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ANTIOXIDATIVE DEFENSES OF SOYBEAN [*Glycine max* (L.) Merr. cv. Grobogan] AGAINST PURPLE NUTSEDGE (*Cyperus rotundus* L.) INTERFERENCE DURING DROUGHT STRESS

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INTRODUCTION

Soybeans are commonly cultivated at the end of the rainy season in Indonesia. Consequently, weed problems usually arise at the same time as low availability of soil water causing drought stress to the plant. Soybean cv Grobogan, a local soybean cultivar from Grobogan district, Central Java province, Indonesia, experiences similar problem during cultivating season (Anonymous, 2010). Direct negative impacts of weeds on cultivated plants are via allelopathy and competition toward water, nutrient and light sources (Qasem and Foy, 2001; Morvillo *et al*. 2011). In nature, the phenomena of allelopathy and competition are very hard to be separate, hence the term "interference" is used, which covers both definitions (Qasem and Foy, 2001; Junaedi *et al*., 2006).

Allelopathy is a mechanism of interaction between two different plants which can have either a positive or negative influence via production and release of secondary metabolites (allelochemicals). However, observation results indicate that allelopathy mostly inhibits the target organism (Weir *et al*., 2004; Gniazdowska and Bogatek, 2005; Narwal and Sampietro, 2009). Purple nutsedge is an important weed at soybean plantation sites since this weed is very difficult to be controled. Production loss caused by purple nutsedge up to 89% had been reported (Kavitha *et al*., 2012).

As a sessile organism, the plant has developed various strategies for avoiding, tolerating or adapting to a diverse range of unfavorable environmental conditions (Perez-Clemente *et al*., 2013). In nature, it is very rare that a plant is only exposed to a single stress factor (Pedrol *et al*., 2006). However, plant responses to multiple stress usually are different compared to that of single stress factor. Hence, research results achieved by addressing various single stresses independently do not represent the real conditions that occur in nature (Akinson and Urwin, 2012).

Reactive Oxygen Species (ROS) is a group of free radicals formed as a by-product of plant metabolism. Under normal conditions, this compound is produced at low quantities and acts as a secondary messenger of intracellular signal, which mediates various plant responses towards biotic and abiotic conditions. However, various biotic and abiotic stresses trigger the synthesis of large amounts of ROS. This condition often puts the plant into oxidative stress (Apel and Hirt, 2004; Bhattacharjee, 2005). Oxidative stress is a condition that

may cause cell damage due to oxidation of lipids, proteins and DNA (Mittler, 2002; Gill andTuteja, 2010). Lipid peroxidation by ROS occurs on phospholipid of non-saturated fatty acids, the components of cell membrane and organelles. This phenomenon directly increases cell membrane instability and permeability, as well as producing lipid radicals which bind to protein and DNA and damage lipid radicals-protein and DNA bounds (Sharma *et al*., 2012). ROS may also cause oxidation of protein by modifying protein covalent bond. The main targets of ROS are proteins with sulfur and those from the thiol group. Oxidation of protein is usually indicated by protein carboxylation. ROS can trigger oxidation of deoxyribose, damage of the DNA chain and deletion of nucleotides. Furthermore, DNA damage affects overall plant growth and development (Gill and Tuteja, 2010; Sharma *et al*., 2012).

Plants develop antioxidan defence mechanism systems which include enzymatic and non-enzymatic antioxidat. Both systems play important roles in protecting the plant from damage caused by oxidative stress by scavenging ROS. In addition, the antioxidant system may also be able to control the amount of ROS to not detrimental level to the plant (Mittler, 2002; Gill and Tuteja, 2010; Akinson and Urwi, 2012). Sharma *et al*. (2012) mentioned that the ability of antioxidant defence systems to reduce ROS indicates plant tolerance towards various environmental stresses. The enzymatic components of the antioxidant defence system consist of various antioxidant enzymes such as superoxide dismutases (SOD), catalase (CAT), guaiacol peroxidase (GPX); as well as enzymes involved in the gluthatione-ascorbate cycle, such as ascorbate peroxidases (APX), monodehydroascorbate (DHR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). Non-enzymatic components of the antioxidant defence system are compounds of cellular redox buffer, such as ascorbate, glutathione, tocopherol, carotenoid and phenolic compounds (Sharma *et al*., 2012). In addition, proline plays a role as antioxidant and osmoliticum (Gill and Tuteja, 2010).

The objectives of this research were to understand the antioxidative defenses of soybean cv Grobogan against multiple stress factor treatments, i.e. purple nutsedge interference (biotic stress) and drought stress (abiotic stress).

MATERIALS AND METHODS

Materials and equipment: Soybean [*Glycine max* (L.) Merr. cv. Grobogan] seeds were obtained from Balai Penelitian Kacang-Kacangan dan Umbi-Umbian (BALITKABI) Malang, East Java. Purple nutsedge (*Cyperus rotundus* L.) tubers were collected from the field of the Tinjomoyo village, Semarang, Central Java. The main equipments used in this reseach were centrifuge and spectrophotometer.

Research design: This research was designed factorially using Completely Randomized Design (CRD) with two factors, i.e. level of drought stress (P_0 = fraction of transpirable soil water (FTSW) 1/control), P_1 = FTSW0.5/ mild stress and P_2 = FTSW0.25 / severe stress) and level of purple nutsedge interference (T_1 = 0 purple nutsedge per pot /control), T_1 = three purple nutsedge per pot and T_2 = six purple nutsedge per pot). Each treatment unit was made in five replicated.

Methods:

Planting and treatment: Soybean seeds and purple nutsedge tubers were selected based on uniformity of size and weight. Furthermore, after purple nutsedge tubers had sprouted, only tubers with one buds were used. Seedlings of soybean and purple nutsedge were planted at the same time in plastic pots (diameter 25 cm) containing 3 kg of latosol soil with base fertilizer (1 g TSP; 0.5 g KCL and 0.3 g urea). Each pot was planted with one soybean seedling and three or six of purple nutsedge tubers according to the treatments. Drought stress treatment was started two weeks after planting and terminated three weeks after treatment.

Drought stress treatment was determined based on the value of FTSW (Hainemann *et al*., 2011). Quantification of drought stress treatment was obtained by measuring the total weight of pot and plants at FTSW 1 (control) = 4.2 kg, FTSW 0.5 = 3.8 kg and FTSW 0.25= 3.6 kg. Watering was performed every day and the water volume used was determined by weighing the pot and its contents until the total weight was equal to the treated unit.

Determination of anti-oxidative enzyme activity:

Extraction: Enzyme extraction was carried out based on the protocol developed by Jiang and Zhang (2002) with some modification. Soybean leaf (0.3 g) was frozen in liquid nitrogen and subsequently ground using mortar and pestle. Furthermore, the sample was homogenized by adding three mL sodium phosphate buffer (50mMpH 7.8) containing 1 mM EDTA and 2% polyvinylpyrrolidone (PVP). Furthermore, homogenate was centrifuged at 12,000 g for 20 minutes at 4°C. Supernatant obtained was the enzyme extract used for enzyme activity test. The amount of protein was determined based on method by Bradford (1976).

Superoxide dismutase (SOD) activity: This enzyme activity was tested by monitoring exhibition of nitro blue tetrazolium (NBT) using a modified method of Jiang and Zhang (2002) with some modification. The three mL solution mixture (consisted of 2.35 mL sodium phosphate buffer 50 mM pH 7.8; 0.3 mL methionine 10 mM; 0.1 mL NBT 33 µM; 0.05 mL riboflavin 0.0033 mM and 0.2 mL EDTA 0.66 mM) was added to 200 µl extract buffer

and 200 µl enzyme extract. The mixed solution was exposed to the light (5000 lux) for 15 minutes. Reduction of the absorbance was measured at λ 560 nm. One unit activity of SOD was determined as the amount of enzyme needed to inhibit NBT reduction by 50% compared to the rate of NBT reduction without enzyme at the same wave length.

Ascorbate peroxidase (APX) activity: Measurement of APX was performed using Nakano and Asada's method, as described by Jiang and Zhang (2002) with some modifications. APX activity was determined based on oxidized ascorbic acid, showed by reduction of absorbance at 290 nm for one minute. 3.5 mL mixed solution consisted of 1.8 mL phosphate buffer (50 mM pH 7.0); 0.8 mL ascorbic acid (2.625 mM); 0.4 ml enzyme extract and 0.5 mL H_2O_2 (10 mM). The reaction was started when H_2O_2 was added to the solution.

Catalase (CAT) activity: This enzyme activity was measured using the method of Aebi as described by Jiang and Zhang (2002) with some modifications. Enzyme activity was expressed as reduction of H_2O_2 and determined by measuring the absorbance reduction of H_2O_2 at 240 nm for one minutes. CAT activity was counted as the amount of H_2O_2 (µmol) degraded in one minute/mg protein. CAT activity was described as U/mg protein. The 3.5 mL mix solution consisted of 2.5 mL phosphate buffer (50 mM pH 7); 0.2 mL enzyme extract and 0.5 mL H_2O_2 (12.0588 mM).

Determination of non-enzymatic antioxidant

Extraction: Glutathione and ascorbic acid extraction was carried out using Ozer's method as described by Panda and Petra (2007) with modification. Leaf sample (0.5 g) was frozen using liquid nitrogen and subsequently ground with mortar and pestle. Furthermore, the sample was homogenized by adding 3 mL of 7% sulfosalicylic acid and centrifuged at 10,000 g for 10 minutes. Supernatant obtained was treated as enzyme extract.

Glutathione: This compound was determined based on the method of Diwan *et al*. (2010) with modification, by defining reduction of glutathione in the reaction with 5-5'-dithiobis[2-nitrobenzoic acid] (DTNB) based on the appearance of yellow color. The mixed solution consisted of 0.1 mL enzyme extract; 0.9 mL sodium phosphate buffer 0.2 M and 2 mL DTNB. This subsequently was incubated for one minute. Absorbance was measure at 412 nm. Glutathione content was quantified based on the standard curve.

Ascorbic acid: Content of ascorbic acid was determined using Ozer's method (Panda and Petra, 2007) with some modification. The mixed solution consisted of 2 mL Na-molybdate 2%; 2 mL $H_2SO_4$0.15 M; 1 mL $NaHPO_4$15 mM and 1 mL enzyme extract. This solution was incubated for 40 minutes at 60°C and then cooled off at room temperature. Subsequently, the solution was centrifuged for 10 minutes at 3000 g. Absorbance value of the supernatant was measured at 660 nm Ascorbic content was determined using the standard curve.

Total phenol: Measurement of total phenol content was performed using method by Rameshkumar and Sivasuda (2012) with some modifications. 0.5 g leaf sample was frozen using liquid nitrogen and then ground with mortar and pestle. Subsequently, this sample was homogenized with 2.5 mL methanol, filtered with filter paper and the final volume was adjusted to 5 mL by adding methanol. A mixed solution of 20 µL extract; 1.58 mL sterile water and 100 µL folin-ciocalteau was incubated for eight minutes. Furthermore, 300 µl sodium carbonate7.5% was added and then the mixed solution was incubated at 30°C for 30 minutes. Absorbance value was measured at 769 nm. Total content of phenol was quantified using the standard curve.

Carotenoid: Determination of this compound was performed using spectrophotometry as described by Wang *et al*. (2008) with modification. A half gram of leaf was soaked in 10 mL dimethyl sulfoxide (DMSO) for 48 hours in the dark, at room temperature. Absorbance values of chlorophyll a, b and carotenoid were measured using spectrophotometer at 665 nm, 649 nm and 480 nm respectively. The carotenoid conten twas calculated according to Wellburn (1994).

Data Analysis: Quantitative data obtained from this research was analyzed using analysis of variant (Anova) to determine the effect of single treatment and the correlation between treatments toward measured parameters. In addition, Duncan's Multiple Range Test (DMRT) was used to determine the significant differences between treatments at 95% confidence level.

RESULTS AND DISCUSSION

A weed is a plant considered undesirable in a particular situation and generally is very consumptive with regard to the growth requirement of the crop plant, competitive and invasive (Kohli *et al*., 2006). Competition between weed and plant can result in the reduction of water availability in the soil, both for the weed and the plant (Ratnayaka *et al*., 2003). Moreover, the competition between weed and plant (Gawronska and Golisz, 2006; Marvilo *et al*., 2011), as well as the low water availability in the soil (Pedrol *et al*., 2006) stimulates the synthesis and accumulation of allelochemicals by the weed and crop.

The growth of the soybean plant shoot shows that at all levels of a single purple nutsedge interference, a single drought stress and a combination of both stress resulted in a decrease in wet weight, dry weight and length of the canopy. The higher the stress level, the greater the reduction of those three parameters measured.

The same thing also happened on the growth of leaves with parameters of leaf number, leaf area and leaf fresh weight (Figure 1 and2).

Results from our research indicated that interference of three purple nutsedge tubers per pot and six purple nutsedge tubers per pot reduced the activity of SOD, APX and CAT. In contrast, both mild and severe drought stress increased the activity of SOD, APX and CAT. At multiple stress treatment, mild drought stress and three purple nutsedge interference per pot increased SOD activity, whereas at other combinations of multiple stress treatment decreased SOD activity compared to control treatment was observed. Activity of APX and CAT decreased at both mild and severe drought stress combinations with interference of three purple nutsedge tubers per pot and six purple nutsedge tubers per pot (Table 1).

Plants regulates the activity of antioxidant enzymes, such as SOD, APX and CAT at high levels to degrade ROS in order to stop oxidative stress (Singh *et al*., 2009). SOD acts as the first defense against ROS by converting O_2^- to H_2O_2. Subsequently, H_2O_2 will be detoxified by APX and CAT. Contrary to CAT, APX requires a regeneration system of ascorbate and glutathione in the form of ascorbate-glutathione cycle. Detoxification of H_2O_2 to H_2O by APX occurs when ascorbate is oxidized to malondialdehyde (MDA), which can be regenerated by MDA reductase (MDAR) using NAD(P)H. Subsequently, MDA can spontaneously dismutate to dehydroascorbate form. Regeneration of ascorbate was mediated by dehydroascorbate reductase (DHAR) and stimulated by oxidation of glutathione (GSH) to become oxidized glutathione (GSSG). At the final step, glutathione reductase (GR) can regenerate GSH from GSSG using NAD(P)H as the reduction agent.The cycle is closed by regenerating GSH from GSSG by GR (Mittler, 2002; Apel and Hirt, 2004; Bhattacharjee, 2005; Gill andTuteja, 2010).

The reduction of anti-oxidative enzyme activity showed by soybean plants as the result of purple nutsedge interference was also reported as occurring on *Brassica campestris*, *Oryza sativa* and *Sorghum vulgare* treated with secalonic acid (allelochemical isolated from fungus *Aspergilu japonicus*). Treatment with 0.3 mM secolanic acid reduced SOD activity by 49, 71, 22.5% on *B.campestris*, *O. sativa* and *S. vulgare*, respectively. In addition, POD activity on *S. vulgare* declined by 21% (Zeng *et al*., 2001). Benzoic acid treatment at the concentration of 1.5 and 10 mM decreased SOD activity from the tomato root during the first five days after treatment, whereas cinnamic acid treatment decreased SOD activity at five and 10 days after treatment (Zhang *et al*., 2010). This condition was consistent with the findings of Lou (2013) since allelochemicals decreased the anti-oxidative enzymes SOD and POD. In addition, decreasing anti-oxidative enzyme activity during the stress was caused by accumulation of large amounts of toxic H_2O_2 inside the target cells. Consequently anti-oxidative enzymes failed to control H_2O_2 at a safe concentration.

At the same level of purple nutsedge interference, severe drought stress treatment caused higher levels of activity of SOD, APX and CAT compared to mild drought stress, although their activity was much lower when compared to the control treatment. This condition indicated that soybean plants are more tolerant toward purple nutsege interference if it is combined with drought stress compared to purple nutsedge interference treatment alone. This result concurs with the findings of Mittler (2006) and Akinson and Urwin (2012), as they mentioned that abiotic stress can decrease or increase the sensitivity of the plant towards biotic stress and vice versa. Hence the resulting effects can not be simply predicted based on the effect of a single stress factor.The effect of multiple stress treatment might increase detrimental impacts on the plant to a level that exceeds the total detrimental impact of all stresses. In contrast, in a different case a plant exposed to a single stress factor was able to increase its resistance against other stress factors, which occurr later (Alexieva *at al*., 2003; Li and Gong, 2011). Pedrol *et al*. (2006) stated that those response interactions were caused by plant centralization system against various stresses. Moreover, drought stress increased the activity of SOD, APX and CAT on corn plants (*Zea mays*) treated with 25% extract of leaf of *Nicotiana plumbagifolia*. The increased enzyme activity was reduced at treatments with higher levels of tobacco leaf extract (Singh *et al*,. 2009).

Mild and severe drought stress, interference of three purple nutsedge tubers per pot and six purple nutsedge tubers per pot, and combinations of both stress factors increased the content of non enzymatic anti-oxidants measured, i.e. glutathione, ascorbic acid and total phenol, whereas carotenoid content decrease in the same treatment (Table 2). This phenomenon indicated that non-enzymatic antioxidant compounds, i.e glutathione, ascorbic acid and total phenol played an important role in determining the tolerance of soybean plants against combined biotic (purple nutsedge interference) and abiotic (drought) stress. In another study, Chakraborty and Pradhan (2012) reported that five varieties of wheat plants (*Triticum aestivum* L.) KW, UP2752, PBW343, SO and LV treated with drought stress increased the production of ascorbic acid, phenol, proline and carotenoid. Moreover, *Zea. mays* treated with the same stress increased glutathione and ascorbic acid content (Chugh *et al*., 2011).

Ascorbic acid and glutathione degrade ROS by acting as electron donor or ROS reductor. Glutathione oxidised by ROS forms oxidased glutathione, whereas ascorbic acid oxidased by the same compound forms monodehydroascorbate (MDA) and dehydroascorbate

(DHA). Reduced glutathione (GSSG), through the ascorbate-glutathione cycle, can be reduced again to form glutathione and ascorbic acid. A high ratio of MDA to glutathione in reduced, rather than oxidized form, is important to detoxify ROS in the plant cell. Reduced status of antioxidants such as glutathione and ascorbic acid is maintained by glutathione reductase (GR), monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR), using NADPH as areducing agent (Apel and Hirt, 2004; Bhattacharjee, 2005; Gill andTuteja, 2010).

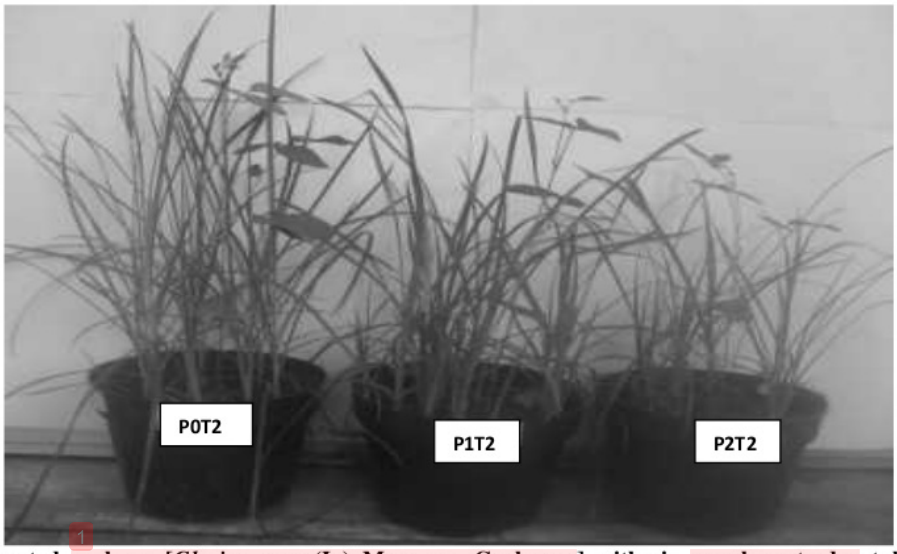

Figure 1. Treated soybean [*Glycine max* (L.) Merr. cv. Grobogan] with six purple nutsedge tubers per pot interference (T_2) combined with control (P_0), mild (P_1) and severe (P_2) drought stress

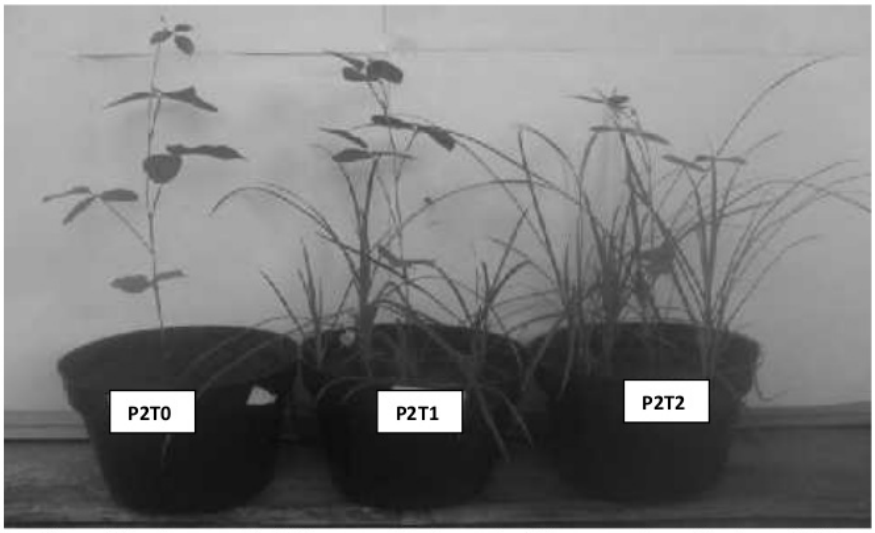

Figure 2.Treated soybean [*Glycine max* (L.) Merr. cv. Grobogan] with severe drought stress (P_2) in combination with various purple nutsedge interferences: control (T_0), three purple nutsedge (T_1), six purple nutsedge (T_2)

Carotenoid is a lipophilic organic compound which exists in plastids of both photosynthetic and non-photosynthetic plant tissues. This compound plays an important role in tolerance towards oxidative stress through several different mechanisms, such as reacting with the product of lipid peroxidase to end the chain reaction and degrading ROS formed by the plant during various stress conditions (Karuppanapandian *et al.*,

2011). Phenolic compounds, including flavonoid, tannin, hydroxycinnamate ester and lignin, are secondary metabolites and play roles as antioxidants through their ability to donate electrons or hydrogen atoms to ROS. Polyphenol has multiple roles, such as chelating transition metal ions to prevent Fenton's reaction, preventing lipid peroxidase by trapping lipid alkoxy radicals (RO) and modifying lipid structure to reduce membrane fluidity. These roles decrease free radical diffusion and restrict peroxidase reaction. Flavonoid and phenylpropanoids are oxidized by peroxidase enzyme and act as a degrading agent of ROS (Sharma *et al*., 2012).

Our results indicated that the tolerance of soybean cv. Grobogan toward purple nutsedge interference in various drought stress conditions was determined by non-enzymatic antioxidants glutathione, ascorbic acid and phenolic compound. The tolerance ability of this plant toward purple nutsedge interference was higher when interference occurred at the same time with drought stress compared to that without drought stress treatments.

Table 1. Enzyme activities of SOD, APX and CAT on leaf of Soybean [*Glycine max* (L.) Merr. cv. Grobogan] treated with purple nutsedge (*Cyperus rotundus* L.) interference under drought stress

Enzyme	Watering	Purple nutsedge Interference			
		T_0	T_1	T_2	Average
SOD (unit enzim /g)	P_0	4.84^{bc}	1.45^{ef}	0.96^{f}	3.00^{z}
	P_1	4.91^{bc}	3.58^{cd}	2.71^{de}	3.73^{y}
	P_2	8.19^{a}	8.15^{a}	5.66^{b}	7.33^{x}
	Average	5.98^{p}	4.39^{q}	3.69^{r}	
APX (μmol/g)	P_0	13.00^{c}	3.75^{f}	1.94^{g}	6.23^{z}
	P_1	14.84^{b}	5.78^{e}	5.52^{e}	8.71^{y}
	P_2	16.86^{a}	6.85^{d}	6.23^{de}	9.98^{x}
	Average	14.90^{p}	5.46^{q}	4.56^{r}	
CAT (μmol/g)	P_0	12.32^{c}	5.56^{d}	3.86^{d}	7.25^{z}
	P_1	12.90^{c}	11.90^{c}	9.87^{c}	11.56^{y}
	P_2	25.38^{a}	18.49^{b}	16.98^{b}	20.28^{x}
	Average	16.86^{p}	11.98^{q}	10.24^{r}	

P_0= control, P_1= mild drought stress; P_2= severe drought stress; T_0= without, T_1= three, T_2= six purple nutsedge interference. Numbers in the same of parameter followed the different letters in the same parameter indicate significant differences by DMRT at ($p < 0.05$).

Table 2. Contents of glutathione, ascorbic acid, phenol total and carotenoid on leaf of soybean [*Glycine max* (L.) Merr. cv. Grobogan] treated with purple nutsedge (*Cyperus rotundus* L.) interference under drought stress conditions

Antioxidant	Watering	Purple nutsedge Interference			
		T_0	T_1	T_2	Average
Glutathione (µM/g)	P_0	2.01^{c}	3.74^{b}	5.13^{a}	3.63^{z}
	P_1	3.48^{b}	3.88^{b}	5.35^{a}	4.23^{y}
	P_2	5.02^{a}	5.35^{a}	5.93^{a}	5.43^{x}
	Average	3.50^{r}	4.32^{q}	5.47^{p}	
Ascorbic acid (µM/g)	P_0	2.33^{c}	3.45^{b}	4.31^{ab}	3.36^{z}
	P_1	3.56^{b}	3.89^{b}	4.42^{ab}	3.96^{xy}
	P_2	5.19^{a}	4.33^{ab}	4.44^{ab}	4.66^{x}
	Average	3.69^{q}	3.89^{p}	4.39^{0}	
Phenol Total (mg/g)	P_0	119.96^{g}	153.69^{f}	211.75^{bcd}	161.80^{z}
	P_1	189.17^{e}	202.07^{cde}	220.25^{bc}	203.83^{y}
	P_2	198.26^{de}	225.53^{b}	272.16^{a}	231.99^{x}
	Average	169.13^{r}	193.77^{q}	234.72^{p}	
Carotenoid (mg/g)	P_0	0.37^{a}	0.28^{c}	0.23^{d}	0.29^{x}
	P_1	0.33^{b}	0.22^{de}	0.21^{de}	0.25^{y}
	P_2	0.31^{b}	0.20^{ef}	0.18^{f}	0.23^{z}
	Average	0.34^{o}	0.23^{p}	0.21^{q}	

P_0= control; P_1= mild drought stress; P_2= severe drought stress; T_0= control, T_1= three, T_2= six purple nutsedge interference. Numbers in the same of parameter followed the different letters indicate significant differences by DMRT at ($p < 0.05$).

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