ELEMENTAL ANALYSES OF CALLI AND DEVELOPING SOAMATIC EMBRYO OF HYBRID *LIRIODENDRON*

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Abstract

Somatic embryogenesis is a complex biological process in which somatic cells transform into an embryonic state. Understanding the mineral nutrition requirements is important to optimize development and growth of somatic embryos. In this study, we used inductively coupled plasma-optical emission spectroscopy to compare the key elemental profiles of tissues of *Liriodendron chinense × Liriodendron tulipifera* hybrids during the induction and expression phases of somatic embryogenesis. The maximum levels of Ca, Fe, Mn, and Al were detected on day 9 of embryo development. Phosphate and Zn accumulations peaked at day 13. Sodium content was highest on day 5 and sharply decreased at day 9. Potassium content showed three peaks; on day 5, 13, and 20. Boron was only detected on day 5 during somatic embryogenesis. High levels of Cu were detected on days 5 and 9. The Mg, Ca, K, Mn, Fe, and Al levels were higher in embryogenic callus than in non-embryogenic callus; Cu levels were higher in non-embryogenic callus than in embryogenic callus. Information about the mineral composition of calli provides important background data to improve existing media by adding minerals that favor the induction of embryogenesis and embryo development. This will be useful for clonal propagation of *L. chinense ×L. tulipifera* hybrids.

Key words: Elemental analysis, *Liriodendron chinense × Liriodendron tulipifera*, Embryogenic callus, Non-embryogenic callus, Somatic embryogenesis.

Introduction

The genes *Liriodendron* belongs to the family *Magnoliaceae* is a genus comprising two species, *Liriodendron chinense,* which is native to China and Vietnam, and *Liriodendron tulipifera,* which is distributed in the eastern United States (Smith *et al.*, 2004; Van Stan & Levia, 2010). A hybrid between these two species was first bred by Prof. PeiZhong Ye and colleagues in 1963, in China. Chemical analyses of interspecific crosses between *L. chinense* and *L. tulipifera* were first reported in 1972 (Santamour, 1972). Hybrid *Liriodendrons* are very useful trees both for commercial plantations and urban landscaping, because they grow rapidly, are resistant to pests and diseases, produce strong wood, and are attractive ornamental trees (Ye & Wang, 2002; Li *et al.*, 2012). However, the expansion of *L. chinense × tulipifera* plantations has been restricted because of a low of high-quality seeds. Somatic embryogenesis is an alternative strategy to produce materials for propagation of species with long reproductive cycle or shortage seed set (Garshasbi *et al.*, 2012; Smertenko & Bozhkov, 2014). Despite the tremendous progress made in developing somatic embryogenesis methods and in industrial production of seedlings from somatic embryos, many problems still remain. Such problems include low conversion frequencies, malformed somatic embryos, asynchronous development, and failure to reach maturity (Dai *et al.*, 2004; Hussain *et al.*, 2009).

Development of somatic embryo are controlled by nutritional, osmotic, hormonal, and gas environments surrounding. Hence, it is critical to optimize the culture environment for successful growth and development of high-quality embryos (Pullman & Buchanan, 2003; Desai

et al., 2006). In tissue culture, the composition of the culture medium is one of the most important factors affecting the growth and development of plant materials. The mineral elements are one of the most basic components for plant tissue culture (Niedz & Evens, 2008; Abbas *et al.*, 2013). The mineral nutrients have an important factor for callus induction and are necessary nutrients for embryogenic differentiation and development (Kintzios *et al.*, 2001), and they are required for many enzymatic reactions associated with various physiological and biochemical processes. Minerals also affect cell sensitivity and interact with plant growth regulators to affect somatic embryogenesis (Ramage & Williams, 2002). Changes in the medium parameters in *In vitro* cultures can affect the make-up, structure, and ultimately the functional processes in plant tissues (Saha *et al.*, 2011). During somatic embryogenesis, explants are induced to form callus tissues/embryogenic cell clusters, which then undergo a series of morphological and biochemical changes and restructure into the embryonic state. It is important to understand the mineral nutrition requirements of these tissues to improve the somatic embryogenesis process.

In this study, we analyzed the mineral composition of *Liriodendron* tissues at different stages during the somatic embryogenesis process, from the callus stage to the cotyledon-embryo stage. The aim was to investigate the element profile of embryos during different developmental stages of the induction and expression phases. These data will provide useful information to formulate suitable nutrient media to induce somatic embryo development and nourish the developing embryo. These results may also help us to understand the genetic regulation of somatic embryogenesis.

Materials and Methods

Embryogenic and non-embryogenic materials: *Liriodendron chinense × Liriodendron tulipifera* embryogenic and non-embryogenic cell lines (Fig. 1) were obtained as *In vitro* callus cultures from the Key Laboratory of Forest Genetics & Biotechnology, Nanjing Forestry University, Nanjing, China. The callus cultures were initiated from immature seeds derived from a hybrid of *L. chinense ×L. tulipifera* (genotype No.163024). The culture medium for callus induction and proliferation was ¾-strength Murashige and Skoog medium (MS) (Murashige & Skoog, 1962) supplemented with 2.0 mg/L 2, 4-dichlorophenoxyacetic acid, 0.2 mg/L benzyladenine (BA), 5 mg/L vitamin C (VC), 500 mg/L lactalbumin hydrolysate, and 30 mg/L sucrose (pH 5.8). The callus cultures were induced and proliferated in a growth chamber at 25°C in the dark. Embryogenic and non-embryogenic calli were identified based on their morphological features observed under a stereomicroscope (Leica, Germany). To confirm the different embryogenic abilities of the calli, portions of each type of callus were transferred to development medium MSI (0.7 MS, 5 mg/L VC, 40 g/L sucrose, 2 g/L active carbon, and agar, pH 5.8). The two tissue types were separated manually and sub-cultured under identical culture conditions. The calli were transferred onto fresh medium each month, and cultures were maintained at 25°C in the dark. For elemental analyses, we collected embryogenic and non-embryogenic calli 2 weeks after transfer to fresh medium. At least three replicates of each sample were collected in vials, quickly frozen in liquid nitrogen, and stored at –70°C until analysis.
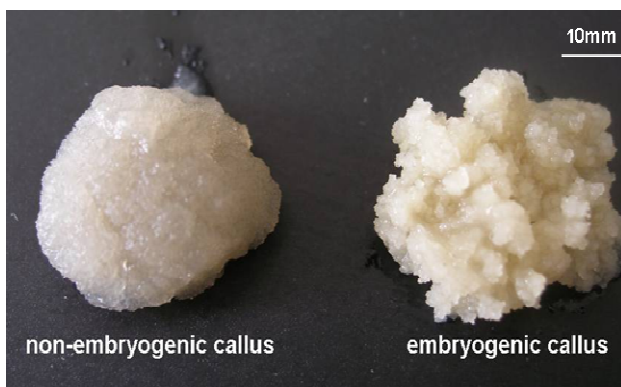

Fig. 1. Embryogenic and non-embryogenic calli of *L. chinense × tulipifera* genotype 163024.

Somatic embryogenesis: After two weeks of subculture, approximately 1–2 g embryonal tissue (embryogenic line genotype No.163024) was suspended by vigorous shaking for 5–8 d in liquid callus induction and proliferation medium. The suspended cells were size-fractionated between 100 and 400 mesh steel sieves as described elsewhere (Merkle *et al*., 1993), and washed with growth regulator-free medium. The cells were resuspended in

fresh 36 liquid medium (0.7 MS, 0.2 mg/L naphthaleneacetic acid, 0.5 mg/L kinetin, 0.2 mg/L BA, 500 mg/L casein hydrolysate, 5 mg/L VC, and 50 g/L sucrose; pH 5.8), and shaken for 2 days. A 1-mL aliquot of the suspension was evenly spread on the surface of a 7-cm filter, and the medium was removed by vacuum. The filtrate with the embryogenic tissue was then placed on MSI medium. Embryos that represented developmental stages 5, 9, 13, 15, and 20 days were harvested after the appropriate period of culture on abscisic acid-containing medium (1.5 mg/L ABA). At each stage, embryos were collected from the filter papers for analysis. These embryogenic samples were collected in a vial, quickly frozen in liquid nitrogen, and stored at –70°C until use.

Elemental analysis by inductively coupled plasma-optical emission spectroscopy: Elemental analysis was performed with inductively coupled plasma-optical emission spectroscopy (ICP-OES), as described by Pullman and Buchanan (Pullman & Buchanan, 2003). A callus cluster of approximately 50 mg was weighed and placed into a screw-capped polyethylene tube. Concentrated, high-purity nitric acid (5 mL) was added to each tube. The tube was capped and placed in a fume hood for 6 h at room temperature. Then, 2 ml of high-purity 30% hydrogen peroxide was added to each tube, and the mixture was inverted twice and vented to release any evolved oxygen. The cap was replaced loosely to avoid pressure from evolved oxygen. Each sample was digested in a fume hood for 24 h at room temperature. After digestion, Millipore-Q Gradient water was added to each tube to complete the volume to 10.0 mL. To remove impurities, each sample was filtered through a 0.45-μm membrane before analysis.

Elemental analysis of the samples was conducted using an OPTIMA 4300DV ICP-OES instrument (Perkin-Elmer, Boston, MA, USA). This instrument was equipped with automatic sampling equipment and an integral computer workstation that facilitated rapid detection of elements. The ICP-OES was equipped with a high-temperature argon plasma torch, and element identification was based on detecting characteristic spectra from UV and visible light emissions. To improve performance, the instrument was calibrated with a custom, multi-element standard (SPEX Certi Prep Inc., Metuchen, NJ, USA) and a blank.

The main operating parameters were as follows: transmitting torch power: 1200 W; atomization gas pressure: 30 psi; pump sampling rate: 1.5 ml/min; plasma gas flow: 15 L/min; auxiliary gas flow: 1.5 L/min; washing time: 15 s; and sampling time: 25 s.

The selected wavelength spectra of the analyzed elements were as follows: Al: 396.153, B: 249.677, Ca: 317.933, Co: 228.616, Cu: 327.393, K: 766.490, Mg: 285.213, Mn: 257.610, Mo: 202.031, Na: 589.592, Ni: 231.604, P: 213.617, Zn: 206.200, and Fe: 238.204.

Statistical analysis: The data of elemental content in different stages of embryogenic and non-embryogenic calli were analyzed by Student's *t*-Test at a significant level of at $p < 0.05$ or $p < 0.01$.Values are means of at least three replicates.

Results and Discussion

Embryogenesis test and embryo development: Induction of immature *L. chinense ×L. tulipifera* embryos on callus induction medium yielded two types of tissues, embryogenic callus and non-embryogenic callus, which were isolated based on their morphological characteristics. The embryogenic callus was tightly compacted but soft and friable, and was typically white or light yellow. In contrast, non-embryogenic callus was loose, translucent, and watery (Fig. 1). Morphological differences between embryogenic and non-embryogenic calli were observed previously in *Dioscorea bulbifera* (Hong *et al.*, 2009), *Centella asiatica* (Lai *et al.*, 2011) and *Musa acuminata* (Jalil *et al.*, 2008). Both embryogenic and non-embryogenic calli actively proliferate in medium. The non-embryogenic callus, which is incapable of somatic embryogenesis, proliferate faster than does embryogenic callus. Both tissues retain their characteristics in successive subcultures. To test their embryogenic capacity, the embryogenic and non-embryogenic calli were used to establish suspension cultures. Then, the suspended cells were cultured on differentiation medium. The results showed that embryogenic calli were capable of somatic embryogenesis, while non-embryogenic calli were not capable (Fig. 1s). The establishment of highly embryogenic and non-embryogenic calli provides new perspectives for comparative studies and for interpreting the process of somatic embryogenesis (Varhaníková *et al.*, 2014). Embryogenic calli were cultured on differentiation medium, where they enlarged and differentiated into embryonal tissues, including cotyledons that were visible at later stages (Fig. 2). In the present study, after culturing for 5 days on differentiation medium, embryogenic masses appeared to be at the globular stage; this was designated as the 5-day stage. At the 9- and 13-day stages, the head regions gradually enlarged. At the 15-day stage, the embryos showed bipolar elongation and primordial cotyledons. The cotyledons had begun to elongate in embryos at the 20-day stage. These embryos successfully regenerated into intact plantlets in culture media (Fig. 2s).
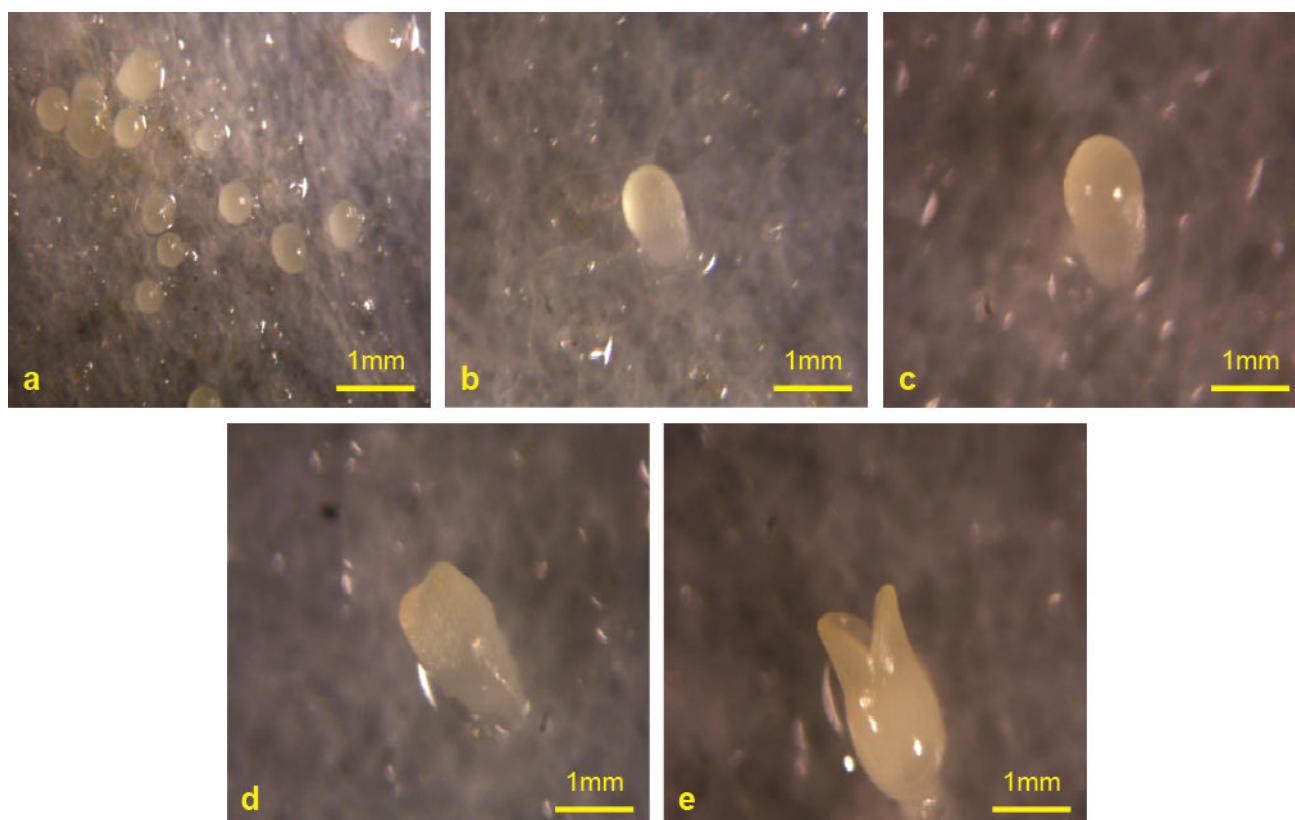

Fig. 2 Development of cultured *L. chinense × tulipifera* somatic embryos. Images of somatic embryos at various developmental stages: (A) 5-day stage, (B) 9-day stage, (C) 13-day stage, (D) 15-day stage, and (E) 20-day stage.

Elemental analysis during somatic embryogenesis: We used ICP-OES to identify key elements in tissues during developmental somatic embryogenesis (Fig. 3A–K) and elements in embryogenic and non-embryogenic calli (Fig. 4A–C). We did not detect Mo, Co, and Ni in tissues undergoing developmental somatic embryogenesis, nor did we detect Mo, Co, and B in embryogenic or non-embryogenic calli. The concentrations of these elements may have been below the lower detection limits of the OPTIMA 4300DV ICP-OES. These results suggested that different elements were required at different developmental stages and in different tissue types.
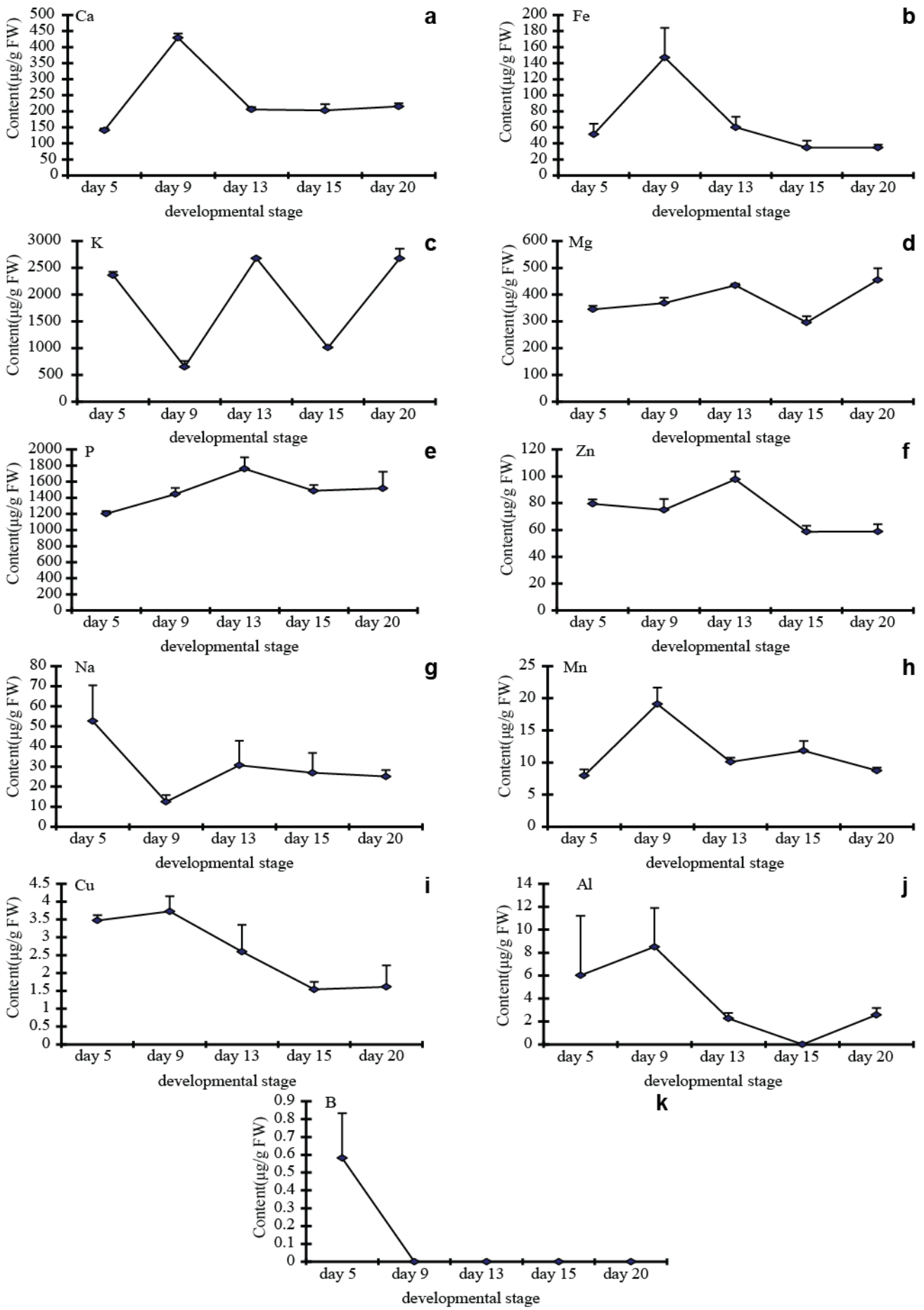
Fig. 3. Elemental analyses of developing *L. chinense × tulipifera* somatic embryos. Vertical bars indicate SE.

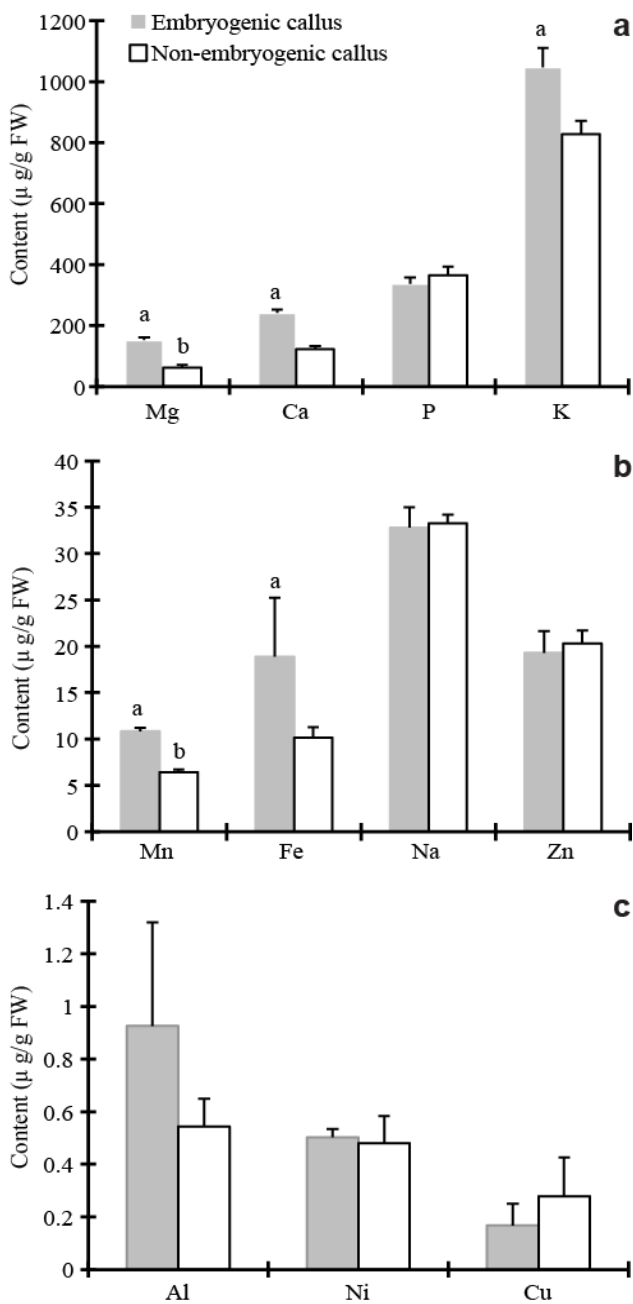Fig. 4. Elemental analyses of embryogenic and non-embryogenic calli in *L. chinense × tulipifera*. Vertical bars indicate SE; "a" indicates significant difference at *p*<0.05 and "b" indicates significant difference at *p*<0.01.

We detected maximum levels of Ca, Fe, Mn, and Al at the 9-day stage of embryo development. Ca is an important signaling molecule in plants. In another study, it was reported that culture conditions conducive for embryo development elicited the transient movement of exogenous Ca into the cytosol of sandalwood pro-embryogenic cells (Anil & Rao, 2000). Stimulatory effects of different levels of Ca were also observed in *Pinus* and sugarcane (Pullman & Buchanan, 2003; Desai *et al.*, 2006). In *C. canephora,* exogenous calcium induce cellular machinery to somatic embryogenesis (Ramakrishna *et al.*, 2012). Our results showed a sharp increase in the Ca concentration at the 9-day stage, suggesting that a transient influx of Ca prompted organogenesis. The Ca content was higher in

embryogenic callus than in non-embryogenic callus, suggesting that Ca might contribute to morphogenesis and induction of somatic embryogenesis. Iron is an indispensable micronutrient, but its insolubility restricts its uptake. Extracellular acidification through the plasma membrane H^+–ATPase can release bound iron (Pullman & Buchanan, 2003). In previous studies, cells required Fe to produce a multicellular somatic embryoid, and reduced concentrations of Fe in the culture media provisionally suppressed embryogenesis (Loh & Lim, 1992; Saha *et al.*, 2011). In the present study, we found higher levels of Fe in developmental somatic embryos than in the calli, consistent with the observations of Saha *et al.* (2010). This suggested that Fe might be a major element affecting somatic embryogenesis. Aluminum is generally not considered to be a vital element for plant growth (Kvaalen *et al.*, 2005), but it is a cofactor required by many enzymes. We found that Al was present in tissues undergoing developmental somatic embryogenesis and in the calli of *L. chinense ×L. tulipifera*. We observed that P and Zn levels peaked at the 13-day stage. P is an essential macronutrient for plant growth and development; it is involved in energy transfer, metabolic regulation, and protein activation (Shen *et al.*, 2008). P is necessary for growth and regeneration of tissues. In the present study, P concentrations were markedly higher in tissues undergoing developmental somatic embryogenesis than in embryogenic and non-embryogenic calli.

This result suggested that P favored somatic embryogenesis, as proposed by Elkonin and Pakhomova (Elkonin & Pakhomova, 2000). Zinc is an important element for processes that control cell fate. It is involved in maintaining the strictly coordinated balance between cell death and survival in plants; thus, a specific concentration of Zn is essential to avoid undergoing cell death (Helmersson *et al.*, 2008; Hill *et al.*, 2013). For example, in previous studies, Zn inhibited programmed cell death (PCD); 10 μM Zn^{2+} caused a 50% inhibition of the apoptotic enzyme, VEIDase (Bozhkov *et al.*, 2004). In the present study, the lowest Zn concentrations were in calli. The Zn concentration peaked at the 13-day stage, and then decreased in tissues undergoing developmental somatic embryogenesis. It was reported that PCD was responsible for the degradation of proliferating proembryogenic masses when they gave rise to somatic embryos in Norway spruce (Filonova *et al.*, 2000). Thus, low Zn concentrations might allow the correct transition from callus to the somatic embryo. The highest Na content was at the 5-day stage, and then it sharply decreased at the 9-day stage. Potassium showed three peaks at the 5-, 13-, and 20-day stages. Na^+ and K^+ are important for counterbalancing negative anions; however, excess Na^+ is more toxic than excess K^+. In late embryonic development or drought conditions, K^+ can regulate the pH and the osmotic environment (Zhu, 2002). Cell elongation was associated with increased K^+ levels. In the present work, there were higher concentrations of K^+ in embryogenic callus than in non-embryogenic callus. In gymnosperms, boron is an essential factor for suspensor formation. For example, during somatic embryogenesis of *Larix deciduas*, when the B concentration was lower than 35 μM, suspensor formation was completely repressed. However, when the B

concentration was increased to 1 mM, the suspensor formed successfully (Behrendt & Zoglauer, 1996). In this study, B was only detected at the 5-day stage during developmental somatic embryogenesis, and it was not detected at all in embryogenic and non-embryogenic calli. Cu is a component of many metallothionein proteins, which are involved in electron transport and protein and carbohydrate biosynthesis (Saha *et al.*, 2010). Previous reports showed that increases in Cu levels in culture medium favored somatic embryogenesis (Kintzios *et al.*, 2001); Cu-depleted medium had little effect on the induction of embryogenic epiblast calli (He *et al.*, 1991)，however, the increase concentration of Cu promoted green haploid plant regeneration for microspore embryogenesis in barley (Jacquard *et al.*, 2009). The ICP-OES data revealed higher concentrations of Cu in developmental somatic embryos than in calli. Moreover, Cu levels were higher in non-embryogenic callus than in embryogenic callus. These results suggested that Cu may be more important for somatic embryogenesis development than for embryogenic induction.

Our elemental analysis of embryogenic and non-embryogenic calli revealed similar P, Na, Zn, and Ni contents; however, Mg, Ca, K, Mn, Fe, and Al levels were higher in embryogenic callus than in non-embryogenic callus. Conversely, Cu levels were higher in non-embryogenic callus than in embryogenic callus. Statistical analyses indicated that the differences between embryogenic and non-embryogenic calli were significant at 0.01 and 0.05 levels for Mg, Ca, K, and Mn, but only at the 0.05 level for Fe (Fig. 4).
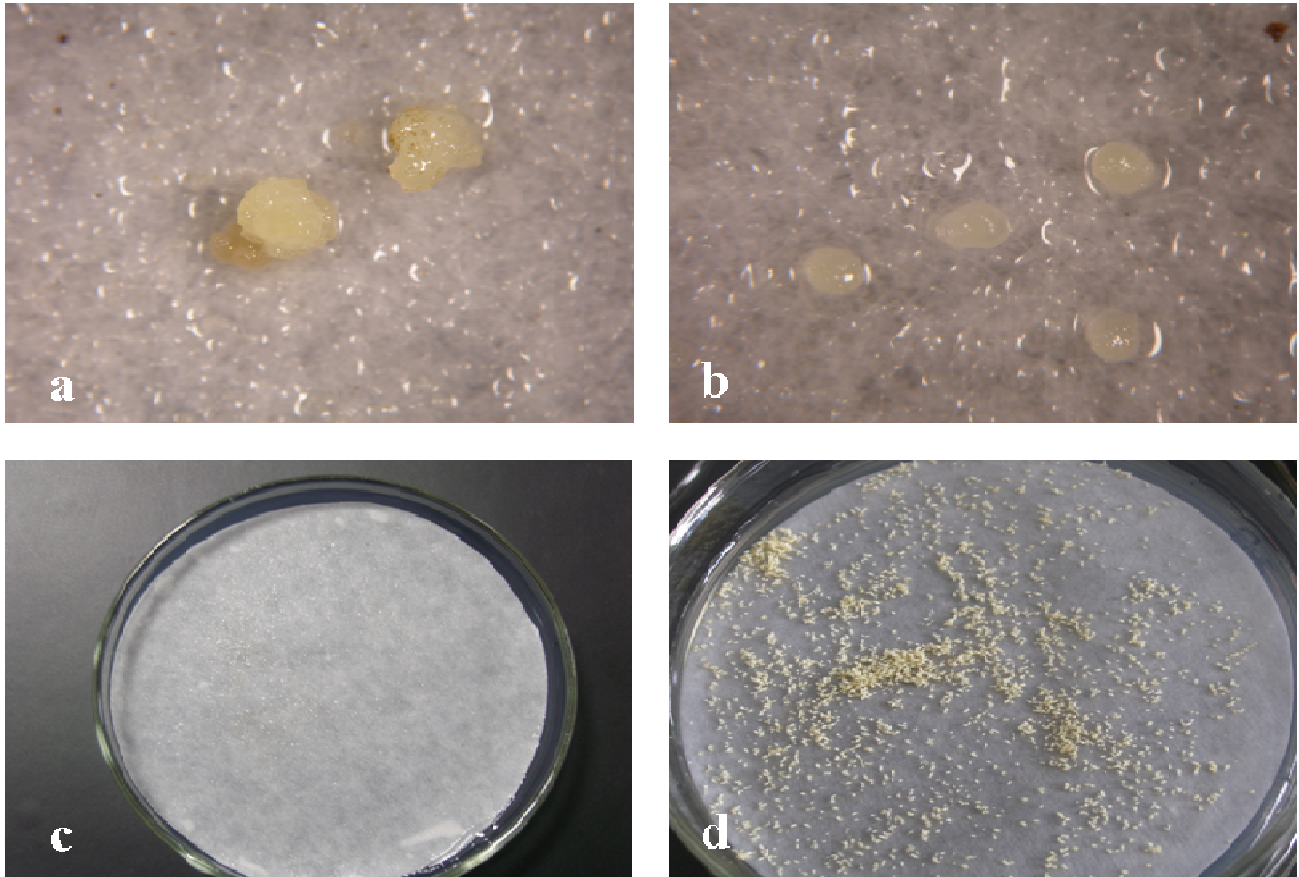

Fig. 1s. The following development of embryogenic and non-embryogenic callus of genotype 163024. (a and c were induced for 5 and 25 d non-embryogenic callus, repespectly; b and d represent inducing for 5 and 25 d embryogenic callus, repespectly).

Fig. 2s. Cotyledon embryos and regenerated shoots of *Liriodendron* hybrid genotype 163024. (a and b: cotyledon embryos; c: regenerated shoots).

Conclusion

In conclusion, we analyzed 14 different elements during the induction and expression phases of *L. chinense × tulipifera* somatic embryogenesis using ICP-OES. The results suggested that different elements and different concentrations of elements played important roles in different stages of somatic embryogenesis and embryo development. The elemental profiles determined in this study might be useful to improve existing media or to formulate new media according to the specific requirements of these woody plant tissues. This could optimize embryonic induction and development of somatic embryos of *L. chinense ×L. tulipifera*. The changes observed in these elemental profiles suggested that different developmental stages of embryogenesis require specific elements at particular concentrations to catalyze and promote reactions and control multiple regulation networks for various biological processes. Optimizing the medium according to the specific mineral requirements of these tissues will increase the quantity and quality of embryos.

Acknowledgments

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