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Full Length Research Paper

Osmotic potential of *Zinnia elegans* plant material affects the yield and morphology of tracheary elements produced *in vitro*

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The *Zinnia elegans* cell suspension culture is excellent for xylogenesis studies at the cellular and molecular level, due to the high and synchronous *in vitro* differentiation of tracheary elements (TEs). The percentage TE differentiation (%TE) in the culture is, however, influenced by a number of factors before and during cell differentiation. One of the factors that is potentially important but has not gotten much attention is the initial osmolarity of the plant material. To examine whether the growth conditions that determine leaf osmolarity (LO) affect the final %TE, we used three light intensities (50, 70 and 100 µmol.m^{-2}s^{-1}) and three electrical conductivity (EC) levels (EC 2, 4 and 6 dS.m^{-1}) in hydroponic systems to induce different osmolarities in leaf materials from two cultivars (cvs) of *Z. elegans*, Envy and Purple Prince. The isolated leaf mesophyll cells were subsequently cultured in a liquid medium (300 mOsm extracellular osmolarity) containing α-naphthalene acetic acid (NAA) (1 mg.l^{-1}) and benzylaminopurine (BA) (1 mg.l^{-1}). The LO increased in both *Zinnia* cvs with increasing light intensity and increasing EC during growth. Mesophyll cell size correlated negatively with EC, but the correlation was positive with light intensity in both *Zinnia* cvs. There was an overall positive correlation between %TE and LO although the degree of %TE change versus LO differed between light and EC treatments and also between the two *Zinnia* cvs. Envy cv is the best known *Z. elegans* cv for establishing xylogenic cultures. However, it turned out that by subjecting the plants to different growth conditions, the Purple Prince cv produces a higher %TE as compared to the Envy cv. At EC4 the TE differentiation for the Purple Prince cv was 75%, a level that is 25 to 60% higher than those earlier reported. We conclude that light intensity and EC of the root environment affect the LO of *Z. elegans* which in turn influences the development and therefore dimensions of TEs in an *in vitro* xylogenic culture. Thus, proper optimization of the growth conditions for the *Zinnia* plants prior to establishment of xylogenic cultures leads to enhancement of *in vitro* TE formation.

Key words: Electrical conductivity, *in vitro* culture, leaf osmolarity, light intensity, osmotic potential, tracheary element, xylogenesis, *Zinnia elegans*.

INTRODUCTION

Xylogenesis is an important research area in plant science, due to the significance of the vascular development in plant performance (Fukuda, 1996). All higher plants rely on the vascular system for long distance transport of water and minerals and the photosynthetically manufactured sugars. The xylem is an important component of the vascular system, composed mainly of conduits built from stacks of tracheary elements (TEs).

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These TEs are derived from procambium or cambium cells consisting of dead, hollow and lignified cellulose cell walls with local secondary thickenings. Fusion of these TEs into conduits allow both horizontal and vertical water conductance necessary for the transport of water and minerals from the soil to the leaves (Comstock and Sperry, 2000). The involvement of programmed cell death (PCD), a part of the xylogenesis process, is under intense study to understand the well orchestrated death of cells necessary for development of a specific functional structure. In xylogenesis, PCD is the last process that facilitates the onset of autolysis, a final stage of TE differentiation in the xylem (Fukuda, 1996; McCann, 1997; Fukuda, 2000; Roberts and McCann, 2000).

For decades, xylem formation has been vigorously studied in many plants (Mellerowicz et al., 2001; Ye, 2002). Due to the complexity of the whole plant system, *in vitro* techniques have been employed to unravel the physiology and biochemistry of secondary cell wall and lignin biosynthesis at the cellular level (Pesquet et al., 2003). In the past 50 years, many callus and single cell cultures from several plant species have been adopted for xylogenesis studies (Yen and Yen, 1999). Introduction of the *in vitro* xylogenic *Zinnia elegans* mesophyll cell suspension culture approximately two decades ago has revolutionalized our understanding of the xylogenesis process (Fukuda and Komamine, 1980a; Pesquet et al., 2003). A comparable xylogenic culture has been established from *Arabidopsis thaliana* (Oda et al., 2005). However, the *Arabidopsis* culture requires further technical optimization to improve the efficiency (currently %TE of 30 is possible) and the degree of synchronization during TE differentiation. Therefore, the xylogenic *Z. elegan*s mesophyll culture, for now, remains the best alternative for xylogenesis studies. It has a higher degree of synchronicity and also efficiency in TE differentiation (Church and Galston, 1988). Such a system is not only suitable for cell biological studies but also for molecular genetic characterization of these cellular processes. At the moment a large genetic library/expressed sequence tags (ESTs) from the xylogenic *Zinnia* culture is available (Pesquet et al., 2005).

In spite of the superiority of the *Z. elegans* culture system, there have been huge inconsistencies in the final yield of TEs as reported by different authors (Church and Galston, 1988; Gabaldon et al., 2005a; Tokunaga et al., 2005; Oda and Hasezawa, 2006). These differences appear to be partly caused by the extracellular osmolarity of the culture medium in relation to that of the mesophyll cells or the leaf material (Lee and Roberts, 2004). One other potentially important factor to consider is the initial intracellular osmotic potential (OP) or leaf osmolarity (LO) of the leaf material that is used for establishing the xylogenic culture. Osmotic preconditioning of the plant material by certain environmental conditions during the growth period of the *Zinnia* plants may affect the osmoregulation of the isolated mesophyll cells in the culture with effect on the differentiation of the TEs. This could mean that, the initial LO or leaf cell sap osmolarity must be well controlled to maximize the degree of synchronicity of TE differentiation coupled with superior dimensional characteristics of TEs formed.

The intracellular OP of a plant is largely affected by plant water status, which is influenced by transpiration and physical water availability and salinity in the root environment (soil EC). The accumulation of the soil water salts (enhanced by transpiration) and photosynthetically synthesized organic compounds (sugars and proteins) in the plant tissues increase the local or systemic OP of the plant (Vaadia et al., 1961; Barlow et al., 1977; Meyer and Boyer, 1981; Morgan, 1984; Boyer and Silk, 2004). Thus, application of different soil ECs and/or imposition of different transpirational pressures via light intensities on the growing plants will have consequent effects on tissue osmolarities in the plant tissues.

In this paper, we present the results of the effect of different LO's, as induced by three different light treatments in soil-grown plants, and three different electrical conductivity (EC) treatments in hydroponic plants of two *Z. elegans L.* cultivars (cvs), Envy and Purple Prince, on the *in vitro* differentiation of TEs in suspension cell cultures.

MATERIALS AND METHODS

Plant material

Light treatment

Seedlings of the two *Z. elegans* cvs, Envy and Purple Prince, were germinated and allowed to grow in peat-based commercial potting compost (Lentse Potgrond nr. 4; 85% peat, 15% clay, Lentse Potgrond, Lent, Netherlands) in four different compartments in climate rooms at Horticultural Production Chain Group of Wageningen University and Research Centre, Netherlands, set at 16 h light and 8 h darkness with 25/20°C temperature, respectively. The relative humidity was maintained at 70% throughout the growth period. Each of the three compartments was given different light levels of 50, 70 and 100 $\mu mol.m^{-2}.s^{-1}$, respectively. Higher light intensity could not be used due to the burning effects on the seedlings. Constant amount of water was supplied to maintain the soil water content at 70% (v/v). Fully expanded first set of leaves from the seedlings was harvested between 13 and 15 days after sowing for leaf sap osmolarity measurements and mesophyll cell isolation to establish the cell culture.

EC treatments

Seeds of the two *Z. elegans* cvs were sown in cotton plugs in a hydroponic system in a climate room. The system consisted of 30 x 50 x 10 cm trays containing rockwool plugs, partly (0.5 cm depth) immersed in (or floating on) the liquid growth medium set at the different ECs (2, 4 and 6 dS/m). Further conditions were a 16 h light and 8 h darkness period, d/n temperatures of 25 and 20°C, 70% relative humidity (RH) and a liquid medium pH of 5.5. Application of a water pump (250 to 300 L/h) ensured even distribution of the solutes in the growth medium. The first true leaves (FTL) were harvested at H_t (harvest-time at which only FTL are present and fully expanded) for LO measurements and mesophyll isolation.

Leaf osmolarity measurements

Eight randomly selected pairs of FTL of seedlings per cv per treatment (from both light and hydroponic treatments) were sampled for the LO measurements using a Vapro® Vapor Pressure Osmometer (model 5520, Wescor Inc., Logan, UT, USA). Each set of leaves was separately folded into a 4 x 4 cm nylon mesh of pore size µm. By using a Zwick Press machine (Zwicki 1120, Zwick, Germany), the leaves in the nylon mesh were pressed individually against the bottom of an Eppendorf tube. The sap from the pressed leaves was collected in a new Eppendorf tube and from this two samples of 10 µl sap extract were independently measured in the osmometer and averaged.

Leaf mesophyll isolation and culture

Approximately 30 pairs of FTL were harvested and surface sterilized in cold (4 °C) 0.15% NaOCl solution containing 0.001% Triton X-100 for 10 min. The mesophyll cells were isolated mechanically and purified as described by Sugiyama and Fukuda (1995), with few modifications (Twumasi et al, 2009). The osmolarity of the culture medium was adjusted to 300 mOsm by addition of D-mannitol. The cell density in the final culture was adjusted to 10^5 cells/ml in the culture medium. Four samples of 3 ml each of the cell suspension per treatment were cultured in six-well culture plates (Sigma; n = 4 independently cultured wells). Induction of TE differentiation in the cultures was achieved by exogenous application of α-naphthalene acetic acid (NAA) (1 mg/l) and benzylaminopurine (BA) (1 mg/l) at the onset of the mesophyll cell isolation (t = 0 h). The control experiments were devoid of these growth regulators. The cultures were incubated in the dark at 25°C on a rotary shaker at 100 rpm speed.

Microscopy of TE differentiation and size measurements

The viability of the mesophyll cells was determined by staining with 0.005% (final concentration) of fluorescein diacetate (FDA). Observation of the yellow fluorescent cells was done by the use of a Diaphot-Nikon fluorescent microscope (Zeiss, Netherlands) equipped with a UV lamp, 488nm filter and a LCD Kappa DX 20L camera. In addition, a Nikon Diaphot TMD 200 inverted microscope, equipped with differential interference contrast (DIC) optics and a Sony 3CCD color video camera, was used for the direct monitoring of cellulose band formation in the differentiating cells. Image J software (Wayne Rasband National Institutes of Health, USA) was used for processing and measuring the physical parameters (length and area) of the differentiated TEs from the microscopic images. The yield of TEs or %TE was calculated as the percentage of mesophyll cells that had differentiated at 120 h after induction with growth regulators, with a correction factor for the initial dead cells in the culture. Thus, %TE was calculated as:

$$\%TE_{actual} = \frac{100\alpha}{\beta\gamma}$$

Where, $\%TE_{actual}$ is the true yield of TEs caused by only the viable mesophyll cells in the initial culture, α is the number of TEs counted in a representative fraction of the culture, β is the total number of differentiated and non-differentiated (dead or live) cells in the sample fraction and γ is the initial fractional viability of the culture sample.

Statistical analysis

All experiments were accomplished in triplicate. For every cell culture, a minimum of 30 cells or tracheary elements that were randomly chosen were used for calculations. Analysis of variance for the effects of different light, EC, and cv (genetic) on the final yield of TEs in the suspension cultures were performed by Prism v.4 (GraphPad Software, Inc.). For each analysis, a significance level of 5% was maintained.

RESULTS

Electrical conductivity and light intensity effects on plant morphology and leaf osmolarity

H_t increased with decreasing light intensity, ranging from 3 to 5 days earlier in 100 µmol.m^{-2}.s^{-1} light intensity compared with the 50 or 70 µmol.m^{-2}.s^{-1} light intensities (Figure 1A). Purple Prince plants were larger than Envy at all the three light treatments. In the EC treatments, H_t increased with increasing EC in the root environment (Figure 1B). Generally, the leaf area increased with decreasing EC in the hydroponic media and the plants growing in EC2 (2dS/m) medium were harvested 1 to 5 days earlier than the plants growing in EC4 growth medium. Plants from the Purple Prince cv grew slightly faster and larger than the Envy cv at all the three EC treatments. A minimal change in mesophyll cell size by differences in EC of the growth media was observed in both *Zinnia* cvs (Figure 2A and B). Significant differences in LO within cv were found only between EC6 and EC2 or EC4 in both *Zinnia* cvs (Figure 2C). LOs were different between the two cv at ECs 2 and 6 (dS/m) where in each case Envy cv recorded higher LO over the Purple Prince cv. An increase of medium EC from 2 to 6 caused 32 and 23% increase in LO of Envy cv and Purple Prince cv, respectively.

The effect of light intensity on LO and mesophyll cell size (length and area) is shown in Figure 3. The Envy cv acquired the highest mean mesophyll cell size in plant materials obtained from the 7 µ m^{-2}.s^{-1} illumination, while in Purple Prince cv the highest mean mesophyll cell size was associated with the 100 µ m^{-2}.s^{-1} illumination (Figure 3A and B). The mean LO was highest in leaf materials from 100 µ mol.m^{-2}.s^{-1} illumination (Envy, 303.0 mOsm; Purple Prince, 276.5 mOsm) and lowest in 50 µ m^{-2}.s^{-1} illumination (Envy, 261.4 mOsm; Purple Prince, 260.1) in both *Zinnia* cvs (Figure 3C). Again, the Envy cv recorded higher mean LO at 100 µ mol.m^{-2}.s^{-1} illumination as compared with the Purple Prince cv. An increase of light intensity from 50 to 100 µm^{-2}.s^{-1} caused the LO to increase by 17 and 3% in Envy cv and Purple Prince cv, respectively (Figure 3C).

Tracheary element differentiation and dimensions

EC treatment

DIC images of the differentiated TEs at 120 h from EC2 and EC6 treatments in both *Z. elegans* cvs are shown in

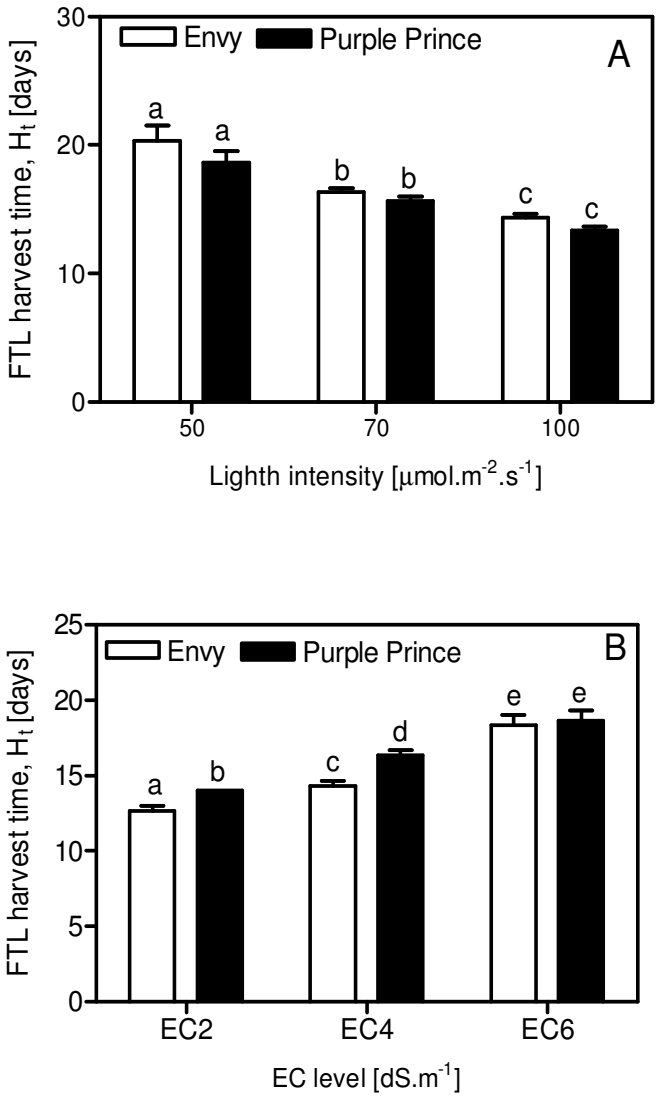

Figure 1. The effects of different (A) light intensities and (B) root medium electrical conductivities on the harvest time (H_t) for the expanded FTL in two *Z. elegans* cvs. Different letters indicate statistically significant differences tested at 5% probability level. (N = 60 seedling x 3 repetitions; bar = SEM).

Figure 4A. In both cvs, the TE size increased proportionally with the level of EC in the root environment (Figure 4B II and III). There were generally no differences in TE sizes (length and area) between Envy and Purple Prince at the three EC treatments. However, significant differences in %TE were found between Envy and Purple Prince at EC 2 and 4 (Figure 4B I). At EC 4, the Purple Prince cv gave the highest %TE of 75% (Figure 4B I). An increase in LO osmolarity by 32% in Envy and 23% measured in Purple Prince produced 68 and 66% increments in TE differentiation, respectively (Figure 4B I). There was a strong interaction between the genotype of *Z. elegans* and the EC treatments with respect to TE differentiation.

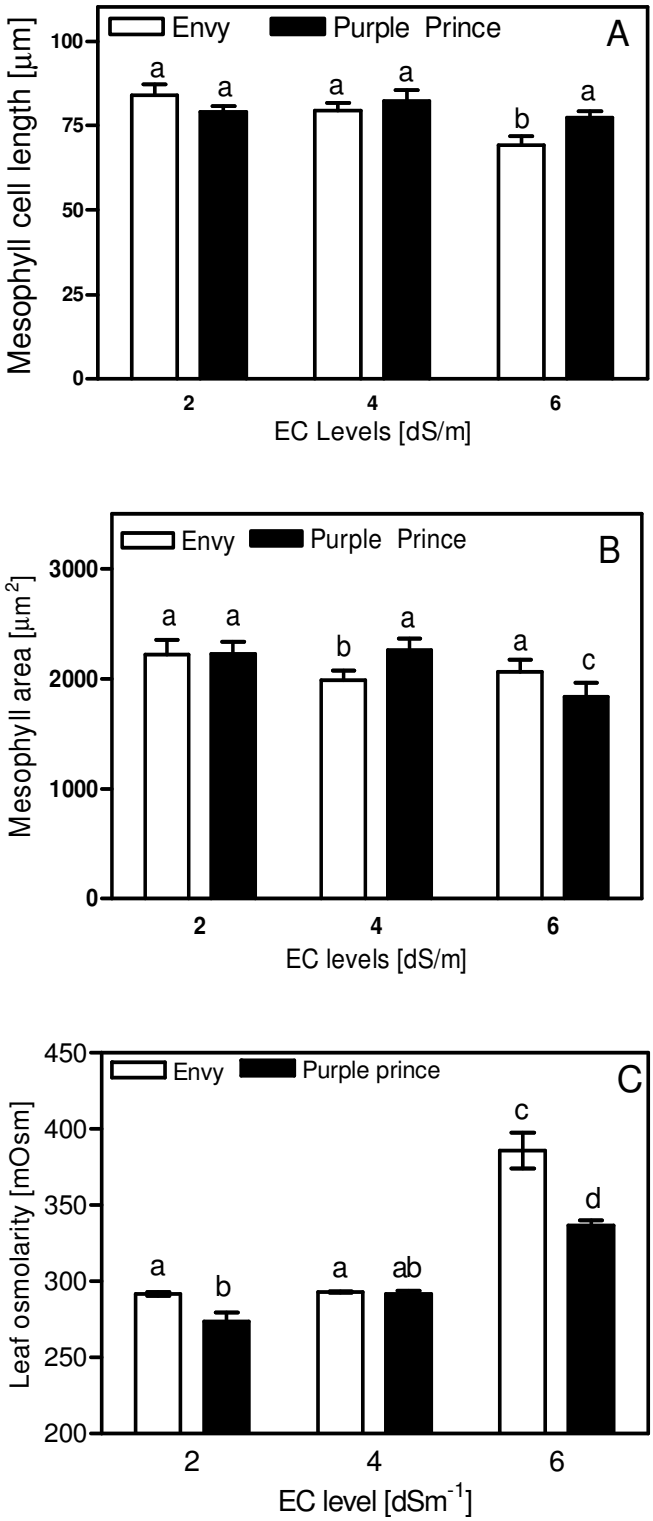

Figure 2. Effect of EC in the root environment on the leaf mesophyll size (A, length; B, area) and LO (C) in two cvs of *Z. elegans* seedlings at H_t. Different letters indicate statistically significant differences. LO, Leaf osmolarity; H_t = harvest time for expanded FTL without second set of leaves, bar = SEM, N = 8 for LO measurements; N = 30 for mesophyll cell size measurements.

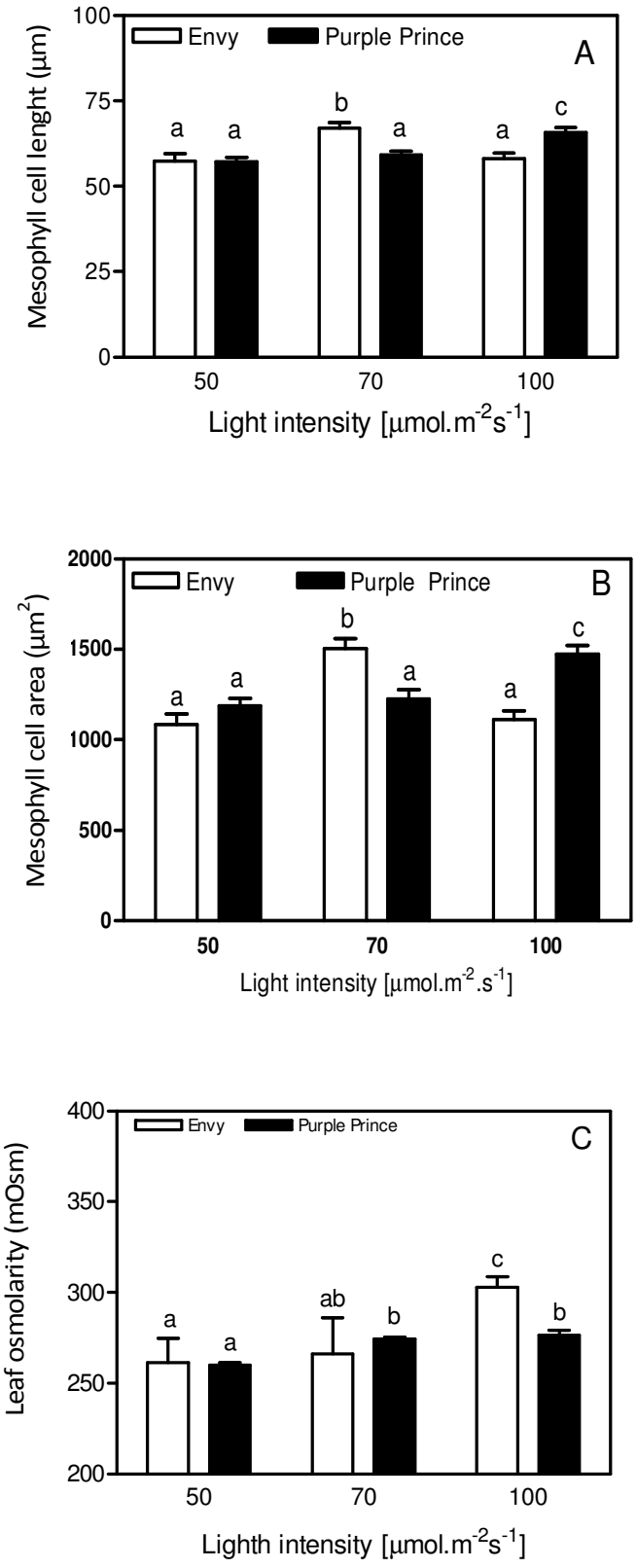

Figure 3. Effect of different light intensities on the leaf mesophyll size (A, length; B, area) and LO (C) in two cvs of *Z. elegans* seedlings at H_t. Different letters indicate statistically significant differences. Bar = SEM, N = 8 for LO measurements; N = 30 for mesophyll cell size measurements.

Light treatment

The DIC images of differentiated TEs at 120 h are shown in Figure 5A. Higher %TEs were observed in the xylogenic mesophyll cultures derived from plant materials grown with 100µmol.m^{-2}-s^{-1} illumination as compared with the 50 or 70µmol.m^{-2}-s^{-1} in both *Z. elegans* cvs (Figure 5B I). Thus a positive correlation is found between light intensity provided during growth of the plants and the final %TE in suspension cultures from both *Z. elegans* cvs. It was found that an increase in LO osmolarity by 17% in Envy and 3% in Purple Prince with light caused an increase of about 116 and 130% TE differentiation, respectively. The light intensity used in the glass house did not significantly affected the TE yield in both *Zinnia* cvs, therefore indicating minimal effect of genetic diversity on TE formation.

Anatomical measurements indicated a positive correlation between TE size (area and length) and light intensities used in growing plants of the two *Z. elegans* cvs from which *in vitro* cultures were established (Figure 5B - II and III). Furthermore, visual observations during *in vitro* TE differentiation indicated an earlier and more rapid differentiation in mesophyll cell cultures originating from leaf materials of LO equal or slightly above the 300 mOsm extracellular medium osmolarity. Generally, the %TE increased with increasing LO above that of the medium, irrespective of the method used for inducing this higher LO in both *Z. elegans* cvs. At all light and EC levels, the LO was higher in the Envy cv as compared with the Purple Prince cv.

DISCUSSION

Due to the suitability of the xylogenic *Z. elegans* cell suspension culture for cytological and molecular research, lots of efforts have been focused on improvements in establishing this system for various applications (Lee et al., 2000; McCann et al., 2001; Lee and Roberts, 2004; Shinohara et al., 2006; Tokunaga et al., 2006). The obvious expectation from such improvements is to achieve higher percentage and more synchronous TE differentiation. Unfortunately, the majority of these improvement techniques are being focused on the xylogenic cell suspension culture itself, that is, processes occurring after isolation of the leaf mesophyll cells. Almost none of these improvements involve preparation of the plant materials. For instance, there was no work about the effect of growth conditions during plant development on the performance of the xylogenic culture established from the mesophyll cells. A unique property of the xylogenic *Zinnia* suspension cell culture is the lack of a mother culture from which an adequate TE differentiation can be maintained. Thus, the originally developed mesophyll cells isolated from the leaves are directly differentiated into the TEs in the culture. This implies that, any influence

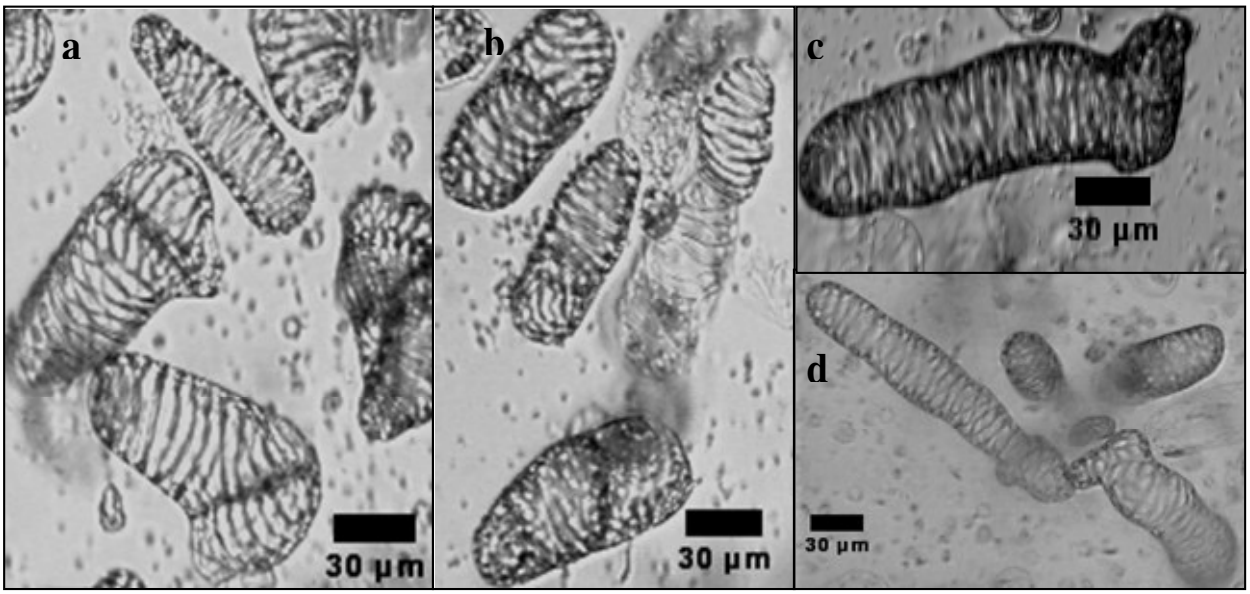

Figure 4A. DIC images of differentiated TEs in suspension cell cultures from plants of two Z. elegans L. cvs, Envy (a, d) and Purple prince (b, c) grown with media with EC 2 (a, b) and EC 6 (c, d). (Bar = 30 µm).

of the developmental process of the mesophyll cells *in planta* will have consequences on the performance of the xylogenic *Zinnia* culture established from these cells. A factor with potential effect on the performance of the xylogenic culture is the OP or the LO of the plant material. Lee and Roberts (2004) have shown that due to the differences in internal osmolarity of the isolated mesophyll cells, high heterogeneity in TE differentiation exists in xylogenic *Zinnia* cultures. To prevent these inequalities in OP, a choice of growth conditions aimed at adjusting the LO is necessary.

We found in our study that growing plants with low light intensity delays the harvest time for the leaves required for mesophyll cell isolation. A similar effect was found when the level of EC in the growth medium was increased. Apart from the reduction in leaf area caused by an increasing EC, the leaf mesophyll cell viability, shape and size were neither affected by light nor EC treatment in both *Z. elegans* cvs. This is an especially important consideration since the quality of mesophyll cells influence the TE differentiation process (Gabaldon et al., 2005b). Even with such low light levels, a small increase in intensity was enough to induce higher LO. Similarly, the LO increased with increasing EC in the growth medium in both Envy cv and Purple Prince cv. However, in the LO measurements, the response of Envy cv to LO change caused by changes in the growth conditions was higher as compared to the Purple Prince cv. By increasing light intensity, transpiration of the plant is increased as the stomatal conductance increases (Morgan, 1984; Wei et al., 1999). Two processes could be identified for this increase in LO by the light intensity: (1) The photosynthetic rate that correlates with light intensity leads to synthesis and accumulation of sugars in the plant tissues especially in the leaf tissue causing elevation of the intracellular OP (Flesch et al., 1991); (2) transpiration rate is dependent on light intensity. Thus, a higher light intensity means an increase in the uptake of water together with dissolved minerals. While the water continuously evaporates into the atmosphere via the stomata, the mineral components accumulate in tissues resulting in higher intracellular OP (Vaadia et al., 1961).

In both *Z. elegans* cvs, the increase in LO by either light or EC, resulted in an increase in the percentage of TEs formed in the culture after induction with the phytohormones (1 mg/l NAA and 1 mg/l BA). In the Envy cv, an increase in the light intensity from 50 to 100 µ $mol.m^{-2}.s^{-1}$ caused 17% increase in LO which resulted in about 116% increase in TE differentiation. In Purple Prince, however, only 3% increase in LO was achieved, but such a minor osmotic change caused the %TE to rise by about 130%. Also, in the EC treatment, an increase from 2 to 6 dS/m achieved 32 and 23% increase in LO in Envy and in Purple Prince, respectively. The corresponding increase in %TE was 68 and 66% in Envy cv and Purple Prince cv, respectively. Thus, although the LO achieved by the two sets of growth conditions (that is, light intensity and EC) were lower in Purple Prince as compared to the Envy, the corresponding increase in TE differentiation was even higher in the Purple Prince cv. It is intriguing to see that despite the differences in LO in response to changes in the growth conditions in the two cvs of *Z. elegans*, a similar change was observed in the final %TE in the culture. Thus, an increase in the overall LO osmolarity does not necessarily determine the final TE differentiation. This suggests that other cellular

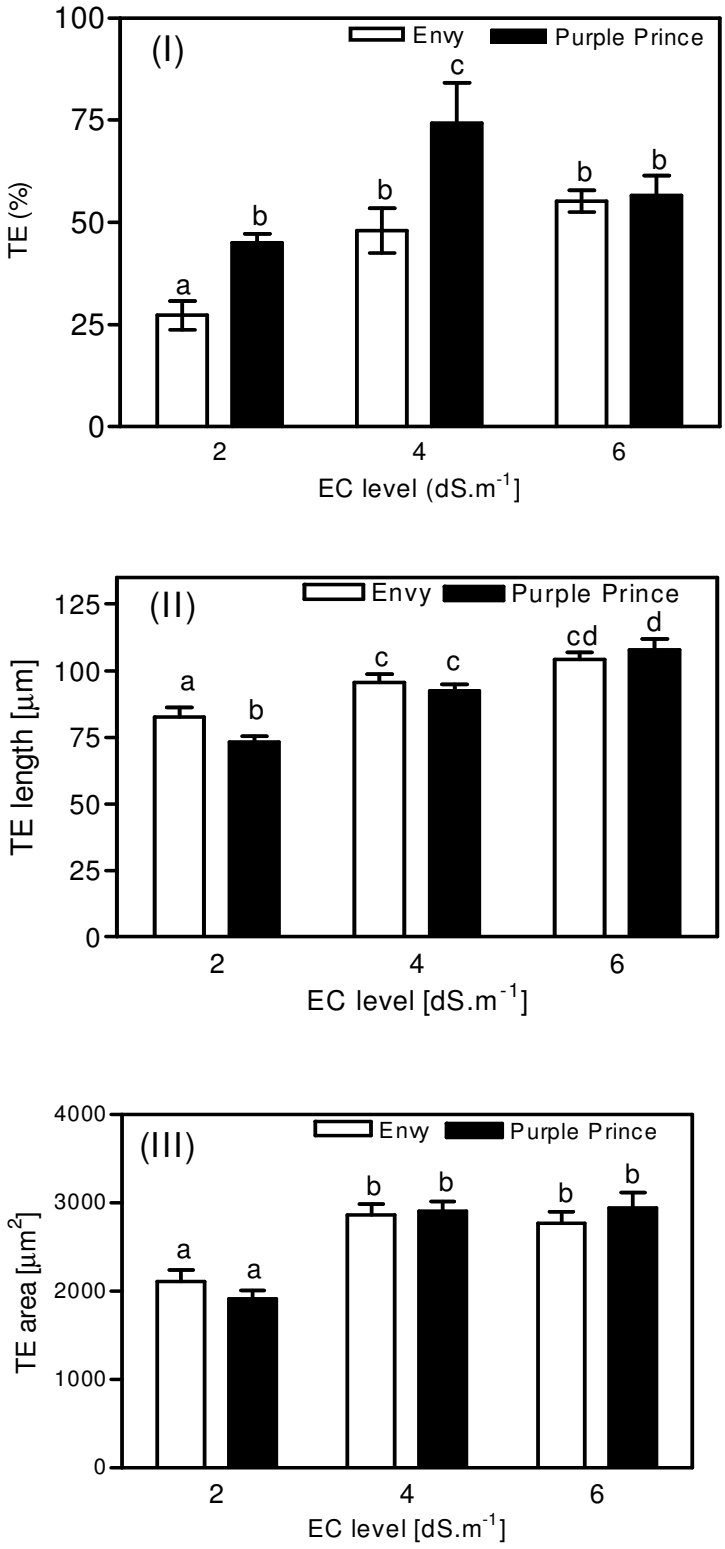

Figure 4B. Effect of different EC in the root environment during growth periods of two *Z. elegans L.* cvs on the (I) final percentage TE differentiation, (II) TE length, and (Jacobsen et al. 2005) TE area in an *in vitro* mesophyll cell cultures measured at 120 h after TE induction with NAA (1 mg/l) and BA (1 mg/l). Values represent the mean of 3 replicates. Different letters indicate statistically significant differences. Bar = SEM, N = 100 or more TEs from 3 independent culture repetitions.

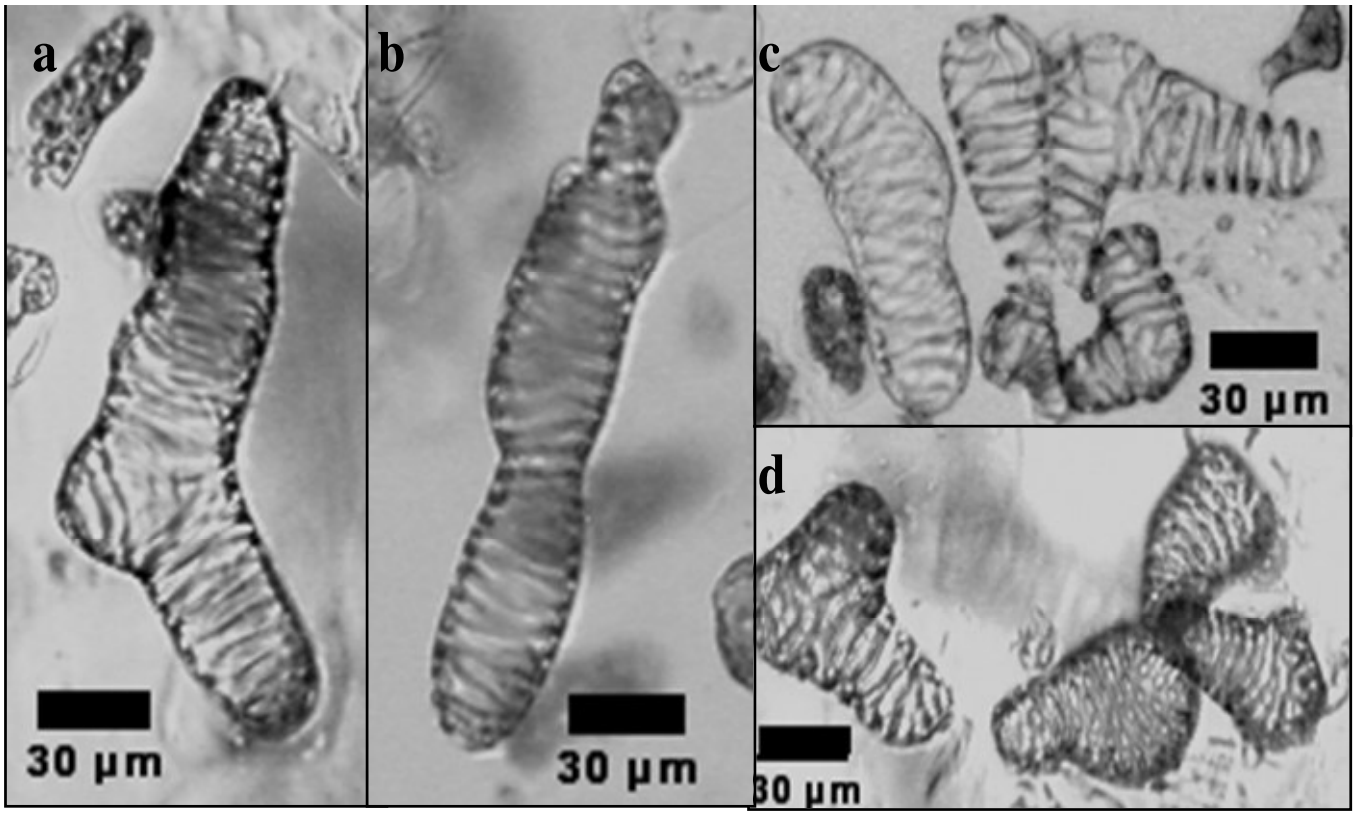

Figure 5A. DIC images of differentiated TEs in suspension cell cultures from plants of two *Z. elegans L.* cvs, Envy (a, d) and Purple Prince (b, c) grown with 50 µ mol.m^{-2}.s^{-1} (c, d) and 100 µ mol.m^{-2}.s^{-1} (a, b) of light intensity in climate rooms. (Bar = 30µm).

processes or factors with effects on TE differentiation might be influenced by these growth conditions.

The observed increase in final %TE by the preconditioning of the materials might be a result of establishing osmotic equilibrium across the cell membrane, that is, intracellular environment in relation to the extracellular medium OP, with an associated minimal cell expansion that can delay TE differentiation (Lee and Roberts, 2004). On the other hand, these treatments applied during plant development eliminated the osmotic differences commonly found between cells especially at different developmental stages or in different locations in the same tissue (Molz and Boyer, 1978). The highest %TE was from cv Purple Prince at EC 4 (75%). This is 25 to 60% higher than those earlier reported (Lopez-Serrano et al., 2004; Oda et al., 2005; Tokunaga et al., 2005). Why this could not be improved further, for example at EC 6 dS/cm, might be a result of the cellular homeostasis which prevents extreme OP (Romero, 2004). Although our results show that an increase in OP in the cells using growth conditions improves %TE differentiation, specific growth conditions are necessary for each kind of *Zinnia* genotype.

Comparison of the length and area measurements in TEs from various cultures established from leaf mesophyll cells at different LOs showed a positive correlation between LO and TE size. This suggests that TE initials with higher OP initially undergo extensive expansion to reach a new OP which is equal to the OP of the xylogenic medium (extracellular OP) before the phytohormones could induce TE differentiation. Extreme growth conditions were avoided in our experiments, but it is expected that large changes in OPs of the mesophyll cells as compared to the extracellular osmolarity of the growth media will negatively affect the performance of the xylogenic culture. Expected excessive explosions or plasmolysis of the mesophyll cells in such a culture could counter the positive effect of growth conditions on TE differentiation. It is therefore, established from this study that plant growth conditions have effects on *in vitro* tracheary element differentiation of *Z. elegans* mesophyll cells, and that initial leaf osmolarity is an important factor to be considered in the differentiation process.

Abbreviations

BA, Benzylaminopurine; **cv,** cultivar; **DIC,** differential interference contrast; **EC,** electrical conductivity; **FTL,** first true leaves; **H_t,** harvest time; **LO,** leaf osmolarity; **NAA,** α-naphthalene acetic acid; **OP,** osmotic potential; **PCD,** programmed cell death; **TE,** tracheary element;

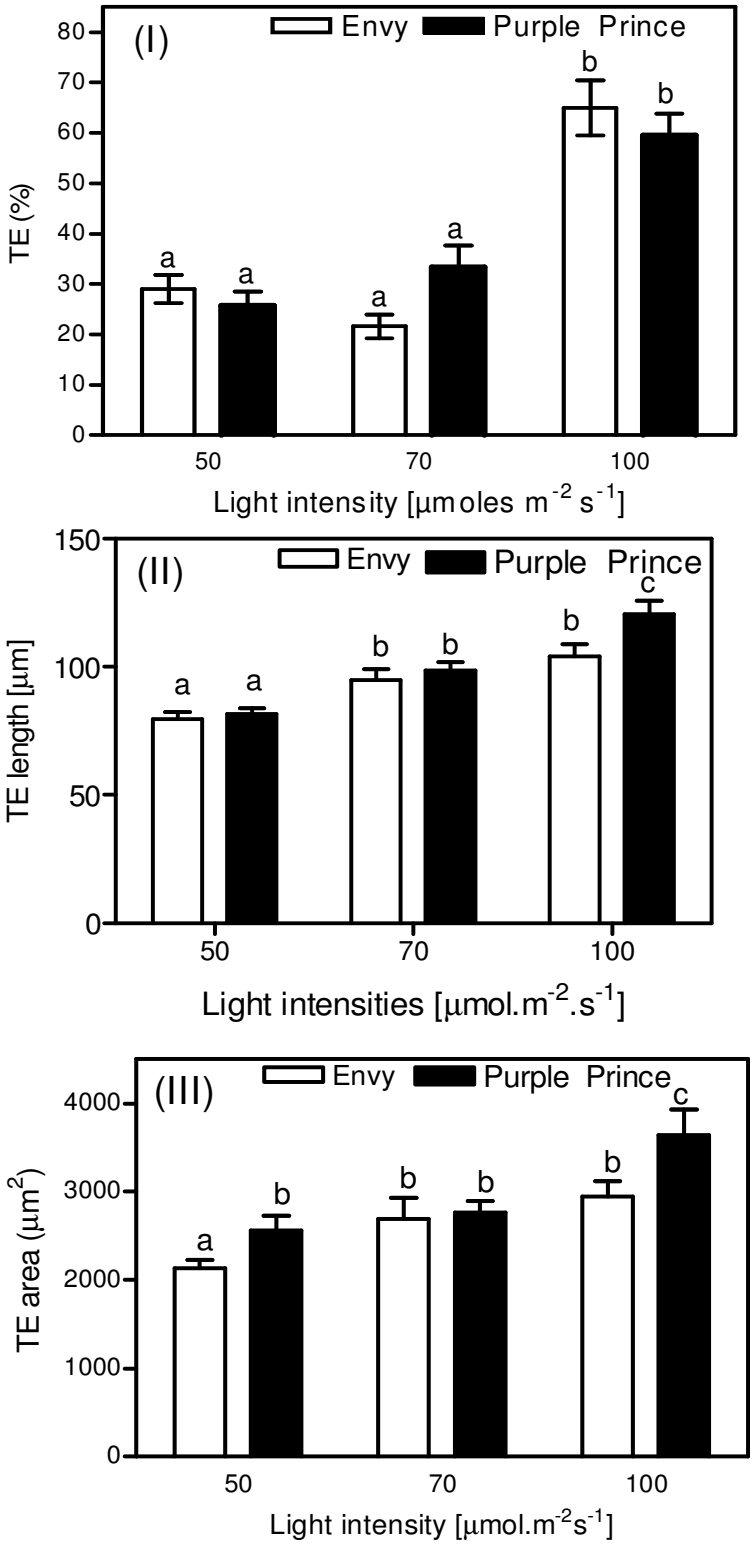

Figure 5B. Effect of different light intensities during growth periods of seedlings of two *Zinnia elegans L.* cvs on (I) final percentage TE differentiation, (II) TE length, and (III.) TE area in *in vitro* mesophyll cell cultures measured at 120 h after TE induction with NAA (1 mg/l) and BA (1 mg/l). Values represent the mean of 3 replicates. Different letters indicate statistically significant differences. Error bars = SEM, N > 100 total cells from 3 independent wells.

ESTs, expressed sequence tags; **FDA,** fluorescein diacetate.

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