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

* M. Lucchesini[1], A. Bertoli[2], A. Mensuali- Sodi[3], L.Pistelli[2]

[1] Dipartimento di Biologia delle Piante Agrarie, Università di Pisa, Viale delle Piagge 23, Pisa, Italy

[2] Dipartimento di Chimica Bioorganica e Biofarmacia, Università di Pisa , via Bonanno 33, Pisa, Italy

[3] Scuola Superiore di Studi e Perfezionamento Sant'Anna, piazza Martiri della Libertà 33, Pisa, Italy

* Corresponding author. Fax: +39 050 2216524 E-mail address: mlucchesini@agr.unipi.it

Abstract

The establishment of *in vitro* cultures of *Echinacea angustifolia* was obtained directly from section of flower stalks of adult plants. The vegetative propagation was obtained directly from section of flower stalks placed on a mineral MS basal medium with B5 vitamins supplemented with 0.5 mgL^{-1} 6-benzylaminopurine (BA) while callus regenerative masses were established from leaf sections cultured on the same basal medium supplemented with 3 mg L^{-1} BA and 0.5 mg L^{-1} indole-3-butyric acid (IBA). The secondary metabolite contents of shoots proliferating on different culture media and callus masses were compared with *in vitro* or *in vivo* seedlings. The quali-quantitative LC-DAD-ESI-MS analyses both on the *n*-hexanic and methanolic extracts demonstrated that significant production of caffeic acid derivatives, echinacoside and alkamides from different *in vitro E. angustifolia* tissues was possible. Choosing the appropriate plant material from different *in vitro* cultures, the plant metabolite pathway might be addressed towards the alkamides or the caffeic acid derivatives productions.

Keywords: alkamides; flavonoids; caffeic acid derivatives; *Echinacea angustifolia*; flower stalk; *in vitro* shoots; LC-DAD-ESI-MS.

Introduction

The genus *Echinacea* belongs to the *Asteraceae* family and has nine species (McGregor, 1968). At present, only three species are used in phytotherapy: *E. 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 had 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; Bauer et al., 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 Extraeuropean 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).

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 to cloning *E. angustifolia*.

Till now only few reports showed attempts concerning the *in vitro* procedures for the establishment of *E. angustifolia* clones from adult plants (Harbage, 2001; Lakshmanan et al., 2001).

However there are no reports of regeneration using flower stalk sections as an explant source for this species.

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. In this work a regeneration protocols from flower stalk explants was established. The use of this type of explants allowed to reduce contamination problems and could be linked to previous analysis of plant during their vegetative growth. The selection of important medicinal species is an essential step to improve the agronomic and pharmaceutical features in particular when a high phenotypic variability is present as in plant belonging to *Echinacea* family Regarding the biosynthesis of the typical secondary metabolites, the presence of echinacoside, caffeic acid derivatives, and polysaccharides were shown only in cell suspension cultures from seedling tissues of *E. angustifolia* (Smith *et al*., 2002), but most of the works about the production of caffeic acid derivatives, alkamides and anthocyanins were carried on mainly on extracts of other species of the *Echinacea* family cultivated *in vitro* (Schollhorn et al., 1993; Sicha et al., 1991 Luczkiewcz and Cisowski, 2001 Luczkiewicz et al. 2002). Moreover, no data on the influence of the origin of the *in vitro* plantlets and their multiplication over time on secondary metabolites are available in the literature.

In this work *E. angustifolia in vitro* cultures were established from adult plants. Extraction and LC-DAD-ESI-MS protocols were performed in order to evaluate the main secondary metabolites production in shoots collected in different phases of the *in vitro* culture. Secondary metabolites content was compared with that of greenhouse flowering plants and with *in vitro* germinated seedlings.

Deleted: ¶
¶

Materials and methods

Plant material

Open field *E. angustifolia* mother plants were furnished by the Department of Agronomy of the University of Pisa. Plants were transferred to a greenhouse and conditioned (phase 0 of micropropagation process according with Debergh and Maene (1981) by spraying them twice with 0.10 g/l 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.

Echinacea 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 hours then, 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 portions were excised containing the central vein (0.25 cm^2). Each type of explants was placed in a climatic chamber 25 ± 1°C either in the darkness or at 16 hours of photoperiod with irradiance of 50 µmol $sec^{-1}m^{-2}$. The culture medium employed was the basal medium, named CH, consisting of MS macro and micro elements, B5

113 Vitamines (Gamborg, 1968), 300 mg L^{-1} reduced Gluthatione (GSH), 500 mg L^{-1} 2-(N-
114 Morpholino) ethanesulfonic acid (MES), 30 g L^{-1}sucrose, 7 g L^{-1} agar, pH 5.8. Two
115 arrangements of growth regulators were used: 0.01 mg L^{-1} 1-naphtaleneacetic acid (NAA)
116 plus 1 mg L^{-1} BA, and 0.5 mg L^{-1} BA. Both media were added with 0.3% of *Plant*
117 *Preservative Mixture*, Plant Cell Technology Inc., U.S.A. (PPM).
118 *E. angustifolia* regenerated shoots were sequentially subcultured in vessels containing CH
119 medium with 0.25 mg L^{-1} (initial proliferating shoots: IP shoots) and 0.5 mg L^{-1} BA (axillary
120 proliferating shoots: AP shoots) interleaved by the CH medium with half mineral strength,
121 vitamins and hormones free, 15 g L^{-1} sucrose and 5 g L^{-1} active charcoal.
122 Leaves from *in vitro* shoots derived from flower stalk regeneration were excised and explants
123 (0.5 cm^2) were cut from the middle area of the lamina. Two different culture media were
124 used named CHe and CHe* containing basal medium CH added with 3 mg L^{-1} BA and 0.5
125 IBA or 6 mg L^{-1} BA 1 mg L^{-1} IBA respectively.
126 Regenerated shoot were subcultured on the same basal medium added with 0.5 mg L^{-1} BA in
127 Magenta vessels (LR).
128 All media tested in these experiments were sterilized by autoclaving at 121°C at 1 atm. for 20
129 min.
130 *In vitro* cultures were maintained in a growth chamber at 22 ± 1°C with an irradiance of 80
131 μmol sec^{-1} m^{-2} and photoperiod of 16 hours.
132
133 ***Echinacea angustifolia*** **greenhouse plants**
134 *Echinacea angustifolia D.C.* achenes were obtained from Gargini Sementi S.n.c. (Lucca,
135 Italy).Achenes were sowed in Petri dishes and incubated at 25 ±1°C with a 16 h photoperiod
136 (cool white fluorescent light 70 μmol m^{-2} s^{-1}). To overcome seed dormancy the inoculated
137 achenes were previously subjected to stratification at 4°C in the dark for 11 days in the
138 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 were collected at the beginning of the flowering period.

***Echinacea angustifolia* tissue cultures from seedlings.**

Seeds from the same source above cited, pre-treated to avoid seed dormancy with the same procedure above described, were sterilized by immersion in 70% ethanol for 30 s followed by soaking in 15% sodium hypochlorite solution (8% active chlorine) containing two drops of Tween 20® for 18 min and followed by three rinses in sterile water. Achenes were sowed on half strength inorganic basic nutrient MS (Murashige and Skoog, 1965), sucrose (15 g L^{-1}), agar (7 g L^{-1}), without vitamins or growth regulators. The medium was sterilized by autoclaving at 121°C at 1 atm. for 20 min. After the cold stratification period sowed seeds were transferred to a growth chamber and maintained at 25 ±1°C with a 16 h photoperiod (cool white fluorescent light 70 µmol m^{-2} s^{-1}).

To induce shoot proliferation, *in vitro* germinated seeds were deprived of the root system and placed on the basal medium CH and, to promote shoot proliferation, 0.5 mg L^{-1} BA was used. *In vitro* cultures were maintained in a growth chamber at 22 ± 1°C with an irradiance of 80 µmol sec^{-1} m^{-2} and photoperiod of 16 hours.

Plant Experiments and Statistical analysis

Explants used for shoot induction from adult plants were positioned in Petri ø 6 cm dishes (5 explant/dish, 5 dishes/treatment). During the proliferation and growing phase explants were subcultured into 175 ml glass culture vessels (5explants/vessel; 10 vessels/treatment) and in G7 Magenta vessels (6 explants/vessel; 5 vessels/treatment). Shoot number per explant and length during the proliferation were expressed as mean ± standard error.

All the experiments were repeated twice and all data were recorded after three weeks (one subcultures).

In vitro and *in vivo* germination data were recorded on the day 2, 4, and 6 after the pre-treatments which were used to avoid seed dormancy. Germination ability was expressed as germination percentages on the total seeds; mean germination time (MGT) was calculated as reported to Ellis e Roberts (1980):

$$\frac{\sum(t_i \cdot n_i)}{\sum n_i}$$

where t_i represents the day number from the root emission and n_i the seed number germinated in each time intervals (n=50; 5 seeds/Petri dishes). As regards shoot induction from aseptic seedlings, each explant was placed into a disposable 30 ml vial (n=25). Germination percentages, MGT, shoot number and length during the proliferation from *in vitro* seedling were recorded and reported as mean values ± Standard Error (SE).

Phytochemical investigation

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%).

189 Alkamide (**6**) [dodeca-2E,4E,8Z,10E-tetraenoic acid isobuthylamide] was isolated and
190 characterized during a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var.
191 *bravado*) cultivated in Sanremo (Italy) (Table 1).
192
193 **Sample preparation and LC-DAD-ESI-MS analysis**
194 The aerial parts (1.3 g) of the *E.angustifolia* plant material were freeze-dried and extracted by
195 ultrasonic apparatus with *n*-exane and methanol in turn (100 ml x 2h, 3 times).
196 Each fraction was analysed by LC-DAD-ESI-MS. LC system consisted of a Surveyor
197 Thermofinnigan liquid chromatograph pump equipped with an analytical Lichrosorb RP-18
198 column (250 x 4.6 mm i.d., 5mm, Merck), a Thermofinnigan Photodiode Array Detector and
199 an ion trap LCQ Advantage mass spectrometer. The analyses were carried out by a linear
200 gradient using water with 0.1% HCOOH (solvent A), CH_3CN (solvent B) from 10:90 v/v (B-
201 A) to 70:30 (15 min) (flow 0.7 ml/min, run time 40 min). The spectral data from the DAD
202 detector were collected during the whole run in the range 210-700 nm and the peaks were
203 detected at 254 (alkamides, flavonoids) and 330 nm (caffeolquinic derivatives , flavonoids)
204 for all analysed samples.
205 LC-ESI-MS analyses [negative ion mode for caffeoilquinic derivatives (**1-5**) and flavonoids
206 (**7-14**), positive mode for alkamide (**6**)], SRM Selected Reaction Monitoring, TIC Total Ion
207 Current , m/z 100 to 800 amu) were performed in the same chromatographic conditions using
208 the specific ESI values for caffeic acid and alkamides (sheath gas flow-rate 62 arbitrary units,
209 auxiliary gas flow 9 arbitrary units, capillary voltage -16 V and capillary temperature 280°C).
210 The qualitative results of LC-DAD-ESI-MS were showed in Table 1.The amounts of the
211 compounds (**1-6**) were estimated by using a multilevel external standard procedure:

212 Caftaric acid (**1**)	Y = 1.0347 + 2.5974 E-05 X	r = 0.9977
213 Chlorogenic acid (**2**)	Y = 1.9601 + 1.6762 E-05 X	r = 0.9985
214 Echinacoside (**3**)	Y = 0.1024 + 6.5431 E-05X	r = 0.9966

Cichoric acid (**4**)	Y = 2.1424 + 1.2889 E-05 X	r = 0.9988
Caffeic acid (**5**)	Y = 1.7444 + 8.4818 E-06 X	r = 0.9994
Alkamide (**6**)	Y = 1.668 + 1.2277 E-05 X	r= 0.9978

Results

Echinacea angustifolia tissue cultures from adult plants

The contamination of *E. angustifolia* explants was a serious problem that required a lot of time consuming tentative 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 both tested media in the in light and darkness, but the callus developed on the BA medium was necrotic as that on the NAA with BA medium in the light. The flower stalk slices give rise to cell proliferation when cultured on 0.5 mg L^{-1} BA in the light (Table 2 and Fig. 1). These green calli showed purple spots which generally developed shoot primordial so they could be considered as markers of the regeneration process (Table 2). The shoots derived from these spots were subcultured on the same medium (Table 3) maintaining, at the end of the second subculture, their morphogenetic capacity (Table 3) with a sustained growth of regenerating callus. The leaf explants cultured on medium with NAA combined with BA in dark condition, developed a conspicuous white friable callus during the successive subculture but didn't show shoot regeneration at all (Table 3). During this induction phase, hyperhydricity occurred in the new developed 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 4), but the successive subcultures showed a progressive culture deterioration which encountered a high hyperhydricity again (Figure 2A).

241 The successive culture on medium with active charcoal t lacking growth regulators
242 determined the callus reduction at the shoot basal end, a drastic decrease of the shoot number
243 and an increase in shoot length and quality (Table 4). When hyperhydricity was reduced,
244 *Echinacea* shoots were cultured again on the initial medium with 0.5 mg l^{-1} BA. During this
245 multiplication phase the explants produced a low number of new axillary shoots (axillary
246 proliferating shoots: AP shoots) (Table 4 and Figure 2B). These shoots, not only showed
247 normal leaf features, no hyperhydric symptoms and less callus amount at the basal end but
248 also were able to regenerate, *de novo* shoots from the central vein of the intact leaves (Figure
249 3).
250 An analogous high morphogenetic potential was observed when leaf sections excised from
251 AP shoots were cultured.
252 From the results summarized in Table 5 we can notice that CHe medium including BA 3
253 mgL^{-1} and IBA 0.5 mgL^{-1} produced a high percentage of callus with purple spots which
254 could be considered "differentiation spots" as above described. Growth regulators in a double
255 concentration (CHe*) didn't improve the callus formation and shoot differentiation.
256 Regenerated shoots from the callus cultures on CHe medium were subcultured (Figure 4) on
257 the same basal medium CH with 0.5 mgL^{-1} BA (leaf regenerated shoots: LR shoots). The
258 results described in Table 5 demonstrated that shoot regeneration from *in vitro* growth leaves
259 could provide a good regeneration rate (1 leaf portion: 16 new shoots) useful to increase the
260 *E. angustifolia* shoot biomass.
261
262 ***Echinacea angustifolia* from seedlings**
263 To compare the phytochemical contents of *in vitro* cultures with those of tissues from *E.*
264 *angustifolia* propagated by seeds, *in vitro* and *in vivo* seedling cultivations were established.
265 For this purpose, germination was tested to provide the starting material for the greenhouse
266 and the *in vitro* cultivation. Pre-treatment with ethephon together with stratification of *E.*

angustifolia achenes was useful as demonstrated by the high percentage of germination (69.7± 4.03)and a low mean germination time (MGT, 2.47±0-11).

New shoots (Proliferating Seedling shoots: PS shoots) was obtained culturing *E. angustifolia* seedling explants on the CH basal culture medium with 0,5 mg/l BA (2,1 ± 0.3 number of shoots per explants with an average length of 1.72 ± 0.24cm).

Deleted: ¶

Phytochemical analysis

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

- Shoots from flower stalk of *E. angustifolia* plants and collected in the different culture phases (IP shoots, AP shoots and LR shoots);
- Shoots from *in vitro* germinated seedlings named Proliferated Seedlings (PS shoots);
- Leaves from green-house plants collected at the beginning of the flowering period named (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 extract samples with those of the reference compounds (**1-14**) (Table 1). A summary of the quantitative results (µg/g $_{\text{dry plant material}}$) for the selected constituents (**1-6**, Fig. 5) in the analysed samples is given in Table 7.

Alkamide (**6**) was detected as the main constituent in the *n*-hexane extracts of *in vitro* IP shoots and LR shoots. Phenolic acids (**1**, **2**, **4**, **5**) and echinacoside (**3**) were produced in much lower amounts in comparison with the alkamide (**6**) in the same samples. An opposite result was observed in the AP shoots and *in vivo* GH plants which produced caffeic acid derivatives especially. The AP samples reduced the production of alkamide (**6**) by half in comparison with the IP samples, but they were characterized by a much larger amount of chlorogenic acid

293 (**2**), echinacoside (**3**), and cichoric acid (**4**). The *in vitro* proliferating seedlings (PS shoots)
294 contained only cichoric acid (**4**).
295
296 **Discussion**
297
298 The aim of this work was to perform a standardized protocol for the massive multiplication of
299 *E. angustifolia* plants able to synthesize their typical secondary metabolites.
300 In this work it was settled up the active shoot organogenesis of flower stalks from adult
301 plants: the possibility to use this type of explant from a particular medicinal plant, previously
302 selected for their yield during the vegetative phase, could be very convenient for the growers.
303 This method could be applied without causing damage or completely destroying individual
304 plants as it occurred when the apical buds were excised from the rosettes. Plant propagation
305 from flower stalks was employed for *in vitro* regeneration of several species (Bajaj et al,
306 1983; Tan Nhut et al., 2001; Martin, 2005) but it is an unusual techniques for tissue culture of
307 *Compositae* plants. Direct and indirect regeneration appeared at the same time on flower stalk
308 explants exposed to light on media supplemented with 0.5 BA. This type of explant produced
309 a a satisfied shoot proliferation comparable with those observed on petioles and leaves of *E.*
310 *purpurea* (Choffe et al.,2000; Koroch et al. 2002). During these experiments this phenomenon
311 occurred together with a high shoot hyperhydricity and with the development of abundant
312 callus at the shoot basal end as also observed by Lakshmanan et al. (2001) on *in vitro* seedling
313 leaves of different *Echinacea* species.
314 Hyperhydricity was considered as a physiological response to simultaneous stress factors
315 determined by the *in vitro* culture conditions as high cytokinin treatments combined with the
316 high relative humidity in the closed flask atmosphere (Kevers et al., 2003). Therefore, in order
317 to obtain *E. angustifolia* plantlets more suitable to develop a continuous multiple shoot

318 production, the BA content was halved. The procedure gave a temporary improvement of the
319 culture but the successive subcultures on the same medium showed again a progressive
320 deterioration . A mid-step culture phase using active charcoal (Debergh and Maene, 1981)
321 was necessary to restore shoot quality. These plantlets, cultured again on 0.5 mg L-1 BA CH
322 basal medium restore completely the shoot quality but maintained a poor multiplication
323 capacity (1.7 shoots per explant). This multi-step protocol, improved the quality of the shoots,
324 their elongation and it reduced the callus production at the basal end, giving suitable explants
325 for the successive proliferation phases. The choice to increase the *in vitro* biomass and the
326 previous observations concerning the direct organogenesis on shoot foliage, lead to perform
327 leaf cultures on media with auxins and cytokinins . Auxins alone or in combination with
328 cytokinins has been frequently associated with the induction and expression of regeneration in
329 plant tissue culture (Steward et al., 1964). Zobayed and Saxena (2003), observed somatic
330 embryogenesis from leaves of E. purpurea *in vitro* seedling when IBA (0.5 mgL-1) was used
331 together with BA (2.5 mg L-1). In this work the combination of analogous IBA with BA
332 concentrations in the CHe medium was useful to produce a conspicuous biomass of shoot
333 regenerating callus confirming that the use of IBA might be effective in regeneration of
334 recalcitrant species to the *in vitro* culture (Zobayed and Saxena, 2003). Summarizing, the *in*
335 *vitro* progagation process of *E. angustifolia* from flower stalk consisted of three distinct
336 phases: an adventitious regeneration phase from stalk sections, an axillary proliferation phase
337 of the previous regenerated shoots and an adventitious regeneration phase from leaf pieces of
338 the axillary shoots. The shoots sampled from these different differentiation process were
339 subjected to the phytochemical analysis. Concerning to the analysis of the active constituents,
340 LC-DAD-ESI-MS screening was carried out on the *in vitro* and *in vivo* plant material to
341 evaluate the production of the typical antioxidant constituents (**1-14**) of *Echinacea* species
342 (Table 1). The results showed that none of the well-known in *Echinacea* adult plants
343 Flavonoids (**7-14**) (Bauer and Foster, 1991; Bauer and Wagner, 1991; Bauer and Reminger,

1989; Bauer *et al.,* 1989; Bauer et al., 1988a) were present in the different plant material analysed in this work.

A significant difference between shoots from seedlings growing *in vitro* (PS shoots) and *in vivo* (GH) was showed. A lower secondary metabolite production was observed in PS (in vitro proliferating seedlings) shoots in comparison with the GH plants and it could be related to the *in vitro* growth conditions. In particular, between all the antioxidant constituents (**1-14**), the *in vitro* seedling accumulated only cichoric acid.

The *in vitro* shoots deriving from adult plants in the axillary proliferation phase (AP shoots) were characterized by a high amount of caffeic acid derivatives. They showed the production of caftaric, chlorogenic, cichoric acids, and echinacoside but no caffeic acid. AP shoots are plantlets well developed and showed phenotypic features similar to the greenhouse plants , for this reason, both cultures produced a significant amount of caffeolquinic derivatives. The yields of these secondary metabolites were similar or higher than those reported in leaves of *E. angustifolia* adult plants showed in Table 7. In particular, echinacoside (**3**), the main active constituent found only in wild or cultivated *E. angustifolia* roots (Bauer and Wagner, 1991; Bauer, 1998), was accumulated in a similar amount in the leaves of the AP shoots. In addition it could be remarked that cichoric acid was the main bioactive constituent in AP shoots and it showed much higher yields (3.05 %) in comparison with those reported in Table 7 from different organs of *E. angustifolia* adult plants (Bauer and Wagner, 1991; Bauer, 1998).

On the contrary, shoot at the end of the initial proliferating phase (IP shoots) accumulated a lower amount of phenolic metabolites in comparison with AP shoots and with the greenhouse plants but were able to produce a large quantity of alkamide (**6**). In addition, it was also evident that leaf regenerating shoots (LR) lost the ability to synthesize caffeic acid derivatives since they produced almost exclusively alkamide (**6**).It is well-known that alkamides are present in the roots, leaves, and stalks of wild or cultivated E. angustifolia plants (Bauer, et al. 1989). In this work the initial shoots from flower stalk and the regenerating shoots from leaf

370 explants (IP and LR) supplied an amount (0.023 and 0.036 % respectively) of alkamides (**6**)
371 comparable to the typical content of wild *E. angustifolia* leaves and stalks showed in Table 7.
372 This behaviour might be due to the IP and LR shoots physiological status in which a primary
373 metabolism, directed towards the regeneration process, was dominant rather than a secondary
374 one. Moreover, the higher propagation rates observed in IP and LR, might cause an
375 hyperhydric status as observed by other Authors (Kevers et al. 2003; Hazarika, 2006) .
376 Hyperhydricity, considered as a stress response, might lead IP and LR shoots towards several
377 biochemical changes associated with a different pattern of metabolite accumulation opposite
378 to AP shoots and GH plants.
379 The AP shoots drastically reduced the production of alkamide (**6**) in comparison with the IP
380 and LR shoots. Furthermore, the leaves of *E. angustifolia* GH plants produced at least ten
381 folds lower amounts of alkamide than the IP and LR shoots.
382 In conclusion, from our knowledge, this is the first report on significant production caffeic
383 acid derivatives and alkamides from *in vitro* regenerated shoots of *E. angustifolia*. It was
384 pointed out how the micropropagation of *E. angustifolia* plantlets from adult plants and the
385 careful development of the proper multiplication procedures could allow us to get plant
386 biomass able to produce active compounds at a rate comparable to that of the original plants.
387 The different *in vitro* conditions, affect the plant metabolite pathway operating as a switch
388 eliciting for the alkamide or the caffeic acid derivatives production. Therefore, the shoot
389 regeneration protocols developed in the current study permit to choice the best culture phase
390 to produce either caffeic acid derivatives or alkamides.
391
392 **Acknowledgments**
393
394 The research was financed by The Sixth Framework Programme of European Commission-
395 Food Quality and Safety Project- titled “Ready to eat food for breakfast and sport activity

with high content of nutraceutics reducing a disease risk and promoting public health" (Nutra-Snacks) and by the research Project of the Italian Space Agency (A.S.I.) "Medicine and Biotechnology - Biotechnological Applications" Feasibility Study Contract (June 2006): "From Molecules To Man: Biotechnological Applications of Space Research" (MoMa).

Deleted: Page Break

Formatted

References

Bajaj, Y. P. S., Sidhu, M. M. S., Gill, A. P. S., 1983. Some factors affecting the *in vitro* propagation of gladiolus. Scientia Horticulturae 18, 269-275.

Baskin, C.C., Baskin, J.M., Hoffman, G.R., 1992. Seed dormancy in the prairie forb *Echinacea angustifolia* var. Angustifolia (*Asteraceae*): After ripening pattern during cold stratification. Int J Plant Sci. 153, 239-243.

Bauer, R., 1998. *Echinacea*: Biological effects and active principles. In Lawson, L.D., Bauer R., (eds.), Phytomedicines of Europe: Chemistry and Biological activity, ACS Symposium Series 691. American Chemical Society, Washington, DC, pp. 140-157.

Bauer, R., Foster, S., 1991. Analysis of Alkamides and Caffeic Acid Derivatives from *Echinacea simulata* and *E. paradoxa* roots. Planta Med. 57, 447-449.

Bauer, R., Khan, I.A., Wagner, H., 1988a. TLC and HPLC analysis of *Echinacea pallida* and *E. Angustifolia roots.* Planta Med. 54, 426-430.

Bauer, R., Remiger, P., Wagner, H., 1989. Alkamides from the roots of *Echinacea angustifolia*", Phytochemistry 28, 505-508.

Bauer, R., Reminger, P. 1989. TLC and HPLC analysis of Alkamides in *Echinacea* Drugs. Planta Med. 55, 367-371.

Bauer, R., Wagner, H., 1991b. Echinacea species as potential immunostimulatory drugs. In: Farnsworth, N. R. and Wagner, H. (eds.) Economic and medicinal plant research 5. New York, NY: Academic Press, pp. 253-321.

421 Choffe, K.L., Victor, J.M.R., Murch, S.J., Saxena, P.K., 2000. In vitro regeneration of
422 *Echinacea purpurea* L.: direct somatic embryogenesis and indirect shoot organogenesis
423 in petiole culture. Vitro Cell Dev Biol Plant. 36, 30-36.

424 Debergh, P.C., Maene, L.J., 1981. A scheme for commercial propagation of ornamental plants
425 by tissue culture. Scientia Hort. 14, 335-345.

426 Ellis, R.H., Roberts, E.H., 1980. The influence of temperature and moisture on the seed
427 viability period in barley (*Horedum disticum* L.). Ann Bot. 45: 31-37.

428 Feghahati, S.M.J., Reese, R.N., 1994. Ethylene-, Light-, and Prechill-enhanced Germination
429 of *Echinacea angustifolia* Seeds. J Amer Soc Hort Sci. 119, 853-858.

430 Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrient requirements of suspension cultures
431 of soybean root cells. Exp Cell Res. 50, 151-158;

432 Harbage, J.F., 2001. Micropropagation of *Echinacea angustifolia*, *E. pallida* and *E. purpurea*
433 from Stem and Seed Explants. HortScience 36, 360-364.

434 Hazarika, B.N., 2006. Morpho-physiological disorders in *in vitro* culture of plant. Sci. Hortic.
435 108, 105–120.

436 Kevers, C., Franck, T., Strasser, R.J., Dommes, J., Gaspar, T., 2003. Hyperhydricity of
437 micropropagated shoot: a typically stress-induced change of physiological state. Plant
438 Cell Tissue and Organ Cult. 77, 181-191.

439 Koroch, A., Juliani, H.R., Kapteyn, J., Simon, J.E., 2002. *In vitro* regeneration of *Echinacea*
440 *purpurea* from leaf explants. Plant Cell Tissue Organ Cult. 69, 79-83.

441 Lakshmanan, P., Danesh, Taji, M., 2001. Production of four commercially cultivated
442 *Echinacea* species by different methods of *in vitro* regeneration. Journal of Horticultural
443 Science and Biotechnology 77, 158-163.

444 Li, T.S.C., 1998. *Echinacea:* Cultivation and medicinal value. HortTecnology. 8, 122-129.

445 Lloyd, J.U., 1921. A Treatise on *Echinacea*. In: Drug Treatise No. 30. Lloyd Brothers,
446 Pharmacists, Inc., Cincinnati Ohio,USA.

447 Luczkiewcz, M., Cisowski, W., 2001. Optimization of the second phase of a two phase
448 growth system for anthocyanin accumulation in callus cultures of *Rudbeckia hirta.* Plant
449 Cell Tissue Organ Cult. 65, 57-68.

450 Luczkiewicz, M., Zarate, R., Migas, W.D., Migas, P., Verpoorte, R. 2002. Production of
451 *pulchelin E* in hairy roots, callus and suspension cultures of *Rudbeckia hirta* L. Plant
452 Sci. 163, 91–100.

453 Macchia, M., Angelini, L.G., Ceccarini, L., 2001. Methods to overcome seed dormancy in
454 *Echinacea angustifolia* D.C. Scientia Horticulturae 89, 317-324.

455 Martin, K. P., Geevarghese, J., Joseph, D., Madassery, J., 2005. *In vitro* propagation of
456 Dendrobium hybrids using flower stalk node explants. Indian J. Exp. Biol. 43, 280-5.

457 McGregor, R.L., 1968. The Taxonomy of the Genus *Echinacea (Compositae)*. Univ. Kansas
458 Sci Bull 48, 113-142;

459 Mensuali Sodi, A., Lucchesini, M., Mittempergher, L., 1997. Elm tissue culture:
460 micropropagation of clones resistant to Dutch elm disease. Acta Hort. 457, 235-241.

461 Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with
462 tobacco tissue cultures. Physiol. Plant. 15: 473-493.

463 Sari, A. O., Morales, M.R., Simon, J. E., 2001. Ethephon can overcome seed dormancy and
464 improve seed germination in Purple coneflower species *Echinacea angustifolia* and
465 *Echinacea pallida.* HortTechnology 11, 202-205.

466 Schollhorn, C., Schecklies, E., Wagner, H. 1993. Immunochemical investigations of
467 polysaccharides from *E. purpurea* cell suspension cultures. Planta Med. 59, A662-A663.

Sicha, J., Becker, H., Dusek, J., Hubia, J., Siatka, T., Hrones, I., 1991. Callus cultures of the genus *Echinacea*. Effect of phenylalanine on the growth of cultures and the production of cinnamic acids. Pharmazie 46, 363-364.

Smith, M. A. L., Kobayashi, H., Gawienowski, M., Briskin, D. P., 2002. An *in vitro* approach to investigate medicinal chemical syntesis by three herbal plants. Plant Cell Tissue Organ Cult. 70, 105-111.

Steward, F. C., Mapes, M. O., Kent, A., Holsten, R. D., 1964. Growth and development of cultured plant cells. Science 143, 20–27.

Tan Nhut, D., Van Le, B., Tanaka, M., Tran Thanh Van, K., 2001. Shoot induction and plant regeneration from receptacle tissues of *Lilium longiflorum*. Sci. Hortic. 87**,** 131-138.

Zobayed, S. M., Saxena, P. K., 2003. *In vitro* regeneration of *Echinacea purpurea L. :* enhancement of somatic embryogenesis by indolbutyric acid and dark pre-incubation. *In vitro* Cell Dev. Biol. 39 , 605-612.

Deleted: ¶
Page Break

Deleted: **Table 1** ¶
Home-made database of natural compounds, alkamide (**6**), flavonoids and caffeic acid derivatives (**1-14**), isolated an identified by NMR and MS experiments by a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var. *bravado*) cultivated in Sanremo (Italy).¶
N° ... [1]

Figure captions

Fig. 1 The *E. angustifolia* flower stalk slices give rise to cell proliferation and shoot regeneration when cultured on CH basal medium with 0.5 mg L^{-1} BA in the light.

Fig 2 Distinct type of *E. angustifolia* shoots during the multi-step propagation phases. **A :** Initial proliferating shoots (IP shoots); **B:** Axillary proliferating shoots (AP shoots);

Fig. 3 *E. angustifolia* shoot regeneration from the leaf central vein. Shoots were cultured on CH basal medium with 0.5 mg L^{-1} BA.

Fig. 4 Leaf regenerating shoots (LR shoots) on the CH basal medium with BA (3 mg L^{-1}) and IBA (0.5 mg L^{-1}) named CHe.

Fig. 5 Chemical structures of the selected constituents (**1-6**) of *E. angustifolia* leaf analysed samples.

Table 1

Home-made database of natural compounds, alkamide (**6**), flavonoids and caffeic acid derivatives (**1-14**), isolated an identified by NMR and MS experiments by a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var. *bravado*) cultivated in Sanremo (Italy).

N°	COMPOUND	Rt (min)	UV (λ nm)	MW	Base peak m/z	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m\z) (collisional fragment ions)	Collision energy (%)
1	caftaric acid	6.80	210, 240, 294, 329sh	312	$[M-H]^-$ 311.1	179.1 $[M-H-132]^-$	23	135.2 $[M-H-132-CO_2]^-$	32		
2	chlorogenic acid	10.2	234sh, 244, 297sh, 328	354	$[M-H]^-$ 353.1	191.1 $[M-H-162]^-$	25				
3	echinacoside	15.1	220, 247sh, 292sh, 330	786	$[M-H]^-$ 785.3	623.0 $[M-H-glc]^-$	30	477.1 $[M-H-glc-rha]^-$	32		
4	cichoric acid	18.8	210, 244, 295, 330sh	474	$[M-H]^-$ 473.1	310.8 $[M-H-162]^-$	23	179.0 $[M-H-162-132]^-$	23	135.1 (44) $[M-H-162-132-CO_2]^-$	32
5	caffeic acid	13.4	220, 247sh, 292sh, 330	180	$[M-H]^-$ 179.1	135.2 $[M-H-CO_2]^-$	33				
6	dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide	45.4	235, 260	247	$M+H]^-$ 248.3	149.1 $[M+H-99]^-$	33	121.1 $[M+H-99-C_2H_4]^-$	30	105.9 (15) $[M+H-99-C_2H_4-CH_3]^-$	33
7	quercetin	29.6	255, 267, 301sh, 298sh, 370	302	$[M-H]^-$ 301.2	179.1 $[M-H-122]^-$	40	151.0 $[M-H-122-CO]^-$	38		
8	luteolin	30.2	253, 267, 242sh, 291sh, 349	286	$[M-H]^-$ 285.2	241.2 $[M-H-CO_2]^-$	48	199.7 $[M-H-CO_2-41]^-$	43		
9	apigenin	33.3	267, 269sh, 336	270	$[M-H]^-$ 269.4						
10	kaempferol	14.9	253sh, 266, 294sh, 322sh, 367	286	$[M-H]^-$ 285.3						
11	p-coumaric acid	52.0	223, 286	164	$[M-H]^-$ 163.2	119.1 $[M-H-CO_2]^-$	31				

12	betulinic acid	52.0	220, 307	456	[M-H+HCOOH]$^-$ 501.2			
13	apigenin 7 O β glucoside	32.7	286, 333	432	[M-H]$^-$ 431.1	269.3 [M-H-glc]$^-$	35	
14	isorhamnetin 3 O rutinoside	18.6	253, 267sh, 306sh, 326sh, 370	624	[M-H]$^-$ 623.1			

Table 2

Influence of the plant growth regulators (PGR) and the growth conditions on the regeneration tendency and callus initiation from leaves and flower stalks of *E. angustifolia* adult plants.

		regeneration tendency		**callus induction**		**callus amount***		**callus colour**	
PGR	explant	dark	light	dark	light	dark	light	dark	light
BA	flower stalk	none	direct from purple spots	none	yes	–	+++	–	green with purple spots, compact
NAA+BA	flower stalk	none	none	none	none	–	–	–	–
BA	leaf	none	none	yes	yes	++	++	necrotic	necrotic
NAA +BA	leaf	direct etiolated	none	yes	yes	++	++	white, friable	necrotic

*Scale to quantify callus amount; += scarcely developed at the explant margins, ++ = medium developed covering half of the explant, +++ = largely developed covering all the explant

Page Break

Table 3

Shoot regeneration (number and length of new formed shoots) and callus formation (colour and texture) from flower stalk and leaf explants in different growth conditions. Data are presented as means ± SE.

Explants and growth conditions	Subcultures	N°shoots/exp	Length (cm)	Callus amount*	Colour, texture
Flower stalk- Light (0.5 mg L^{-1} BA)	I	2.67 ± 0,33	0.67 ± 0,20	+++	green, compact
	II	3.00 ± 0.58	0.73 ± 0.15	+++	green, compact
Leaf - Dark (0.01 mg L^{-1} NAA+ 1 mg L^{-1} BA)	I	1.67 ± 0.33	0.50 ± 0.01	++	white, friable
	II	0.00	/	++	white, friable

*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

Page Break

Table 4

Shoot proliferation (shoot number and length of new formed shoots), and callus development at the basal end of *E. angustifolia* shoots in different phases of the propagation process. Data are presented as means ± standard error. AP: Growth Phase shoots; IP: Proliferation Phase shoots.

	N° shoot/exp	Length (cm)	Callus Amount*	Callus quality
IP shoots: BA (0.25 mgL^{-1})	2,36 ± 0,40	1,31 ± 0,14	+/++	friable light green
Active charcoal (5 g/L)	1,10 ± 0,06	1,63 ± 0,25	+	compact green
AP shoots: 0.5 mg L^{-1} BA	1,77 ± 0,79	1,66 ± 0,39	+	compact green

*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

Page Break

Table 5

Callus formation and shoot regeneration 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 medium (LR shoots). Shoot proliferation was performed on CH basal medium with 0.5 mg L^{-1} BA.

	N° shoot/exp	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
LR shoots (0.5 mg L^{-1} BA)	3.60 ± 0.54	2.65± 0.36	+	Friable at the shoot base

*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

Page Break

Table 6

LC-DAD-ESI-MS analysis of the aerial part of *E. angustifolia* selected plant material.

IP: initial proliferating shoots; AP: axillary proliferating shoots; LR: Leaf Regenerated shoots; PS: Proliferating seedlings; GH plants: plants cultivated in greenhouse. Standard error (n = 3)

COMPOUNDS	*In vitro* proliferation from flower stalk			seedlings	
(μμg/g $_{\text{dried plant}}$)	**IP shoots**	**AP shoots**	**LR shoots**	**PS 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	–	110.63±1.32	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	367.95 ± 10.75	–	26.83±0.94

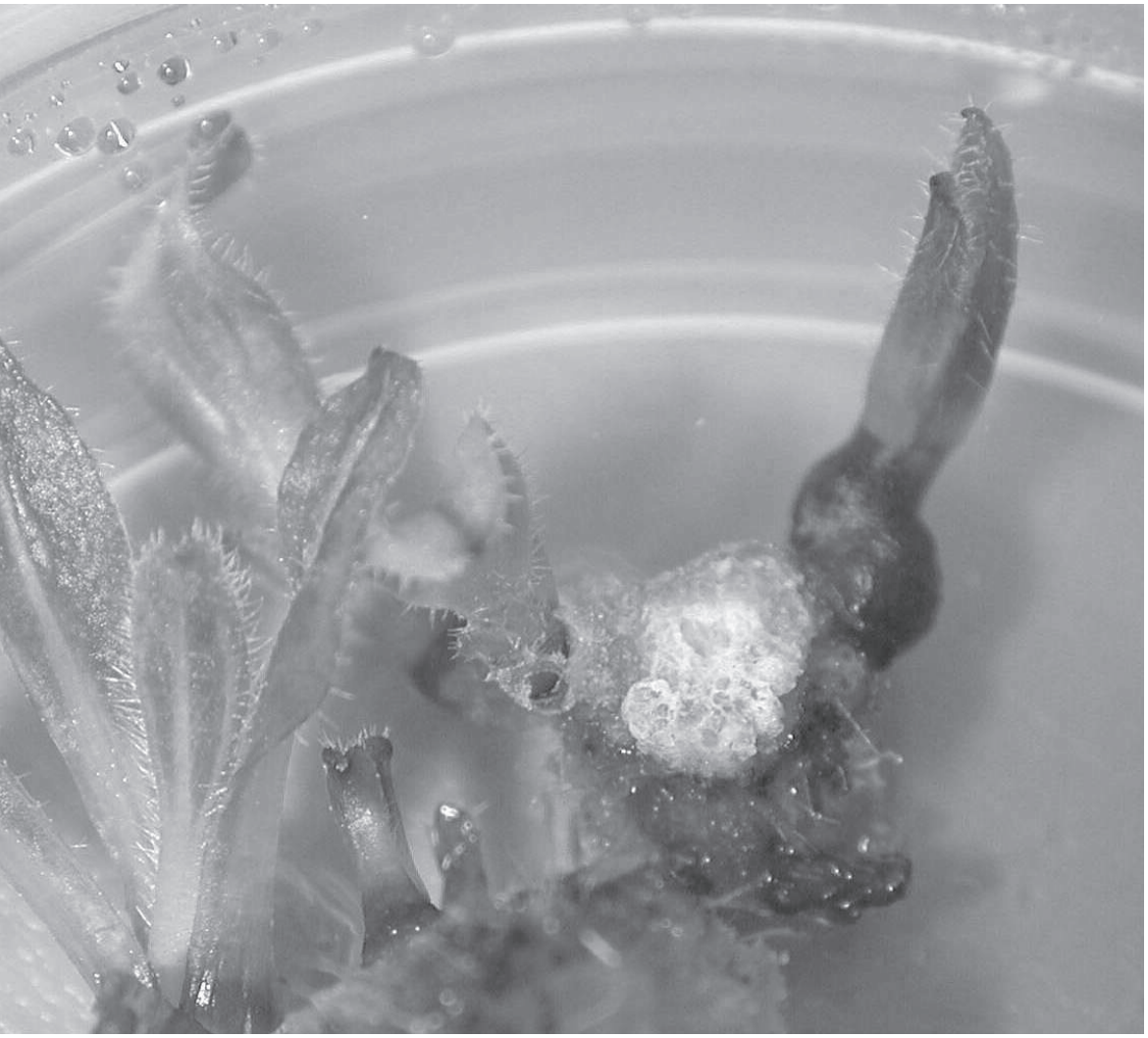
Page Break

Table 7

Data from literature on the main active substance yields (%) in different organs of *Echinacea angustifolia* plants (*4*; *29*).

Plant material	Echinacoside	Cichoric acid	Alkamides	Flavonoides	Glicoproteines /polisaccarides (μg/mg)	Essential oil (%/fresh plant material)
leaves		0.1	0.001-0.03	0.38		< 0.1
flowers	0.1-1.0	0.15	0.001-0.03			< 0.1
stalks		0.05	0.001-0.03			< 0.1
roots	0.3-1.3	traces	0.009-0.151		220.01	< 0.1

Figure

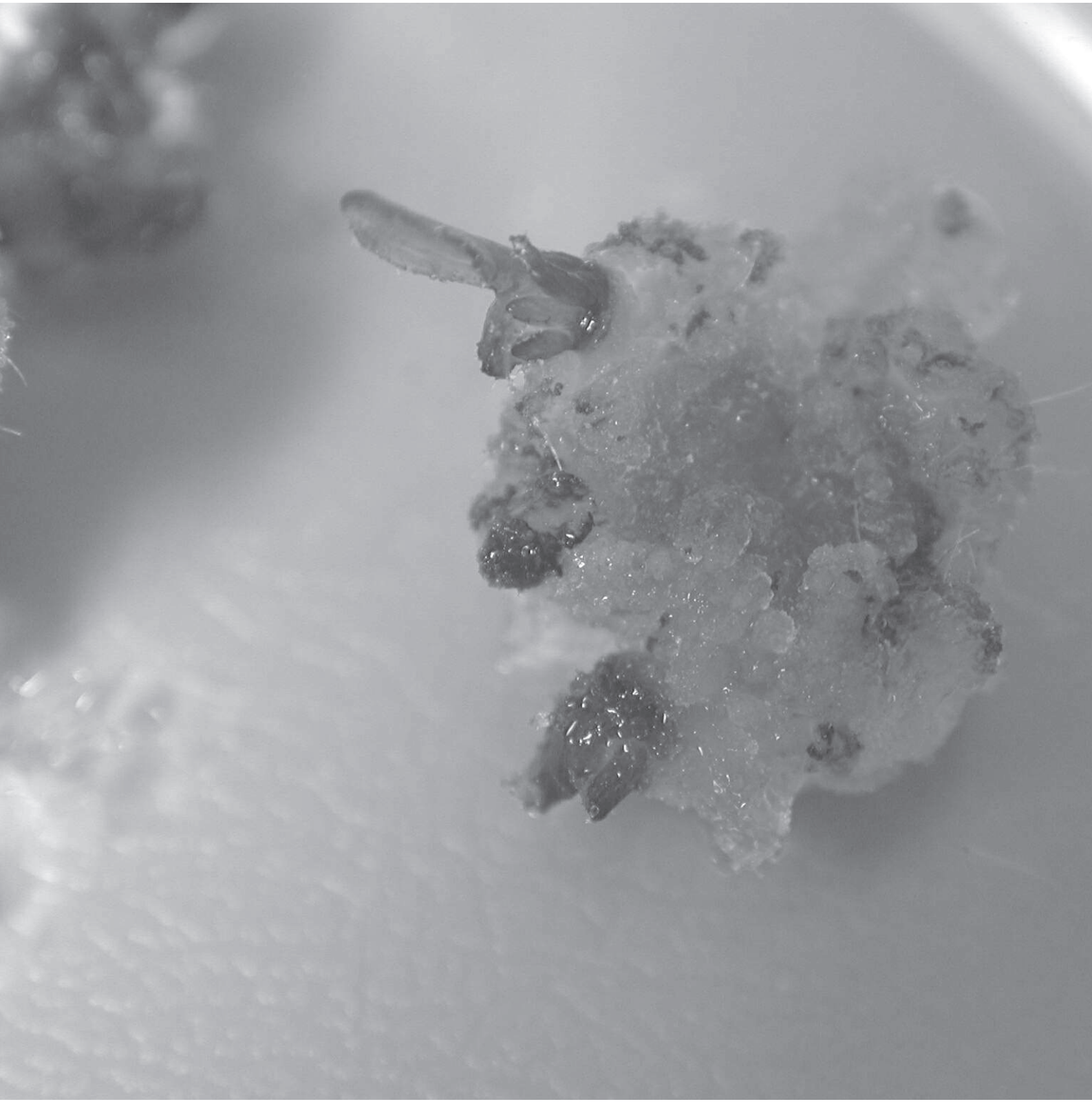


Figure

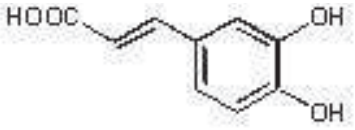
Figure

Figure

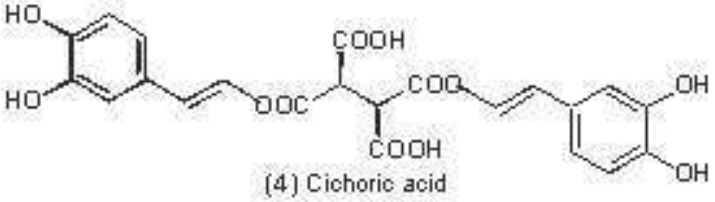
Figure



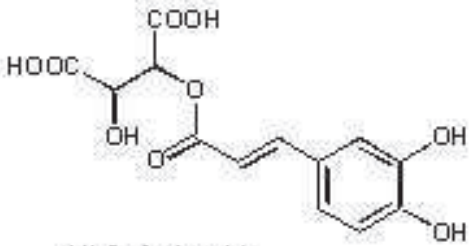
Figure



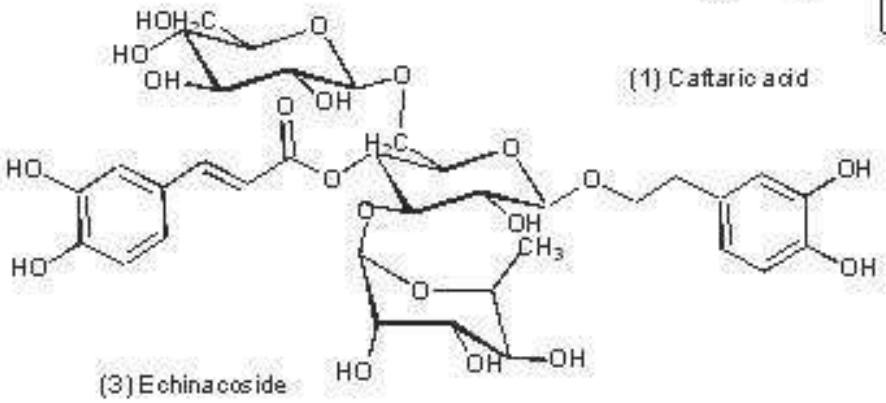
(5) Caffeic acid



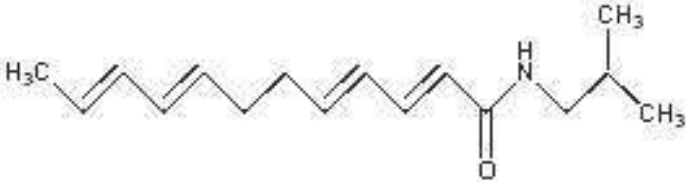
(4) Cichoric acid



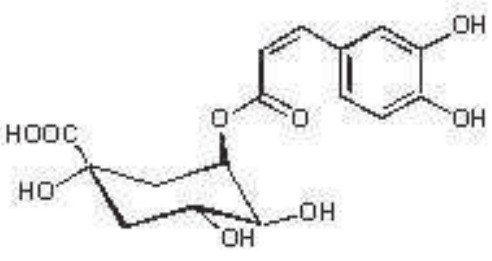
(1) Caftaric acid



(3) Echinacoside



(6) Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide



(2) Chlorogenic acid

Tables

Table 1

Home-made database of natural compounds, alkamide (**6**), flavonoids and caffeic acid derivatives (**1-14**), isolated an identified by NMR and MS experiments by a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var. *bravado*) cultivated in Sanremo (Italy).

N°	COMPOUND	Rt (min)	UV (λ nm)	MW	Base peak m/z	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m\z) (collisional fragment ions)	Collision energy (%)
1	caftaric acid	6.80	210, 240, 294, 329sh	312	$[M-H]^-$ 311.1	179.1 $[M-H-132]^-$	23	135.2 $[M-H-132-CO_2]^-$	32		
2	chlorogenic acid	10.2	234sh, 244, 297sh, 328	354	$[M-H]^-$ 353.1	191.1 $[M-H-162]^-$	25				
3	echinacoside	15.1	220, 247sh, 292sh, 330	786	$[M-H]^-$ 785.3	623.0 $[M-H-glc]^-$	30	477.1 $[M-H-glc-rha]^-$	32		
4	cichoric acid	18.8	210, 244, 295, 330sh	474	$[M-H]^-$ 473.1	310.8 $[M-H-162]^-$	23	179.0 $[M-H-162-132]^-$	23	135.1 (44) $[M-H-162-132-CO_2]^-$	32
5	caffeic acid	13.4	220, 247sh, 292sh, 330	180	$[M-H]^-$ 179.1	135.2 $[M-H-CO_2]^-$	33				
6	dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide	45.4	235, 260	247	$M+H]^-$ 248.3	149.1 $[M+H-99]^-$	33	121.1 $[M+H-99-C_2H_4]^-$	30	105.9 (15) $[M+H-99-C_2H_4-CH_3]^-$	33
7	quercetin	29.6	255, 267, 301sh, 298sh, 370	302	$[M-H]^-$ 301.2	179.1 $[M-H-122]^-$	40	151.0 $[M-H-122-CO]^-$	38		
8	luteolin	30.2	253, 267, 242sh, 291sh, 349	286	$[M-H]^-$ 285.2	241.2 $[M-H-CO_2]^-$	48	199.7 $[M-H-CO_2-41]^-$	43		
9	apigenin	33.3	267, 269sh, 336	270	$[M-H]^-$ 269.4						
10	kaempferol	14.9	253sh, 266, 294sh, 322sh, 367	286	$[M-H]^-$ 285.3						
11	p-coumaric acid	52.0	223, 286	164	$[M-H]^-$ 163.2	119.1 $[M-H-CO_2]^-$	31				
12	betulinic acid	52.0	220, 307	456	$[M-H+HCOOH]^-$ 501.2						
13	apigenin 7 O β glucoside	32.7	286, 333	432	$[M-H]^-$ 431.1	269.3 $[M-H-glc]^-$	35				
14	isorhamnetin 3 O rutinoside	18.6	253, 267sh, 306sh, 326sh, 370	624	$[M-H]^-$ 623.1						

Table 2

Influence of the plant growth regulators (PGR) and the growth conditions on the regeneration tendency and callus initiation from leaves and flower stalks of *E. angustifolia* adult plants.

		regeneration tendency		**callus induction**		**callus amount***		**callus colour**	
PGR	explant	dark	light	dark	light	dark	light	dark	light
BA	flower stalk	none	direct from purple spots	none	yes	–	+++	–	green with purple spots, compact
NAA+BA	flower stalk	none	none	none	none	–	–	–	–
BA	leaf	none	none	yes	yes	++	++	necrotic	necrotic
NAA +BA	leaf	direct etiolated	none	yes	yes	++	++	white, friable	necrotic

*Scale to quantify callus amount; += scarcely developed at the explant margins, ++ = medium developed covering half of the explant, +++ = largely developed covering all the explant

Table 3

Shoot regeneration (number and length of new formed shoots) and callus formation (colour and texture) from flower stalk and leaf explants in different growth conditions. Data are presented as means ± SE.

Explants and growth conditions	Subcultures	N°shoots/exp	Length (cm)	Callus amount*	Colour, texture
Flower stalk- Light (0.5 mg L^{-1} BA)	I	2.67 ± 0,33	0.67 ± 0,20	+++	green, compact
	II	3.00 ± 0.58	0.73 ± 0.15	+++	green, compact
Leaf - Dark (0.01 mg L^{-1} NAA+ 1 mg L^{-1} BA)	I	1.67 ± 0.33	0.50 ± 0.01	++	white, friable
	II	0.00	/	++	white, friable

*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

Table 4

Shoot proliferation (shoot number and length of new formed shoots), and callus development at the basal end of *E. angustifolia* shoots in different phases of the propagation process. Data are presented as means ± standard error. AP: Growth Phase shoots; IP: Proliferation Phase shoots.

	N° shoot/exp	**Length (cm)**	**Callus Amount***	**Callus quality**
IP shoots: BA (0.25 mgL^{-1})	2,36 ± 0,40	1,31 ± 0,14	+/++	friable light green
Active charcoal (5 g/L)	1,10 ± 0,06	1,63 ± 0,25	+	compact green
AP shoots: 0.5 mg L^{-1} BA	1,77 ± 0,79	1,66 ± 0,39	+	compact green

*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

Table 5

Callus formation and shoot regeneration 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 medium (LR shoots). Shoot proliferation was performed on CH basal medium with 0.5 mg L^{-1} BA.

	N° shoot/exp	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
LR shoots (0.5 mg L^{-1} BA)	3.60 ± 0.54	2.65± 0.36	+	Friable at the shoot base

*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

Table 6

LC-DAD-ESI-MS analysis of the aerial part of *E. angustifolia* selected plant material.

IP: initial proliferating shoots; AP: axillary proliferating shoots; LR: Leaf Regenerated shoots; PS: Proliferating seedlings; GH plants: plants cultivated in greenhouse. Standard error (n = 3)

COMPOUNDS	*In vitro* proliferation from flower stalk			seedlings	
(μμg/g $_{\text{dried plant}}$)	**IP shoots**	**AP shoots**	**LR shoots**	**PS 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	–	110.63±1.32	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	367.95 ± 10.75	–	26.83±0.94

Table 7

Data from literature on the main active substance yields (%) in different organs of *Echinacea angustifolia* plants (*4*; *29*).

Plant material	Echinacoside	Cichoric acid	Alkamides	Flavonoides	Glicoproteines /polisaccarides (μg/mg)	Essential oil (%/fresh plant material)
leaves		0.1	0.001-0.03	0.38		< 0.1
flowers	0.1-1.0	0.15	0.001-0.03			< 0.1
stalks		0.05	0.001-0.03			< 0.1
roots	0.3-1.3	traces	0.009-0.151		220.01	< 0.1