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Full Length Research Paper

Influence of $AgNO_3$ on somatic embryo induction and development in Manchurian ash (*Fraxinus mandshurica* Rupr.)

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In this present study, we explored the effects of silver nitrate ($AgNO_3$) on somatic embryo induction and the development from immature zygotic embryos in Manchurian ash (*Fraxinus mandshurica* Rupr.). $AgNO_3$ played a minor role on *in vitro* embryo induction frequency and in the number of somatic embryos per explant. However, 1 mg L^{-1} $AgNO_3$ enhanced synchronization and significantly inhibited abnormal somatic embryo formation suggesting that $AgNO_3$ might serve an important function in controlling the development of somatic embryos in Manchurian ash. Our results provided foundation for a future more efficient somatic embryogenesis and regeneration protocol.

Key words: Abnormality, *Fraxinus mandshurica*, silver nitrate, somatic embryogenesis, synchronization.

INTRODUCTION

Manchurian ash (*Fraxinus mandshurica* Rupr.), a member of the Oleaceae, is one of the three most valuable hardwood trees in Northeast China. The wood is an excellent choice for furniture due to its moderate hardness and beautiful texture. In addition, it is a popular ornamental tree with attractive compound leaves and elegant crowns. However, the species has been suffering a notable decline during the past few decades due to increased wood harvest. Therefore, it is crucial to have conservation measures to protect the important remaining tree populations.

Numerous efforts have been successful at implementing plantation and breeding programs for the national endangered species. To date, seed propagation is the standard method for propagation of this species. However, traditional propagation methods are limited by the long intervals between seed crops, dormancy and complex stratification systems prior to germination. Recent studies on cuttage and grafting in Manchurian ash have showed little success. In comparison, *in vitro* culture is known to circumvent the problems associated with seeding and vegetative propagation by cuttings, and can exploit maximum genetic gain achieved in breeding programs (Thorpe et al., 1991; Miguel et al., 2004; Akin-Idowu et al., 2009; Payghamzadeh and Kazemitabar, 2011). Therefore, *in vitro* culture is considered the most promising means to protect and propagate this important tree species.

Organogenesis (Tan and Shen, 2003) and young zygotic embryo culture protocols (Zhang and Luo, 2003) have been previously described for Manchurian ash. Somatic embryogenesis and regeneration have also been recently reported (Kong et al., 2006, 2011; Li et al., 2009). However, the somatic embryogenesis system required substantial improvement and was therefore impractical. Factors currently limiting commercial release of somatic embryogenesis, specifically for Manchurian ash include low induction rates, asynchronous development, high abnormal embryo frequency, low germination rates and slow initial growth of somatic plantlets.

Somatic embryogenesis is affected by numerous factors, notably ethylene. Ethylene is known to reduce somatic embryogenic competence in many plants. The use of silver nitrate ($AgNO_3$), an ethylene action inhibitor (Beyer, 1976), has been shown to increase *in vitro*

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embryogenesis and regeneration rates. However, the influence of ethylene on embryogenic response is genotype-specific (Fuentes et al., 2000; Al-Khayri and Al-Bahrany, 2004) and therefore must be elucidated on a species-specific basis.

We are not aware of any data on the role of ethylene and its inhibitor in Manchurian ash tissue culture protocols. The objective of this study was to evaluate the effects of $AgNO_3$ on somatic embryo formation and development in Manchurian ash, particularly synchronization and morphology.

MATERIALS AND METHODS

Materials

Immature seeds of Manchurian ash were collected from three healthy trees growing in the University Forest of Northeast Forestry University (Harbin, China) on 23 July 2007. The seeds from different maternal trees were equally mixed before use.

Explant preparation

The seeds were removed from the pericarps and disinfested in 70% (v/v) ethanol for 20 to 30 s, followed by stirring in 2% (v/v) sodium hypochlorite solution for 15 min with five final rinses in sterile distilled water. Embryos were extracted from the sterilized seeds and the cotyledons were excised as explants. Only one cotyledon was used per embryo.

Somatic embryo induction

The explants were cultured on ½ Murashige and Skoog (MS; Murashige and Skoog, 1962) medium (half-strength MS salts and vitamins) containing 8.0 µM naphthaleneacetic acid (NAA), 2.2 µM benzyladenine (BA), 400 mg L^{-1} casein hydrolysate (CH), 70 g L^{-1} sucrose and 6 g L^{-1} agar (initiation medium). The pH of the medium was adjusted to 5.8 prior to the addition of agar, and autoclaved at 120°C for 20 min. After 2 weeks of culture initiation, the tissues were transferred onto ½ MS medium and supplemented with 1, 2.5, 5 and 10 mg L^{-1} $AgNO_3$ (induction medium) to induce somatic embryos. The composition of induction medium was identical to that of initiation medium but supplemented with $AgNO_3$. A ½ MS medium without $AgNO_3$ was used as a control. The culture was incubated at 24 ± 2°C in the dark, with 60 to 70% relative humidity.

Experimental design and statistical analysis

Fifty cotyledon explants were applied to each $AgNO_3$ treatment. Ten explants were used per 90 mm diameter plastic Petri dish, and each dish contained 30 ml of the medium. The experiment was arranged in a completely random design. Four weeks after transfer to the induction medium, results were scored with the aid of a stereomicroscope according to the following formulas:

Mean somatic embryos per explant = Total somatic embryos induced / Explants that produced somatic embryos;

Mean cotyledonary somatic embryos per explant = Total cotyledonary somatic embryos induced / Explants that produced somatic embryos;

Somatic embryo induction frequency (%) = Explants that produced somatic embryos / Total explants cultured on the induction medium ×100;

Abnormal somatic embryo induction ratio (%) = Abnormal cotyledonary somatic embryos produced/ Total cotyledonary somatic embryos produced ×100;

Cotyledonary somatic embryo induction ratio (%) = Cotyledonary somatic embryos produced / Total somatic embryos produced ×100;

All experiments were repeated three times. Data were analyzed using Analysis of Variance (ANOVA) in SPSS software. Significant differences among group means were assessed using the post hoc Least Significant Differences test (LSD).

RESULTS

Effect of $AgNO_3$ on somatic embryo induction

The results of somatic embryogenesis are presented in Figures 1 and 2. The number of somatic embryos per explant fluctuated with increasing $AgNO_3$ concentration (Figure 1). When 10 mg L^{-1} $AgNO_3$ was included in the medium, the total number of somatic embryos was the highest, with an average of 3.86 embryos per explant. The lowest number of embryos per explant (one) was induced at 2.5 mg L^{-1} $AgNO_3$. A significant difference was detected between the number of somatic embryos per explant at 2.5 mg L^{-1} $AgNO_3$ and 10 mg L^{-1} $AgNO_3$ ($p < 0.05$). However, significant differences among other $AgNO_3$ concentrations were not revealed ($p > 0.05$).

Somatic embryo induction frequency increased with increasing $AgNO_3$ concentration (Figure 2). The lowest induction rate was observed in the control (10.00%). The highest induction rate of 23.33% was obtained with a medium supplemented with 10 mg L^{-1} $AgNO_3$. However, a significant effect was not detected for different $AgNO_3$ levels for total somatic embryo induction frequency ($p > 0.05$).

Effect of $AgNO_3$ on synchronization of somatic embryo development

Some somatic embryos developed to the cotyledonary stage following 4 weeks in the induction medium. However, an extended culture period did not increase the number of cotyledonary somatic embryos. The largest number of cotyledonary somatic embryos per explant (1.42) was obtained under 1 mg L^{-1} $AgNO_3$, higher than the number (0.74) at 5 mg L^{-1} $AgNO_3$; but no significant difference was detected between the two values ($p > 0.05$). The number of cotyledonary embryos per explant did not exceed 0.2 at other $AgNO_3$ levels, significantly lower than the 1 mg L^{-1} $AgNO_3$ treatment ($p < 0.05$) (Figure 1).

The proportion of somatic embryos at a particular stage reflected the synchronization of somatic embryo

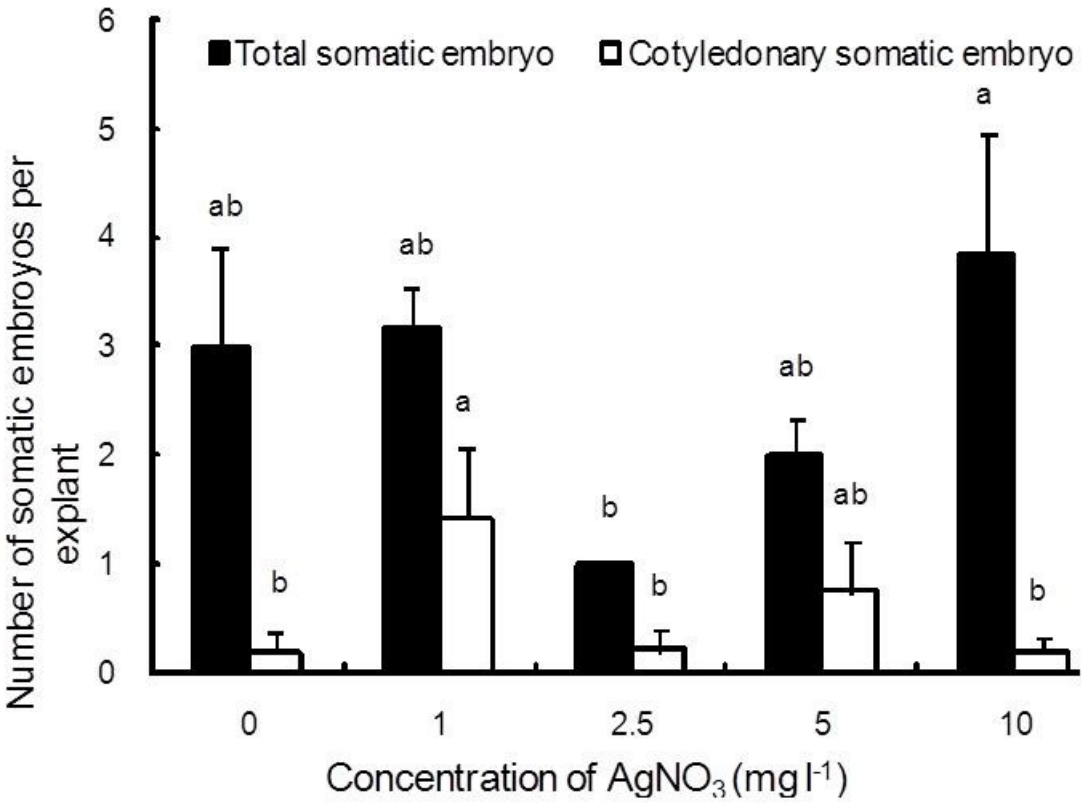

Figure 1. Effect of $AgNO_3$ on the number of somatic embryos per explant in Manchurian ash. Each mean ± standard error, means with the same letters are not significantly different according to the LSD test ($p > 0.05$).

development. In our study, the percentage of cotyledonary somatic embryos changed with increasing $AgNO_3$ concentration (Figure 2). No significant difference among different $AgNO_3$ concentrations was observed, but the cotyledonary percent was higher in the treatment groups relative to the control. The cotyledonary rate was highest at 41% with $AgNO_3$ at 1 mg L^{-1}, significantly higher ($p < 0.05$) than the control, where cotyledonary somatic embryos accounted for 2.71%. This result indicated that supplementing the medium with 1 mg L^{-1} $AgNO_3$ promoted more somatic embryos to develop synchronously into the cotyledonary stage.

Effect of $AgNO_3$ on morphology of somatic embryo development

Prior to cotyledon stage, somatic embryos were very small and morphological differentiation was not distinguishable until development into the cotyledonary stage. Therefore, we scored morphologically normal and abnormal somatic embryos only for those at the cotyledonary stages. Frequent abnormal somatic embryos, which included fused hypocotyls, trumpeted cotyledons and altered number of cotyledons, were observed (Figure 3). To simplify our data, we assumed the somatic embryos with two cotyledons and one hypocotyl at the appropriate sizes to be normal, whereas all other forms were grouped as abnormal. In the absence of $AgNO_3$ in the induction medium, or $AgNO_3$ concentration higher than 1 mg L^{-1}, all cotyledonary somatic embryos were morphologically abnormal. Normal embryos were obtained only on medium containing 1 mg L^{-1} $AgNO_3$, that is, abnormality frequency was 69.00%, significantly lower than embryos under other $AgNO_3$ concentration treatments and the control (100%) (Figure 2). The results demonstrate that 1 mg L^{-1} $AgNO_3$ inhibited abnormal development to a certain extent and stimulated normal somatic embryo formation.

DISCUSSION

In most plants, ethylene acts either as a promoter or inhibitor of somatic embryogenesis, depending on the species and the concentration (Biddington, 1992; Ptak et al., 2010). Ethylene has been shown to accumulate during embryogenesis in *Medicago sativa* (Meijer, 1989) and *Coffea canephora* (Hatanaka et al., 1995). A biochemical analysis on five coniferous species indicated

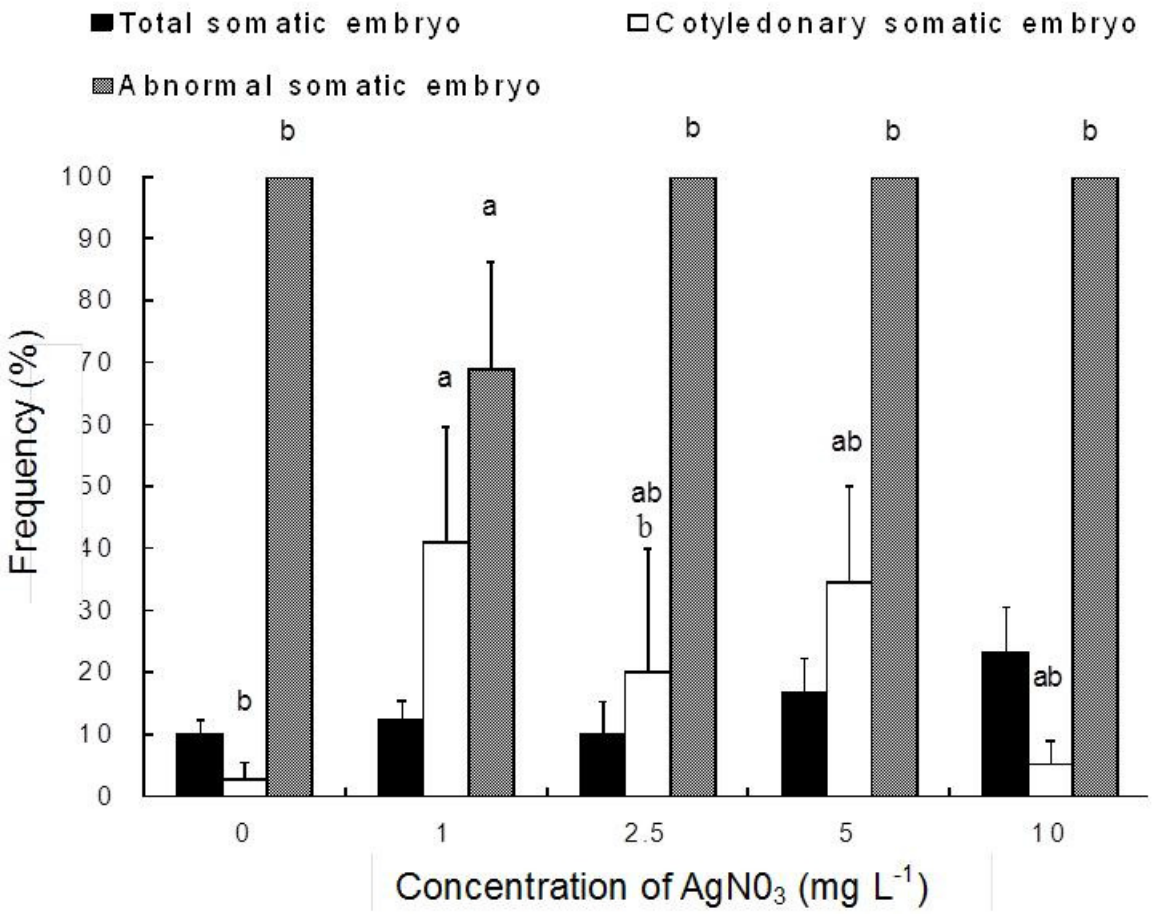

Figure 2. Effect of $AgNO_3$ on frequency of somatic embryogenesis in Manchurian ash. Each mean ± standard error, means with the same letters are not significantly different according to the LSD test ($p > 0.05$).

that embryogenic tissue evolved ethylene at a lower rate relative to non-embryogenic tissue (Wann et al., 1989). Pullman et al. (2003) indicated that in conifers, low ethylene exposure was necessary for somatic embryo initiation.

$AgNO_3$ has been shown to be a very potent inhibitor of ethylene action (Beyer, 1976; Kumar et al., 2009). Media supplemented with $AgNO_3$ improved embryogenesis or regeneration in numerous angiosperms and gymnosperms, including *Carya illinoensis* (Juglandaceae) (Mathews and Wetzstein, 1993), *Triticum durum* (Poaceae) (Fernandez et al., 1999), *Coffea canephora* (Rubiaceae) (Fuentes et al., 2000), *Carthamus tinctorius* (Asteraceae) (Mandal et al., 2001), *Paspalum scrobiculatum* (Poaceae) (Vikrant and Rashid, 2002), *Bactris gasipaes* (Arecaceae) (Steinmacher et al., 2007), *Paspalum scrobiculatum* (Poaceae) and *Eleusine coracana* (Poaceae) (Kothari-Chajer et al., 2008), *Hedychiurn bousigonianum* (Gingeberacea) (Sakhanokho et al., 2009), *Gossypium nelsonii* (Malvaceae) and *G. australe* (Yan et al., 2010), and *Pinus taeda* (Pinaceae) (Pullman et al., 2003). However, Hatanaka et al. (1995) reported that addition of $AgNO_3$ inhibited somatic embryo formation in leaf explants of *Coffea canephora*. In addition, the effects of $AgNO_3$ on somatic embryogenesis varied with the compound concentration.

The exact mechanism by which $AgNO_3$ affects somatic embryogenesis is not completely understood. Strong evidence indicates that $AgNO_3$ inhibits ethylene function; however, Kong and Yeung (1995) demonstrated that by increasing endogenous ABA levels to 100 µM in white spruce, $AgNO_3$ enhanced embryo maturation. Fuentes et al. (2000) found that the addition of $AgNO_3$ caused only small modifications in the ionic equilibrium of the medium and concluded the effects of the compound on somatic embryogenesis were not attributable to any substantial changes in available nutrients.

Our study showed that 1 to 10 mg L^{-1} $AgNO_3$ exhibited no significant acceleration in somatic embryogenesis frequency. In addition, the number of embryos per responded explant in Manchurian ash was not significantly different. Therefore, poor somatic embryo induction in Manchurian ash is likely not the result of accumulated ethylene and the effect of $AgNO_3$ on embryogenic response is species specific.

Many studies have focused on the effects of $AgNO_3$ on

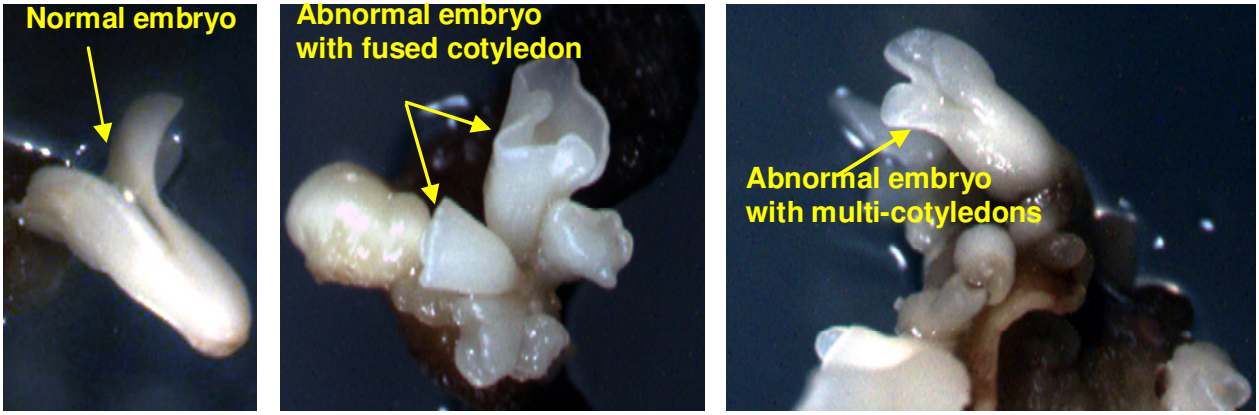

Figure 3. Examples of normal and abnormal cotyledonary somatic embryos in Manchurian ash.

somatic embryo initiation frequency and the number of somatic embryos generated, whereas few investigations have addressed the relationship between $AgNO_3$ and the synchronization of somatic embryos. In one of the few studies examining the effects of ethylene and ethylene inhibition on somatic embryo morphology, the use of $AgNO_3$ during the induction process led to a three-fold increase in the frequency of normal somatic embryos in the soybean cultivar "Bragg"; but two additional cultivars "IAS-5" and "RS-7", which had fairly high rates of normal embryos formation，were not affected by $AgNO_3$ (Santos et al., 1997).

The ratio of cotyledonary embryos to all somatic embryos must, to some extent, reflect synchronization. In this current study, somatic embryo induction and development was carried out under the same medium. A medium with 1 mg L^{-1} $AgNO_3$ resulted in the number of cotyledonary embryos and its ratio to all embryos significantly higher than the control. In addition, normally formed cotyledonary embryos were only observed on this medium. This result suggested that $AgNO_3$ at low concentration (1 mg L^{-1}) was advantageous for synchronization of somatic embryo development and inhibited abnormal embryo formation in Manchurian ash.

Morphological abnormalities are common in plant somatic embryogenesis. However, some studies have demonstrated that malformation does not always inhibit normal regeneration. Frequent abnormal somatic embryos such as fused cotyledons and/or an altered number of cotyledons were induced from young zygotic embryos in *Cinnamomum camphora*, while the plantlets derived from these abnormal somatic embryos had normal appearance similar to zygotic embryos (Shi et al., 2009). In *Quercus suber*, both morphologically normal and abnormal somatic embryos conversed into plantlets with normal appearance. Investigations into DNA ploidy levels and nuclear DNA content indicated that these morphological abnormalities did not reflect major genetic differences (Pinto et al., 2002; Loureiro et al., 2005). In soybean, a high percentage of abnormal somatic embryos occurred in mature somatic embryos (Buchheim et al., 1989; Bailey et al., 1993; Santo et al., 1997). Santo et al. (1997) considered this the rule rather than the exception, because a large number of abnormal embryos in soybean were capable of germination into normal plantlets and the frequency of conversion for all embryos was higher than the frequency for morphologically normal embryos. The high percentage of abnormal somatic embryos found in our study is congruent with previous reports (Kong et al., 2006). In this present study, 1 mg L^{-1} $AgNO_3$ stimulated normal embryo formation, but the frequency of abnormal embryos was still very high. This fact suggested that abnormal somatic embryos appeared to be the rule in this species. Therefore, special efforts are needed to germinate abnormal somatic embryos into normal plantlets in future studies.

In summary, $AgNO_3$ played a minor role on *in vitro* embryo induction frequency and in the number of somatic embryos per explant in Manchurian ash. However, the fact that 1 mg L^{-1} $AgNO_3$ enhanced synchronization and significantly inhibited abnormal somatic embryo formation suggests that $AgNO_3$ might serve an important function in controlling the development of somatic embryos in Manchurian ash. Our results provide the foundation for a more efficient somatic embryogenesis and regeneration protocol in the future.

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