

APPLICATION OF SiO₂ NANOPARTICLES AS PRETREATMENT ALLEVIATES THE IMPACT OF DROUGHT ON THE PHYSIOLOGICAL PERFORMANCE OF *PRUNUS MAHALEB* (ROSACEAE)

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Summary: We studied the physiological responses of *Prunus mahaleb* (Mahaleb) seedlings to drought stress when previously irrigated (or not) with different concentrations of SiO₂ nanoparticles (SNPs). SNPs were applied at four concentrations (0, 10, 50 and 100 mg L⁻¹) for 45 days, and then seedlings were subjected to three watering treatments including low (300 mL water every 3 d), moderate (150 mL water every 3 d) and severe drought stress (no irrigation) for 19 days. Results showed that gas exchange – photosynthesis, stomatal conductance, and transpiration rate – were significantly less impacted by severe drought stress when seedlings were pretreated with SNPs at high concentrations. Beneficial effects of SNPs pretreatment were evident in the nutritional status of the plants as the concentration of N, P and K, were maintained at similar levels than in well-watered seedlings. Pretreated seedlings were able to maintain the root length and to reduce the impact of severe drought on root dry mass accumulation. Therefore, application of SNPs as pretreatment should be considered as a promising agronomic practice in sites prone to suffer from water deficit.

Key words: Pre-treatment, Silica nanoparticles, drought, stress alleviation, photosynthesis.

Resumen: La aplicación de nanopartículas de SiO₂ como pretratamiento disminuye el impacto de la sequía en la performance fisiológica de *Prunus mahaleb* (Rosaceae). En este trabajo se estudiaron respuestas fisiológicas de *Prunus mahaleb* (Mahaleb) a la sequía luego de la aplicación de diferentes concentraciones de nanopartículas de SiO₂ (SNPs) por irrigación como pretratamientos. Se aplicaron 4 concentraciones de SNPs (0, 10, 50 and 100 mg L⁻¹) durante 45 días y, a posteriori, las plantas fueron sujetas a tres regímenes hídricos que incluyeron control (300 mL cada 3 días), estrés hídrico moderado (150 mL cada 3 días) y estrés hídrico severo (sin riego) por 19 días. El intercambio de gases – fotosíntesis, conductancia estomática y transpiración – se redujo menos frente a la sequía en las plantas que recibieron pretratamientos con SNPs. El estado nutricional de las plantas tratadas con SNPs visto por la concentración de N, P y K se mantuvo bajo sequía moderada. Las plantas pretratadas con SNPs mantuvieron el largo de sus raíces y sufrieron menor impacto en su biomasa radical ante sequía. Se concluye que la aplicación de SNPs como pretratamiento podría ser una práctica agronómica para sitios propensos a déficit hídricos en épocas cercanas a la plantación.

Palabras clave: Nanopartículas de sílice, fotosíntesis, sequía, pre-tratamiento, mitigación de estrés.

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INTRODUCTION

Drought stress, as multidimensional abiotic stress, strongly affects growth, development, and yield of plants (Mahajan & Tuteja, 2005). Undoubtedly, understanding mechanisms that plants use to deal with drought stress are important. However, equally important are cultural methods enhancing natural drought tolerance (Sacala, 2009). Given that conventional fertilisers can have adverse effects on the environment and potentially on food quality, researchers are now investigating the potential use of nanotechnology to reduce the negative impact of abiotic stresses in plants (Asadzade *et al.*, 2015; Chen & Yada, 2011; Haghighi & Pessarakli, 2013). Nanosciences have led to the development of a wide range of applications for enhancing of plant growth (Nair *et al.*, 2010). Carbon-based, metal oxides, quantum dots, nano-sized polymers and biocomposites materials in plant science are being developed (Khot *et al.*, 2012). Nanomaterials are materials with a particle size between 1 and 100 nm and implicate new physical, chemical and biological properties compared to bulk size materials (Monica & Cremonini, 2009). Also, some researchers think that absorption of nanoparticles in plants is greater than the same chemicals applied to the plant in bulk size (Braunack, 1995; Suriyaprabha *et al.*, 2012). Although some recent studies on the effects of SiO₂ nanoparticles (hereafter SNPs) on plant growth have been performed, these studies are still scarce. For instance, Bao-shan *et al.* (2004) immersed the roots of Changbai larch (*Larix olgensis*) seedlings in 62 to 2000 µl L⁻¹ concentrations of nanosilica for 6 hours and showed positive effects of silicon nanoparticles on seedling's growth. In addition, Haghighi *et al.* (2012) applied nano-silicon to tomato seeds and seedlings subjected to salt stress and concluded that nano-silicon application reduced the deleterious effects of salinity on germination; root length and plant dry weight. Also, Haghighi & Pessarakli (2013) showed that application of silicon in nano and bulk size was beneficial in improving the salt tolerance of tomato plants. SNPs improved seed germination and seedling growth of lentil (*Lens culinaris* Medik) under salinity stress (Sabaghnia & Janmohammadi, 2015). Zarafshar *et al.* (2015) reported no toxic effects of SNPs on pear seedlings even when the seedlings were irrigated with high concentrations of SNPs. Nevertheless, the silicon nanoparticles role on

plant physiological behaviour is poorly understood (Lee *et al.*, 2010; da Silva Lobato *et al.*, 2013). In fact, Si is the second most abundant element in soil, however, is not considered as an essential plant mineral nutrient; but its beneficial effects on growth of many plants, especially growing under biotic and abiotic stress conditions have been demonstrated (Chalmardi *et al.*, 2014; Ma & Yamaji, 2006, 2015). In previous research, investigators have shown that silicon enhances plants resistance to drought, salinity, cold, heat, and metal toxicity. For example, Ashkavand *et al.* (2015) found that SNPs play a positive role in maintaining critical physiological and biochemical functions in Hawthorn seedlings subjected to drought stress. Thus, it seems that the application of silicon nanoparticles looks promising (and non-expensive) agronomic practice to reduce detrimental environmental effects due to drought (Xie *et al.*, 2015; Balakhnina & Borkowska, 2013).

The present study aimed to test the effects of SNPs pre-treatments on subsequent drought stress responses of *Prunus mahaleb* seedlings, a woody species widely distributed in western and central Asia, and Mediterranean countries (Özçelik *et al.*, 2012). Our working hypothesis is that SNPs pretreatment alleviates the detrimental effects of a subsequent drought due to a reduced impact on root growth and elongation (concerning non-SNPs pre-treated seedlings). We expect that under severe drought SNPs pretreated seedlings have a better physiological performance regarding higher photosynthesis and stomatal conductance, lower accumulation of proline, reduced lipid peroxidation and lower chlorophyll degradation concerning that of seedlings non-pretreated with SNPs.

MATERIALS AND METHODS

Experimental materials

In late winter 108 dormant (uniformly-sized) one-year-old Mahaleb seedlings (*Prunus mahaleb* L. or syn. *Cerasus mahaleb* L. Mill. Rosaceae) were obtained from an Iranian forest nursery, and transferred to the experimental garden facility at the Faculty of Natural Resources and Marine Sciences of Tarbiat Modares University, Noor, Mazandaran, IRAN (Latitude 35° 43' 46" N, longitude 51° 23' 15" E). The seedlings were transplanted to plastic pots (7 L) containing a mixture of forest brown soil,

river sand, and clay (2:1:1 v/v/v) and grown in a greenhouse with day/night average temperatures of 30/21 °C. The soil contained 28%, 46% and 26% of silt, sand and clay (respectively), 0.87% of organic carbon, and 30 ppm of available phosphorus.

Examined material. IRAN, Mazandaran, Noor, 14-II-2015, P. Ashkavand 81-85 (HKS, Research Center of Agricultural and Natural Resources Kurdistan Province).

Nanoparticles pre-treatments and imposition of drought stress

After potting, SNPs (acquired from Tecnología Navarra de Nanoproductos S.L., Spain) were applied at four concentrations (0, 10, 50 and 100 mg L⁻¹) for 45 d. The SNPs were white coloured, within a size range of 10 to 15 nm, and specific surface area ranging from 180 to 270 m²g⁻¹. The seedlings were irrigated to field capacity (300 mL pot⁻¹) with SNPs suspensions every three days. There were 27 seedlings in each SNPs treatment. At the end of the SNPs treatments (day 45), seedlings in each SNPs treatment were randomly allocated to one of three soil moisture stress groups ($n=9$). Seedlings were then subjected to three watering treatments consisting on irrigation with tap water every three d with (i) 300 mL pot⁻¹ (*i.e.* control/low stress), (ii) 150 mL pot⁻¹ (*i.e.* moderate stress) and (iii) no irrigation (*i.e.* severe stress). The criterion used to finish the experiment was the beginning of leaf rolling in seedlings subjected to severe drought, which occurred after 19 d of treatments.

Plant physiological parameters measurements

Net photosynthesis (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$) and transpiration rate (E , $\text{mmol m}^{-2} \text{s}^{-1}$) were measured at 7, 14 and 19 d after the watering treatments began. They were made on 2-3 leaves from the upper third of each plant of six randomly selected individuals. Measurements were done on sunny days (between 09:00 and 11:00h) at temperatures ranging from 22 to 28 °C, using a portable infrared gas analyser (Model LCpro+, ADC BioScientific Ltd., Hertfordshire, UK). Average values of leaf temperature and internal CO₂ concentrations were 27.5±3.1 °C and 340±11.9 ppm, respectively.

Predawn xylem stem water potential (ψ stem, MPa) was measured with a pressure chamber system supplied with compressed nitrogen (Skye, SKPM 1400, UK) on day 19. Complementarily, relative water

content (RWC) of leaves was determined at midday (from 13 to 15 pm where maximum evaporative demand potentially occur) according to the following description: four leaves (located in the upper third of plants) were removed from randomly selected plants in each treatment, immediately weighed (W_i), and placed in tubes with deionized water for 24 h at room temperature under low light. After that, individual leaves were reweighed to determine their turgid weights (W_f). Finally, the samples were placed in an oven at 60 °C for 48 h and then reweighed to obtain their dry weights (W_d). RWC was calculated by the following equation:

$$\text{RWC} = [(W_i - W_d) / (W_f - W_d)] * 100$$

Plant morphological parameters and growth

At the end of the experiment (*i.e.* day 19 after a drought), the primary stem length, collar diameter, longest root, and root volume of all seedlings were measured. Root length was measured using a scaled ruler, and root volumes were measured through water displacement in graduated cylinders. Afterwards, seedlings were harvested separating roots and shoots (*i.e.* aerial organs), and then all tissues were oven dried for 48 h at 70 °C to obtain their corresponding dry weights.

Determination of thiobarbituric acid-reactive-substances (TBARs) and membrane electrolyte leakage (ELI)

Thiobarbituric acid reaction (TBA) was measured as described by Heath & Packer (1968). Leaf fresh mass (200 mg) was homogenised in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 12.000 × g for 20 min. The supernatant (1 mL) was mixed with an equal volume of TCA (10%) containing 0.5% (w/v) TBA or no TBA as the blank and heated at 95°C for 30 min and then cooled in ice. The reaction product was centrifuged at 12.000 × g for 15 min, and the supernatant absorbance was measured at 400, 532 and 600 nm.

Leaves were cut into 1-to-2 cm² pieces and placed in test tubes with 20 mL deionised distilled water (0.5-0.8 g fresh leaf tissue per sample). After vortexing, the samples for 3 s, the initial electrical conductivity (EC_0) of each sample was measured. The samples were stored at 4°C for 24 h, and conductivity (EC_1) was measured again. Samples

were then autoclaved for 15 min, cooled to room temperature, and conductivity (EC_2) was measured for the third time. The electrolyte leakage index (ELI) of cell membranes was calculated using a modification of the method of Zhao *et al.* (1992) as:

$$ELI (\%) = [(EC_1 - EC_0) / (EC_2 - EC_0)] * 100$$

Measurements of biochemical parameters

At the end of the experiment, fresh leaf samples were covered with aluminium foil, frozen in liquid nitrogen and stored at $-85^\circ C$ until used for biochemical analysis. Chlorophylls and carotenoids were extracted from leaf samples in 80% v/v acetone, and their contents were determined by spectrophotometry according to Gholami *et al.* (2012). Free proline content in leaves was quantified following the procedure of Bates *et al.* (1973) as cited by Nikolaev *et al.* (2010).

Microscopic observations

At the end of the experiment, the fresh root sections were taken for microscopic analysis. The adsorption of SNPs to fresh roots was observed by scanning electron microscopy (SEM) (KYKY-EM3200) in the laboratory of Tarbiat Modares University.

Measurements of leaf nutrient elements

Oven-dried leaves were pulverised in an electric mill. The powdered leaf tissues were transmitted to the atomic energy organisation of Iran (AEOI). The concentrations of Si, N, P, and K were detected by X-ray fluorescence analysis (XRF; ED 2000 Oxford Instruments Corporation) following the methodological considerations by Towet *et al.* (2016).

Statistical analysis

Physiological data were analysed through repeated measures ANOVA (rmANOVA). All other variables were assessed using two-way ANOVAs in a fixed factor model. For comparison between groups, Duncan's multiple range tests were applied at 0.05 probability level. In case of percentage data, arcsine transformation was applied before ANOVA analyses. All data were tested for normality, homogeneity of variance and Mauchly's test before ANOVA. Statistical analyses were performed using SPSS software (IBM SPSS Statistics).

RESULTS

Confirmation of the presence of SNPs in treated roots

Elements consistent in size with the SNPs were adsorbed by the roots in the treated seedlings but not in the untreated ones (Data not shown). Observation of the root system of treated plants revealed the presence of nanoparticles attached to the roots at the highest SNPs concentration (100 mg L^{-1}) while few nanoparticles were observed in roots treated with SNPs of 10 and 50 mg L^{-1} .

Effect of SNPs pre-treatments on leaf physiological parameters

There were no differences in leaf physiological parameters between SNP-treated and untreated seedlings before drought stress. The photosynthesis rate (A), stomatal conductance (gs) and transpiration (E) were affected by SNPs treatments after drought stress (repeated measures ANOVA; treatment and treatment x time effect: $P < 0.001$). The positive effect of SNPs pre-treatments on A and gs was evident after 19 days of no irrigation (severe water stress; Fig. 1 right panels), where seedlings pretreated with 50, and 100 mg L^{-1} SNPs registered significantly higher values for such parameters than those of control plants (i.e. with no addition of SNPs). Under moderate water stress (150 mL every 3 d), the beneficial effects of SNPs application were less notorious for all variables than under severe drought. Under well-watered conditions (300 mL every 3 d), application of SNPs did not provoke any significant effect on the physiological parameters measured (Fig. 1 left panels). Responses in E paralleled those of gs in most of the cases under either water treatment condition.

Root morphology and biomass responses to drought as affected by SNPs pre-treatments

Under severe water stress (no irrigation for 19d), seedlings pretreated with any concentration of SNPs had longer roots than those of non treated with SNPs (Fig. 2A). Under moderate stress conditions (150 mL every 3 d) the pattern was less obvious although longer roots were recorded on seedlings pretreated with 50 mg L^{-1} SNPs with respect to all other concentrations. Interestingly, the use of SNPs on well-watered conditions (300 mL every 3 d) was also positive on the length of roots, where longer roots were attained at increasing concentration of SNPs

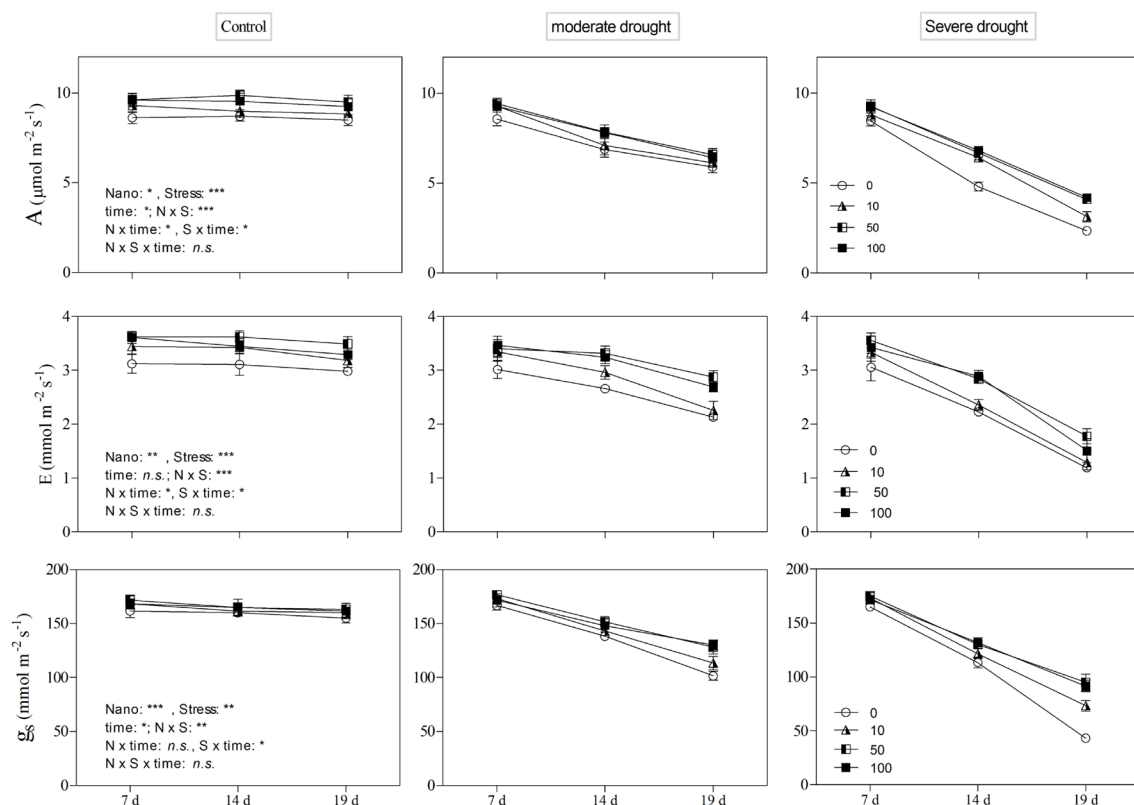


Fig. 1. Responses of photosynthesis rate (A), stomatal conductance (g_s) and leaf transpiration rate (E) of *Prunus mahaleb* seedlings pretreated with different concentration of SNPs during 45 days, and subsequently subjected to 19 days to three irrigation treatments (0, 150 and 300 mL of water every 3 days [severe drought, moderate drought and well-watered controls, respectively]). Gas exchange measurements were done using a portable infrared gas analyser (Model LCpro+, ADC BioScientific Ltd., Hertfordshire, UK) on fully expanded leaves located in the upper third of the plant of six randomly selected individuals at days 7, 14 and 19 after drought. (Mean \pm SE; n=6). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; n.s.: $P > 0.05$.

applied (Fig. 2A). Root volume of plants subjected to moderate drought was less enhanced by SNPs application than root length, suggesting that these roots also had a lower diameter (Fig. 2B). In contrast, seedlings growing under both moderated stress and well-watered conditions displayed an increase in root volume than root length when SNPs were applied at 50 and 100 mg L⁻¹ (Fig. 2B), which suggest an increase in root diameter. In general, positive effects of SNPs on the whole plant dry weights under three irrigation regimes were observed. Root biomass was slightly higher in plants pretreated with SNPs, but shoot biomass was not (Fig. 2C). These responses viewed in dry mass terms were in line with the better physiological performance and the increase in root

lengthening due to the use of SNPs previous to the drought treatment application.

Water relations and biochemical parameters responses to drought in seedlings pretreated with SNPs

Relative leaf water content (RWC) decreased with increasing severity of the drought, but it was not affected by SNPs pre-treatments (Fig. 3A). Xylem water potential (XWP) was affected by drought depending on SiO₂NPs pre-treatment. On this note, higher XWP was detected in seedlings subjected to severe drought at increasing application of SNPs, thereby a better water status was seen particularly in those seedlings pretreated with 100 mg L⁻¹ of

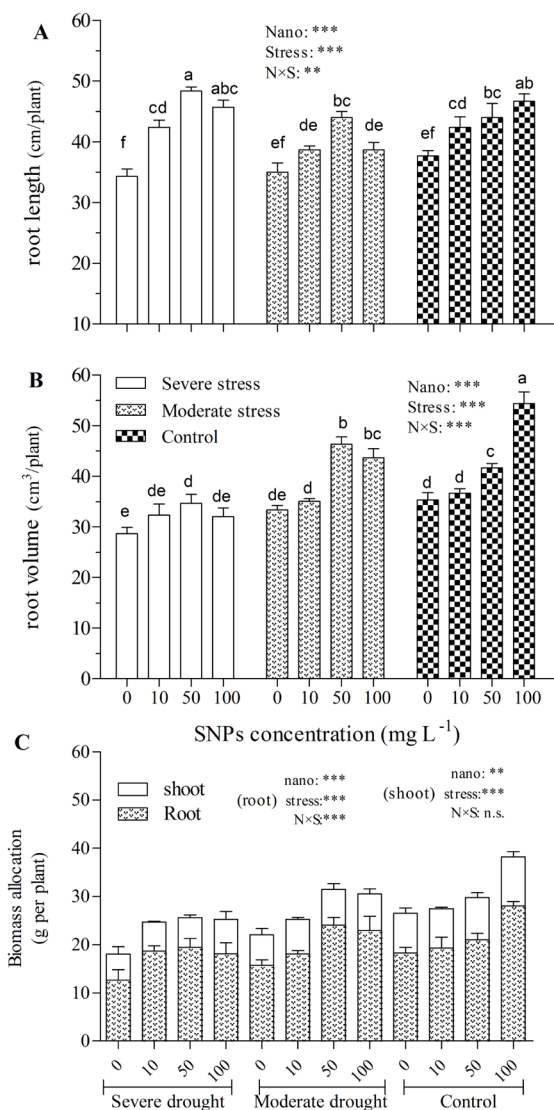


Fig. 2. A: Root length, **B:** root volume, **C:** and biomass allocation of *Prunus mahaleb* seedlings pre-treated with different concentration of SNPs during 45 days, and subsequently subjected to 19 days to three irrigation treatments (0, 150 and 300 mL of water every 3 days [severe drought, moderate drought and well-watered controls, respectively]).

SNPs in the severe drought regime (Fig. 3B). Under moderate stress conditions, the beneficial effect of nanoparticles on XWP was true for those seedling pretreated with 50 and 100 mg L⁻¹ of SNPs under severe or moderate drought. Under well-watered conditions, XWP – naturally higher on average

than under water stress – was similar irrespective of SNPs pretreatment (Fig. 3B). The electrolyte leakage index (ELI) values registered in tissues of seedlings subjected to severe drought were more than 2-fold higher than those of seedlings exposed to moderate stress or irrigated conditions, which did not differ for this parameter (Fig. 3C). Application of SNPs did not change ELI under either irrigation treatment. TBARs in leaf tissues, as an indicator of lipid peroxidation, was affected by SNPs depending on the irrigation regime (see the significant interaction between factors in Fig. 3D). The highest values for TBARs were registered under severe drought in non-SNPs pretreated plants. Also, SNPs application at higher concentration (100 mg L⁻¹) under severe drought determined a significant reduction in TBARs comparable to the values obtained under moderate drought (Fig. 3D). These results are in line with the slightly better water status of plants pretreated with SNPs at higher concentration under stressful drought conditions. It should be noticed that TBARs values allowed to distinguish the protection by SNPs when under severe drought conditions while ELI did not (compare Fig. 3C and Fig. 3D).

Total chlorophyll concentration in non-SNPs pretreated seedlings decreased by 33% under severe drought (Table 1). SNPs pretreatment (50 and 100 mg L⁻¹) enhanced chlorophyll concentration in seedlings subjected to severe drought. Moderate drought conditions did not affect this parameter when SNPs under either concentration was previously applied (Table 1). Under severe drought, carotenoids were progressively higher at increasing SNPs concentrations as pretreatments (1.40 vs 2.77 mg/g in seedlings non-SNPs-treated and treated with 100 mg L⁻¹ of SNPs), even clearer than when under moderate drought conditions (Table 1). The concentration of free proline was highest under severe stress in seedling that did not receive SNPs application. Interestingly, SNPs used as a pretreatment at 50 and 100 mg L⁻¹ determined a 24-27% reduction in proline concentration in leaves (Table 1). Under moderate drought, the values for this parameter were similar than those obtained from control seedlings.

Effect of SNPs pre-treatments on leaf concentrations of N, P, K and Si after drought

To verify the effect of SNPs pretreatments on the nutrient uptake in Mahaleb seedlings, we studied the concentrations of three main mineral elements such

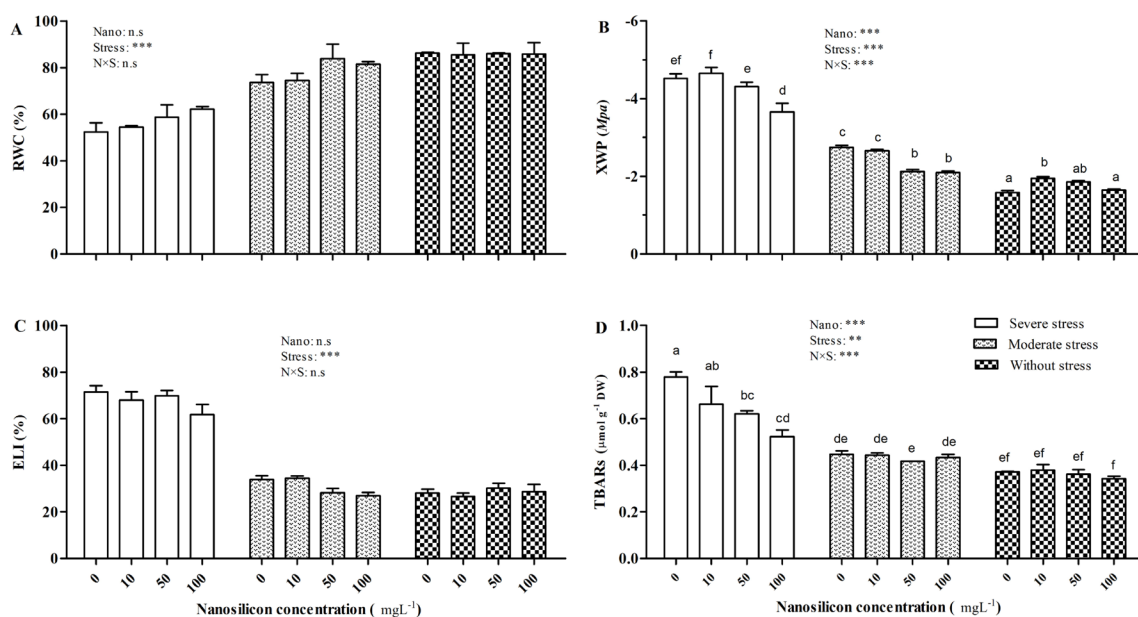


Fig. 3. Water relations parameters (A: relative water content, B: xylem water potential, C: electrolyte leakage index-ELI and D: thiobarbituric acid-reactive-substances (TBARS) of *Prunus mahaleb* seedlings pretreated with different concentration of SNPs during 45 days, and subsequently subjected to 19 days to three irrigation treatments (0, 150 and 300 mL of water every 3 days [severe drought, moderate drought and well-watered controls, respectively]). Different letters indicate significant differences ($P < 0.05$) among treatments based on the Duncan tests. (Mean \pm SE; $n=6$). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; *n.s.*: $P > 0.05$.

as N, P and K, and also Si in seedlings subjected to severe drought (no irrigation during 19 d) and well-watered control by applying X-ray fluorescence. The concentration of N in leaves under severe drought was higher in seedlings pretreated with 50 and 100 mg L⁻¹ SNPs compared to those non-treated with SNPs (Fig. 4B). Furthermore, improvement in leaf N concentration by SNPs when in severe drought determined that such parameter reached similar values than those of well-watered control seedlings (Fig. 4B). The concentration of P in leaf tissues in seedlings exposed to drought was enhanced by pretreatment with high doses of SNPs (*i.e.* 50 and 100 mg L⁻¹) compared to those non-pretreated with SNPs (Fig. 4C). Interestingly, seedlings growing under well-watered conditions had higher leaf P concentrations when pretreated with either SNPs concentration with respect to those that did not receive SNPs application as a pretreatment (Fig. 4C). In the case of K, SNPs application did not affect the concentration of K in leaves, except 100 mg L⁻¹ SNPs pretreatment of

well-watered seedlings, which attained a slightly higher K concentration with respect to all other treatment combinations (Fig. 4D). Finally, Si concentration in leaf tissues was significantly higher at increasing SNPs concentrations when seedlings grew under control conditions (Fig. 4A) whereas in seedlings subjected to drought a similar increase in leaf Si concentration was observed at either SNPs pretreatment (10, 50 and 100 mg L⁻¹), which was higher than of seedlings non-pretreated with SNPs (Fig. 4A).

DISCUSSION

The application of Si nanoparticles (SNPs) can improve the growth of several crops and increase their tolerance to biotic and abiotic stresses (Richmond & Sussman, 2003; Ma, 2004; Ahmed *et al.*, 2014) but data on woody plants (and particularly in tree fruit species) were scarce. In our research, we have applied different concentrations

Table 1. Total chlorophyll, carotenoids and free proline concentrations (dry weight basis) of *Prunus mahaleb* seedlings pretreated with different concentration of SNPs during 45 days, and subsequently subjected to 19 days to three irrigation treatments (0, 150 and 300 mL of water every 3 days [severe drought, moderate drought, and well-watered controls, respectively]). Different letters indicate significant differences ($P < 0.05$) among treatments based on the Duncan tests. (Mean \pm SE; $n=6$).

Treatment	Control	Moderate Drought	Severe Drought
Chlorophyll a+b ($\mu\text{g/g}$)			
0 mgL ⁻¹	9.38 \pm 0.34 cd	7.53 \pm 0.52 de	6.26 \pm 0.47 e
10 mgL ⁻¹	10.79 \pm 0.16 bc	10.29 \pm 0.45 ab	7.19 \pm 0.57 de
50 mgL ⁻¹	10.39 \pm 0.32 bc	9.99 \pm 0.3 bc	9.09 \pm 0.52 bc
100 mgL ⁻¹	11.11 \pm 0.09 ab	12.04 \pm 0.35 a	9.76 \pm 0.53 ab
Carotenoid (mg/g)			
0 mgL ⁻¹	2.21 \pm 0.07 ce	1.60 \pm 0.09 g	1.40 \pm 0.06 g
10 mgL ⁻¹	2.63 \pm 0.3 b	1.93 \pm 0.06 ef	1.48 \pm 0.09 fg
50 mgL ⁻¹	2.39 \pm 0.07 bd	2.08 \pm 0.07 e	2.10 \pm 0.15 bc
100 mgL ⁻¹	2.41 \pm 0.08 bd	1.74 \pm 0.08 fg	2.77 \pm 0.08 a
Free proline ($\mu\text{g/g}$)			
0 mgL ⁻¹	105.83 \pm 1.89 ef	108.12 \pm 0.68 cd	141.45 \pm 1.55 a
10 mgL ⁻¹	102.60 \pm 2.65 fg	105.50 \pm 0.57 de	132.87 \pm 0.43 b
50 mgL ⁻¹	109.61 \pm 2.10 de	110.61 \pm 0.07 de	102.81 \pm 0.23 c
100 mgL ⁻¹	99.96 \pm 4.72 g	96.01 \pm 1.74g	106.57 \pm 1.53 c

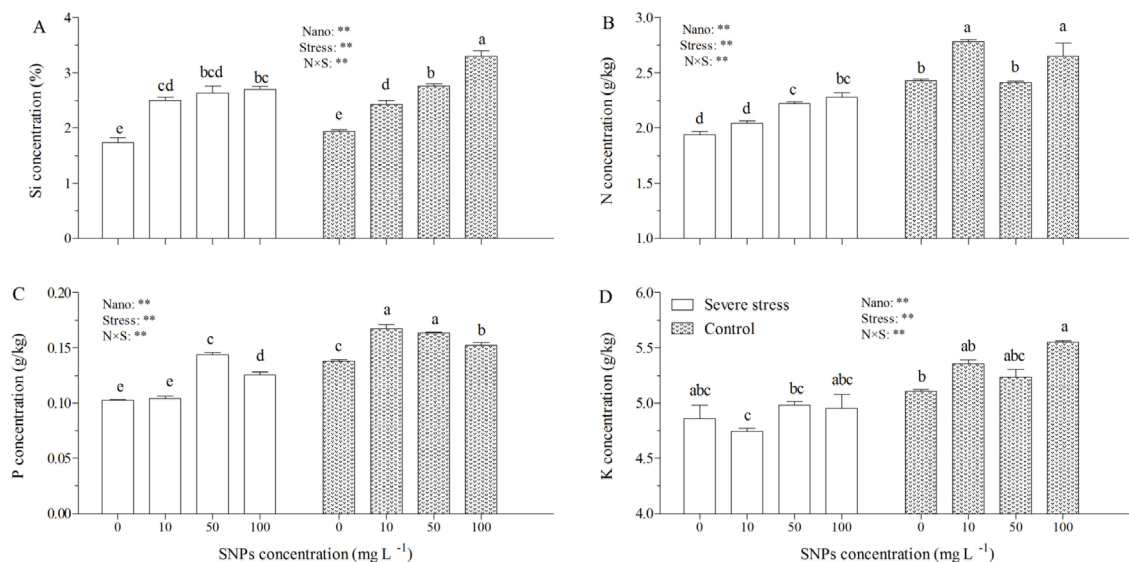


Fig. 4. Concentration of Si, N, P and K in leaf tissues of *Prunus mahaleb* seedlings (dry weight basis) pretreated with different concentration of SNPs during 45 days, and subsequently subjected to 19 days of three irrigation treatments (0, 150 and 300 mL of water every 3 days [severe drought, moderate drought and well-watered controls, respectively]). Different letters indicate significant differences ($p < 0.05$) among treatments based on the Duncan tests. (Mean \pm SE; $n=6$). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; n.s.: $P > 0.05$.

of SNPs for 45 days to *Prunus mahaleb* (Mahaleb) seedlings, and afterwards, they were subjected to drought to assess the role of SNP pre-treatment on plant physiological responses of this woody species. Based on our results, SNP application at concentrations of 50 and 100 mg L⁻¹ was clearly beneficial to Mahaleb seedlings given the improvement of its drought tolerance. Positive effects of Si were reported in many plant species such as wheat, rice, cucumber, maize and bamboo, in particular, under stressful conditions (Ma & Takahashi, 2002; Shi *et al.*, 2005; Zhang *et al.*, 2008; Vaculik *et al.*, 2009; Feng *et al.*, 2010; Collin *et al.*, 2014; Xu *et al.*, 2015). In the case of *P. mahaleb*, we found that the positive effects of SNPs were evident when seedlings were later exposed to drought stress. Seedlings pretreated with SNPs showed less impact of severe drought (no irrigation) on root length – as an indicator of potential for water and nutrient uptake – and displayed a better physiological behaviour regarding photosynthesis, stomatal conductance, and xylem water potential when compared to seedlings non-pretreated with SNPs. Such better physiological performance of SNPs pretreated seedlings was related to the maintenance of leaf nutritional status, which showed comparable concentrations of N and P than those of never-stressed (control) seedlings. Along the experiment, the improved tolerance by SNPs to drought was reflected in higher biomass accumulation, particularly of roots. Therefore, we can accept our hypothesis by which we proposed that SNPs pre-treatments alleviate the detrimental effects of a subsequent severe drought. Also, it is interesting to notice that there were no apparent associated biological costs of SNPs application regarding growth when plants grew under control conditions as well watered seedlings pretreated with the highest SNPs concentration (100 mg L⁻¹) attained the highest dry mass. The mechanisms underlying this growth promotion even when seedlings were well irrigated deserves further experimental investigation.

Cell expansion and, consequently, shoot and root elongation are sensitive responses to drought stress (Lambers *et al.*, 2008). Important responses to SNPs pretreatment on seedlings exposed to severe drought were the maintenance of the root length and the amelioration of the negative impact of water deficit on root volume and root biomass

accumulation with respect to non-pretreated seedlings. These results are in line with the better physiological water status of seedlings in terms of xylem water potential (XWP) due to the presence of SNPs (100 mg L⁻¹) as shown also in adult plants of this species when subjected to summer drought given its deep roots compared to other coexistent woody species like *Quercus pubescens* and *Ostrya carpinifolia* (see Figure 2 in Nardini *et al.*, 2015). This better water status indicated by XWP was not reflected in improved leaf relative water content (RWC) as could be expected (see also Zarafshar *et al.*, 2014). So, SNPs aided plants to maintain root length and decreased the impact on XWP but not on RWR. In this respect, Zhang *et al.* (2013) found that silicon application did not enhance leaf RWC of Chestnut plants subjected to water deficit as well as it was found by Ashkavand *et al.* (2015) on hawthorn seedlings. Curiously, it seems that SNPs application did not aid to improve leaf RWC in woody plants (*eg.* Zhang *et al.*, 2013; Ashkavand *et al.*, 2015) but it does it in herbaceous crop species such as sorghum (Kafi *et al.*, 2011), cotton, canola, and wheat (Mehrabanjoubani *et al.*, 2015). Gadallah (2000) proposed that RWC improvement could be due to (i) an enhanced water uptake resulting from a more developed root system and (ii) a reduction of water loss by transpiration. In our experiment, RWC was not improved by SNPs application as leaf transpiration was not reduced as stomatal conductance remained higher despite Mahaleb seedlings growing under severe drought developed longer roots, which increased the potential for water uptake and, explained – at least partially - the amelioration of drought impact on XWP.

Leaf gas exchange was negatively affected by drought intensity along time but to a lesser extent when seedlings were pretreated with SNPs at high concentrations (50 and 100 mg L⁻¹ SNPs; Fig. 1). In agreement with findings by Matoh *et al.* (1986) for rice, we found that under severe drought conditions, stomatal conductance was 2-fold higher in seedlings that received SNPs application than those that did not. In this sense, besides the relation between the water status, cell turgor, and stomatal aperture, Agarie *et al.* (1998) reported positive effects of silicon (Si) on stomatal conductance in rice, which likely play a role in the responses of stomata cells to blue light (Agarie *et al.*, 1999). Nevertheless, the

specific mechanism by which Si regulates stomatal responses in woody species remains unclear and needs further experimental investigation (Gao *et al.*, 2005). The magnitude of the retained stomatal conductance of SNPs pre-treated seedlings were the same than the retained capacity for carbon fixation of such seedlings (Figure 2) as it was previously informed for maize (Kaya *et al.*, 2006), cherry tomatoes (Haghighi & Pessarakli, 2013), chestnut (Zhang *et al.*, 2013) and Hawthorn (Ashkavand *et al.*, 2015). Moreover, such less impacted photosynthesis matched with the maintenance of chlorophyll and carotenoids levels when compared to that of controls seedlings (Table 1). Therefore, seedlings pretreated with SNPs displayed a less negative impact on drought on stomatal aperture, carbon fixation, and photosynthetic pigments showing the beneficial effects of SNPs pre-treatments on physiological performance when facing a subsequent drought period.

Alleviation of drought stress by SNP application was also clear in biochemical parameters as free proline, and TBARs had lower concentrations when seedlings were pretreated with SNPs than when they were not (Table 1 and Figure 3D, respectively). In general, we found that free proline in leaves at all watering regimes decreased at increasing of SNPs concentration similarly to what has been documented in soybean plants under salinity stress (Lee *et al.*, 2010) and drought stress (Shen *et al.*, 2010). The accumulation of free proline under stressful conditions (no irrigation plus non-SNPs pretreatment) might be regarded as indicative of the osmotic adjustment capability of this species. Also, seedlings that received an application of SNPs previous to water withholding were less stressed than those without SNPs pretreatment as indicated the leaf gas exchange responses already discussed. On the other hand, TBARs, an indicator of oxidative damage to membrane lipids under stress (Ozkur *et al.*, 2009), was the highest under severe drought conditions in seedlings non-pretreated with SNPs while those pretreated with silicon nanoparticles registered progressively lower concentrations of TBARs at increasing SNPs application. Again, this finding illustrates that exogenous SNPs application relieves drought-induced injury in Mahaleb seedlings. Such relief would be associated with the maintenance of root length and its functionality (Fig. 3A; see also Zarafshar *et al.*, 2015).

The application of SNPs not only alleviated the effects of drought on plant physiological activity but also enable them to continue with nutrient uptaking of N and P and to a lesser extent K, which was reflected in a similar leaf concentration of these nutrients with respect to that of non-stressed seedlings (Fig. 4). The maintenance of the concentration of N and P in high levels of seedlings pretreated with SNPs under drought conditions might explain the lowered impact on photosynthesis and plant growth (see also reviews by Zlatev & Lindon, 2012, and by Ashraf & Harris, 2013). In this sense, the better status of N and P likely to have an important role in sustaining the photosynthetic rate (Lambers *et al.*, 2008). So, the beneficial effects of SNPs application were also evident in the leaf nutritional status of this species.

In conclusion, as discussed above, the application of silica nanoparticles (SNPs in this report) as pre-treatments should be considered as a promising agronomic practice to be tested at field scale in sites prone to suffer from water deficit as SNPs appear to be able to alleviate the common physiological deleterious effects of drought on plants as demonstrated here for Mahaleb.

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