

REACTIVE OXYGEN SPECIES
ACCUMULATIONS,
PHENYLALANINE
AMMONIALYASE ACTIVITY
AND PHENOLIC ACID
COMPOSITION OF SOYBEAN
[GLYCINE MAX (L.) MERR.] CV.
GROBOGAN THAT EXPOSED
TO MULTIPLE STRESS OF
PURPLE NUTS

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REACTIVE OXYGEN SPECIES ACCUMULATIONS, PHENYLALANINE AMMONIA-LYASE ACTIVITY AND PHENOLIC ACID COMPOSITION OF SOYBEAN [*GLYCINE MAX* (L.) MERR.] CV. GROBOGAN THAT EXPOSED TO MULTIPLE STRESS OF PURPLE NUTSEDGE (*CYPERUS ROTUNDUS* L.) INTERFERENCE AND DROUGHT

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INTRODUCTION

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Reactive Oxygen Species (ROS) are produced in abundance by the plant receiving environmental biotic and abiotic stresses (Akinson and Urwin 2012). According to Mittler (2002) and Gill and Tuteja (2011) ROS accumulation in large amounts can cause cell damage, but as sessile organism plants have a mechanism to be tolerant to unfavorable environmental conditions by the formation of secondary metabolites. Phenylalanine ammonia-lyase (PAL) is a key enzyme in diverting primary metabolism pathways (shikimic pathway) to secondary metabolism pathways (phenylpropanoid pathway). PAL with cinnamic 4-hydroxylase enzyme will form a group of enzymes important to allocate a portion of the carbon of phenylalanine to biosynthesis of secondary metabolites by forming various compounds of cinnamic acid and its derivatives (Maldonado *et al.*, 2007; Khan *et al.*, 2011).

Secondary metabolites play a role in plants adaptation to environmental changes and a defense mechanism against various biotic and abiotic stress by acting as antioxidants as well as allelochemicals (Edreva *et al.*, 2008; Sirikantaramas *et al.*, 2008; Sharma *et al.*, 2012). De Albuquerque *et al.* (2011) classifies secondary metabolites into three groups, namely: phenolic, terpenoids and compounds containing sulfur and

nitrogen. Phenol is a chemical compound composed of hydroxyl (OH) attached directly to the ring of aromatic hydrocarbons and plants subjected to this stress will produce more abundantly (Li and Capple, 2010). Phenolic compounds which are classified as allelochemicals are specifically derived from cinnamic acid, benzoic acid, coumaric acid, tannins, polyphenols and complex flavonoids. Each derivative of these compounds showed a similar mechanism of action in inhibiting growth (Einhellig, 2004). In general, allelochemicals inhibits the other plants but does not affect the activity of cells that synthesize and store because plants have a mechanism of resistance to toxic compounds that they produce themselves with the biosynthesis of extracellular allelochemicals. The same mechanism saves allelochemicals in the vacuole; activates the excretion of extracellular allelochemicals; enables transport by vesicles of allelochemicals from the cytoplasm to the vacuole and activates enzymatic detoxification of allelochemicals; and triggers mutations of genes that encode proteins that become targets of allelochemicals and which are accumulated in non-toxic form in the vacuole (Sirikantaramas *et al.*, 2008). Phenolic compounds act as antioxidants for the plant because of their ability (1) to donate electrons or hydrogen atoms to ROS, (2) to bind transition of metal ions, thus preventing Fenton reactions, (3) to degrade

ROS directly and inhibit lipid peroxidation by trapping lipid alkoxy radical (RO[•]), (4) to modify the lipid composition thus lowering membrane fluidity and causing a decreased diffusion of free radicals and limit the peroxidation reaction (Sharma *et al.*, 2012).

Soybean [*Glycine max* (L.) Merr.] is crops a source of vegetable protein of high economic value. In regions with limited water resources, generally soybeans are planted at the end of the rainy season. A consequence of this pattern, if the weed management is not perfect then soybeans potentially suffer multiple stress in the form of weed interference and drought simultaneously (Partohardjono, 2005; Atman, 2006). According to Akinson and Urwin (2012), plants' response to multiple stress will vary with the sole stress response. Purple nutsedge (*Cyperus rotundus* L.) is one of the dominant weeds in soybean because it can decrease the production of soybeans by up to 89% (Kavitha *et al.*, 2012). According to Baloch *et al.* (2015), purple nutsedge is invasive and difficult to eradicate, either manually or with herbicides, because they have bulbs that can be dormant in unfavorable environmental conditions. Soybean cv. Grobogan is one of the local cultivars in Grobogan district, Central Java province, Indonesia (Anonymous, 2010). This study aims to assess the rate of accumulation of ROS such as superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), PAL enzyme activity and phenolic compounds composition in the leaves of soybean cv. Grobogan which suffered a multiple stress of purple nutsedge interference (biotic stress) and drought (abiotic stress).

MATERIALS AND METHODS

Materials and equipment: Soybean cv. Grobogan seeds were obtained from Balai Penelitian Kacang-Kacangan dan Umbi-Umbian (BALITKABI) Malang, East Java. Purple nutsedge tubers were collected from the field in Semarang, Central Java. The main equipment used in this research were centrifuge with refrigerator, spectrophotometer and High Performance Liquid Chromatography (HPLC).

Research design: This research was designed using Completely Randomized Design (CRD) with two factors, i.e. level of purple nutsedge interference (C₀ = control, C₁ = three purple nutsedge per pot and C₂ = six purple nutsedge per pot) and level of drought (D₀ = control (FTSW 1), D₁ = mild stress (FTSW 0,5) and D₂ = severe stress (FTSW 0,25). Each treatment unit contained five replications.

Method: Planting and treatment: Purple nutsedge tubers were selected based on the uniformity of weight and soybean seeds were selected based on the uniformity of seed size. Purple nutsedge tubers had sprouted and selected tubers with one bud only.

Seedlings of soybean and purple nutsedge were planted at the same time in plastic pots with a diameter of 25 cm which were filled with 3 kg of latosol soil and base fertilizer i.e. 1 gr TSP, 0,5 gr KCL and 0,3 gr ZA. Each pot was planted with one soybean seedling and various numbers of purple nutsedge tubers according to the treatments. Drought stress treatment was started two weeks after planting and terminated five weeks after planting. Drought stress treatment was determined based on the value of The Fraction of Transpirable Soil Water. Watering was performed every day with volume used was determined by weighing the pot and its contents the total weight was equal to the treated unit (Hainemann *et al.*, 2011; Darmanti *et al.*, 2016).

Determination of ROS accumulations:

Superoxide (O₂⁻). Analysis was by using methods of Malecka *et al.* (2014) with modifications. A soybean leaf sample of 0.5 g was inserted in a test tube. Seven mL of 50 mM phosphate buffer pH 7.8 containing 0.05% NBT and 10 mM NaN₃ was added. The sample was incubated in the dark and at room temperature for 5 minutes. Two mL of solution was taken and heated in a water bath with temperature of 85°C for 10 minutes. The sample was then cooled in an ice bath for 5 minutes. Absorbance value was read at λ 589 nm. O₂⁻ level was expressed in Absorbance/g fresh weight of sample (A/g).

Hydrogen peroxide (H₂O₂). Analysis was conducted using methods of Bouazizi *et al.* (2007) with some modifications. Samples of fresh leaves 0.5 g were ground by mortar in liquid nitrogen. These were homogenized with 5 mL TCA 0.1% in cold conditions. Samples were centrifuged at 12,000 g for 15 min at 4 °C. Supernatant was an H₂O₂ extract. The reaction mixture was composed of 0.5 mL of the supernatant, 0.5 mL of 10 mM phosphate buffer pH 7 and 1 mL solution of KI 1 M. The absorbance of the solution was determined at λ 390 nm. For the control treatment, supernatant was replaced with 0.1% TCA. The content of H₂O₂ was determined by H₂O₂ standard curve.

Determination of PAL enzyme activity: PAL activity was determined by the amount of trans-cinnamic acid which formed as products of the reaction catalyzed by PAL, which was detected by HPLC according to the method of Ferrarese *et al.* (2000) with some modifications.

Enzyme extraction. Soybean leaf samples weighing 0.5 g were ground in liquid nitrogen, then homogenized with 1.5 mL of 0.2 M borate buffer, pH 8.8. Extracts were centrifuged at a speed of 12,000 g for 15 min at 4 °C. Supernatant was an enzyme extract.

Determination of the total protein: Soybean leaf samples weighing 0.1 g were ground in liquid nitrogen and then homogenized in 1 mL of 50 mM Na phosphate

buffer pH 7.8 containing 0.1 mM EDTA and 2% PVP. These were then centrifuged at a speed of 12,000 g for 20 min at 4 °C. One and a half mL of Bradford reagent was added to the reaction solution consisting of 30 mL of protein extract, then vortexed and incubated for 10 minutes. The protein level was determined by spectrophotometer at absorbance at λ 595. On the control treatment, the extract was replaced by phosphate buffer. Total protein was calculated with albumin standard curve (Bradford, 1976).

Determining trans-cinnamic acid content: Samples of 1 mL enzyme extract were incubated at 40 °C in a water bath for 5 minutes, then 0.5 mL of L-phenylalanine 30 mM were added and incubated for 1 hour. Fifty mL of HCl 5 N was then added to the mixture. The extract was stored in a dark room at 4 °C. HPLC conditions were as follows: Triat column YMC-C18 Reversed - Phase (250 mm x 4.6 mm, 5 m), Phase Motion: Water: Methanol (30:70), flow rate: 0.5 mL/ min, Detector: RID - 10A, Pump: LC - 20AD. PAL activity was expressed as the amount of cinnamic acid formed per unit amount of protein for a certain time.

Determination of phenolic compound compositions:

Extraction. Based on the method of Proestos and Komaitis (2013) with some modifications. Samples of 1g leaves were dried at room temperature in the dark for 24 hours. The leaves were ground in liquid nitrogen, and homogenized with 40 mL of methanol containing 62.5% BHT (1g BHT in 1L methanol) and 10 mL 6M HCl. These were mixed in a stirrer, then were sonificated for 5 minutes and heated in a water bath at a temperature of 90 °C for 2 hours. The mixture was filtered through two sheets of rough filter paper, then the filtrate was added with methanol to a volume of 100 mL. One hundred mL of distilled water was added to the filtrate. This was then partitioned three times with the volume of ethyl acetate 60, 20, 20 mL, respectively. The organic phase was collected, then evaporated at room temperature with the help of the fan until the filtrate volume was about 10 mL. Filtrate was then added to the excess of Na₂SO₄

anhydrous to remove water. To complete removal of all remaining water content, the filtrate was evaporated to dryness at room temperature. The precipitate was dissolved in 2 mL of ethyl acetate and centrifuged at cold temperature at a speed of 12,000 g. The supernatant was filtered with a 0.45 μ m microfilter.

Phenolic compound analysis by HPLC: HPLC conditions were as follows: YMC column - Triat C18 (250 mm x 4.6 mm, 5 m). Mobile phase: Eluent A (acetic acid: water (2: 9.8 v/v), eluent B: acetonitrile acetic acid: water (2: 30: 68, v/v), gradient: eluent B from 10% to 100 % for 30 minutes. Flow rate: 1 mL/ min. temperature of 25 °C. Detector: SPD - 20 AD. λ 280 nm. Pump HPLC: LC - 20 AD, standard phenolic compounds used were: acid 4-Hydroxybenzoate, vanillic acid, 2,5-dihydroxybenzoate, caffeic acid, syringic acid, coumaric acid, trans-ferulic acid, trans-cinnamic acid and salicylic acid (Merck).

Data Analysis: Quantitative data was analyzed using analysis of variant to determine the effect of single treatment and the correlation between treatments. In addition, Duncan's Multiple Range Test was used to determine the significant differences between treatments at 95% confidence level. (Gomez and Gomez, 2010)

RESULTS AND DISCUSSION

There was an interaction between purple nutsedge interference and drought treatment on the average accumulation of ROS such as O₂⁻ and H₂O₂. Except for the treatment of interference by three purple nutsedge on soybean without drought stress conditions, all combinations of multiple stress interference by three or six purple nutsedge and mild or severe drought increased the accumulation of superoxide (O₂⁻). The greater the weight of the combination of stress levels, the higher the accumulation of O₂⁻ observed (Table 1).

Table 1. Average ROS accumulation such as superoxide (O₂⁻) (Abs/g) and hydrogen peroxide (H₂O₂) (nmol/g) of leaves of soybean [*Glycine max* (L.) Merr.] cv. Grobogan due to multiple stress of purple nutsedge (*Cyperus rotundus* L.) interference and drought.

ROS	Drought	The number of <i>Cyperus rotundus</i> L.			
		Control	3	6	Average
O ₂ ⁻ (Abs/g)	Control	0,70 ^e	0,75 ^e	0,91 ^c	0,79
	Mild	0,83 ^d	0,97 ^b	1,09 ^a	0,96
	Severe	0,84 ^d	1,02 ^b	1,13 ^a	1,00
	Average	0,79	0,91	1,04	(+)
H ₂ O ₂ (nmol/g)	Control	3,25 ^e	5,17 ^a	5,26 ^a	4,56
	Mild	3,70 ^e	4,23 ^b	5,41 ^a	4,45
	Severe	2,35 ^d	2,39 ^d	2,75 ^d	2,49
	Average	3,10	3,93	4,47	(+)

- Numbers followed by different letters on the same parameters showed a significant difference with Duncan Multiple Test at level of 95%. Each treatment was applied with 5 replications

O_2^- is one of the ROS formed from oxygen (O_2) through experiencing electron transfer. Furthermore, O_2^- will be converted to hydrogen peroxide (H_2O_2) through spontaneous reaction or reaction catalyzed by the SOD. At the same purple level of nutsedge interference, increasing drought stress causes a decrease of H_2O_2 accumulation. In contrast to the conditions of mild or no drought stress, increased purple nutsedge drought stress causes increased H_2O_2 accumulation. In conditions of severe drought stress, with or without purple nutsedge interference, results showed content of H_2O_2 lower than control. In conditions of severe drought stress, with treatments either without purple nutsedge, or with interference by three or six purple nutsedge, results did not show significant difference in H_2O_2 levels. This result indicates that in conditions of multiple stress of purple nutsedge interference and drought, the purple nutsedge interference factor causes an increase of H_2O_2 accumulation, whereas drought stress causes a decrease of H_2O_2 accumulation.

H_2O_2 accumulation in plant organs is the difference between the amount of H_2O_2 synthesized from O_2 and the amount of H_2O_2 scavenged by the antioxidative defense system (Sharma *et al.* 2012). The decrease of H_2O_2 accumulation is suspected to be due to the high CAT activity that contributes to the degradation of H_2O_2 , and high levels of non-enzymatic antioxidants that play a role in scavenging various of forms of ROS including H_2O_2 in soybeans with the same treatment as

reported by Darmanti *et al.* (2016). The decrease content of ROS such as H_2O_2 is also reported by Bubna *et al.* (2011). They found that caffeic acid treatment in hydroponic medium at a concentration of 0.25 μ M to 2 μ M decreases the amount of H_2O_2 and increases the concentration of lignin in the roots of *Glycine max* L. This decrease is thought to occur because H_2O_2 acts as a precursor in the synthesis of lignin. Lignin is a major phenolic polymer in plants that is accumulated in vacuoles or deposited in the secondary cell wall (Kafeli *et al.*, 2003). However the increased content of ROS such as H_2O_2 is reported to occur in sprouts of *Synapses alba* L. as a result treatment with water extract of leaves of *Helianthus annuus* L. containing allelochemical (Oracz *et al.*, 2004). Similar increase in $O_2^{\bullet-}$ and H_2O_2 at the root of *Cucumis sativus* L. cv. Jinyan No. 4 was reported due to treatment with cinnamic acid in hydroponic medium with a concentration of 0.005 μ M to 0.25 μ M (Ding *et al.*, 2007).

There was an interaction between the purple nutsedge interference and drought stress on the PAL enzyme activity. Multiple stress increases the activity of PAL, but interference by three or six purple nutsedge combined with increasing intensity of drought from mild to severe did not cause an increase in PAL activity (Figure 1). The increase of PAL activity was also reported by Gholizadeh (2011) occurs in the leaves of maize (*Zea mays* L.) inbred lines A-18 and A-19 that experiencing drought.

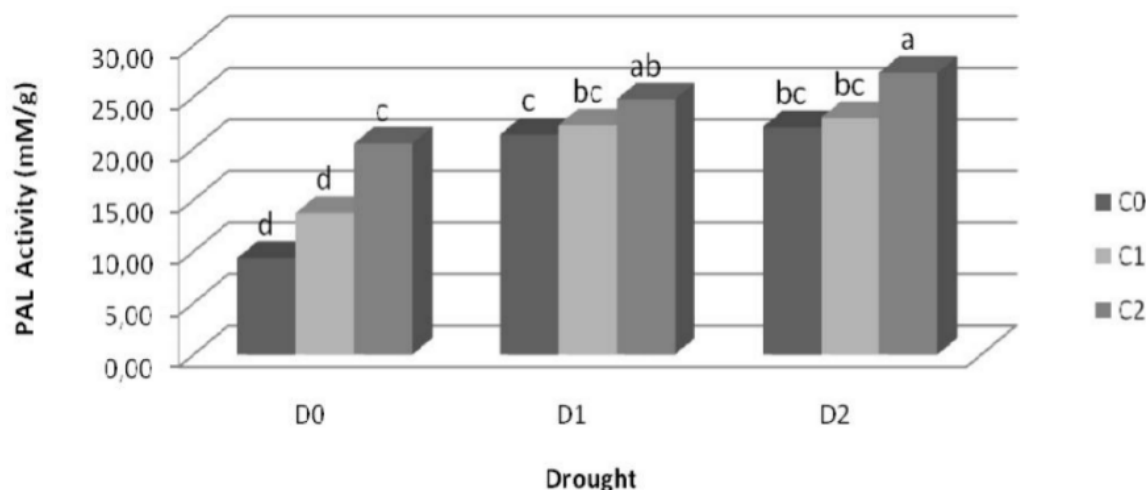


Figure 1. Average PAL enzyme activity of soybean [*Glycine max* (L.) Merr.] cv. Grobogan leaf due to multiple stress of purple nutsedge (*Cyperus rotundus* L.) interference and drought. C₀: control without purple nutsedge interference; C₁: three purple nutsedge interference; C₂: six purple nutsedge interference. D₀: control, D₁: mild drought; D₂: severe drought.

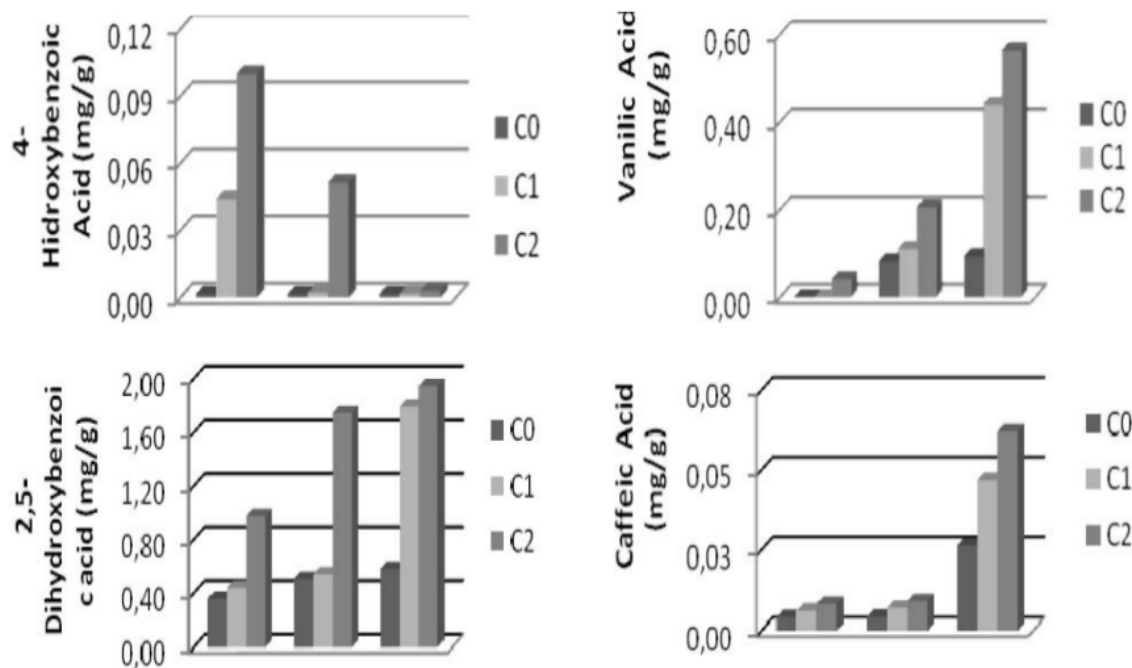
PAL is a key enzyme which deflects primary metabolic pathways (shikimic) to secondary metabolic pathways (phenylpropanoid) (Khan *et al.*, 2011). Various

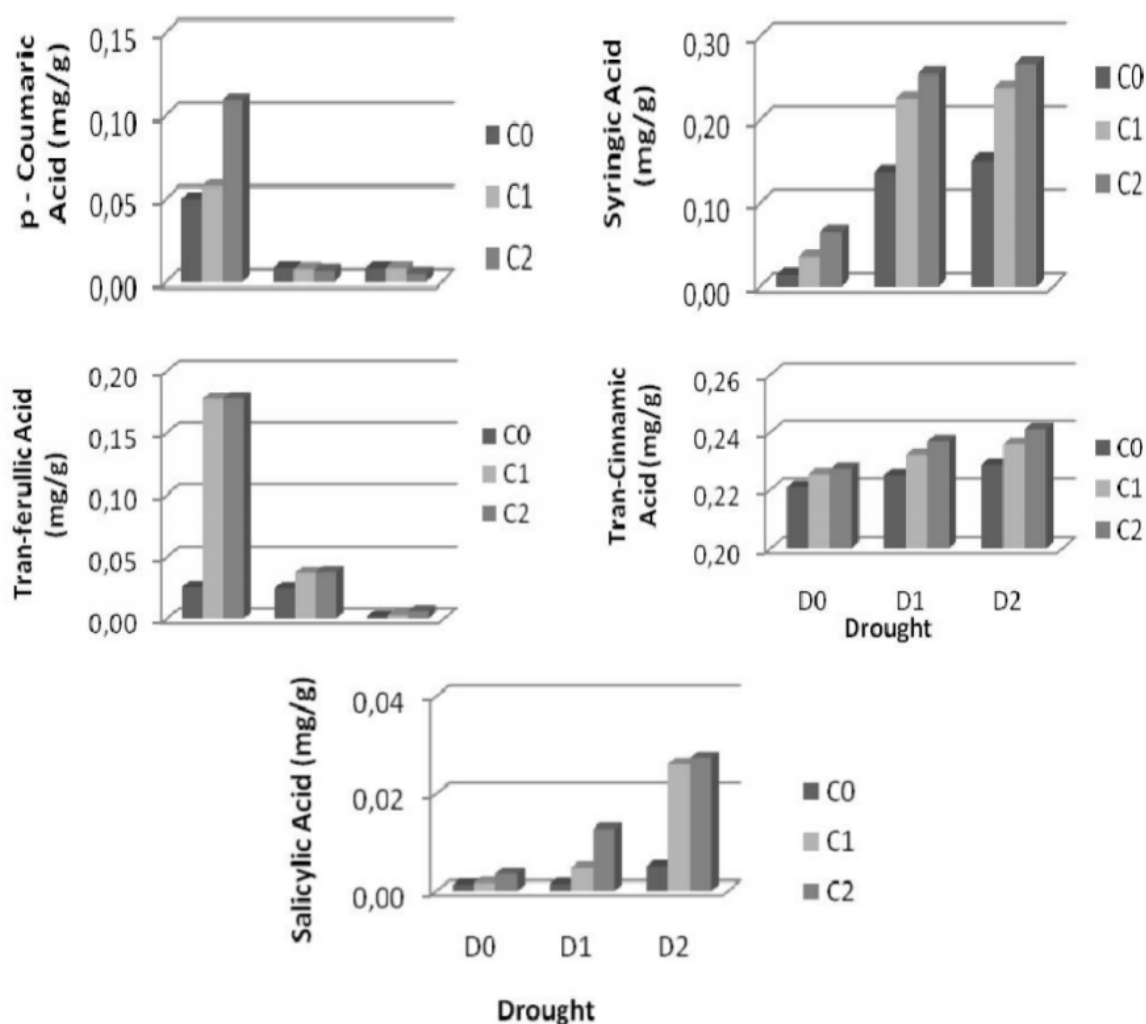
environmental stresses are known to increase the expression of genes that encode PAL enzymes and induce an increase of PAL enzyme activity (Kelij *et al.*, 2013) so that the synthesis of secondary metabolites increases.

Secondary metabolites that are formed of mainly phenolic groups will act as electron donors in enzymatic antioxidant systems that uses a variety of redox enzymes to catalyze the transfer of electrons to ROS (Maldonado *et al.*, 2007; Gholizadeh, 2011). In addition to functioning as antioxidants, phenolic compounds are also secreted by the roots to the rhizosphere and act as allelochemicals in allelopathic process to suppress the growth of competitor organisms such as weeds (He *et al.*, 2012; Iannucci *et al.*, 2012). The increase in total phenol content in the leaves of soybean due to multiple stress treatment of purple nutsedge interference and drought have also been reported by Darmanti *et al.* (2016).

Composition analysis of soybean leaf phenolic compounds was performed by HPLC using nine standard phenolic compounds that are known to be allelopathic at the root, stem and root exudates of soybean plants, i.e: 4-hydroxybenzoic acid, vanillic acid, 2,5-dihydroxybenzoic acid, caffeic acid, syringic acid, coumaric acid, trans-ferulic acid, trans-cinnamic acid and salicylic acid (Porter *et al.*, 1986). The results of this study showed that nine of the phenolic compounds contained in soybean leaves were exposed to multiple stresses of purple nutsedge

interference and drought as well as in the control (Figure 2), with an average content of vanillic acid, 2,5-dihydroxybenzoic acid, caffeic acid, syringic acid, trans-cinnamic acid and salicylic acid increased in all treatment combinations than control. The higher the stress level, the more the average content of these compounds increased. The average of 4-hydroxybenzoic acid and trans-ferulic acid content decreased with treatment of the same level of drought stress and with increase of purple nutsedge interference. Conversely, concentration of both compounds increased at the same level of purple nutsedge interference with increasing drought stress. Average coumaric acid content increased in drought stress conditions without three or six purple nutsedge interference. In mild or severe drought, three or six purple nutsedge interference caused the average coumaric acid content to decrease (Figure 2). However, it is not yet known what causes the decrease of 4-hydroxybenzoic acid, trans-ferulic acid and coumaric acid content in conditions of purple nutsedge interference in drought conditions. Further research is necessary to study the expression of genes that encode enzymes involved in each stage of the phenylpropanoid pathway.





Figur 2. Average content of allelopathic phenolic compound of soybean [*Glycine max* (L.) Merr. cv. Grobogan] leaf due multiple stress of purple nutsedge (*Cyperus rotundus* L.) interference and drought. C₀: control without purple nutsedge interference; C₁: three purple nutsedge interference; C₂: six purple nutsedge interference. D₀: control, D₁: mild drought; D₂: severe drought.

Phenylpropanoids are all compound trans-cinnamic acid derivatives, which are formed by reaction of deamination of L-phenylalanine with PAL catalyst. According to Solecka (1997) in drought stress treatment, the expression of genes that encode enzymes in the phenylpropanoid synthesis pathway generally increased so that the synthesis of phenylpropanoid also increased. But according to Krol *et al.* (2014), various environmental stresses can influence increase or decrease in the number of phenolic compounds in plant cells. This is evidenced by the decrease in the number of total phenols by 20% to 25% of the roots and leaves of *Vitis vinifera* L. exposed to drought stress over a long time.

Phenols found in *V. vinifera* are caffeic acid, coumaric acid and ferulic acid. It was conversely reported by Weidner *et al.* (2009), that drought conditions lasting a week increased the amount of p-coumaric acid, ferulic acid and caffeic acid in the root sprouts of *V. vinifera*. The difference in the results of the experiments as above may have been caused by different types of stress, the intensity of the stress, the duration of the stress, phases of plant growth or biological material that was used, whether the whole plant or plant organs (Krol *et al.*, 2014) or differences in expression levels of genes that encode enzymes which act on the phenylpropanoid pathway (PAL, C4H, F5H and COMT).

due to stress (He *et al.*, 2012).

From the results observed in this study, it can be concluded that the multiple stress condition of purple nutsedge interference and drought, increase the level of of purple nutsedge interference factors leads increase of ROS types of H_2O_2 accumulation, whereas the increase the level of drought factor lead decrease of ROS types of H_2O_2 accumulation. Whereas, ROS types of O_2^- accumulation increased in all combinations treatment. ROS accumulation conditions in all these treatments cause an increase the PAL activity compared with controls, but on the same level of purple nutsedge interference increasing levels of drought factors do not lead an increase in PAL activity. Although the PAL activity increased in all treatments compared with control, but the composition of phenolic compounds showed that the same of multiple stress combination effect differently on different types of phenolic compounds, as well as the different multiple stress combinations effects differently on the same type of phenol compounds.

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