

RESEARCH NOTE

Osmotic stress on genetically transformed tobacco plant seeds¹

Michele Trombin-Souza^{2*}, Camila Ribeiro de Souza Grzybowski²,
Yohana de Oliveira-Cauduro², Elisa Serra Negra Vieira³, Maristela Panobianco²

ABSTRACT - Salinity and water deficit limit the productivity of several crops; thus, studies related to the genetic transformation of seeds in a model plant, such as tobacco, can be an alternative to minimize negative impacts caused by environmental conditions. The purpose of this work was to evaluate the tolerance to osmotic stress of seeds from genetically transformed tobacco plants, with the introduction of the proline-synthesizer gene (*p5csf129a*), under salinity and water deficit conditions. To do so, five events with differences in proline content were selected, ranging from 0.70 to 10.47 $\mu\text{moles.g}^{-1}$ of fresh mass. The used saline concentrations were: zero (distilled water); 50; 100; 150 and 200 mmol.L^{-1} of NaCl, whereas for the water deficit, simulated with PEG 6000, the following osmotic potentials were used: zero (distilled water); -0.2; -0.4; -0.6 and -0.8 MPa. Each tested treatment was evaluated through germination, first germination count and germination speed index tests. It is possible to conclude that seeds from genetically transformed tobacco plants with overexpression of the gene *p5csf129a*, a proline synthesizer, are more tolerant to osmotic stresses. Tobacco seeds with a proline content of 10.47 $\mu\text{mol.g}^{-1}$ showed a better performance, revealing higher physiological potential.

Index terms: *Nicotiana tabacum* L., proline, saline stress, water deficit, physiological quality.

Estresse osmótico em sementes de plantas de tabaco transformadas geneticamente

RESUMO – Salinidade e déficit hídrico limitam a produtividade de várias culturas; assim, estudos relacionados à transformação genética de sementes de planta modelo, como a do tabaco, podem ser uma alternativa para minimizar os impactos negativos causados pelas condições ambientais. Objetivou-se avaliar a tolerância a estresses osmóticos de sementes de tabaco de plantas transformadas geneticamente com a introdução do gene sintetizador da prolina (*p5csf129a*), sob condições de salinidade e déficit hídrico. No presente experimento foram selecionados cinco eventos com diferenças no teor de prolina, variando de 0,70 a 10,47 $\mu\text{moles.g}^{-1}$ de massa fresca. As concentrações salinas utilizadas foram: zero (água destilada); 50; 100; 150 e 200 mmol.L^{-1} de NaCl, enquanto para o estresse hídrico, simulado com PEG 6000, trabalhou-se com os seguintes potenciais osmóticos: zero (água destilada); -0,2; -0,4; -0,6 e -0,8 MPa. Cada tratamento testado foi avaliado pelos testes de germinação, de primeira contagem de germinação e pelo índice de velocidade de germinação. Pode-se concluir que sementes de tabaco de plantas transformadas geneticamente com superexpressão do gene *p5csf129a*, sintetizador da prolina, são mais tolerantes a estresses osmóticos. Sementes de tabaco com teor de prolina de 10,47 $\mu\text{mol.g}^{-1}$ apresentam desempenho superior, revelando maior potencial fisiológico.

Termos para indexação: *Nicotiana tabacum* L., prolina, estresse salino, déficit hídrico, qualidade fisiológica.

Introduction

Genetic seed transformation with the introduction of low molecular mass osmolytes, such as the amino acid L-proline,

may be a promising alternative to increase seedling capacity to tolerate osmotic stresses in the establishment of the stand. Proline acts as an osmoprotectant in the osmotic adjustment and contributes to the stabilization of subcellular structures and

¹Submitted on 06/07/2017. Accepted for publication on 10/17/2017.

²Departamento de Fitotecnia e Fitossanitarismo, Universidade Federal do Paraná, 80035-050 - Curitiba, PR, Brasil.

³Embrapa Florestas, Caixa Postal 319, 83411-000 - Colombo, PR, Brasil.

*Corresponding author <micheletrombin@gmail.com>

to the increase of osmolarity, providing the necessary turgidity for the expansion of cells under stress conditions (Taiz and Zeiger, 2009). Moreover, it is also related to preventing the production of free radicals or to the sequestration of reactive oxygen species (ROS), which are involved in a series of degenerative processes (Miller et al., 2009)

Proline accumulation occurs normally in the cytoplasm, where this amino acid acts to stabilize subcellular structures (such as membranes and proteins), to eliminate free radicals, and to buffer the cellular redox potential under stress conditions (Posmyk et al., 2009). It has also been reported that proline accumulation may be part of an adaptive strategy to saline and water stress (Hayat et al., 2012). There is evidence that genotypes which are tolerant to osmotic stress accumulate more proline than sensitive genotypes (Yu and Lu, 2016; Kumar et al., 2010).

Proline synthesis in higher plants occurs via glutamate or via ornithine, and under conditions of osmotic stress, the highest biosynthetic rate is achieved via glutamate. Proline is synthesized from glutamic acid by Δ^1 -pyrroline-5-carboxylate (P5C), by two successive reductions catalyzed by the enzymes Δ^1 -pyrroline-5-carboxylate synthase (P5CS) and Δ^1 -pyrroline-5-carboxylate reductase (P5CR) (Sekhar et al., 2007).

The mechanism by which osmotic stress induces P5CS gene expression and proline accumulation in transgenic plants has been partially postulated. The understanding of gene expression varies according to species, period of exposure to adverse conditions, stress level, environmental conditions (water supply and air relative humidity), transpiration rate and leaf water potential (Munns and Tester, 2008).

Salinity and water deficit are the main osmotic stresses affecting seed germination, seedling establishment in the field and crop productivity. Environments with water restriction and salt excess produce harmful effects on seeds, such as a significant reduction in the germination percentage and rate in the root length and fresh mass and in the shoot of seedlings (Yan, 2015; Conus et al., 2009); they may even cause a complete inhibition of the germination process of seeds (Atia et al., 2009).

Nicotiana tabacum L. is a model species in genetic transformation works, because its plants have a short cycle, great seed production, high transformation efficiency, low operational costs of transformation protocols, well established regeneration and *in vitro* cultivation (Brasileiro and Lacorte, 2000). In addition, tobacco is considered a glycophyte plant; this makes it possible to verify if tolerance to osmotic stress is induced by differences in the expression of genes, forms or gene functions (Deinlein et al., 2014).

Despite the advances in studies about plant responses to environmental stresses, there is a lack of investigations on the

use of genetic transformation in seeds, in order to increase their tolerance to unfavorable conditions. In this sense, the purpose of this work was to evaluate the tolerance to osmotic stress of genetically modified tobacco seeds, with the introduction of the proline synthesizer gene (*p5csf129a*), when submitted to saline and water stress.

Material and Methods

Obtaining seeds from genetically modified tobacco plants: *Nicotiana tabacum* cv. Petit Havana SR-1 seeds were used, provided by the Instituto Agronômico do Paraná (IAPAR). The *Agrobacterium tumefaciens* strain used in the transformation was EHA15, with the inserted gene *p5csf129a*, for the overexpression of the amino acid proline; 35S is the promoter. The determination of the proline content was performed according to the methodology described by Bates et al. (1973). The analysis of genetically transformed plants was performed by PCR, as described by Borgo et al. (2015).

In this research, five events with the following proline levels (Pro) were used: Pro 0.70 (control treatment); Pro 2.25; Pro 4.42; Pro 7.73 and Pro 10.47 $\mu\text{mol.g}^{-1}$ of fresh weight.

Saline and water stress conditions: For saline stress, sodium chloride (NaCl) was used as the osmotic agent, moistening the substrate used for seed germination with saline solutions at the concentrations: zero (distilled water, composing the control treatment); 50; 100; 150 and 200 mmol.L^{-1} . As for water stress, simulated with Polyethylene-Glycol - (PEG 6000), solutions of five osmotic potentials were used: zero (distilled water, composing the control treatment); -0.2; -0.4; -0.6 and -0.8 MPa.

The physiological potential of seeds was evaluated by the tests:

Germination: four replicates with 50 seeds per treatment were used, sown in transparent plastic boxes (11.0 x 11.0 x 3.5 cm) containing two sheets of blotting paper moistened with distilled water (control treatment) or with the tested solutions (NaCl and PEG), in an amount equivalent to 2.5 times the weight of the dry substrate. The plastic boxes were maintained in a Mangelsdorf-type germinator at 20-30 °C, providing light at the highest temperature for eight hours. The final count of the test was performed on the 16th day after sowing (Brasil, 2009), and the results were expressed as mean percentage of normal seedlings for each treatment.

First germination count: conducted together with the germination test; the evaluation was performed seven days after sowing, and the results were expressed as mean percentage of normal seedlings.

Germination speed index (GSI): conducted together with the germination test; daily seedling counts were performed at the same time, from the observation of the first normal

seedling until the 16th day, when the test was closed. The results were calculated according to the formula proposed by Maguire (1962).

The used experimental design was completely randomized, with four replications. Data were submitted to analysis of variance by F test ($p < 0.01$) and to regression analysis, with better trend curve fitting of the NaCl and PEG 6000 concentrations; the models were chosen based on the determination coefficient and its significance. The ASSISTAT software was used (Silva and Azevedo, 2002).

Results and Discussion

Salinity affected the germination percentage of tobacco seeds, obtaining a linear equation adjustment for all studied proline concentrations (Figure 1). By analyzing data about the initial germination percentage, that is, when seeds were sown in a substrate that was dampened only with water, it is possible to observe that seeds presented high germination values: 90% (Pro 10.47), 83% (Pro 7.73), 85% (Pro 4.42) and 89% (Pro 2.25 and Pro 0.70), all being above the minimum standard for selling tobacco seeds, which is 80% (MAPA, 2013). The events Pro 10.47; 7.73 and 4.42 presented tolerance to saline stress up to the 100 mmol.L⁻¹ NaCl concentration, not differing statistically from the control treatment. It is worth mentioning the proline content of 10.47, which was significantly higher than the others, maintaining 90% germination at the mentioned NaCl concentration. At the NaCl concentration of 150 mmol.L⁻¹, seeds still presented 80% germination, while the other two contents fell to 69% and 66%, respectively. On the other hand, seeds from the Pro 2.25 event showed similar behavior to the control treatment (Pro 0.70), revealing tolerance only up to 50 mmol.L⁻¹ of NaCl. This reduction was more drastic in the NaCl concentration of 200 mmol.L⁻¹, where there was a germination of 48% (Pro 2.25) and 10% (Pro 0.70), whereas in the 10.47 Pro event, it was still possible to observe a 76% germination after stress.

With the increase of the saline concentration in the substrate, there is a reduction in the germination percentage of seeds; thus, it is possible to infer that, in addition to the ionic toxicity effect, the NaCl excess caused a reduction in the cellular water potential (Taiz and Zeiger, 2009), reducing the capacity to absorb water during the imbibition stage of both genetically and non-genetically transformed seeds. In glycophytes such as tobacco, increased Na⁺ concentrations in the cytosol cause changes in the Ca²⁺ uptake and metabolism of cell membranes, thus reducing their permeability (Pourrut et al., 2011). Additionally, the decrease in seed germination with the exogenous increase of salt may be related to changes in the signal transduction process of

proteins, from the stress perception site to the synthesis site of proteins encoded by genes that are regulated in response to stress (Türkan and Demiral, 2009).

The evaluation of the first germination count test of tobacco seeds under conditions of saline stress (Figure 2a) revealed that for seed with proline contents of 10.47; 7.73; 4.42 and 2.25, seed vigor was maintained up to the NaCl concentration of 100 mmol.L⁻¹, without presenting statistical differences from the NaCl concentration of 0 mmol.L⁻¹. On the other hand, in the control treatment (Pro 0.70) seed vigor was already reduced significantly at the NaCl concentration of 50 mmol.L⁻¹. Again, it is worth mentioning the outstanding behavior of Pro 10.47, which showed 65% of normal seedlings during the first germination count test at the highest NaCl concentration (200 mmol.L⁻¹), whereas the control treatment (Pro 0.70) presented only 9%.

Similarly to the results found for germination and first germination count, seed vigor was evaluated through the germination speed index (GSI), under saline stress conditions (Figure 2b), and remained stable up to the NaCl concentration of 100 mmol.L⁻¹. All events showed a GSI decrease from 150 mmol.L⁻¹ of NaCl, except for the control treatment (Pro 0.70), which showed a GSI decrease already at the NaCl concentration of 50 mmol.L⁻¹.

As for water stress, it is possible to observe significant reductions in the germination percentage of tobacco seeds soaked with PEG solutions at different concentrations

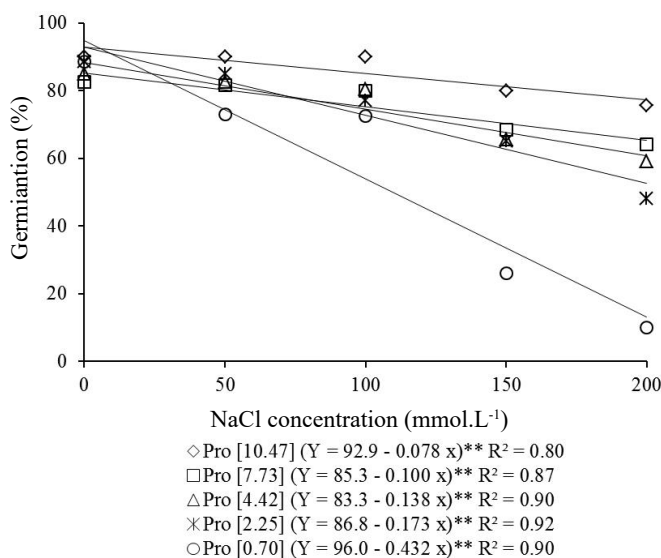


Figure 1. Germination of genetically transformed tobacco seeds under saline stress conditions. Events with proline contents (Pro) of 10.47; 7.73; 4.42; 2.25 and 0.70 $\mu\text{mol.g}^{-1}$ of fresh proline weight. (**) significance level, where $p < 0.01$.

(Figure 3). Pro events 10.47; 7.73; 4.42 and 2.25 allowed water stress tolerance up to -0.2 MPa, without significant reductions in relation to the PEG concentration of 0 MPa, whereas seeds from the control treatment (Pro 0.70) already showed a significant germination reduction at the -0.2 MPa concentration of PEG. Again, seeds from the Pro 10.47 event are worth mentioning; even at the highest osmotic potential (-0.8 MPa), they still provided a germination of 51%, while the control treatment (Pro 0.70) presented only 8% of normal

seedlings in this unfavorable environment.

Under water stress conditions, the decrease in the germination percentage is probably due to the lower water availability for seeds, since polyethylene glycol compounds are inert and non-toxic polymers. Thus, the osmotic agent inhibited the absorption of water by tissues, preventing the activation and maintenance of the germination metabolism. In order for seed with cotyledonary reserves to initiate the re-growth of the embryonic stem, it is necessary to reach water levels between 35.0 and 40.0% (Carvalho and Nakagawa, 2012); however, the substrate limitation of water availability and the decrease in the water movement to seeds caused by the osmotic solution may reduce or prevent the event sequence of the germination process.

It has been suggested that the decrease in seed hydration levels may increase the production of ROS (Gill and Tuteja, 2010), and they may cause damages to the physical structure of the cell membranes (Sharma et al., 2012), thus reducing germination. Genetically transformed plants for proline synthesis are tolerant to water deficits because they inhibit the excessive accumulation of ROS and protect seeds from oxidative damages, as reported in literature (Sharma et al., 2012; Chen and Arora, 2011).

As for the evaluation of the first germination count test of tobacco seeds under water stress conditions (Figure 4a), the events Pro 10.47; 7.73 and 4.42 did not present a significant reduction in the physiological quality up to the osmotic potential of -0.6 MPa. On the other hand, the Pro 2.25 event did not differ

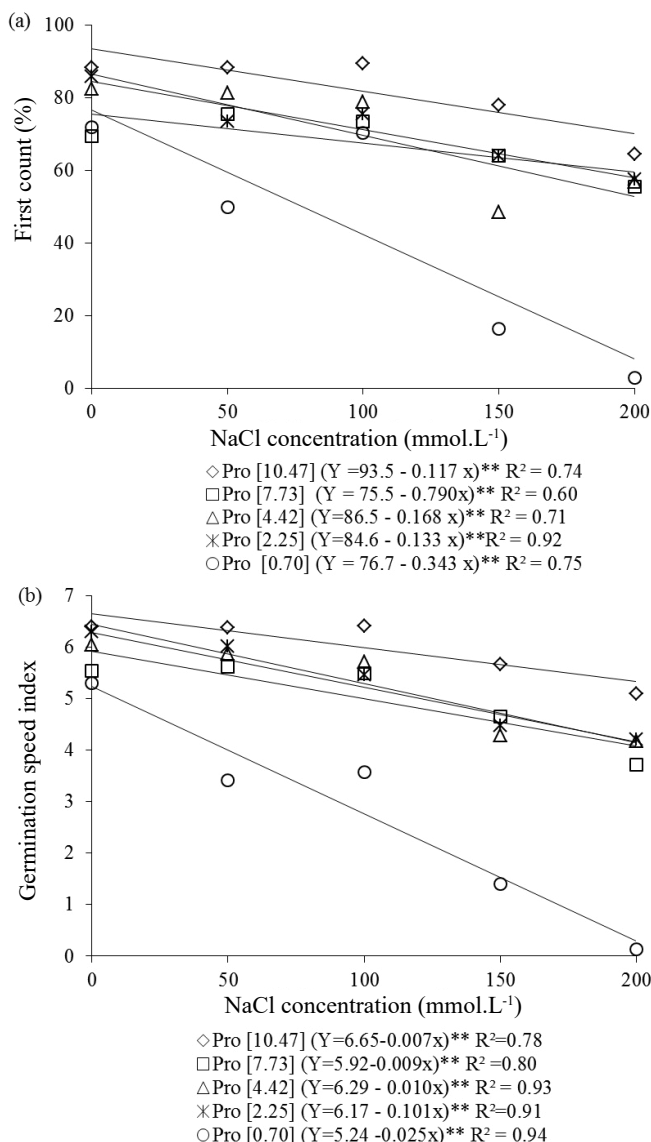


Figure 2. Germination speed index and first count of genetically transformed tobacco seeds under saline stress conditions. Events with proline contents (Pro) of 10.47; 7.73; 4.42; 2.25 and 0.70 $\mu\text{mol.g}^{-1}$ of fresh proline weight: (a) first count. (b) germination speed index. (**) significance level, where $p < 0.01$.

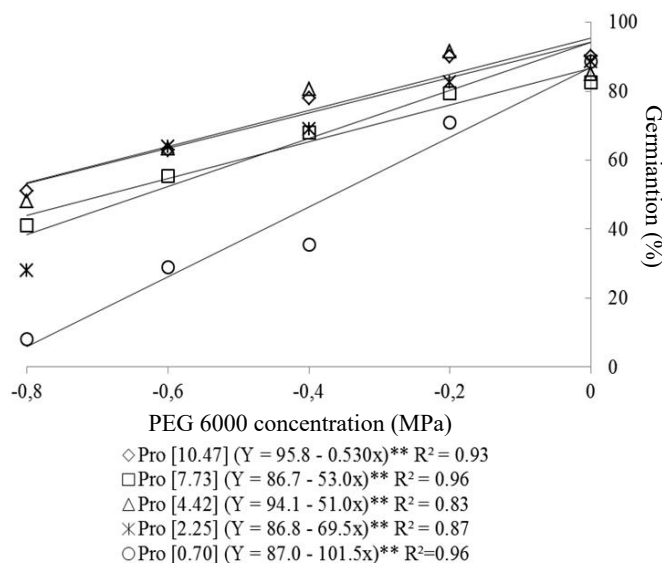


Figure 3. Germination of genetically transformed tobacco seeds under conditions of water stress. Events with proline contents (Pro) of 10.47; 7.73; 4.42; 2.25 and 0.70 $\mu\text{mol.g}^{-1}$ of fresh proline weight. (**) significance level, where $p < 0.01$.

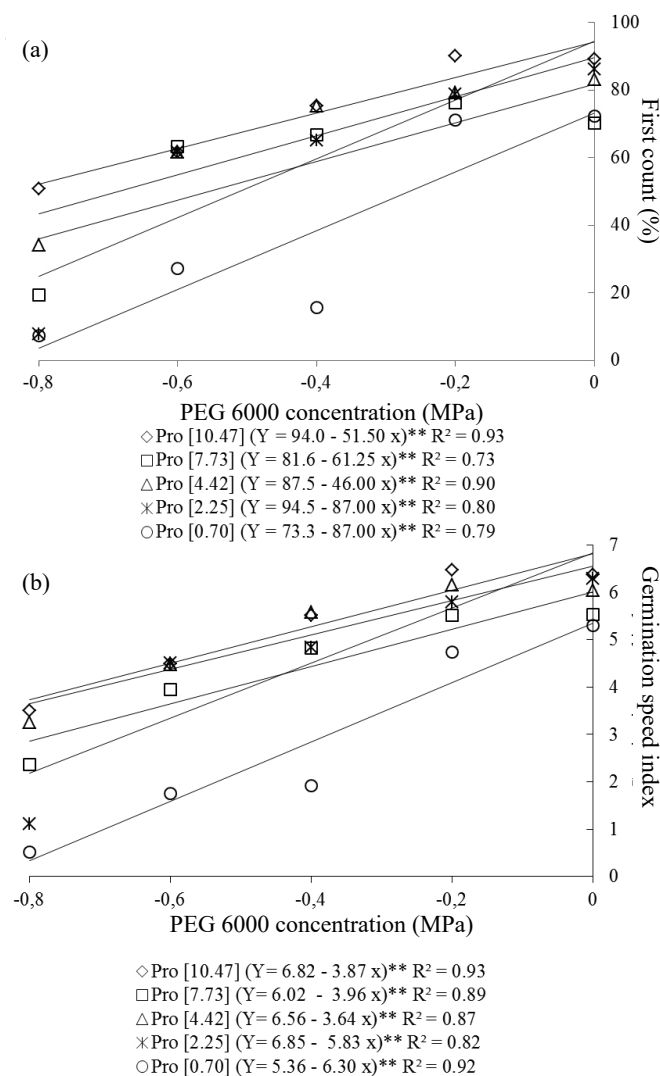


Figure 4. Germination speed index and first count of genetically modified tobacco seeds under water stress conditions. Events with proline contents (Pro) of 10.47; 7.73; 4.42; 2.25 and 0.70 $\mu\text{mol}\cdot\text{g}^{-1}$ of fresh proline weight: (a) first count. (b) germination speed index. (**) significance level, where $p < 0.01$.

from the control treatment up to -0.2 MPa of PEG, and at a potential of -0.4 MPa, there was a decrease in seed vigor.

The vigor reduction may be attributed to the fact that a high solute concentration in the solution reduces the osmotic potential of the substrate, making water less available to seeds. The protein synthesis of the embryo is drastically reduced when there is water unavailability in the substrate, causing alterations in cellular turgescence, in the membrane structure and in the regulation of protein synthesis stages (Gulen et al., 2006). Thus, a probable explanation for the decrease in the vigor of tobacco seeds, when subjected to saline and water stresses, is that the

osmotic agents act reducing the speed of the physiological and biochemical processes.

In a water deficit situation, the GSI remained statistically unchanged up to -0.2 MPa (Figure 4b), showing a significant fall by -0.4 MPa for all the evaluated events. Seeds probably had enough water for the initial imbibition stages (Stages I and II) without, however, being able to start Stage III (Weitbrecht et al., 2011), consequently reducing the germination speed and extending the necessary period to reach the minimum water content required for the beginning of the embryo's own growth.

The higher the GSI value, the higher the seed's ability to express its potential (Nakagawa et al., 1999). Thus, it has been reported that the vigor reduction due to high solute concentrations in the solution reduces its osmotic potential and restricts the amount of water, reducing the metabolic process of the seed, due to lower water availability for the digestion of reserves and translocation of metabolized products (Bewley et al., 2013).

Both saline and water stress led to reductions in the viability and vigor of tobacco seeds; however, the presence of higher levels of proline in seeds genetically transformed with the *p5csf129a* gene allowed an increase in osmotic stress tolerance. Thus, there would be greater chances for these seeds to germinate and develop normal seedlings in areas where genotypes that are intolerant to salinity and water deficit would not be able to establish.

Conclusions

Genetically transformed tobacco seeds with overexpression of the *p5csf129a* gene, a proline synthesizer, are tolerant to osmotic stresses.

Tobacco seeds with a proline content of $10.47 \mu\text{mol}\cdot\text{g}^{-1}$ showed a better performance under salinity and water deficit conditions, revealing a higher physiological potential.

Acknowledgments

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarships that were granted.

References

ATIA, A.; DEBEZ, A.; RABHI, M.; SMAOUI, A.; ABDELLY, C. Interactive effects of salinity, nitrate, light, and seed weight on the germination of the halophyte *Crithmum maritimum*. *Acta Biologica Hungarica*, v.60, n.4, p.433-439, 2009. <https://www.researchgate.net/publication/40693216>

- BATES, L.S.; WALDREN, R. P.; TEARE, I.D. Rapid determination of free proline for water-stress studies. *Plant and Soil*, v.39, n.1, p.205-207, 1973. <https://link.springer.com/article/10.1007/BF00018060>
- BEWLEY, J. D.; BRADFORD, K. J.; HILHORST, H. W. M.; NONOGAKI, H. *Seeds: physiology of development, germination and dormancy*. 3.ed. New York: Springer, 2013. 392p
- BORGIO, L.; MARUR, C.J.; VIEIRA, L.G.E. Effects of high proline accumulation on chloroplast and mitochondrial ultrastructure and on osmotic adjustment in tobacco plants. *Acta Scientiarum. Agronomy*, v.37, n.2, p. 191-199, 2015. <http://www.scielo.br/pdf/asagr/v37n2/1807-8621-asagr-37-02-00191.pdf>
- BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. *Regras para análise de sementes*. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Brasília: MAPA/ACS, 2009. 395p. http://www.agricultura.gov.br/arq_editor/file/2946_regras_analise_sementes.pdf
- BRASILEIRO, A.C.M.; LACORTE, C. Agrobacterium: um sistema natural de transferência de genes para plantas. *Biociência e Desenvolvimento*, v.15, n.15, p.12-15, 2000. <http://www.cestadual.com/2012/agrobacterium.pdf>
- CARVALHO, N. M.; NAKAGAWA, J. *Sementes: ciência, tecnologia e produção*. 5.ed. FUNEP: Jaboticabal, 2012. 590 p.
- CHEN, K.; ARORA, R. Dynamics of the antioxidant system during seed osmopriming, post-priming germination, and seedling establishment in Spinach (*Spinacia oleracea*). *Plant Science*, v.180, n.2, p.212-220, 2011. <http://www.sciencedirect.com/science/article/pii/S0168945210002311>
- CONUS, L.A.; CARDOSO, P.C.; VENTUROSO, L.R.; SCALON, S.P.Q. Germinação de sementes e vigor de plântulas de milho submetidas ao estresse salino induzido por diferentes sais. *Revista Brasileira de Sementes*, v.31, n.4, p.67-74, 2009. www.scielo.br/scielo.php?script=sci_arttext&pid=S0101-31222009000400008
- DEINLEIN, U.; STEPHAN, A.B.; SCHROEDER, J.I. Plant salt-tolerance mechanisms. *Trends in Plant Science*, v.19, n.6, p.371-379, 2014. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4041829/>
- GILL, S.S.; TUTEJA, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, v.48, n.12, p.909-930, 2010. <https://www.researchgate.net/publication/46426925>
- GULEN, H.; TURHAN, E.; ERIS, A. Changes in peroxidase activities and soluble proteins in strawberry varieties under salt-stress. *Acta Physiologica Plantarum*, v.28, n.2, p.109-116, 2006. <https://www.researchgate.net/publication/226869459>
- HAYAT, S.; HAYAT, Q.; ALYEMENI, M.; AHMAD, A. Role of proline under changing environments: A review. *Plant Signaling & Behavior*, v.7, n.11, p.1456-1466, 2012. <https://www.researchgate.net/publication/285684673>
- KUMAR, V.; SHRIRAMP, V.; KISHOR, B.K.; JAWALI, N.; SHITOLE, M.G. Enhanced proline accumulation and salt stress tolerance of transgenic *Indica rice* by over-expressing *p5csf129a* gene. *Plant Biotechnology Reports*, v.4, p.37-48, 2010. <https://link.springer.com/article/10.1007/s11816-009-0118-3>
- MAGUIRE, J. D. Speed of germination-aid in selection and evaluation for seedling and vigour. *Crop Science*, v.2, p.176-177, 1962.
- MAPA. Ministério da Agricultura, Pecuária e Abastecimento. *Instrução Normativa n.º 45, de 13 de setembro de 2013: padrão para a produção e a comercialização de sementes de tabaco*. Anexo XXVI. <<http://www.abrasem.com.br/wp-content/uploads/2012/10/Instru%C3%A7%C3%A3o-Normativa-n%C2%BA-45-de-17-de-Setembro-de-2013-Padr%C3%B5es-de-Identidade-e-Qualidade-Prod-e-Comerc-de-Sementes-Grandes-Culturas-Republica%C3%A7%C3%A3o-DOU-20.09.13.pdf>>.
- MILLER, G.; HONIG, A.; STEIN, H.; SUZUKI, N.; MITTLER, R.; ZILBERSTEIN, A. Unraveling $\Delta 1$ -pyrroline-5-carboxylate-proline cycle in plants by uncoupled expression of proline oxidation enzymes. *Journal of Biological Chemistry*, v.284, n.39, p.26482-26492, 2009. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2785336/pdf/zbc26482.pdf>
- MUNNS, R.; TESTER, M. Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, v.59, n.58, p.651-681, 2008. <https://www.ncbi.nlm.nih.gov/pubmed/18444910>
- NAKAGAWA, J. Testes de vigor baseados no desempenho de plântulas. In: KRZYŻANOWSKI, F. C.; VIEIRA, R.D.; FRANÇA-NETO, J. B. *Vigor de sementes: conceito e testes*. Londrina: ABRATES, 1999. cap. 2: 1-24 p.
- POSMYK, M.M.; KONTEK, R.; JANAS, K.M. Antioxidant enzymes activity and phenolic compounds content in red cabbage seedlings exposed to copper stress. *Ecotoxicology and Environmental Safety*, v.72, p.596-602, 2009. <https://www.researchgate.net/publication/23267123>
- POURRUT, B.; SHAHID M.; DUMAT, C.; WINTERTON P.; PINELLI, E. Lead uptake, toxicity, and detoxification in plants. *Reviews of Environmental Contamination and Toxicology*, v.213, p.113-36, 2011. <https://www.researchgate.net/publication/51097636>
- SEKHAR, P.N.; AMRUTHA, R.N.; SANGAM, S.; VERMA, D.P.S.; KAVI KISHOR, P.B. Biochemical characterization, homology modeling and docking studies of ornithine delta-aminotransferase-an important enzyme in proline bio-synthesis of plants. *Journal of Molecular Graphics and Modelling*, v.26, n.4, p.709-19, 2007. <http://www.sciencedirect.com/science/article/pii/S1093326307000782>
- SHARMA, P.; JHA, A.B.; DUBEY, R.S.; PESSARAKLI, M. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany*, v.12, p.1-26, 2012. <https://www.hindawi.com/journals/jb/2012/217037/>
- SILVA, F.A.S.; AZEVEDO, C.A.V. Versão do programa computacional Assistat para o sistema operacional Windows. *Revista Brasileira de Produtos Agroindustriais*, v.4, n.1, p.71-78, 2002. <http://www.deag.ufcg.edu.br/rbpa/rev41/Art410.pdf>
- TAIZ, L.; ZEIGER, E. *Fisiologia vegetal*. 4.ed. Porto Alegre: Artmed, 2009. 819p.

TÜRKAN, I.; DEMIRAL, T. Recent developments in understanding salinity tolerance. *Environmental and Experimental Botany*, v.67, p.2-9, 2009. https://www.researchgate.net/publication/223507077_Recent_developments_in_understanding_salinity_tolerance_Environ_Exp_Bot_1Special_issue2-9

WEITBRECHT, K.; MULLER, K.; LEUBNER, G. First off the mark: early seed germination. *Journal of Experimental Botany*, v.62, n.10, p.3289–3309, 2011. <https://www.researchgate.net/publication/50831680>

YAN, M. Seed priming stimulate germination and early seedling growth of *Chinese cabbage* under drought stress. *South African Journal of Botany*, v.99, p.88–92, 2015. <http://agri.ckcest.cn/ass/NK002-20160215004.pdf>

YU, X.Z.; LU, M.R. Responses of endogenous proline in rice seedlings under chromium exposure. *Global Journal of Environmental Science and Management*, v.2, n.4, p.319-326, 2016. http://www.gjesm.net/article_20999_c4558e7142673b12eb43599255748d7d.pdf