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Effects of silver nanoparticles on *Tecomella undulata* (Roxb.) Seem. micropropagation

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Abstract: Plant tissue culture is a reliable tool for conservation and multiplication of many plants, including medicinal plants. *Tecomella undulata* (Roxb.) Seem. is a plant native to tropical regions such as Iran, India and Pakistan; this precious plant which contains lapachol (a strong antiseptic used against jaundice) is an endangered species, therefore its conservation is of prime importance. The aim of this experiment was to evaluate the effects of silver nanoparticles (SNPs) at concentrations ranging from 5 to 80 mg l^{-1} alone or combined with 6-benzyl-amino-purine (BAP) and indoleacetic acid (IAA) on growth properties of *T. undulata* in aseptic condition. Thidiazuron (TDZ) at concentrations from 0.001 to 20 mg l^{-1} was used in proliferation medium of *T. undulata* single nodes; combinations of BAP (from 0.3 to 1.2 mg l^{-1}), and 2,4-dichloro-phenoxy-acetic acid (2,4-D, 0.2 and 0.4 mg l^{-1}) were also used in callus production and in indirect bud regeneration media. Explants were surface sterilized using 10% Clorox for 7-8 minutes. Results indicated that adding of SNPs in MS medium increased the mean number of fresh shoots per explants (MNFS/E), the percentage of explants producing shoots (PEPS) and also plant survival, due to its action on ethylene blockage. TDZ at the concentration of 0.1 mg l^{-1} increased bud proliferation up to two buds per explants, however higher concentration inhibited growth and in some cases caused death of the explants.

1. Introduction

Tecomella undulata (Roxb.) Seem., known as Rohida tree, is an ornamental and medicinal shrub species of the Bignoniaceae family widespread in tropical regions such as Iran, India, and Pakistan. This endangered plant species currently grows in arid and semi-arid regions of southern parts of Iran. Its bark contains lapachol, a naphthoquinone with anticancer, antibacterial, antifungal, antivirus (Consolacao *et al*., 1975; Guiraud *et al*., 1994; Hussain *et al.,* 2007) and analgesic (Ahmad *et al*., 1994) activities. It may have a pivotal role in environmental conservation in the arid parts of Iran; furthermore it is an accepted tree species in agroforestry systems (Tewari and Singh, 2009). Due to its beautiful flowers and semi-deciduous habit, it can be used in landscaping with some limitations due to its sterility and low potential germination of the few seeds produced in Iran. Karami and Salehi (2010) reported that rooting of stem cuttings is limited from spring to autumn in this species and it is included in the list of hard to root woody plant species. Therefore, micropropagation is necessary to protect this endangered species. Plant tissue culture is one of the most important steps in genetic transformation and plant biotechnology studies to produce plantlets from stock plants as a rapid and efficient procedure throughout the year (Giri *et al*., 2004; Sarmast *et al*., 2009). There are few reports about micropropagation of *T. undulata*; *in vitro* seasonal effect on shoot proliferation was reported (Rathore *et al*., 1991). Attempts have been made to multiply *T. undulata* plants through micropropagation. Rathore *et al*. (1991) reported that about 8-10 shoots were obtained over two to three weeks at 31ºC in MS medium supplemented with BAP (2 mg l^{-1}) and IAA (0.05 mg l^{-1}). Robinson *et al.* (2005) claimed that MS basal medium supplemented with 1.5 mg l^{-1} BAP and 0.02 mg l^{-1} IAA was the most effective medium for maximum (95%) regeneration of nodal explants. They reported that 29 shoots per nodal segment were observed on MS medium supplemented with 0.75 mg l^{-1} BAP and 0.01 mg l^{-1} IAA within 3 weeks. Furthermore, they observed about 27% mortality after transfer of plantlets to soil mixture. Aslam *et al*. (2009) developed a transformation protocol for *osmotin* gene in *T. undulata*. The effects of silver nanoparticles (SNPs) on decontamination in tissue culture systems have been reported (Abdi *et al*., 2008; Sarmast *et al*., 2011). However, the effect of silver-based material on ethylene mode of action is not well acknowledged in plants (Taiz and Zeiger, 2006). Explants grown in media supplemented with silver ions (Eapen and George, 1997; Zhang *et al*., 2001) and SNPs (Sarmast *et al*., 2011) were relatively healthier than control. The most important step of any *in vitro* propagation system is mass multiplication of plantlets that are genetically homogenous and phenotypically uniform (Sarmast *et al*., 2012). Therefore, micropropagation is restricted to direct regeneration.

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The aim of the present work was to assess SNPs as ethylene inhibitor for the improvement of micropropagation in *T. undulata.* A secondary objective was to examine TDZ and BAP effects on direct and indirect regeneration in *T. undulata* through single node and callus obtained from the proximal part of the explants.

2. Materials and Methods

More than 15-year-old Iranian *Tecomella undulata* plants were chosen for the present study. Stem pieces (4 cm long) were cut and prewashed in tap water for 15 min. Explants were then treated with 10% Clorox (containing 5.25% sodium hypochlorite) plus 0.2% household detergent for 7 min for surface sterilization and then rinsed six times with sterilized distilled water. The stem pieces were finally cut into nearly 10 mm long segments (including a single node) and placed with their proximal ends on MS (Murashige and Skoog, 1962) basal medium with 3.0% sucrose and 0.8% agar. Mean number of fresh shoots per explant (MNFS/E), mean length of shoots per explant (MLS/E), mean diameter of callus per explant (MDC/E) and percentage of explants producing shoots (PEPS) on MS medium supplemented with SNPs (5 to 80 mg l^{-1}) and combination of BAP (2.5 mg l^{-1}) and IAA (0.1 mg l^{-1}) (Aslam *et al.,* 2009) along with SNPs (5 to 80 mg l^{-1}) were evaluated. Thidiazuron (TDZ) was used at concentrations ranging from 0.001 to 10 mg l^{-1} for assessment mean number and length of shoots per explant, after two weeks of implementation. The average size of nanoparticles used in this study was 18.5 nm and they were synthesized by nanotechnologies, Inc. (Nanocid Company, Tehran, Iran) (Fig. 1). Callus derived from TDZ primary treatments was cultured for one week on MS hormone-free medium and then transferred to MS medium supplemented with BAP (0.3 to 1.2 mg l^{-1}) in combination with 2,4-D (0.2 and 0.4 mg l^{-1}) for indirect bud formation. The explants were subcultured every two weeks. The pH of the media was adjusted to 5.8 before autoclaving for 15 min at 121°C and 1.5 kg cm^{-2} pressure.

Cultures were kept at 25±2°C under cool white fluorescent light (30 µmol m^{-2} s^{-1}) with 16/8 h photoperiod. The experiment was conducted as a completely randomized design. Means were compared using LSD at p≤0.05 with SPSS software (SPSS Inc., Chicago, USA).

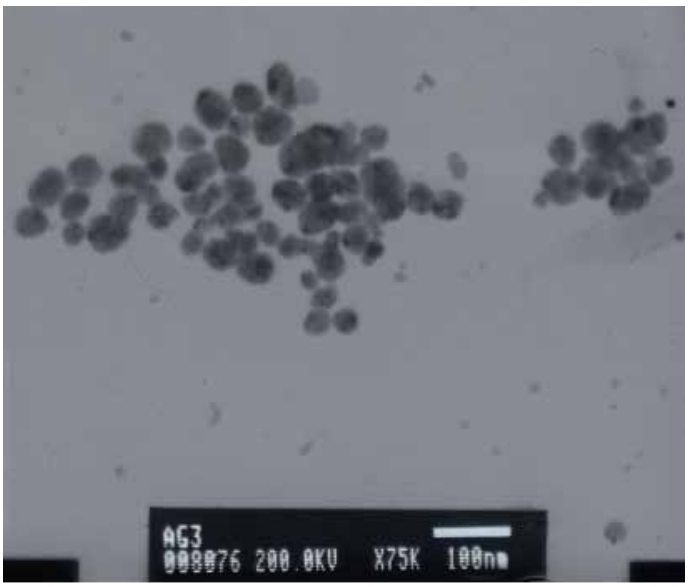

Fig. 1 - Transmission electron microscopy (TEM) micrograph of Ag nanoparticles (scale bar of 100 nm).

3. Results and Discussion

Completely healthy and disinfected explants were achieved following treatment with 10% Clorox for 7 min. Explants cultured on MS basal medium supplemented with different concentrations of SNPs had a higher MNFS/E, MLS/E and PEPS than control (Table 1). In other words, SNPs had positive effects on single node explants of *T. undulata,* but MDC/E in the control was higher than in other treatments. For this reason, there were not significant differences between 10 mg l^{-1} SNPs compared to 80 mg l^{-1} on MNFS/E and MLS/E and, in order to save on cost, the use of 10 mg l^{-1} SNPs in tissue culture of *T. undulata* was proposed. In another experiment (Table 1) there were

Table 1 - Comparing SNPs and combination of SNPs along with BAP (2.5 mg l^{-1}) and IAA (0.1 mg l^{-1}) after two weeks on *T. undulata* (RoxB.) single node explants

SNP (mg l-1)	Mean number of shoots per explants		Mean length of shoots per explants MLS/E		Mean diameter of callus per explants		Percentage of explants producing shoots	
	SNP	SNP+BAP+IAA	SNP	SNP+BAP+IAA	SNP	SNP+BAP+IAA	SNP	SNP+BAP+IAA
0	1.06 cd [z]	1.06 cd	2.03 a-c	2.03 a-c	4.27 a	4.27 a	62 ab	62 ab
5	1.00 c	1.24 b-d	1.81 bc	3.01 ab	0.39 c	5.10 a	50 b	67 ab
10	1.65 ab	0.37 e	3.59 ab	1.07 c	0.75 c	2.10 bc	77 a	27 c
20	1.06 cd	1.16 cd	2.33 a-c	2.92 ab	1.19 c	5.02 a	70 ab	70 ab
40	1.82 a	1.41 a-d	3.74 a	1.94 a-c	2.22 bc	5.17 a	80 a	70 ab
80	1.68 a	1.49 a-c	3.02 ab	2.73 a-c	1.27 c	3.81 ab	85 a	70 ab
Mean	1.37 a	1.12 b	2.75 a	2.28 a	1.68 a	4.24 b	70 a	61 b

[z] In each column, means with the same letters are not significantly different at ≤ 0.05 level of probability using LSD.

not significant effects between explants grown in medium supplemented with BAP (2.5 mg l^{-1}) and IAA (0.1 mg l^{-1}) compared with medium supplemented with different concentrations of SNPs. When we compared data of SNPs with SNPs in combination with plant growth regulators (BAP and IAA), there were significant effects between MNFS/E, MDC/E and PEPS (Table 1). Our results demonstrate that Iranian *Tecomella undulata* (Roxb.) Seem. had high potential for producing callus from stem segments even in hormone-free medium, but after a couple of days they turned brown and died. Even single node explants, after nearly three weeks, had necrotic leaves. The use of common antioxidants such as ascorbic and citric acid or activated charcoal (data not shown), did not have significant effects on survival of single node explants and callus derived from stem segments.

Leaves of *T. undulata* produced callus in MS medium but these, when attached to a single node, directly started callus formation. BAP at the concentration of 0.9 mg l^{-1} allowed regeneration of few buds on callus produced from stem segments (data not shown). Results indicate that TDZ at the concentration of 0.1 mg l^{-1} increased MNS/E and MLS/E up to 2 and 3.33 mm respectively from samples taken in autumn (Table 2, Fig. 2). However TDZ at low concentration had a positive effect on callus production, but concentrations of more than 0.01 mg l^{-1} decreased the dimensions of MDC/E.

Table 2 - Regeneration response of *T. undulata* (RoxB.) single node explants on MS medium supplemented with TDZ after two weeks

SNP (mg l^{-1})	Number of shoots per explants	Mean length of shoots per explants (mm)	Mean diameter of callus per explants (mm)
0.000	0.85 b-d [(z)]	2.28 a-c	4.23 a-d
0.001	0.90 bc	1.66 a-d	4.80 ab
0.010	1.13 b	2.83 ab	5.33 a
0.100	2.00 a	3.33 a	3.46 a-d
1.000	0.53 b-d	1.66 a-d	4.40 a-c
10.000	0.10 cd	0.40 d	0.16 c-d
Mean	0.93	1.98	3.63

[(z)] In each column, means with the same letters are not significantly different at ≤ 0.05 level of probability using LSD.

Fig. 2 - Proliferating single node explants of *T. undulata* in medium supplemented with TDZ (0.1 mg l^{-1}).

As desert ecosystems currently cover about 35% of the earth's land surface and also global warming and water deficiency have become worldwide problems, it is urgent to focus on tolerant plant species in these areas (Hellen, 1991). Utilization of plants for medicinal purposes in Iran has been documented in ancient literature. Avicenna (Abu Ali Sina) was a Persian physician and philosopher whose medical system was for a long time the standard in Europe and in the Middle East (Berman *et al*., 2009). To protect ecosystems from drought, an option is propagation of tolerant plants such as wild medicinal plants and a rapid and efficient technique for plant propagation is micropropagation (Thorpe, 2007). Explants used in the present study were collected in October and we believe their low regeneration potential was due to seasonal effects. TDZ is one of the most active cytokinin-like substance used successfully for regeneration of recalcitrant woody plants (Huetteman and Preece, 1993; Sarmast *et al*., 2012). The present study indicates that BAP along with IAA did not have a high potential to induce bud regeneration, as previously reported (Rathore *et al*., 1991; Robinson *et al*., 2005). The data presented in this work demonstrated that, at increasing SNPs concentrations, the dimensions of MDC/E decreased and that buds derived from callus caused somaclonal variation (Larkin and Scowcroft, 1981; Mondal and Chand, 2002; Sarmast *et al*., 2012). Hence, we conjecture that SNPs may be helpful in micropropagation to avoid indirect regeneration and the connected somaclonal variability. Sarmast *et al*. (2011) reported that *Araucaria excelsa* explants, grown in MS medium supplemented with SNPs, were fresher than in MS medium only and that $AgNO_3$ in *Brassica spp.* significantly affected bud formation on callus induction medium (Akasaka-Kennedy *et al*., 2005) and had positive effects on shoot regeneration in *Brassica oleracea* var. *italica* (Qin *et al*., 2007). Ethylene, the simplest olefin, exists in gaseous state in environmental conditions; it is biologically active in trace amounts and regulates many aspects of the plant life cycle such as senescence (Lin *et al*., 2009; Taiz and Zeiger, 2006). In tissue culture vessels, plantlet growth and development can be severely influenced by gaseous effects, especially of ethylene at elevated level that may damage explants by suppressing their growth and causing hyperhydration (Ziv, 1995). Additionally, SNPs affected ethylene formation and it can be concluded that increasing of durability of explants in culture vessel is due to ethylene blockage. In tissue culture of woody plants, rooting is a severe problem (Giri *et al*., 2004; Sarmast *et al*., 2012), therefore a separate experiment is now in progress to improve rooting of *T. undulata* with mediating *Agrobacterium rhizogenes* (strain K559).

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