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NANO SILVER FUNCTIONALIZATION ON *AGROBACTERIUM*-MEDIATED TRANSFORMATION WITH COMPANIONSHIP OF NANOBIO TECHNOLOGY

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ABSTRACT In genetic transformation studies explants are exposed to antibiotics in the medium for a long time to eradicate their surplus bacteria, that not only loses the explant, but also increases the possible resistance to bacteria because common antibiotics have a single mode of action. In this study, for the first time, the interaction of silver nanoparticle (NS) against *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pBI121 and *A. rhizogenes* strain K599 containing the binary vector pKGWFS7.0-35Sp, that are the common bacteria in genetic transformation studies, is reported. Transmission electron microscopy (TEM) was assessed to study the biocidal action of this nanoparticle on bacteria after 4 hours. Results confirmed that NS can alternatively be used instead of common antibiotic treatments in genetic transformation experiments. Using the present method resulted in a good GUS scorable gene transfer to an ornamental and medicinal plant, *Tecomella* sp. that could be a milestone for future research in the area of genetic engineering.

Keywords: *Agrobacterium*, Genetic transformation, Nanobiotechnology, Transmission electron microscopy (TEM).

INTRODUCTION Nanotechnology is enabling technology that deals with nano-meter (a billionth) sized objects (Fynmen, 1991). While benefits of nanotechnology are widely published, the discussion of adverse effects of their widespread uses on cell and environments is rather unclear. Colloidal silver is widely used in antimicrobial formulation and dressings (Salata, 2004). Among noble-metal nonmaterial, silver nano particles have received considerable attention due to their strong toxicity that silver exhibits in various chemical forms to a wide range of microorganisms is very well known (Hollady, 2006) and silver nano particles have recently been shown to be a promising antimicrobial material (Sondi and Salopek-Sondi, 2004). Plant transformation is an invaluable tool for basic plant research, as well as a useful technique for the direct improvement of commercial crops. In a comparison of particle bombardment and

Agrobacterium- mediated techniques in plants, it was shown that *Agrobacterium*-mediated transformation was more efficient and led to the production of transgenic lines with lower numbers of transgenic copies. In addition, lines produced using *Agrobacterium*-mediated techniques showed more stable transgene expression over generations and fewer cases of transgene silencing (Travella, 2005). However, bacteria growth after cocultivation on explants is one of the most important problems in plant biotechnology and genetic transformation. The uses of antibiotic such as cefotaxime, carbencillin and thimentin is common to eradication exceed bacteria in culture medium in world. Conventional antibiotics and chemotherapeutic agents may be employed to correct persistent problems, but frequently are phytotoxic or may retard plant tissue growth (Dodds and Roberts, 1981) and prolonged exposure increases the risk of resistance and may even progress to dependence on the drug (Falkiner, 1990).

The use of antibiotic may have negative and genotype dependent repercussion on the regeneration capacity of the initial explant and on the genetic stability of the resulting plant materials (Teixeira et al., 2003). As you know that in plant transformation explants are exposure to antibiotic for a long time that it not only lose the explant but increase inferences about possible occurrence persist to bacteria owing to single mode of action of cefotaxime that just have a interaction to cell division and may bacteria persist them. Among the most promising nano materials with antibacterial properties are metallic nano particles, which exhibit increased chemical activity due to their large surface to volume ratios and crystallographic surface structure (Morones et al., 2005). For these reasons, and based upon our previous work regarding interactions of silver nano particles with bacterial contamination in vitro plant cell culture, we decided to study the replacing nano silver to common antibiotic in genetic transformation. The usage of nano silver in plant biotechnology can be a milestone for future research. Resistance of bacteria to bactericides and antibiotics has increased in recent years due to the development of resistant strains. Some antimicrobial agents are extremely irritant and toxic and there is much interest in finding safe and cost effective biocidal materials (Sondi and Salopek-Sondi, 2004). Here we investigate the bactericidal action of nano silver against *A. tumefaciens* and *A. rhizogenes* that is the common bacteria for genetic transformation. The ultimate objectives of the present work were to applied NS replaced common antibiotic for gene transformation in *Tecomella* sp. overthrow trees. To our knowledge the application of NS in plant biotechnology and genetic transformation as an alternative to common antibiotic has not been reported in the literature. To the best of our knowledge, this is the first report of application of NS in cell biology for prevent adverse effect of common antibiotics in genetic transformation.

MATERIALS AND METHODS

Bactericidal test The average size of nano silver (NS) used in this study was 18.5 nm. To examine the effect of NS on *Agrobacterium* approximately 10⁵ Colony forming units (CFU) (0d= 1.5) of *A. tumefaciens* strain LBA4404 (Jefferson et al. 1987) and *Agrobacterium rhizogenes* K599 (was obtained from Dr. Hahn) were culture on LB (Tryptone 10 g/L NaCl 5 g/L, Yeast Extract 5 g/L) medium agar plate supplemented with NS in concentration of zero, 5, 10, 15, 20, 25, 50, 100, 200, 400 and 800 µg/ml. The plates were incubated for 48 h at 28° C and the number of colonies was counted. In another experiment bacteria were grown in 10 cm³ LB liquid medium with NS in

concentration of zero, 5, 10, 15, 20, 25 and 30 µg/ml. Growth rate and bacterial concentration were determined by measuring optical density (OD) at 600 nm with interval 5 h with spectrophotometer.

Kanamycin selection The single nodes of *Tecomella* sp. were cultured on MS medium (Murashige and Skoog, 1962) containing a series of kanamycin (Sigma Co.) (0, 65, 85 and 105 µmol) to find optimum concentration for selection of transgenic explants.

NS selection MS media containing a series of NS (0, 25, 50, 100, 200, 400, 800, 1000, 1500 µg/ml) were used to find concentrations maximum that plant can growth them

Plant materials For proven our hypothesis we conduct a practical experiment on two trees species. The Agrobacterium-mediated transformation of two species including *Tecomella undulata* and *Araucaria excelsa* R. Br. was performed as follows. In this experiment we use to micropropagated single nod In vitro explant for transformation with *A. tumefaciens* and *A. rhizogenes*.

Agrobacterium strains and binary vectors *A. tumefaciens* strain LBA4404 harboring a plasmid contained the binary vector pBI121 contains the GUS gene under control of the cauliflower mosaic virus (CaMV) 35S promoter and the selectable marker neomycin phosphotransferase II (nptII) under control of the nopaline synthase (NOS) promoter and NOS terminator for both gene. Another bacterium was *A. rhizogenes* (K599) including binary vector pKGWFS7.0-35SP. Bacteria was grown and selected in rotatory (200 rpm) in LB liquid medium containing 50 mg/L kanamycin and 50 mg/l rifampicin for *A. tumefaciens* and 50 mg \L spectinomycine for *A. rhizogenes* overnight at 28°C. The cells were harvested by centrifugation (4500g, 10 min) and further re-suspended in 10 ml MS medium. Acetosyringone was added to the medium up to 200 µmol. For transformation we perform two procedure 1) explants were Inoculated with bacteria for 24 h without co-cultivation (Aslam et al., 2009) then after blotting, explants were cultured to medium supplemented with 11.09 µm BA, 0.57 µm IAA, and NS, then after one weeks cultured in selective medium contain kanamycin and 2) explant inoculated to bacteria for 20-60 min in 10 ml MS medium, then cultured on cocultivation MS medium to nearly 3 days.

After cocultivation, explants washed and cultured on MS medium supplemented with NS, BA and IAA for 7 days and then explants cultured on kanamycin and NS selection medium supplemented with BA and IAA.

DNA extraction and polymerase chain reaction (PCR) PCR amplification of the GUS genes that is exist in two bacteria, using their genes specific primers (GSPs) was carry out to check the present of transgenes in the plant genome. Total genomic DNA was isolated (Stange et al. 1998), with more modifying from young leaf tissue after 40- 50 days And DNA concentration were measured using Nano drop (Thermo. ND1000. USA). The PCR was carry out for screening of regenerated plantlets with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C, 1 min, 55 °C, 1 min, 72 °C for 1 min, and a final extension, 75 °C for 10 min, using gene specific primers for GUS (.5'- GGT GGG AAA GCG CGT TAC AAG -3' and 5'- TGG ATT CCG GCA TAG TTA AA -3') gene.

TEM analysis The interaction of *A. tumefaciens* and *A. rhizogenes* with silver nano particle was analyzed with transmission electron microscope (TEM) according to (Bechtel et al. 1976) with highly modifying.

RESULTS

Bioassay of bacteria exposed to NS Results demonstrated that *Agrobacterium* growth impeded in liquid LB mediums containing more than 10-20 $\mu\text{g/ml}$ NS after 20h. Bacterial growth in LB solid medium in concentrations more than 15 $\mu\text{g/ml}$ were suppressed (Fig. 1 and 2).

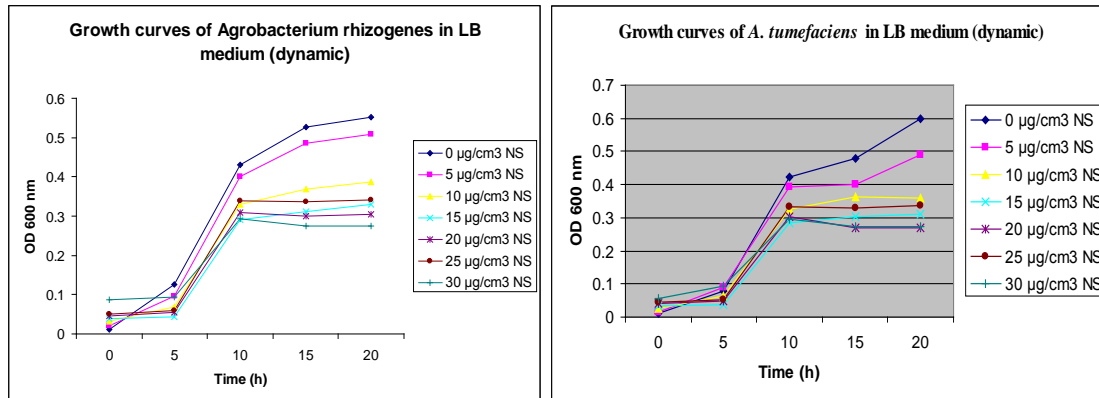


Figure 1. Growth curves of *A. rhizogenes* and *A. tumefaciens* in LB liquid medium supplemented with different concentration of NS.

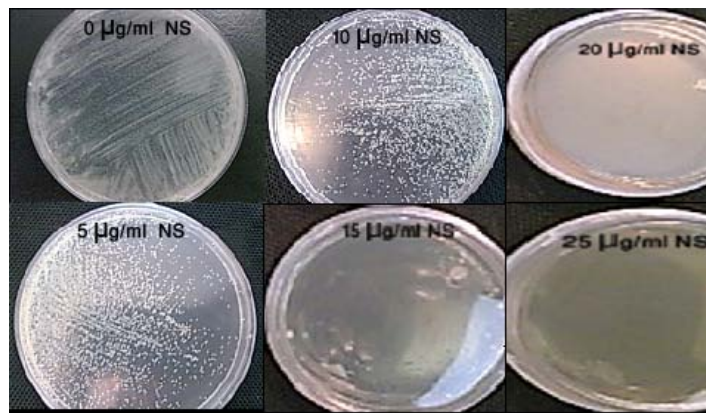


Figure 2. Single colony of *A. tumefaciens* in LB solid medium supplemented with different concentration of NS.

NS selection Explant can be grown in medium supplemented with NS nearly 600 $\mu\text{g/ml}$. Hence, to likely prevent explant losses we used 20 $\mu\text{g/ml}$ from NS.

Kanamycin selection Kanamycin was chosen as the selective agent in this study, because the binary vectors pBI121 and pKGWFS7.0-35SP both contained the neomycin phosphotransferase (*nptII*) gene. More than 20% of explants

regenerated on the media with 65 μmol kanamycin concentration. A very low regeneration rate was observed with kanamycin at 85 μmol , while no regeneration occurred on the media containing 105 μmol . Thus 85 μmol kanamycin were the best concentration for selecting regenerating explants of *Tecomella* sp.

Agrobacterium mediated transformation After cocultivation in the medium supplemented to 30 $\mu\text{g/ml}$ NS bacteria could attractively generate. Hence we augmented dos of NS up to 600 $\mu\text{g/ml}$. After one weeks explants subcultured to medium supplemented with 500 $\mu\text{g/ml}$ NS, BA, IAA and 85 μmol kanamycin. Cultures were kept at 25°C under cool white fluorescent light (30 $\mu\text{m m}^{-2} \text{s}^{-1}$), for 16 h each day. A relatively short co-cultivation (3 days) was optimal for *T. undulata* transformation. In contract to Aslam et al. (2009) no explant survived in treatment without cocultivation (data not shown). But in treatment with cocultivation some explant grown in selective medium.

Polymerase chain reaction (PCR) The frequency of transformation in *T. undulate* was 1% with *A. tumefaciens* (Fig. 3). But explants of *Araucaria excelsa* R. Br. is not regenerated in kanamycin selection medium, however 600 $\mu\text{g/ml}$ NS suppressed bacterial growth.

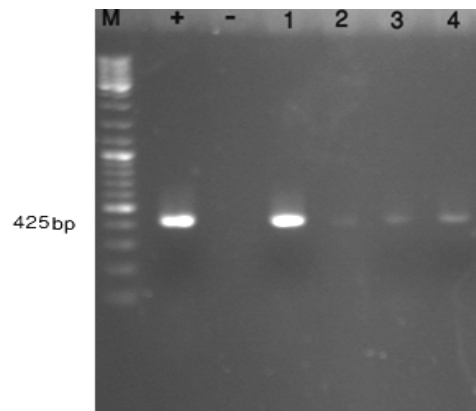


Figure 3. Molecular analysis of putative transgenic plants of *Tecommella* sp. Electrophoretic analysis of PCR products of transgenic *Tecommella* sp. plants showing the presence of an expected 425 bp of *GUS* gene amplified using GSPs. Lane M is the marker, Lane + is positive control (cloned pbi 121 DNA), Lane – is untransformed plant DNA. Lane 1-4 are independent transgenic lines of *Tecommella* sp. plants.

TEM analysis The actual mechanism by which silver nano particles interfere with bacteria is as yet unclear. Fig. 4 shows Transmission electron microscopy (TEM) micrograph of NS in interaction with *A. tumefaciens* and *A. rhizogenes*.

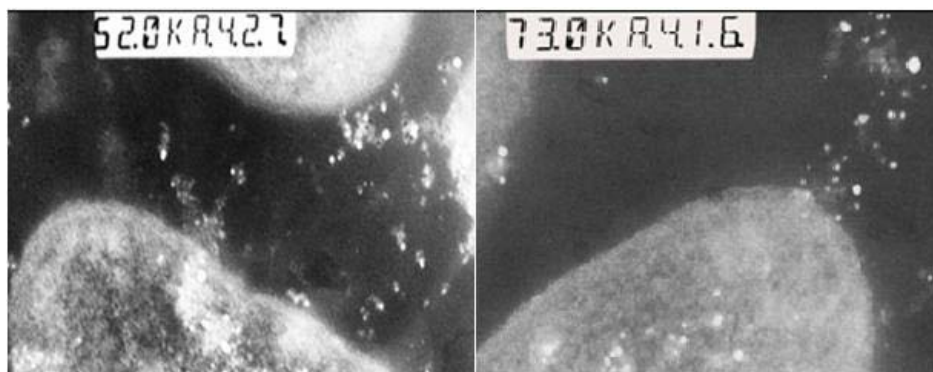


Figure 4. Transmission electron microscopy (TEM) micrograph of NS in interaction with *A. tumefaciens* (Left) and *A. rhizogenes* (Right).

DISCUSSION In recent years much perfunctory literature has been reported that exhibited Nan silver has a high considerable collision on bacteria but it never practical. Our study in three years ago demonstrate that when in genetic transformation technique explants cocultivated with bacteria, low concentration of NS is not efficacious to eradication bacteria contamination. Hence high levels of NS with a small size needed to decontaminate explant without any side effects on explant. Our results prove conceivable that NS displaced with widespread antibiotic in the culture medium. Due to bacterial contiguity to explant it is make possible growth on high level of MS medium supplemented with NS. Whereas *A. rhizogenes* and *A. tumefaciens* not enable separately in MS medium supplemented with NS. Insofar as we know Silver ions (Ag⁺) are potent inhibitors of ethylene action (Taiz and Zeiger., 2006). Ethylene produced by plants that this Cause concussioned explants. Our previous study on tissue culture of *Araucaria excelsa* R. Br. var. *glauca* that is a conifers showed that grown explants in the medium supplemented to NS is better than control plant that this hypothesis confirmed by several reports. The explants grown in NS medium were relatively healthier than to the control. (Akasaka, 2005; Qin, 2007; Eapen, 1997; Zang, 2001). It is demonstrated that nano silver particle expunge microbial infection by a several action. silver ions interact with sulfydryl (-SH) groups of proteins as well as with the bases of bacterial DNA leading either to the inhibition of respiratory processes or DNA unwinding (Bragg and Rannie, 1974; Batarseh, 2004) and its interaction with hydrogen bonding processes has been demonstrate to occur (Russell and Hugo 1994). It is believed that the mechanism of the antibacterial effects of silver ions involves shrinkage of the cytoplasm membrane or it's detachment from the cell wall. DNA molecules become condensed and lose their ability to replicate upon the infiltration of Ag ions (Geong et al., 2005). The silver ions also interact with the thiol groups of proteins, which induce the inactivation of bacterial proteins (Geong et al., 2005). The major mechanism through which silver particles manifested antibacterial properties was by anchoring to and penetrating the bacterial cell wall, and modulating cellular signaling by dephosphorylating putative key peptide substrates on tyrosine residues (Braydich-Stolle et al., 2005; Shrivastava et al., 2007). However further study in genetic transformation must be conducted in other woody and herbaceous plants. This report is a milestone to new threshold in nanobitechnology.

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