

## Effects of Silver Nanoparticle Exposure on Germination and Early Growth of *Pinus sylvestris* and *Alnus subcordata*

(Kesan Pendedahan Nanozarah Perak kepada Percambahan dan Pertumbuhan Awal *Pinus sylvestris* dan *Alnus subcordata*)

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### ABSTRACT

The possible ecological toxicity of silver nanoparticles (AgNP) was evaluated based on germination and growth characteristics of *Pinus sylvestris* and *Alnus subcordata*. Seeds were exposed to different concentrations of AgNP in soil (0, 10, 20, 40, 80 and 100 mg/kg) and aqueous suspension (0, 10 and 20 mg/L). Then, seed germination percentage (GP%), speed of germination (S.G), seedling length (SL), as well as fresh and dry weights (FW and DW) were measured. The results showed that low concentration of AgNP (under 80 mg/kg) could be used without detrimental effects on the germination characteristics of *P. sylvestris* in the soil. Nevertheless, inhibitory effect of AgNP was observed at 10 mg/L for *P. sylvestris* in aqueous suspension. The dissimilar results in soil and aqueous suspension were due to the organic matters and clay minerals in the soil. There was no significant difference ( $p < 0.01$ ) among the treatments of *A. subcordata*, not only in soil but also in the aqueous suspension.

**Keywords:** Aqueous suspension; inhibitory effect; seed germination; silver nanoparticles; soil; woody plants

### ABSTRAK

Ketoksikan ekologi nanozarah perak (AgNP) dinilai berdasarkan ciri-ciri percambahan dan pertumbuhan *Pinus sylvestris* dan *Alnus subcordata*. Benih didedahkan kepada kepekatan berbeza AgNP dalam tanah (0, 10, 20, 40, 80 dan 100 mg/kg) dan gantungan akues (0, 10 dan 20 mg/L). Kemudian, peratusan percambahan biji benih (GP%), kelajuan percambahan (S.G), panjang semaian (SL), serta berat kering dan segar (FW dan DW) diukur. Hasil kajian menunjukkan bahawa kepekatan rendah AgNP (di bawah 80 mg/kg) boleh digunakan tanpa kesan-kesan yang memudaratkan terhadap ciri-ciri percambahan *P. sylvestris* dalam tanah. Walau bagaimanapun, kesan rencatan AgNP diperhatikan pada 10 mg/L bagi *P. sylvestris* pada penggantungan akues. Keputusan yang berbeza dalam tanah dan penggantungan akues adalah disebabkan oleh bahan organik dan mineral tanah liat dalam tanah. Tiada perbezaan yang signifikan ( $p < 0.01$ ) bagi rawatan *A. subcordata*, di dalam tanah mahupun di dalam penggantungan akues.

**Kata kunci:** Kesan rencatan; penggantungan akues; percambahan benih; nanozarah perak; tanah; tumbuhan berkayu

### INTRODUCTION

Nanotechnology is described as the consideration and control of matter at size of 1-100 nm, where unique physical and chemical properties make novel applications achievable (EPA 2007). The special properties, such as high specific area and mobility, could potentially direct to unpredicted health or environmental hazards (Maynard et al. 2006; Wiesner et al. 2006). Many factors have been increasing the importance of the risk determination related to a nanomaterial discharge event, such as its changing properties and condition of scattering (Handy & Shaw 2007).

One of the substances used in nano-formulation is silver. It is used in a growing number of consumers and medical products. It is utilized just about in all places, and in a wide range to stop the spread of microorganisms. The strong antimicrobial action is the major direction for the development of nanosilver products. About 25% of the silver production is lost to terrestrial and aquatic

ecosystems accidentally or intentionally (approximately 2/3 and 1/3, respectively) (Purcell & Peters 1998), resulting in toxic effects to organisms. The extensive use of nanosilver has directed to a growing number of research on their toxicity and ecotoxicity.

The greater parts of the toxicity studies on nanomaterial have been focusing on mammals and aquatic species, and relatively few studies have been accomplished using terrestrial species (Christian et al. 2008; EPA 2007; Klaine et al. 2008). The few toxicity studies of nanomaterial on terrestrial species have yielded conflicting results (El-Temsa & Joner 2010; Jiang et al. 2012; Kumari et al. 2009; Lee et al. 2012; Stampoulis et al. 2009; Yin et al. 2011). For example, Stampoulis et al. (2009) observed that AgNP reduced the biomass and transpiration rates in *Cucurbita pepo* compared to control plants. Similar results were reported for *Allium cepa*, *Lolium multiflorum*, ryegrass (*Lolium perenne*), barley (*Hordeum vulgare*) and flax (*Linum usitatissimum*) (El-Temsa & Joner 2010; Kumari et

al. 2009; Yin et al. 2011). However, differing result in the growth rate of *Phaseolus rabiatus* was reported by Lee et al. (2012) while testing in a soil medium. Therefore, there are still many unanswered issues and challenges relating to the effects of AgNP on terrestrial organisms, and mainly woody plants, which may be specifically at risk, due to the large porous in the woody sections.

We studied the effect of AgNP on seed germination and growth characteristics of *A. subcordata* and *P. sylvestris*, since seed germination is the earliest stage in plant growth. Seed germination and seedling elongation are widely used for the phytotoxicity tests due to several advantages like sensitivity, simplicity, low cost, and suitability for reactive chemicals and contaminated soil samples (Lin & Xing 2007; Munzuroglu & Geckil 2002; Wang et al. 2001). These two woody species were chosen as they represent both needle- and broad-leaved trees. In addition, plantations with *A. subcordata* and *P. sylvestris* are present in plain areas near the cities in Iran (Gharachorlou et al. 2010; Tabari et al. 2011). Plain areas are polluted with different hazardous materials in varying extents in Iran (Tabari et al. 2008), and yet no results of AgNP toxicity tests on trees of plantation have been reported.

## MATERIALS AND METHODS

### PLANT MATERIALS AND EXPERIMENTAL DESIGN

Seeds of two woody species, *P. sylvestris* and *A. subcordata*, were used in the experiment. The seeds were collected and stored in cool (4°C) dry condition away from direct light for three months. Seeds were placed in the silty clay and sandy loam textured soil and aqueous suspension in the petri dishes. Seeds were then exposed to the following AgNP concentrations: 0, 10, 20, 40, 80 and 100 mg AgNP/kg soil (El-Temseh & Joner 2010; Kumari et al. 2009; Lee et al. 2012) as well as 0, 10 and 20 mg AgNP per liter of water (El-Temseh & Joner 2010; Lee et al. 2012; Yin et al. 2011). The experiment was performed in a completely randomized design (RCBD) and each treatment was replicated five times.

### NANOSILVER PREPARATION

Silver nanoparticles (100 nm, 99.5% metal) were produced using the method described by El-Temseh and Joner (2010). Primary particle diameter was calculated based on Scanning Electron Microscopy and image-processing software (Image J; National Institute of Mental Health) (Figure 1).

### SOIL ANALYSIS

Visually observed silty clay and sandy loam soil were collected from the northern forests of Iran. Soil samples were air dried and ground to enable passage through a 2 mm sieve. Soil analyses were carried out by the following methods: particle size distribution by the hydrometer method (Gee & Bauder 1986); organic matter (OM) content

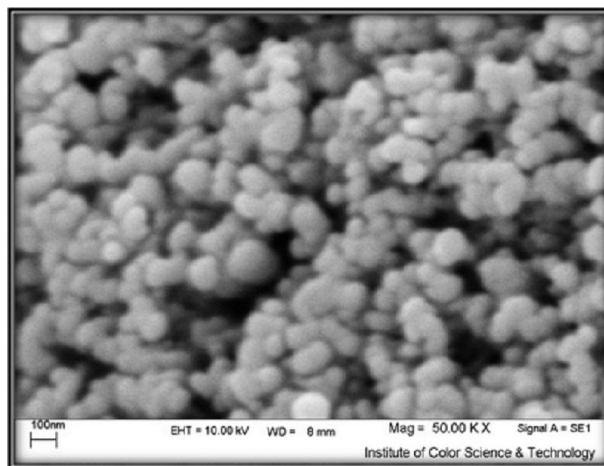


FIGURE 1. Scanning Electron Microscopy image of nanosilver. Bar = 100 nm

by the Walkley-Black (1934) procedure; pH values using a glass electrode in mixture of soil and deionized water (1:5, w/v) and electrical conductivity (EC) of soil samples estimated by Richards (1954). The results of soil analysis are shown in Table 1.

### SEED GERMINATION

Test conditions and germination criteria used were those specified for each species in rules of testing seeds (Association Official Seed Analysts 1970) and international rules for seed testing (International Seed Testing Association 1976). For the seed germination in aqueous suspension, seeds were rinsed with deionized water and then 50 of them were placed in a Petri dish with a disc of filter paper and 5 mL water or nano-particle suspension with the above-mentioned concentrations per dish (three replicates). Petri dishes were covered and sealed with tape and incubated for 15 days at 22°C in a dark growth chamber (MB-60B, USA).

The same plant species and seed densities were used for testing seed germination in the soil. The seeds were placed in Petri dishes and covered with 50 g of air-dry soil (silty clay and sandy loam). The Petri dishes were subsequently wetted to 75% of their respective water holding capacities, covered for preventing loss of water and placed in the growth chamber with 16 h light and 8

TABLE 1. Soil characteristics

Soil	Silty clay	Sandy loam
EC(μs/cm)	0.72	0.80
pH(water)	5.00	8.30
Water holding capacity (%)	36.50	6.90
Organic matter content (%)	0.50	0.10
Sand (%)	12.00	76.00
Silt (%)	41.00	16.00
Clay (%)	47.00	8.00

h dark cycle at 22°C to roughly approximate day length during the spring and early summer in Iran. Loss of water was checked by weighting, and deionized water was added when required. After 15 days, the seed germination and growth were measured.

#### PARAMETERS

Germination was counted for 48 h intervals and continued until no further germination occurred. At the end of the 15th day, the following parameters were calculated:

- a) Seed germination percentage (GP%) =  $\frac{\text{germinated seeds}}{\text{total seeds}} \times 100$
- b) Speed of germination (SG) was calculated based on the following formula of Maguire (1962)
- $$S.G. = \frac{X_1}{Y_1} + \frac{X_2 - X_1}{Y_2} + \dots + \frac{X_n - X_{n-1}}{Y_n} \quad (1)$$
- where; X<sub>1</sub>, X<sub>2</sub> and X<sub>n</sub> are numbers of seeds germinated on 1<sup>st</sup>, 2<sup>nd</sup>, and n<sup>th</sup> day, respectively. Y<sub>1</sub>, Y<sub>2</sub> and Y<sub>n</sub> are numbers of days from sowing to 1<sup>st</sup>, 2<sup>nd</sup> and n<sup>th</sup> count.
- c) Seedling length (SL) in millimeter (mm)
- d) Fresh weight (FW) in gram (g)
- e) Dry weight (DW, in g): seedlings were placed in a paper cover, dried in the oven at 85±2°C for 48 h and were weighed to obtain the dry weight

#### STATISTICAL ANALYSIS

The data were analyzed by Statgraphics Plus Professional 16.0.3 software (Bayramzadeh et al. 2008; Roschewitz et al. 2005). The comparison of means was done using Fisher's Least Significant Difference (LSD) Test ( $p < 0.01$ ) for aqueous suspension and Tukey's Honestly Significant Differences (HSD) for soil environment ( $p < 0.01$ ). Spearman's rank order correlation coefficient was used to test the relationship between the studied growth and germination characteristics and different concentrations of AgNP.

#### RESULTS

The AgNP affected seed germination and growth characteristics in a different way for the two woody plants. These species showed dissimilar responses to AgNP due to their anatomical (e.g. types of cell) and morphological (e.g. leaf form) differences. Therefore, we did not compare them with each other statistically, and our objective was to evaluate the responses of each species unconnectedly.

#### SOIL

AgNP had significant inhibitory effects ( $p < 0.01$ ) on *P. sylvestris* in silty clay and sandy loam textured soils.

The increasing concentrations of AgNP had decreased the characteristics SG%, SG, SL, FW and DW significantly (Figures 2 & 5; Table 2). The reduction of the characteristics was noticeable in 80 to 100 mg/kg in both soil textures. It is interesting to note that the studied parameters in the silty clay soil (even in control) were less than those in the sandy loam soils (Figures 5 & 6). The results also showed that there was no significant difference among the treatments of *A. sabcordata* ( $p < 0.01$ ) in both silty clay and sandy loam textured soils (Figure 3; Table 2). However, *A. sabcordata* showed better growth in silty clay than sandy loam textured soil.

#### AQUEOUS SUSPENSION

Significant ( $p < 0.01$ ) negative effects on germination and growth characteristics of *P. sylvestris* was observed in the aqueous suspension (Figure 4). The significant reductions in GP%, SG, SL, FW and DW was started from 10 mg AgNP/L water (Figure 4). Different concentrations of AgNP in aqueous suspension had no significant ( $p < 0.01$ ) effects on the germination and growth characteristics of *A. sabcordata* (Figure 4).

#### DISCUSSION

In this study, we investigated the effect of AgNP on germination and growth of *A. sabcordata* and *P. sylvestris* in soil and aqueous suspension since few results of toxicity tests with trees have been reported (Seeger et al. 2009). The results showed that AgNP had significant negative effects ( $p < 0.01$ ) on germination and growth characteristics of *P. sylvestris* in both soil textures (silty clay and sandy loam) and aqueous suspension.

The seed germination and growth characteristics of *P. sylvestris* reduced while the concentration of AgNP increased. The noticeable reduction was observed at 80 mg AgNP/kg soil and 10 mg AgNP/L of water. It should be mentioned that AgNP did not impeded the germination completely. This finding is in agreement with those reported for ryegrass, barley, and flax (El-Temseh & Joner 2010). The SG%, SG, SL, FW and DW in the all treatments of the silty clay soil (even in control) were less than sandy loam soils (Figures 2, 5 & 6). These results can be due to the fact that *P. sylvestris* grows well on different soils but optimum growth is on the freely drained sands and gravels (Steven & Carlisle 1959).

The inhibitory effects of AgNP on the studied characteristics of the *P. sylvestris* were observed at very low concentration in aquatic suspension (10 mg/L) when compared to the soil (80 mg/kg). This can be due to the fact that nano-particles are likely to react with constituents of environmental matrices like soil and water (Brant et al. 2007). In addition, organic matters and clay minerals in the soil are known to decrease bioavailability and phytotoxicity of both inorganic (Lombi et al. 2002) and organic pollutants (Roberts et al. 2007).

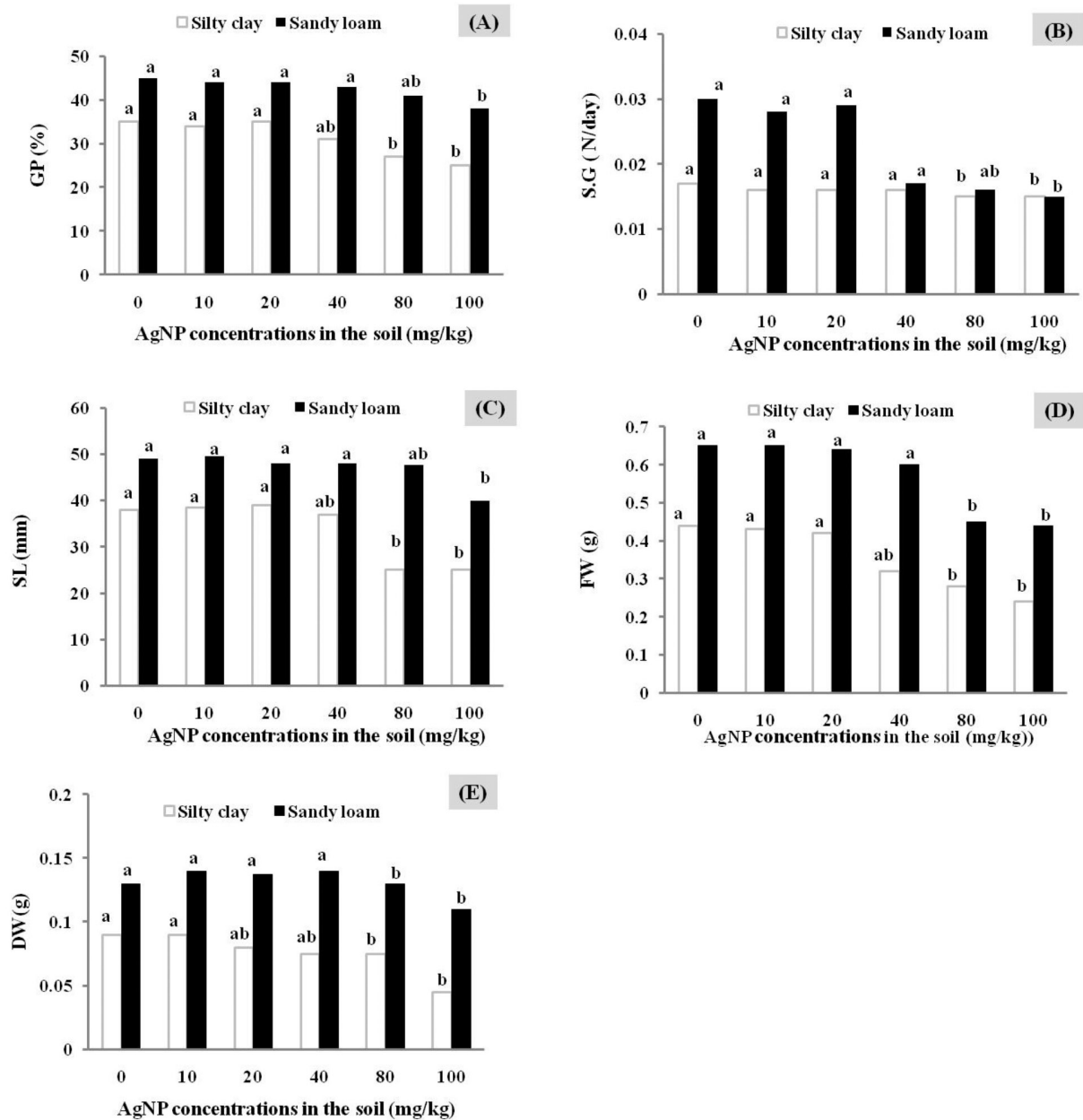


FIGURE 2. Seed germination percentage (GP%, A), speed of seed germination (SG, B), seedling length (SL, C), fresh weight (FW, D) and day weight (DW, E) of *P. sylvestris* in the soil.  $n = 5$ . Bars show S.D. means with the same letters are not significantly different at  $P < 0.01$  by Tukey's HSD procedure

TABLE 2. Correlation analysis (Spearman Rank Order Correlations) between the growth and germination characteristics of *P. sylvestris* and *A. subcordata* with AgNP concentrations in the soil and aqueous suspension

Woody species	Environmental media		Correlation coefficient				
			Growth and germination characteristics				
			GP%	SG	SL	FW	DW
<i>P. sylvestris</i>	Soil	Silty clay	-0.87**	-0.57*	-0.75**	-0.95**	-0.83**
		Sandy loam	-0.91**	-0.86**	-0.89**	-0.90**	-0.47*
	Aqueous suspension	-0.94**	-0.71*	0.94**	0.95**	0.95**	
<i>A. subcordata</i>	Soil	Silty clay	0.50	0.23	-0.32	-0.16	-0.23
		Sandy loam	-0.22	-0.16	-0.19	0.17	-0.19
	Aqueous suspension	-0.59	-0.20	0.01	0.32	-0.43	

GP% (Seed germination percentage), S.G (speed of seed germination), SL (seedling length), FW (fresh weight) and DW (day weight); \*\* Significant at  $p < 0.01$ ; \* Significant at  $p < 0.05$

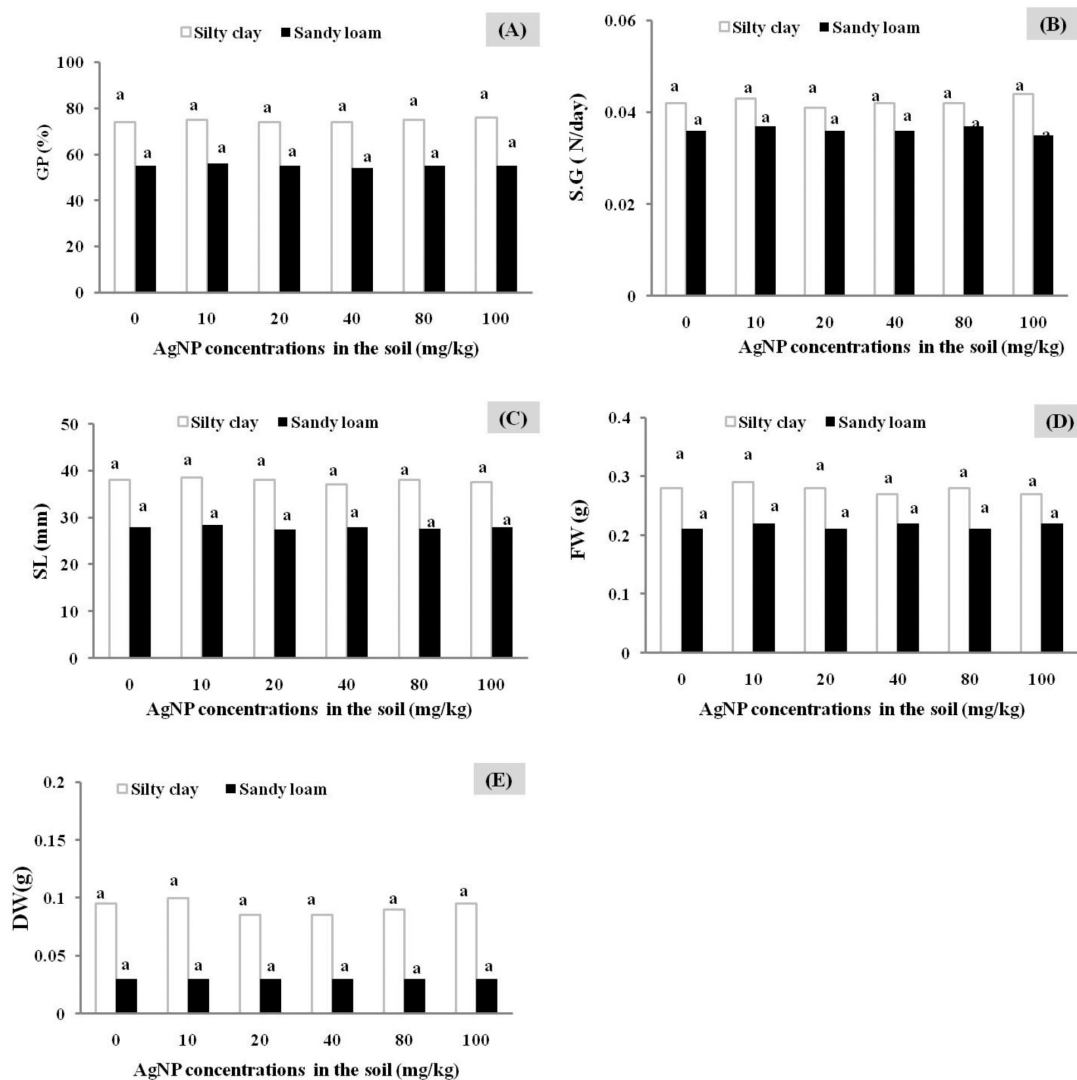


FIGURE 3. Seed germination percentage (GP%, A), speed of seed germination (SG, B), seedling length (SL, C), fresh weight (FW, D) and day weight (DW, E) of *A. subcordata* in the soil (. n = 5). Bars show S.D. means with the same letters are not significantly different at  $p < 0.01$  by Tukey's HSD procedure

The phytotoxicity profile of AgNP has been frequently investigated via seed germination and growth characteristics for annual plants (El-Temseh & Joner 2010; Jiang et al. 2012; Lee et al. 2012). The findings for the annual plants reported by El-Temseh and Jiang (2012), Jiang et al. (2012), Kumari et al. (2009), Lee et al. (2012), Stampoulis et al. (2009) and Yin et al. (2011), were very similar to our findings for *P. sylvestris*. The toxicities of the AgNP in the *P. sylvestris* and other annual plants are likely to be attributed to three mechanisms: Generation of reactive oxygen species (ROS) (Navarro et al. 2008), which can damage the cell membrane; Penetration of nanoparticles into the cell where they interfere with intracellular metabolism (a nano-Trojan-horse type mechanism) (Limbach et al. 2007; Luoma 2008); and Release of metal ions that hinder enzyme functions (Hwang et al. 2007).

The results for *A. subcordata* completely differed from those obtained for *P. sylvestris*. The lack of restrictive impact can be likely due to the paucity of the absorbance or

resistance of *A. subcordata* against the AgNP. The results of *A. subcordata* is comparable to the findings of Seeger et al. (2009) who studied the toxicity of TiO<sub>2</sub> nanoparticle on willow trees and came to the conclusion that willow trees are not in particular vulnerable to nanosized TiO<sub>2</sub>-particles.

We conclude that in contrast to *P. sylvestris*, *A. subcordata* is not sensitive to nanosized silver particles in the conditions, concentrations, and time periods used in this study. However, the preliminary outcomes of this study should be verified by other types of nanoparticles, its concentrations, duration, and other tree species.

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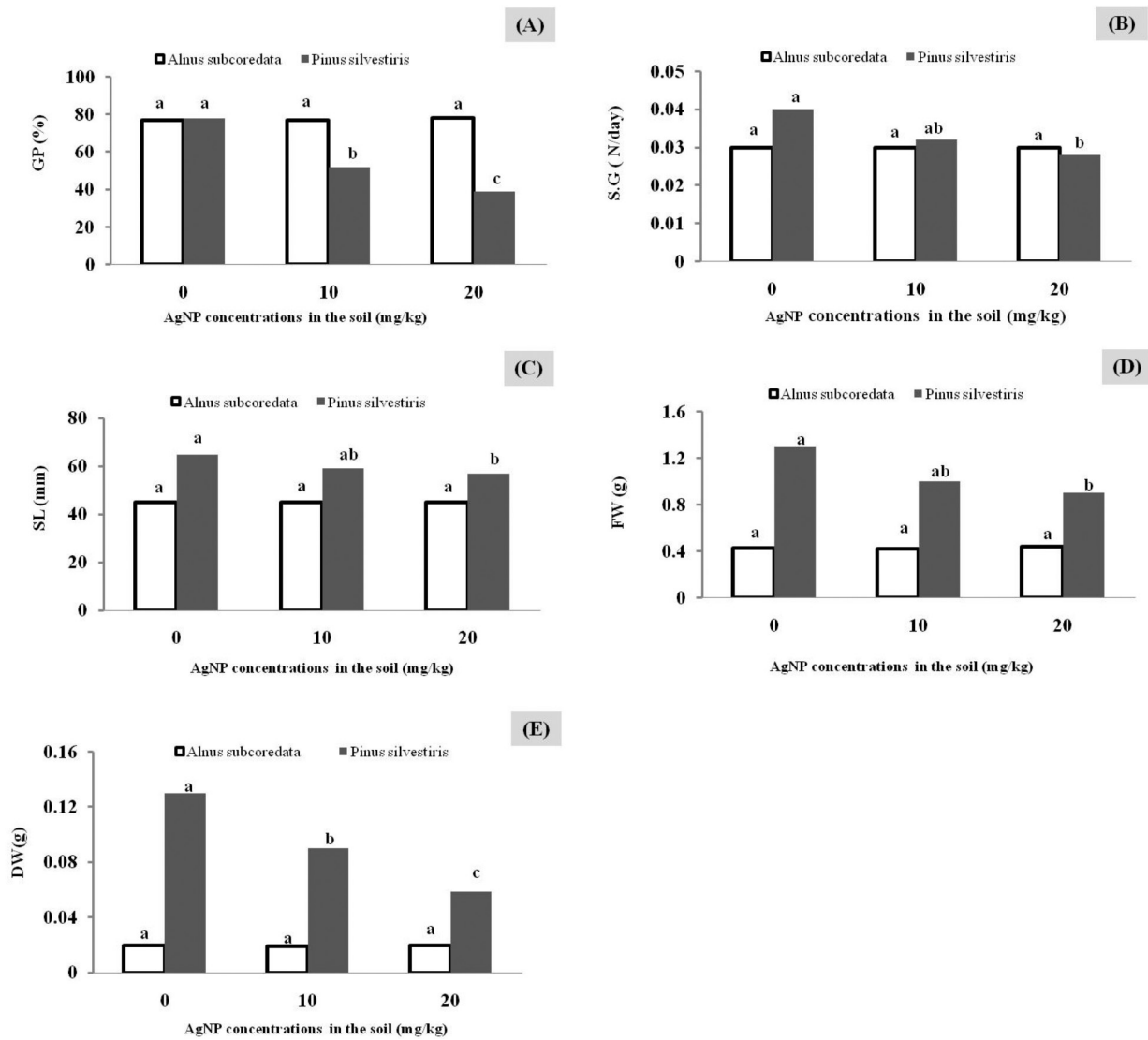


FIGURE 4. Seed germination percentage (GP%, A), speed of seed germination (SG, B), seedling length (SL, C), fresh weight (FW, D) and day weight (DW, E) of *P. sylvestris* and *A. subcordata* in the water.  $n = 5$ . Bars show S.D. means with the same letters are not significantly different at  $p < 0.01$  by Fisher's LSD procedure

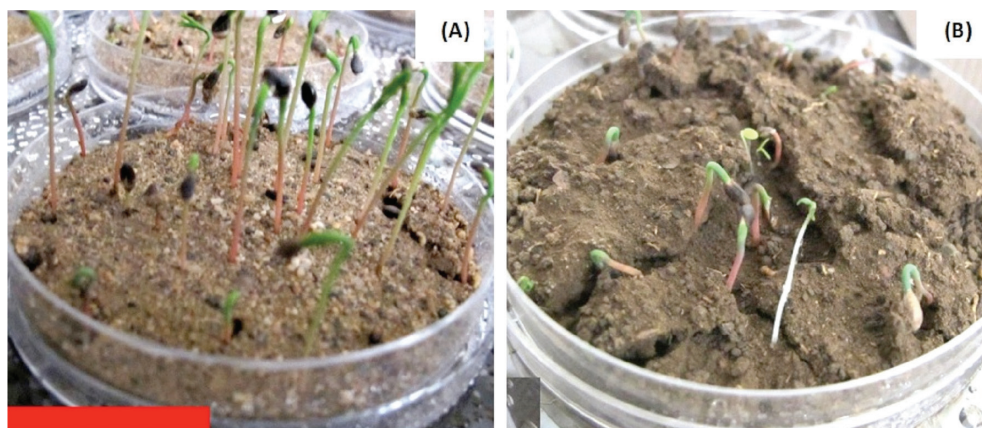


FIGURE 5. Seed germination of *P. sylvestris* in 0 mg/Kg (A) and 80 mg/Kg (B) of AgNP in sandy loam soil. Scale bar = 50 mm

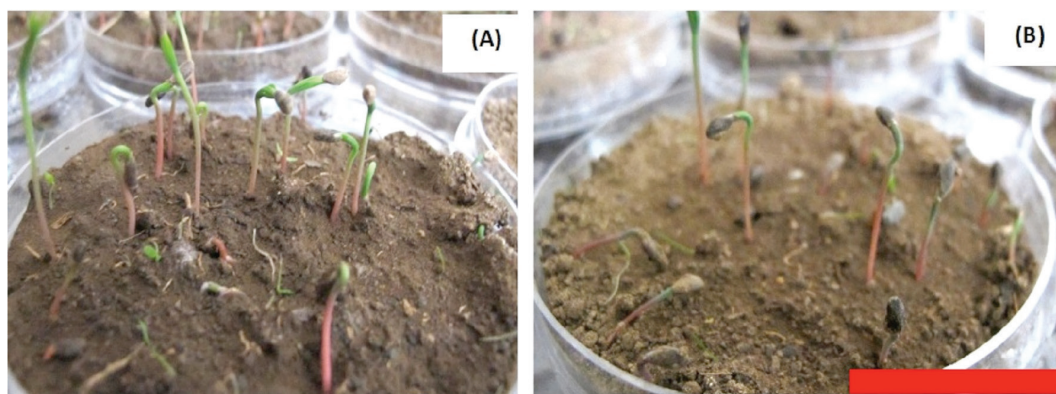


FIGURE 6. Seed germination of *P. sylvestris* in 0 mg/Kg (A) and 80 mg/Kg (B) of AgNP in silty clay soil. Scale bar = 50 mm

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