



Expression of genes related to soil flooding tolerance in soybeans

Gabriele Casarotto^{1*}, Tiago Edu Kaspar², Luan Cutti³, André Luis Thomas³ and José Fernandes Barbosa Neto³

¹Pesquisa, Desenvolvimento e Inovação, Três Tentos Agroindustrial S/A, Estrada da Figueira, 1300, 98240-000, Santa Bárbara do Sul, Rio Grande do Sul, Brazil. ²Instituto Nacional de Investigación Agropecuaria, INIA La Estanzuela, Colonia, Uruguay. ³Programa de Pós-Graduação em Fitotecnia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. *Author for correspondence. E-mail: gabrielecasarotto@gmail.com

ABSTRACT. The flooded environment brings about injuries to soybeans that vary depending on the adaptation ability of the genotype. Oxygen deprivation promotes the induction of the expression of genes related to glycolysis and fermentation pathways to maintain energy metabolism and, in addition to reducing-power consuming processes, act in the formation of adaptive structures and the maintenance of the redox status of the plant. The aim of this work was to evaluate the relative expression of genes related to soil flooding response in two contrasting soybean cultivars. Soybean plants of the sensitive (BRS 154) and tolerant (I27) cultivars at the V1 development stage were submitted to the flooding and control conditions (without flooding) for 0, 24, 48, and 96 hours. The relative expression of genes associated with flooding, including enolase (*ENO*), alcohol dehydrogenase 1 (*ADH1*), alanine aminotransferase 2 (*ALAT2*), hemoglobin 1 (*GLB1*), LOB41 domain-containing protein (*LBD41*), xyloglucan endotransglycosylase (*XETP*) and ascorbate peroxidase (*APX2*), was evaluated by means of RT-qPCR. The relative expression, in general, increased with flooding, especially in the root tissue. Cultivar I27 responded positively as observed by the expression of the maintenance genes of energy metabolism, structural changes and detoxification, suggesting the presence of three tolerance mechanisms in the flooding response.

Keywords: *Glycine max* (L.) Merr.; hypoxia; abiotic stress; metabolic alterations; RT-qPCR.

Received on December 10, 2017.

Accepted on March 19, 2018.

Introduction

The soybean (*Glycine max* (L.) Merr.) is the chief legume plant grown worldwide, standing out as a raw material in human and animal feeding, in addition to being used in industry and in obtaining biofuels. Recently, its cultivation in lowland areas appeared as an alternative to irrigated rice plantations, seeking thus to decrease the incidence of pests and diseases, manage weeds and increase the economic gains of the production system. However, the lowland environment brings about injuries to soybeans through frequent floodings, and these injuries vary depending on the development stage of the crop. During germination, the fast water absorption can cause physical rupture and seed deterioration (Sayama et al., 2009), and after primary root emission, the damages vary depending on the intensity and prolongation of stress (Komatsu et al., 2009). The occurrence of that stress results in serious reductions, which become more intense as the flooding period increases (Rhine, Stevens, Shannon, Wrather, & Sleper, 2010). Thus, the preferential cropping of the species occurs in flooding-free regions.

The identification of soybean cultivars with either the absence or insignificant alterations in grain yield under flooding are reported (Rhine et al., 2010), as well as the genetic variability for that trait (Nanjo et al., 2014). These studies present alterations in the metabolism that enable the adaptation to environments with low oxygen availability. Nevertheless, oxygen deprivation and the impossibility of obtaining energy by means of the oxidative phosphorylation pathway is one of the major hindrances to soil flooding tolerance in plants (Irfan, Hayat, Hayat, Afroz, & Ahmad, 2010). In that sense, plants in the presence of this stress induce the expression of genes related to reserve degradation, glycolysis and fermentation, aiming to maintain energy metabolism and warranting survival during flooding periods (Voesebeck & Bailey-Serres, 2015). Nevertheless, the energetic efficiency of these processes is poor and does not warrant the survival of the plant under these circumstances, suggesting that the soil flooding tolerance mechanism is far more complex.

Flooding tolerance demands the activation of reduced-power consuming processes ($\text{NADH} + \text{H}^+$ - reduced nicotinamide adenine dinucleotide) and collaborates with glycolytic process conservation. The genes coding

for the enzymes pyruvate decarboxylase (*PDC*) and alcohol dehydrogenase (*ADH*), which act on the alcoholic fermentation pathway, are expressed as many as 71 to 10,000 times, respectively, in soybean hypocotyls and roots 12 hours after the seedlings are subjected to flooding conditions compared with control conditions (Komatsu et al., 2009). These processes also result in non-tricarboxylic amino acid production and nitric oxide oxidation by non-symbiotic hemoglobins (Bailey-Serres & Voeselek, 2008; Gupta & Igamberdiev, 2016). The greatest alanine accumulation, compared with other amino acids formed in the tricarboxylic acid cycle, is found in *Lotus japonicus* (Rocha et al., 2010). In agreement with these findings, in soybeans, the greatest expression of the gene alanine aminotransferase 2 (*ALAT2*) is also associated with flooding stress (Valliyodan et al., 2014).

Furthermore, the signaling cascade, which triggers the soil flooding responses in plants, has as active participants reactive oxygen species (ROS) and the plant hormone ethylene and is involved both in the formation of aerenchyma and adventitious roots and in the development of the shoot under this condition (Voeselek & Sasidharan, 2013). Aerenchyma formation involves the expression and activity of the enzymes expansin, xyloglucan endotransglycosylase, pectin, etc., which are associated with programmed cell death (Irfan et al., 2010).

The elucidation of the mechanisms involved in the response of plants to flooding stress is indispensable and is the focus of a number of studies on soybean crops (Komatsu, Kobayashi, Nishizawa, Nanjo, & Furukawa, 2010; Khatoon, Rehman, Oh, Woo, & Komatsu, 2012; Nanjo et al., 2014), as well as many different species (Magneschi & Perata, 2009; Sasidharan et al., 2013; van Veen et al., 2016). RT-qPCR analysis (*reverse transcription - quantitative polymerase chain reaction*) is a tool utilized to evaluate the expression of genes involved in the response of soybean plants to flooding conditions (Nakayama et al., 2014; Valliyodan et al., 2014). Thus, the objective of this work was to evaluate the relative expression of genes related to the energy pathways, the formation of adaptive structures and ROS detoxification in response to soil flooding in soybeans.

Material and methods

Experimental material

The experiment was conducted in a greenhouse of the Department of Crops of the Federal University of Rio Grande do Sul (Departamento de Plantas de Lavoura da Universidade Federal do Rio Grande do Sul) between April and May 2016 in a completely randomized design with three replications. Soybean seeds of cultivars Introduction 27 (I27, tolerant) and BRS 154 (sensitive) were sown in plastic cups (200 mL) containing a mixture of commercial substrate and vermiculite at a ratio of 3:1. The cups were maintained with moisture close to the field capacity until the plants reached the V1 development stage, and then, they were subjected to the treatments. At this stage, half of the plants were subjected to stress, which consisted in the maintenance of a water depth of 2 cm above the soil level, and the others were maintained with the soil in the field capacity (control). The plants were maintained under these conditions for 0, 24, 48, and 96 hours. At each of these times, samples from the root and shoot tissues were collected, immediately frozen in liquid nitrogen and stored at -80°C. The total RNA was extracted from the samples utilizing the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was measured in a Genesys 2 spectrophotometer (Thermo Spectronic) and, afterwards, the samples were subjected to the DNase I (Invitrogen) treatment, eliminating DNA contaminants that might influence the posterior analyses. The cDNA synthesis was carried out by using the reverse transcriptase enzyme M₋MLV (Invitrogen).

RT-qPCR

A real-time PCR analysis was utilized for assessing the differential expression of the gene candidates to soil flooding tolerance among the sensitive (BRS 154) and tolerant soybean genotypes (Introduction 27). In this study, the reference endogenous genes utilized were the elongation factor 1- β (*ELF1- β*), actin 11 (*ACTB*) and ubiquitin (*UBI*), since they present expression stable under the flooding condition (Byfield, Xue, & Upchurch, 2006; Jian et al., 2008; Nakayama et al., 2014; Valliyodan et al., 2014). The genes enolase (*ENO*), alcohol dehydrogenase 1 (*ADH1*), alanine aminotransferase 2 (*ALAT2*), hemoglobin 1 (*GLB1*), LBD41 domain-containing protein (*LBD41*), xyloglucan endotransglycosylase (*XETP*), and ascorbate peroxidase (*APX2*) were investigated as candidates to flooding response, since they are correlated with the adequacy of energy metabolism, structural modifications and ROS detoxification under flooding conditions (Nakayama

et al., 2014; Valliyodan et al., 2014). The sequences of the primers of the candidate reference genes and their pairing temperatures are presented in Table 1.

Table 1. Reference and candidate genes to soil flooding tolerance, the locus, and the sequence of the *primers* (5'-3') and pairing temperature (Tm) of the primers utilized for the RT-qPCR analysis.

Gene*	Locus	Forward sequence	Reverse sequence	Tm
<i>ELF1-β</i> ^A	Glyma.02g276600	GTTGAAAAGCCAGGGGACA	TCTTACCCCTTGAGCGTGG	64
<i>ACTB</i> ^B	Glyma.15g050200	GAGCTATGAATTGCCTGATGG	CGTTTCATGAATTCCAGTAGC	62
<i>UBI</i> ^C	Glyma.10g251900	AGCTATTCGCAGTCCCAAAT	CAGAGACGAACCTTGAGGAGA	64
<i>ENO</i> ^C	Glyma.19g190900	ATCTCAATTGCATCGCTGCC	CATCGGAGCAAGTCAAATCCA	64
<i>ADH1</i> ^D	Glyma.04g240800	CCTGTGAGGGACTGAGTTCTCTTTC	ACAGCAGCAAAGGTCCTATCATTTCT	64
<i>ALAT2</i> ^C	Glyma.01g026700	TTGCAAGTGATCCACGACACA	AAAGGCTTTGGTGGCATGG	64
<i>GLB1</i> ^C	Glyma.11g121800	CCATTTTAGAACCGGCGTAGC	TAGCCGGTGACCACATTTCTG	65
<i>LBD41</i> ^C	Glyma.18g297100	AAAACGCTTTGAAAGGCGCA	CTTTGGACACGTGGCGTATGT	64
<i>XETP</i> ^C	Glyma.16g045000	GAGTGAAATTTCCCATTCGACCA	CGACCAATCCGTTTCTCCA	64
<i>APX2</i> ^D	Glyma.12g073100	CACGGTGCCCATATTTCTCTC	CAACCCAACTCCAATCATCATCAC	62

**ELF1-β*: elongation factor 1-β; *ACTB*: actin 11; *UBI*: ubiquitin; *ENO*: enolase; *ADH1*: alcohol dehydrogenase 1; *ALAT2*: alanine aminotransferase 2; *GLB1*: hemoglobin1; *LBD41*: LOB41 domain-containing protein; *XETP*: xyloglucan endotransglycosylase; *APX2*: ascorbate peroxidase. ^A(Jian et al., 2008),

^B(Byfield, Xue, & Upchurch, 2006), ^C(Valliyodan et al., 2014), ^D(Nakayama et al., 2014).

For the RT-qPCR reaction, 10 µL of the cDNA sample (2 ng µL⁻¹), 0.4 µL of each *primer* (10 µM), 2 µL of 1X *SYBER Green Master Mix* (Invitrogen), 2 µL 10X buffer, 0.5 µL dNTPs (10 mM), 1.2 µL of MgCl₂ (50 mM), 0.2 µL of *ROX Reference Dye* (Invitrogen), 0.1 µL of *Taq Platinum* (Invitrogen), and autoclaved ultrapure water were utilized to complete the reaction volume of 20 µL. The amplifications were evaluated in the 7300 Real Time Thermocycler System (Applied Biosystems) using the following conditions: 50°C for 2 min.; 95°C for 10 min.; 40 cycles of 95°C for 15 seconds; 62 to 65°C (depending on the *primer* pair) for 1 min., and 72°C for 30 seconds. The expression levels of the target and endogenous reference genes were analyzed in triplicate for each of the three biological replications. Concomitantly, a blank sample (only water) was utilized to discard contamination, and the dissociation curves confirmed the amplification specificity.

Analysis of the relative expression of the candidate genes

The stability of the reference genes was evaluated from the Ct values (cycle threshold) obtained in the RT-qPCR reaction and utilized the RefFinder tool, which integrates the computational algorithms geNorm, Normfinder, BestKeeper and the Ct method to compare and rank the reference genes (Xie, Xiao, Chen, Xu, & Zhang, 2012). The most encompassing classification of the stability coefficient provided by the program was utilized for selecting the normalizer genes.

For the target genes, we calculated the mean, standard deviation and the confidence interval of the Ct values of each treatment. The adjustment of the amplification curves and efficiency of the RT-qPCR analysis was conducted using LinRegPCR v.12.2 software. Values of R > 0.99, with an efficiency between 1.8 and 2 and number of points greater than 4, were accepted (Tuomi, Voorbraak, Jones, & Ruijter, 2010). In turn, the relative expression analysis was carried out using the equation proposed by Dussault and Pouliot (2006), where: $\Delta\Delta Ct = (Ct_{flooded} - Ct_{reference}) - (Ct_{control} - Ct_{reference})$ and $\Delta\Delta Ct$ was the relative expression of the gene and the application of the result in $2^{(\Delta\Delta Ct)}$ gave the variation dimension. The amount of the transcripts of cultivars Introduction 27 and BRS 154 in the different flooding or control condition (0, 24, 48, and 96 hours) was compared with the abundance in the control (time zero hours of cultivar BRS 154, flooding sensitive). Thus, it was possible to compare the flooding effects in the different periods and the ability to affect the increase and/or reduction of the target gene transcription in each of the cultivars.

Results and discussion

Stability of the reference genes

The amplification of the *ACTB* gene presented a greater amount of transcripts with a minor number of cycles in the real-time *PCR* reaction, demonstrating an intense constitutive presence and resulting in a lower average Ct value compared with the other two reference genes (Figure 1A). Nevertheless, the amplification of that gene for the different flooding times and conditions demonstrated an elevated variation, which resulted in a greater range of the confidence interval (Figure 1A). The *ELF1-β* and *UBI* genes had Ct values that were higher than *ACTB*, with lower amounts in the evaluated conditions. However, these two genes demonstrated less variation compared to the treatments (flooding and control) in which they

were evaluated resulting in a smaller range of the confidence interval. The average *Ct* values obtained for the *ELF1-β*, *UBI*, and *ACTB* genes were of 24.55, 27.48, and 22.13, respectively (Figure 1A).

The reference gene stability, measured by the computational program RefFinder, from the four algorithms indicated a better performance for the *ELF1-β* gene in the treatments utilized in the study (Figure 1B). It is important to emphasize that the lower the stability coefficient is, the lesser the influence of the treatments utilized in the study over their expression and the more stable the gene is under the conditions evaluated (Xie et al., 2012). Therefore, the lower the value of the stability coefficient is, the more the gene becomes an indicator for utilization as a normalizer.

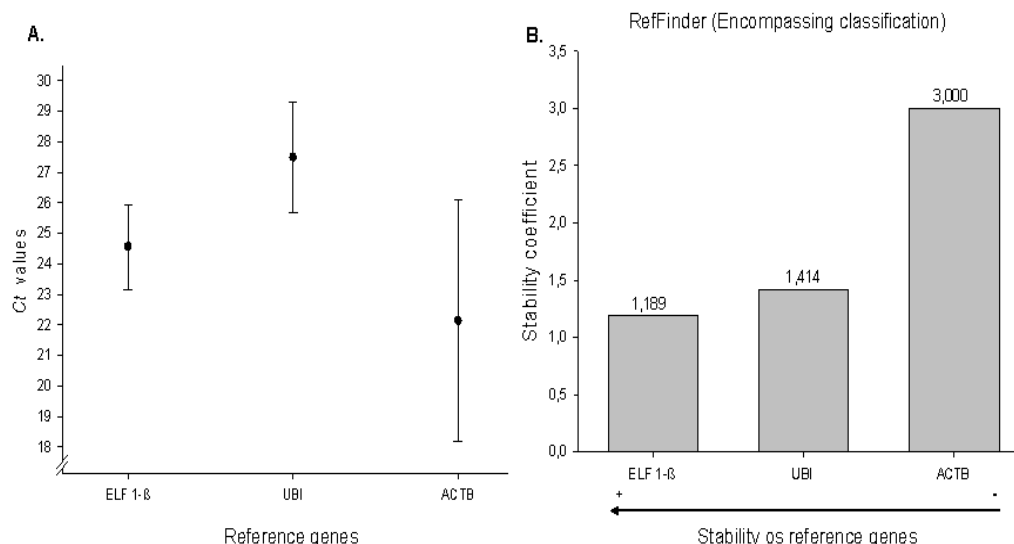


Figure 1. Variation in the abundance of the transcripts (A); the stability coefficient of the reference RefFinder (B) Elongation Factor 1- β (*ELF1-β*), Ubiquitine (*UBI*), and Actine 11 (*ACTB*) expressed in the shoot and roots of cultivars Introduction 27 and BRS 154 subjected to 0, 24, 48, and 96 hours of flooding and the control condition. The points indicate the average *Ct* value estimated, and the vertical bars are the confidence interval ($\alpha = 0.05$).

In a study about the stability of reference genes with soybean crops under flooding conditions, the *ELF1-β* and *ACTB* genes presented a greater stability (Nakayama et al., 2014). However, in the current study, only *ELF1-β* presented a higher stability, whereas *ACTB* was less stable than *UBI*. Still, in this context, the utilization of the mean of the *Ct* of two genes for the normalization of the relative expression is indicated, improving the standard deviation parameter and making the identification of the differences in the expression of the target gene more reliable (Nakayama et al., 2014). Thus, from the evaluation of the stability, the *ELF1-β* and *UBI* genes were utilized for the normalization of the relative expression of the candidate genes involved in flooding tolerance.

Relative expression of the genes related to energy metabolism

The analysis of the expression of the genes related to the energy metabolism, including *ENO*, *ADH1*, *ALAT2*, and *GLB1*, demonstrated, in general, an increase in the presence of transcripts under the flooding condition (Figures 2 and 3). The rise in the expression of the genes involved in the anaerobic energy metabolism is a response of the tolerant plant, which, under the oxygen deprivation condition, alters the metabolism, with the purpose of capturing energy for the maintenance of biological functions and survival (Irfan et al., 2010). In this sense, glycolysis is the main energy source for the plant under stress and therefore needs to be maintained. The conversion of 2-phosphoglycerate to phosphoenolpyruvate is one of the final steps of glycolysis, which culminates with pyruvate formation. This step is performed by the glycolytic enzyme resulting from the expression of the *ENO* gene (Hossain, Khatoon, & Komatsu, 2013). Thus, the greatest expression of this gene indicates a greater maintenance ability of the glycolytic pathway under the flooding condition.

The *ENO* gene presented no significant alteration in the expression when evaluated in the shoot tissue of cultivar BRS 154 during the first 48 hours, whether in the control or the flooded condition (Figure 2A). For the same cultivar, in the 96-hour period, a reduced expression, approximately 90% compared with the control, was observed. Nevertheless, the reduction in both conditions indicated that the flooding did not

contribute to modifying the *ENO* expression in the shoot of cultivar BRS 154. However, in cultivar I27, the expression of that gene in the leaves during the initial time (zero) was poor, and little increase was found after 48 hours of flooding, with values of 0.14 and 0.71 times when compared those of the control (Figure 2A). Thus, for the shoot, flooding induced only modest gains in the expression of this gene in the tolerant genotype, revealing its low response in the tissues, which was not directly caused by stress.

In the root tissue, the *ENO* expression for the sensitive cultivar (BRS 154) did not differ among the checking periods and for the control and flooded conditions, except in the 96-hour time point, when the relative expression in the control condition was twice as high as the flooded one (Figure 2B). In that way, the prolongation of stress reduced its expression in the root system of the stress-sensitive cultivar. However, for the flooded tolerant cultivar, the results showed an increase of approximately 14 times for the *ENO* expression 48 hours after flooding (Figure 2B).

The difference in the expression among the cultivars, mainly the increased expression in the root tissue of cultivar I27 was attributed to the effect of the flooding and the ability of the tolerant cultivar to respond adaptively to stress. Nevertheless, when evaluating contrasting soybean cultivars for soil tolerance to soil flooding, the level of *ENO* gene expression increased in the stress-sensitive cultivar by 1.64 times and reduced to 0.60 times in the tolerant one, only in the 24-hour period after the onset of stress when compared to the control condition (Valliyodan et al., 2014). However, the tolerance mechanism of the tolerant cultivar is based on a structural modification rather than the energy metabolism adjustments (Valliyodan et al., 2014).

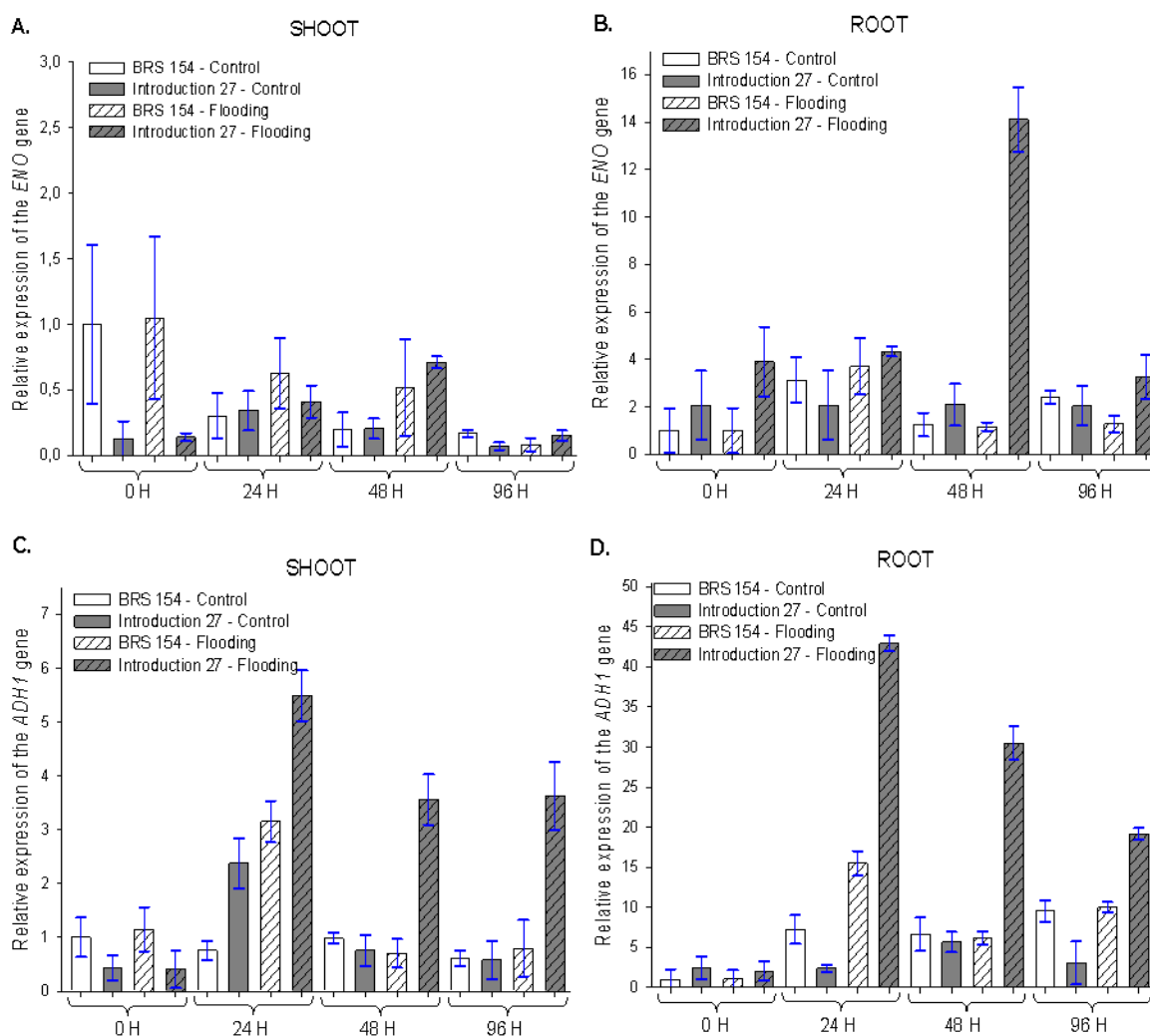


Figure 2. Relative expression of genes enolase (*ENO*) and alcohol dehydrogenase 1 (*ADH1*) in soybean plants of cultivars Introduction 27 and BRS 154 that were subjected to 0, 24, 48, and 96 hours of flooding or the control condition. Relative expression of *ENO* in the shoot (A) and root (B). Relative expression of *ADH1* in the shoot (C) and root (D). Vertical bars indicate the confidence interval ($\alpha = 0.05$).

The maintenance of the glycolytic pathway of energy production requires the consumption of pyruvate and the regeneration of NAD^+ , which occur via lactic and/or ethanolic fermentation. In the latter, the alcohol

dehydrogenase 1 (*ADH1*) gene plays a key role in the formation of ethanol and pyruvate exhaustion. In the present work, cultivar I27 presented general gains in the expression of *ADH1* in both evaluated tissues coming from the plants under the stress condition, indicating the participation of this stress-response mechanism (Figure 2C and D). The induction of *ADH1* expression observed in the 24-hour flooding period for cultivar I27 was 5.49 and 42.92 as great as the control in the leaves and roots, respectively (Figure 2C and D). The participation of this gene in the response of the plants under flooding and/or hypoxia is reported in rice and soybeans, with an increased expression in the first 12 hours of stress of over 10,000 times (Ismail, Ella, Vergara, & Mackill, 2009; Komatsu et al., 2009). In another work with soybeans, however, the greater expression of the *ADH1* gene (10.17 times) was verified in plants after 24 hours of flooding of the sensitive cultivar (Valliyodan et al., 2014). It is noteworthy that in this work, the authors promoted the comparison with a flooding-tolerant cultivar by the presence of adventitious roots that is different from the mechanism investigated in the present study.

In proteomics studies, the analysis of the differentially expressed proteins in cultivar Enrei, under 72 hours of flooding, showed an increase of 1.5 and 3.6-fold in the concentration of the enzymes enolase and alcohol dehydrogenase 1, respectively (Khatoon et al., 2012). The cultivar studied presents a moderate tolerance to soil flooding (Nanjo et al., 2014), corroborating with the results of the present study, which identified higher levels of transcripts in tolerant soybean cultivars (Figure 2). However, the positive response of only one or few genes or mechanisms does not necessarily indicate that the genotype is stress-tolerant and maintains its development, necessitating a more thorough investigation of the flood response mechanism.

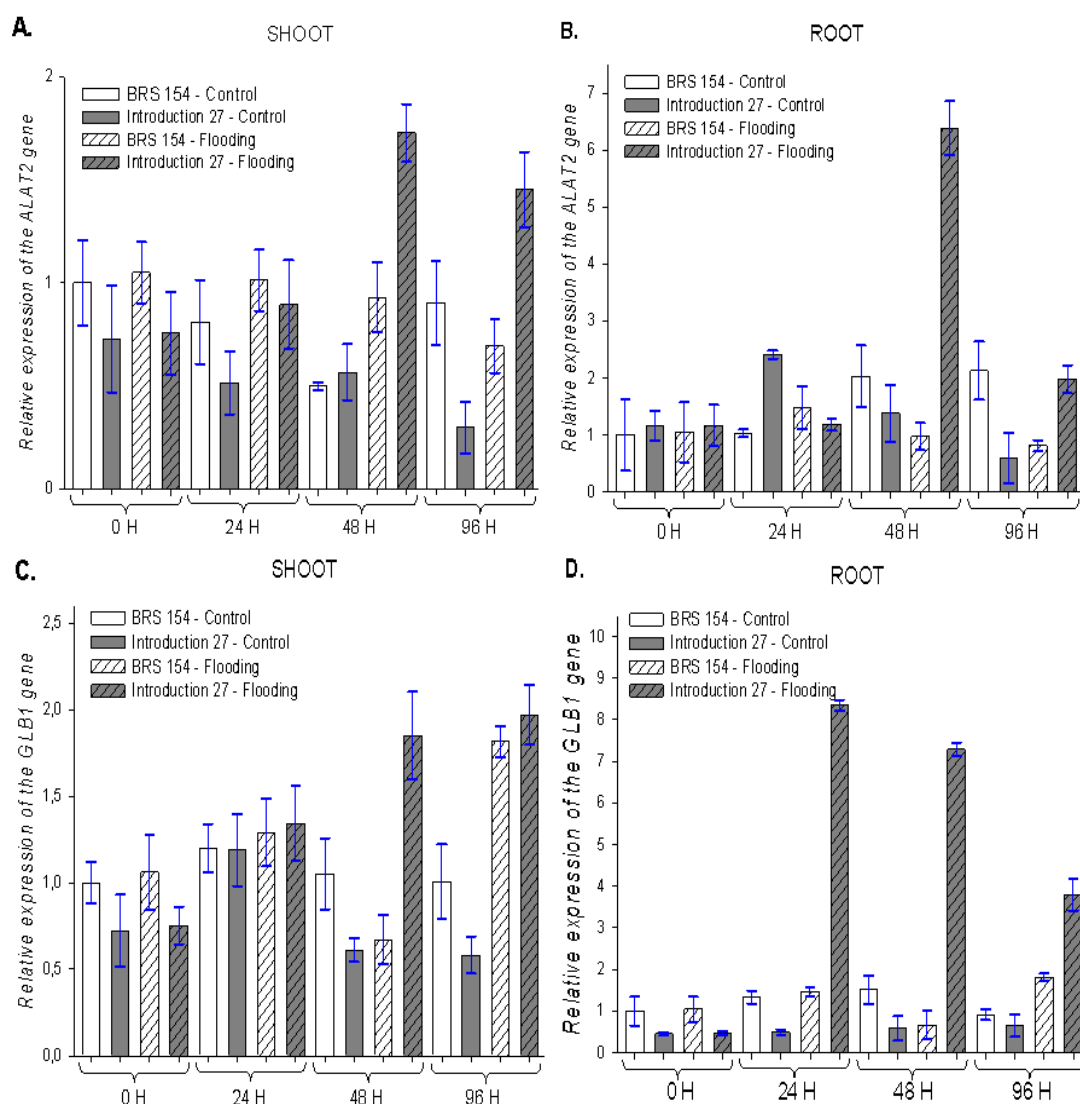


Figure 3. Relative expression of gene alanine aminotransferase (*ALAT2*) and non-symbiotic hemoglobin (*GLB1*) in soybean plants of cultivars Introduction 27 and BRS 154, which were subjected to 0, 24, 48, and 96 hours of flooding or the control condition. Relative expression of *ALAT2* in the shoot (A) and root (B). Relative expression of *GLB1* in the shoot (C) and root (D). Vertical bars indicate the confidence interval ($\alpha = 0.05$).

Other candidate genes involved in the maintenance of energy metabolism, as a response to flooding, were investigated in the present study, including alanine aminotransferase 2 (*ALAT2*) and non-symbiotic hemoglobin *GLB1* (Figure 3). The expression of *ALAT2* showed, in general, a significant difference only for cultivar I27 in the evaluations of 48 and 96 hours of flooding for the shoots and for 48 hours for the root system when compared to the control (Figure 3A and B). The increase in the relative expression in the shoot was inferior to the expression in the roots, indicating also a greater response of this gene in the tissue directly affected by flooding. In work evaluating the *ALAT2* expression in soybeans under flooding, differences only in the first day of flooding were found, with higher values in the susceptible genotypes and a reduction in tolerance for the tolerant plant at 1.38 and 1.24 times when compared to that of the control, respectively (Valliyodan et al., 2014). In the same work, a 3.86-fold increase in the *ALAT1* gene expression in the tolerant cultivar, from the stress prolongation until the tenth day, is reported (Valliyodan et al., 2014).

The action of the *ALAT2* gene in the flooding response occurs from the participation of the enzyme encoded by alanine aminotransferase 2 in obtaining energy via the formation of NADH. Thus, flooding tolerant cultivars, as in the case of I27, may be capable of obtaining energy complementary to the amount coming from glycolysis (Rocha et al., 2010). Still, in this sense, oxygen recycling via the nitric oxide cycle and non-symbiotic hemoglobins plays key roles in the obtaining of energy for the plant (Riquelme & Hinrichsen, 2015). The *GLB1* gene encodes non-symbiotic hemoglobin, and in the present study, in the absence of flooding, the relative expression of this gene was superior in cultivar BRS 154, at approximately 1- to 1.5-fold, not differing from the control (Figure 3C and D). However, the flooding stimulated the expression of this gene in the shoot of this cultivar BRS 154, reaching a relative expression that was 1.82 as great as that in the control at 96 hours after the onset of stress.

The expression of *GLB1* in the leaves of cultivar I27 was stimulated in the 48-hour evaluation and was maintained at a high level until 96 hours of flooding, and the values of relative expression were 1.85 and 1.97 as great as those of the control, respectively (Figure 3C). In the root tissue, the increase in *GLB1* expression in cultivar I27 was more marked, reaching 8.34, 7.29, and 3.80 times greater than the expression of the control after 24, 48 and 96 hours of evaluation, respectively, under the flooding condition (Figure 3D). The transcriptional and non-symbiotic hemoglobin protein levels were positively regulated by flooding in the early development stages of cultivar Enrei (Komatsu et al., 2009). In only 12 hours of flooding, the amount of transcript was 95 times higher in the flooded condition compared to the control condition.

In the 24-hour period after the onset of the flooding, the highest presence of *GLB1* is reported when compared to that of the control condition, and for the tolerant genotype, it was surpassed by more than 1,000 times the initial value (Komatsu et al., 2009). These results corroborate the present work and refer to the high importance of non-symbiotic hemoglobin in the response of the soybean plants to soil flooding. However, the results obtained in the present work also demonstrate a reduction in the relative expression of the *GLB1* gene in cultivar I27 in the longest period of stress (Figure 3D), suggesting that it participates in an initial response to flooding. Thus, the expression of the *ALAT2* and *GLB1* genes becomes an important bottleneck related to the response of soybean plants to obtain energy under soil flooding conditions.

The increase in the transcriptional level of the *ENO*, *ADH1*, *ALAT2*, *GLB1* genes, mainly in the tolerant cultivar, suggests that it is capable of overcoming soil flooding through energy metabolism reorganization. Alam et al. (2010), evaluating the soybean proteome under flooding for 3 and 7 days of stress, verified the presence of isoforms of enzymes Enolase and Alcohol Dehydrogenase, demonstrating their important participation in the response of soybean plants to stress. The induction of the genes of the glycolytic and fermentation pathways and the increase of non-symbiotic hemoglobin levels was verified by Komatsu et al. (2009), supporting the results of this experiment.

Relative expression of structural modification-related genes

The evaluation of two genes, LOB41 domain-containing protein (*LBD41*) and xyloglucan endotransglycosylase (*XETP*), with effects on the structural modifications in soybean crops reveals their participation in the flooding response in the tolerant cultivar (Figure 4). Structural modifications, such as the formation of aerenchyma and adventitious roots, are common responses to soil flooding. The *LBD41* gene proved responsive to stress in the soybean leaves and roots of the evaluated cultivars (Figure 4A and B), and the increased expression was visualized mainly in the tolerant cultivar (I27) already in the first 24 hours of flooding. In the leaves, this increase was approximately 4, 12, and 8 times for 24, 48, and 96 hours of soil flooding, respectively (Figure 4A).

In the roots, the flooding brought about an increased relative expression in both cultivars (Figure 4B). After 24 hours from the onset of flooding, the *LBD41* expression was similar for both cultivars BRS 154 and I27, with values 7.65 and 8.68 as great as the control, respectively. In the 48-hour evaluation, the expression of this gene remained high only in the tolerant cultivar and was 13.88 times higher than that of the control

and decreased in the susceptible cultivar to 2.16 times. With 96 hours of flooding, there was an increase in the expression of *LBD41* in the sensitive cultivar and maintenance of high expression in the tolerant cultivar, being 6.87 and 13.00 times higher than that of the control, respectively (Figure 4B). In that sense, the family of *LOB* transcription factors (*lateral organ boundary*), of which *LBD41* is a part, is plant-specific, and among its attributions is the formation of lateral roots in arabidopsis (Okushima, Fukaki, Onoda, Theologis, & Tasaka, 2007). A higher gene expression of the *LOB* family is associated with higher adventitious root formation in tolerant soybean cultivars, where the *LBD41* gene showed a greater expression with stress prolongation (Valliyodan et al., 2014).

In turn, xyloglucan endotransglycosylase gene (*XETP*) encodes an enzyme that acts on cell wall degradation and relaxation. In the present experiment, *XETP* was influenced by flooding in both leaf and root tissue (Figure 4C and D). In general, the highest expression of *XETP* was in roots of cultivar I27 (Figure 4B). Flooding caused an increase in the expression of this gene in all the flooding periods, especially in the first 24 hours, when the expression was approximately 24-fold higher than the control and was maintained at a high level until the 96-hour evaluation (Figure 4B). Higher levels of expression were identified in sensitive soybean cultivars by Valliyodan et al. (2014), who also reported the differential expression only after 24 hours of flooding.

The analysis of the expression of the genes related to the formation of adaptive structures showed a higher expression of the *LBD41* and *XETP* genes under the flooding condition in the root system of the tolerant cultivar (Figure 4). This result was contrary to the expectations, since cultivar I27 was not characterized by a high formation of aerenchymas and adventitious roots. However, the higher levels of the relative expression of these two genes after 24 hours of flooding and the persistence during the 96-hour assessment (Figure 4) suggest that the mechanisms of alteration of the structures may be acting, with less capacity, concomitantly in response to the flooding tolerance by cultivar I27.

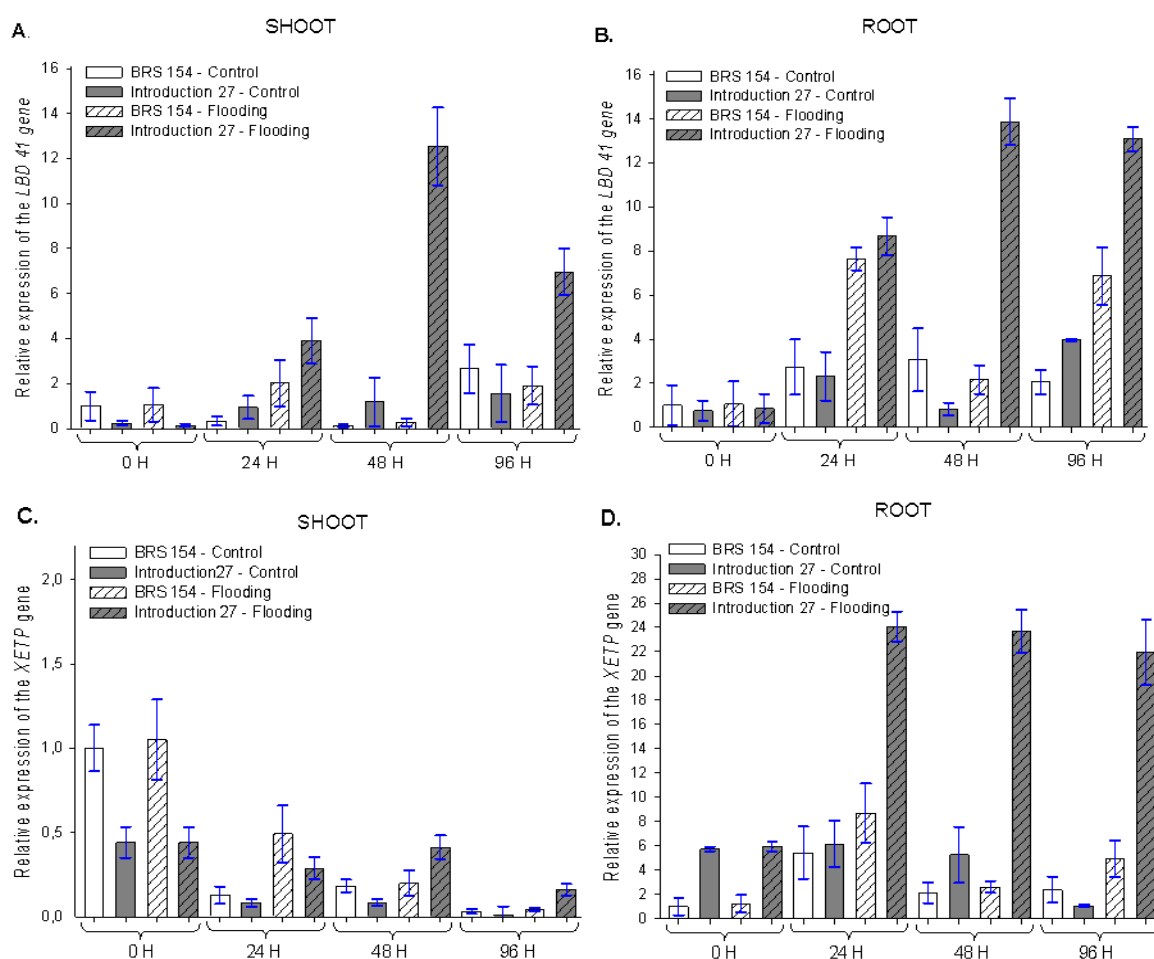


Figure 4. Relative expression of the genes *LOB41* domain-containing protein (*LBD41*) and xyloglucan endotransglycosylase (*XETP*) in soybean plants of the cultivars Introduction 27 and BRS 154, which were subjected to 0, 24, 48, and 96 hours of flooding or the control condition. Relative expression of *LBD41* in the shoot (A) and root (B). Relative expression of *XETP* in the shoot (C) and root (D).

Vertical bars indicate the confidence interval ($\alpha = 0.05$).

Relative expression of detoxification-related genes of reactive oxygen species

Out of the candidate genes with the ROS detoxification response to soil flooding in the soybean crop, the differential expression of the ascorbate peroxidase (*APX2*) gene among the genotypes evaluated, mainly in the root system (Figure 5), was stressed. In general, flooding caused a reduction in the relative expression of the gene in the root system of the sensitive cultivar, and it increased in the roots of cultivar I27 (Figure 5B). The increase in expression in the tolerant cultivar was approximately four times at 48 hours from the onset of stress and was three times after 96 hours (Figure 5B). The enzyme encoded by this gene participates in the ROS detoxification pathway, such as hydrogen peroxide (Kausar, Hossain, Makino, & Komatsu, 2012). In this sense, the greater expression of the *APX2* gene encoding this enzyme may contribute to the adaptive responses of the plant against stress.

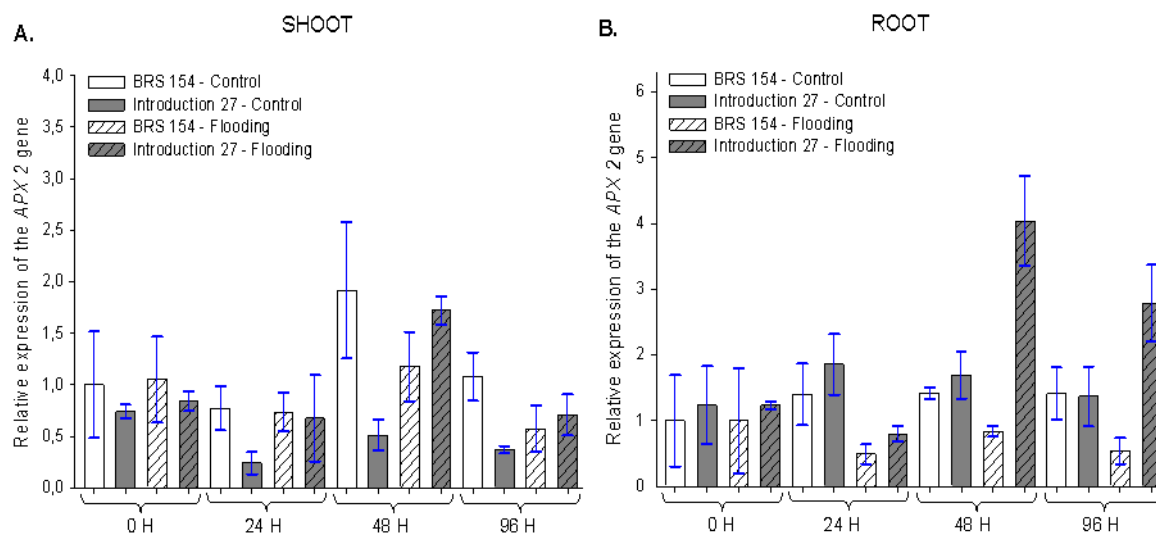


Figure 5. Relative expression of the gene ascorbate peroxidase (*APX2*) in soybean plants of cultivars Introduction 27 and BRS 154, which were subjected to 0, 24, 48, and 96 hours of flooding or the control condition. Relative expression of *APX2* in the shoot (A) and root (B). Vertical bars indicate the confidence interval ($\alpha = 0.05$).

Previous studies show the reduction of protein expression and peroxidase activity under flooding conditions in soybean (Shi et al., 2008; Alam et al., 2010; Wang, Oh, Sakata, & Komatsu, 2016). The reduction of the levels of *APX2* may, in these cases, be associated with the accumulation of reactive oxygen species. Thus, the results of this work suggest that the increased level of transcripts in cultivar I27 and the maintenance of higher levels with the continuity of the flooding period (Figure 5B) may play an important role in the response of the tolerant cultivar compared to oxidative stress brought about by soil flooding.

Conclusion

Flooding-tolerant soybean cultivar I27 responded positively, showing the expression of the genes related to energy metabolism maintenance, structural changes and ROS detoxification. The participation, in a concomitant manner, of the three flooding tolerance mechanisms can be inferred from the results obtained for cultivar I27.

References

- Alam, I., Lee, D. G., Kim, K. H., Park, C. H., Sharmin, S. A., Lee, H., ... Lee, B. H. (2010). Proteome analysis of soybean roots under waterlogging stress at an early vegetative stage. *Journal of Bioscience*, 35(1), 49-62. DOI: 10.1007/s12038-010-0007-5
- Bailey-Serres, J., & Voesenek, L. A. C. J. (2008). Flooding stress: acclimations and genetic diversity. *Annual Review of Plant Biology*, 59(1), 313-339. DOI: 10.1146/annurev.arplant.59.032607.092752
- Byfield, G. E., Xue, H., & Upchurch, R. G. (2006). Two genes from soybean encoding soluble $\Delta 9$ stearoyl-ACP desaturases. *Crop Science*, 46(2), 840-846. DOI: 10.2135/cropsci2005.06-0172
- Dussault, A. A., & Pouliot, M. (2006). Rapid and simple comparison of messenger RNA levels using real-time PCR. *Biological Procedures Online*, 8(1), 1-10. DOI: 10.1251/bpo114

- Gupta, K. J., & Igamberdiev, A. U. (2016). Reactive nitrogen species in mitochondria and their implications in plant energy status and hypoxic stress tolerance. *Frontiers in Plant Science*, 7(369), 1-6. DOI: 10.3389/fpls.2016.00369
- Hossain, Z., Khatoon, A., & Komatsu, S. (2013). Soybean proteomics for unraveling abiotic stress response mechanism. *Journal of Proteome Research*, 12(1), 4670-4684. DOI: 10.1021/pr400604b
- Irfan, M., Hayat, S., Hayat, Q., Afroz, S., & Ahmad, A. (2010). Physiological and biochemical changes in plants under waterlogging. *Protoplasma*, 241(1), 3-17. DOI: 10.1007/s00709-009-0098-8
- Ismail, A. M., Ella, E. S., Vergara, G. V., & Mackill, D. J. (2009). Mechanisms associated with tolerance to flooding during germination and early seedling growth in rice (*Oryza sativa*). *Annals of Botany*, 103(2), 197-209. DOI: 10.1093/aob/mcn211
- Jian, B., Liu, B., Bi, Y., Hou, W., Wu, C., & Han, T. (2008). Validation of internal control for gene expression study in soybean by quantitative real-time PCR. *BMC Molecular Biology*, 9(59), 1-14. DOI: 10.1186/1471-2199-9-59
- Kausar, R., Hossain, Z., Makino, T., & Komatsu, S. (2012). Characterization of ascorbate peroxidase in soybean under flooding and drought stresses. *Molecular Biology Reports*, 39(12), 10573-10579. DOI: 10.1007/s11033-012-1945-9
- Khatoon, A., Rehman, S., Oh, M. W., Woo, S. H., & Komatsu, S. (2012). Analysis of response mechanism in soybean under low oxygen and flooding stresses using gel-base proteomics technique. *Molecular Biology Reports*, 39(12), 10581-10594. DOI: 10.1007/s11033-012-1946-8
- Komatsu, S., Yamamoto, R., Nanjo, Y., Mikami, Y., Yunokawa, H., & Sakata, K. (2009). A comprehensive analysis of the soybean genes and proteins expressed under flooding stress using transcriptome and proteome techniques. *Journal of Proteome Research*, 8(1), 4766-4778. DOI: 10.1021/pr900460x
- Komatsu, S., Kobayashi, Y., Nishizawa, K., Nanjo, Y., & Furukawa, K. (2010). Comparative proteomics analysis of differentially expressed proteins in soybean cell wall during flooding stress. *Amino Acids*, 39(5), 1435-1449. DOI: 10.1007/s00726-010-0608-1
- Magneshi, L., & Perata, P. (2009). Rice germination and seedling growth in the absence of oxygen. *Annals of Botany*, 103(2), 181-196. DOI: 10.1093/aob/mcn121
- Nakayama, T. J., Rodrigues, F. A., Neumaier N., Marcelino-Guimarães, F. C., Farias, J. R. B., Oliveira, M. C. N., ... Nepomuceno, A. L. (2014). Reference genes for quantitative real-time polymerase chain reaction studies in soybean plants under hypoxic conditions. *Genetics and Molecular Research*, 13(1), 860-871. DOI: 10.4238/2014
- Nanjo, Y., Jang, H. Y., Kim, H. S., Hiraga, S., Woo, S. W., & Komatsu, S. (2014). Analyses of flooding tolerance of soybean varieties at emergence and varietal differences in their proteomes. *Phytochemistry*, 106(1), 25-36. DOI: 10.1016/j.phytochem.2014.06.017
- Okushima, Y., Fukaki, H., Onoda, M., Theologis, A., & Tasaka, M. (2007). *ARF7* and *ARF19* regulate lateral root formation via direct activation of LBD/ASL genes in Arabidopsis. *Plant Cell*, 19(1), 118-130. DOI: 10.1105/tpc.106.047761
- Rhine M. D., Stevens, G., Shannon, G., Wrather, A., & Sleper, D. (2010). Yield and nutritional responses to waterlogging of soybean cultivars. *Irrigation Science*, 28(1), 135-142. DOI: 10.1007/s00271-009-0168-x
- Riquelme, A., & Hinrichsen, P. (2015). Non-symbiotic hemoglobin and its relation with hypoxic stress. *Chilean Journal of Agricultural Research*, 75(1), 80-89. DOI: 10.4067/S0718-58392015000300009
- Rocha, M., Licausi, F., Araújo, W. L., Nunes-Nesi, A., Sodek, L., Fernie, A. R., & Dongen, J. T. V. (2010). Glycolysis and the tricarboxylic acid cycle are linked by alanine aminotransferase during hypoxia induced by waterlogging of *Lotus japonicus*. *Plant Physiology*, 152(3), 1501-1513. DOI: 10.1104/pp.109.150045
- Sasidharan, R., Mustroph, A., Boonman, A., Akman, M., Ammerlaan, A. M. H., Breit, T., ... Tienderen P. H. V. (2013). Root transcript profiling of two *Rorippa* species reveals gene clusters associated with extreme submergence tolerance. *Plant Physiology*, 163(3), 1277-1292. DOI: 10.1104/pp.113.222588
- Sayama, T., Nakazaki, T., Ishikawa, G., Yagasaki, K., Yamada, N., Hirota, N., ... Tanisaka, T. (2009). QTL analysis of seed-flooding tolerance in soybean (*Glycine max* [L.] Merr.). *Plant Science*, 176(4), 514-521. DOI: 10.1016/j.plantsci.2009.01.007
- Shi, F., Yamamoto, R., Shimamura, S., Hiraga, S., Nakayama, N., Nakamura, T., ... Komatsu, S. (2008). Cytosolic ascorbate peroxidase 2 (*cAPX2*) is involved in the soybean response to flooding. *Phytochemistry*, 69(6), 1295-1303. DOI: 10.1016/j.phytochem.2008.01.007

- Tuomi, J. M., Voorbraak, F., Jones, D. L., & Ruijter, J. M. (2010). Bias in the C_q value observed with hydrolysis probe based quantitative PCR can be corrected with the estimated PCR efficiency value. *Methods*, 50(4), 313-322. DOI: 10.1016/j.ymeth.2010.02.003
- Valliyodan, B., Toai, T. T. V., Alves, J. D., Goulart, P. F. P., Lee, J. D., Fritschi, F. B., ... Nguyen, H. T. (2014). Expression of root-related transcription factors associated with flooding tolerance of soybean (*Glycine max*). *International Journal of Molecular Sciences*, 15(1), 17622-17643. DOI: 10.3390/ijms151017622
- van Veen, H., Vashisht, D., Akman, M., Girke, T., Mustroph, A., Reinen, E., ... Sasidharan, R. (2016). Transcriptomes of eight *Arabidopsis thaliana* accessions reveal core conserved, genotype- and organ-specific responses to flooding stress. *Plant Physiology*, 172(2), 668-689. DOI: 10.1104/pp.16.00472
- Voesenek, L. A. C. J., & Bailey-Serres, J. (2015). Flood adaptive traits and processes: an overview. *New Phytologist*, 206(1), 57-73. DOI: 10.1111/nph.13209
- Voesenek, L. A. C. J., & Sasidharan, R. (2013). Ethylene-and oxygen signaling-drive plant survival during flooding. *Plant Biology*, 15(3), 426-435. DOI: 10.1111/plb.12014
- Wang, X., Oh, M. W., Sakata, K., & Komatsu, S. (2016) Gel-free/label-free proteomic analysis of root tip of soybean over time under flooding and drought stresses. *Journal of Proteomics*, 130(1), 42-55. DOI: 10.1016/j.jprot.2015.09.007
- Xie, F., Xiao, P., Chen, D., Xu, L., & Zhang, B. (2012). miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Molecular Biology*, 80(1), 75-84. DOI: 10.1007/s11103-012-9885-2