International Journal of Plant Developmental Biology ©2010 Global Science Books

Inhibition of *Agrobacterium tumefaciens* Growth by Silver Nitrate

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ABSTRACT

Silver nitrate has been found to enhance plant regeneration frequency probably due to its anti-ethylene activity. It has also been found to be a potential inhibitor of *Agrobacterium tumefaciens* growth after co-cultivation due to its bactericidal properties. *A. tumefaciens* strain GV2260, harboring a plasmid with the marker gene neomycin phosphotransferase II (*npt*II) and the chimeric gene Def H9-*iaaM* was used to assess the extent of susceptibility to $AgNO_3$. Bacterial growth curves were generated using turbidimetric measurements of bacterial density to evaluate bacterial response in the presence of different $AgNO_3$ concentrations. At low initial cell densities (OD_{600} = 0.01 and 0.03) bacteria completely stopped growing at 8 and 10 mg l^{-1} of $AgNO_3$, respectively while at the initial cell density of OD_{600} = 0.1, silver ions strongly retarded cell growth at 25 mg l^{-1}; however, after 48 hrs they continued to grow, although at very slow rates. Also, bacterial fitness was assessed in the presence of kanamycin (Kan) after treatment with cefotaxime (0, 50, 100, 200 mg l^{-1}) (Cef) in combination with $AgNO_3$ to evaluate the frequency of plasmid loss. The presence of $AgNO_3$ was correlated with a decrease in plasmid stability in the presence of Cef (50 mg l^{-1}). Colonies that developed on non-Kan plates were analyzed by PCR to determine if functional copies of the *npt*II gene were still present after treatment with $AgNO_3$ and Cef. The effect on the growth and survival of bacterial cells investigated here may provide a useful clue to the interaction between plants and *Agrobacterium* during and after co-cultivation in the presence of $AgNO_3$, taking advantage that this chemical also stimulates plant regeneration.

Keywords: *Agrobacterium* growth inhibition, plant regeneration, plant transformation, plasmid loss

INTRODUCTION

Silver nitrate ($AgNO_3$) is known to be very toxic to organisms. Silver ions are effective inhibitors of microbial growth, including important pathogens (Lin *et al*. 1998). They have been used in disease control and water sterilization (Davies and Etris 1997). There is an increase interest in studying the role of $AgNO_3$ in plant regeneration (Orlikowska 1997; Zhong *et al*. 2002; Seong *et al*. 2005; Qin *et al*. 2005; Wang and Xu 2008). According to most publications, the addition of $AgNO_3$ (2-10 mg l^{-1}) to the medium markedly enhanced regeneration frequency as well as the number of shoots obtained per explant, probably due to its anti-ethylene activity (Biddington 1992). In addition to stimulating shoot regeneration *in vitro*, $AgNO_3$ can also be used to inhibit *Agrobacterium* growth after co-cultivation due to its bactericidal properties. In fact, it is difficult to eliminate *Agrobacterium* from plant tissues after transformation. The use of antibiotics often fails in the elimination and high concentrations can cause necrosis in tissues (Sjahril and Mii 2006). Latent contamination of *Agrobacterium* in plant tissues after co-cultivation often represents a serious problem since the presence of its DNA can interfere with results obtained by PCR analysis of putative transgenic lines (Shackelford and Chlan 1996). It also represents a possible vehicle for gene escape resulting in transfer of genes to non-target plants or other microorganisms in the environment (Barrett *et al*. 1996; Leifert and Cassells 2001). The excessive growth of *Agrobacterium* and its extended time of action during the course of transformation can also influence the T-DNA transfer process leading to a high number of copies of the transgene into the plant cell. There is a higher incidence of genetic instability in terms of gene silencing or low expression of transgenes correlated with multiple copy gene insertion (Assaad *et al*. 1993). Controlling the exposure of explants to *Agrobacterium* using appropriate levels of $AgNO_3$ could provide a balanced action for adjusting an accurate and proper transfer of T-DNA with a limited number of copies of the introduced DNA.

In order to assess the extent of susceptibility of *Agrobacterium* to $AgNO_3$, the following experiments were carried out to investigate its effect on the growth and survival of bacterial cells as well as the frequency of plasmid loss. This study will provide a useful clue as to the interaction between plants and *Agrobacterium* during and after co-cultivation in the presence of silver ions.

MATERIALS AND METHODS

Bacterial strain, culture conditions and growth measurements

A set of experiments was performed using *Agrobacterium tumefaciens* strain GV2260, harboring a plasmid with the marker gene neomycin phosphotransferase II (*npt*II) and the chimeric gene Def H9-*iaaM* (Rotino *et al*. 1997). The strain (kindly provided by Prof. Spena from Verona University, Italy) has been widely used in tomato, olive and kiwifruit transformation trials in our laboratory (Ficadenti 1999). Culture medium consisted of Luria Bertani (LB) broth, which was supplemented with 100 mg l^{-1} kanamycin (Kan) only in starter cultures. Cultures were diluted according to a specific starting optical density (OD_{600}). The diluting medium was a solution of the same composition used for the growth medium.

Received: 6 August, 2009. Accepted: 11 July, 2010.

Original Research Paper

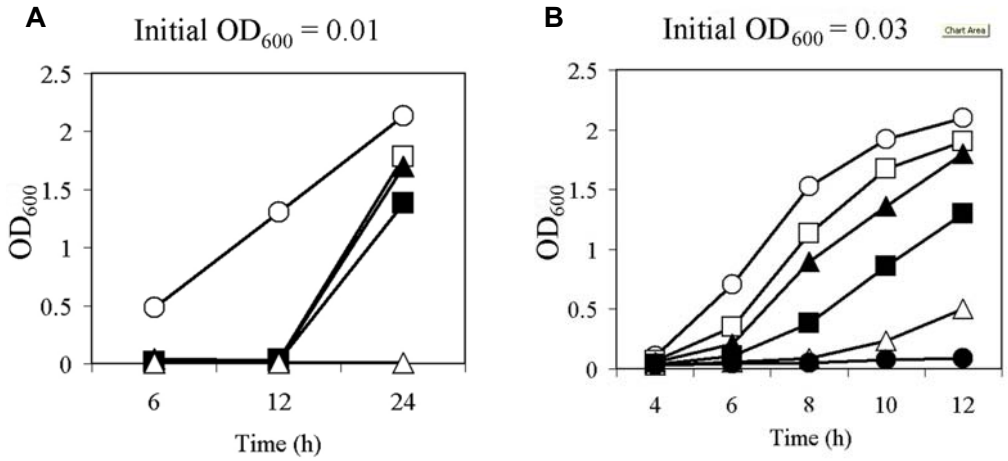

Fig. 1 Toxicant effect of $AgNO_3$ on growth curve patterns of *Agrobacterium tumefaciens*, strain GV2260 at different initial densities. (○), growth control; (□), 2 mg l^{-1}; (▲), 4 mg l^{-1}; (■), 6 mgl^{-1}; (Δ), 8 mg l^{-1}; (●), 10 mg l^{-1}. At time zero, overnight cultures were diluted in LB at a starting OD_{600} of 0.01 (**A**) and 0.03 (**B**) and then incubated at 28°C with shaking. At the indicated times, the OD_{600} was measured and plotted against time. Note the longer lag phase of cultures with lower initial density.

Growth was measured turbidimetrically on a spectrophotometer (Lambda 3B, Perkin Elmer). Readings were taken at variable intervals, depending on the slope of the curve being generated. Cultures were maintained at 28°C in 100 ml flasks containing 50 ml culture and shaken at 150 rpm on an orbital shaker in the dark. The pH of the medium was adjusted to 7 before autoclaving at 121°C for 20 min. Each treatment was carried out in triplicate. Each series of treatments was repeated twice. $AgNO_3$ crystals (Carlo Erba Reagenti) were sterilized by filtration using a disposable sterile Millex syringe filter (0.2 µm and 25 mm diameter) and stored in the dark, protected from light.

Bacterial growth experiments

For the initial test of $AgNO_3$ on *A. tumefaciens* inhibition, bacterial growth curves were generated to evaluate the presence of different concentrations of $AgNO_3$. Since the frequency of transformation can be influenced by the *Agrobacterium* density, bacterial inoculation was adjusted to three different initial cell densities: OD_{600} = 0.01, 0.03 and 0.1 from liquid cultures in the mid-log-phase growth (8 hrs). $AgNO_3$ concentrations were applied according to initial optical density as follows: 0, 2, 4, 6, 8, 10 mg l^{-1} for the first two initial densities and 0, 10, 15, 20, 25, 30 mg l^{-1} for the third one. The turbidities of the bacteria were compared to evaluate the toxic effects of $AgNO_3$.

A subsequent experiment was performed by using cefotaxime sodium salt (Cef) (Sigma-Aldrich, USA), one of the most used antibiotics in transformation protocols against *Agrobacterium*. Cef was added at 0, 50, 100, and 200 mg l^{-1}, in combination with 10 mg $AgNO_3$ l^{-1} and initial cell densities were adjusted to OD_{600} = 0.06. The preliminary results of this experiment indicated that bacteria were able to grow at 50 mg l^{-1} Cef and 10 mg l^{-1} $AgNO_3$, therefore a further analysis was carried out using the same concentration of Cef in combination with a range of $AgNO_3$ concentrations (0, 10, 20, 30 mg l^{-1}); however, this time initial cell densities were adjusted to OD_{600} = 0.10. After 4 days of culture, 100 µl aliquots from each treatment were plated onto LB solid medium containing either 100 mg l^{-1} Kan or free of it, using polystyrene Petri dishes (90 mm Ø) (Sterilin Ltd., UK). The inoculation of dishes was followed by a 4-day incubation at 28°C in the dark. The number of colonies that formed in Kan-containing media was compared to that of Kan-free media. Serial dilutions (10^{-6}) were prepared before plating only on control cultures (0 mg l^{-1} AgNO3) in order to facilitate colony counts. Individual colony samples were used for assessing the presence or absence of plasmid by PCR amplification of the *npt*II gene by using specific primers to test for its presence as reported in Velten and Schell (1985) (forward: ATG GAT TGC ACG CAG GTT CTC; reverse: CCA ACG CTA TGT CCT GAT AGC), in Perkin Elmer thermal cycler. The standard of 25 µl PCR reaction contained 2.5 µl dNTPs (250 µM), 2.5 µl of each primer (50 mM), 2.5 µl 10X PCR buffer, 0.75 µl of $MgCl_2$ (25 mM) and 0.2 µl *Taq* polymerase (Invitrogen, USA) with 13.05 µl water and 1 µl of template DNA (20 ng μl^{-1}). Reaction conditions were 95°C for initial step of 5' at 94°C, PCR was carried out for 30 cycles of 1 min at 94°C, 1 min 58°C; then 1: 30 min at 72°C and a final incubation of 5 min at 72°C.

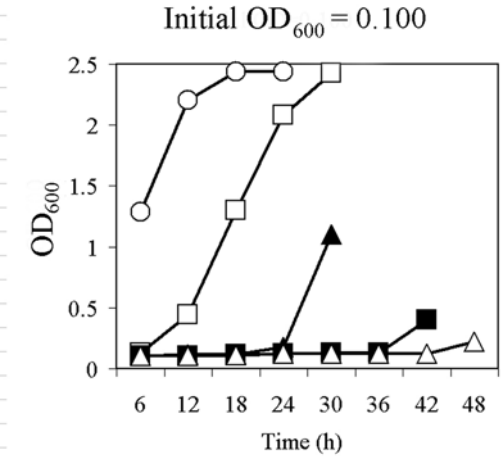

Fig. 2 Toxicant effect of $AgNO_3$ on growth curve patterns of *Agrobacterium tumefaciens*, strain GV2260. (○), growth control; (□), 10 mg l^{-1}; (▲), 15 mg l^{-1}; (■), 20 mg l^{-1}; (Δ), 25 mg l^{-1}. At time zero, overnight cultures were diluted in LB at a starting OD_{600} of 0.100 and incubated at 28°C with shaking. At the indicated times, the OD_{600} was measured and plotted against time. At 25 mg silver nitrate l^{-1} (Δ), bacteria seemed to be died; however, they presented a long lag phase followed by growth although at very slow rates.

Data were analyzed using the student's *t*-test for independent samples at P = 0.05 (SigmaStat program). All experiments were repeated twice.

RESULTS AND DISCUSSION

$AgNO_3$ strongly inhibited *Agrobacterium* cell growth at 10 mg l^{-1} starting at OD_{600} = 0.03. When $AgNO_3$ was applied at lower initial bacterial concentrations (OD_{600} = 0.01), inhibition of bacterial growth occurred at 8 mg l^{-1} $AgNO_3$. Lag phases were longer at OD_{600} = 0.03 (**Fig. 1A, 1B**). Higher concentrations were markedly toxic to the bacterial cells. In contrast, the bacterial cells were not inhibited at the doses where the culture started with a higher turbidity (**Fig. 2**). When initial density was OD_{600} = 0.1, complete inhibition of bacterial growth occurred only at 30 mg l^{-1}. At 20 mg l^{-1} $AgNO_3$, bacteria seemed to stop growing, although, after 48 hrs they continued to grow but at a very slow rate (OD_{600} < 0.5). At this initial density, *Agrobacterium* grew as well as the control in the presence of < 8 mg l^{-1} (data not shown).

When bacterial growth was not completely inhibited, long lag phases followed by growth were observed. This is probably due to the adaptation of the microorganism to the chemical with the help of a selective mutation (Alsheikh *et al*. 2002). $AgNO_3$ strongly influences bacterial viability under a low level of initial cell density and short-term exposure but not under high density and long-term exposure.

$AgNO_3$-Cef interaction

From previous trials working with GV2260 strain in our lab, we have noticed that the use of only Cef (up to 200 mg l^{-1}) is not enough to completely suppress this strain, while only retarding its growth. However, we also noticed that silver particles were able to enhance the antibacterial action of Cef. The combination of 100 and 200 mg l^{-1} Cef with $AgNO_3$ inhibited completely cell growth (data not shown). Therefore, we wanted to analyse in the present experiment, the response of GV2260 strain to the presence of low amounts of Cef (50 mg l^{-1}) in combination with $AgNO_3$. **Fig. 3** shows the bacterial growth behaviour using 50 mg l^{-1} Cef in combination with 10 mg l^{-1} $AgNO_3$. Although growth at this concentration was not completely inhibited, cells grew at very slow rates and prevailed over adverse conditions with difficulty. Instead, cells under treatment with only 10 mg $AgNO_3$ l^{-1} overcame unfavourable conditions after 10 hrs of resistance, time in which cells started growing normally. We also wanted to ascertain the possible plasmid loss in surviving cells as a result of the strong treatment of Cef + $AgNO_3$ following Kan resistance screening in surviving cells after treatment.

Surviving bacterial cells exposed to Cef (50 mg l^{-1}) plus a range of $AgNO_3$ in liquid medium were transferred onto solid medium; a few colonies formed in Kan-free LB solid medium but often none formed in medium containing Kan (**Fig. 4**). The presence of the *npt*II gene was not often detected by PCR amplification in cells sampled from colonies that formed on Kan-free medium observing the *npt*II gene only around of 30% from colonies sampled.

These conditions, i.e. that bacteria were not often able to grow under Kan selective pressure and that the *npt*II gene was not always amplified by PCR, provide reasonable evidence of an increase in the frequency of plasmid loss as a response to stress (**Fig. 5**). Chemical compounds may provoke a high selective pressure in cells during cell division; consequently, some daughter cells may not receive a copy of the plasmid as found in other recombinant bacteria (Smith and Bidochka 1998; Cazorla *et al*. 2001).

Heavy metals are toxic for all living cells; however, many organisms find a way to overcome this toxicity by avoiding toxic metals before they can damage them. This slowing of growth was also clearly visible when cultures were kept for a long period (48 hrs) with only $AgNO_3$. For this reason, it is not advisable to replace Cef with $AgNO_3$ after co-cultivation, although the former is often phytotoxic. The integration of both compounds should be applied at least during the first passage after co-cultivation with *Agrobacterium*. Apparently, silver ions inhibit growth by attaching to the bacterial cell wall, which is negatively charged by interrupting its normal impermeable activity. Bacterial cells die by disruption of their bounding membrane allowing the cell contents to escape (Ratte 1999). Cef may aid in suppressing definitely the bacterial cell before it can overcome severe damage by silver particles.

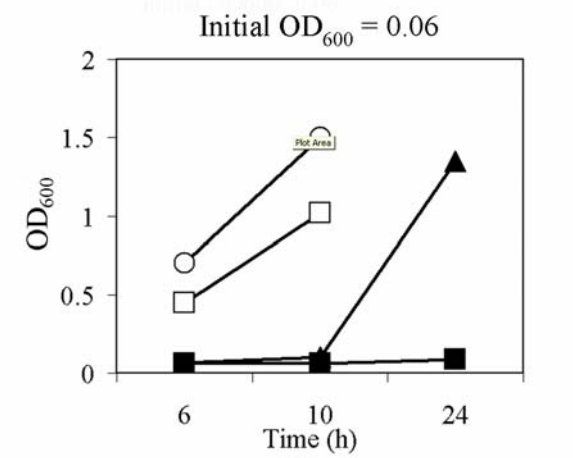

Fig. 3 Toxicant effect of $AgNO_3$, alone or in combination with cefotaxime (cef), on *Agrobacterium tumefaciens* growth, strain GV2260. (○), growth control; (□), 50 mg cef l^{-1}; (▲), 10 mg silver nitrate l^{-1}; (■), 50 mg cef l^{-1} + 10 mg silver nitrate l^{-1}. At time zero, overnight cultures were diluted in LB at a starting OD_{600} of 0.060 and incubated at 28°C with shaking. At the indicated times, the OD_{600} was measured and plotted against time. Treatment of cef + silver nitrate (■), showed survival cells after 24 hours (OD_{600}=0.089) growing at very slow rates.

Advantages of silver nitrate in plant tissue culture

$AgNO_3$ improves regeneration and transformation efficiency (Zhong *et al*. 2002; Seong *et al*. 2005; Qin *et al*. 2005; Wang and Xu 2008). A large number of protocols reporting the use of $AgNO_3$ on *in vitro* plant regeneration have been published during the last two decades. Its use has been described for over 30 plant species in ethylene inhibition. Petri *et al*. (2005) found an important participation of silver ions (as thiosulphate form) in stability enhancement of transformation events. $AgNO_3$ has also been used in yeast decontamination in shoots (Kolozsvari *et al*. 2005) and it seems to be innocuous for the plant cells at reported concentrations. On the other hand, $AgNO_3$ can be used for suppressing *Agrobacterium* post co-cultivation. It is well known that latent contamination of *Agrobacterium* in plant

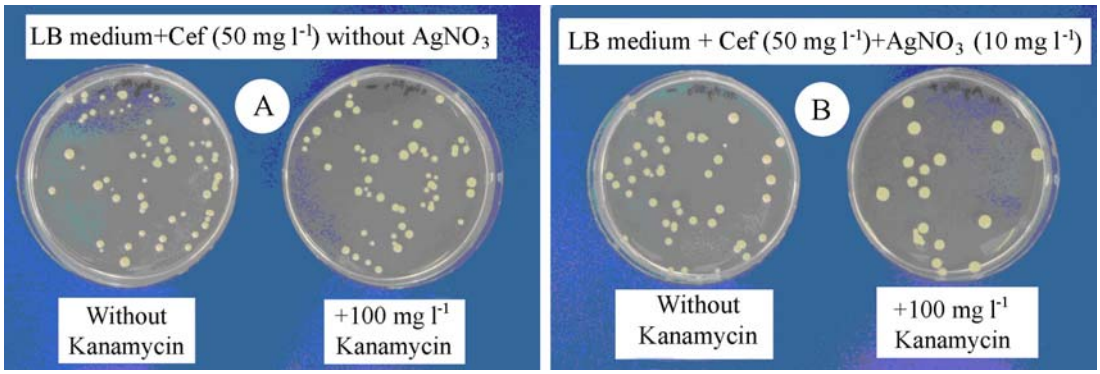

Fig. 4 (**A**) Comparison on bacteria growth culture between LB free-kanamycin culture (left dish) and LB-kanamycin (right dish) where surviving bacterial cells were plated after treatment with 50 mg cef l^{-1} without silver nitrate. (**B**) Comparison on bacteria growth culture between LB free-kanamycin culture (left dish) and LB-kanamycin (right dish) where surviving bacterial cells were plated after treatment with 50 mg cef l^{-1} + 10 mg silver nitrate l^{-1}. The difference in the number of arisen colonies between the dishes was considered as an indication that makes plasmid loss evident. Values were statistically analyzed and some colonies were sampled to prove the missing of plasmid by amplifying *npt*II gene.

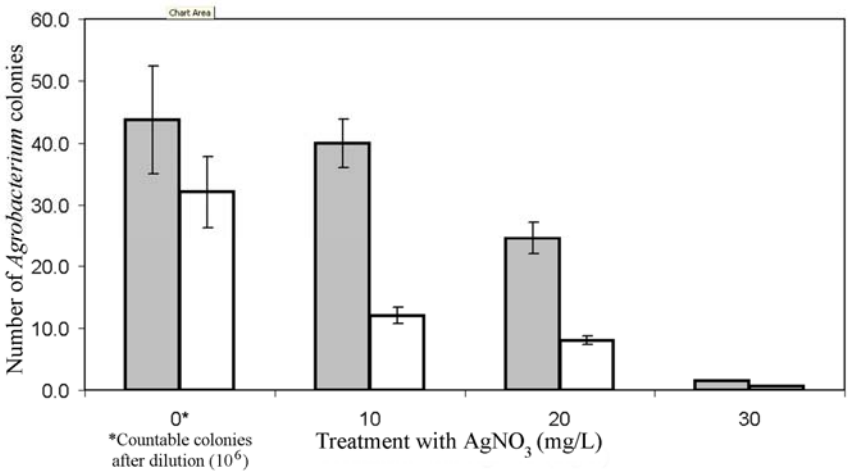

Fig. 5 Frequency of plasmid loss occurrence. Comparison between LB and LB-Kan (kanamycin-containing) selective medium. Effect of Kan on *Agrobacterium* cells recovered on LB solid medium after treatment with a range of $AgNO_3$ concentrations (0, 10, 20 and 30 mg l^{-1}) in LB liquid medium enriched with 50 mg cefotaxime l^{-1}. Colonies arose on LB-Kan medium are represented by white bars and are compared with their respective treatment represented by grey bars LB (Kan-free). Serial dilutions (10^{-6}) were prepared before plating only on control cultures (0 mg 1^{-1} $AgNO_3$) in order to facilitate the count of colonies. Values are means of three replicates and bars represent standard errors. At 0 mg 1^{-1} of $AgNO_3$ (control dishes), the number of colonies arisen in LB-Kan dishes did not show significant difference respect to the number of colonies arisen in LB dishes. While significant differences were determined between LB and LB-Kan in treatments containing $AgNO_3$ according to Student's *t*-test ($P = 0.05$). The participation of $AgNO_3$ was evident in the difference about the overall number of colonies that grew on the Kan plates and non-Kan plates. Compared to the plates with Kan, more colonies grew on the plates with only LB media because any strain could grow on those plates without being inhibited by Kan. Consequently, fewer bacteria grew on the plates with Kan because only the bacteria that retained the plasmid after treatment with $AgNO_3$ could survive.

tissues after co-cultivation can interfere with results obtained by PCR analysis of putative transgenic lines. Current methods for detecting this contamination are not sufficiently sensitive or may require radioactivity and large amounts of tissue and long analytical processes (reverse transcription or Southern blot analysis). Recently, a new method was proposed for preventing *Agrobacterium* to be amplified by PCR (Nain *et al*. 2005), but this method involves the use of specific restriction enzymes for cutting the unexpected *Agrobacterium* DNA. A novel antibiotic, meropenem, with no phytotoxic effect, has also been proposed for suppressing *Agrobacterium* (Sjahril and Mii 2006). $AgNO_3$ enhances the potential of Cef as a better control of explants exposed to *Agrobacterium*. It may provide a balanced action for the accurate transfer of T-DNA with a limited number of copies of the introduced DNA and also avoid possible gene escape for latent contamination after plants are potted in soil. Some reports have mentioned the slowing of bacterial growth in response to $AgNO_3$ (Orlikowska 1997; Zhong *et al*. 2002), but the behaviour of bacteria against soluble silver ions has never been reported.

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