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Elicitation of Pharmacologically Active Substances in an Intact Medical Plant

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The quality of medical plants used for the production of galenics or pharmacologically useful compounds is usually assessed by the content of biologically active compounds. Because most of these plants are grown in fields, this study focused on stimulation of active compounds by in vivo elicitation. Foliar application of elicitors on the immunostimulating medical plant purple coneflower (*Echinacea purpurea* L. Moench.) grown on soil was used to increase the content of biologically active phenolics. Natural plant stress mediators and their derivatives (acetylsalicylic acid, salicylic acid, and methyl salicylate) as well as newly introduced biocompatible metal elicitor [titanium(IV) ascorbate] were chosen as active components of foliar sprays. A tremendous increase of phenolics (up to 10 times compared to control) and stimulation of the biomass yield were achieved. Tuning of organ specificity by modulation of the concentration of elicitor was also observed. This methodology represents a convenient alternative to cell suspension or hydroponic cultures being applicable in wide agricultural practice.

KEYWORDS: Elicitation; medical plant; *Echinacea purpurea*; secondary metabolite; soil; foliar application; phenolics

INTRODUCTION

Plants have been used for curative or palliative treatment of miscellaneous diseases since ancient times. Despite the development of modern synthetic drugs, medical plants maintain their considerable importance in medicine in the form of galenics for phytotherapy, nutraceuticals, and cosmetics. A large part of the production of medical plants is employed in the isolation of active substances (*1*), and considerable attention has been devoted to the production of biologically active substances by plant cell cultures grown in vitro (*2*).

The pharmacologically interesting compounds of plant origin are mostly, beyond some exceptions (e.g., polysaccharides), products of secondary metabolism. Such metabolites are toxic or inconsumable for herbivores, detoxify heavy metals, or possess bactericidal or fungicidal activities and thus protect the plant against external threats (*3*). Elicitation, that is, the stimulation of any type of plant defense response by apparently stressing factors, so-called elicitors, is often used for the induction of secondary metabolites' biosynthesis (*4*). The addition of elicitors (which could be of biotic or abiotic origin) to the nutrient medium is commonly exploited in plant cell cultures (*5*). Recently, the stimulation of plants by addition of elicitors to hydroponic media has also been investigated (*6*). However, despite the fact that the majority of medical plants are currently grown in field conditions, very little research has focused on elicitation of plants grown on soil to improve the production of pharmaceutically active substances.

In our attempts to develop useful technology for large-scale production of plant secondary metabolites, we suggested that application of certain plant stress hormones and/or metal stressing factors directly on the whole intact plants can increase the content of secondary metabolites (*7*), and we also developed a commercialized elicitor foliar formulation (EliTic, Agra Group, Inc., Strelske Hostice, Czech Republic). A similar approach was successfully used by other groups for effective stimulation of isoflavones in the soybean plant (*Glycine max* L. Merr.) (*8*) and red clover (*Trifolium pratense* L.) (*9*), as well as phenolics in black chokeberry [*Aronia melanocarpa* (Michx) Elliot] (*10*, *11*), black currant (*Ribes nigrum* L.) (*11*), nettle (*Urtica dioica* L.), dandelion (*Taraxacum officinale*), and purple coneflower (*Echinacea purpurea* L. Moench.) (*12*).

The last mentioned medical plant is well-known for its immunostimulating effects. Over time, many bioactive substances were isolated from *Echinacea* species (*13*). A single substance definitively responsible for the described effects on human beings has been elusive, and the stimulatory action is probably based on the synergic effects of multiple components (*14*). Phenolics are the most potent bioactive compounds present in all parts of *E. purpurea* other than alkylamides, polysaccharides, and glycoproteins (*13*). Among the most remarkable representatives of

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antioxidant phenolics are the caffeic acid derivatives (cichoric, caftaric, and chlorogenic acid) and flavonoid rutin.

In this paper, we report the massive induction of secondary metabolites (caffeic acid-derived phenolics) in whole intact plants of *E. purpurea*. We show that such stimulation can be achieved by simple foliar application of inexpensive natural or novel titanium-based biocompatible elicitors. We also demonstrate the concentration-dependent organ specificity of elicitor action and effective shoot-to-root transmission of the stress signal in the plant.

MATERIALS AND METHODS

Chemicals. Caftaric acid standard was purchased from Dalton Chemical Laboratories, Inc., Canada. All of the other standards and chemicals were purchased form Sigma-Aldrich Ltd. (Prague, Czech Republic) and were used as obtained without additional purification.

Solution of Titanium(IV) Ascorbate (10.0 g of Ti L^{-1}). $TiCl_4$ (39.6 g) was slowly added to the cooled and stirred solution of ascorbic acid (100 g) in 300 mL of H_2O under nitrogen atmosphere. A deep red-brown complex was formed. The resulting solution was cooled and vigorously stirred while a 40% KOH solution was slowly added to partially neutralize the solution (final pH 5.0). The volume was then adjusted to 1000 mL with water. This solution was stable for months when stored at 5 °C [some turbidity caused by TiO_2 precipitation could appear in the course of time, but the change of Ti(IV) ascorbate concentration is negligible, as proven by UV−vis spectroscopy].

Elicitors. All elicitors were dissolved in distilled water, and these solutions were sprayed directly on plants (100 mL per plant). Acetylsalicylic acid (ASA), salicylic acid (SA), and methylsalicylate (MS) were applied in three different concentrations (10, 100, and 1000 μM). Titanium(IV) ascorbate was also applied in three different concentrations (10, 25, and 50 ppm Ti). Water was sprayed as a negative control.

Plant Culture. Plants of *E. purpurea* (wild type) were grown in pots (four plants per experimental alternative), 1 plant per pot, 6 kg of cambisol fertilized with NPK [0.2 g kg^{-1} N (NH_4NO_3), 0.03 g kg^{-1} P, and 0.08 g kg^{-1} K (KH_2PO_4)] per pot. The plants were grown outdoors. The first year the plants were grown without any applications of the elicitors. The second year the elicitors (see above) were sprayed four times with 20 day delays between subsequent applications, the first application just before bloom. The third year the elicitors were sprayed three times with 20 day delays between subsequent applications, the first application just before bloom. The plants were harvested the third year 20 days after the last spray of the elicitors, tops and roots separately.

Analysis of Plant Material. Top and root dry weights (g $plant^{-1}$) were determined after gentle washing of the plants with water and drying. All phenolic substances were determined in dry mass via reversed-phase HPLC using the method and equipment described in ref *15*. The dried plant material was extracted sequentialy by methanol and water, and the contents of phenolics and rutin were analyzed on a C18 column (Luna, Phenomenex) using gradient mobile phase acetonitrile/water (0.15% of trifluoroacetic acid). Mobile phase A consisted of 5% acetonitrile with 0.15% of trifluoroacetic acid and mobile phase B of 80% acetonitrile with 0.15% of trifluoroacetic acid. The separation of all phenolic compounds was carried out using the following gradient: 0−20% B (25 min), 20−40% B (35 min), with a flow rate 0.25 mL/min. The addition of 0.1% of *o*-phosphoric acid instead trifluoroacetic acid will shorten retention times by about 25% without worsening the separation efficiency. The analytes were quantified by diode array detector at 330 nm using external standard calibration. The spectra were recorded in the range from 190 to 600 nm. The content of cichoric acid, which was not available as a standard, was calculated using the calibration curve for caftaric acid.

Statistical Analysis. Differences among the experimental alternatives were tested using the analysis of variance (ANOVA), calculated on the Microcal Origin, version 6.0 software. The significance levels are represented by α.

RESULTS

The contents of caftaric, cichoric, and chlorogenic acids (caffeic acid derivatives) and rutin (flavonoid) were determined in tops and roots (see **Figure 1** for results). Top and root biomass yields were also estimated (**Figure 2**). Because of comparison between individual trends for different parameters observed, the values of variables in the following text are stated in percent of untreated controls that are listed in **Table 1**.

Tops (See Figure 1a and Table 2 for Data). The cichoric acid content was significantly ($\alpha = 0.05$) increased by the application of salicylic acid, the most effective elicitor (171% of untreated control) at the concentration of 10 μM. The effect of titanium followed the same trend but was not statistically significant. The caftaric acid content was significantly ($\alpha = 0.05$) increased by the application of all salicylic acid derivatives. Salicylic acid was again the most effective elicitor at the concentration of 10 μM (158% of untreated control). The contents of chlorogenic acid and rutin were not significantly changed by application of any elicitor.

Because of the low quantity of plants per treatment, the variance of yield was high and the trends caused by the application of inhibitors, although apparently positive, were not statistically significant. Interestingly, salicylic acid at the concentration of 1000 μM also induced flowering (the salicylic acid-sprayed controls had 1.9 times more flowers than untreated controls; data not shown).

Roots (See Figure 1b and Table 2 for Data). The cichoric acid content was strongly increased by all elicitors, and the effect was statistically significant ($\alpha = 0.10$) for salicylic acid and methyl salicylate, being the strongest for 1000 μM salicylic acid (237% of untreated control, see **Figure 1b**). The caftaric acid content was also elicited by all elicitors. Although the trend was statistically significant for titanium ascorbate only ($\alpha = 0.10$), the strongest elicitation was observed for 1000 μM salicylic acid (193% of untreated control).

The chlorogenic acid content was tremendously increased by all elicitors. Most active was titanium acsorbate, which caused an increase of 1 order of magnitude ($\alpha = 0.10$, 1000% of control for 10 ppm Ti). The caffeic acid content was not significantly ($\alpha = 0.05$) influenced by any elicitor.

Despite the low quantity of plants per treatment, the yield of root dry weight was also strongly increased by different elicitors (see **Figure 2**), the trends being statistically significant ($\alpha = 0.05$) for salicylic acid and titanium ascorbate. The strongest stimulants in this case were 1000 μM salicylic acid (253% of untreated control) and 100 μM methyl salicylate (254% of untreated control).

DISCUSSION

The quality of plants intended for processing by the pharmaceutical industry is usually judged on the basis of secondary metabolite content, which can strongly vary depending on weather, growth conditions, and damage by pathogens and pests. In most cases the effect of genetic heterogeneity among the individual plants also plays a role (*16*). The low reproducibility and variability of content of active compounds are key limitations in using plants as resources for the pharmaceutical industry. These complications could be overcome by the transfer of plants in more isolated and defined conditions using hydroponics (*6*, *17*−*24*), cell culture media (*5*), or explantate cultures (*25*−*28*), which was also described for plants of the genus *Echinacea* studied in this paper. However, there is still a lack of the facilities necessary for such a sophisticated culture, and the majority of medical plants worldwide are nowadays still cultivated in fields.

Elicitation of plants grown in soil was predicted to be problematic, because of limited uptake of the elicitors by the plant cuticle (*6*). However, this obstacle can be overcome by varying the concentration of the respective elicitor and by proper formulation

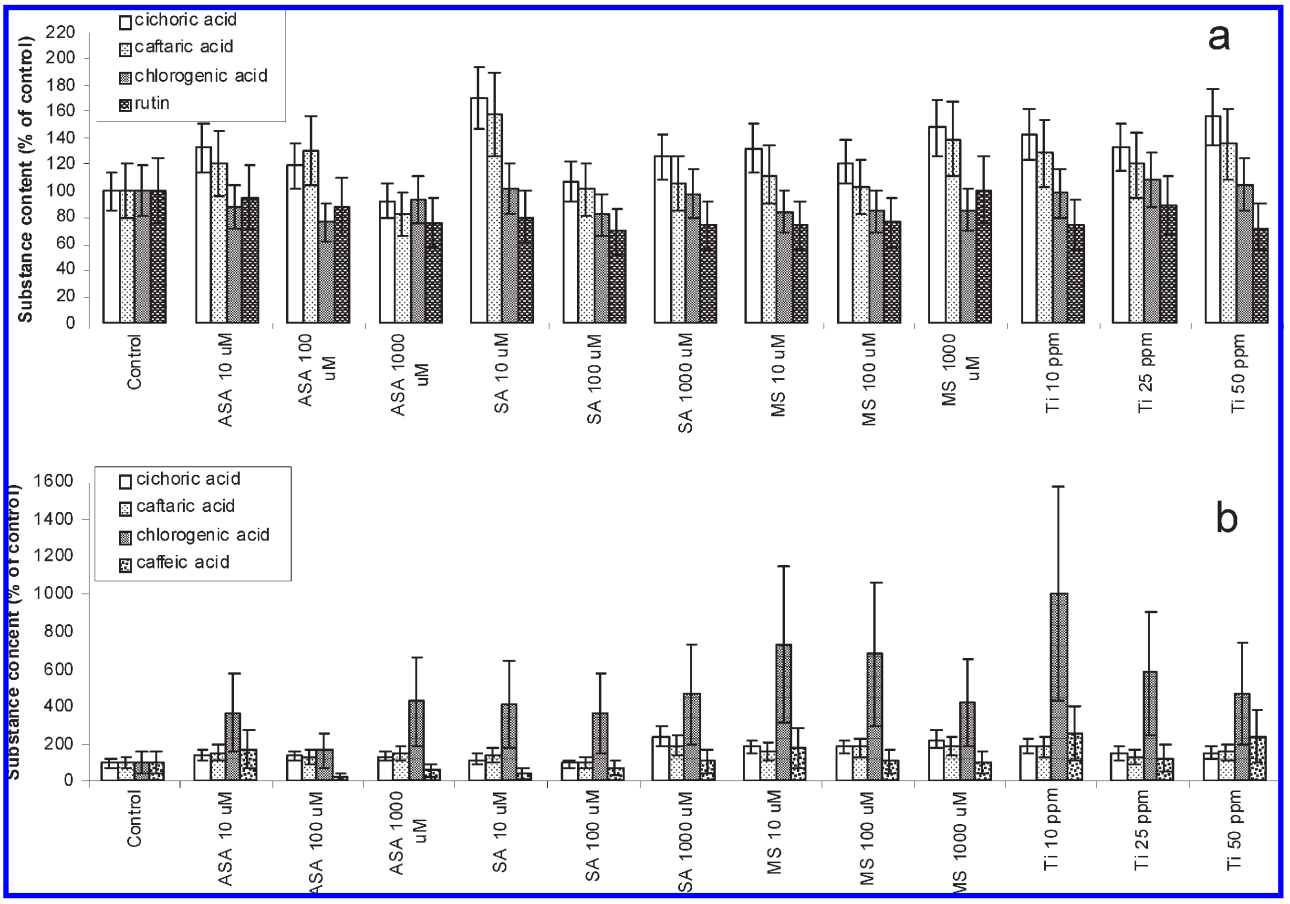

Figure 1. Contents of caftaric, cichoric, chlorogenic, and caffeic acids and of rutin in (**a**) tops and (**b**) roots of *Echinacea purpurea* (L.) Moench. The values are stated in percent of untreated controls that are listed in **Table 1**. ASA, acetylsalicylic acid; SA, salicylic acid; MS, methyl salicylate; Ti, Ti(IV) ascorbate. The alternatives are marked as <elicitor> <concentration>, for example, ASA 100 μM corresponds to the acetylsalicylic acid in the concentration 10^{-4} mol L^{-1}. Error bars represent standard deviations.

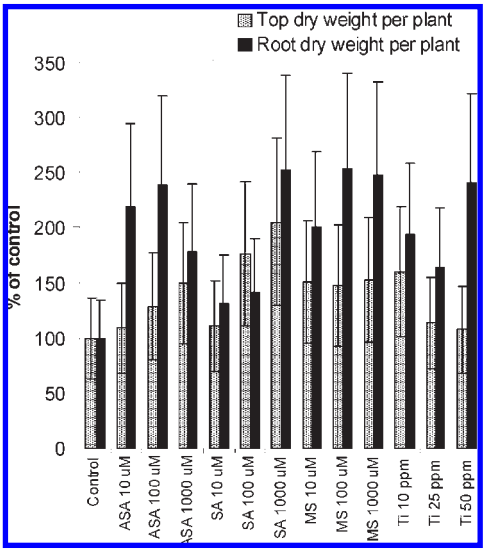

Figure 2. Top and root dry weights of the *Echinacea purpurea* (L.) Moench. plants.

of spray composition. For testing of this approach, we have chosen purple coneflower (*E. purpurea* L. Moench.) representing the genus *Echinacea*.

We followed the effect of elicitor foliar sprays on the production of phenolics in intact plants growing in soil. Natural plant stress mediators and their derivatives (acetylsalicylic acid, salicylic acid, and methyl salicylate) (*4*) as well as an abiotic metal stressing agent (titanium ascorbate) were chosen as active components of sprays. Titanium(IV) complexes have been known for a long time to possess significant beneficial effects on plants at low concentrations (*29*), and now a strong effort continues for their exploitation in agronomy. The main advantage of titanium is in its innoxiousness for animals and man (*29*). We have offered an explanation for this specific behavior by means of hormesis, an induced stress response that results in a beneficial increase in plant metabolism (*30*−*33*). Generally, salicylates and Ti(IV) complexes with natural ligands (ascorbic or citric acid) are natural or biocompatible compounds that fulfill the current ecotoxicological criteria of application in agriculture.

Table 1. Contents of the Followed Biologically Active Substances in Tops and Roots of *E. purpurea* (L.) Moench. in Untreated Controls (= 100% Hereinafter in This Paper)

substance	content in tops (mg g^{-1} of dry mass)	content in roots (mg g^{-1} of dry mass)
cichoric acid	16.7 ± 4.5	13.9 ± 4.1
caftaric acid	5.1 ± 1.0	3.1 ± 1.0
chlorogenic acid	0.152 ± 0.020	0.011 ± 0.006
caffeic acid	nd[a]	0.080 ± 0.033
rutin	0.51 ± 0.15	nd

[a] Not determined.

Table 2. Summary of ANOVA for Elicitor Types Affecting Individual Content of Phenolics or Dry Weight

		significance of the treatment effect[a]			
		acetylsalicylic acid	salicylic acid	methyl salicylate	titanium(IV) ascorbate
tops	cichoric acid	NS	++	NS	NS
	caftaric acid	++	++	++	NS
	chlorogenic acid	NS	NS	NS	NS
	rutin	NS	NS	NS	NS
	dry weight per plant	NS	NS	NS	NS
roots	cichoric acid	NS	++	++	NS
	caftaric acid	NS	NS	NS	+
	chlorogenic acid	NS	NS	+	++
	caffeic acid	NS	NS	NS	NS
	dry weight per plant	NS	++	+	++

[a] ++ indicates a significant difference at $\alpha = 0.05$, + indicates a significant difference at $\alpha = 0.10$, NS indicates no significant difference $\alpha = 0.10$.

Here we show the highly effective in vivo elicitation of pharmacologically interesting phenolics in whole intact medical plants grown in soil. Although the effect of elicitor application was generally distorted by high variability of the pot experiment, massive, up to 10 times, increases in active substance content could be achieved by application of elicitor spray (for chlorogenic acid in roots, see **Figure 1b**). Similar tremendous production of phenolics (cichoric acid) was also observed for roots of *E. purpurea* after foliar application of carboxymethyl chitin glucan, an elicitor produced from *Penicillium chrizogenum* mycelia (*12*). As a notable serendipitous effect, the stimulation of the root biomass yield is also very strong, based obviously on hormetic action of elicitors (*30*, *31*, *34*).

The best elicitor from the tested scale is salicylic acid (natural plant stress mediator). Whereas the diluted solutions (10 μM) act better for the elicitation of the phenolics content in tops, the more concentrated solutions (1000 μM) are most effective for the elicitation of phenolics in roots and total biomass yield.

Notably, the results suggest the complexity and organ-specificity of the defense reaction of the plant on the elicitor leaf spray—for example, the chlorogenic acid content in tops remains almost unchanged by elicitors, whereas extremely elicited in roots of the same plants. Furthermore, although the contents of most caffeic acid derivatives were increased in roots by the application of elicitors, the content of the caffeic acid itself (metabolic intermediate of cichoric, caftaric, and chlorogenic acids biosynthesis) is rather unchanged. Also, the content of flavonoid rutin is not substantially influenced by the elicitors. Other authors have found that a foliar spray of elicitor can also cause the action in roots (*12*). This demonstrates a practical consequence for the production of medical plants: the finding of optimal elicitor and its concentration can selectively influence not only the total content but also the ratio of targeted active substances and, therefore, the quality of the final product.

The higher concentration necessary for induction of stress response in roots corresponds with the known delay and limited effectiveness of shoot-to-root transmission of the stress signal (*35*). Importantly, foliar application of chosen elicitors can achieve the desired effect at concentrations below their toxicity threshold, despite the limitations connected with such long-distance signal transmission.

Interestingly, at the most active concentrations, the elicitors showed a propensity to induce similar patterns of elicitation (see **Figure 1**)—practically, only the quantitative strength of the effect was affected by the nature of the elicitor, whether it was a plant stress hormone (salicylic acid, methyl salicylate), its derivative (acetylsalicylic acid), or a metal stressor (titanium ascorbate). One can speculate that these elicitors influence the plant's metabolism by a similar stress response that includes the increased production of phenolics.

Introduction of *Echinacea* plants into abnormal conditions such as the action of drought stress for a certain time of the plant's development (*36*) or high salinity (*37*) has been shown to positively influence the formation of secondary metabolites. However, use of real stressing factors usually damages the plants and also decreases yields. On the other hand, certain types of fertilization (*20*, *38*) represent an interesting possibility to increase the content of secondary metabolites in field conditions. Application of elicitor sprays thus extends the list of available stimulatory techniques without decreasing and, often, even increasing the yield. This approach is becoming a convenient alternative to recent technonologies that comprise cell suspension cultures of *E. purpurea* (*39*) as well as stimulation of its adventitious (*40*) or hairy roots (*41*). Scaled-up field experiments on different medical plants using the described elicitation methodology are in progress.

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LITERATURE CITED

(1) Balunas, M. J.; Kinghorn, A. D. Drug discovery from medicinal plants. *Life Sci.* **2005**, *78*, 431–441.

(2) Kolewe, M. E.; Gaurav, V.; Roberts, S. C. Pharmaceutically active natural product synthesis and supply via plant cell culture technology. *Mol. Pharm.* **2008**, *5*, 243–256.

(3) Sudha, G.; Ravishankar, G. A. Involvement and interaction of various signaling compounds on the plant metabolic events during defense response, resistance to stress factors, formation of secondary metabolites and their molecular aspects. *Plant Cell, Tissue Organ Cult.* **2002**, *71*, 181–212.

(4) Szpitter, A.; Krolicka, A. Stimulating effect of biotic elicitors on the production of pharmacologically active secondary metabolites in plant in vitro cultures. *Biotechnologia* **2005**, *4*, 82–108.

(5) Namdeo, A. G. Plant cell elicitation for production of secondary metabolites: a review. *Pharmacogn. Rev.* **2007**, *1*, 69–79.

(6) Poulev, A.; O'Neal, J. M.; Logendra, S.; Pouleva, R. B.; Timeva, V.; Garvey, A. S.; Gleba, D.; Jenkins, I. S.; Halpern, B. T.; Kneer, R.; Cragg, G. M.; Raskin, I. Elicitation, a new window into plant chemodiversity and phytochemical drug discovery. *J. Med. Chem.* **2003**, *46*, 2542–2547.

(7) Cigler, P.; Hruby, M.; Kuzel, S. Preparation for induction of increased production of bioactive compounds in plants. International Patent WO 2005118508.

(8) Zhang, B.; Hettiarachchy, N.; Chen, P.; Horax, R.; Cornelious, B.; Zhu, D. Influence of the application of three different elicitors on soybean plants on the concentrations of several isoflavones in soybean seeds. *J. Agric. Food Chem.* **2006**, *54*, 5548–5554.
(9) Sivesind, E.; Seguin, P. Effects of foliar application of elicitors on red clover isoflavone content. *J. Agron. Crop Sci.* **2006**, *192*, 50–54.
(10) Hudec, J.; Bakos, D.; Mravec, D.; Kobida, L.; Burdova, M.; Turianica, I.; Hlusek, J. Content of phenolic compounds and free polyamines in black chokeberry (*Aronia melanocarpa*) after application of polyamine biosynthesis regulators. *J. Agric. Food Chem.* **2006**, *54*, 3625–3628.
(11) Hudec, J.; Kochanova, R.; Burdova, M.; Kobida, L.; Kogan, G.; Turianica, I.; Chlebo, P.; Hanackova, E.; Slamka, P. Regulation of the phenolic profile of berries can increase their antioxidant activity. *J. Agric. Food Chem.* **2009**, *57*, 2022–2029.
(12) Hudec, J.; Burdova, M.; Kobida, L.; Komora, L.; Macho, V.; Kogan, G.; Turianica, I.; Kochanova, R.; Lozek, O.; Haban, M.; Chlebo, P. Antioxidant capacity changes and phenolic profile of *Echinacea purpurea*, nettle (*Urtica dioica* L.), and dandelion (*Taraxacum officinale*) after application of polyamine and phenolic biosynthesis regulators. *J. Agric. Food Chem.* **2007**, *55*, 5689–5696.
(13) Barnes, J.; Anderson, L. A.; Gibbons, S.; Phillipson, J. D. *Echinacea* species (*Echinacea angustifolia* (DC.) hell., *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. *J. Pharm. Pharmacol.* **2005**, *57*, 929–954.
(14) Dalby-Brown, L.; Barsett, H.; Landbo, A. K.; Meyer, A. S.; Molgaard, P. Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on in vitro oxidation of human low-density lipoproteins. *J. Agric. Food Chem.* **2005**, *53*, 9413–9423.
(15) Vrchotova, N.; Kuzel, S.; Triska, J.; Kolar, L.; Totusek, J. Extraction and analysis of phenolic compounds from *Echinacea purpurea* (L.) Moench. *Chem. Listy* **2002**, *96*, 636–639.
(16) Binns, S. E.; Livesey, J. F.; Arnason, J. T.; Baum, B. R. Phytochemical variation in *Echinacea* from roots and flowerheads of wild and cultivated populations. *J. Agric. Food Chem.* **2002**, *50*, 3673–3687.
(17) Deschamps, C.; Raskin, I.; Simon, J. E. Regulation of essential oil accumulation in basil (*Ocimum basilicum* L.) in response to elicitation. *Int. J. Plant Sci.* (*Chicago, IL*) **2008**, *169*, 981–986.
(18) Zheng, Y.; Dixon, M.; Saxena, P. Greenhouse production of *Echinacea purpurea* (L.) and *E. angustifolia* using different growing media, NO_3^-/NH_4^+ ratios and watering regimes. *Can. J. Plant Sci.* **2006**, *86*, 809–815.
(19) Zheng, Y.; Jones, M. P. A.; Dixon, M. A.; Saxena, P. K. Conventional and emerging cultivation strategies for medicinal *Echinacea* production. In *Advances in Medicinal Plant Research*; Acharya, S. N., Thomas, J. E., Eds.; Research Signpost: Kerala, India, 2007; pp 513−528.
(20) Zheng, Y.; Dixon, M.; Saxena, P. K. Growing environment and nutrient availability affect the content of some phenolic compounds in *Echinacea purpurea* and *Echinacea angustifolia*. *Planta Med.* **2006**, *72*, 1407–1414.
(21) Kirakosyan, A.; Kaufman, P. B.; Chang, S. C.; Warber, S.; Bolling, S.; Vardapetyan, H. Regulation of isoflavone production in hydroponically grown *Pueraria montana* (kudzu) by cork pieces, XAD-4, and methyl jasmonate. *Plant Cell Rep.* **2006**, *25*, 1387–1391.
(22) Kneer, R.; Poulev, A. A.; Olesinski, A.; Raskin, I. Characterization of the elicitor-induced biosynthesis and secretion of genistein from roots of *Lupinus luteus* L. *J. Exp. Bot.* **1999**, *50*, 1553–1559.
(23) Shotipruk, A.; Wang, H. Y.; Kaufman, P. B. Production of secondary metabolites of medicinal value from plants using a two-stage programmable photobioreactor. *Recent Prog. Med. Plants* **2004**, *4*, 225–236.
(24) Smetanska, I.; Krumbein, A.; Schreiner, M.; Knorr, D. Influence of salicylic acid and methyl jasmonate on glucosinolate levels in turnip. *J. Hortic. Sci. Biotechnol.* **2007**, *82*, 690–694.
(25) Koroch, A.; Juliani, H. R.; Kapteyn, J.; Simon, J. E. In vitro regeneration of *Echinacea purpurea* from leaf explants. *Plant Cell, Tissue Organ Cult.* **2002**, *69*, 79–83.
(26) Abbasi, B. H.; Saxena, P. K.; Murch, S. J.; Liu, C. Z. *Echinacea* biotechnology: challenges and opportunities. *In Vitro Cell. Dev. Biol.: Plant* **2007**, *43*, 481–492.
(27) Pan, Z. G.; Liu, C. Z.; Zobayed, S. M. A.; Saxena, P. K. Plant regeneration from mesophyll protoplasts of *Echinacea purpurea*. *Plant Cell, Tissue Organ Cult.* **2004**, *77*, 251–255.
(28) Zobayed, S. M. A.; Saxena, P. K. In vitro regeneration of *Echinacea purpurea* L.: enhancement of somatic embryogenesis by indolebutyric acid and dark pre-incubation. *In Vitro Cell. Dev. Biol.: Plant* **2003**, *39*, 605–612.
(29) Pais, I. The biological importance of titanium. *J. Plant Nutr.* **1983**, *6*, 3–131.
(30) Hruby, M.; Cigler, P.; Kuzel, S. Contribution to understanding the mechanism of titanium action in plant. *J. Plant Nutr.* **2002**, *25*, 577–598.
(31) Kuzel, S.; Hruby, M.; Cigler, P.; Tlustos, P.; Van, P. N. Mechanism of physiological effects of titanium leaf sprays on plants grown on soil. *Biol. Trace Elem. Res.* **2003**, *91*, 179–189.
(32) Tlustos, P.; Cigler, P.; Hruby, M.; Kuzel, S.; Szakova, J.; Balik, J. The role of titanium in biomass production and its influence on essential elements' contents in field growing crops. *Plant, Soil Environ.* **2005**, *51*, 19–25.
(33) Kuzel, S.; Cigler, P.; Hruby, M.; Vydra, J.; Pavlikova, D.; Tlustos, P. The effect of simultaneous magnesium application on the biological effects of titanium. *Plant, Soil Environ.* **2007**, *53*, 16–23.
(34) Stebbing, A. R. A theory for growth hormesis. *Mutat. Res.* **1998**, *403*, 249–258.
(35) Heil, M.; Ton, J. Long-distance signalling in plant defence. *Trends Plant Sci.* **2008**, *13*, 264–272.
(36) Gray, D. E.; Pallardy, S. G.; Garrett, H. E.; Rottinghaus, G. E. Acute drought stress and plant age effects on alkamide and phenolic acid content in purple coneflower roots. *Planta Med.* **2003**, *69*, 50–55.
(37) Montanari, M.; Degl'Innocenti, E.; Maggini, R.; Pacifici, S.; Pardossi, A.; Guidi, L. Effect of nitrate fertilization and saline stress on the contents of active constituents of *Echinacea angustifolia* DC. *Food Chem.* **2007**, *107*, 1461–1466.
(38) Kolar, L.; Ledvina, L.; Kuzel, S.; Pasek, J. The effect of nitrogen surplus in fertilizing rates applied to *Echinacea purpurea* on the production of its active substances. *Rostl. Vyroba* **1998**, *44*, 489–495.
(39) Li, W. W.; Barz, W. Structure and accumulation of phenolics in elicited *Echinacea purpurea* cell cultures. *Planta Med.* **2006**, *72*, 248–254.
(40) Wu, C. H.; Tewari, R. K.; Hahn, E. J.; Paek, K. Y. Nitric oxide elicitation induces the accumulation of secondary metabolites and antioxidant defense in adventitious roots of *Echinacea purpurea*. *J. Plant Biol.* **2007**, *50*, 636–643.
(41) Liu, C. Z.; Abbasi, B. H.; Gao, M.; Murch, S. J.; Saxena, P. K. Caffeic acid derivatives production by hairy root cultures of *Echinacea purpurea*. *J. Agric. Food Chem.* **2006**, *54*, 8456–8460.

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