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Amino Acids as Stress Reducers in Soybean Plant Growth Under Different Water-Deficit Conditions

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Abstract

Soybean is one of the most important crops of economic value, and among the factors that can alter its productivity is the water-deficit. Studies show that amino acids such as proline and glutamate can help protect plants against abiotic stresses, such as water restriction. Therefore, the present study aimed to investigate the improvement of tolerance to water deficit in soybean plants when submitted to the application of proline and glutamate. The application of these amino acids was carried out as seed treatment (ST) or as foliar application (FA). Three irrigation levels (80, 60, and 40% of the pot field capacity) were used, which corresponded to treatments without water deficit, moderate, and high deficits, respectively. The high water deficit provided a significant reduction in plant growth and productivity. Under these conditions, glutamate as ST was effective in increasing the plant dry mass and yield (21% increase in relation to control). In plants without water restriction, the application of glutamate as ST reduced the lipid peroxidation and increased the dry mass of the plants, volume, and root projection area (PA). On the other hand, for plants submitted to the low water deficit, the FA of proline increased the dry mass of the plants, nitrate reductase, and PA. Therefore, in soybean plants without water restriction and with high water deficit, the best response was obtained with glutamate as ST. For plants submitted to low water deficit, the best procedure was the application of proline as FA.

Keywords Proline · Glutamate · Drought · *Glycine max* (L.) Merrill

Introduction

Soybeans are one of the most important plants grown throughout the world, mainly because the beans contain approximately 40% protein and 18–20% oil. This makes this crop fundamental for human and animal feeding.

However, this crop is often exposed to climatic adversities in the major soybean-producing regions of the world. About 90% of world soybean production comes from the United States, Brazil, Argentina, and China, mostly under rain-fed conditions, which increases the risks of variations in the world production of this grain. During the 2018/2019 harvest, Paraná, which is considered the second largest soybean-producing state in Brazil, suffered intensely with low water availability. This caused an average productivity reduction of 534 kg ha^{-1}, causing approximately 15% of the total production loss in this state.

In addition, drought spells (veranicos) are common in the area of the Brazilian Cerrado, where most of the soybean production in Brazil is located. These dry spells of 1–3 days occur during the rainy summer, sometimes being more prolonged, as occurred in the 2018/2019 cropping year. They are sometimes milder, in the middle of the harvest, mainly affecting flowering and grain filling of the crop, soybean phases that are very sensitive to water deficit. Such problems caused the production drop of 2.9 million tons in the 2018/2019 harvest, in relation to the 2017/2018 crop, even with a 2.1% increase in the cultivated area.

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Thus, it is necessary to adopt strategies in the soybean production fields, in order to minimize the effects of recurrent water deficits. These strategies go through two main concepts. The first is to increase the water-retention capacity of the soil, which could be achieved with the increasing soil organic matter content and the maintenance of the soil being covered with a straw mulch. The second one seeks to maximize the ability of plants to explore soil water, increasing the depth of the root system, and stimulating metabolisms that are important to minimize the effect of water stress, especially the antioxidant metabolism.

We have already demonstrated in Teixeira et al. (2017a, b and 2018) that the use of glutamate in seed treatment (ST) or in foliar application (FA) during the vegetative phase of soybean cultivation increases root development and accumulation of dry matter mass; reduces stress; and increases productivity. However, there are not yet any references in the literature on the application of this amino acid under water-deficit condition. In addition, it is known that proline is one of the major amino acids related to water-stress tolerance in plants. This amino acid plays a key role in the antioxidant defense system (Gill and Tuteja 2010). It can also function as an osmoprotector, acting in drought, saline, and excess metal stresses, and also acting as a chelator of metals avoiding the production of OH^- (Smirnoff and Cumbes 1989; Ashraf and Foolad 2007; Gill and Tuteja 2010). Some studies have shown that proline may also act as a signaling molecule, and may induce increased activity of antioxidant enzymes in response to different types of stresses (Hoque et al. 2007; Rejeb et al. 2014).

However, there are few studies that show these effects for the soybean crop. In this way, we try to demonstrate here that, depending on the stress levels of the plants and the application of these amino acids, the soybean plants present changes in biochemical, morphological, and phenometric parameters, which may provide higher grain yield. Therefore, the objective of this work was to evaluate the effects of glutamate and proline applications on soybean plants subjected to water-deficient conditions and to evaluate possible attenuation of the effects of this type of stress and the influence of these amino acids on the enzymatic antioxidant system, and also on the plant development and grain production.

Materials and Methods

The experiment was carried out in a greenhouse located in the University Center of Patos de Minas (Unipam), municipality of Patos de Minas, MG, Brazil. Soybean plants [*Glicyne max* (L.) Merrill], cultivar RK6813RR of medium cycle, and indeterminate growth habit were used. The experiment was conducted in a randomized block design, consisting of the application of amino acids as seed treatment (ST) or leaf treatment (FA, foliar application), using eight replicates for each treatment.

For ST treatments, the amino acids were diluted in distilled water and applied to seeds at the concentration of 12 mg kg^{-1} [seed], with a volume of 4 mL kg^{-1} [seed]. This represents 26 mM for glutamate treatments and 21 mM for proline treatments. In addition, before sowing, seeds were treated with fungicide and insecticide (fipronil + piraclostrobin + methyl thiophanate) at the rate of 1 mL kg^{-1} [seeds]. The foliar application of amino acids was performed at a concentration of 120 mg ha^{-1} (0.005 mM glutamate or 0.004 mM proline), with a syrup volume of 200 L ha^{-1}, when plants were at the growth stage V_4 (four nodes on the main stem). In the control treatments, distilled water was applied to the seeds or leaves.

The plants were grown in pots of 10 dm^3 capacity, filled with washed sand. During the conduction of the experiment, the pots were irrigated daily according to the water requirement. A weekly application of nutrient solution was also applied as proposed by Johnson et al. (1957).

For the application of water-deficit levels (Table 1), the pot field capacity (CC) was initially determined, according to the substrate used in the experiment. From this result,

Table 1 Description of the amino acid and water-deficiency treatments that were used in the experiment with the RK6813RR soybean. Unipam, Patos de Minas, MG, Brazil, 2018

Treatment[1]	Water deficit (% of field (pot) capacity)
Control (ST)	WWD (80)
	LWD (60)
	HWD (40)
Glutamate (ST)	WWD (80)
	LWD (60)
	HWD (40)
Proline (ST)	WWD (80)
	LWD (60)
	HWD (40)
Control (FA)	WWD (80)
	LWD (60)
	HWD (40)
Glutamate (FA)	WWD (80)
	LWD (60)
	HWD (40)
Proline (FA)	WWD (80)
	LWD (60)
	HWD (40)

The sources used correspond to the pure amino acids Sigma Aldrich®, with optical isomerism levogiro (L-amino acid)

ST seed treatment, *FA* foliar application, *WWD* without water deficit, *LWD* low water deficit, *HWD* high water deficit the value corresponding to 80% of the field capacity (taken as water potential of − 0.05 MPa) was adopted as control, without water deficit (WWD). The other treatments with water deficiency were determined by reducing the amount of water to 60% of the field capacity (low water deficit LWD, − 1 MPa) and 40% of the field capacity (high water deficit HWD, − 1.5 MPa). The water-deficit levels were initiated at stage V_4 (four nodes on the main stem), and maintained for a period of 15 days. Substrate water content was monitored by means of daily pot weighing and also with tensiometers. The two ways of control were used together, because tensiometers stopped to function for water potentials of − 1 Mpa.

Evaluations

For the biochemical determinations, samplings were performed when the plants were in the V_6 (six nodes on the main stem) growth stage. Completely expanded leaves were collected from the middle third of the plants. The fresh material was used to determine the activity of nitrate reductase—NR (Mulder et al. 1959), urease (Hogan et al. 1983; McCullough 1967), quantitation of hydrogen peroxide—H_2O_2 (Alexieva et al. 2001), lipid peroxidation—LP (Heath and Packer 1968), and proline levels (Bates et al. 1973). Quantitation of antioxidant enzymes was performed with fresh leaves and frozen in liquid nitrogen shortly after collection. This material was extracted according to the protocol proposed by Kar and Mishra (1976). Then, the determination of total soluble protein content (Bradford 1976) and the enzymes catalase—CAT (CAT, Peixoto et al. 1999), peroxidase—POD (Teisseire and Guy 2000), and superoxide dismutase—SOD (Beauchamp and Fridovich 1971) were performed.

Root growth was evaluated at the V_6 growth stage. Two plants from each replication were used and analyzed in a computational root analysis system, using the Winrhizo® software, version 4.1, coupled to an Epson XL 10000 scanner. The analysis followed the procedures proposed by Bouma et al. (2000). The digital images were obtained at a resolution of 600 dpi. Roots were arranged completely without overlapping on acrylic vats containing 1000 dm^3 of water. The analysis was performed based on a gray tonality classification of each of the pixels that compose the image. The program establishes a gray tonality value automatically, from which it is possible to identify each plant tissue. The parameters main length root (MLR, cm), total root length (TLR, cm), projection area (PA, cm^2), root volume (cm^3), and number of secondary roots (NSR) were obtained by means of these data.

Root (RDM), stem (SDM), leaf (LDM), and pod (PDM) dry matter mass determinations were performed at the V_6 and R_8 growth stages. Two plants were used per replicate, when each organ of the plant was separately packed in paper bags, and the dry experiment was carried out using an oven with forced air circulation and with a temperature of 65 °C, until reaching a constant mass.

For yield, the plants were harvested manually considering three plants per replicate. The grains harvested from each plant were weighed in a digital scale with an accuracy of 0.01 g. The water content of the grains was determined, and the productivity was calculated with the water content corrected to 13% (0.13 g g^{-1}). The final result was presented in grams per plant.

Evaluation of the data for normality and homogeneity was made using the Shapiro–Wilk and Levene tests, respectively, both at the 5% significance level. Variance analysis was performed and, when significant, the Duncan test was applied at the 5% level of significance. Using the data that presented the highest correlation, a multivariate analysis was performed, by means of the principal components method. All analyses were carried out using statistical software SAS 9.3 (SAS Institute 2011).

Results

V_6 Growth Stage Evaluations: 15 Days After the Water-Restriction Period

Plants with no water deficit and with moderate deficit had a greater accumulation of root dry mass (RDM, Fig. 1a) and total dry mass (TDM, Fig. 1d) when proline was applied in seed treatment (ST) and on the leaves (FA). Plants without water restriction also showed higher RDM (Fig. 1a), stem (SDM, Fig. 1b), leaves (LDM, Fig. 1c), and TDM (Fig. 1c), through the application of glutamate in the seed treatment.

Under conditions of severe water deficit (high), none of the amino acids was effective for the mass increment of the plants. In addition, the use of glutamate in the seed treatment reduced the dry mass of the plants.

Amino acids did not increase the development of secondary roots (NSR) and total root length (TLR). However, the control treatment with higher water deficit presented increase of these parameters (Figs. 2a, b).

The application of glutamate in ST led to increased volume (Fig. 2c) and projection area (PA) (Fig. 2d) of the roots in plants without water restriction. In plants with low and high water deficits, the application of proline in the leaves increased the PA of the roots (Fig. 2d).

The two modes of proline application provided higher nodule formation in the roots of plants with low water deficit (NDM, Fig. 3d). In addition, under these same water conditions, the plants showed higher nitrate reductase (NR) activity by foliar application of proline (Fig. 3c), and urease,

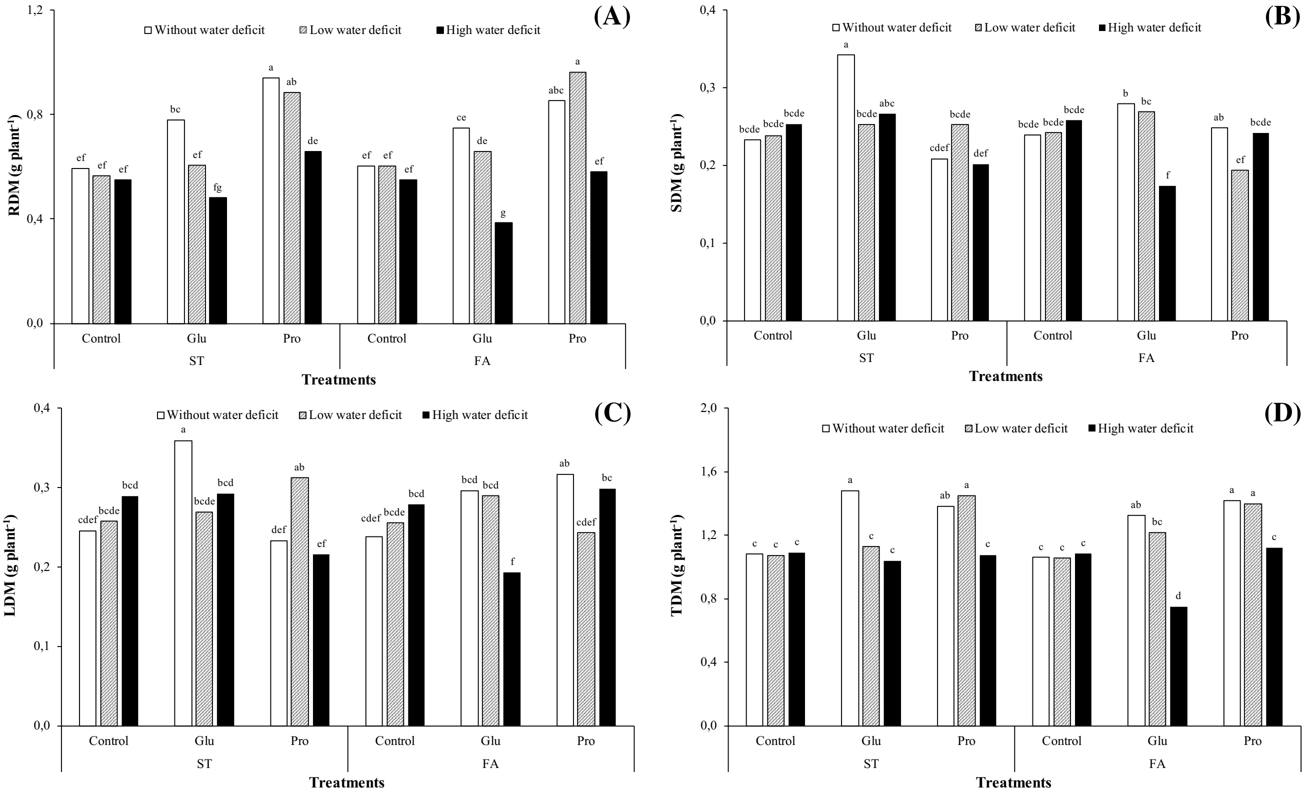


Fig. 1 Root dry mass (RDM, **a**), stem dry mass (SDM, **b**), leaf dry mass (LDM, **c**), and total dry mass (TDM, **d**) at the V_6 stage, for soybean cultivar RK6813RR, submitted to the application of glutamate (Glu) and proline (Pro) on seed treatment (ST) or foliar application (FA), associated to water-deficit levels (WD): high WD (40% of field capacity—FC), low WD (60% FC), and without WD (80% WD). Means followed by the same letters do not differ significantly from each other, using the Duncan test at 5% significance

with the application of this amino acid in the treatment of seeds (Fig. 3b).

In relation to the antioxidant metabolism, the application of glutamate on the leaves induced the increase of the enzymes peroxidase (POD) and superoxide dismutase (SOD) enzymes in plants without water deficit (Figs. 4a, b). The plants under the same water conditions presented reduction of the POD activity in the control treatment (Fig. 4a).

Plants without water restriction submitted to the application of proline in the treatment of seeds presented higher catalase (CAT) activity (Fig. 4c). Under low water deficit, this enzyme activity was reduced in plants submitted to glutamate application in the seed treatment. In contrast, this same treatment provided an increase in H_2O_2 levels in the plants (Fig. 5a).

The plants with higher water deficit had an increase in lipid peroxidation (LP) in the control and proline treatments, applied to the leaves (Fig. 4d). On the other hand, the treatment of seeds with glutamate and proline, and leaf glutamate maintained LP of the plants in a similar level, regardless of the conditions of water deficit.

The application of glutamate in the seed treatment increased the amount of proline in the leaves of the plants submitted to high water deficit (Fig. 5b), and also increased the relative water content (RWC) of the leaves (Fig. 5c. This same amino acid, when applied to leaves, provided an increase in RWC of plants submitted to low water deficit. Plants of the control treatments with high water deficit presented reduction of the relative amount of water in the leaves (Fig. 5c).

Evaluations at the R_8 Growth Stage

The highest accumulation of RDM was observed at the R_8 growth stage, as observed in V_6 for plants without water restriction, submitted to the treatment of proline to seeds (Fig. 6a). However, this same amino acid, applied to leaves, reduced RDM.

For the plants with low water deficit, lower RDM, SDM, and LDM occurred when glutamate had been applied to the seeds (Fig. 6a–c). Under these same water conditions, there were increases of LDM (Fig. 6c) and TDM (Fig. 7a) with foliar application of proline.

Plants with the highest water deficit presented higher LDM (Fig. 6c) and TDM (Fig. 7a), when glutamate was

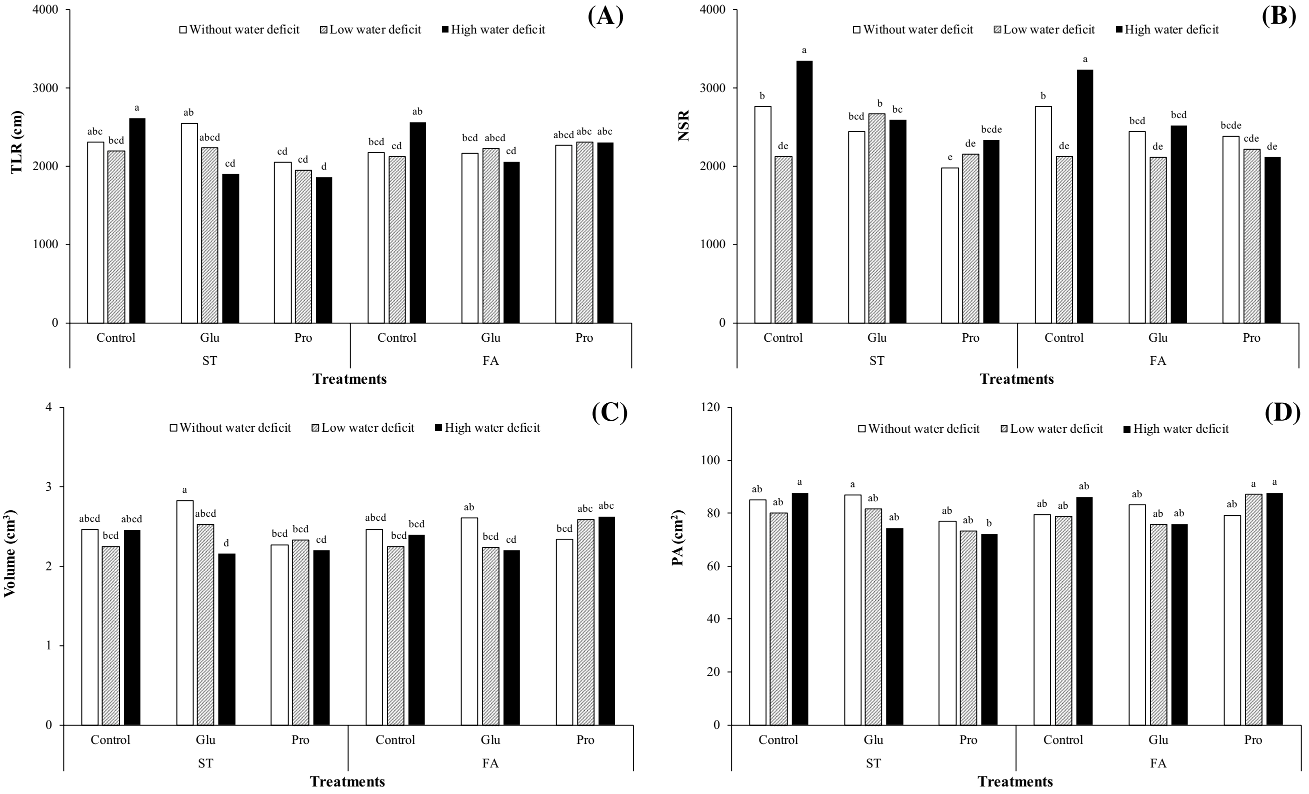


Fig. 2 Total length of root (TLR, cm plant^{-1}), number of secondary roots (NSR), root volume (cm^3 plant^{-1}), and project area (PA, cm^2 plant^{-1}), at the V_6 stage, for soybean cultivar RK6813RR, submitted to the application of glutamate (Glu) and proline (Pro) on seed treatment (ST) or foliar application (FA), associated to water-deficit levels (WD): high WD (40% of field capacity—FC), low WD (60% FC), and without WD (80% FC). Means followed by the same letters do not differ significantly from each other, using the Duncan test at 5% significance

applied to the seed (Fig. 6c), and higher PDM (Fig. 6d) with the application of glutamate in both treatments ST and FA.

The lowest number of pods (NP) and productivity occurred in plants with highest water deficit submitted to proline application on seeds (Fig. 7b, c).

A summary of the results can be observed for the application of glutamate in Fig. 8a, proline in Fig. 8b, and control in Fig. 8c.

The multivariate analysis by means of main components (PC) indicated two variables with greater representativeness: total dry mass as PC1 and productivity as PC2 (Fig. 9). In addition, there was a positive correlation between the variables productivity, nitrate reductase, relative water content, and dry mass of pods, indicating that the increment of these variables provides higher productivity. Based on this analysis, the highest productivity occurred with the use of glutamate, for the seed treatment and foliar application, under conditions of high water deficit; proline, in both modes of application, under conditions of low water deficit; and proline, for the seed treatment, in plants without water restriction.

Discussion

Plants Without Water Restriction

Plants that did not undergo water restriction at the growth, presented a more effective use of amino acids in the seed treatment (Fig. 8a). The application of 12 mg kg^{-1} [26 mM] glutamate increased stem dry mass, total plant mass, and volume and root projection area (Figs. 1b, d, 2c, d) at the V_6 stage.

The glutamate can play many roles in plants. Some of these roles have been gaining prominence in the literature as, for example, their action in the regulation of gene expression. In a hydroponic rice system, the application of 2.5 mM glutamate provided the expression of at least 122 genes in roots. Among these are amino acid permeases, phenylalanine ammonium lyase, calcium transporters, genes for systemic resistance to diseases, stress genes, and genes involved in metabolism, transport, and growth (Kan et al. 2017). For soybean, we did not find reports that demonstrate the activation of genes due to glutamate

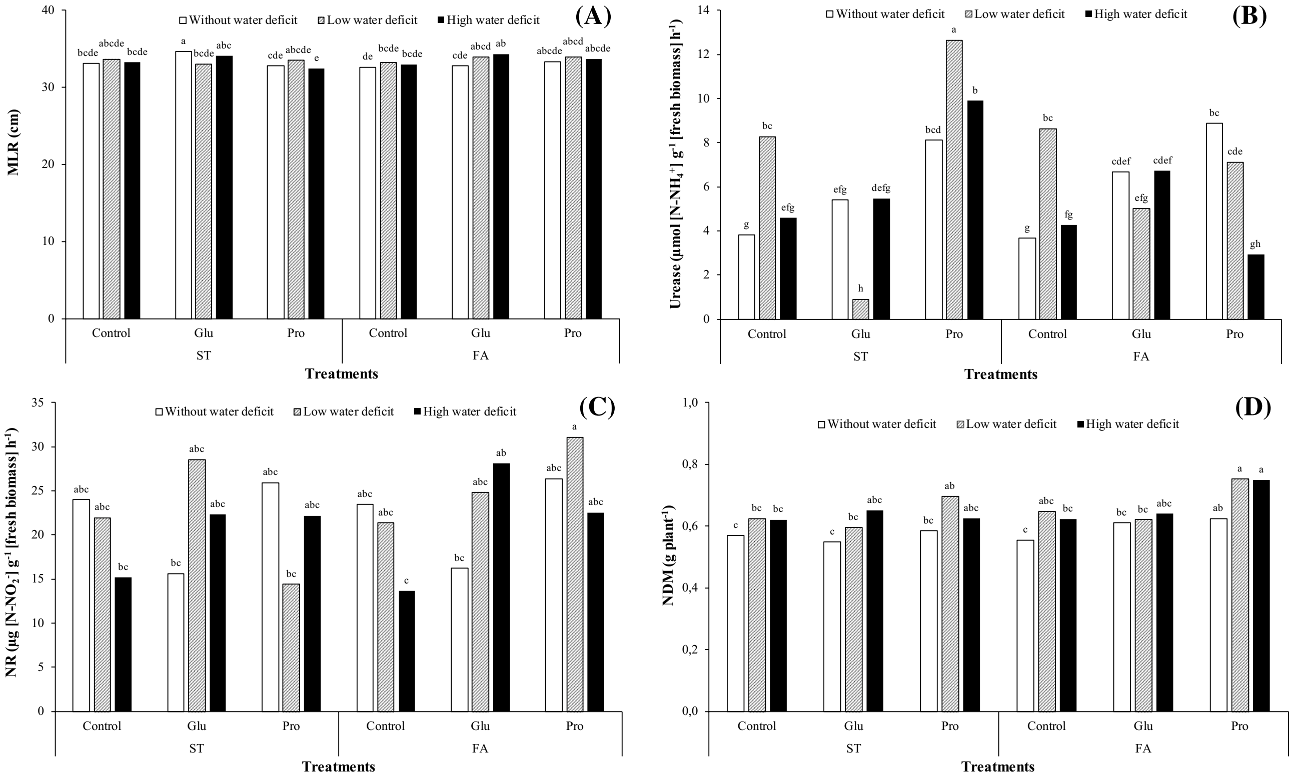


Fig. 3 Main length root (MLR, **a**), activity of the enzymes urease (**b**), nitrate reductase (NR, **c**), and nodule dry mass (NDM, **d**), at the V_6 stage, for soybean cultivar RK6813RR, submitted to the application of glutamate (Glu) and proline (Pro) for seed treatment or foliar application, associated to water-deficit levels (WD): high WD (40% of field capacity—FC), low WD (60% FC), and without WD (80% FC). Means followed by the same letters do not differ significantly from each other, using the Duncan test at 5% significance

application, however, this fact may have occurred and favored the greater accumulation of dry mass in the plants.

For Arabidopsis, application of glutamate to the root at concentrations above 1 mM promotes the reduction of the main root and increase of root branching (Walch-Liu et al. 2006). In our experiment, no difference was observed in the number of secondary roots and length of the main root, with the application of 26 mM glutamate; however, there was a larger volume and area of root projection, evidencing that this amino acid can alter the growth pattern of root. In an experiment with another soybean cultivar (NS 7901 RR), the same root growth pattern was observed with the application of 26 mM glutamate (Teixeira et al. 2018).

The change in growth pattern is explained by the role of glutamate in signaling physiological processes (Forde and Roberts 2014; Weiland et al. 2015). In roots there are several receptor-like genes (GLR), which are glutamate receptors and may activate several physiological processes (Vincill et al. 2012; Forde and Roberts 2014). These responses are dependent on plant phenotype, but include the regulations of carbon and nitrogen metabolism (Kang and Turano 2003); and the regulations of abscisic acid (ABA) biosynthesis and signaling (Kang and Turano 2003; Kang et al. 2004) and ion Ca^{2+} (Kim et al. 2001, Toyota et al. 2018). The signals received by the GLRs are converted into the increase in the concentration of calcium that is propagated through the phloem and intercellular channels called plasmodesms. Therefore, glutamate is essential for the activation of these channels eliciting defense signals that propagate through the change of cytosolic Ca^{2+} from cell to cell (Toyota et al. 2018).

In addition, glutamate inhibits the growth of the main root due to reduced mitotic divisions and induces lateral root formation (Walch-Liu and Forde 2008). However, in our experiment, there was no reduction in the development of the main root (Fig. 3a). This may have occurred for two reasons, one of which is that the response to glutamate application is dependent on the genetic characteristics of the plant (Forde and Walch-Liu 2009). The second is that in the presence of nitrate in the medium, the sensitivity to the inhibitory effect of glutamate can be altered (Walch-Liu and Forde 2008). In our experiment, a nutrient solution was applied in the presence of nitrate, which may have caused a different response of glutamate to the development of the main root.

Another positive feature of glutamate is that it can be converted into other amino acids like glutamine, alanine, serine,

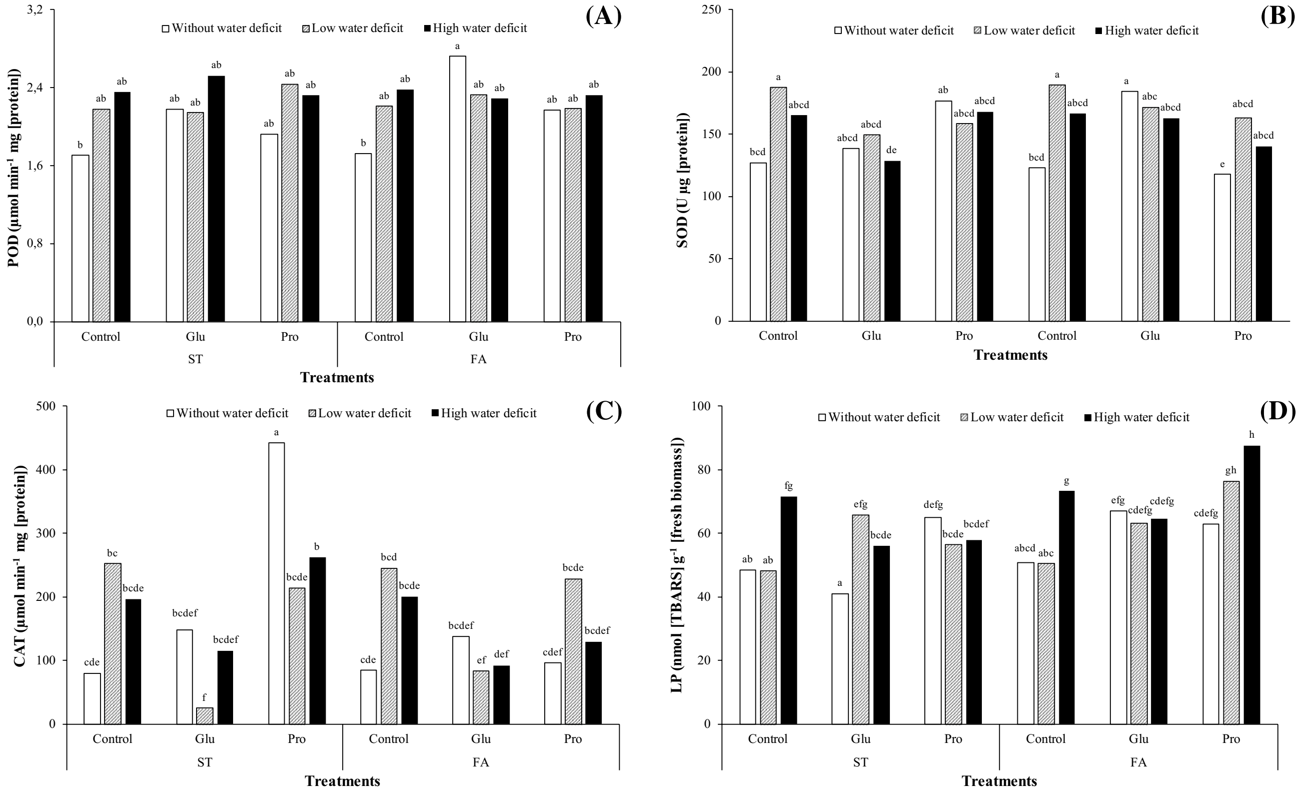


Fig. 4 Activities of the enzymes peroxidase (POD, **a**), superoxide dismutase (SOD, **b**), and catalase (CAT, **c**), and lipid peroxidation (LP, **d**), at the V_6 stage, for the soybean cultivar RK6813RR, submitted to the application of glutamate (Glu) and proline (Pro) in seed treatment or foliar application, associated to water-deficit levels (WD): high WD (40% of field capacity—FC), low WD (60% FC), and without WD (80% FC). Means followed by the same letters do not differ significantly from each other, using the Duncan test at 5% significance

and asparagine, since it is part of the production route of several amino acids. In rice, a higher accumulation of these amino acids was reported 24 h after application of 2.5 mM glutamate (Kan et al. 2017). In addition, there was a greater accumulation of gamma-amino butyric acid (GABA), a nitrogen-containing compound, which is directly bound to the metabolism of this amino acid (Kan et al. 2017).

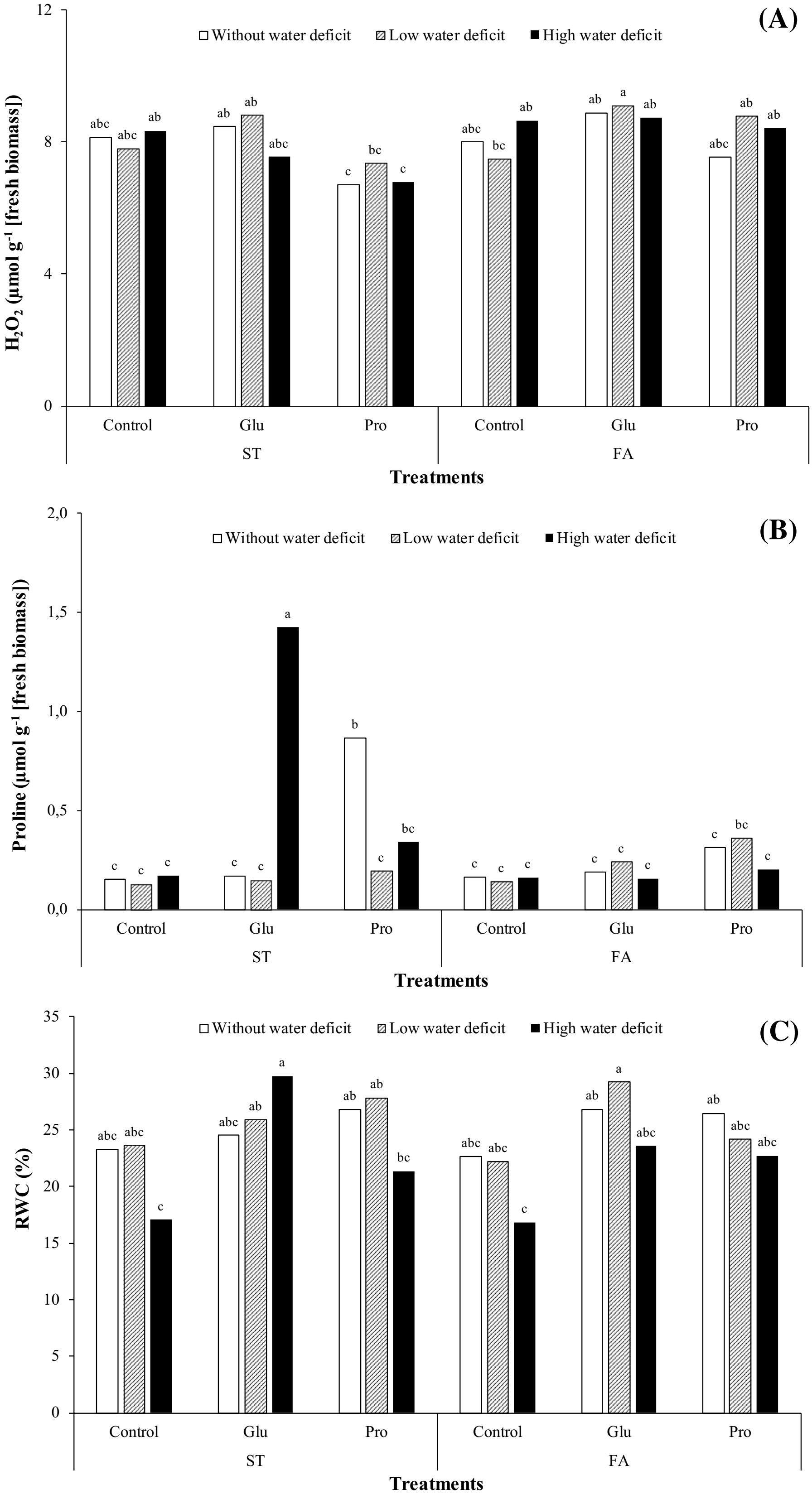
In relation to the antioxidant metabolism, the use of glutamate in the treatment of seeds provided a reduction of lipid peroxidation and, through leaf growth, increased the activities of POD and SOD enzymes (Figs. 4a, b, d). This may have occurred due to indirect effects of glutamate, which acts on the production of proline, arginine, and glutathione, compounds that may aid in metabolic routes that reduce plant stress (Mittler 2002; Gill and Tuteja 2010; Rejeb et al. 2014).

Proline application was not as effective as glutamate, and presented effect only in the seed treatment (Fig. 8b). Under these conditions, plants showed higher CAT activity (Fig. 4c), reduction of lipid peroxidation (Fig. 4d), as well as the higher proline concentration in the leaves (Fig. 5b), and higher dry mass of roots (Fig. 1a) This amino acid can act directly on the reduction of reactive oxygen species in plants. Some studies have shown that proline acts as a signaling molecule, and may induce an increase in the activity of antioxidant enzymes in response to different types of stress (Hoque et al. 2007; Carvalho et al. 2013; Rejeb et al. 2014). In Arabidopsis, the exogenous application of 1 mM proline to roots reduced the level of reactive oxygen species (ROS) (Cuin and Shabala 2007), indicating its potential to eliminate ROS.

With respect to root dry mass, more recent studies show that proline can aid to early plant development, modulating growth by acting on the root meristem (Ford et al. 2013; Biancucci et al. 2015). In Arabidopsis, proline can affect root elongation, modulating the rate of cell division (Biancucci et al. 2015); therefore, the application of proline can induce an increase in root dry mass.

Plants with no amino acid application and with high water deficit had lower total dry mass, dry mass of nodules, and lower peroxidase activity (Fig. 8c). This shows the benefits provided by the application of proline or glutamate. In an experiment developed with *Avena sativa*, the authors observed that when plants were subjected to water stress

Fig. 5 Hydrogen peroxide content (H_2O_2, **a**), proline content (**b**), and relative water content (RWC, **c**), at the V_6 stage, in the soybean cultivar RK6813RR, submitted to the application of glutamate (Glu) and proline (Pro) of seed treatment or foliar application, associated to water-deficit levels (WD): high WD (40% of field capacity—FC), low WD (60% FC), and without WD (80% FC). Means followed by the same letters do not differ significantly from each other, using the Duncan test at 5% significance



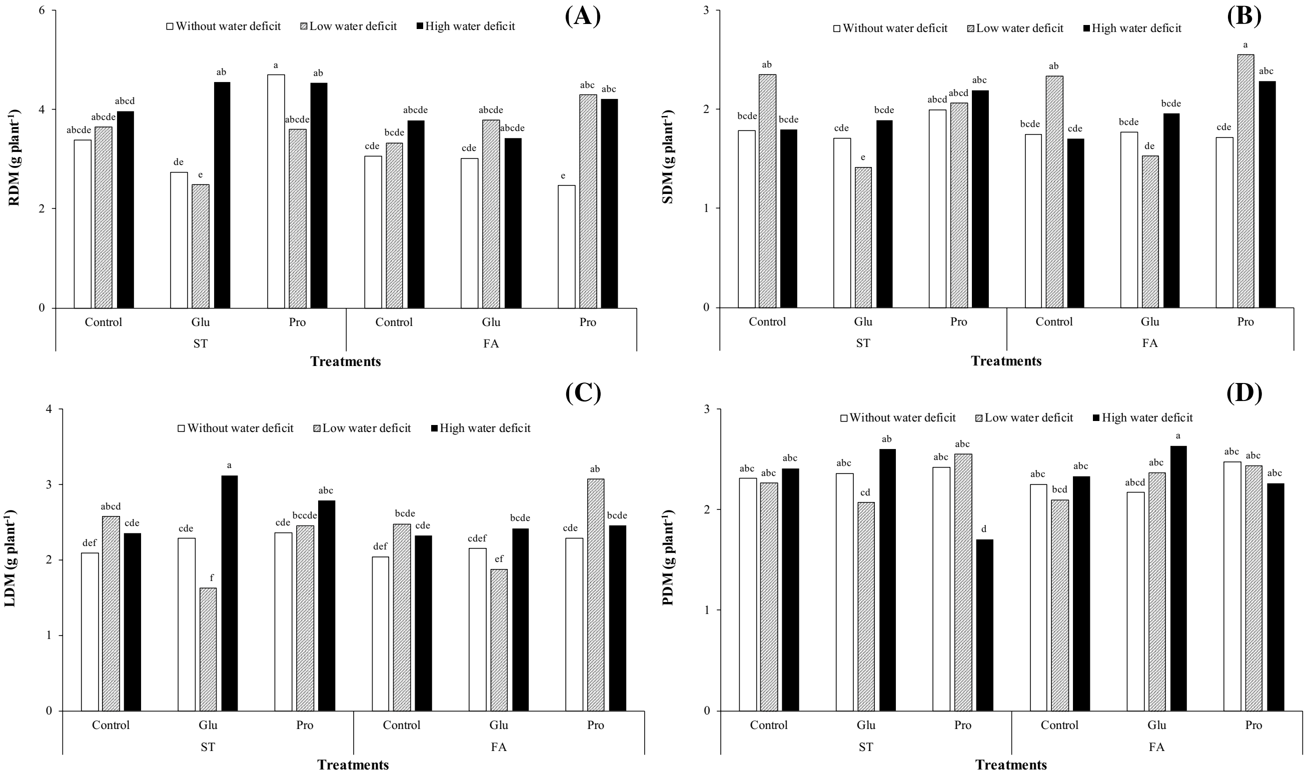


Fig. 6 Root dry mass (RDM, **a**), stem dry mass (SDM, **b**), leaf dry mass (LDM, **c**), and pod dry mass (PDM, **d**), at the R_8 stage, for the soybean cultivar RK6813RR, submitted to the application of glutamate (Glu) and proline (Pro) on seed treatment or foliar application, associated to water-deficit levels (WD): high WD (40% of field capacity—FC), low WD (60% FC), and without WD (80% FC). Means followed by the same letters do not differ significantly from each other, using the Duncan test at 5% significance

(− 2.7 bars), they showed reduction of the activity of the enzymes: CAT, SOD, and ascorbate peroxidase, besides the lower production of free amino acids, including proline, due to the reduction of the activity of the aminotransferases (Ahanger et al. 2015). These characteristics may lead to lower resistance of the plant to water-stress conditions, which may affect the lower growth and development of plants.

Low Water Deficit in Plants

Under conditions of low water deficit, the application of glutamate was not very positive. Seed treatment with 26 mM of this amino acid increased only the number of secondary plant roots (NSR, Fig. 2b). In Arabidopsis from the exogenous application of glutamate at concentrations between 1 and 50 µM a reduction in the development of the main root was shown in relation to the control, and induction of secondary root formation (Walch-Liu and Forde 2007). This reduction occurs due to the inhibition of cell division of the apical meristem of the main root, and the other regions of the root are not sensitive to glutamate, which provides an increase in the development of secondary roots (Forde 2014).

Plants derived from seeds treated with glutamate presented lower accumulation of total dry mass (TDM, Fig. 1d), 15 days after the period of water deficit, as well as the plants of the control treatment, which shows that glutamate, under conditions of low deficit did not provide benefits on the accumulation of dry mass of the plants. The water deficit may lead to a reduction of the photosynthetic activity, in addition to the reduction of biomass production (Baroowa and Gogoi 2013). In addition, the magnitude of the changes depends on the duration and intensity of the stress, the genetic material and the stage of development of the plant (Farooq et al. 2016).

When the application of glutamate was performed on the leaves, a reduction of catalase activity (CAT, Fig. 4c) occurred and increased the content of hydrogen peroxide and relative water content (RWC, Fig. 5c). This latter characteristic may be linked to an indirect effect of glutamate, which is the production of GABA, a nonprotein amino acid. Some studies report that GABA is a compound that can assist in the maintenance of membrane integrity under stress conditions (Roychoudhury and Chakraborty 2013; Roychoudhury

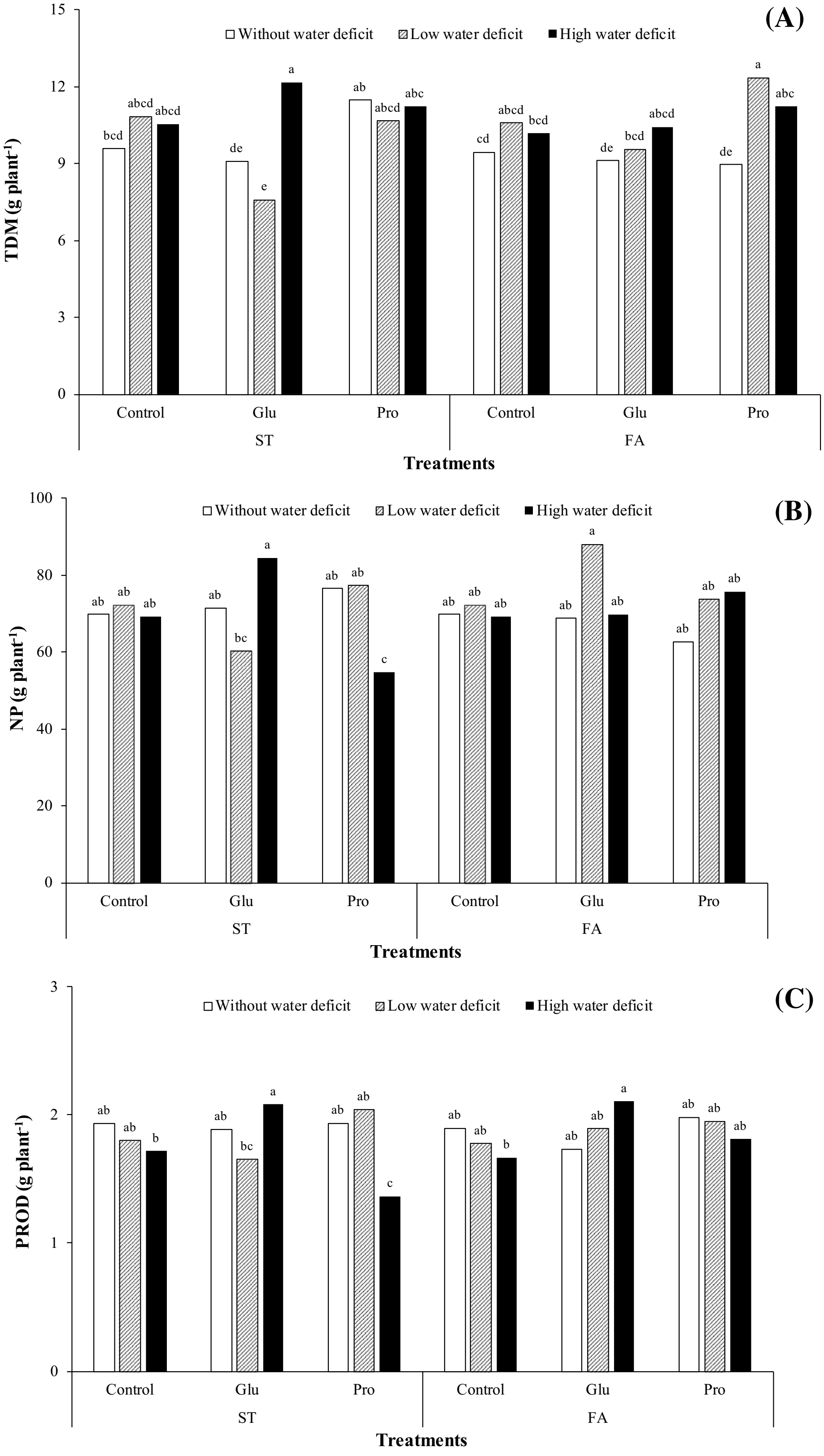


Fig. 7 Total dry mass (TDM, **a**), number of pod (NP, **b**), and productivity (PROD, **c**), at R_8 stage, for the soybean cultivar RK6813RR, submitted to the application of glutamate (Glu) and proline (Pro) on seed treatment or foliar application, associated to water-deficit levels (WD): high WD (40% of field capacity—FC), low WD (60% FC), and without WD (80% FC). Means followed by the same letters do not differ significantly from each other, using the Duncan test at 5% significance

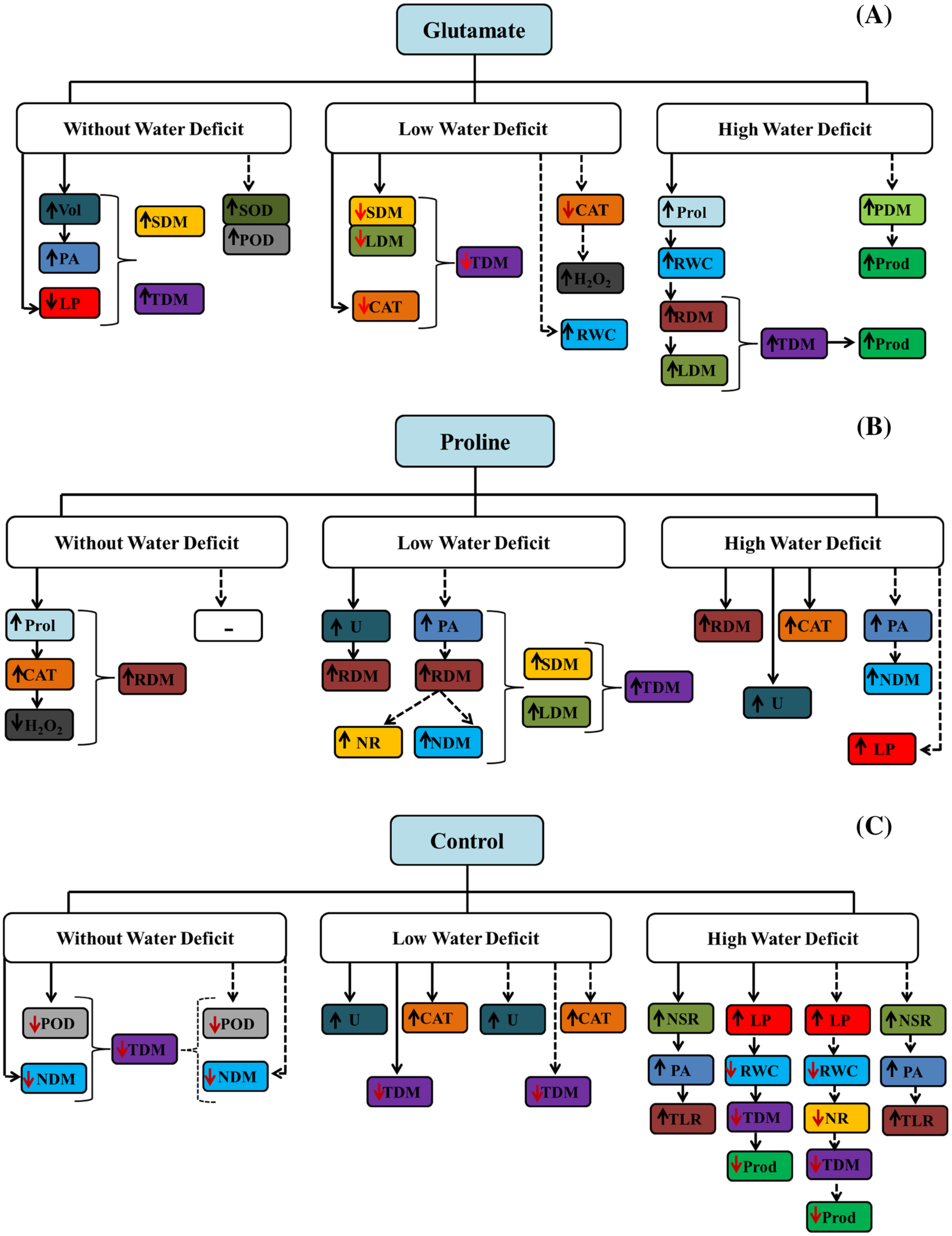
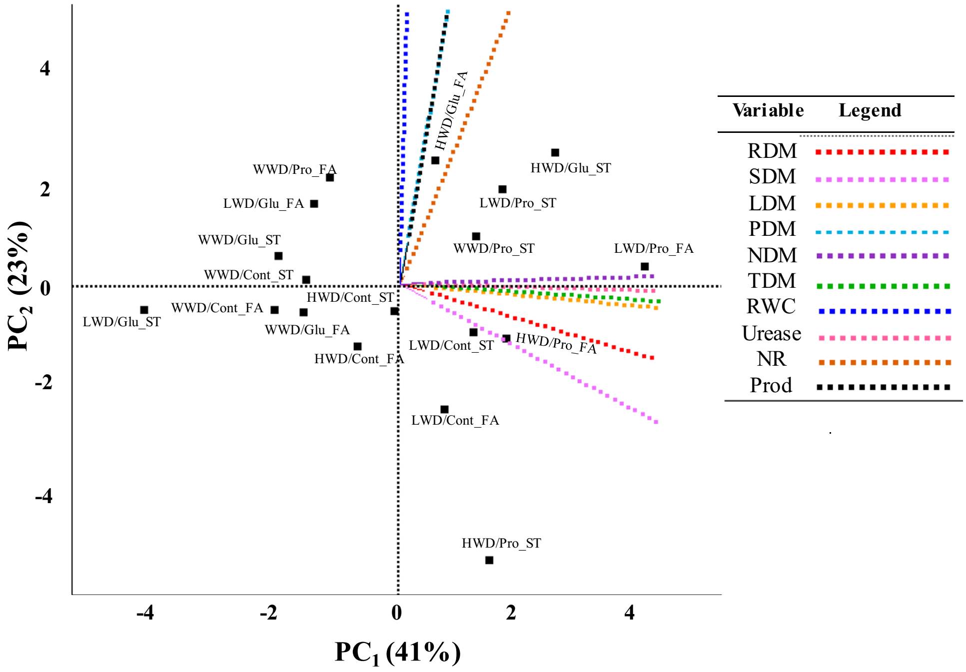


Fig. 8 Summary of the effects of glutamate (**a**), proline (**b**), and control (**c**) applied in the seed treatment (continuous line) or foliar application (dotted line), for the soybean cultivar RK6813RR, submitted to water-deficit levels: high water deficit (HWD 40); low water deficit (LWD 60); and without water deficit (WWD 80). CAT—catalase, H_2O_2—hidrogen peroxide, LP—lipid peroxidation, LDM—leaf dry mass, NDM—nodule dry mass, NSR—number of secundary roots, NR—nitrate reductase, PDM—pod dry mass, Prol—proline content, POD—peroxidase, PA—project area of roots, Prod—productivity, RDM—root dry mass, RWC—relative water content, SOD—superoxide dismutase, SDM—steam dry mass, TDM—total dry mass, TLR—total length roots, U—urease, Vol—volume of roots

Fig. 9 Byplot obtained from the principal components analysis (PCA) of the results of the variables RDM- root dry mass, SDM—steam dry mass, LDM—leaf dry mass, PDM—pod dry mass, NDM—nodule dry mass, TDM—total dry mass, RWC—relative water content, urease, NR—nitrate reductase, and Prod—productivity, evaluated in the soybean crop submitted to the application of amino acids (Glutamate—glu or proline—pro) as seed treatment—ST or foliar application—FA, associated to water-deficit levels: high—(HWD 40), low (LWD 60), and without (WWD 80). Cont—control



et al. 2015), which may have provided retention of RWC during water deficit. With regard to CAT activity, it is not clear whether this reduction occurred due to the application of glutamate or not. However, in a field experiment with soybean crop (without water deficit), a reduction of CAT was also observed after foliar application of 12 mg ha^{-1} of glutamate (Teixeira et al. 2017a). Therefore, what can be said is that the reduction of the activity of CAT was responsible for the increase of the H_2O_2 content, because this enzyme is one of the responsible for the degradation of this reactive oxygen species (Gill and Tuteja 2010).

On the other hand, under low water deficit, the application of proline (0.004 mM) was more positive when carried out on the leaves, where it increased nitrate reductase enzyme activity (Fig. 3c), leaf dry mass (Fig. 7a), in addition to the increase of the projection area of the roots (Fig. 2d). Some studies have shown that proline may play the role of protecting proteins and enzymes under stress conditions (Sharma and Dubey 2005; Hayat et al. 2012; Liang et al. 2013). In an in vitro experiment, application of 1 mM proline protected the nitrate reductase from rice plants under water stress (Sharma and Dubey 2005). Exogenous application of 30 mM proline in two-week-old *Zea mays* exposed to water stress (60% of field capacity) increased growth and also the maintained nutritional status by promoting uptake of K^+, Ca^{2+}, P and N (Ali et al. 2008). Gadallah (1999) reported that the content of soluble sugars, and soluble proteins increased significantly when proline was supplied to the leaves of *Vicia faba* under saline stress conditions. There are, however, still few studies that show if these previously listed effects occur in soybean, due to application of proline. However, if this occurs, it may be the justification for the increment of dry mass of the plants of our experiment.

In the treatment of seeds, proline only increased urease and root dry mass (Fig. 6a). This amino acid can aid the early development of plants, modulating plant growth by acting on the root meristem. In Arabidopsis, proline may affect root elongation modulating the rate of cell division (Biancucci et al. 2015).

Therefore, when evaluating proline application, it is suggested that the effect from amino acid is more effective when applied directly to the leaves, in the period next to the water deficit, making the signaling promoted by this amino acid to provide more benefits. On the other hand, the application of proline in the treatment of seeds does not seem to promote residual systemic effect for a long period and, when the water stress was induced, there were no signs of this amino acid to increase the tolerance for stress. Another hypothesis is that in the case of seed treatment, the greater amount of proline may have led to ProDH proline degradation to prevent damage to plant cells by excess proline, and proline accumulation as a compatible solute is suppressed by feedback. Thus, proline tolerance to water stress as a compatible solute can be suppressed.

High Water Deficit in Plants

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Therefore, when evaluating proline application, it is suggested that the effect coming from amino acid is more effective when applied directly to the leaves, in the period next to the water deficit, making the signaling promoted by this amino acid provide more benefits. On the other hand, the application of proline in the treatment of seeds does not seem to promote residual systemic effect for a long period and, when the water stress was induced, there were no signs of this amino acid to increase tolerance to stress.

The application of glutamate in the seed treatment provided good results in the plants that were submitted to the high water deficit, increasing the proline content (Fig. 5b) and RWC in the leaves (Fig. 5c), RDM (Fig. 6a), LDM (Fig. 6c), and TDM (Fig. 7a); and productivity (Fig. 7c).

Glutamine can help plant resistance to stress by being a precursor of glutathione and chlorophyll, and by being involved in the production of other amino acids such as glutamine, arginine, and proline. The accumulation of this last amino acid from the application of glutamate was observed in our experiment (Fig. 5b). Under water-deficit conditions, proline is synthesized from glutamate by the enzymes P5CS and P5CR (Yoshiba et al. 1997). Therefore, when the amount of glutamate is increased by treatment with this amino acid, proline accumulation as a compatible solute may be promoted, and water-stress tolerance may be strengthened. Proline plays a key role in resistance to water deficit (Gill and Tuteja 2010), acting as an osmoprotector, and its accumulation reduces osmotic potential and therefore helps in restoration and maintenance of the potential gradient between the plant cell and the external soil solution (Ahanger et al. 2017). This amino acid also acts when saline stresses prevail, and also stresses caused by excess metals, acting as a chelator of metals avoiding the production of OH^- (Smirnoff and Cumbes 1989; Ashraf and Foolad 2007). Increased accumulation of osmotic constituents like proline mediated the active water uptake resulting in the maintenance of RWC (Ahanger et al. 2017), characteristic that was observed in our experiment (Fig. 5c).

Furthermore, application of glutamate can provide expression of the GDC1 gene responsible for the conversion of glutamate into GABA (Kan et al. 2017). GABA is a nonprotein amino acid that accumulates in plants under conditions of biotic and abiotic stresses, and modulates plant growth. This can occur through the regulation of carbon metabolism or by signaling pathways (Bouché et al. 2004; Ramesh et al. 2015; Kan et al. 2017). This characteristic may have favored an increase in the accumulation of dry mass by the plants (Figs. 6a, c and 7a).

All these benefits provided by the application of glutamate in the seed treatment led to higher plant productivity (Figs. 7c and 8a). Similar results were also observed in the soybean crop, where the application of glutamate in seeds or leaves increased root development, nutrient uptake, and, consequently, productivity (Soares et al. 2016; Teixeira et al. 2017a).

The application of glutamate to the leaves increased PDM and productivity. However, it did not affect any other of the evaluated physiological characteristics, which makes it difficult to explain the event. Probably, the role of this amino acid as a marker may have increased some metabolic pathway of the plant, leading to increased productivity. For wheat plants (*Triticum aestivum* L.) it was observed that foliar application of amino acid products increases plant resistance to water deficiency and also leads to increased productivity (Hammad and Ali 2014).

Proline was less effective than the application of glutamate in plants induced to a period of high water deficit, as already observed for plants without water restriction. Seed treatment with this amino acid increased CAT (Fig. 4c), Urease (Fig. 3b), and RDM (Fig. 6a). Foliar use of proline increased PA (Fig. 2d) and NDM (Fig. 3d). However, in other crops, as *Triticum aestivum*, the use of proline at concentrations of 25 and 50 mM increased the activity of antioxidant enzymes, dry mass of plants, and crop yield (Kibria et al. 2016). In our experiment, only 21 and 0.004 mM proline were used on seeds and leaves, respectively. These concentrations may have been low for plants induced to high water deficiency.

Plants with no application of amino acids indicate that the water deficit affected the dry mass, RWC, NR and, consequently, reduced productivity (Fig. 8c). This may be due to the reduction of the photosynthetic activity, as well as the reduction of biomass production, as discussed previously.

Results suggest that more studies should be developed at genetic levels, aiming to evaluate the role of glutamate and proline in the regulation of soybean gene expression with and without water restriction. In this way, it will be possible to understand more clearly how these amino acids can signal changes in plants.

Conclusion

- In plants without water restriction, the use of glutamate on seeds was more effective in relation to proline and control;
- Plants with moderate water deficit showed only response to proline application to leaves;
- In plants subjected to high water deficit, glutamate applied to the seeds increased the relative water content, dry mass of the plants, and productivity. Foliar application of this amino acid also increased plant productivity;

- In general, it can be said that the application of glutamate and proline protects the soybean crop from water stresses mainly due to an improvement in the health of the crop.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

References

Ahanger MA, Agarwal RM, Tomar NS, Shrivastava M (2015) Potassium induces positive changes in nitrogen metabolism and antioxidant system of oat (*Avena sativa* L. cultivar Kent). J Plant Interact 10(1):211–223

Ahanger MA, Tomar NS, Tittal M, Argal S, Agarwal RM (2017) Plant growth under water/salt stress: ROS production; antioxidants and significance of added potassium under such conditions. Physiol Mol Biol Plants 23(4):731–744

Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24:1337–1344

Ali Q, Ashraf M, Shahbaz M, Humera H (2008) Ameliorating effect of foliar applied proline on nutrient uptake in water stressed maize (*Zea mays* L.) plants. Pak J Bot 40:211–219

Ashraf M, Foolad MR (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59:206–216

Baroowa B, Gogoi N (2013) Biochemical changes in two *Vigna* spp. During drought and subsequent recovery. Indian J Plant Physiol 18:319–325

Bates L, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and applicable to acrylamide gels. Anal Biochem 44(1):276–287

Biancucci M, Mattioli R, Moubayidin L, Sabatini S, Costantino P, Trovato M (2015) Proline affects the size of the root meristematic zone in *Arabidopsis*. BMC Plant Biol 15:263

Bouché N, Fait A, Zik M, Fromm H (2004) The root specific glutamate decarboxylase (GAD1) is essential for sustaining GABA levels in *Arabidopsis*. Plant Mol Biol 55:315–325

Bouma TJ, Nielson KL, Koutstaal BAS (2000) Sample preparation and scanning protocol for computerized analysis of root length and diameter. Plant Soil 218:185–196

Bradford MM (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem 72:248–254

Carvalho K, de Campos MKF, Domingues DS, Pereira LFP, Vieira LGE (2013) The accumulation of endogenous proline induces changes in gene expression of several antioxidant enzymes in leaves of transgenic *Swingle citrumelo*. Mol Biol Rep 40:3269–3279

Cuin TA, Shabala S (2007) Compatible solutes reduce ROS-induced potassium efflux in Arabidopsis roots. Plant Cell Environ 30:875–885

Farooq M, Gogoi N, Barthakur S, Baroowa B, Bharadwaj N, Alghamdi SS, Siddique KHM (2016) Drought stress in grain legumes during reproduction and grain filling. J Agron Crop Sci 203:1–23

Forde BG (2014) Glutamate signalling in roots. J Exp Bot 65(3):779–787

Forde BG, Roberts MR (2014) Glutamate receptor-like channels in plants: a role as amino acid sensors in plant defense? F1000Prime Rep 37:6–37

Forde BG, Walch-Liu P (2009) Nitrate and glutamate as environmental cues for behavioural responses in plant roots. Plant Cell Environ 32:682–693

Forde BG, Cutler SR, Zaman N, Krysan PJ (2013) Glutamate signalling via a MEKK1 kinase-dependent pathway induces changes in *Arabidopsis* root architecture. Plant J 75:1–10

Gadallah MAA (1999) Effects of proline and glycine betaine on *Vicia faba* responses to salt stress. Biol Plant 42:249–257

Gill S, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930

Hammad SAR, Ali OAM (2014) Physiological and biochemical studies on drought tolerance of wheat plants by application of amino acids and yeast extract. Ann Agric Sci 59:133–145

Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A (2012) Role of proline under changing environments: a review. Plant Signal Behav 7(11):1456–1466

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Hogan ME, Swift IE, Done HJ (1983) Urease assay and ammonia release from tissue. Phytochemistry 22:663–667

Hoque MAOE, Banu MNA, Nakamura Y, Shimoishi Y, Murata Y (2007) Exogenous proline mitigates the detrimental effects of salt stress more than the betaine by increasing antioxidant enzyme activities. J Plant Physiol 164:553–561

Johnson CM, Stout PR, Broyer TC, Carlton AB (1957) Comparative chlorine requirement of different plant species. Plant Soil 8:337–353

Kan CC, Chung TY, Wu HY, Juo YA, Hsieh MH (2017) Exogenous glutamate rapidly induces the expression of genes involved in metabolism and defense responses in rice roots. BMC Genom 18:186

Kang JM, Turano FJ (2003) The putative glutamate receptor 1.1 (AtGLR1.1) functions as a regulator of carbon and nitrogen metabolism in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 100:6872–6877

Kang JM, Mehta S, Turano FJ (2004) The putative glutamate receptor 1.1 (AtGLR1.1) in Arabidopsis thaliana regulates abscisic acid biosynthesis and signaling to control development and water loss. Plant Cell Physiol 45:1380–1389

Kar M, Mishra D (1976) Catalase, peroxidase, and polyphenol oxidase activities during rice leaf senescence. Plant Physiol 57:315–319

Kibria MG, Farzana K, Matin MdA, Hoque MdA (2016) Mitigating water stress in wheat (BARI Gom-26) by exogenous application of proline. Fundam Appl Agric 1(3):118–123

Kim SA, Kwak JM, Jae SK, Wang MH, Nam HG (2001) Overexpression of the AtGluR2 gene encoding an arabidopsis homolog of mammalian glutamate receptors impairs calcium utilization and sensitivity to ionic stress in transgenic plants. Plant Cell Physiol 42:74–84

Liang X, Zhang L, Natarajan SK, Becker DF (2013) Proline mechanisms of stress survival. Antioxid Redox Signal 19(9):998–1011

McCullough H (1967) The determination of ammonia in whole blood by direct colorimetric method. Clin Chim Acta 17:297–298

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Mulder EG, Boxma R, Veen WLV (1959) The effect of molybdenum and nitrogen deficiencies on nitrate reduction in plant tissues. Plant Soil 10:335–355

Peixoto HPP, Cambraia J, Sant'ana R, Mosquim PR, Moreira AM (1999) Aluminum effects on lipid peroxidation and the activities of enzymes of oxidative metabolism in sorghum. Revista Brasileira de Fisiologia Vegetal 11(3):137–143

Sas Institute (2011) SAS/STAT statistical analysis system manual (v.9.3). SAS Institute, Cary

Ramesh SA, Tyerman SD, Xu B, Bose J, Kaur S, Conn V et al (2015) GABA signaling modulates plant growth by directly regulating the activity of plant-specific anion transporters. Nat Commun 6:7879

Rejeb KB, Abdelly C, Savouré A (2014) How reactive oxygen species and proline face stress together. Plant Physiol Biochem 80:278–284

Roychoudhury A, Chakraborty M (2013) Biochemical and molecular basis of varietal difference in plant salt tolerance. Annu Rev Res Biol 3:422–454

Roychoudhury A, Banerjee A, Lahiri V (2015) Metabolic and molecular-genetic regulation of proline signalling and its cross-talk with major effectors mediates abiotic stress tolerance in plants. Turk J Bot 39:887–910

Sharma P, Dubey RS (2005) Modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress: role of osmolytes as enzyme protectant. J Plant Physiol 162:854–864

Smirnoff N, Cumbes QJ (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28:1057–1060

Soares LH, Dourado-Neto D, Fagan EB, Teixeira WF, Reis MR, Reichardt K (2016) Soybean seed treatment with micronutrients, hormones and amino acids on physiological characteristics of plants. Afr J Agric Res 11(35):3314–3319

Teisseire H, Guy V (2000) Copper-induced changes in antioxidant enzymes activities in fronds of duckweed (*Lemna minor*). Plant Sci 153:65–72

Teixeira WF, Fagan EB, Soares LH, Umburanas RC, Reichardt K, Dourado-Neto D (2017a) Foliar and seed application of amino acids affects the antioxidant metabolism of the soybean crop. Front Plant Sci 8:1–14

Teixeira WF, Fagan EB, Soares LH, Soares JN, Reichardt K, Dourado-Neto D (2017b) Seed and foliar application of amino acids improve variables of nitrogen metabolism and productivity in soybean crop. Front Plant Sci 9:1–12

Teixeira WF, Fagan EB, Soares LH, Cabral EMA, Dourado-Neto D (2018) Changes in root architecture after amino acid application in a soybean crop. J Agric Sci 11(1):325–334

Toyota M, Spencer D, Sawai-Toyota S, Jiaqi W, Zhang T, Koo AJ, Howe GA, Gilroy S (2018) Glutamate triggers long-distance, calcium-based plant defense signaling. Science 14(361):1112–1115

Vincill ED, Bieck AM, Spalding EP (2012) Ca^{2+} conduction by an amino acid-gated ion channel related to glutamate receptors. Plant Physiol 159:40–46

Walch-Liu P, Forde BG (2007) L-Glutamate as a novel modifier of root growth and branching: what's the sensor? Plant Signal Behav 2:284–286

Walch-Liu P, Forde BG (2008) Nitrate signaling mediated by the NRT1.1 nitrate transporter antagonises l-glutamate-induced changes in root architecture. Plant J 54:820–828

Walch-Liu P, Liu LH, Remans T, Tester M, Forde BG (2006) Evidence that L-glutamate can act as an exogenous signal to modulate root growth and branching in *Arabidopsis thaliana*. Plant Cell Physiol 47(8):1045–1057

Weiland M, Mancuso S, Baluska F (2015) Signalling via glutamate and GLRs in *Arabidopsis thaliana*. Funct Plant Biol 43:1–25

YoshibaY Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K (1997) Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol 38(10):1095–1102