# SILICON INDUCES POLYUBIQUITIN AND PEROXIDASE AND INCREASES PROTEIN CONTENT AND PHENOLS IN PROSO MILLET

Adriana Basilio-Apolinar, Luis Eugenio González-de la Vara, Jorge Covarrubias-Prieto, J. Gabriel Ramírez-Pimentel, Cesar L. Aguirre-Mancilla, Gabriel Iturriaga and Juan Carlos Raya-Pérez

*SUMMARY*

*Results of several researches about the application of Si (as $Na_2SiO_3$) in agriculture indicate that it improves the agronomic crops performance, especially under stress conditions. The objective of this research was to evaluate Si effect on protein expression, phenolic content, antioxidant capacity of seed from plants subjected to stress due to drought and salinity in the vegetative stage, in proso millet (*Panicum miliaceum *L) cultivation. Silicon fertilization in millet plants had an effect on root development of plants subjected to irrigation and treated with Si; roots were collected 30 days after sowing (DAS) for proteomic analysis by mass spectrometry. At 60 DAS seeds were harvested and storage protein and phenolic compounds content were quantified; antioxidant activity of seed extracts were determined by the DPPH and ABTS methods. Proteomic analysis showed that Si promoted expression of a polyubiquitin 11-like protein and two peroxidases. Fertilization with Si in millet crops developed in the different environments (drought, salinity and irrigation) promoted higher content of the albumin, globulin and prolamin fractions. Si also promoted higher phenolic content in seeds grown under irrigation and higher antioxidant activity in seeds grown under drought conditions. Si fertilization in proso millet improved the nutritional potential of seeds under drought, salinity and irrigation environments evaluated in this research.*

## Introduction

Proso millet (*Panicum miliaceum*) was one of the first domesticated cereals by man 10,000 years ago in China (Habiyaremye *et al*., 2017). Millet has a C4-type photosynthetic metabolism; the efficiency in carbon fixation and in the use of water are attributes that allow it to be established in semi-arid regions, where there are low precipitation and high temperatures (Li *et al*., 2011). Due to its short cycle, millet can be included in rotation of summer crops such as maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* Moench); It competes favorably with weeds and keeps soil moisture (Das *et al*., 2019). Millet grain offers multiple benefits to the human diet; some genotypes contain up to 17% protein and its nutritional value is compared to other cereals such as wheat (Wiedemair *et al*., 2020). It is a rich source of minerals, polyphenols, vitamins and fiber; millet is gluten-free and has a low glycemic index; it is ideal for people intolerant to gluten or with diabetes (Das *et al*., 2019). The ability of millets to establish in arid regions and its nutritional value is an alternative for food security. In 2022 there was a world production of 30 million t; the main producers were India and China (FOASTAT, 2023).

In the last decade, different researches have been developed to demonstrate Si ($Si(OH)_4$) benefits on crops development. Si provides benefits to plant defense mechanisms against biotic and abiotic stress, through absorption, transport and accumulation in tissues (Basilio-Apolinar *et al*., 2021; Mundada *et al*., 2021). In maize, Si promoted an increase in epidermal cells thickness, as well as stomatal density and stomatal conductance; under water deficit conditions, Si allowed them to maintain the photosynthetic rate (Marques *et al*., 2020). Si role in plant stress tolerance is not based only on a physical barrier that forms during Si accumulation; this element, somehow, affects antioxidant enzymes activity such as catalase and peroxidase

**KEYWORDS / ABTS / Drought / *P. Miliaceum* / Proteomics / Salinity /**

Received: 09/13/2023. Modified: 01/11/2024. Accepted: 01/13/2024.

**Adriana Basilio-Apolinar**. MSc . in Seed Production and Technologies, Tecnológico Nacional de México/IT Roque (TecNM/ITRoque), Mexico. PH.D. TecNM/ITRoque, Mexico. Graduate of Doctoral Program, TecNM/ITRoque, Mexico.

**Luis Eugenio González-de la Vara**. PhD. and MSc. in Biochemistry, Centro de Investigación y de Estudios Avanzados (CINVESTAV), Mexico. Professor-Researcher, Centro de Investigación y de Estudios Avanzados (CINVESTAV), Instituto Politécnico Nacional (IPN), Mexico.

**Jorge Covarrubias-Prieto**. MSc. in Genetics, Colegio de Postgraduados (COLPOS), Mexico. PhD. in Plant Breeding and Cytogenetics, Iowa State University, USA. Professor-Researcher, TecNM/ITRoque, Mexico. e-mail: jocovarrubias@itroque.edu.mx.

**J. Gabriel Ramírez-Pimentel**. Biochemical Engineer, TecNM/ITCelaya, Mexico. Doctor in Plant Biotechnology, CINVESTAV, Mexico. Professor-Researcher, TecNM/ITRoque, México. e-mail: garamirez@itroque.edu.mx.

**Cesar L. Aguirre-Mancilla**. Biochemical Engineer, TecNM/ITColima, México. MSc. and Doctor in Plant Biotechnology, CINVESTAV, México. Professor-Researcher, TecNM/ITRoque, México. e-mail: ceaguirre@itroque.edu.mx.

**Gabriel Iturriaga**. MSc. in Molecular Biology, CINVESTAV, Mexico. PhD. in Biology, Cambridge University, UK. Professor-Researcher, TecNM/ITRoque, Mexico.

**Juan Carlos Raya-Pérez** (Corresponding author). Biologist, Universidad Nacional Autónoma de México. MSc in Botany, COLPOS, México. Doctor in Plant Biotechnology, CINVESTAV, México. Professor-Researcher, TecNM/ITRoque, México. Address: División de Estudios de Posgrado e Investigación, TecNM/ITRoque. Km 8 Carretera Celaya-Juventino Rosas, C.P.38110, Celaya, Gto., México. e-mail: juan.rp2@roque.tecnm.mx.

## EL SILICIO INDUCE POLIUBIQUITINA Y PEROXIDASA Y ELEVA EL CONTENIDO DE PROTEÍNA Y FENOLES EN MIJO PROSO

Adriana Basilio-Apolinar, Luis Eugenio González-de la Vara, Jorge Covarrubias-Prieto, J. Gabriel Ramírez-Pimentel, Cesar L. Aguirre-Mancilla, Gabriel Iturriaga y Juan Carlos Raya-Pérez

*RESUMEN*

*Los resultados de varias investigaciones sobre la aplicación de Si (como $Na_2SiO_3$) en agricultura, indican mejoras en el rendimiento agronómico de los cultivos especialmente en condiciones de estrés. El objetivo de esta investigación fue evaluar el efecto del Silicio sobre la expresión proteica, el contenido fenólico y la capacidad antioxidante de semillas de plantas sometidas a estrés por sequía y salinidad en la etapa vegetativa, en el cultivo de mijo proso (*Panicum miliaceum *L). La fertilización con Si en plantas de mijo tuvo un efecto sobre el desarrollo radicular de plantas sometidas a riego y tratadas con Si; las raíces se recolectaron 30 días después de la siembra (DDS) para su análisis proteómico mediante espectrometría de masas. A los 60 DDS se cosecharon las semillas y se cuantificó el contenido en proteínas de almacenamiento y compuestos fenólicos; la actividad antioxidante de los extractos de semillas se determinó mediate métodos DPPH y ABTS. El análisis proteómico mostró que el Silicio favorece la producción de una proteína similar a la poliubiquitina-11 y de dos peroxidasas. La fertilización con Si en cultivos de mijo desarrollados en los diferentes ambientes (sequía, salinidad e irrigación) indujo un mayor contenido de las fracciones albúmina, globulina y prolamina. El Si también generó un mayor contenido fenólico en las semillas cultivadas bajo riego y mayor actividad antioxidante en las semillas cultivadas bajo condiciones de sequía. La fertilización con Silicio en mijo proso mejoró el potencial nutricional de las semillas en los ambientes de sequía, salinidad y riego evaluados en esta investigación.*

## O SILÍCIO INDUZ A POLIUBIQUITINA E A PEROXIDASE E AUMENTA O TEOR DE PROTEÍNA E FENOL NO PAINÇO PROSO

Adriana Basilio-Apolinar, Luis Eugenio González-de la Vara, Jorge Covarrubias-Prieto, J. Gabriel Ramírez-Pimentel, Cesar L. Aguirre-Mancilla, Gabriel Iturriaga e Juan Carlos Raya-Pérez

*RESUMO*

*Os resultados de várias pesquisas sobre a aplicação de Si (como $Na_2SiO_3$) na agricultura indicam que ele melhora o desempenho agronômico das plantações, especialmente em condições de estresse. O objetivo desta pesquisa foi avaliar o efeito do Si na expressão de proteínas, no conteúdo fenólico e na capacidade antioxidante das sementes de plantas submetidas a estresse devido à seca e à salinidade no estágio vegetativo, no cultivo de painço (*Panicum miliaceum *L). A fertilização com silício em plantas de painço teve um efeito sobre o desenvolvimento da raiz das plantas submetidas à irrigação e tratadas com Si; as raízes foram coletadas 30 dias após a semeadura (DAS) para análise proteômica por espectrometria de massa. Aos 60 DAS, as sementes foram colhidas e o conteúdo de proteína de armazenamento e compostos fenólicos foram quantificados; a atividade antioxidante dos extratos de sementes foi determinada pelos métodos DPPH e ABTS. A análise proteômica mostrou que o Si promoveu a produção de uma proteína semelhante à polubiquitina 11 e duas peroxidases. A fertilização com Si em culturas de milheto desenvolvidas em diferentes ambientes (seca, salinidade e irrigação) promoveu maior conteúdo das frações de albumina, globulina e prolamina. O Si também promoveu maior conteúdo fenólico em sementes cultivadas sob irrigação e maior atividade antioxidante em sementes cultivadas sob condições de seca. A fertilização com Si no painço Proso melhorou o potencial nutricional das sementes em ambientes de seca, salinidade e irrigação avaliados nesta pesquisa.*

---

(CAT, POD) for redox state maintenance (Jadhao and Rout, 2020). Si activates transcription of genes coding for enzymes involved lignin and suberin metabolism, such as acetyltransferase, ABC-type transporter, and POD. Lignin and suberin synthesis increases cell wall mechanical resistance (Fleck *et al*., 2011). Si inclusion in crops fertilization significantly improves nutritional seed value (Biju *et al*., 2020). The effect of Si on nutritional quality of the grain has been poorly evaluated. The effect of Si fertilization on storage protein and phenolic compounds contents and antioxidant capacity of millet seeds grown under drought and salinity environments was also investigated (Basilio-Apolinar *et al*., 2022). In order to contribute in crops metabolism understanding of Si role, in this research, a proteomic analysis on root tissue of millet plants treated with Si was carried out. Most of the research on the role of Si in plants is limited to evaluate the different crop models at seedling stage; here, the effect of Si on the nutritional quality of the grain was evaluated.

### Materials and Methods

The experiment was carried out at Tecnológico Nacional de México campus Roque, Celaya, Guanajuato, México and Departamento de Biotecnología. Proteomic analysis was carried out at Bioquímica del Centro de Investigacion y Estudios Avanzados (CINVESTAV), Irapuato, Guanajuato, México. Millet (*Panicum miliaceum*) seeds were obtained from local market. The experiment was established in the spring-summer 2019 cycle. Two Si doses was evaluated in three environments (drought, salinity and irrigation (control). A randomized complete blocks design and five replications were considered; a 2*3 factorial

experiment was conducted; factor A were Si ($Na_2SiO_3$, Sigma Aldrich) doses with two levels (0 and 1.8mM) and factor B were environments with three levels (drought, salinity and irrigation). The concentration Si 1.8mM was chosen based on previous experiments, where concentration 0 to 4mM were tested. Experiment was established under greenhouse conditions; seeds were sowed in greenhouse polyethylene bags (40 x40 x22cm) filled with Pelic Vertisol type soil (IUSS Working Group WRB, 2015) and they were fertilized with 17-17-17 (NPK) using the recommended dose by commercial manufacturer (10g per bag), each bag contained five plants. Temperature was 30°C/23°C (day/night) inside a greenhose. Eight das plants were treated with 0 and 1.8mM Si by applying a pH 5.8 solution via roots. To drought simulation, irrigation was suspended 15 to 30 das; for salinity treatment, plants were treated with a saline solution (350mM NaCl) at field capacity at 15 and 22DAS; control plants were irrigated with tap water every 8 days. At 30 das, roots of Si-treated and untreated plants were collected for proteomic analysis. The concentration 350mM NaCl was chosen based on a previous experiment where concentrations from 0 to 400mM NaCl were tested. At 60 DAS, seeds from treated plants were harvested and storage protein content and electrophoretic profile of protein fractions were determined; total phenolic content in the seed and antioxidant activity of the extracts on the ABTS•+ radicals and DPPH•, were determined.

*Protein analysis on microsomal fraction.*

Fresh roots from plants treated with Si at 1.8mM and untreated plants were collected, rinsed with distilled water to remove dirt and any foreign matter; samples were then stored at -80°C until they were processed. Samples were macerated with an extraction buffer containing: 250mM sucrose, 70mM Tris-HCl pH 8, 4mM dithiothreitol (DTT) (Sigma Aldrich), 0.1% bovine serum albumin (BSA), 0.5 % polyvinylpyrrolidone (PVP) and 3mM ethylenediaminetetraacetic acid (EDTA); 300mg of tissue per ml of buffer were used (González and Medina, 1998). Subsequently, samples were centrifuged at 9000rpm during 10 minutes (IEC Micromax RF Refrigerated Microcentrifuge); recovered supernatant was centrifuged again under same conditions. Recovered supernatant was centrifuged at 60,000 rpm during 20 minutes (Beckman Optima TLX Ultracentrifuge) to recover microsomal fraction, this was suspended in a buffer containing 25mM Tris-MES, 2mM EDTA, 1mM DTT pH 7, and 45% glycerol. Protein content was quantified according Bradford (1976) method, standard curve was constructed using BSA as a standard using microplates reader xMarkTM BIO-RAD.

*SDS-PAGE and mass spectrometry*

Separation of proteins by electrophoresis on SDS-PAGE and gel staining were done as indicated by Schägger and von Jagow (1987). To identify proteins by mass spectrometry, the bands of interest were excised from gels stained with Coomassie blue (differentially expressed bands). These gel fragments were washed with water and acetonitrile as indicated by Lino *et al*. (2006). Purified proteins were digested with trypsin, and the resulting peptides were extracted according to Lino *et al*. (2006). Peptides were separated in a nanoAcquity nanoflow chromatograph (Waters Corp. Milford, MA; USA) coupled to the ESI ionizer of LTQ velos linear ion trap mass spectrometer (ThermoFisher Scientific, Bremen, Germany). Fragmentation spectra were analyzed to identify proteins by Trans Proteomic Pipeline (TPP) software as indicated by Lino *et al*. (2016).

*Seed storage proteins*

Storage proteins of millet seeds were extracted according to Agboola *et al*. (2005), based on fractions solubility. Albumin fraction was extracted with distilled water using a 1:4 ratio (0.25g of fluor: 100ml of water); mixture was shaken during one hour, and centrifuged at 14,000 x g for 15 minutes; globulin fraction was extracted with 0.5M NaCl, 50mM Tris pH 8, under the same conditions as previous fraction; prolamin fraction was extracted with 70% ethanol under the same conditions as previous fraction and finally, glutelin was extracted with 0.5% SDS and 0.1M sodium borate pH 9 under the same conditions. Protein content was quantified according to Bradford method (1976). Protein fractions were separated based on their molecular weight on 8% SDS-PAGE as indicated.

*Phenols extraction and quantification*

Total phenolic compounds content of millet seeds were extracted according to Kalam *et al*. (2019). Flour (50mg) were mixed with 1ml of 80% ethanol, then sonicated for 40 minutes and centrifuged at 4000 x g for 5 minutes. Phenols content was quantified by the Folin-Ciocalteu method (Georgé *et al*., 2005). Reaction consisted of 25µl of Folin-Ciocalteu reagent, 25µl of $NaCO_3$ ($75g{\cdot}l^{-1}$). Reaction mixture was incubated at 40°C for 30 minutes. Then 200µl of water was added and the absorbance was read at 750nm. A standard curve was made using gallic acid as a standard. Total phenol content was expressed as equivalents in milligrams of gallic acid per gram of flour (EAG mg/g).

*Antioxidant activity*

Proso millet seed antioxidant activity of extracts on the ABTS•+ radical was evaluated according to Van Den Berg *et al*. (1999). First, cationic radical was generated by oxidation of ABTS (7mM) with potassium persulfate (140mM); mixture was incubated at dark for 12h and the absorbance value was adjusted to 0.700 ±0.002 with absolute ethanol. For the assay, 20µL of extract were mixed with 230µl of ABTS•+ radical, homogenized and absorbance was read at 734nm. Antioxidant activity of millet seed extracts on the DPPH• radical was evaluated according to Brand-Williams *et al*. (1995). For the reaction, 5µl of extract was taken and homogenized with 295µl of DPPH reagent (60mM in methanol). Mixture was incubated under dark during 30 minutes; subsequently, absorbance was read at 517nm xMarkTM BIO-RAD. A standard curve was constructed using Trolox as a standard. Inhibition of ABTS•+ and DPPH• was expressed as equivalents in milligrams of Trolox per g of flour (ETmg/g).

*Statistical analysis*

Data analysis was performed using ANOVA and means comparison with Tukey test ($\alpha \leq 0.05$) using SAS software version 9.4 (SAS Institute, Cary, North Caroline, USA). Interaction (environment*Si) were analyzed by RStudio program, R version 4.2.2 using "metan" package for interaction analysis.

**Results and Discussions**

*Differentially expressed proteins*

Proteomic analysis of Si-fertilized compared non-Si-fertilized millet root tissue shown the expression of a 70 kDa polyubichitin-11-like protein and expression of two 35 kDa peroxidases in irrigated plants (Table I). Si treatment in irrigated proso millet plants promoted expression of these proteins. Farouk *et al*. (2020) indicated that Si treatment in basil (*Ocimun basilicum* L.) plants promoted expression of peroxidase-encoding genes. In soybean (*Glycine max* L.), Si promoted expression of a gene

TABLE I
DIFFERENTIALLY EXPRESSED PROTEINS IN MILLET ROOT FERTILIZED WITH SI VS. WITHOUT SI. CELAYA, GTO. S-S, 2019

| Protein | Calculated MW (kDa) | Best matching proteins | Coverage (%) | Total independent spectra |
|---|---|---|---|---|
| A0A3L6PF99_PANMI | 229 aa (25.675) | Polyubiquitin 11-like | 44.6 | 55 |
| A0A3L6Q246_PANMI | 328 aa (34.139) | Peroxidase CE: 1.11.1.7 | 62.8 | 25 |
| A0A3L6Q046_PANMI | 328 aa (34.199) | Peroxidase CE: 1.11.1.7 | 62.8 | 282 |

coding for peroxidase, as well as other genes related to lignin biosynthesis: cinnamoyl alcohol dehydrogenase (CAD), 4-coumarin ligase (4CL), and phenylalanine ammonia lyase (PAL) (Hussain *et al*., 2021). Authors argue that Si-promoted peroxidase gene expression is probably dedicated to lignin biosynthesis to improve plant mechanical strength (Hussain *et al*., 2021). Peroxidase is an enzyme that fulfills its antioxidant function through $H_2O_2$ dismutation, and also participates in lignin biosynthesis; it is active in cytosol, vacuole and in cell wall (Das and Roychoudhury, 2014). Si alleviated the effects of salinity in cotton through antioxidant activity of catalase (CAT) and peroxidase (POD) by enhancing POD and CAT gene expression (Li *et al*., 2022). In tomato plants (*S. lycopersicum*), genes expression related to antioxidant enzyme system, SlPOD, significantly increased its expression due to Si treatment (Khan *et al*., 2020).

Peroxidase expression in proso millet root, could be related to lignin biosynthesis. It is known that Si improves plant mechanical resistance; deposition of Si in cells of tissues improves strength and rigidity; therefore, it gives greater resistance to plants (Yamaji *et al*., 2008). Si binds to epidermal cells and the cell wall, contributing to cross-linking of cell wall components (Javaid *et al*., 2019). Cell wall organosilicones can alter cell wall biosynthesis, structures, and functions; possible cell wall ligands are pectin, lignin, and hemicellulose (Sheng and Chen, 2020). Si treatment promoted expression of POD, phenylalanine ammonia lyase (PAL), 4-coumarate-CoA ligase (4CL) and ABC-type transporter, as well as POD activity in cell wall of apical and basal zones root rice (*Oriza sativa* L.); POD activity would probably balance $H_2O_2$ levels or limit excessive Fe uptake through lignification and/or suberization of cell walls through polymerization of monolignols, since thickening of the endodermis and cortex were also observed (Mehrabanjoubani *et al*., 2019). Fleck *et al*. (2011) also indicated that rice Si treatment increased suberization and lignification in the roots; this was accompanied by genes related to lignin and suberin metabolism. High transcription of a LRR-RLK gene could play a central role in the Si perception of signals for the promotion of suberin and lignin synthesis (Fleck *et al*., 2011). The monolignols are transported to apoplast probably through the ATP-binding cassette (ABC) transporter, where the PODs catalyze lignin formation by their polymerization; POD also catalyzes suberin formation (Mehrabanjoubani *et al*., 2019).

Ubiquitination is a post-translational modification, which determines protein turnover, regulation, and molecular function (Zang *et al*., 2012). The Ubiquitin ligand is a protein with seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) to form chains binding the target protein (Johnson and Vert, 2019). Ubiquitin-conjugated proteins fate depends on whether it is monoubiquitination or polyubiquitination and on topology of polyubiquitination chain (Stone, 2018). K63 polyubiquitination is a proteasome-directed activity (Li and Ye, 2008) and it fulfills non-proteolytic functions in at least four pathways: DNA damage repair, cell signaling, intracellular trafficking, and ribosomal biogenesis (Johnson and Vert, 2019).

Manivannan *et al*. (2016) indicated that Si promoted proteins expression related to ubiquitin-proteasome pathway in *Capsicum annuum* plants subjected to salinity: E3 ubiquitin ligase, culin 1D protein and F-box type 8 protein, possibly for stress-damaged proteins removal. Millet plants Si-treated promoted expression of a ubiquitin-like protein K11. K11 binding to proteins can either provide protein stability or lead to proteosome degradation (Grice *et al*., 2015). The best characterized role of K11 polyubiquitin binding is in the cell cycle; K11 ubiquitin binding increases in a UBES2-dependent manner on salinity during mitosis, for temporal organization in substrate degradation of ubiquitin (Min *et al*., 2015). K11 ubiquitin binding is also proposed to participate on membrane trafficking activity and DNA repair pathways (Boughton *et al*., 2019). Expression of polyubiquitin-11-like protein in millet root was induced by silicon, it could have activity in non-proteolytic pathways. Although this fact cannot be rule out. Since millet plants grew under irrigated conditions; therefore, there would be fewer damaged proteins targeting the proteasome.

Si fertilization in plants affects the ubiquitin system through regulation of protein expression as reported by some authors; Si-mediated E2 8 and E2 36 ubiquitin-conjugating enzyme expression in *Rosa hybrida* plants under salinity probably enhanced process of regulation of proteins in post-translational modification process, protein specificity or selectivity (Soundararajan *et al*., 2017). Expression of the ubiquitin ligase protein in tomato (*Solanum lycopersicum* L.) inoculated with *Rastolnia solanacearum* may have contributed to signaling of defense responses, that is, in negative regulator of JA (jasmonic acid) degradation, since a positive regulation was observed in JA signaling pathways (Ghareeb *et al*., 2011).

*Storage proteins*

Significant differences were observed in both two factors (Si and environment) for albumin, globulin and glutelin content variables (ANOVA, not showed); in the prolamin content variable, factors interaction was significant. This indicates that Si effect on protein fractions content depends on environment. Means comparison ($p \leq 0.05$) (Table II) indicated that silicon fertilization in millet plants increases albumin and globulin content in seeds, while glutelin content decreased. Millet plants exposed to drought and salinity caused a significant increase in albumin content; globulins and glutelins content decreased significantly in seeds produced under salinity. Environment affect the proso

TABLE II
MEANS COMPARISON BY TUKEY'S TEST FOR ALBUMIN, GLOBULIN, PROLAMIN AND GLUTELIN CONTENT VARIABLES IN MILLET GRAIN DEVELOPED IN THREE ENVIRONMENTS AND FERTILIZED WITH SI. ROQUE, CELAYA, GTO. S-S 2019

| Factors | Treatment | Albumin (mg·100 mg$^{-1}$) | Globulin (mg·100 mg$^{-1}$) | Prolamin (mg·100 mg$^{-1}$) | Glutelin (mg·100 mg$^{-1}$) |
|---|---|---|---|---|---|
| Silicon | 1.8mM | 0.655 a | 0.477 a | 0.230 a | 0.422 b |
| | 0mM | 0.307 b | 0.350 b | 0.230 a | 0.632 a |
| Environment | Drought | 0.559 a | 0.441 a | 0.285 a | 0.583 a |
| | NaCl 350mM | 0.485 b | 0.372 b | 0.305 a | 0.397 b |
| | Control | 0.399 c | 0.425 a | 0.299 a | 0.602 a |

Means with the same letter within the same variable are statistically equal (Tukey, p≤0.05).

millet protein content and quality (Kalinova and Moudry, 2006). Drought stress promoted higher protein content. Leonova *et al*. (2019) indicated that a mild drought seems to induce remobilization of amino acids and polysaccharides for grain filling. Xu *et al*. (2020) indicated that low protein content in *Setaria italica* seeds developed in a dry environment was due to interruption of nitrogen metabolism since there was a glutamine synthase low expression.

Factors interaction (environment*Si) was highly significant (ANOVA not showed) for prolamin and glutelin content variables, and it was significant for albumin and globulin content variables. This indicates that Si response depends on the environmental conditions (Basilio-Apolinar *et al*., 2021).

Si fertilization in millet contributed to a higher content of albumin and globulin fractions in seeds harvested in three environments (drought, salinity and irrigation) (Figure 1a and 1b). Prolamin fraction increased its levels with Si fertilization only in seeds under drought and salinity treatments (Figure 1c, 1d). In irrigated treatment, Si fertilization decreased prolamin and glutelin content fractions (Figure 1a and 1b, Figure 2a, 2b). Millet Si fertilization had a stronger effect on seed protein content subjected to abiotic stress. Biju *et al*. (2021) also observed Si effect on protein content in plants subjected to drought: high protein content mediated by Si is attributed to activation of enzymes associated with protein synthesis / amino acids storage in seeds.

Figure 2a shows the electrophoretic profile of albumin fraction of millet seed, where bands of different molecular weight can be seen, the major bands observed at 25kDa, 40kDa and 55kDa. Figure 2b shows the electrophoretic profile of globulin fraction, where two main patterns were observed at 40kDa and another two at 55kDa. Figure 3a shows the electrophoretic profile of prolamin fraction, where four bands were observed; two between 10kDa and 15kDa, another at 25kDa and the other at 40kDa, which present a molecular mass close to maize prolamins that yielded a band slightly below 4 kDa, two bands below 20, and one around 14kDa (Aguirre-Mancilla *et al*., 2020). Figure 3b shows the electrophoretic profile of glutelin fraction, where a main band was observed between 15kDa and 25kDa, among the environments and Si fertilization, no significant changes were observed in the four fractions.

Storage proteins are the main grain proteins; albumins are a source of sulfur and nitrogen during seed germination (Akharume *et al*., 2019). Albumin content can improve the germinatve quatility of millet proso seeds. Globulins are rich in arginine (Kalinova, 2007). Proso millet prolamines occur in two groups, first (24kDa) is rich in glutamic acid, leucine and alanine; second group (14-17kDa) is rich in methionine and cysteine; in general, cereals are deficient in sulfur amino acids (Kalinova, 2007). Prolamines content in proso millet seeds can be used in the human diet. Glutelins

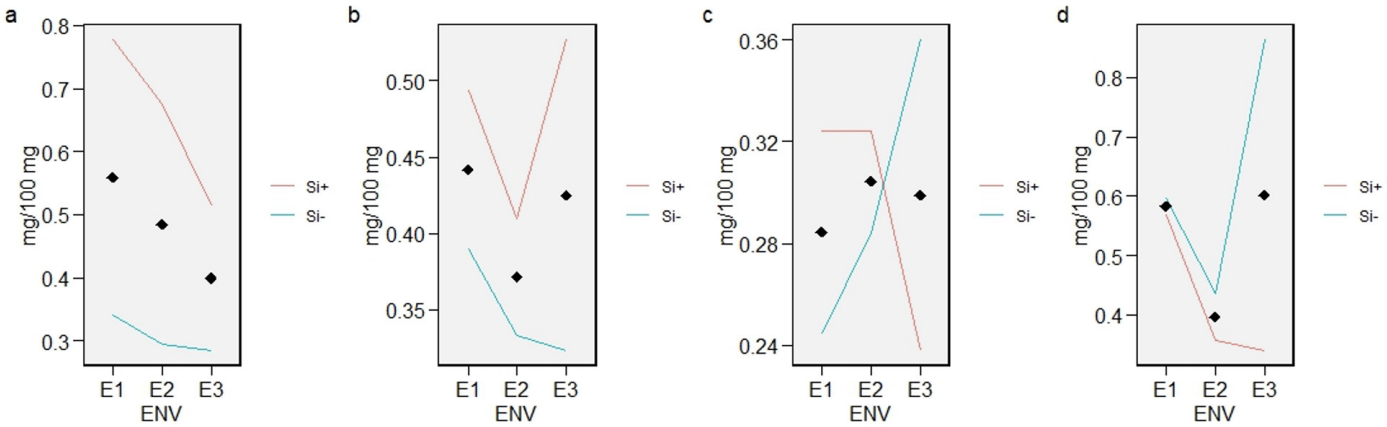


Figure 1. Interaction line graph (Environment*Si) for: albumin (a), globulin (b), prolamin (c) and glutelin content (d) variables in millet seeds. ENV: environment; E1: drought, E2: salinity, E3: irrigation. Diamonds: means.

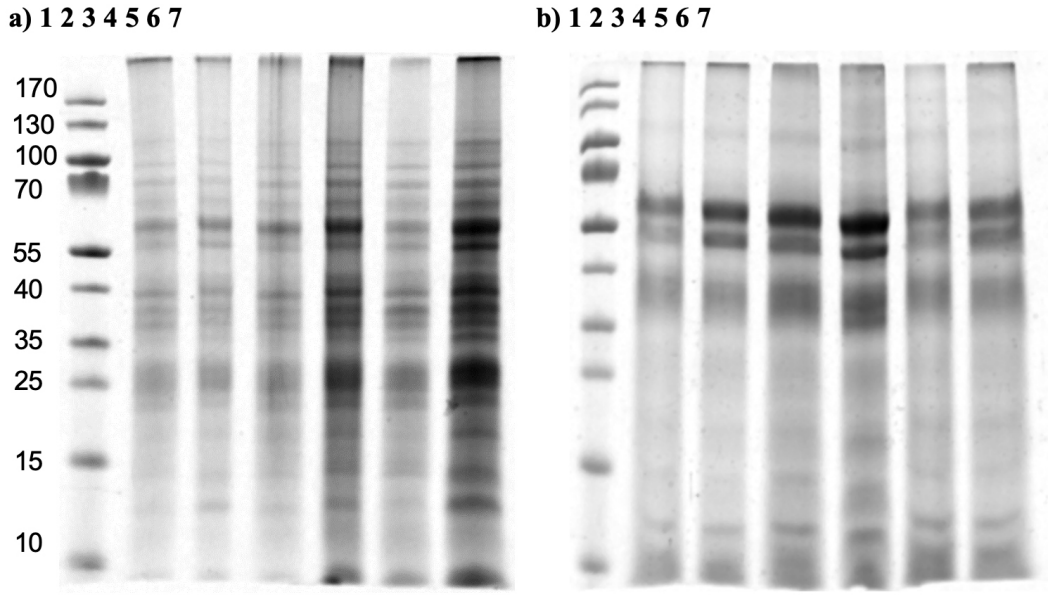


Figure 2. Electrophoretic patterns of albumin fraction (a) and the globulin fraction (b). Lane 1: molecular weight marker (kDa); lane 2: drought + Si; lane 3: drought; lane 4: salinity + Si; line 5: salinity; line 6: millet+ Si; line 7: control (left to right).

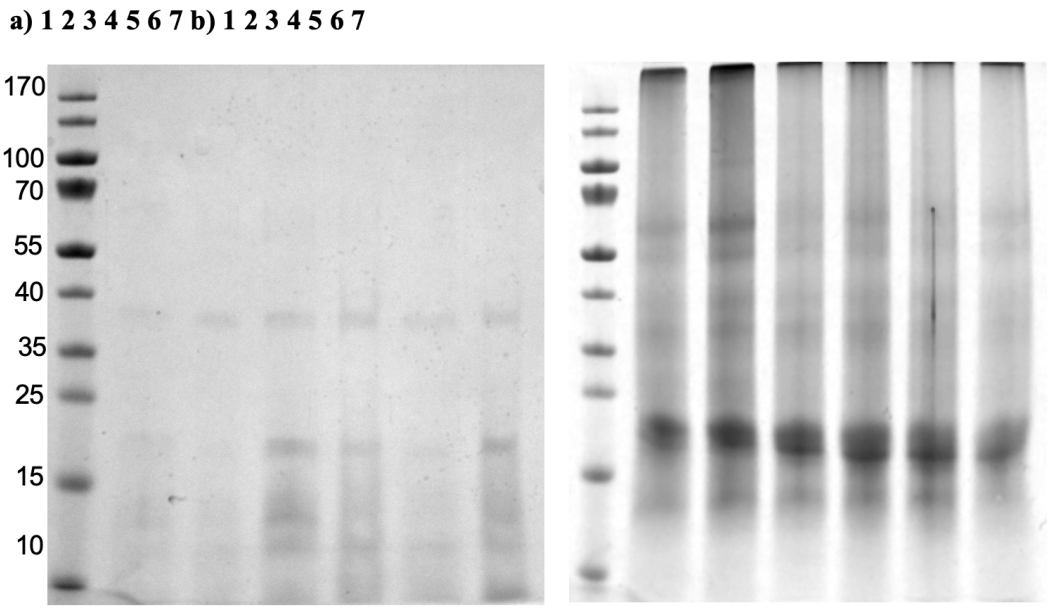


Figure 3. Electrophoretic patterns of prolamin fraction (a) and the glutelin fraction (b). Line 1: molecular weight marker (kDa); line 2: drought + Si; line 3: drought; lane 4: salinity + Si; line 5: salinity; line 6: millet+ Si; line 7: control (left to right).

are rich in hydrophobic amino acids such as phenylalanine, valine, proline and tyrosine (Akharume *et al*., 2019). Prolamines low percentage are indicators of seed protein quality: the first two fractions are rich in essential amino acids such as lysine and tryptophan in maize (Aguirre-Mancilla *et al*., 2020). Storage proteins are a source of bioactive peptides with potential for human health (Akharume *et al*., 2019).

*Total phenolic content and antioxidant activity of seed extracts*

Highly significant differences were observed among environments and in the interaction of factors for total phenols content variable (Table III). This indicates that the phenolic content is affected by the environment. Means comparison (Table IV) indicated that drought and salinity stress significantly decreased seeds phenolic content. Phenolic content differences are attributed to factors such as plant genetics, soil composition and environment (Hosseini-Boldaji *et al*., 2020). In this research, seeds developed under drought, salinity and irrigation environments showed differences in phenolic content. Polyphenols profile can change with plants plasticity subjetec to abiotic stresses (Francini *et al*., 2021). Factors interaction, showed that Si fertilization in irrigated plants contributed to a higher phenolic content in millet seeds (Figure 4a, Figure 4b). Si could stimulate accumulation of some nutritional components in millet seeds, as indicated by Biju *et al*. (2021); lentil Si fertilization significantly increased seeds phenolic content.

Antioxidant activity by the DPHH method was highly significant in factors interaction and antioxidant activity by the ABTS method, it was highly significant in the Si factor, environment factor, and in factors interaction. Seeds antioxidant activity is both, influenced by environment, and Si fertilization. Means comparison (Table IV) showed that Si fertilization significantly increased antioxidant seeds activity on ABTS radical; stress increased ($p \leq 0.05$) seeds antioxidant capacity (Table IV). Factors interaction indicated that Si fertilization increased millet seeds antioxidant activity on the DPPH• and ABTS•+ radicals under drought and salinity treatments (Figure 4b and 4c). Si positive effect in millet was observed in higher seeds phenolic content developed under irrigation and a greater seeds antioxidant capacity developed under drought and salinity. Millets are an excellent source of natural antioxidants; they have more than 50 phenolic compounds:

TABLE III
MEAN SQUARES, DEGREES OF FREEDOM AND STATISTICAL SIGNIFICANCE FOR THE VARIABLES TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF MILLET GRAIN EXTRACTS DEVELOPED IN THREE ENVIRONMENTS AND FERTILIZED WITH SI. CELAYA, GTO. S-S. 2019

| Source | Degrees of Freedom | Total Phenolic Content | DPPH | ABTS |
|---|---|---|---|---|
| Block | 2 | 7.76 | 205.37 | 224.84 |
| Silicon (S) | 1 | 24.27ns | 324.36ns | 2012.74** |
| Environment (E) | 2 | 156.63** | 258.02ns | 909.76** |
| S*E | 2 | 184.65** | 2018.7** | 2921.14** |
| Error | 12 | 6.29 | 180.65 | 86.52 |
| Total | 17 | | | |
| CV (%) | | 3.1 | 10.3 | 6.92 |

CV: coefficient of variation. ns: not significant. *, ** indicates statistical significance at the 0.05 and 0.01 probability level, respectively.

TABLE IV
TUKEY'S TEST MEANS COMPARISON FOR TOTAL PHENOL CONTENT AND ANTIOXIDANT ACTIVITY VARIABLES OF MILLET GRAIN EXTRACTS DEVELOPED UNDER THREE ENVIRONMENTS AND FERTILIZED WITH SI. ROQUE, CELAYA, GTO. S-S. 2019

| Factors | Treatments | Total phenolic compounds (mg EAG·100 $g^{-1}$) | DPPH (mg ET·100 $g^{-1}$) | ABTS (mg ET·100 $g^{-1}$) |
|---|---|---|---|---|
| Silicon | 1.8mM | 79.68 a | 134.01 a | 144.94 a |
| | 0mM | 82.00 a | 125.60 a | 123.82 b |
| Enviroment | Drought | 75.92 c | 124.38 a | 142.37 a |
| | NaCl 350mM | 80.48 b | 128.02 a | 149.60 a |
| | Control | 86.12 a | 137.12 a | 120.21 b |

EAG: Gallic Acid Equivalents. ET: Trolox Equivalents. Means with the same letter within same variable are statistically equal (Tukey, $p \leq 0.05$).

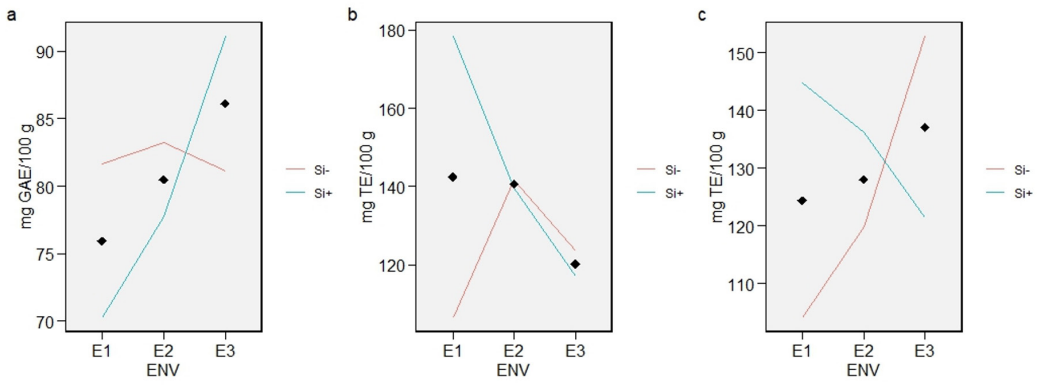


Figure 4. Line graph factors interaction (environment*Si) for the: phenolic content (a), antioxidant activity on the DPPH radical (b) and ABTS (c) variables. ENV: environment; E1: drought, E2: salinity, E3: irrigation. Diamonds: means.

flavonoids, phenolic acids and derivatives (Kalam *et al*., 2019). Phenolic compounds exert a wide range of biological and protective effects, antioxidant and antimicrobial activity (Cosme *et al*., 2020). Millet bran fraction have inhibitory activity on α-amylase and α-glucosidase activity; such effect is attributed to ferulic acid, p-coumaric acid and quercetin, possibly due to complexes formation with enzymes and/or starch (Pradeed and Sreerama, 2017).

Nutritional characteristics of millet grains: antioxidant capacity, high resistant starch content, high protein content, and adequate amino acid constitution, can be used as healthy food ingredients and/or functional foods (Shen *et al*., 2018).

## Conclusion

Si fertilization in millet plants caused significant changes. Si promoted expression of a polyubiquitin-11-like protein and two peroxidases. Si fertilization promoted albumin, globulin, and prolamin fractions higher content; Si also increases phenolic content, and antioxidant activity of millet seed extracts. Si effects were observed in both, irrigated and stress environments.


### ACKNOWLEDGMENTS

We thank to CONAHCYT, fellowship to Adriana Basilio-Apolinar and to TecNM (Project No.13488.21-P).

## REFERENCES


Agboola S, Ng D, Mills D (2005) Characterization and functional properties of Australian rice protein isolates. *Journal of Cereal Science 41*: 283-290.

Aguirre-Mancilla CL, Godinez-Galán RY, Raya-Pérez JC, Gutiérrez-Benicio GM, Ramírez-Pimentel JG, Covarrubias-Prieto J, García-Rodríguez G (2020) Protein content and quality of seeds in Central Mexican maize (*Zea mays*) accessions. *UNCuyo 52*: 14-25. http://revistas.uncu.edu.ar/ojs3/index.php/RFCA/article/view/3065

Akharume F, Santra D, Adedeji A (2019) Physicochemical and functional properties of proso millet storage protein fractions. *Food hydrocolloids 108*: 105497.

Basilio-Apolinar A, Gonzalez de la Vara LE, Ramirez-Pimentel JG, Aguirre-Mancilla CL, Iturriaga G, Covarrubias-Prieto J, Raya-Perez JC (2021) Silicon induces changes in the antioxidant system of millet cultivated in drought and salinity. *Chilean JAR 81*: 655-663. https://oes.chileanjar.cl/files/cjar210176-81-4-2021.pdf

Biju S, Fuentes S, González Viejo C, Torrico DD, Inayat S, Gupta D (2020) Silicon supplementation improves the nutritional and sensory characteristics of lentil seeds obtained from drought-stressed plants. *Journal of the Science of Food and Agriculture 101*: 1454-1466.

Bradford MM (1976) A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry 72*: 248-254.

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology 28*: 25-30.

Cosme P, Rodríguez AB, Espino J, Garrido M (2020) Plant phenolics: Bioavailability as a key determinant of their potential health-promoting applications. *Antioxidants 9*: 1263.

Das K, Roychoudhury A (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science 2*: 53.

Das S, Khound R, Santra M, Santra D (2019) Beyond Bird Feed: Proso Millet for Human Health and Environment. *Agriculture 9*: 64. https://doi.org/10.3390/agriculture9030064

FAO (2023) FAOSTAT Producción agrícola [online]. https://www.fao.org/faostat/es/#data/QCL

Farouk S, Elhindi KM, Alotaibi MA (2020) Silicon supplementation mitigates salinity stress on *Ocimum basilicum* L. via improving water balance, ion homeostasis, and antioxidant defense system. *Ecotoxicology and Environmental Safety 206*: 111396.

Fleck AT, Nye T, Repenning C, Stahl F, Zahn M, Schenk MK (2011) Silicon enhances suberization and lignification in roots of rice (*Oryza sativa*). *Journal of Experimental Botany 62*: 2001–2011.

Francini A, Fidalgo-Illesca C, Raffaelli A, Sebastiani L (2021) Phenolics and mineral elements composition in underutilized apple varieties. *Horticulturae 8*: 40.

Georgé S, Brat P, Alter P, Amiot MJ (2005) Rapid determination of polyphenols and vitamin C in plant-derived products. *Journal of Agricultural and Food Chemistry 53*: 1370-1373.

Ghareeb H, Bozsó Z, Ott PG, Repenning C, Stahl F, Wydra K (2011) Transcriptome of silicon-induced resistance against *Rastolnia solanacearum* in the silicon non-accumulator tomato implicates priming effect. *Physiological and Molecular Plant Pathology 75*: 83-89.

González de la Vara LE, Medina G (1988) Effects of Inorganic Phosphate on the Plasma Membrane H-ATPase from Red Beet (*Beta vulgaris* L.). *Plant Physiology 88*: 1073-1076.

Habiyaremye C, Matanguihan JB, D'Alpoim Guedes J, Ganjyal GM, Whiteman MR, Kidwell KK, Murphy KM (2017) Proso Millet (*Panicum miliaceum* L.) and its potential for cultivation in the pacific Northwest, U.S.: A Review. *Frontiers in Plant Science 7*: 1961.

Hosseini-Boldaji SA, Babakhani B, Hassan-Sajedi R, Houshani M (2020) Antioxidant properties of two alfalfa (*Medicago sativa* L.) ecotypes in response to sodium chloride salinity stress. *Journal of Plant Physiology and Breeding 10*: 45-58.

Hussain S, Shuxian L, Mumtaz M, Shafiq I, Iqbal N, Brestic M, Wenyu Y (2021) Foliar application of silicon improves stem strength under low light stress by regulating lignin biosynthesis genes in soybean (*Glycine max* (L.) Merr.). *Journal of Hazardous Materials 401*: 123256.

IUSS Working Group WRB. (2015) Base referencial mundial del recurso suelo 2014. Actualización 2015. *Sistema internacional de clasificación de suelos para la nomenclatura de suelos y la creación de leyendas de mapas de suelos*. Informes sobre Recursos Mundiales de Suelos 106. FAO, Roma, Italia.

Jadhao KR, Rout GR (2020) Silicon (Si) enhances the resistance in finger millet genotypes against blast disease. *Journal of Plant Pathology. 102*: 985-1006, https://doi.org/10.1007/s42161-020-00622-2

Javaid T, Ansar FM, Akhtar J, Ahmad S, Anwar-ul-Haq M (2019) Silicon nutrition improves growht of salt-stressed wheat by modulating flows and partitioning of $Na^+$, $Cl^-$ and mineral ions. *Plant Physiology and Biochemistry 141*: 291-299.

Johnson A, Vert A (2016) Unraveling K63 Polyubiquitination networks by sensor-based proteomics. *Plant Physiology 171*: 1808-1820.

Kalam Azad MO, Jeong DI, Adnan M, Salitxay T, Heo JW, Naznin MT, Lim JD, Cho DH, Park BJ, Park CH (2019) Effect of different processing methods on the accumulation of the phenolic compounds and antioxidant profile of broomcorn millet (*Panicum miliaceum* L.) flour. *Foods 8*: 230.

Kalinova J (2007) Nutritionally important components of proso millet (*Panicum miliaceum* L.). *Food. Global Science Books 1*: 91-100.

Kalinova J, Moudry J (2006) Content and quality in proso millet (*Panicum miliaceum* L.) varieties. *Plant Foods for Human Nutrition 61*: 45-49.

Khan A, Khan AL, Imran M, Asaf M, Kim YH, Bilal S, Numan M, Al-Harris A, Al-Rawahi A, Lee IJ (2020) Silicon-induced thermotolerance in *Solanum lycopersicum* L. via activation of antioxidant system, heat shock proteins, and endogenous phytohormones. *BMC Plant Biol 20*: 248. https://doi.org/10.1186/s12870-020-02456-7

Leonova T, Popova V, Tsarev A, Henning C, Antonova K, Rogovskaya N, Vikhnina M, Baldensperger T, Soboleva A, Dinastia E, Dorn M, Shiroglasova O, Grishina T, Balcke GU, Ihling C, Smolikova G, Medvedev S, Zhukov VA, Babakov V, Tikhonovich IA, Frolov A (2020) Does Protein Glycation Impact on the Drought-Related Changes in Metabolism and Nutritional Properties of Mature Pea (*Pisum sativum* L.) Seeds? *International Journal of Molecular Sciences 21*: 567.

Li L, Qi Q, Zhang H, Dong Q, Iqbal A, Gui H, Kayoumu M, Song M, Zhang X, Wang X (2022) Ameliorative Effects of Silicon against Salt Stress in *Gossypium hirsutum* L. MDPI AG. *Antioxidants 11*: 1520.

Li P, Brutnell TP (2011) *Setaria viridis* and *Setaria italica*, model genetic systems for the Panicoid grasses. *Journal of Experimental Botany 62*: 3031-3037.

Li W, Ye Y (2008) Polyubiquitin chains: functions, structures, and mechanisms. *Cell. Mol. Life Science 65*: 2397-2406.

Lino B, Carrillo-Rayas MT, Chagolla A, González de la Vara LE (2006) Purification and characterization of a calcium-dependent protein kinase from beetroot plasma membranes. *Planta 225*: 255-268.

Lino B, Chagolla A, González de la Vara L (2016) Membrane proteins involved in transport, vesicle traffic and $Ca^{2+}$ signaling increase in beetroots grown in saline soils. *Planta 244*: 87-101.

Manivannan A, Soundararajan P, Munner S, Ho KC, Ryong JB (2016) Silicon mitigates salinity stress by regulating the physiology, antioxidant enzyme activities, and protein expression in *Capsicum annuum* "Buwang". *BioMed Research International 2016*: 3076357.

Mehrabanjoubani P, Abdolzadeh A, Sadeghipour HR, Aghdasi M, Bagherieh-Najjar MB, Barzegargolchini B (2019) Silicon increases cell wall thickening and lignification in rice (*Oryza sativa*) root tip under excess Fe nutrition. *Plant Physiology and Biochemistry: PPB 144*: 264-273.

Pradeep PM, Sreerama YN (2017) Soluble and bound phenolics of two different millet genera and their millet fractions: comparative evaluation of antioxidant properties and inhibitory effects on starch hydrolyzing enzyme activities. *Journal of Functional Foods 35*: 682-693.

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry 166*: 368-379.

Shen R, Ma Y, Jiang L, Dong J, Zhu Y, Ren G (2018) Chemical

composition, antioxidant, and antiproliferative activities of nine Chinese proso millet varieties. *Food and Agricultural Immunology 29*: 625-637.

Sheng H, Chen S (2020) Plant silicon-cell wall complexes: Identification, model of covalent bond formation and biofunction. *Plant Physiology and Biochemistry 155*: 13-19.

Soundararajan P, Manivannann A, Ko CH, Muneer S, Jeong BR (2017) Leaf Physiological and Proteomic Analysis to Elucidate Silicon Induced Adaptive Response under Salt Stress in Rosa hybrida 'Rock Fire'. I*nternational Journal of Molecular Sciences 18*: 1768.

Stone LS (2018) Role of the ubiquitin proteasome system in plant response to abiotic stress. *International Review of Cell and Molecular Biology 343*: 65-10.

Van Den Berg R, Haenen GR, Van Den Berg H, Bast AALT (1999) Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chemistry 66*: 511-517.

Wiedemair V, Scholl-Burgi S, Karall D, Huck CW (2020) Amino acid profiles and compositions of different cultivars of *Panicum miliaceum* L. *Chromatographia 83*: 829-837.

Xu B, Gao X, Dong K, Li X, Yang P, Yang T, Feng B (2020) Grain protein content comparison and proteomic analysis of foxtail Millet (*Setaria italica* L.) seed response to different drought stress levels. *Acta Physiologiae Plantarum 42*: 20.

Yamaji N, Mitatni N, Ma JF (2008) A transporter regulating silicon distribution in rice shoots. *The Plant Cell 20*: 1381-1389.

Zang Y, Wang Q, Xue C, Li M, Wen R, Xiao W (2012) Rice UBC13, a candidate housekeeping gene, is required for K63-linked polyubiquitination and tolerance to DNA damage. *Rice 5*: 24.