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Fulvic acid affects proliferation and maturation phases in Abies cephalonica embryogenic cells

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ABSTRACT

Embryogenic cell masses (ECM) of Abies cephalonica were grown on proliferation media in the presence and absence of fulvic acid (FA), whose molecular composition and conformational rigidity were evaluated by CPMAS-13C NMR spectroscopy. To assess the physiological effects of this humic material during proliferation and maturation stages of somatic embryogenesis (SE), proliferation rate, proportion of consecutive developmental stages of pro-embryogenic masses (PEM), cellular ATP and glucose-6-phosphate were evaluated at regular intervals. FA increased the proliferation rate, especially during the early sampling days, and the percentage of PEM in their advanced developmental stage. Cellular ATP and glucose-6-phosphate were increased by FA pre-treatment during the maturation phase. Furthermore, the effects of the anti-auxin p-chlorophenoxyisobutyric acid (PCIB), such as a decrease of growth and the enhancement of PEM III induction, were inverted by FA. Proton pumping ATPase and PPase activities were decreased in microsomes from PCIB-treated ECM, while they increased in the presence of FA. This fulvic matter also induced a delay in somatic embryo formation during the maturation phase. Both the improvement of the PEM proliferation and the reduction of the subsequent maturation process of A. cephalonica are explained by a release from the complex humic structure of low molecular-weight molecules, which may interact with the plant hormonal signaling pathway. These effects appear to be related to the hydrophilic and conformationally labile nature of FA. The structure–activity relationship observed here suggests that the influence of FA on ECM may be attributed to specific bioactive molecules that are preferentially released from the FA loose superstructure.

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Introduction

Somatic embryogenesis (SE) is a process by which somatic cells differentiate into somatic embryos (von Arnold et al., 2002). Somatic embryos do not develop directly, but from pro-embryogenic masses (PEM), which pass through a series of three characteristic stages, distinguished by cellular organization and cell number (PEM I, PEM II, and PEM III) (Fig. 1) (Filonova et al., 2000b). Pro-embryogenic mass will not differentiate to somatic embryos (embryogenesis).

At the PEM I stage, cell aggregates are composed of a small compact clump of densely cytoplasmic cells adjacent to a single enlarged and vacuolated cell (panel A). Similar cell aggregates, but possessing more than one vacuolated cell, have been classified as PEM II (panel B). At the PEM III stage, enlarged clumps of densely cytoplasmic cells appear loose rather than compact (panel C).

The biochemical mechanisms governing induction, proliferation and maturation during SE are still poorly understood. Among the signals that directly participate in the regulation of the different phases of this process, plant hormones have emerged as major candidates (von Arnold et al., 2002; Stasolla and Yeung, 2003). Auxins are considered to be the most important hormones in regulating SE in vitro. In conifers, auxin is required for the proliferation phases of PEM, but its presence is inhibitory to further development into somatic embryos (Filonova et al., 2000a). The degree of proliferation, which takes place in the presence of auxin, varies among different species: in the genus Abies, the induction and proliferation of SE differs from most other coniferous species because cytokinin is required as the sole plant growth regulator (Nørgaard...
and Krogstrup, 1995). Furthermore, antiauxins, in association with abscisic acid, are reported to be very effective on the maturation process of embryogenic cultures of Abies nordmanniana (Find et al., 2002) and Picea morrisonicola (Liao et al., 2008). Larsson et al. (2008) showed that the addition of 1-N-naphthylphtalamic acid, an inhibitor of polar auxin transport, during early embryo development, leads to abnormal embryo formation. Moreover, it has been recently suggested that PIN efflux proteins could be involved in auxin transport during cotyledon initiation related to somatic embryo development in Picea abies (Hakman et al., 2009). These results suggest that polar auxin transport is an essential process for the correct development of embryos and that its role is similar in angiosperms and gymnosperms.

Humic substances (HS) have been included among the natural molecules that exert physiological influences on plant growth (Nardi et al., 2002). HS are derived from the degradation and decomposition of dead biological material in soils and are considered as supramolecular associations of self-assembling heterogenous and relatively small molecules (Piccolo, 2001). Marked effects of HS on plant growth have been reported, and are induced especially by the low molecular components of HS (LMS) (Piccolo et al., 1992; Muscolo et al., 1993, 1999, 2007; Nardi et al., 1994; Zancani et al., 2009). Although the biochemical pathways followed by LMS and natural auxins could be different, the effects of LMS and auxins on root growth and development are very similar (Nardi et al., 1994; Canellas et al., 2002; Zandonadi et al., 2007). These effects are reversed by the presence of auxin inhibitors, such as 2,3,5-triodobenzoic acid and p-chlorophenoxyisobutyric acid (PCIB) (Nardi et al., 1994), and are related to interactions of LMS with plasma membrane (Muscolo et al., 2007). The increase in root growth and the induction of lateral root development induced by both LMS and indolacetic acid are paralleled by an increase of plasma membrane and tonoplast proton pumps (Canellas et al., 2002, 2008; Zandonadi et al., 2007). These authors reported that the microstructure of humic acid derived from earthworm compost also possesses exchangeable auxin groups (Canellas et al., 2002, 2009, 2010). Therefore, it has been hypothesized that these HS could act as environmental factors able to regulate the root architecture (Zandonadi et al., 2007; Canellas et al., 2008). On the other hand, other authors have also reported that water-extractable humic substances are able to induce an ordered remodeling of the root morphology in Arabidopsis, but have concluded that such effects are not linked to changes in plant auxin homeostasis (Schmidt et al., 2007).

The aim of this work was to evaluate the physiological (hormone-like) effects of fulvic acid (FA) on different stages occurring during SE of Abies cephalonica, chosen as a model system. This species does not require exogenous auxins during the proliferation and maturation phases (Nørgaard and Krogstrup, 1995). Thus, the cell masses could grow on solid culture medium without auxin addition, avoiding possible interference between this hormone and humic matter. The effects of FA were evaluated on: (i) proliferation rate and proportion of consecutive developmental stages of PEM; (ii) ATP and glucose-6-phosphate (Glu-6-P) levels of cell masses grown in the presence of this humic material in solid media during the proliferation and maturation phases of SE; (iii) plasma membrane and tonoplast proton pumping (H+-ATPases and H+-PPases) activities in microsomes obtained from FA-treated cell masses in the proliferation phase.

Materials and methods

Extraction and purification of humic matter

The FA was extracted from a sandy loam agricultural soil classified as Fluventic Xerochrept. Humic substances were extracted by standard procedures (Piccolo and Spiteller, 2003). The soil was shaken overnight in a 0.5 M NaOH and 0.1 M Na2P2O7 solution under N2 atmosphere. The humic acid was precipitated from alkaline extracts by lowering the pH to 1 with 6 M HCl. The FA, the humic material left in solution after the soil extracting alkaline solution was reconstituted to pH 1, was purified by absorbing on an Amberlite XAD8 resin, eluting by a 1 M NaOH solution and, after adjusting the pH to 5, dialyzing in Spectrapore 3 tubes against distilled water until chloride-free, and freeze-dried. The FA was then redissolved...
in 0.5 M NaOH and passed through a strong cation-exchange resin (Dowex 50) to further eliminate divalent and trivalent metals and freeze-dried again. FA samples (50 mg, corresponding to 16.5 mg of OC) were subsequently suspended in distilled water (50 mL) and titrated for 2 h at pH 7 with a CO2-free solution of 0.5 M KOH by an automatic titrator (VIT 90 Videotitrator, Radiometer, Copenhagen) under N2 atmosphere and stirring. The resulting potassium fulvates were then filtered through a Millipore 0.45 μm and freeze-dried.

Characterization of humic matter

The FA was characterized for elemental content using a Fisons EA 1108 Elemental Analyzer. The ash content was less than 5%. Cross-polarization magic angle spinning (CPMAS) 13C spectra were acquired as described previously (Nebbioso and Piccolo, 2009) on a Bruker AVANCEIII 300, equipped with a 4 mm wide-bore MAS probe, operating at the 13C resonating frequencies of 75.4, and a probe, operating at the 13C resonating frequencies of 75.4, and a rotor spin rate of 13,000 ± 2 Hz. Samples were packed in 4 mm zirconia rotors with Kel-F caps and 1510 data points were collected over an acquisition time of 20 ms, a recycle delay of 3.0 s, and 2200 scans. A variable spin lock pulse sequence was applied with a 1H ramp to account for inhomogeneity of the Hartmann–Hahn condition at high rotor spin rates. An average spin lock frequency of 60 MHz was applied during the ramped cross-polarization time. Spin lock was varied in intervals from 0.01 to 75.0 ms in 21 increments. Spectra processing was conducted by Mestre-C version 4.9.9.9 software. All free induction decays (FID) were transformed by applying a 2 k zero filling and an exponential filter function with a line broadening (LB) of 100 Hz. Manual baseline correction was carried out with a zero-order function using Bernstein polynomials and filter set at the value of 2. Spectra were integrated in the intervals of 185–150 ppm (carboxyl carbons), 150–95 ppm (aromatic carbons), and 65–0 ppm (plain or substituted alkyl carbons). The values of 185–150 ppm (carboxyl carbons), 150–95 ppm (aromatic carbons), and 65–0 ppm (plain or substituted alkyl carbons). The 95–65 ppm (anomeric carbons) region showed no significant signal for this lignite HA. Proton spin–lattice relaxation time (T1,ρ(H)) and signal area in absence of any relaxation (I0) are related by Eq. (1):

\[
I(t) = I_0 \exp \left[ -\frac{-t_{SL}}{T_1,\rho(H)} \right]
\]

where \(t_{SL}\) is the variable spin lock time and I(t) is the signal area at t\(_n\) spin lock time. Values of T1,ρ(H) and I0 were calculated by OriginPro 7.5 SR5 vs 7.5870 (B870) (MA, USA) software.

Plant material

Embryogenic cell line 6 of Abies cephalonica Loud. was initiated and sub-cultured as described by Krajňáková et al. (2008). Briefly, proliferation medium was composed of half-strength Murashige and Skoog medium with full-strength vitamins (Murashige and Skoog, 1962), supplemented with 58 mM sucrose, 4.44 μM benzyl adenine, 0.1% (w/v) casein hydrolysate, 3.4 mM L-glutamine (sterile filtered and added into the cooled media) and solidified with 0.3% (w/v) Phytagel (pH 5.7). Regular sub-culturing of the proliferating cultures was performed every 2 weeks.

Proliferation rate

For determination of the proliferation rate, the Murashige and Skoog proliferation medium was used. Five pieces ECM were weighed (200 ± 25 mg, fresh weight, w0) and placed equidistant from each other on one Petri plate. For each sampling day (3, 5, 7, 14 and 21), 3 Petri plates (e.g. 15 numbered pieces of ECM) were used. The experiment was repeated 3–5 times. During the sampling days, ECM were detached from the medium and weighted (w3, w5, w7, w14 and w21). The proliferation rate was determined as ratio between fresh weight of sampling day and the initial weight (w3/w0, w5/w0, w7/w0, w14/w0 and w21/w0). The addition of FA and/or PCIB was made by adding 1 mL solution containing 100 μg of lyophilized FA (corresponding to 33 μg of OC) or 50, 100 and 200 μg PCIB (filter sterilized) in each plate. The control contained 1 mL of ultrapure sterilized water.

Morphological assessment of ECM

The percentage composition of PEM (at stage I, II and III) and early somatic embryos from embryogenic cell line of A. cephalonica was determined in sampling days as stated for proliferation rate using double staining technique with acetocarmine and Evan’s blue (Gupta et al., 1997). The morphological evaluation of cell aggregates was based on the description given by Filonova et al. (2000b) for Picea abies. On each sampling day, at least 500 individual cell aggregates were determined.

Maturation of embryogenic cultures

ECM were pre-treated for 2 weeks with FA (as described in Proliferation rate section) and then transferred to maturation medium. At the beginning of the maturation experiment, 4 g of fresh ECM was transferred to the sterile Falcon flasks with 20 mL of liquid proliferation medium without cytokinin. The suspension was gently mixed by vortex and allowed to settle. After removal of supernatant, 1 mL of suspension containing approximately 250 mg ECM (fresh weight) was plated onto sterile Whatman filter paper on maturation media. The basic Murashige and Skoog maturation medium (Salaj et al., 2005) was supplemented with 0.05% (w/v) casein hydrolysate and 1.7 mM L-glutamine, pH 5.7. The medium was solidified with 0.25% (w/v) Phytagel and supplemented with 83.3 mM maltose. The medium was supplemented with 10% (w/v) PEG-4000, and 32 μM of abscisic acid. Sub-culturing was done every 2 weeks. The first three transfers were performed on maturation media as described above, while the following transfers were performed on the same media without PEG–4000. The maturation phase took 12 weeks in total. Cultures were monitored at the time of sub-culturing.

Measurement of cellular ATP and glucose-6-phosphate

ATP and Glu-6-P content of ECM was determined by means of the luciferin–luciferase luminometric assay and reduction of β-NADP\(^+\) catalyzed by Glu-6-P dehydrogenase, respectively, as described by Petruzza et al. (2009).

Microsome preparation and measurement of proton pumping activities

Approximately 30 g of ECM, collected after 14 days in proliferation medium, was frozen with liquid N\(_2\) ground to fine powder and then homogenized in a mortar with 120 mL of 0.3 M sucrose, 50 mM Tris–HCl (pH 8.0), 8 mM ethylenediaminetetraacetic acid, 5 mM dithioerythritol, 0.1 M MgCl\(_2\), 1 mM phenylmethylsulfonyl fluoride, and 0.5% (w/v) BSA at 4 °C. The homogenate was filtered through 100 μm nylon gauze and centrifuged at 2800 g for 5 min in a Sorvall RC-5B centrifuge (SS-34 rotor). The supernatant was re-centrifuged at 13,000 g for 12 min and then at 100,000 × g for 40 min in a Beckman L7-55 centrifuge (Ty 70i rotor). The microsomal fraction was resuspended in 0.3 M sucrose, 20 mM Tris–HCl (pH 7.5) and 0.1% (w/v) BSA at a final protein concentration of 2–4 mg mL\(^{-1}\).

ATP- or PPI-dependent H\(^+\) transport was monitored as acidine orange fluorescence quenching as described by Zancani et al. (1998), with minor changes. The incubation medium was 25 mM
Tris–Mes (pH 7.3), 125 mM KCl, 5 mM MgSO₄, 1 mM EDTA, 0.1% (w/v) BSA, 2.5 μM acridine orange and 50 μg protein mL⁻¹ of micromosomal vesicles in a final volume of 2 mL. The reactions were started by the addition of 1 mM ATP or 200 μM PiPP.

**Experimental design and data analysis**

The effect of FA was tested during the proliferation period (four sampling days: 3, 7, 14, and 21), according to proliferation activities, formation of different morphological aggregates and determination of cellular levels of ATP (nmol mg⁻¹ fresh weight) and Glu-6-P (nmol mg⁻¹ fresh weight) in three independent experiments. Proliferation rates (ratios between ECM fresh weight of sampling day and the ECM initial weight) were analyzed by two-way ANOVA and the entire experiment was repeated twice. The cellular levels of ATP, Glu-6-P and number of developing somatic embryos were detected. Maturing ECM were monitored six times, at 2-week intervals, using material from three independent Petri plates, and the data of cellular levels of ATP and Glu-6-P were collected. ANOVA was used for the evaluation (see in Proliferation rate section).

**Results**

**Characteristics of FA**

FA revealed the presence of nitrogen and high oxygen content (Table 1). The presence of more oxygenated and nitrogenated compounds in FA was also shown by its large H/C ratio. The CPMAS-¹³C NMR spectrum (Fig. 2), further indicated that FA was rich in nitrogen containing compounds (C–N signals at around 56 ppm), carbohydrate moieties (C–O signals in the 76–107 ppm), and carboxyl groups (COO signals at about 178 ppm) (Fig. 2). These characteristics are confirmed by the distribution of different carbons as shown by signal integration (Table 1). Moreover, this carbon distribution confers a great degree of hydrophilicity to FA (Table 1).

CPMAS-¹³C NMR spectroscopy is also used to characterize the dynamics of solids at the molecular level, being very sensitive to g organic carbon (OC)/plate). A significant effect on the formation of different morphological aggregates and determination of cellular levels of ATP (nmol mg⁻¹ fresh weight) and Glu-6-P (nmol mg⁻¹ fresh weight) in three independent experiments. Proliferation rates (ratios between ECM fresh weight of sampling day and the ECM initial weight) were analyzed by two-way ANOVA and the entire experiment was repeated twice. The cellular levels of ATP, Glu-6-P and number of developing somatic embryos were detected. Maturing ECM were monitored six times, at 2-week intervals, using material from three independent Petri plates, and the data of cellular levels of ATP and Glu-6-P were collected. ANOVA was used for the evaluation (see in Proliferation rate section).

**Effect of FA on proliferation and maturation phases**

The embryogenic cell masses (ECM) of *A. cephalonica* were grown for 21 days on proliferation media (Fig. 3), in the absence (open columns) or the presence of FA (black columns), to verify the effect of such a humic substance on cell growth. Preliminary experiments (results not shown) suggested that the optimal concentration of FA for cell growth was around 100 μg/plate (corresponding to 33 μg organic carbon (OC)/plate). A significant effect on proliferation rate was achieved in the presence of FA (100 μg/plate) on days 3 and 7, with this stimulation limited to the first sampling days. Statistical analysis showed that treatment and sampling days significantly affected the proliferation rate (*P* < 0.001, Table 2).

![Fig. 2. CPMAS-¹³C NMR spectrum of FA used in this study.](image-url)
entire experiment (being 3.3), with respect to control (proliferation rate 2.8). Furthermore, FA was able to increase the percentage of PEM II from 44 to 52.

During the proliferation phase, two main bioenergetic markers were also evaluated, namely cellular ATP and Glu-6-P. Both showed a decrease from 0.65 to 0.31 nmol mg$^{-1}$FW (ATP) and from 55 to 25 nmol mg$^{-1}$FW (Glu-6-P), respectively, after 21 days of sub-culture. FA was only slightly effective, as the increases in ATP and Glu-6-P were very limited (results not shown).

The effect of FA was also tested on the subsequent maturation period, where ECM were pre-treated with FA only during a 2-week proliferation period before maturation. After the first 6 weeks on maturation media, induction of somatic embryos was observed. The first structures observed were early pre-cotyledonary embryos, which further developed into cotyledonary embryos. At this stage, the embryos might either continue differentiation or cease development. After a 12-week maturation, the average number of somatic embryos per 1 g of fresh ECM was counted, according to two categories (early pre-cotyledonary embryos and cotyledonary embryos). The pre-treatment with FA significantly affected the number of developed somatic embryos, which varied from 154.6 to 45 (not shown).

The levels of ATP and Glu-6-P were significantly affected by duration of the maturation period, maturation pre-treatment and, in the case of Glu-6-P, even by the interaction maturation period × maturation pre-treatment (Table 3). Pairwise comparisons for cellular levels of ATP and Glu-6-P, determined in ECM pre-treated with FA and control during 6 sequential maturation periods, are presented in Fig. 4. The numbers of developed somatic embryos tended to increase with both treatments, particularly when comparing the effects of control and FA.

**Effect of FA on proliferation rate phase**

To compare the effect of an anti-auxin compound with that obtained with FA, PCIB was added to the proliferation media (Fig. 5). As shown in panel A, PCIB inhibited the proliferation rate of ECM in a concentration-dependent manner during 14 days of sub-culture. After 14 days, PCIB, at a concentration of 200 μg/plate, caused a 60% inhibition of proliferation and a significant increase in PEM III formation (not shown). To exclude any possible interference between the auxin material and PCIB added on the same plate (e.g. the formation of complexes between FA and PCIB that would lower the availability of the auxin inhibitor for the cell aggregates), ECM were transferred to a fresh medium without (open columns) or with 100 μg of FA/plate (black columns). Then, the cell masses, after 7, 10 and 14 days, showed that FA was able to increase the cell growth significantly. Furthermore, the negative effect exerted by PCIB on ECM proliferation rate (panel C, grey columns) was partially reversed by the presence of FA at a concentration of 100 μg/plate, especially after 7 and 14 days of sub-culture (dashed columns). There was also a marked effect of FA on the formation of morphological aggregates: the percentage of PEM III was increased by PCIB treatment (from 43 to 58%), but lowered in the presence of FA to 48%, a value comparable to the control.

**Effect of FA on microsomal ATPase and PPase activities**

The positive effects of FA on ECM growth at the proliferation stage were confirmed by the determination of proton pumping activities of ATPase and PPase (Fig. 6). The ATPase activity of microsomes, obtained from FA-treated ECM (panel A, dotted lines), was increased with respect to the control (solid line), while that of microsomes of PCIB-treated ECM (dashed line) showed no significant effect. Pyrophosphatase activity of microsomes from PCIB-treated ECM (panel B, dashed line) was significantly lower with respect to the control (solid line), but was increased by FA either when present alone (dotted line) or together with PCIB (dash-dotted line).

**Table 3**

<table>
<thead>
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<th>DF</th>
<th>F-value</th>
<th>Pr &gt; F</th>
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<td></td>
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<tr>
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<tr>
<td>Maturation pre-treatment</td>
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</tr>
<tr>
<td>Maturation period × maturation pre-treatment</td>
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<td>NS</td>
</tr>
<tr>
<td>Error</td>
<td>52</td>
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<td></td>
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<tr>
<td>Maturation period</td>
<td>5</td>
<td>4.58</td>
<td>**</td>
</tr>
<tr>
<td>Maturation pre-treatment</td>
<td>1</td>
<td>5.06</td>
<td>*</td>
</tr>
<tr>
<td>Maturation period × maturation pre-treatment</td>
<td>5</td>
<td>4.09</td>
<td>**</td>
</tr>
<tr>
<td>Error</td>
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<td></td>
<td></td>
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<tr>
<td>NS, P &gt; 0.05 (non-significant).</td>
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<tr>
<td>P &lt; 0.05.</td>
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<tr>
<td><strong>P &lt; 0.01.</strong></td>
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<tr>
<td>***P &lt; 0.001.</td>
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FS and Glu-6-P (59.57–62.57 nmol mg$^{-1}$FW), but a significant increase was observed at the 8th week, and remained high during the last weeks of maturation. Taken as a whole, these data (average) show that the highest levels of ATP were observed with samples pre-treated with FA (0.82 nmol mg$^{-1}$FW). FA also increased the levels of Glu-6-P (being 69.28 nmol mg$^{-1}$FW) when compared to controls (60.76 nmol mg$^{-1}$FW).

**Table 2**

<table>
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NS, P > 0.05 (non-significant).  
***P < 0.001.
Fig. 4. Statistical evaluation of pairwise comparisons for cellular levels of ATP (panel A) and Glu-6-P (panel B) determined in ECM of *A. cephalonica* during six sequential maturation periods. Columns with different letters are significantly different (*P* < 0.05). Data reported as averages are pairwise comparisons among treatments (control and 100 µg/plate FA, corresponding to 33 µg of OC).

**Discussion**

This work was undertaken to investigate the physiological (hormone-like) effects of FA on *A. cephalonica*, during both proliferation and maturation phases. Since no information is available on the energetic status of cells during SE, a preliminary part of the experiments dealt with this aspect.

Our results suggest that the proliferation phase is characterized by a high level of cellular ATP and Glu-6-P, indicating a high requirement for energy for the initial cell growth. Such energetic metabolites decreased during the 21 days of cultivation in the proliferation phase, followed by an increase for ATP and a transient increase for Glu-6-P, reaching a maximum at week 10 during the maturation phase (Fig. 4). The general decrease of ATP and Glu-6-P, after 3 weeks of growth on solid media, could be explained by a nutrient stress induced by limited availability of micro- and/or macro-elements, as well as a possible increase of ethylene in the cultivation media. This confirms that, according to general protocols (Klimaszewska and Cyr, 2002), the correct period of subculture on solid media was around 14 days, when the cell cultures were still unstressed. Furthermore, ATP and Glu-6-P levels seem to be higher during the initial phase of cell mass growth, when the metabolic activity is high. This phase is followed by a lower...
cell growth rate and by the differentiation of the cells in PEM and finally SE. We suggest that the limited concentrations of ATP and Glu–6-P, possibly caused by the decreased availability of nutrients from the media, would represent one of the several factors involved in embryo development during the initial phase of SE.

The correlation between the increase of proliferation rate and the higher level of ATP (and partially of Glu–6-P), when FA was added to the culture media (Fig. 3), seems to confirm this hypothesis. When the ATP level was higher, the differentiation was delayed and the ECM growth rate was increased. Furthermore, this effect was not limited to the proliferation, since the presence of humic materials during this phase caused a delay in embryo differentiation during the following maturation phase.

The development of PEM and SE is strictly dependent on the presence and balance of plant growth regulators. Once auxin is applied to PEM II or PEM III (e.g. when cells are sub-cultured into fresh proliferation medium), the ECM develop new PEM I and delay the differentiation (Filanova et al., 2000b). The application of anti-auxins, such as 2,3,5-triiodobenzoic acid or PCIB, has frequently been used to alter the activity or balance of endogenous plant growth regulators. These inhibitors have been successfully used to reduce the proliferation of ECM of *A. nordmanniana* where PCIB, in association with ABA, promotes the development of mature embryos (Find et al., 2002). Furthermore, anti-auxins are able to significantly decrease the amount of endogenous indolacetic acid in *P. morrisonicola* embryogen lines (Liao et al., 2008), and the presence of an inhibitor of auxin polar transport during early embryo development leads to abnormal embryo formation (Larsson et al., 2008). Therefore, the auxin level and its polar transport seem to be crucial factors able to modulate the development of PEM and, as a consequence, the final embryo formation.

This work also shows how FA was able to influence the growth and development of ECM of *A. cephalonica*. These effects may be explained through the ability of FA to act as an auxin-like molecule, although the inherent mechanism is still not completely understood. While the auxin-like activity of humic matter has been since long hypothesized, it is still under debate, since other authors have refuted this possibility and proposed alternative explanations for the direct and indirect physiological effects of humic matter (Varanini and Pinton, 2001; Chen et al., 2004). Nevertheless, recent evidence indicates that some morphological and biochemical parameters are influenced by HS, which seem to mimic the effect of natural auxins. In particular, humic acid enhanced root elongation, lateral root emergence (Canellas et al., 2002, 2009, 2010) and, as also shown in this work by FA (Fig. 6), the activity of membrane-bound proton pumps such as ATPase and PPhase (Canellas et al., 2002, 2009, 2010; Zandonadi et al., 2007). The involvement of these proton pumps, which are capable of generating an electrochemical gradient to energize the secondary transport and maintain a high cell osmotic pressure, also suggests a possible role of humic materials in the cell expansion process. Indeed, when FA was added after incubation with PCIB, a significant increase in both growth (Fig. 5, panel C) and proton pumping activities was observed (Fig. 6).

It has been reported that these effects are concomitant with an increase of the oxalic and citric acids efflux that, in turn, favors a modification of structural conformation of humic acids (Canellas et al., 2008). The observed effects of FA on ECM proliferation rate (Fig. 5, panels B and C), proportion of different developmental aggregates, and proton pumping activities in microsomes (Fig. 6) are consistent with the hypothesis that small molecules present in the humic complex structure could interact with the plant hormonal signaling pathway (Canellas et al., 2002, 2009, 2010). The hydrophilic, conformationally loose FA appeared to be active, thereby suggesting that a combination of properties may confer a specific bioactivity to humic structures. In particular, the substantial structural flexibility and heterogeneous composition of FA is likely to release the mobile hormone-like molecules, which may enter the biological pathways related to auxin activities.

Therefore, we suggest that some molecular components released by FA may succeed in crossing the cell wall (Muscolo et al., 2007) and interfere with the auxin metabolism, either directly or indirectly, thus inducing significant changes in both the proliferation and maturation processes. This interpretation is corroborated by the counteraction exerted by FA with respect to PCIB, which was able to inhibit the proliferation in embryogenic tissue cultures of *A. nordmanniana* (Find et al., 2002). Indeed, PCIB seems to act as an auxin antagonist, enabling reduction of the activity of endogenous auxins by competitive binding to the TIR/Aux auxin receptor (Find et al., 2002; Oono et al., 2003). We suggest that the observed effects of FA on proliferation and maturation of ECM of *A. cephalonica* are linked to their auxin-like activity, similarly to what has been previously observed for low molecular weight HA (Piccolo et al., 1992; Nardi et al., 1994; Muscolo et al., 1999, 2007; Canellas et al., 2009, 2010). Nevertheless, the precise mechanism by which FA are bioactive, by either influencing auxin transport and signaling pathway or affecting alternative hormonal pathways, is still to be elucidated. New research should also be conducted to isolate different FA fractions where to identify the molecule(s) possessing specific biological activity. The identification of such “natural” molecules that induce cell mass increase during the proliferation phase, may...
be useful to enhance the amount of such cells and promote the following maturation phase of somatic embryos.

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References