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Establishment of *in vitro* tissue cultures from *Echinacea angustifolia* D.C. adult plants for the production of phytochemical compounds

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ABSTRACT

The establishment of *in vitro* cultures of *Echinacea angustifolia* D.C. was obtained directly from sections of flower stalks of adult plants. The shoot formation was obtained from this plant material placed on a modified MS basal medium named CH supplemented with 0.5 mg L^{-1} 6-benzylaminopurine (BA). The *in vitro* propagation procedure of *E. angustifolia* consisted of three distinct phases: an initial regeneration phase from stalk sections (IP shoots on basal medium with 0.25 mg L^{-1} BA), an elongation phase on active charcoal and an axillary proliferation of the shoots (AP shoots on basal medium with 0.5 mg L^{-1} BA).

Regenerating calli were established from leaves of *in vitro* shoots cultured on CH medium supplemented with 3 mg L^{-1} BA and 0.5 mg L^{-1} indole-3-butyric acid (IBA). Developed shoots from the callus cultures were subcultured on the CH medium with 0.5 mg L^{-1} BA (leaf regenerated shoots: LR shoots). The secondary metabolite content of the *in vitro* plant material was compared with that of the greenhouse growing plants. The quali-quantitative LC-DAD-ESI-MS analysis on the extracts from axillary proliferation shoots (AP shoots) showed significant production of caffeic acid derivatives while leaf callus and LR shoots, accumulated mainly alkamides. These results showed that the proper choice of the procedures for *in vitro* multiplication allowed us to obtain plant biomass able to produce the active compounds typical of *E. angustifolia* plants.

1. Introduction

The genus *Echinacea* belongs to the *Asteraceae* family and has nine species (McGregor, 1968). At present, only three species are used in phytotherapy: *Echinacea angustifolia* D.C. (De Candolle) var. *angustifolia* (syn. *Rudbeckia angustifolia L.*), *E. pallida* (Nutt.) Nutt. and *E. purpurea* (*L.*) Moench. *Echinacea* spp. are native of North America (McGregor, 1968) and belonged to the rich Pharmacopoeia of the native Americans, who used them for hundreds of years for infections, inflammations and insect bites (Lloyd, 1921).

The chemistry of *Echinacea* species is well known and caffeic acid derivatives, flavonoids, polyacetylenes, alkamides, pyrrolizidine alkaloids, polysaccharides and glycoproteins were isolated and characterized (Bauer and Foster, 1991; Bauer and Wagner, 1991; Bauer and Reminger, 1989; Bauer et al., 1989, 1988a).

In the last few years, the increased demand of natural remedies in Europe has caused an enhancing industrial request in the production of standardized plant material and extracts.

Echinacea is an Extra European genus and the certified plant material for propagation of *E. angustifolia* is not available yet (Li, 1998). Moreover, plants of the genus *Echinacea* are characterized by their difficult germination caused mainly by seed dormancy (Baskin et al., 1992; Macchia et al., 2001; Feghahati and Reese, 1994; Sari et al., 2001) and by elevated population variability among the species (Binns et al., 2002).

Progress in medicinal plant clonal propagation has been requested, especially for species such as *Echinacea* with an agricultural production not sufficient for the growing pharmaceutical industry demand. For this purpose, it is important to develop a reproducible protocol for cloning *E. angustifolia*.

Till now, the reports concerning the *in vitro* procedures for the establishment of *E. angustifolia*, describe protocols starting from seedling (Harbage, 2001; Lakshmanan et al., 2002) whereas there are no reports about the *E. angustifolia in vitro* regeneration using flower stalk sections as explant source.

Difficulties to promote *E. angustifolia* tissue cultures from selected adult plants were mainly caused by the rosette habit of this species. The short internodes and the vegetative apices located near the ground caused initial contaminations difficult to eradicate. The selection of important medicinal species is an

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essential step to improve the agronomic and pharmaceutical features in particular when a high phenotypic variability is present as in plant belonging to the *Echinacea* genus. Regarding the biosynthesis of the typical secondary metabolites, the presence of echinacoside, caffeic acid derivatives, and polysaccharides was shown only in cell suspension cultures from seedling tissues of *E. angustifolia* (Smith et al., 2002). Most of the works about the production of caffeic acid derivatives, alkamides and anthocyanins were carried on other species of the *Echinacea* genus cultivated *in vitro* (Schollhorn et al., 1993; Sicha et al., 1991; Luczkiewicz and Cisowski, 2001; Łuczkiewicz et al., 2002). Moreover, no data on the influence of different *in vitro* culture systems of *E. angustifolia* on secondary metabolite production are available in the literature. In this work *E. angustifolia in vitro* cultures were established from flower stalks of adult plants. Extractions and LC-DAD-ESI-MS analysis were performed in order to evaluate the main secondary metabolite production in shoots derived from two different *in vitro* regeneration protocols. Secondary metabolite content was compared with that of the greenhouse plant material harvested in reproductive phase.

2. Materials and methods

2.1. Plant material

Open field *E. angustifolia* mother plants were furnished by the Department of Agronomy of the University of Pisa. Plants were transferred to the greenhouse and conditioned (phase 0 of micropropagation process according with Debergh and Maene (1981) by spraying them twice with 0.10 g L^{-1} Benomyl fungicide (Du Pont Agricultural Products, Wilmington, Delaware, UK) every 15 days; the shoots were cut after an additional period of five days without treatments (Mensuali Sodi et al., 1997). Leaf explants and flower stalks were employed as starting material for tissue culture.

2.2. E. angustifolia tissue cultures from adult plants

Leaves and flower stalks, removed from mother plants, were subjected to a first washing in tap water for 16 h. After reducing explant size, they were sterilized in a 15% of sodium hypochlorite (8% Cl active) aqueous solution stirred for 15 min followed by three final rinses in sterile distilled water. Under laminar flow cabinet the flower stalks were cut in slices 1–2 mm thick and the leaf explant were cut in portions of 0.25 cm^2 containing the central vein. To the basal medium, named CH, which consisted in MS (Murashige and Skoog, 1962) macro and micro elements, B5 vitamins (Gamborg et al., 1968), 300 mg L^{-1} reduced gluthatione (GSH), 500 mg L^{-1} 2-(N-morpholino) ethanesulfonic acid (MES), 30 g L^{-1}sucrose, 7 g L^{-1} agar, pH 5.8, two arrangements of growth regulators were added: 0.01 mg L^{-1} 1-naphthaleneacetic acid (NAA) plus 1 mg L^{-1} 6-benzylaminopurine (BA) or 0.5 mg L^{-1} BA alone. Both media were added with 0.3% of *Plant Preservative Mixture*, Plant Cell Technology Inc., U.S.A. (PPM). Each type of explants was placed in a climatic chamber 25 ± 1 °C either in the darkness or at 16 h of photoperiod with irradiance of 50 μmol s^{-1} m^{-2}.

Callus formation and shoot regeneration were detected after three weeks of cultures. *E. angustifolia* regenerated shoots were subcultured in vessels containing CH medium with 0.25 mg L^{-1} BA (initial proliferating shoots: IP shoots) and then with 0.5 mg L^{-1} BA (axillary proliferating shoots: AP shoots). Between the two cultures on BA enriched media, it was inserted an another one on a hormones-free CH medium with half mineral and vitamins strength, 15 g L^{-1} sucrose and 5 g L^{-1} active charcoal.

To study a different protocol for shoot regeneration, leaves from *in vitro* growing shoots were excised and explants (0.5 cm^2) were cut from the middle area of the lamina (four pieces/leaf). Two different culture media were used named CHe and CHe* containing basal medium CH added with 3 mg L^{-1} BA/0.5 mg L^{-1} indole-3-butyric acid (IBA) or 6 mg L^{-1} BA 1 mg L^{-1} IBA, under light conditions. Regenerated buds from this experiment were then proliferated on the same basal medium added with 0.5 mg L^{-1} BA (LR shoots).

All media tested in these experiments were sterilized by autoclaving at 121 °C at 1 atm for 20 min. *In vitro* cultures were maintained in a growth chamber at 22 ± 1 °C with an irradiance of 80 μmol s^{-1} m^{-2} and photoperiod of 16 h.

2.3. E. angustifolia greenhouse plants

E. angustifolia D.C. achenes were obtained from Gargini Sementi S.n.c. (Lucca, Italy). Achenes were sowed in Petri dishes and incubated at 25 ± 1 °C with a 16 h photoperiod (cool white fluorescent light 70 μmol m^{-2} s^{-1}). To overcome seed dormancy the inoculated achenes were previously subjected to stratification at 4 °C in the dark for 11 days in the presence of 1 mM ethephon (2-chloroethylphosphonic acid) (Macchia et al., 2001). After germination seedlings were transplanted in multi-pots containing pit-perlite soil (50:50, v:v) under greenhouse conditions. Leaf samples for extraction were collected at the beginning of the flowering period.

2.4. Plant experiments and statistical analysis

Explants used for shoot induction from adult plants were positioned in Petri Ø 6 cm dishes (5 explants/dish, 10 dishes/treatment). During the proliferation and growing phase explants were subcultured into 175 mL glass culture vessels (5 explants/vessel; 10 vessels/treatment) and in G7 Magenta vessels (6 explants/vessel; 5 vessels/treatment). *In vitro* leaf segments to induce shoot regeneration were positioned in Petri Ø 6 cm dishes (5 explants/dish, 10 dishes/treatment). Shoot number per explant and length during the proliferation stages were expressed as mean ± standard error. ANOVA statistical analysis ($P \leq 0.05$) was performed and the Tukey test was used to separate means calculated for the different parameters.

All the experiments were repeated twice and data were recorded after three weeks of culture.

2.5. Phytochemical investigation

2.5.1. Chemicals

LC grade water, acetonitrile, methanol and formic acid (Backer) were used for LC-DAD-MS Liquid Chromatography Diode Array Detector Electrospray Ionization Mass analysis. Commercial compounds were used as some reference materials: caftaric acid (**1**) (10 mg, ChromaDex, lot: 01-03028-301), chlorogenic acid (**2**) (10 mg, Extrasynthese, lot: 327-97-9), echinacoside (**3**) (10 mg, ChromaDex, lot: 01-05020-101), cichoric acid (**4**) (10 mg, ChromaDex, lot: 00-03640-300) and, caffeic acid (**5**) (10 mg, Sigma–Aldrich, lot: 60018). The flavonoids [quercetin (**7**), luteolin (**8**), apigenin (**9**), kaempferol (**10**), *p*-cumaric acid (**11**), betulinic acid (**12**), apigenin 7-*O*-β-glucoside (**13**), isorhamnetin 3-rutinoside (**14**)] used as standard compounds were part of a home-made database of natural compounds, isolated and identified by NMR and MS experiments in our laboratory (HPLC purity grade 97–98%). Alkamide (**6**) [dodeca-2E,4E,8Z,10E-tetraenoic acid isobuthylamide] was isolated and characterized during a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var. *bravado*) cultivated in Sanremo (Italy). Its spectroscopic data are in good agreement with that reported in the literature for the same compounds.

2.5.2. Sample preparation and LC-DAD-ESI-MS analysis

The aerial parts (1.3 g) of the *E. angustifolia* plant material were freeze-dried and extracted by ultrasonic apparatus with *n*-hexane and methanol in turn (100 mL × 2 h, 3 times).

Each fraction was analysed by LC-DAD-ESI-MS. LC system consisted of a Surveyor Thermofinnigan liquid chromatograph pump equipped with an analytical Lichrosorb RP-18 column (250 mm × 4.6 mm i.d., 5 mm, Merck), a Thermofinnigan Photodiode Array Detector and an ion trap LCQ Advantage mass spectrometer. The analyses were carried out by a linear gradient using water with 0.1% HCOOH (solvent A), CH_3CN (solvent B) from 10:90 (v/v) (B–A) to 70:30 (15 min) (flow 0.7 mL/min, run time 40 min). The spectral data from the DAD detector were collected during the whole run in the range 210–700 nm and the peaks were detected at 254 (alkamides, flavonoids) and 330 nm (caffeolquinic derivatives, flavonoids) for all analysed samples.

LC-ESI-MS analyses [negative ion mode for caffeoilquinic derivatives (**1**–**5**) and flavonoids (**7**–**14**), positive mode for alkamide (**6**)], SRM: Selected Reaction Monitoring, TIC: Total Ion Current (*m*/*z* 100 to 800 amu) were performed in the same chromatographic conditions using the specific ESI values for caffeic acid and alkamides (sheath gas flow-rate 62 arbitrary units, auxiliary gas flow 9 arbitrary units, capillary voltage −16 V and capillary temperature 280 °C) (Yasuda et al., 1981).

3. Results

3.1. In vitro shoots from adult plants of E. angustifolia

The contamination of *E. angustifolia* explants was a serious problem consuming time to establish a protocol for the mother plant management to pull down the micro flora of these plants. Several treatments with fungicides on the *in vivo* mother plants and the presence of the biocide PPM in the culture medium gave 40% of non-contaminated explants, which was sufficient to start the culture. Callus formation was induced from leaf explants cultured on the tested media in light and dark condition, but only the white friable calli developed on the NAA with BA medium in darkness showed shoot regeneration (Table 1). However these calli, during the successive subculture, stopped to provide shoot regeneration at all (data not shown). The flower stalk slices give rise to cell proliferation when cultured on 0.5 mg L^{-1} BA in the light (Table 1 and Fig. 1A). These green calli showed purple spots which generally matched the outset of the shoots so they could be considered as visible markers of the regeneration process.

The new shoots developed in the light from flower stalk slices placed on 0.5 mg L^{-1} BA medium were subcultured on the same medium. During this first culture phase, hyperhydricity occurred in new shoots. To reduce this phenomenon, the shoots were subcultured in the same medium with half BA amount (0.25 mg L^{-1}) (initial proliferating shoots: IP shoots) (Table 2), but the successive subcultures showed a progressive deterioration of the culture with abundant callus masses at the base of the shoots which encountered hyperhydricity again. The successive culture on the hormone-free medium with active charcoal, reduced the callus formation, decreased the shoot number (Table 2) and therefore increased the shoot quality. Subsequently *E. angustifolia* shoots were cultured again on the initial medium with 0.5 mg L^{-1} BA. During this multiplication phase the explants produced new shoots (axillary proliferating shoots: AP shoots) longer than IP shoots (Table 2 and Fig. 1B) and the shoot quality was maintained without the developing of hyperhydric symptoms and callus formations. Interestingly, during this propagation stages both IP and AP shoots showed a recurrent regeneration of *de novo* shoots and ectopic leaf formations from the central vein of the intact leaves (Fig. 1C).

A high morphogenetic potential was also observed when leaf sections excised from AP shoots were cultured on CHe and CHe* media. From the results summarized in Table 3 it was detected that CHe medium with BA 3 mg L^{-1} and IBA 0.5 mg L^{-1} produced a high percentage of callus with vegetative buds and with purple spots which could be considered "differentiation spots" as observed also during the induction phase on flower stalk sections (Fig. 1D). Growth regulators in a double concentration (CHe*) did not improve the callus formation and the buds differentiated from this type of callus did not develop into normal shoots (data not shown). Developed shoots from the CHe callus cultures were subcultured on the same basal medium with 0.5 mg L^{-1} BA (leaf regenerated shoots: LR shoots). The results described in Table 3 demonstrated that shoot regenerated from *in vitro* leaves could provide a good multiplication rate (one leaf could provide up to 60 new shoots). These type of shoots continued to proliferate morphogenetic callus at their basal end which could be a useful source to increase the further *E. angustifolia* shoot proliferation.

3.2. Phytochemical analysis

Echinacea plant material, listed below, was investigated for the production of secondary metabolites:

- Shoots from flower stalk of *E. angustifolia* plants and collected in different culture phases (IP shoots and AP shoots);
- Regenerating calli from *in vitro* shoot leaves;
- Shoot developed from the regenerating calli (LR shoots);
- Leaves from greenhouse plants collected at the beginning of the flowering period (GH plants).

The *n*-hexane and methanolic extracts were obtained by ultrasonic apparatus from fresh plant material after freeze-drying. LC-DAD-ESI-MS analyses were performed in order to evaluate the production of alkamides, flavonoids and caffeolquinic derivatives.

The phytochemical screening was carried out by the comparison of the retention time, UV and MS spectra for each peak in the

Table 1
Influence of the plant growth regulators (PGR), explant source (flower stalk or leaf) and growth conditions (light or dark) on the callus formation and the initial regeneration tendency (shoot number per explant) of *E. angustifolia* adult plants. Data are presented as coefficient (+ or −) or means ± SE (*n* = 50).

	Callus amount*		Callus quality		Shoot number	
	Dark	Light	Dark	Light	Dark	Light
Flower stalk						
BA	–	+++	–	Green, purple spots	–	2.83 ± 0.45
NAA + BA	–	–	–	–	–	–
Leaf						
BA	++	++	Necrotic	Necrotic	–	–
NAA + BA	++	++	White, friable	Necrotic	1.67 ± 0.33	–

* Callus amount; + = scarcely developed at the explant margins, ++ = medium developed covering half of the explant, +++ = largely developed covering all the explant.

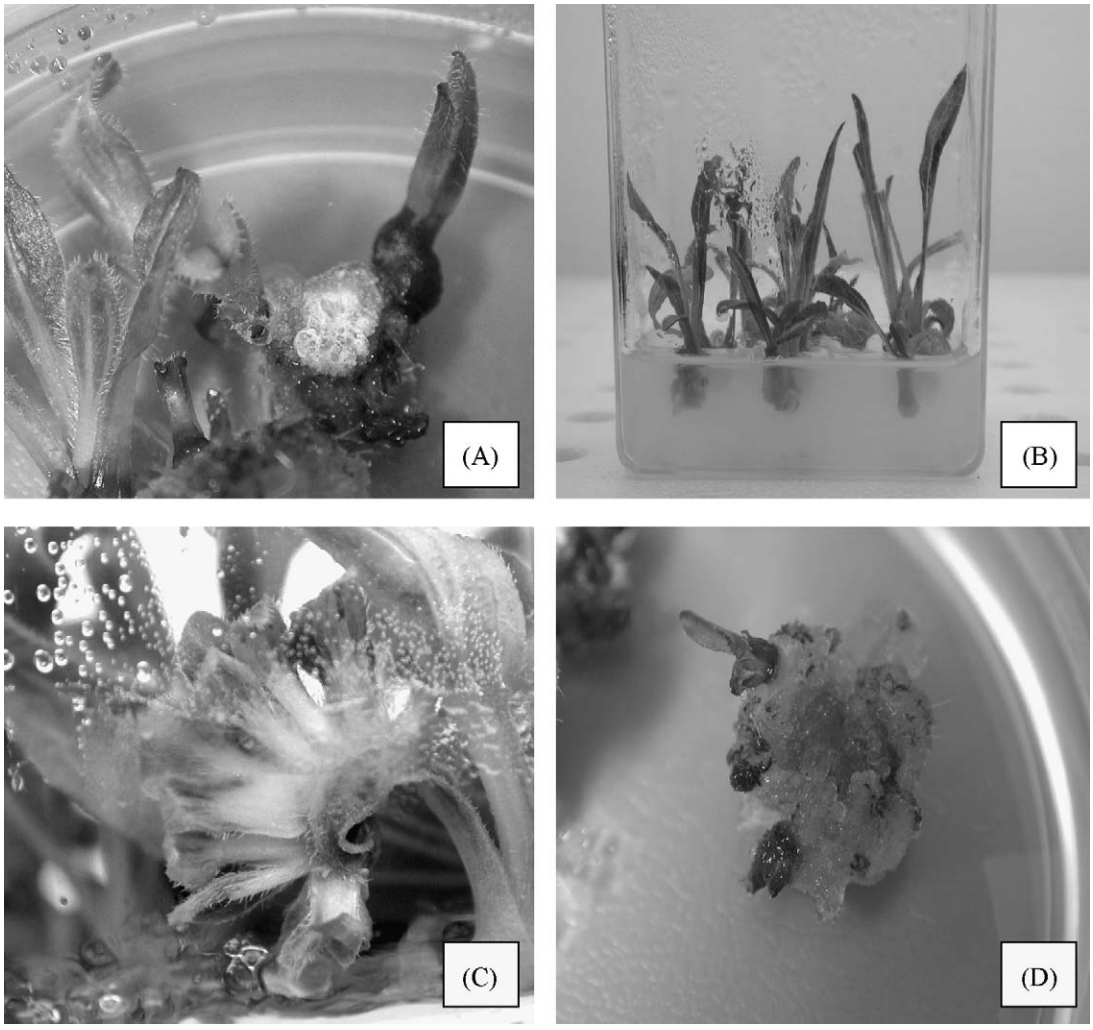

Fig. 1. (A) *E. angustifolia* flower stalk slice which gave rise to cell proliferation and shoot regeneration when cultured on CH basal medium with 0.5 mg L^{-1} BA in the light. (B) *E. angustifolia* axillary proliferating shoots (AP shoots) during the propagation phase on CH basal medium with 0.5 mg L^{-1} BA. (C) *E. angustifolia* shoot regeneration from the leaf central vein. Shoots in proliferation were cultured on CH basal medium with 0.5 mg L^{-1} BA. (D) *E. angustifolia* leaf segment of *in vitro* shoots cultured on the CH basal medium with BA (3 mg L^{-1}) and IBA (0.5 mg L^{-1}) named CHe. This explant showed several buds arisen from the callus mass.

Table 2
Shoot proliferation (shoot number and length of new formed shoots), and callus development at the base of *E. angustifolia* shoots in different phases of the propagation process: initial proliferating shoots (IP shoots), hormone-free shoots and axillary proliferating shoots (AP shoots). The basal medium was added with different BA concentrations. ANOVA statistical analysis ($P \leq 0.05$) was performed. Mean values ($n = 50$) the different parameters were separated by the Tukey test and different letters corresponded to significant differences ($P \leq 0.05$).

	Shoot number	Shoot length (cm)	Callus amount*	Callus quality
BA (0.25 mg L^{-1}) (IP shoots)	2.36 a	1.31 a	+/++	Friable light green
Hormone-free (active charcoal)	1.10 b	1.63 ab	+	Compact green
BA (0.5 mg L^{-1}) (AP shoots)	1.77 ab	1.66 b	+	Compact green

* Callus amount; + = scarcely, ++ = medium, +++ = largely.

extract samples with those of the reference compounds (**1**–**14**). The results showed that the flavonoids (**7**–**14**) were not present in the extracts coming from *E. angustifolia in vitro* plant material and in plants grown in the greenhouse. A summary of the quantitative results ($\mu g/g_{dry\ plant\ material}$) for the selected constituents (**1**–**6**) in the analysed samples is given in Table 4.

A smaller amount of alkamide (**6**) was accumulated in GH plants in comparison with the *in vitro* grown material. In detail it

Table 3
Callus formation and shoot regeneration (shoot number per explant and length) of *in vitro E. angustifolia* leaf portions on CH basal medium with different amounts of growth regulators (CHe and CHe*) and proliferation of shoots derived from CHe callus on basal medium with 0.5 mg L^{-1} BA (LR shoots). Data are presented as means ± SE ($n = 50$).

	Shoot number	Shoot length (cm)	Callus amount*	Callus quality
CHe (3 mg L^{-1} BA + 0.5 mg L^{-1} IBA)	3.98 ± 0.69	0.54 ± 0.10	+++	Friable white + purple spots
CHe* (6 mg L^{-1} BA + 1 mg L^{-1} IBA)	2.72 ± 1.57	0.42 ± 0.08	+++	Friable white
0.5 mg L^{-1} BA (LR shoots)	3.60 ± 0.54	2.65 ± 0.36	+	Friable at the shoot base

* Callus amount; + = scarcely, ++ = medium, +++ = largely.

Table 4
LC-DAD-ESI-MS analysis of the aerial part of *E. angustifolia in vitro* and *in vivo* plant material. IP: Initial proliferating shoots; AP: axillary proliferating shoots; LR: leaf regenerated shoots; GH plants: plants cultivated in greenhouse. Standard error ($n = 3$).

Compounds	*In vitro*				*In vivo*
($\mu g/g_{dry\ plant}$)	IP shoots	AP shoots	Callus	LR shoots	GH plants
Caftaric acid (**1**)	16.54 ± 0.17	2551.5 ± 33.6	–	–	4283.7 ± 24.3
Chlorogenic acid (**2**)	54.61 ± 2.11	11230.5 ± 145.5	–	–	1176.4 ± 6.5
Echinacoside (**3**)	47.34 ± 0.98	5813.3 ± 53.7	–	–	5991.1 ± 46.1
Cichoric acid (**4**)	46.13 ± 1.23	30530.8 ± 456.0	–	–	1534.3 ±10.6
Caffeic acid (**5**)	4.11 ± 0.09	–	–	16.78 ± 0.16	116.4 ± 2.45
Alkamide (**6**)	235.63 ± 9.86	107.35 ± 3.39	103.7 ± 0.36	367.95 ± 10.75	26.83 ± 0.94

was detected as the unique metabolite in callus cultures and as the main constituent in the *n*-hexane extracts of *in vitro* IP shoots and LR shoots. Caffeic acid derivatives (**1**, **2**, **3**, **4**) completely lacked in callus and LR shoot samples.

Phenolic acids (**1**, **2**, **4**, **5**) and echinacoside (**3**) were produced in much higher amounts in comparison with the alkamide (**6**) in the AP shoots and *in vivo* GH plants. In particular the AP shoot samples were characterized by a larger amount of cichoric acid (**4**) than GH plant samples but they did not show any detectable content of caffeic acid (**5**). Echinacoside (**3**) amount contained in AP shoots was comparable with that detected in GH plants.

4. Discussion

In this work it was settled up the active shoot organogenesis from flower stalks of *E. angustifolia* adult plants. The use of flower stalk explants could be very convenient for growers and herbal product companies to clone selected superior individuals for enhancing *E. angustifolia* cultivation. In fact this method can be applied without causing damage or completely destroying individual plants as it occurs when the apical buds are excised from the rosettes. Moreover, vegetative propagation from selected plants is a reliable procedure because chemical profiles varied significantly among seedlings within the species (Binns et al., 2002; Murch et al., 2006). Plant propagation from flower stalks was employed for *in vitro* regeneration of several species (Bajaj et al., 1983; Tan Nhut et al., 2001; Martin et al., 2005) but it is an unusual technique for tissue culture of *Asteraceae* plants. Shoot regeneration was induced on flower stalk explants exposed to light on a medium supplemented with 0.5 BA. This type of explant produced a satisfied shoot proliferation in comparison with those observed on *E. purpurea* petioles and leaves (Choffe et al., 2000; Koroch et al., 2002). However, after the first subcultures *E. angustifolia* shoots showed a high hyperhydricity, the development of abundant callus and *de novo* shoot formations on the veins of the intact leaves (Fig. 1C) as also observed by Lakshmanan et al. (2002) on *in vitro* seedling leaves of different *Echinacea* species. Therefore, in order to obtain *E. angustifolia* plantlets more suitable to produce continuous multiple shoots, the BA content was halved. The procedure gave a temporary improvement of the culture (IP shoots), but the successive subcultures on the same medium showed again a progressive deterioration. A mid-step culture phase without growth regulators and using active charcoal (Debergh and Maene, 1981) was necessary to restore the shoot quality. These plantlets, cultured again on 0.5 mg L^{-1} BA CH basal medium, gave appropriate explants for the successive proliferation phases (AP shoots). Summarizing, the *in vitro* propagation procedure of *E. angustifolia* from flower stalk consisted of three distinct phases: an initial regeneration phase from stalk sections (IP shoots), an elongation phase on active charcoal and an axillary proliferation of the shoots (AP shoots).

On the basis of the high organogenetic potential observed in shoot foliage during the initial phase of the culture, another regeneration pattern was investigated starting from AP leaf explants. Zobayed and Saxena (2003) observed somatic embryogenesis from leaves of *E. purpurea in vitro* seedling when IBA (0.5 mg L^{-1}) was used together with BA (2.5 mg L^{-1}). In this work an analogous combination of IBA and BA added to the CH basal medium was useful to produce conspicuous callus biomass with vegetative buds. This *in vitro* plant material successively developed shoots (LR shoots).

Concerning to the analysis of the active constituents, LC-DAD-ESI-MS screening was carried out on the *in vitro* and *in vivo* plant material to evaluate the production of the typical antioxidant substances (**1**–**14**) of *Echinacea* species. The results showed that none of the well-known flavonoids (**7**–**14**) of *Echinacea* adult plants were present in the extracts coming from *in vitro* plant material as well as in plants grown in the greenhouse (Bauer and Wagner, 1991; Lin et al., 2002). This behaviour is similar to that observed in *Genista* spp. where the typical flavonoids (apigenin, luteolin) were not detectable in callus cultures (Łuczkiewicz and Głód, 2003) of this species. The lack of flavonoids could be related to the light conditions in the growth environments. It is well known that flavonoids are UV-B-inducible (Cockell and Knowland, 1999) while the lamps used in the *in vitro* growth chamber did not provide wavelengths in the range of the UV radiation. An analogous behaviour was showed in callus cultures of *Passiflora* spp. where the UV-B irradiation was able to increase the production of flavonoids (Antognoli et al., 2007). The *Echinacea* plants grown under greenhouse, were subjected to light conditions similar to the *in vitro* ones since greenhouse covering materials screen UV radiations below 340 nm (Nelson, 1991).

The phytochemical profiles of the *E. angustifolia* (caffeic acid derivatives and alkamides) showed differences among the *in vitro* (IP, AP, LR shoots and callus) and *in vivo* plantlets (GH). Also for other plant species it was often observed that secondary metabolite patterns changed with the introduction of plant material into *in vitro* conditions (Łuczkiewicz and Głód, 2003, 2005).

The main difference regarded the alkamide content of *E. angustifolia in vitro* cultures. A larger amount of alkamide (**6**) was accumulated in cultured tissues in comparison with the leaves of plants grown in greenhouse (GH). The regenerated shoots (IP, AP, and LR) produced alkamide (**6**) amounts (0.023, 0.017 and 0.036%, respectively) comparable to the typical content of wild *E. angustifolia* leaves and stalks (Bauer and Wagner, 1991; Bauer, 1998). As reported in the literature regarding to the localization of alkamides in the aerial portion of *Echinacea* plants, the reproductive stems of *E. purpurea* showed alkamide contents much higher than the leaves (Perry et al., 1997). In this study the reproductive origin (flower stalk explants) of IP, AP and LR shoots might explain the presence of the higher alkamide content than in GH plants. On the other hand, the presence of alkamides could be discussed on the basis of their potential roles in plant morphogenesis. López-Bucio

et al. (2006) demonstrated that alkamides regulate many aspects of plant development by altering cell division and differentiation process but these compounds required cytokinin receptors for normal cellular response. Exogenous treatments of *Arabidopsis in vitro* plantlets with alkamides alter cell determination, leading to the production of ectopic leaf blades along the petioles (López-Bucio et al., 2007). In our experiments a high level of alkamides was present in BA-induced shoots which frequently showed the formation of similar ectopic leaves. This phenomenon was ascribed by Lakshaman et al. (2002) to the accumulation of cytokinines but on the basis of these results, it might be suggested a possible interaction of plant hormones with alkamides on the differentiation processes. The high levels of these compounds found in *E. angustifolia* callus and LR shoots, might be ascribed to the high cytokinin content (3 mg L^{-1}) used for the callus culture induction. The hormonal composition of the culture media is known to be one of the main causes of morphological and physiological modification in regenerated plantlets (Van Staden et al., 2006) and in turn it could alter the plant biomass capacity to produce secondary metabolites (Patnaik et al., 1999).

Regarding to the caffeic acid derivatives, the *in vitro* shoots in the axillary proliferation phase (AP shoots) showed production of caftaric, chlorogenic, and cichoric acids, together with echinacoside but no caffeic acid was evidenced. The yields of these secondary metabolites were the highest among the *in vitro* cultures and they were similar or higher than those reported in the literature for the leaves of *E. angustifolia* adult plants (Bauer and Wagner, 1991; Bauer, 1998; Pacifici et al., 2007). AP shoots were well developed and showed phenotypic features similar to the *in vivo* plants. This might be due to the appropriate *in vitro* mixotrophic condition where the shoots could take advantage of the high sucrose availability in the culture medium and to the possibility to perform photosynthesis (data not show). It was noted that a relationship occurs between different tissue culture systems and the accumulation of secondary metabolites. In particular, Murch et al. (2006) detected bioactive compounds in well developed shoots growing in a temporary immersion system. On the contrary, they did not observe significant productions of secondary metabolites in *E. purpurea* callus, shoot or embryo. In the same way also in *Hypericum perforatum in vitro* cultures, the hypericin and flavonoid production increase during shoot development from callus with vegetative buds to plantlets without roots (Pasqua et al., 2003). These considerations pointed out that the regeneration process chosen to amplify the *in vitro* biomass not always could be right to give the secondary metabolites of interest.

In conclusion, this is the first report on caffeic acid derivative and alkamide production from *in vitro* regenerated shoots of *E. angustifolia* adult plants.

It was underlined how the *in vitro* production of *E. angustifolia* plantlets from adult plants and the development of the proper multiplication procedures allowed us to obtain plant biomass able to produce active compounds characteristic of this species.

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