Plant Biotechnology 31, 71–75 (2014)
DOI: 10.5511/plantbiotechnology.13.1109a

Note

Effects of silver nitrate on shoot regeneration of *Artemisia annua* L.

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Received August 5, 2013; accepted November 9, 2013 (Edited by M. Otani)

Abstract In order to further increase the shoot regeneration frequency of *Artemisia annua* L., the effects of silver nitrate on this process was investigated in this study. Different concentration of silver nitrate was added to the shoot induction medium, which was MS basic medium containing 1.0 mg l^{-1} 6-benzyladenine (6-BA) and 0.05 mg l^{-1} α-napthaleneacetic acid (NAA). When 2 mg l^{-1} silver nitrate was added to the shoot induction medium, the shoot induction frequency and shoot number per explants was significantly higher than that of the control (without silver nitrate). In addition, silver nitrate at all the tested concentrations could significantly reduce callus formation of the explants. Silver nitrate had also positive influence on shoot elongation in the first 20 days. Furthermore, silver nitrate did not affect the sensitivity of *A. annua* shoots to Kanamycin (KM); therefore, silver nitrate could be used to improve shoot regeneration capacity and frequency in *A. annua* genetic transformation.

Key words: *Artemisia annua* L., elongation, growth, shoot induction, silver nitrate.

Artemisia annua L. (wormwood or sweet wormwood) is an important medicinal composite plant used as Chinese traditional medicine to treat hemorrhoids and fevers including malaria (Bhakuni et al. 2001). It evokes wide ranges of interests because it contains artemisinin, an effectively curative anti-malarial drug against both chloroquinine-resistant and chloroquinine-sensitive strains of *Plasmondium falciparum* as well as cerebral malaria (Klayman 1985). Currently, *A. annua* is the only commercial source of artemisinin. But because of its low content (0.01–0.8% DW) in *A. annua* plant, artemisinin is in very short supply on the international market. Therefore, numerous efforts are focusing on improving artemisinin production. Currently, genes encoding key enzymes involving in artemisinin biosynthesis have been cloned and characterized from *A. annua* (Chang et al. 2000; Teoh et al. 2006; Zhang et al. 2008), which makes genetic engineering a potential fast approach for increasing artemisinin content of *A. annua*. Although *Agrobacterium tumefaciens*-mediated transformation system of *A. annua* has been developed by Vergauwe et al. (1996; 1998) and our laboratory (Han et al. 2005), the transformation efficiency is only 4–10%, not high enough to produce large number of transgenic plants. Therefore, it is necessary to try different ways to further increase the transformation efficiency.

In in vitro tissue cultures, plant cells often produce ethylene and the amount of ethylene increased while the explants were treated with *Agrobacterium* in *Agrobacterium*-mediated transformation, which resulted in reduced efficiency of plant regeneration and gene transfer (Ezura et al. 2000; Seong et al. 2005). Silver nitrate, as an inhibitor of ethylene production, can be employed to enhance plant regeneration and *Agrobacterium*-mediated transformation (Seong et al. 2005). Furthermore, silver nitrate addition into culture medium can effectively rehabilitate decreased regeneration potentials of cultured cells and tissues incurring through their subcultures (Ogura and Shimada 1978). In the recent years, silver nitrate has been successfully employed to improve somatic embryogenesis and plant regeneration in tissue culture (Akasaka-Kennedy et al. 2005; Al-Khayri and Al-Bahrany 2004; Fei et al. 2000; Sridevi et al. 2010; Zhang et al. 2001). However, effect of silver nitrate on shoot regeneration of *A. annua* is still unknown. Here we report our results in this topic, which provide the basis of employing silver nitrate in *A. annua* genetic engineering.

A high-artemisinin-yielding strain of *A. annua* L., collected in Sichuan of Province, China was used in this study. The establishment and maintenance of *A. annua* was as described before (Wang et al. 2009).

This article can be found at http://www.jspcmb.jp/
Published online February 14, 2014

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In this work, the shoot induction medium was the MS medium (Murashige and Skoog 1962) supplemented with 1.0 mg l^{-1} 6-BA and 0.05 mg l^{-1} NAA and adjusted to have a pH of 6.1 with 1 mol l^{-1} NaOH (Han et al. 2005). Filter sterilized silver nitrate stock solution (1.0 mg ml^{-1}) was added into the autoclaved shoot induction medium to end concentration of 0, 2, 4, 6, 8 and 10 mg l^{-1}.

The middle or upper leaves of 25-day-old *A. annua* seedlings were taken as the explants in the experiments. The explants were horizontally placed in petri dishes (diameter = 100 mm; depth = 15 mm) filled with 30 ml of the shoot induction medium. Each treatment had four replications and at least 60 explants. Twenty days after they were cultured, the frequency of shoot induction (number of explants forming shoots/total number of the explants) and callus formation (number of explants forming callus/total number of the explants) were calculated. At the same time, the number of regenerated shoots per explants was counted.

Silver nitrate was applied at 2 mg l^{-1} in further experiments according to the results of the above experiment. In order to find out the influences of silver nitrate on shoot elongation, the shoot clusters cultured on the medium with silver nitrate for 20 days were randomly divided into two groups. One group was kept on the same medium, i.e. shoot induction medium with silver nitrate and the other group was transferred on the induction medium without silver nitrate. The shoots cultured on the induction medium without silver nitrate addition were the control. Each group had four replicates. One month later, the shoots longer than 15 mm were counted and the heights of the shoots were recorded. The elongation percentage (No. of the shoots longer than 15 mm long/the total No. of the shoots involved) and the average heights of shoot longer than 15 mm were calculated. Following the shoot development experiment, the individual elongated shoots (around 15 mm long) were aseptically cut down and transferred to hormone free MS media to root and form seedlings. Forty five days after the shoots being cultured, the heights of the seedlings in the two groups were measured and averaged. There were at least fifteen elongated shoots (seedlings) measured in each group.

KM is one of the selective agents widely employed to efficiently select transgenic plants in *A. annua* genetic engineering, so this research observed the effects of silver nitrate on the KM sensitivity of *A. annua* shoots. Explants were inoculated on the shoot induction medium supplemented with silver nitrate (0 and 2 mg l^{-1}) and KM (0, 5, 10, 15 and 20 mg l^{-1}). The shoot induction medium was first autoclaved, then stock solution of Kan (1.0 mg ml^{-1}) and silver nitrate (1.0 mg ml^{-1}) was added into medium to various levels. After the explants were cultured for 30 days, the shoots that the explants formed in the different groups were counted and the shoot induction frequencies were calculated depending on the shoot numbers thus obtained.

All experiments were conducted twice, each time in four replication; the mean ± SE values of the results are presented. The data were analyzed using ANOVA. Significant differences between the control and treatment were analyzed using Student's *t* test with SPSS 11.5 software.

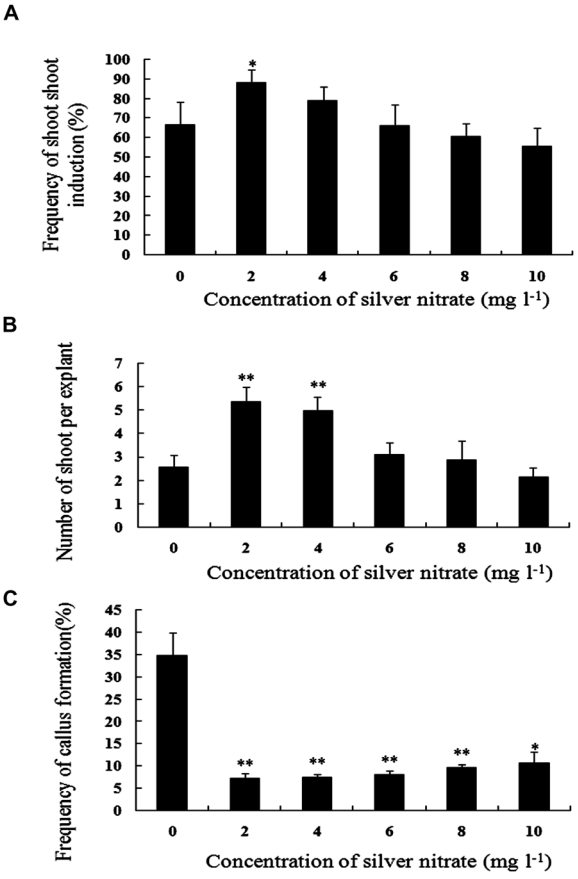

Figure 1. Influence of silver nitrate on *A. annua* shoots induction. A: Shoot induction frequency; B: number of regenerated shoots per explant; C: frequency of callus formation. Clusters of fascicled shoots and callus formed from leaf stalk or other cuts of the explant. One cluster of fascicled shoots corresponds to one leaf stalk and consists of many shoots. Asterisks represent significant differences between treatment and un-treated explants (* $p<0.05$; ** $p<0.01$).

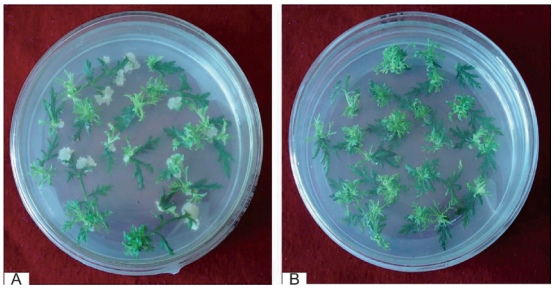

Figure 2. Influence of silver nitrate on callus information. A: Explants cultured on shoot induction medium without silver nitrate for 20 days; B: explants cultured on shoot induction medium with 2 mg l^{-1} silver nitrate for 20 days.

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Table 1. Influence of silver nitrate on *A. annua* regenerated plantlets growth.

Silver nitrate concentration ($mg\,l^{-1}$)	No. of elongation shoots	Frequency of rooting (%)±SE	Heigth of plantlets (cm)±SE
0	12	90.42±3.27 a	9.054±1.29 b
2	12	92.89±3.89 a	13.13±2.08 a

Elongation shoots (>15 mm) were transferred to MS medium and average height of rooted plantlets were measured 45 days after culture. Mean values within the column followed by the different letters are significantly different ($p \leqq 0.05$) according to Student's *t* test.

Figure 1 and Figure 2 show silver nitrate improved shoot induction of *A. annua*. The highest shoot induction frequency (88.2%) was found on medium containing $2\,mg\,l^{-1}$ of silver nitrate, which was 32.5% higher than that of the control (Figure 1-A). Without silver nitrate additions, the regenerated shoots number per explants was 2.54±1.11, but with silver nitrate added at 2 and $4\,mg\,l^{-1}$, the regenerated shoot numbers reached 5.23± 1.21 and 4.97±1.16, respectively (Figure 1-B), which were significantly higher than that of control. The callus formations were significantly inhibited by silver nitrate at all the concentrations tested (Figure 1-C, Figure 2).

Addition silver nitrate in medium for 20 days improved *A. annua* shoot development. The length of the shoots cultured on the silver nitrate containing medium for 20 days were significantly longer than that of the control, indicating that silver nitrate could promote shoot elongation. However, the length of the shoots consecutively cultured on the silver nitrate-added medium for 50 days was shorter than that on the control medium (data not shown).

Table 1 shows the residual effects of silver nitrate additions on rooting and growth. There was no difference in term of rooting between the 2 types of shoots. Most of the shoots (over 90%) were observed to root within 5–8 days after their transferring. However, 45 days after cultured on the MS medium, the height of seedlings from silver nitrate containing medium was significantly than that of the shoots from silver nitrate free medium, indicating the effects of residual of silver nitrate has positive effect on the later growth of regenerated plantlets.

It was observed in this experiment silver nitrate had no influence on sensitivity of *A. annua* shoots to KM. As the KM concentration increased to $10\,mg\,l^{-1}$ or higher, the shoots were completely inhibited on the induction medium either supplemented with silver nitrate or not (data not shown).

Several studies showed that silver nitrate has positive effects on in vitro shoot regenerations of a plenty of plants, e.g. *Punica granatum* (Naik and Chand 2003), *Manihot esculenta* (Zhang et al. 2001), *Coffea canephora* (Sridevi et al. 2010) and *Malaxis acuminate* (Meena et al. 2010). Under certain circumstances, silver nitrate was shown to have negative effects on shoot regeneration of *Saccharum* spp. hybrids (Taylor et al. 1994). In *A. annua* silver nitrate can increase not only its shoot induction frequency but also the regenerated shoot number per explants (Figure 1-A, Figure 1-B). Silver nitrate addition at a concentration above $6\,mg\,l^{-1}$ slightly inhibited the shoot induction of *A. annua*, which was in concurrence with a similar response in other plants (Sridevi et al. 2010; Zhang et al. 2001), indicating that too high level of Ag^+ can be detrimental to its shoot induction. Silver nitrate can affect plant regeneration mode, thus capable of directly inducing shoot formation without intermediate callus phase (Sridevi and Giridhar 2013; Zhang and Hou 1996; Zhang and Ling 1995). In the study, silver nitrate additions at the different concentrations remarkably decreased callus formation (Figure 1-C). Therefore, the study held that the improved shoot induction capacity *of A. annua* was partially related to its callus formation inhibition.

Although silver nitrate enhances in vitro shoot growth (Giridhar et al. 2003), long time exposure to silver nitrate suppresses shoot development of *Brassica campestris* (Palmer 1992). We have found out similar phenomenon in this study. Shoot elongation percentages and average shoots were higher than control when the explants cultured on medium with silver nitrate for 20 days. Fifty days after the explants cultured on the silver nitrate containing medium, their shoot elongation percentages and average shoot lengths decreased (data not shown). The study hypothesized that silver nitrate additions at higher concentrations could lead to stronger toxicities of Ag^+ ions, thus detrimental to shoot elongation. No conclusion has been reached on the optimum culturing time necessary for silver nitrate to induce shoot formation. Shoot formation of *A. annua* mostly occurs after 20 day culture on the MS medium containing $1.0\,mg\,l^{-1}$ 6-BA and $0.05\,mg\,l^{-1}$ NAA. Therefore, a culture period of 20 days was enough for silver nitrate to promote shoot regeneration of *A. annua* explants.

Silver nitrate has positive influences on rooting of *Vanilla planifolia* Andr (Giridhar et al. 2001) and *Rotula aquatica* Lour (Chithra et al. 2004) in tissue culture. However, silver nitrate addition to the regeneration medium of *Pisum sativum* L. (Madsen et al. 1998) and barely (Castillo et al. 1998) inhibits their root formation. The present study showed that within 5–8 days after the elongated shoots of *A. annua* were transferred onto the MS medium, most of them rooted simultaneously, which indicated that explants cultured on the medium with silver nitrate at $2\,mg\,l^{-1}$ for 20 day no longer had any residual effects on rooting of the regenerated shoots, but it did have positive effects on the growth of the

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regenerated shoots, resulted in increased average heights of plantlets (Table 1). Silver nitrate addition at 2 mgl^{-1}, moreover, did not affect the sensitivity of regenerated *A. annua* shoots to KM, the selective agent applied in the genetic transformation (data not shown). This result was consistent with the result on cassava that silver nitrate produced no negative effects on the efficiencies of selective markers necessary for screening transgenic plants (Zhang et al. 2001).

The results in this study indicated that the addition of silver nitrate at 2 mgl^{-1} to the induction medium could improve not only the in vitro regeneration frequencies of *A. annua* but also the growth of the regenerated shoots. Silver nitrate did not affect adversely the efficiency of selectable markers required for screening its transgenic plants. Therefore, silver nitrate could be applied to the *Agrobacterimu*-mediated transformation of *A. annua* to improve transformation frequency.

Acknowledgements

This work was supported by the Public Welfare (agriculture) Research Project China (201303030) and by the Science and Technology Research Projects of Henan Province, China (13B210049).

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