neither in biomass nor in culture media. Unfortunately, the best culture conditions for cell growth showed the minimal grindelic acid accumulation rates. Interestingly, grindelic acid production increased and it was a noticeable positive effect on grindelic acid extracellular accumulation when biomass inoculum was diminished to 5%. Since the rates of extracellular accumulation increased as the intracellular quantities were reduced, it seems that grindelic acid was excreted to the media more efficiently in this condition than in more dense cultures (Figure 3).

Elicitation effects on cell growth

15 monthly subcultures were performed in order to obtain stable cell lines. After this period, not only grindelic acid accumulation rates drastically diminished, but also intra and extracellular ratios changed. These results might have been caused by epigenetic changes or epimutations (Martienssen and Colot, 2001) caused by cell dedifferentiation process that often lead to a lower productivity (Verpoorte et al. 2002).

It has been reported that octadecanoid elicitors affected isoprene metabolite biosynthesis and accumulation in a number of plant species. Namely, the interaction of jasmonates with wounding and/or fungal elicitation in *Hyoscyamus muticus* root cultures (Singh et al. 1998); in *Solanum tuberosum* (Choi et al. 1994) and in elicited cell suspension of *Tessaria absinthioides* (Kurina et al. 2000). Moreover, in the last specie, when the solvent DMSO was added as a permeabilizing agent, it acted as an abiotic elicitor (Kurina and Donadel, 2003).

When DMSO was used to permeabilize the *G. pulchella* cell suspension cultures maintained in MS basal media amended with 20.0 µM IBA/18.0 µM BA, cell growth rates

Table 1. Growth response of *G. pulchella* explants on MS media and different plant growth regulator combinations. Concentrations are expressed in µM. Results come from three independent experiments of 40-60 explants in each one.

2,4-D	IBA	NAA	BA	K	Growth results
4.5	-	-	-	-	roots/shoots
2.2	-	-	-	-	N/d*
-	-	5.4	-	2.3	roots
-	-	21.5	-	2.3	calli
-	2.5	-	-	-	shoots
-	2.5	-	2.2	-	shoots
-	5.0	-	-	-	roots
-	5.0	-	2.2	-	calli
-	5.0	-	4.4	-	calli
-	10.0	-	2.2	-	calli
-	20.0	-	-	-	roots
-	20.0	-	2.2	-	friable calli
-	20.0	-	4.4	-	friable calli
-	20.0	-	9.0	-	friable calli
-	20.0	-	18.0	-	friable calli

*N/d: No development.

did not show statistical significant differences with the controls. Furthermore, the growth of *G. pulchella* cell suspension was not affected by the addition of JA although it was drastically suppressed by $CuSO_4$ treatments as it is shown in Figure 4.

Elicitation effects on grindelic acid accumulation

Grindelic acid contents were determined during logarithmic growth phase after the elicitor addition. Highest grindelic acid accumulations in biomass and culture media were expressed as mg of grindelic acid per cell dry weight and are shown in Figure 5. Surprisingly permeabilization experiments did not result in a statistical significant increase in the grindelic acid rates excreted from cells to media. Furthermore, a slight elicitation effect was observed in cultures treated only with DMSO. Grindelic acid rates were higher in the controls that in the cultures elicited with 1.0 and 2.0 mM of $CuSO_4$, but the highest grindelic acid contents (1.85 mg/gDW^{-1}) were obtained in the biomass when these treatments were combined by the addition of 1 µl DMSO per ml of culture. On the other hand, treatments with 0.005 and 0.010 mM of JA only afforded grindelic acid levels comparable to the controls.

CONCLUDING REMARKS

This work results in a valuable contribution to establish *in vitro* culture conditions for the wild specie *G. pulchella* in order to produce the diterpene grindelic acid. In this sense different IBA/BA combinations resulted in the best phytoregulator supplementation to establish *G. pulchella* callus and cell suspension cultures. Meanwhile the addition of 20.0 µM IBA/4.4 µM BA was found to be optimal for biomass development in submerged culture, MS media with 20.0 µM IBA/18.0 µM BA resulted in a relative higher grindelic acid production. The elicitation experiments demonstrated that the association of $CuSO_4$ and DMSO increases the terpenoid compound production at expense of cell growth. In contrast, neither cell growth nor grindelic acid accumulation was affected with the addition of JA alone and combined with DMSO. Further elicitation

experiments such as the use of biotic agents and combinations of biotic and abiotic elicitors would allow improving grindelic acid production. According to these results, other mediators different of JA should be involved in the transduction of the elicitor signals in the regulation of the expression of diterpenoid metabolites in this system.

ACKNOWLEDGMENTS

Thanks are due to Fabricio Pena for the statistical analyses and Lic. Mónica Ferrari for the technical assistance. This work is part of X.E.H.'s Doctoral thesis and has been partially presented as a poster communication at the XII National Symposium of Organic Chemistry: XII SINAQO (Hernandez et al. 2000).

REFERENCES

BANTHORPE, D.V. Secondary metabolism in plant tissue cultures: scope and limitations. *Natural Product Reports*, 1994, vol. 11, p. 303-328.

CHOI, D.; BOSTOCK, R.M.; AVDIUSHKO, S. and HILDEBRAND, D.F. Lipid derived signals that discriminate wound and pathogen responsive isoprenoid pathways: methyl jasmonate and fungal elicitor arachidonic acid induce different 3-hydroxy3-methenyl glutaril-coenzime A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. *Proceedings of the National Academy of Sciences of the United States of America*, March 1994, vol. 91, no. 6, p. 2329-2333.

GUERREIRO, E.; KAVKA, J.; SAAD, J.R.; ORIENTAL, M. and GIORDANO, O.S. Acidos diterpénicos en *Grindelia pulchella* y *G. chiloensis* Cabr. *Revista Latinoamericana de Química*, 1981, vol. 12, no. 2, p. 77-81.

HERNANDEZ, X.E.; KURINA, M. and GIORDANO, O.S. Production of 6-β-hydroxygrindelic acid from grindelic acid by *Alternaria alternata*. *Biotechnology Letters*, December 1997, vol. 19, no. 12, p. 1223-1225.

HERNANDEZ, X.E.; KURINA, M. and GIORDANO, O.S. Grindelic acid production in *Grindelia pulchella* cell suspension cultures elicited with $CuSO_4$. In: *National Congress: Proceeding of the XII National Symposium of Organic Chemistry* (14-17th November 1999, Los Cocos Cordoba, Argentina) Molecules Ed. Molecular Diversity Preservation. March 2000, vol. 5, p. 614-615.

HERNANDEZ, X.E.; CARRIZO, R.A.; GIORDANO, O.S. and KURINA, M. Hydroxylation of grindelic acid by filamentous fungus.*The Journal of Argentine Chemical Society*, 2002, vol. 90, no. 1-3, p. 27-30.

HOFFMANN, J.J. and McLAUGHLIN, S.P. *Grindelia camporum*: potential cash crop for the arid southwest. *Economic Botany*, 1986, vol. 40, p. 162-169.

KURINA, M.; HERNANDEZ, X.E.; TONN, C.E. and GUERREIRO, E. Enhancement of Tessaric acid production by *Tessaria absinthioides* cell suspension cultures. *Plant Cell Reports*, 2000, vol. 19, no. 8, p. 821-824.

KURINA, M. and DONADEL, O.J. Biotransformation of eudesmanes by *Tessaria absinthioides* cell suspension cultures. Plant Cell, Tissue and Organ Culture, 2003, vol. 73, no. 2, p. 123-129.

MARTIENSSEN, R.A. and COLOT, V. DNA methylation and epigenetic inheritance in plants and filamentous fungi. *Science*, 2001, vol. 293, no. 5532, p. 1070-1074.

MENKE, F.L.H.; PARCHMANN, S.; MUELLER, M.J.; KIJNE, J.W. and MEMELINK, J. Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in *Chataranthus roseus*. *Plant Physiology,* April 1999, vol. 199, no. 4, p.1289-1286.

MITHÖFER, A.; SCHULZE, B. and BOLAND, W. Biotic and heavy metal stress response in plants: evidence for common signals. *FEBS Letters*, 2004, vol. 566, no. 1-3. p. 1-5.

OIKAWA, A.; ISHIHARA, A.; HASEGAWA, M.; KODAMA, O. and IWAMURA, H. Induced accumulation of 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) in maize leaves. Phytochemistry, 2001, vol. 56, no. 7, p. 669-675.

ORDEN, A.A.; CIFUENTE, D.A.; BORKOWSKI, E.J.; TONN, C.E. and KURINA, M.B. Stereo and regioselective hydroxylation of grindelic acid by *Aspergillus niger*. *Natural Product Research*, 2005, vol. 19, no. 6, p. 625-631.

RAKWAL, R.; TOMOGAMI, S. and KODAMA, O. Role of jasmonic acid as signaling molecule in copper chloride-elicited rice phytoalexin production. *Bioscience, Biotechnology and Biochemistry*, 1996, vol. 60, no. 6, p. 1046-1048.

RAVETTA, D.A., ANOUTI, A. and McLAUGHLIN, S.P. Resin production of *Grindelia* accessions under cultivation. *Industrial Crops and Products*, 1996, vol. 5, no. 3, p. 197-201.

RAVETTA, D.A. and SORIANO, A. Alternatives for the development of new industrial crops for Patagonia. *Ecología Austral*, 1998, vol. 8, no. 2, p. 297-307.

SINGH, G.; GAVRIELI, J.; OAKEY, J.S. and CURTIS, W.R. Interaction of methyl jasmonate, wounding and fungal elicitation during sesquiterpene induction in *Hyoscyamus muticus* in root cultures. *Plant Cell Reports*, 1998, vol. 17, no. 5, p. 391-395.

TIMMERMANN, B.N., McLAUGHLIN, S.P. and HOFFMANN, J.J. Quantitative variation of grindelane

diterpene acids in 20 species of North American *Grindelia*. Biochemical Systematics and Ecology, 1987, vol. 15, no. 4, p. 401-410.

VERPOORTE, R.; CONTIN, A. and MEMELINK, J. Biotechnology for the production of plant secondary metabolites. *Phytochemistry Reviews*, January 2002, vol.1, no. 1, p. 13-25.

VOM ENDT, D.; KIJNE, J.W. and MEMELINK, J. Transcription factors controlling plant secondary metabolism: what regulates the regulators? *Phytochemistry*, 2002, vol. 61, no. 2, p. 107-114.

WASSNER, D.F. and RAVETTA, D.A. Vegetative propagation of *Grindelia chiloensis* (Asteraceae). *Industrial Crops and Products*, 2000, vol. 11, no. 1, p. 7-10.

WASSNER, D.F. and RAVETTA, D.A. Temperature effects on leaf properties, resin content and composition in *Grindelia chiloensis* (Asteraceae). *Industrial Crops and Products*, 2005, vol. 21, no. 2, p. 155-163.

ZAVALA, J.A. and RAVETTA, D.A. The effect of irrigation regime on biomass and resin production of *Grindelia chiloensis*. *Field Crop Research*, 2001, vol. 69, no. 3, p. 227-236.

ZAVALA, J.A. and RAVETTA, D.A. The effect of solar radiation on terpenes and biomass production in *Grindelia chiloensis* (Asteraceae), a woody perennial of Patagonia, Argentina. *Plant Ecology*, 2002, vol. 161, no. 2, p. 185-191.