Differential physiology and expression of phenylalanine ammonia lyase (PAL) and universal stress protein (USP) in the endangered species *Astragalus fridae* following seed priming with cold plasma and manipulation of culture medium with silica nanoparticles

Maryam Moghanloo¹ · Alireza Iranbakhsh¹ · Mostafa Ebadi² · Zahra Oraghi Ardebili³

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Abstract

Key message Seed priming with cold plasma in combination with manipulation of culture medium with silica nanoparticle provokes anatomical, physiological and molecular changes, thereby reinforcing the plant growth and protection.

Abstract This study addressed responses of *Astragalus fridae* to seed priming with cold plasma (0.84 W/cm²; 0, 30, 60, and 90 s) and applications of SiO₂ nanoparticle (nSi; 0, 5, 40, and 80 mg l⁻¹) in culture medium (an in vitro study). FE-SEM confirmed nSi uptake and translocation. Bulk Si at high concentrations reduced biomass accumulation (mean = 45%), while nSi did not make significant differences. The growth-enhancing effects of plasma by 41.5% were promoted by the nSi supplementation and reached 71%. Plasma did not make significant changes in Chla, while led to the slightly higher (mean = 14%) Chlb. The presence of nSi at high doses caused slight reductions in Chlb (mean = 25%) which were mitigated by plasma. The plasma and/or nSi treatments modified activities of phenylalanine ammonia lyase (PAL) in both roots (mean = 32%) and leaves (mean = 44%). With a similar trend, both individual and combined treatments of plasma and nSi provoked inductions in peroxidase activities in roots and leaves. The simultaneous treatments of plasma and nSi had the highest expression rates of PAL gene. The individual treatments of plasma did not make a significant difference in the expression of universal stress protein (USP) gene, whereas the nSi-treated seedlings exhibited the higher expression rates of USP. Leaf thicknesses and development of the vascular system (xylem and phloem) were reinforced in response to plasma and nSi. The findings provide evidence on potential benefits and phytotoxicity of nSi and plasma which may be employed as a theoretical basis for possible exploitation.

Keywords Cold plasma · Nanoparticle · In vitro · Silicon · Universal stress protein

Introduction

Silicon (Si) has not been characterized as a plant essential nutrient, despite the existence of several evidence manifesting it may exhibit beneficial effects on plant growth,

biomass, anatomy, physiology, and acclimation to stress condition (Neu et al. 2017; Asgari et al. 2018; Moghanloo et al. 2019). Si at suitable concentrations in a manner dependent on plant species displayed the potential advantages, such as improving growth (Schaller et al. 2012), modifying tissue differentiation (Moghanloo et al. 2019), activating defense system (Ma and Yamaji 2006; Tripathi et al. 2015; Moghanloo et al. 2019), and promoting the antioxidant defense system, (Zhu et al. 2004; Moghanloo et al. 2019), while it may exert toxicity and adversely affect plant at high levels (Schaller et al. 2012; Moghanloo et al. 2019).

Owing to unique physicochemical characteristics of nanoparticles, a novel fictionalization of diverse nano-based products in agriculture-related activities and

✉ Alireza Iranbakhsh
iranbakhsh@iau.ac.ir

¹ Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

² Department of Biology, Damghan Branch, Islamic Azad University, Damghan, Iran

³ Department of Biology, Garmsar Branch, Islamic Azad University, Garmsar, Iran

industries are extensively developing to improve crop productivity, metabolism, and protection (Asgari-Targhi et al. 2018; Babajani et al. 2019a). Besides that, the utilization of the nanoparticles is worldwide under debate, basically taking account of environmental issues (Asgari-Targhi et al. 2018; Babajani et al. 2019a). Si nanoparticle (nSi) is known as one of the most widely applied nanoproducts in different industries (Rui et al. 2014; Moghanloo et al. 2019). Diverse efforts have been made to exploit the nSi-based products in agriculture to improve plant nutrition and protection (Rui et al. 2014). Although, the possible roles of this nano compound on plant growth, physiology, and development are unknown and need to be further explored. It should be noted that the ameliorating effect of nSi had been confirmed in *Pisum sativum* counteracted with chromium (Tripathi et al. 2015). On the other hand, nSi exposure had negative effects on plant growth (Nair et al. 2011; Rui et al. 2014). Recently, it has been reported that the supplementation of culture medium with nSi not only modified growth and physiology in *Astragalus fridae* but also, it associated with anatomical changes, especially in vascular tissues (Moghanloo et al. 2019).

Seed pretreatment with cold (non-thermal) plasma has been introduced as a pollution-free, eco-friendly, fast, and economical method to affect seed germination and seedling performance (Sheteiwy et al. 2018; Iranbakhsh et al. 2018a; Babajani et al. 2019b; Seddighinia et al. 2019). Formation of different bioactive nitrogen and oxygen species (such as nitric oxide) and UV photons has been manifested during the plasma generation as the 4th state of matter (Iranbakhsh et al. 2017; Babajani et al. 2019b). Recent evidence clearly reflected that plasma exposure has efficient potency to activate a specific process by which rectify seed germination (Sadhu et al. 2017) and decontamination (Jiang et al. 2014), affect cellular differentiation (Safari et al. 2017; Moghanloo et al. 2019), improve seedling performance (Ling et al. 2014; Iranbakhsh et al. 2017; Seddighinia et al. 2019), modify physiological traits (Ji et al. 2016; Iranbakhsh et al. 2017; Sheteiwy et al. 2018; Iranbakhsh et al. 2018a, 2018b; Moghanloo et al. 2019), and flowering (Seddighinia et al. 2019). It is interesting to note that cold plasma pretreatment relieved the toxicity signs of nano zinc oxide (Iranbakhsh et al. 2018b), salinity (Iranbakhsh et al. 2018a; Sheteiwy et al. 2018), and nano selenium (Babajani et al. 2019b). Furthermore, the cellular modifications at the molecular level have been supported by some recent studies (Ji et al. 2016; Iranbakhsh et al. 2018a). Interestingly, the plasma treatment changed the concentration of gibberellin phytohormone and expression of several genes contributed to germination phenomenon (Ji et al. 2016). Obviously, knowledge and convincing data on plant-plasma interactions are rare and more studies are required to exploit in related technologies and industries (Babajani et al. 2019b).

It is believed that aseptic culture of the plant cell, tissue, and organs in in vitro controlled condition may provide a theoretical basis for clarifying the underlying mechanisms contributed to plant reactions to the studied factors and exploiting in related technologies and industries. Furthermore, manipulating the culture rooting medium with different chemical ingredients, such as hormones, minerals, and elicitors is identified as an effective method to modify plant behaviors at diverse morphological, physiological, and molecular levels in in vitro condition to enhance efficiency of plant tissue culture and production of secondary metabolites (Asgari-Targhi et al. 2018; Moghanloo et al. 2019). However, data on the effects of supplementations of nutrient culture medium with the nanoparticles on plant subjected for in vitro culture is rare and contradictory, so require to be further figured out.

Astragalus is a large genus of about 3300 species belonging to the legume family of Fabaceae and the subfamily Faboideae. This genus is widely distributed throughout the temperate regions of the world among which Iran is the main source and one of the centers of species diversity in the ancient world (Maassoumi 1998; Podlech 1982). *Astragalus fridae* is an exclusive and endemic species in Iran. According to the classification of the World Conservation Union (IUCN) and using available resources in Iran, this plant is considered an endangered plant (Jalili and Jamzad 1999). However, most of the species are used as forage for livestock and wildlife, thirty-two of its species are used for food, medicine, cosmetics, tea, and coffee, or as a source of herbal gums. *A. fridae* contains several different types of primary and secondary metabolites such as polysaccharides and saponins, and toxic compounds such as indosolidin, alkaloid, and nitroallithic (Li et al. 2014). The plant also has other medicinal effects, including antioxidant, liver protective effects, antiviral and antibacterial effects, and substances that affect the blood vessels of the heart (Ghasemian-Yadegari et al. 2017). The antioxidant components obtained from the root prevent the decrease in the content of liver glycogen, which increases the total protein and albumin of the serum (Du et al. 2003; Feng et al. 2014).

Taking account of the above highlighted significance of nSi, cold plasma, and in vitro culture, the current research was conducted to explore the possible effects of seed priming with the cold plasma and manipulating the culture rooting medium with nSi on the growth, physiology, anatomy, and gene expression in *A. fridae* as an endangered species, for the first time.

Materials and methods

The nSi powder (SiO₂ nanoparticle; US Research nanomaterials Company) was picked out for the application. Its physicochemical characteristics were as follows.

Specific surface area: 600 m²/g; average primary particle size: 20–30 nm; purity: 99%.

Plasma generating device

Dielectric-barrier discharge (DBD) was utilized as an experimental device for cold plasma treatment in this study and argon was applied as the functional gas between dielectrics. It should be noted that the details and schematic plan of the plasma-generating device and the plasma diagnostic data (Iranbakhsh et al. 2018a) were represented elsewhere.

Seed priming with the plasma and nSi treatments for in vitro culture

The surface sterilized seeds were immersed in water for 12 h prior the plasma pretreatment (to activate intracellular metabolism). The seeds were subjected to plasma exposure, where the surface power density and duration times were, respectively, 0.84 W/cm² and different (0, 30, 60, and 90 s). The plasma-primed seeds were grown in hormone-free MS culture medium (Murashige and Skoog 1962) manipulated with different concentrations of nSi (0, 5, 40, and 80 mg l⁻¹) and its bulk counterpart. All samples were kept in a controlled condition (germinator; light intensity of 30 μmol photons m⁻²s⁻¹ (16/8 h light; dark); the temperature of 25 °C). It should be noted that the seedlings were cultured in the medium supplemented with the different levels of the bulk type were only subjected to the growth-related parameters to provide preliminary comparative data. The treatment groups were called as follows: C—Control; BSi5—Bulk Si of 5 mg l⁻¹; BSi40—Bulk Si of 40 mg l⁻¹; BSi80—Bulk Si of 80 mg l⁻¹; nSi5—nSi of 5 mg l⁻¹; nSi40—nSi of 40 mg l⁻¹; nSi80—nSi of 80 mg l⁻¹; P30—Plasma of 30 s; P30 + nSi5—Plasma of 30 s and nSi of 5 mg l⁻¹; P30 + nSi40—Plasma of 30 s and nSi of 40 mg l⁻¹; P30 + nSi80—Plasma of 30 s and nSi of 80 mg l⁻¹; P60—Plasma of 60 s; P60 + nSi5—Plasma of 60 s and nSi of 5 mg l⁻¹; P60 + nSi40—Plasma of 60 s and nSi of 40 mg l⁻¹; P60 + nSi80—Plasma of 60 s and nSi of 80 mg l⁻¹; P90—Plasma of 90 s; P90 + nSi5—Plasma of 90 s and nSi of 5 mg l⁻¹; P90 + nSi40—Plasma of 90 s and nSi of 40 mg l⁻¹; P90 + nSi80—Plasma of 90 s and nSi of 80 mg l⁻¹.

Measurements of photosynthetic pigments

Photosynthetic pigments were extracted using 80% (v/v) acetone as a solvent. Chla and Chlb contents were determined according to the previously presented equations by Arnon (Arnon 1949).

Field emission scanning electron microscopy (FESEM)

FESEM procedure was used to monitor the nanoparticle uptake, transport, and accumulation in the shoot. Stem cross sections of the 21-day old seedlings were subjected to FESEM. Briefly, the prepared samples were dehydrated, fixed (by utilizing freeze dryer method), gold-coated, and photographed.

Enzyme extraction

Enzyme extraction was carried out using the modified phosphate buffer (0.1 M; pH of 7.3) from the well-grounded fresh leaves and roots in liquid nitrogen (Asgari-Targhi et al. 2018). Then, the resulted homogenate was centrifuged at 4 °C and the supernatants subjected for the analysis of enzyme activities.

Quantification of peroxidase and phenylalanine ammonia lyase (PAL) activities

Peroxidase activity was spectrophotometrically assessed at a wavelength of 470 nm by guaiacol (hydrogen donor) and H₂O₂ (substrate). The reaction mixture was composited of H₂O₂ (1%), guaiacol (1%), and phosphate buffer (0.05 M; pH 6.5). The enzyme activity was monitored according to the absorbance differences per min per gram fresh weight.

The reaction mixture for PAL activity consisted of 6 μM phenylalanine, Tris-HCl buffer (0.5 M, pH 8), and 200 μL of enzyme extract. After 60 min at 37 °C, the reaction was ended by adding 50 μL of 5 N HCl. PAL activities were analyzed (the rate of conversion of L-phenylalanine to trans-cinnamic acid) at 290 nm. PAL activity was determined by measuring the amount of cinnamic acid produced and expressed in μg Cin min⁻¹ g⁻¹ fw, according to the method described elsewhere (Beaudoin-Eagan and Thorpe 1985).

Gene expression

RNA extraction

Shoots of *Astragalus fridae* from all treatments were stored in -80 °C until RNA extraction. Samples were ground to powder in liquid nitrogen. Total RNA were extracted from samples using Kit (#74804, Roche, Germany) according to the manufacturer's instructions. The quantity of the extracted RNA was assessed by the Nanodrop.

Preparations of cDNA and quantitative real-time PCR analysis of PAL and universal stress protein (USP)

Complementary DNA (cDNAs) were synthesized from the extracted RNA of shoot tissues using the kit (QIAGEN, USA) by RT-PCR. To design primers for quantitative Real-time PCR (qRT-PCR), the Bicon designer and Primer 3 program (<http://frodo.wi.mit.edu/primer3>). The forward and reverse primer sequences were presented in Table 1.

The qRT-PCR was conducted in 0.5 μ M of each set of primers in 20 μ l final reaction volume of SYBR Green Real-time PCR on a Rotor-Gene Q real-time PCR system (QIAGEN, USA) under the following conditions: 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. All experiments were performed in duplicate and three times.

Histological procedure

Roots of the treated seedlings were stored in fixator solution (ethanol and glycerol (75:25) prior preparing the hand-made cross-section. Then, the samples were stained (methylene blue and carmine), monitored by light microscope, and photographed.

Statistical analysis

The achieved data were statistically analyzed using SPSS software. All data was represented as mean and standard error (SE). Mean differences were conducted according to Duncan's multiple range test ($P \leq 0.05$). In addition, the correlation coefficient (r) was determined to monitor the existence of a possible relationship between the measured traits.

Table 1 Forward and reverse primer sequences for PAL, USP, and 18S rRNA

Primer name	Sequence (5–3)	t_m (°C)
PAL-R	ACACCAAATACACAAAGGGAGT	61
PAL-F	CCGCAACCTTCTAAAGATCTCA	63
USP-R	CACCATACCCATGGCTTCCC	63
USP-F	TGGTGTGTAACGACGTCCAA	58
18S-R	AGCCTGCGTCGACCTTTTAT	58
18S-F	GTGAAACTGCGAATGGCTCA	58

Results

Uptake and accumulation of the nanoparticle

FESEM method was used for monitoring and manifesting the uptake of nSi. The ultra-structural analysis of leaf clearly confirmed the nSi uptake and accumulation (Fig. 1). It should be noted that the ultra-structure studies in stem and root organs similarly manifested these phenomena (data was presented elsewhere).

Biomass accumulation and phototosynthetic pigments

The total dry mass was influenced by different treatments of plasma and nSi. Except for the BSi5, BSi40 and BSi80 treatments had the growth delaying impacts and led to the significant reduction in total dry mass by 34% and 56%. However, their corresponding concentrations of nSi did not make significant differences. The individual treatments of plasma increased total dry mass by 48, 32, and 44.5% for the P30, P60, and P90 groups, respectively. Interestingly, these rates were augmented by the simultaneous application of nSi of 5 mg l^{-1} and reached to 70, 78, and 65%, respectively, for the P60 + nSi5, P30 + nSi5, and P90 + nSi5 groups in comparison to the control (Fig. 2). The seed priming with cold plasma did not make significant changes in Chla content when compared to the control (Fig. 3a). Except for the nSi of 5 mg l^{-1} , the nSi40 (4.7%) and nSi80 (13.77%) treatments slightly diminished Chla level which was partially mitigated by the plasma treatment (Fig. 3b). The P30 (14%) and P90 (14.5%) groups were found to have significantly higher amounts of Chlb, over the control (Fig. 3b). The individual nSi40 and nSi80 treatments significantly decreased the Chlb content by 15.32% and 35%, respectively, which were significantly mitigated by the plasma treatment (Fig. 3b).

Peroxidase and PAL activities

Seed priming with cold plasma individually or in combination with nSi augmented the PAL activity in leaves, compared to the control. The P30 + nSi80, nSi5, P30, nSi80, P60, P60 + nSi5, and nSi40 treatments provoked significant intensive promotions in the leaf PAL activity by 97, 66, 62, 53, 51.8, 42.3, and 39% over the control (Fig. 4a). Also, the moderate significant rises in the leaf PAL activity were recorded for the P30 + nSi5 (25%), P30 + nSi40 (18.5%), P60 + nSi40 (22%), P60 + nSi80 (23.6%), P90 (20.3%), and P90 + nSi5 (19%), relative to the untreated control group. However, the recorded increases in the leaf PAL activity in the P90 + nSi40 (5.3%) and P90 + nSi80 (6.5%) were not

Fig. 1 The ultra-structure photographs based on the electron microscopy (FESEM) for tracing the transportation of nSiO_2 from the roots to leaves. **a–c** Control, **d–f** nSiO_2 40 mg l^{-1} , **g–i** plasma of 60 s, **j–l** plasma of 60 s and nSiO_2 5 mg l^{-1}

found to be the statistically significant changes (Fig. 4a). The negligible significant reductions in the root PAL activities were observed in the nSi80 , P90, P90 + nSi40 , and P90 + nSi80 groups (Fig. 4b), while the differences between

control and P90 + nSi5 was not statistically significant. Interestingly, the dramatic significant inductions in the root PAL activity caused by the P60 (69%), P30 + nSi80 (49%), P60 + nSi80 (42%), and P30 + nSi40 (42.4%) treatments. In

Fig. 2 Changes in the total plant dry weight following seed priming with cold plasma and the manipulation of the MS culture medium with nSi or its bulk counterpart. Data are presented as mean $\pm$ SE of three independent replications. Different letters indicate significant differences at $P \leq 0.05$ according to Duncan's multiple range test

addition, the root PAL activity in the nSi5 (12.9%), nSi40 (29.4%), P30 (30.7%), P30 + nSi5 (32.6%), P60 + nSi5 (31%), and P60 + nSi40 (16%) groups were significantly higher than the control (Fig. 4b). Furthermore, seed priming with the cold plasma and supplementations of rooting medium with nSi led to the severe augmentation in the leaf peroxidase activity when compared to the control (Fig. 4c). Except for the nSi40 group, the significant inductions in the root peroxidase activities were recorded for the plasma and/or nSi-treated seedlings among which the P30 + nSi40 (3 folds), P90 + nSi80 (3 folds), P60 + nSi40 (2.6 folds), P30 + nSi80 (2 folds), and nSi80 (2 folds) had the highest rates in comparison to the control (Fig. 4d). The correlation coefficients (r) between various analyzed traits are depicted in Table 2. The statistically significant correlations were found between root peroxidase activities and the expression rates of PAL and USP in leaves (Table 2). While there were significant negative correlations between leaf peroxidase and

PAL expression or USP transcription in leaves (Table 2). A significant negative correlation was also found between the USP expression trait in leaves and root PAL activity (Table 2).

Gene expression

Except for the nSi80 group, the plasma and/or nSi-treated treatments provoked the inductions in the expression of PAL (Fig. 5a). The P60 + nSi80, P60 + nSi40, P30 + nSi40, P60 + nSi5, P90 + nSi5, and P30 + nSi80 treatments led to the drastic significant increases in PAL expression (Fig. 5a). The individual treatments of plasma did not make a significant change in the expression of USP gene, relative to the untreated control (Fig. 5b). While the supplementation of culture medium with the different levels of nSi altered the expression rate of USP. The P90 + nSi80, nSi5, P60 + nSi80, P90 + nSi40 had the highest expression of USP which was found to be drastic significant differences (Fig. 5b).

Leaf anatomy

As it was depicted in Fig. 6, leaf anatomy, tissue differentiation patterns (especially vascular system) were affected by the seed priming with the plasma and/or supplementation of rooting medium with nSi. The P30 + nSi5, P60 + nSi5, P60 + nSi80, P90, P90 + nSi5, and nSi40 groups exhibited the enhancing roles on the phloem tissues in the midrib of leaf (Fig. 7a). Interestingly, the P60 + nSi5, P60 + nSi40, P90 + nSi80, P90 + nSi5, and nSi40 groups had the highest amounts of differentiated xylem tissues. Furthermore, P60, P60 + nSi5, P90 + nSi80, and P90 + nSi5 possessed the xylem with the higher diameters, when compared to the control (Fig. 7b). The leaf and leaf midrib thicknesses were reinforced in the treated seedlings among which the P30 + nSi5, P30 + nSi5, P90 + nSi5, and P30 treatment groups had the greatest (Fig. 7).

Fig. 3 Induced differences in the Chla (a) and Chlb (b) concentrations following seed priming with cold plasma and the supplementation of the rooting MS medium with nSi. Data are presented as

mean $\pm$ SE of three independent replications. Different letters indicate significant differences at $P \leq 0.05$ according to Duncan's multiple range test

Fig. 4 Induced differences in the activities of PAL (leaves (a) and roots (b)) and peroxidase (leaves (a) and roots (b)) enzymes following the seed priming with cold plasma and the supplementation of

the rooting MS medium with nSi. Data are presented as mean $\pm$ SE of three independent replications. Different letters indicate significant differences at $P \leq 0.05$ according to Duncan's multiple range test

Table 2 The correlation coefficient (r) between different evaluated traits

	PAL expression	USP expression	Leaf PAL activity	Root PAL activity	Leaf peroxidase activity	Root peroxidase activity
PAL gene	1	0.161 ns	-0.159 ns	0.255 ns	-0.324*	0.408**
USP gene		1	-0.217 ns	-0.518**	-0.287*	0.419**
Leaf PAL			1	0.486**	0.190 ns	-0.102 ns
Root PAL				1	0.076 ns	-0.204 ns
Leaf peroxidase					1	-0.149 ns
Root peroxidase						1

ns non-significant

* $P \leq 0.05$

** $P \leq 0.01$

Discussion

The findings of this study provided insight into the potential advantage and toxicity of supplementation of rooting medium with nSi and seed priming with the non-thermal plasma to affect the plant at various physiological and molecular aspects in vitro culture. Moreover, the plant behaviors to nSi were different from the bulk type. It

appears that their differences in physicochemical traits, uptake, and assimilation mechanisms are responsible for occurrence the partially different responses following applications of the bulk or nano sources of Si. For example, there is evidence exhibiting a differential expression of Si transporter (PST) in response to the bulk or nano types of Si in fenugreek (Nazarian et al. 2017). The promoting effects of the plasma priming on biomass accumulation were augmented with the application

Fig. 5 Differential expression of PAL (**a**) and USP (**b**) following the seed priming with the cold plasma and the manipulation of the rooting MS medium with nSi. Data are presented as mean $\pm$ SE of three independent replications. Different letters indicate significant differences at $P \leq 0.05$ according to Duncan's multiple range test

of nSi5. There is limited evidence on the functions of the plasma-generating devices to treat plant cell, tissue, seed, or organs in *in vitro* sterile conditions (Safari et al. 2017; Iranbakhsh et al. 2018b; Moghanloo et al. 2019). It should be noted that we recently reported the plasma and nSi-mediated changes in seedling early performance, physiology, and anatomy of *A. fridae* (Moghanloo et al. 2019). Root and basal stem anatomical traits were influenced by both individual and combined treatments of plasma and nSi. The magnitudes of xylem diameters were found to be improved in response to plasma priming. We introduced the plasma-associated reinforcement in the differentiation of vascular tissues, inductions in antioxidant machinery, and stimulations in nitrate reductase enzyme as an underlying mechanism through which plant growth, nutrition, and physiology can be improved (Moghanloo et al. 2019). The main focus of the majority of researches on plant-plasma interaction is seed germination and seedling early growth; therefore, it seems that the plasma-associated later differences in plant growth and physiology are due to the difference in seed germination

(a critical developmental stage). However, recent findings clearly indicated that plasma effects are not limited to changes in germination. Several lines of evidence and our findings underline this hypothesis that plasma treatment (especially when the seeds were soaked prior exposure to plasma; considering the activation of metabolism) not only may affect germination and seedling early establishment but also, anatomy, tissue differentiation (Moghanloo et al. 2019; Seddighinia et al. 2019), hormonal balances (Ji et al. 2016; Mildažienė et al. 2019), proteome (Mildažienė et al. 2019), signaling cascades (Babajani et al. 2019b), redox status (Sheteiwy et al. 2018), activities of enzymes (Iranbakhsh et al. 2018a, b; Babajani et al. 2019b; Moghanloo et al. 2019), and gene expression (Iranbakhsh et al. 2018a) may be influenced. Each of these mechanisms can mediate upcoming signaling and responses and subsequently make differences in plant growth, anatomy, physiology, metabolism, transcription profile, and protection against stress condition. This hypothesis is more supported by some evidence exhibiting the plasma potency to improve plant protection against stress conditions (Iranbakhsh et al. 2018a, 2018b; Sheteiwy et al. 2018; Babajani et al. 2019b). Our results are in agreement with findings of Safari et al. (2017) who reported the growth-enhancing effect of seed priming with the plasma in pepper seedlings cultured in *in vitro*. The slight toxicity of the individual nSi40 and nSi80 treatments on the photosynthetic pigments were relieved by the plasma treatment. In line with our findings, the plasma treatment of *Capsicum annuum* not only reinforced growth but also, alleviated the growth-inhibiting impact of zinc oxide nanoparticles (Iranbakhsh et al. 2018b). Also, the salinity-induced toxicity in wheat was mitigated by the seed priming with the plasma (Iranbakhsh et al. 2018a). Likewise, seed priming with the plasma reinforced rice resistance against salinity via modulating cellular turgidity, ROS levels, and membrane integrity (Sheteiwy et al. 2018). Moreover, seed priming with plasma rectified plant early growth and relieved toxicity signs of selenium nanoparticles in *Melissa officinalis* (Babajani et al. 2019b). In addition, the nSi exposure exerted the ameliorating roles toward *Cucurbita pepo* counteracted with salinity stress via modification in transpiration, photosynthesis performance, and water use (Siddiqui et al. 2014). Convincing data on phyto-toxicity of nSi is limited. There are several studies exhibiting the phyto-toxicity roles of nSi at high concentrations in different plant species (Nair et al. 2011; Rui et al. 2014; Sun et al. 2014; Karimi and Mohsenzadeh 2016; Moghanloo et al. 2019). While the nSi exposure at suitable doses varied rang dependent on plant species improved plant growth in *Zea mays* (Yuvakkumar et al. 2011).

Fig. 6 The anatomical differences between the various treatment groups in leaves and their vascular system. **a–a'** Control, **b–b'** plasma of 30 s, **c–c'** plasma of 30 s and nSiO₂ 5 mg l⁻¹, **d–d'** plasma of 30 s and nSiO₂ 40 mg l⁻¹, **e–e'** plasma of 30 s and nSiO₂ 80 mg l⁻¹, **f–f'** plasma of 60 s, **g–g'** plasma of 60 s and nSiO₂ 5 mg l⁻¹, **h–h'** plasma

of 60 s and nSiO₂ 40 mg l⁻¹, **i–i'** plasma of 60 s and nSiO₂ 80 mg l⁻¹, **j–j'** plasma of 90 s, **k–k'** plasma of 90 s and nSiO₂ 5 mg l⁻¹, **l–l'** plasma of 90 s and nSiO₂ 40 mg l⁻¹, **m–m'** plasma of 90 s and nSiO₂ 80 mg l⁻¹, **n–n'** nSiO₂ 5 mg l⁻¹, **o–o'** nSiO₂ 40 mg l⁻¹, **p–p'** nSiO₂ 80 mg l⁻¹

Fig. 7 The alterations in leaf midrib thickness (**a**) and leaf thickness (**b**) induced by seed priming with the cold plasma and manipulation of culture medium with nSi. Data are presented as mean $\pm$ SE of three independent replications. Different letters indicate significant differences at $P \leq 0.05$ according to Duncan's multiple range test

PAL and peroxidase activities

The seed priming with the plasma and/or nSi supplementation modified the activities of PAL as a key enzyme contributed to the synthesis of varieties of phenolic secondary metabolites and peroxidase as a pivotal enzyme in antioxidant defense machinery in both roots and leaves. It is worth noting that a regular linear relationship between the different plasma treatments, the concentrations of nSi in the culture medium, and enzyme activities were not found. As alteration rates in activities of the peroxidase enzyme following the plasma and/or nSi treatments are much higher than the PAL, it might be speculated that changes in the cellular oxidant levels and their following signaling may responsible for the upcoming modifications in antioxidant machinery, phenylpropanoid metabolism, or other possible biochemical pathways. The plasma-associated inductions in catalase activities have been recently reported in *A. friidae* (Moghanloo et al. 2019). The nSi application in culture medium induced catalase activities in leaves, whereas it declined catalase activities in roots of *A. friidae* (Moghanloo et al. 2019). Based on the current evidence, it appears

that there are direct correlations between the nanoparticles provoked oxidative burst, changes in several phytohormones (especially salicylic acid, ethylene, and jasmonic acid), and secondary metabolism (Marslin et al. 2017). Furthermore, enhanced accumulations of various active oxygen species, augmentations in cytoplasmic Ca^{2+} , Ca^{+2} -associated signaling proteins, and up-regulation of mitogen-activated protein kinase (MAPK) cascades may occur as the initial plant cellular reactions to nanoparticles (Marslin et al. 2017). Moreover, there is supporting evidence manifested that the plasma priming may alter the transcription of heat shock factors, especially HSFA4A, and HSFA5, which have close cross-links with the MAPK signaling cascade (Iranbakhsh et al. 2018a). Up-regulations in MAPKs induces specific downstream transcription factors, thereby modulating the plant secondary metabolism at transcriptional levels (Schlutenhofer and Yuan 2015; Phukan et al. 2016). Alterations in the expression of heat shock factors and their possible correlations with MAPK cascade has been addressed in wheat plants exposed to selenium nanoparticle (Safari et al. 2017). Several studies in different plant species exhibited the modulating and reinforcing roles of the plasma pretreatments on the activities of various enzymes, such as hydrolytic enzymes (Neu et al. 2017; Chen et al. 2016; Sadhu et al. 2017), antioxidant enzymes, and PAL (Iranbakhsh et al. 2017, 2018a, b; Babajani et al. 2019b). Besides, it has been hypothesized that the bioactive agents derived from the plasma generation may epigenetically trigger specific signaling cascades, such as MAPK, thereby affecting plant cells at different physiological and molecular aspects (Iranbakhsh et al. 2018a; Babajani et al. 2019b). Mildažienė et al. (2019) also provided evidence on the plasma-associated changes in phytohormones and proteome in sunflower. Sivanesan and Park (2014) has stated that Si application in vitro condition may reinforce plant defense system, nutritional status, secondary metabolism, and water use efficiency as well as change phytohormone levels. Furthermore, the ameliorating roles of nSi in *Cucurbita pepo* counteracted with salinity stress had been attributed to underlying mechanisms, including controlling the lipid peroxidation, decreasing the H_2O_2 accumulation, and modulating antioxidant system (Siddiqui et al. 2014).

Gene expression

The simultaneous treatments of plasma and nSi were found to be the most effective method to induce expression of PAL in leaves. Data on possible changes in responses to the simultaneous treatments of plasma and nanoparticles is rare and needs to be further explored. There are several reports highlighted the plasma-associated changes in xylem magnitude (Moghanloo et al. 2019; Seddighinia et al. 2019), phytohormones (Ji et al. 2016; Mildažienė

et al. 2019), proteome (Mildažienė et al. 2019), and gene transcription (Iranbakhsh et al. 2018a). Hence, it appears that plasma treatment may affect the nanoparticle uptake, translocation, and signaling, thereby modifying the nanoparticle-mediated responses in treated plants. The correlation between the expression of PAL and the activity of this enzyme was not statistically found to be significant. Our results showed that there is not necessarily a direct relationship between the rate of expression of the genes and the activity of the enzymes. This can be due to the difference in induction time, posttranscriptional, or posttranslational changes. Hence, we speculate that the expression of this gene influenced much more sooner than the time of our evaluation, especially in the case of the plasma priming. Moreover, another possible reason may be the implications of different post-translational and post-translational factors. The individual application of the plasma treatment did not make significant changes in the USP expression, contrasted with the other treatments. It is possible that the plasma treatment as short temporal stress influenced the USP expression immediately after the exposure, triggered following signaling, and then declined to the control levels after several days. However, the presence of significant correlations between the PAL gene and peroxidase activities in both roots and leaves may exhibit the occurrence of convergent signaling routes. PAL is the pivotal enzyme contributed to phenylpropanoid metabolism which modulates the flux of primary intermediates into secondary metabolites and produces precursors to varieties of phenolics, such as flavonoids and lignin (Yu et al. 2018). PAL activity as part of a defense system is highly modulated in response to plant developmental stage and environmental factors. Different stresses may provoke oxidative burst due to the excess generation of active oxygen species having dual roles (signaling molecules and harmful byproducts), thereby modulating cellular downstream signaling systems, such as transcription factors, phosphatases, protein kinases, chaperones, and stress-responsive proteins in a manner dependent on the doses of these bioactive signaling agents (Jung et al. 2015).

The USP domain known as stress-responsive genes are widely distributed across most living organisms. They are contributed to plant reactions to various abiotic stress conditions, such as oxidative, salinity, and drought (Jung et al. 2015). USP contributes to rice adaptation to abiotic stress through the activation of a downstream signaling cascade (Sauter et al. 2002). Similarly, the implication of USP genes in plant acclimation to environmental factors and stress condition have been confirmed in several plant species such as tomato (Loukehaich et al. 2012), *Astragalus sinicus* (Chou et al. 2007), and *Gossypium arboreum* (Maqbool et al. 2009).

Leaf anatomy

Leaf anatomical traits and differentiation patterns of conducting tissues were altered following the seed priming with the plasma and manipulation of culture medium with nSi. We recently exhibited the plasma- and nSi-mediated modifications in root and stem of *A. fridae* (Moghanloo et al. 2019). Modification in the differentiation process of vascular conducting tissues in response to plasma exposure has been considered as an important mechanism (Moghanloo et al. 2019). Moreover, the plasma-associated improvement in xylem magnitude has been recently reported in stem and root of *Momordica charantia* (Seddighinia et al. 2019). The plasma treatment mitigated the inhibiting effects of individual treatment of nSi on the xylem differentiation. It seems that nSi at high doses prevents plant growth and tissue differentiation through affecting cellular division, growth, and differentiation. It is obvious that conducting tissues play critical roles in nutrient uptake and translocation. In line with our results, the seed priming with plasma reinforced the xylem differentiation, thereby ameliorating the toxicity of nano zinc oxide in pepper in in vitro condition (Iranbakhsh et al. 2018b). In addition, Si utilization in culture medium for micropropagation altered leaf anatomy in banana (Asmar et al. 2013). Manipulations of culture medium by various Si sources rectified leaf anatomy in banana (Magno Queiroz Luz et al. 2012). However, structural and ultra-structural evidence of nSi-plant interaction is rare. Consistently, nSi altered xylem tissue in fenugreek plant (Nazaralian et al. 2017).

In conclusion, the findings provide anatomical, physiological and molecular evidence on potential benefits and phytotoxicity of nSi and plasma as well as introducing several possible contributed mechanisms which may be employed as a theoretical foundation for possible exploitation in the related science and industries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24(1):1
- Asgari F, Majd A, Jonoubi P, Najafi F (2018) Effects of silicon nanoparticles on molecular, chemical, structural and ultrastructural

- characteristics of oat (*Avena sativa* L.). *Plant Physiol Biochem* 127:152–160
- Asgari-Targhi G, Iranbakhsh A, Ardebili ZO (2018) Potential benefits and phytotoxicity of bulk and nano-chitosan on the growth, morphogenesis, physiology, and micropropagation of *Capsicum annuum*. *Plant Physiol Biochem* 127:393–402
- Asmar SA, Castro EM, Pasqual M, Pereira FJ, Soares JDR (2013) Changes in leaf anatomy and photosynthesis of micropropagated banana plantlets under different silicon sources. *Sci Hortic* 161:328–332
- Babajani A, Iranbakhsh A, Oraghi Ardebili Z, Eslami B (2019a) Differential growth, nutrition, physiology, and gene expression in *Melissa officinalis* mediated by zinc oxide and elemental selenium nanoparticles. *Environ Sci Pollut Res*. <https://doi.org/10.1007/s11356-019-05676-z>
- Babajani A, Iranbakhsh A, Oraghi Ardebili Z, Eslami B (2019b) seed priming with non-thermal plasma modified plant reactions to selenium or zinc oxide nanoparticles: cold plasma as a novel emerging tool for plant science. *Plasma Chem Plasma Process* 39:21–34. <https://doi.org/10.1007/s11090-018-9934-y>
- Beaudoin-Eagan LD, Thorpe TA (1985) Tyrosine and phenylalanine ammonia lyase activities during shoot initiation in tobacco callus cultures. *Plant Physiol* 78(3):438–441
- Chen HH, Chang HC, Chen YK, Hung CL, Lin SY, Chen YS (2016) An improved process for high nutrition of germinated brown rice production: low-pressure plasma. *Food Chem* 191:120–127
- Chou MX, Wei XY, Chen DS, Zhou JC (2007) A novel nodule-enhanced gene encoding a putative universal stress protein from *Astragalus sinicus*. *J Plant Physiol* 164(6):764–772
- Du M, Wu XJ, Ding J, Hu ZB, White KN, Branford-White CJ (2003) Astragaloside IV and polysaccharide production by hairy roots of *Astragalus membranaceus* in bioreactors. *Biotech Lett* 25(21):1853–1856
- Feng LM, Lin XH, Huang FX, Cao J, Qiao X, Guo DA, Ye M (2014) Smith degradation, an efficient method for the preparation of cycloastragenol from astragaloside IV. *Fitoterapia* 95:42–50
- Ghasemian-Yadegari J, Nazemiyeh H, Hamedeyazdan S, Fathiazad F (2017) Secondary metabolites from the roots of *Astragalus maximus*. *Res J Pharmacogn (RJP)* 4(2):31–38
- Iranbakhsh A, Ghoranneviss M, Ardebili ZO, Ardebili NO, Tackallou SH, Nikmaram H (2017) Non-thermal plasma modified growth and physiology in *Triticum aestivum* via generated signaling molecules and UV radiation. *Biol Plant* 61(4):702–708
- Iranbakhsh A, Ardebili NO, Ardebili ZO, Shafaati M, Ghoranneviss M (2018a) Non-thermal plasma induced expression of heat shock factor A4A and improved wheat (*Triticum aestivum* L.) growth and resistance against salt stress. *Plasma Chem Plasma Process* 38(1):29–44
- Iranbakhsh A, Ardebili ZO, Ardebili NO, Ghoranneviss M, Safari N (2018b) Cold plasma relieved toxicity signs of nano zinc oxide in *Capsicum annuum* cayenne via modifying growth, differentiation, and physiology. *Acta Physiol Plant* 40(8):154
- Jalili A, Jamzad Z (1999) Red data book of Iran. Research Institute of Forests & Rangelands, Publication. No. 1999–215
- Ji SH, Choi KH, Pengkit A, Im JS, Kim JS, Kim YH et al (2016) Effects of high voltage nanosecond pulsed plasma and micro DBD plasma on seed germination, growth development and physiological activities in spinach. *Arch Biochem Biophys* 605:117–128
- Jiang J, Lu Y, Li J, Ling L, He X, Shao H, Dong Y (2014) Effect of seed treatment by cold plasma on the resistance of tomato to *Ralstonia solanacearum* (bacterial wilt). *PLoS One* 9:e97753
- Jung YJ, Melencion SMB, Lee ES, Park JH, Alinapon CV, Oh HT et al (2015) Universal stress protein exhibits a redox-dependent chaperone function in *Arabidopsis* and enhances plant tolerance to heat shock and oxidative stress. *Front Plant Sci* 6:1141
- Karimi J, Mohsenzadeh S (2016) Effects of silicon oxide nanoparticles on growth and physiology of wheat seedlings. *Russian J Plant Physiol* 63(1):119–123
- Li X, Qu L, Dong Y, Han L, Liu E, Fang S et al (2014) A review of recent research progress on the *Astragalus* genus. *Molecules* 19(11):18850–18880
- Ling L, Jiang J, Jiangang L, Minchong S, Xin H, Hanliang S, Yuanhua D (2014) Effects of cold plasma treatment on seed germination and seedling growth of soybean. *Sci Rep* 4:5859
- Loukehaich R, Wang T, Ouyang B, Ziaf K, Li H, Zhang J, Ye Z (2012) SpUSP, an annexin-interacting universal stress protein, enhances drought tolerance in tomato. *J Exp Bot* 63(15):5593–5606
- Ma JF, Yamaji N (2006) Silicon uptake and accumulation in higher plants. *Trends Plant Sci* 11(8):392–397
- Maassoumi AA (1998) *Astragalus* in the old world: a check-list. Research Institute of Forests and Rangelands Publication, Tehran
- Magno Queiroz Luz J, Abreu Asmar S, Pasqual M, Gomes de Araujo A, Pio LA, Ferreira Resende R (2012) Modifications in leaf anatomy of banana plants cultivar ‘Maca’ subjected to different silicon sources in vitro. In: VII International symposium on in vitro culture and horticultural breeding, vol 961, ISHS Acta Horticulturae, 18 Sep 2011, pp 239–243
- Maqbool A, Zahur M, Husnain T, Riazuddin S (2009) GUSP1 and GUSP2, two drought-responsive genes in *Gossypium arboreum* have homology to universal stress proteins. *Plant Mol Biol Report* 27(1):109–114
- Marslin G, Sheeba CJ, Franklin G (2017) Nanoparticles alter secondary metabolism in plants via ROS burst. *Front Plant Sci* 8:832
- Mildaženė V, Aleknavičiūtė V, Žukienė R, Paužaitė G, Naučienė Z, Filatova I et al (2019) Treatment of common sunflower (*Helianthus annuus* L.) seeds with radio-frequency electromagnetic field and cold plasma induces changes in seed phytohormone balance, seedling development and leaf protein expression. *Sci Rep* 9(1):6437
- Moghanloo M, Iranbakhsh A, Ebadi M, Satari TN, Ardebili ZO (2019) Seed priming with cold plasma and supplementation of culture medium with silicon nanoparticle modified growth, physiology, and anatomy in *Astragalus fridae* as an endangered species. *Acta Physiol Plant* 41(4):54
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15(3):473–497
- Nair R, Poulouse AC, Nagaoka Y, Yoshida Y, Maekawa T, Kumar DS (2011) Uptake of FITC labeled silica nanoparticles and quantum dots by rice seedlings: effects on seed germination and their potential as biolabels for plants. *J Fluoresc* 21(6):2057
- Nazaralian S, Majd A, Irian S, Najafi F, Ghahremaninejad F, Landberg T, Greger M (2017) Comparison of silicon nanoparticles and silicate treatments in fenugreek. *Plant Physiol Biochem* 115:25–33
- Neu S, Schaller J, Dudel EG (2017) Silicon availability modifies nutrient use efficiency and content, C:N:P stoichiometry, and productivity of winter wheat (*Triticum aestivum* L.). *Sci Rep* 7:40829
- Phukan UJ, Jeena GS, Shukla RK (2016) WRKY transcription factors: molecular regulation and stress responses in plants. *Front Plant Sci* 7:760
- Podlech D (1982) Neue aspektezur evolution und gliederung der Gattung *Astragalus* L. *Mitt Bot Staatssamml München* 18:359–378
- Rui Y, Gui X, Li X, Liu S, Han Y (2014) Uptake, transport, distribution and bio-effects of SiO₂ nanoparticles in Bt-transgenic cotton. *J Nanobiotechnol* 12(1):50
- Sadhu S, Thirumdas R, Deshmukh RR, Annapure US (2017) Influence of cold plasma on the enzymatic activity in germinating mung beans (*Vigna radiata*). *LWT-Food Sci Technol* 78:97–104
- Safari N, Iranbakhsh A, Ardebili ZO (2017) Non-thermal plasma modified growth and differentiation process of *Capsicum*

- annuum* PP805 Godiva in in vitro conditions. *Plasma Sci Technol* 19(5):055501
- Sauter M, Rzewuski G, Marwedel T, Lorbiecke R (2002) The novel ethylene-regulated gene *OsUsp1* from rice encodes a member of a plant protein family related to prokaryotic universal stress proteins. *J Exp Bot* 53(379):2325–2331
- Schaller J, Brackhage C, Gessner MO, Bäuerle E, Gert Dudel E (2012) Silicon supply modifies C:N:P stoichiometry and growth of *Phragmites australis*. *Plant Biolo* 14(2):392–396
- Schluttenhofer C, Yuan L (2015) Regulation of specialized metabolism by WRKY transcription factors. *Plant Physiol* 167(2):295–306
- Seddighinia FS, Iranbakhsh A, Ardebili ZO, Satari TN, Soleimanpour S (2019) Seed priming with cold plasma and multi-walled carbon nanotubes modified growth, tissue differentiation, anatomy, and yield in bitter melon (*Momordica charantia*). *J Plant Growth Regul*. <https://doi.org/10.1007/s00344-019-09965-2>
- Sheteiwy MS, An J, Yin M, Jia X, Guan Y, He F, Hu J (2018) Cold plasma treatment and exogenous salicylic acid priming enhances salinity tolerance of *Oryza sativa* seedlings. *Protoplasma* 1:21
- Siddiqui MH, Al-Whaibi MH, Faisal M, Al Sahli AA (2014) Nano-silicon dioxide mitigates the adverse effects of salt stress on *Cucurbita pepo* L. *Environ Toxicol Chem* 33(11):2429–2437
- Sivanesan I, Park SW (2014) The role of silicon in plant tissue culture. *Front Plant Sci* 5:571
- Sun D, Hussain HI, Yi Z, Siegle R, Cresswell T, Kong L, Cahill DM (2014) Uptake and cellular distribution, in four plant species, of fluorescently labeled mesoporous silica nanoparticles. *Plant Cell Rep* 33(8):1389–1402
- Tripathi DK, Singh VP, Prasad SM, Chauhan DK, Dubey NK (2015) Silicon nanoparticles (SiNp) alleviate chromium (VI) phytotoxicity in *Pisum sativum* (L.) seedlings. *Plant Physiol Biochem* 96:189–198
- Yu XZ, Fan WJ, Lin YJ, Zhang FF, Gupta DK (2018) Differential expression of the PAL gene family in rice seedlings exposed to chromium by microarray analysis. *Ecotoxicology* 27(3):325–335
- Yuvakkumar R, Elango V, Rajendran V, Kannan NS, Prabu P (2011) Influence of nanosilica powder on the growth of maize crop (*Zea mays* L.). *Intern J Green Nanotechnol* 3(3):180–190
- Zhu Z, Wei G, Li J, Qian Q, Yu J (2004) Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (*Cucumis sativus* L.). *Plant Sci* 167(3):527–533