Photosynthetic Rate and Lipid Peroxidation of Cultivated (*Glycine max* L.) and wild Soybean (*G. tomentella* L.) Exposed to Drought Stress and Paraquat

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ABSTRACT

*Oxidative stress of three soybean (*Glycine max*, (L.) Merr) cultivars and a wild line soybean (*G. tomentella*) were analyzed in response to drought and paraquat treatment. Drought treatment was performed by withholding water for 12 days (for cultivars) and 22 days (for wild line soybean) in greenhouse experiment during flower initiation. Paraquat treatment was applied using manual sprayer at the same time of drought treatment application. Plant water status and photosynthetic rate were measured every three days during the drought treatment and 2 days after rewatering, and during 5 days after paraquat application. During the treatment, malondialdehyde (MDA) was analyzed to study lipid peroxydase activity. Drought treatment decreased plant relative water content up to 33%*

and 42% in sensitive and tolerant variety,respectively.. Transpiration and photosynthetic rate decreased almost to zero at the end of drought period, while those of control plant were 4.7 µmol m^{-2} s^{-1} and 12.58 µmol m^{-2} s^{-1}, respectively. Malondialdehyde content increased dramatically (5890 nmol/g fresh weight) on drought stressed plant as well as on paraquat treated plant as compared to control plant (3281 nmol/g fresh wight). It indicated that plants underwent oxidative stress due to severe drought stress.

Key Words: Oxidative stress, Photosynthesis, Drought, , MDA, soybean

INTRODUCTION

Drought is considered as an abiotic factor that significantly reduces agriculture production almost every year. Drought stress causes the plant potosynthesis rate reduces by decreasing stomatal conductance (Morison and Lawlor, 1998, Cornic, 2000) and may also cause the damage of the photosynthesis apparatus including Photosystem I (PSI) and PSII of photosynthesis (Genty *et al*., 1987). In addition, partial stomatal closure due to drought stress in C3 plants increases photorespiration (Oliver, 1998) that could decrease carbohydrate accumulation required for growth and seed filling.

The lower CO_2 assimilation rate caused by drought, especially during excessive light exposure, may lead to over-reduction of PSII photosynthesis reaction center (Demming-Adams dan Adams, 1992). This situation may result in enhancement of reactive oxygen species (ROS) such as superoxide radical ions (O_2^-), hydrogen peroxides (H_2O_2), and oxygen singlets (Smirnoff, 1993; Bartoli *et.al* 1999; Loggini *at al*, 1999; Noctor and Foyer,

1998; Noctor *et al*., 2002). Reactive Oxygen Species are unstable substances that are harmful to the cells or tissue and a higher degree of ROS accumulation can cause cell and tissue damage (Sgherri and Navari-Izzo, 1995; Scandalios, 1997) leading to oxidative stress.

A growing body of evidence indicates that drought stress increases the accumulation of ROS in many species including wheat (*Triticum aestivum* L.) (Sairam *et al*. 1998), sunflower (*Helianthus annuus* L.) (Sgherri and Navari-Izzo, 1995) and some perennial plants such as *Coffea canephora* (Lima *et al.*, 2002). In soybean (*Glycine max* L.), the high degree of nodule senescence in response to drought stress is also predicted due to high accumulation of ROS during drought stress (Porcel *et al*., 2003). Water stress-induced ABA accumulation triggers the increased generation of ROS, which in turn, leads to the up-regulation of the antioxidant defense system (Jiang and Zhang 2002).

It is still unclear whether the accumulation of ROS and the elimination of this substance by antioxidative enzymes activities are different in tolerant and sensitive plants. Iturbe-Ormaetxe *et al*. (1998) have concluded that tolerance to water deficit in terms of oxidative damage largely depends on the cultivar, however little data exists indicating the differences in antioxidative enzyme activities of tolerant and sensitive plants. Some experiments in soybean have shown that the accumulation of enzymes such as super oxide dismutase (SOD) and ascorbate peroxidase (APx) were lower in drought stressed nodules of mycorrhizal plants than in nonmycorrhizal plants, whereas glutathione reductase (GR) activity was higher in nodules from mycorrhizal plants than in nonmycorrhizal plants (Porcel *et al*. 2003).

In this experiment we analyzed the photosynthetic rate, ROS accumulation and antoxidative enzyme activities of tolerant and sensitive soybean varieties (*G. max* L.) and

wild soybean (*G. tomentella* L.) in response to drought stress and paraquat, a herbicide that is able to induce oxidative stress (Iturbe-Ormaetxe *et al*. 1998) by accumulation of high ROS in the plants.

MATERIALS AND METHOD

The plants used in this experiment were drought-tolerant, moderately tolerant and sensitive soybean varieties (*Glycine max* L.) and a wild soybean (*Glycine tomentella* L.). A drought-tolerant variety (Tidar), a moderately tolerant variety (Burangrang) and a drought-sensitive variety (Panderman) were provided by The Indonesian Beans and Tubers Research Bureau (BALITKABI) Malang, East Java, Indonesia, while a drought-tolerant wild soybean was provided by The Life Science and Biotechnology Research Center, Bogor Agricultural University, Bogor, Indonesia. The experiment was carried out in a greenhouse at Department of Biology, Faculty of Natural Sciences and Math, Bogor Agricultural University, Bogor, Indonesia from October 2006 to July 2007.

The plants were grown in 8 Kg capacity polybags containing a mixture of soil and compost 2:1 (v/v) as the plant growing media. The plants were fertilized with nitrogen, phosphorus and potassium fertilizer (15:15:15) with the dosage of 2 g per polybag. Four seeds were sown in each polybag and then one-week old seedlings were thinned and selected to become 2 seedlings per polybag.

The experiment carried out using a Completely Randomized Design with two factors. The first factor was plant varieties and species comprised of drought-tolerant [(1) Tidar], moderately tolerant [(2) Burangrang] and drought-sensitive varieties [(3) Panderman], and a drought-tolerant wild soybean [(4) *Glycine tomentella* L.]. The second

93 factor was environmental stress including (1) drought stress, (2) paraquat application, and
94 (3) normal watering (of control plants).

95 The stress treatment (drought and paraquat application) was applied to 25-day old
96 plants, when the plants started to flower. Drought stress was administered by withholding
97 water for 12 days until the plants were heavily wilted. After the drought stress period, the
98 plants were rewatered to recovery (to the condition of the control plants). Paraquat
99 application was performed by spraying paraquat on the leaves in the morning between
100 07:00-08:00 a.m. with a dosage of 90 g of active compound per Ha. Based on a previous
101 experiment (data not shown), this dosage did not kill the plants but it reduced by 35% the
102 biomass dry weight after 2 weeks of treatment.

103 The parameters measured in this experiment were media water content, relative
104 water content of plants, transpiration and photosynthetic rate, and lipid peroxidation activity.
105 Gas exchange analysis was carried out to analyze the transpiration and photosynthetic rate of
106 the youngest fully expanded leaves using Photosynthetic Leaf Chamber Analyzer type of
107 LCA-4 with the light intensity of approximately 950 μmol $m^{-2}s^{-1}$. The photosynthetic rate
108 was measured at 0, 4, 8, 10, and 12 days after drought stress treatment for cultivated
109 soybean, and at 22 days after drought stress treatment for wild soybean. The measurement
110 was again carried out two days after rewatering to analyze photosynthetic rate of recovered
111 plants. For the plants treated with paraquat herbicide, the photosynthetic rate measurement
112 was carried out before paraquat application, 4 hours after application, and 1, 3 and 5 days
113 after application.

114 Lipid peroxidation was estimated as the content of total 2-thiobarbituric acid-(TBA)
115 substances expressed as equivalent to malondialdehyde (MDA) production as described in

Ono *et al*. (1995) with some modifications. Fresh leaves (0.2 g) were extracted in 0.5 ml of 0.1% (w/v) trichloracetic acid (TCA) at 4 °C. The extract then was added to 3 ml of 1% H_3PO_4 and 1 ml of 0,6% TBA dissolved in 20%TCA. The solution was incubated in the oven at a temperature of 100^{o}C for 30 minutes. After cooling to the room temperature, 4 ml n-butanol was added to the solution and followed by cetrifugation at 4200 rpm at 28^{0}C for 20 minutes. The absorbance of supernatant was then measured using a UV-VIS spectrophotometer at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 520 nm. The concentration of MDA was calculated from its extinction coefficient (ε=155 L mmol^{-1} cm^{-1}).

RESULTS AND DISCUSSION

Results

Plant water status during the drought and paraquat treatment

To analyze plant water status, relative water content (RWC) of plant was measured periodically during the drought and paraquat treatments. The average of RWC of the control plants was 80.3% with small variation between 78.0% to 82.8% (Table 1). The drought stress treatment for 12 days significantly decreased RWC of the cultivated plant. At the last day of the drought treatment, the sensitive soybean (Panderman) had lower RWC (33%) than the tolerant (Tidar) and moderate (Burangrang) varieties (42.5 and 42.0% respectively). The most dramatic reduction of RWC occurred after 8 days of drought treatment for cultivated soybean, whereas in wild soybean *G. tomentella* it occurred after 12 days of drought stress (Table 1). *Glycine tomentella* survived 22 days of drought period with the

RWC of 39.4%. Two days after rewatering the RWC of all the plants rose again to that of the control plants.

The RWC of plants treated with paraquat was also reduced significantly one day after treatment. The RWC started to decline 4 hours after paraquat application and the maximum reduction was observed one day after treatment. Three days after paraquat application, the RWC rose again for tolerant, moderate and wild soybeans, while it remained low for the sensitive variety (Table 2). Even though the RWC of wild soybean *G. tomentella* dropped dramatically one day after paraquat application, it recovered very well 3 days after application. The RWC of the paraquat-treated sensitive variety recovered 5 days after the treatment (Table 2).

Gas exchange analysis

Transpiration rate (*E*) varied during the treatment with the average of 4.7 mmol $m^{-2}s^{-1}$ for control plants. During the drought stress period, the *E* decreased significantly and dropped almost to zero after 12 days in cultivated soybeans (Tidar, Burangrang and Panderman), whereas in wild soybean (*G. tomentella*) the maximum reduction of *E* occurred 22 days after the drought stress period. Three days after rewatering, the *E* increased again to the level of control plants (Figure 1). Application of paraquat also reduced *E*, and the reduction became significantly different from that of control plants three days after application (Figure 2).

The photosynthesis rate (*Pn*) measured under green house conditions with average PPFD of 950 mmol m^{-2} fluctuated during the day. The average *Pn* of control plants and both cultivated and wild soybean, measured between 08:00-10:00 a.m., was 12.6 μmol m^{-2} s^{-}

[1]. As the drought stress period increased, the *Pn* reduced gradually with the maximum reduction on 12 and 22 days after drought stress treatment for cultivated and wild soybeans, respectively (Figure 3). The *Pn* of the drought-sensitive soybean (Panderman) decreased more than that of the tolerant (Tidar) and moderate (Burangrang) varieties after 8 and 10 days drought stress treatment (Figure 3). At the period of maximum drought stress, the *Pn* of all plants dropped to near zero. Meanwhile, rewatering increased the *Pn* back to the normal (control) condition 2 days after rewatering (Figure 3).

Paraquat treatment also caused significant reduction of the *Pn* of all treated plants. Even though *Pn* reduction was detected 4 hours after paraquat application, significant reduction of *Pn* occurred one day after application (Figure 4). The reduction of *Pn* continued until it reached the minimum level (approximately 0.14 μmol m^{-2} s^{-1}) on the third day after application. The *Pn* increased again 5 days after the plants recoverd from stress. In contrast to the *Pn* recovery after drought stress, the increase of *Pn* 5 days after paraquat application the level of the control plants (Figure 4).

Lipid peroxidation

Lipid peroxidation was analyzed by the measurement of MDA accumulation in the leaf tissues as a result of membrane lipid degradation. At normal conditions indicated by the control plants, the average MDA level in the leaves was 3281 nmol g^{-1} fresh weight. The MDA level increased in drought-stressed plants (Figure 5). Generally, the MDA level of cultivated soybean significantly increased after 8 days of drought treatment when the plant started wilting. However, in *G. tomentella* MDA levels started to increase when mild drought stress was reached (4 days after drought treatment). The maximum level of MDA

184 (5890 nmol g^{-1} fresh weight), or almost twice that of control plants, was reached at 10 days
185 when severe drought stress occurred (Figure 5). After rewatering, the MDA level decreased
186 to that of the control plants or even lower in sensitive variety Panderman (Figure 5).
187 Application with paraquat herbicide also increased the MDA level in all treated
188 plants one day after treatment (Figure 6). However, the maximum level of MDA was lower
189 in paraquat application compared to that of drought treatment. Five days after paraquat
190 application, the MDA concentration decreased again to the level of that in the control plants,
191 except in *G. tomentella,* which remained unchanged (Figure 6).
192
193
194 **Discussion**
195 *Water balance and gas exchange inside the plant during drought stress*
196 Water deficit in plants occurs when water loss due to the transpiration of the leaves
197 exceeds water absorption by the plant roots (Berkowitz, 1998). When this happens, the
198 plant generally tends to reduce water loss by reducing transpiration rate as indicated by
199 Figure 1. Lower transpiration in response to drought stress is associated with the decrease of
200 stomatal conductance which is sensitive to water deficit even before the water potential of
201 the plant decreases (Davies and Zhang, 1991; Davies, Tardieu and Trejo, 1994). In this
202 experiment, the fluctuation of transpiration rate in the control plants was due to the
203 fluctuation of light intensity during measurement under green house conditions without
204 additional light.
205 The-different responses of *E* and *Pn* to drought stress treatment between cultivated
206 soybeans and wild soybean (*G. tomentella*), where those parameters dramatically reduced

after 12 days in cultivated soybeans varieties, while in *G. tomentella* the reduction occurred after 22 days after drought period, may be explained by differences in growth and canopy development. The cultivated soybeans Tidar, Burangrang and Panderman had similar growth and canopy development, while wild soybean *G tomentella* grew more slowly with smaller leaves than cultivated plants (data not shown). Therefore, the water was lost from the media through transpiration and consequently caused reduction of RWC of cultivated soybeans faster than *G. tomentella*.

The reduction of transpiration rate as a response to drought stress was in accordance with the decrease of photosynthetic rate that dramatically declined after 8 days of drought (Figures 1 and 3). The decrease of stomatal conductance reduced CO_2 supply to the chloroplast and then reduced photosynthetic rate (Berkowitz, 1998). Stomatal conductance is the main factor that influences photosynthesis reduction in plants exposed to drought stress (Cornic, 2000).

The application of paraquat herbicide also reduced transpiration as well as photosynthetic rate, however the reduction of the *Pn* was faster than the reduction of *E* (Figures 2 and 4), even though the maximum reduction of *Pn* occurred as the same time as that of *E* (3 days after paraquat application). Paraquat is an active compound that accepts electrons from the early acceptors of photosystem I and then reacts with oxygen to form superoxide, a free radical (Taiz and Zeiger, 2002). High accumulation of ROS inside the leaf may cause damage to chloroplast components, especially lipids, which consequently can reduce photosynthetic rate.

The increase of *E* and *Pn* back to the level of control plants 2 days after drought-stressed plants were re-watered, indicated that all the plants were capable of recovery after

drought (Figure 2 and 4). On the other hand, the *E* and *Pn* of paraquat treated plants did not recover well until 5 days after paraquat application (Figures 2 and 4). This treatment may cause damage to photosynthetic and other cellular apparati, causing malfunction of these components and the need to be rebuilt by the growth of new shoots. Paraquat is a compound that can induce the accumulation of free radicals that cause cellular damage (Chia *et al*. 1982; Scandalios, 1993).

Oxidative stress can be induced by severe drought stress as well as paraquat

The increase of MDA in all of the soybean plants during severe drought stress indicated that severe drought stress can induce oxidative stress in both cultivated and wild soybean plants. The MDA increased dramatically 8 days after the induction of drought stress and reached the maximum level on the 10th and 12th days of drought when the stress was most severe (Figure 5). MDA is a compound resulting from lipid peroxidation at the cellular level, and is frequently used as an indicator of lipid peroxidation level due to oxidative stress (Iturbe-ormaextse *et al.,* 1998).

The increase of MDA also occurred in the plants treated by paraquat herbicide (Figure 6). This herbicide is a positive compound that can be reduced by photosynthetic photoreaction to become an unstable free radical. This radical compound can be oxidized back by oxygen to form the original ions and hydrogen peroxide (H_2O_2), which destroys cells and tissues (Chia *et al*., 1982). When this happens, the plant undergoes lipid peroxidation, protein degradation, DNA denaturation, and pigment damage (Scandalios, 1993). At the cellular level, it causes damage to the cellular membrane and chloroplast (Chia *et al*., 1982). The fact that the increase of MDA levels after 10 days of drought stress

was approximately the same as that of the paraquat application, indicated that oxidative stress may be induced by severe drought.

The level of MDA decreased to that of the control plant 2 days after the drought-stressed plants were re-watered. This seems to indicate that the plants had recovered from oxidative stress after 2 days of re-watering. The same result has also been demonstrated by Zhang dan Kirkham (1994) on wheat, and Wang dan Huang (2004) on bluegrass.

CONCLUSION

Drought stress caused RWC of soybean to dramatically decrease up to 33% in sensitive and 42% in tolerant varieties, while the RWC of control plants was approximately 80%. The *E* and *Pn* also reduced to almost zero in response to drought stress, whereas in control plants they measured 4.7 mmol m^{-2} s^{-1} and 12.58 μmol m^{-2} s^{-1} , respectively. Rewatering after the drought period effectively improved *E* and *Pn,* suggesting that the plants were able to recover after 12 days of drought stress. Application of paraquat also caused *E* and *Pn* reduction, but the the *Pn* did not recover well even 5 days after the application. The increase of MDA to almost twice that of control plants occurred after severe drought as well as paraquat application, which was evidence of oxidative stress in cultivated and wild soybean due to severe drought.

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Tables and Figures

Table 1. Relative water content (%) of soybean leaves of Tidar, Burangrang, Panderman and *G. tomentella* in response to drought period (days) and control plants

Soybean	Drought period (days)							
	0	4	8	10	12	14	22	24
	..... (%)							
Control	80.8	78.0	84.8	78.7	78.0	82.8	82.5	82.8
Tidar	80.2	72.6	52.0*	49.5*	42.5*	71.9	-	-
Burangrang	79.0	70.4*	50.5*	48.1*	42.0*	76.9	-	-
Panderman	80.9	79.2	52.0*	40.7*	32.5*	83.7	-	-
G. tomentella	82.9	83.0	85.5	87.9	60.6*	43.0*	39.4*	84.0

Note: * The values in the same column significantly different at 5% of t-student

Table 2. Relative water content (%) of soybean leaves of Tidar, Burangrang, Panderman and *G. tomentella* after Paraquat application and control plants

Soybean Varieties	Days after paraquat application				
	0	0.16	1	3	5
	……..(%)……				
control	80.8	80.8	77.9	78.0	77.0
Tidar	80.2	63.6*	63.4*	71.3	74.2
Burangrang	80.9	68.6	37.2*	61.9*	74.4
Panderman	79.0	74.9	54.0*	53.7*	79.9
G. tomentella	82.9	69.2	22.5*	79.2	62.3

Note: * The values in the same column significantly different at 5% of t-student

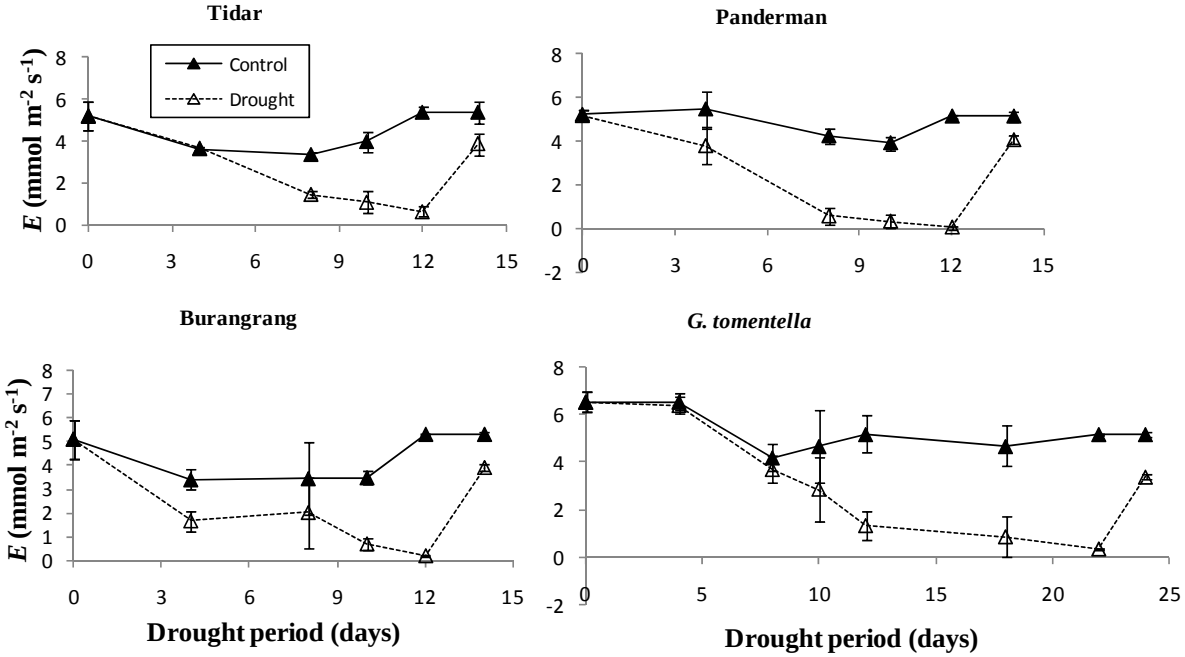

Figure 1. Transpiration rate (*E*) of cultivated soybean varieties Tidar, Burangrang, Panderman and wild soybean *G. tomentella* during drought period (mean ± SE of t-student test at α of 5%, n=3).

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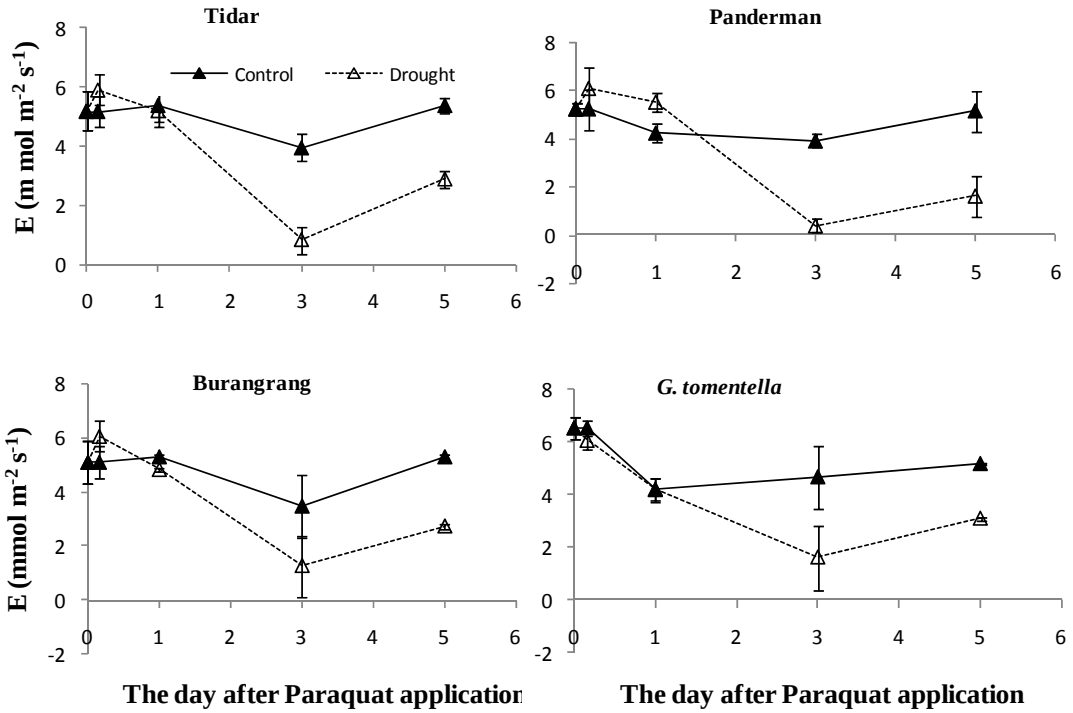

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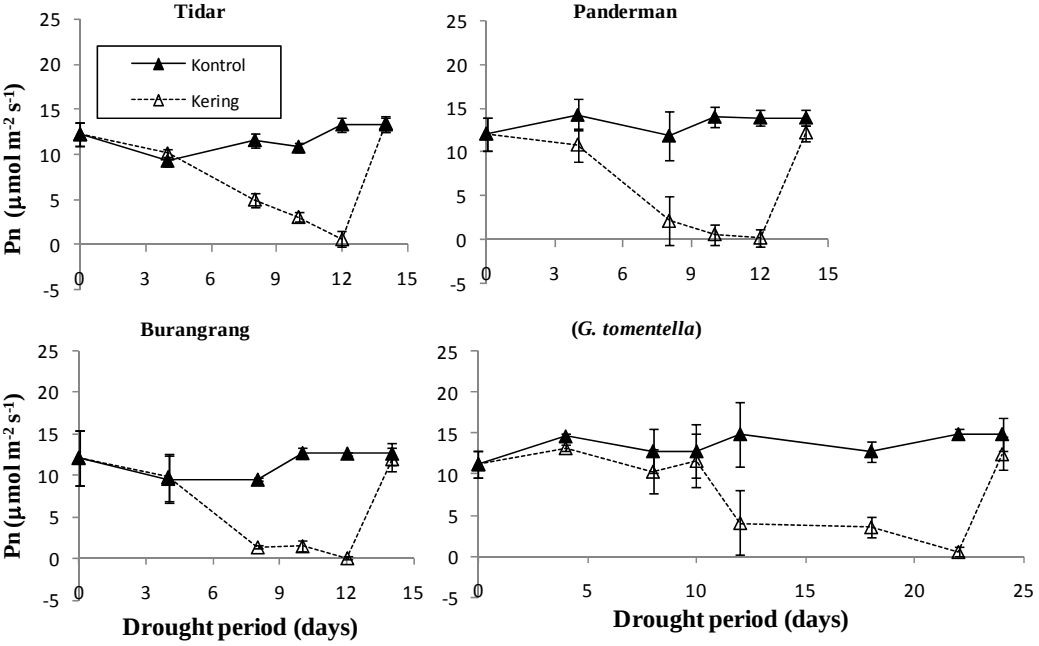
366 Figure 2. Transpiration rate (*E*) of cultivated soybean varieties Tidar, Burangrang,
367 Panderman and wild soybean *G. tomentella* during 5 days after Paraquat application (mean
368 ± SE of t-student test at α of 5%, n=3).



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371 Figure 3. Photosyntetic rate (*Pn*) of cultivated soybean varieties Tidar, Burangrang,
372 Panderman and wild soybean *G. tomentella* during drought period (mean ± SE of t-student
373 test at α of 5%, n=3).

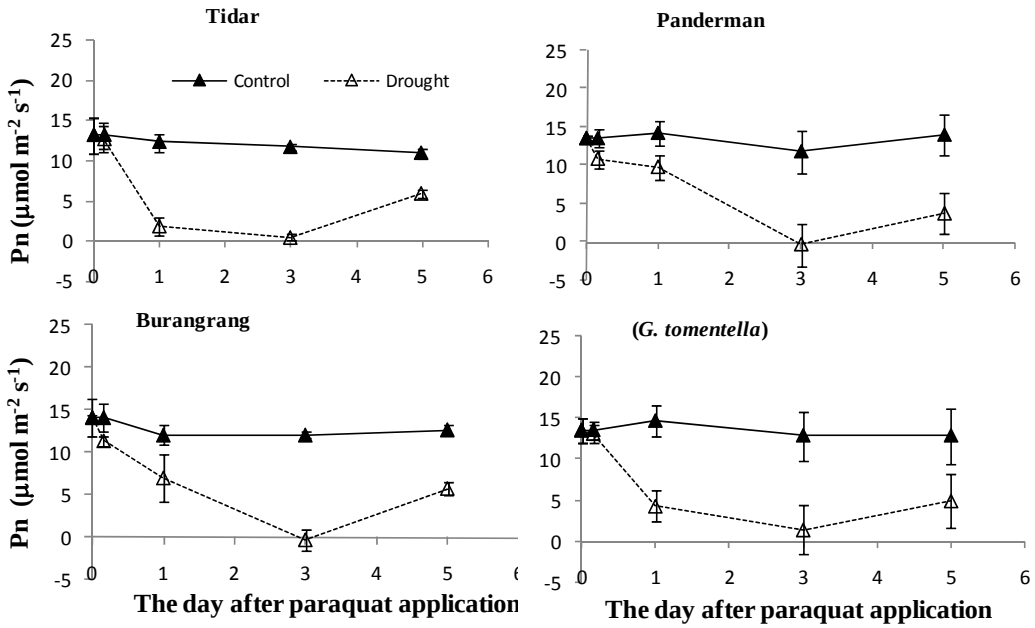

Figure 4. Photosyntetic rate (*Pn*) of cultivated soybean varieties Tidar, Burangrang, Panderman and wild soybean *G. tomentella* during 5 days after Paraquat application (mean ± SE of t-student test at α of 5%, n=3).

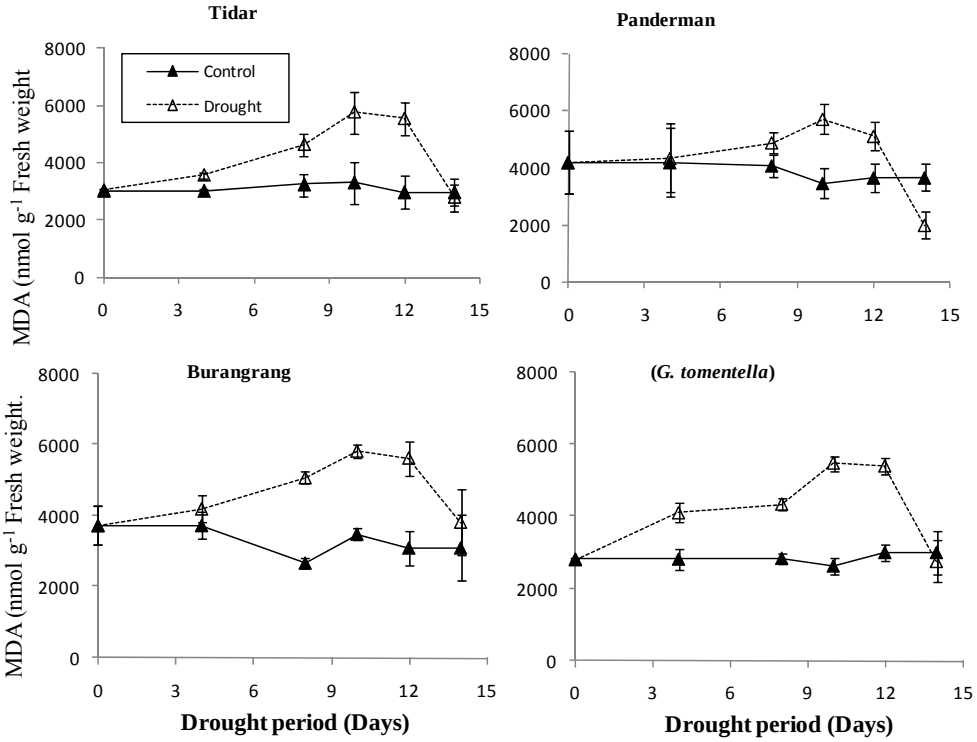

Figure 5. Malondialdehyde (MDA) content of soybean leaves of Tidar, Burangrang, Panderman and wild soybean *G. tomentella* during drought period (mean ± SE of t-student test at α of 5%, n=3).

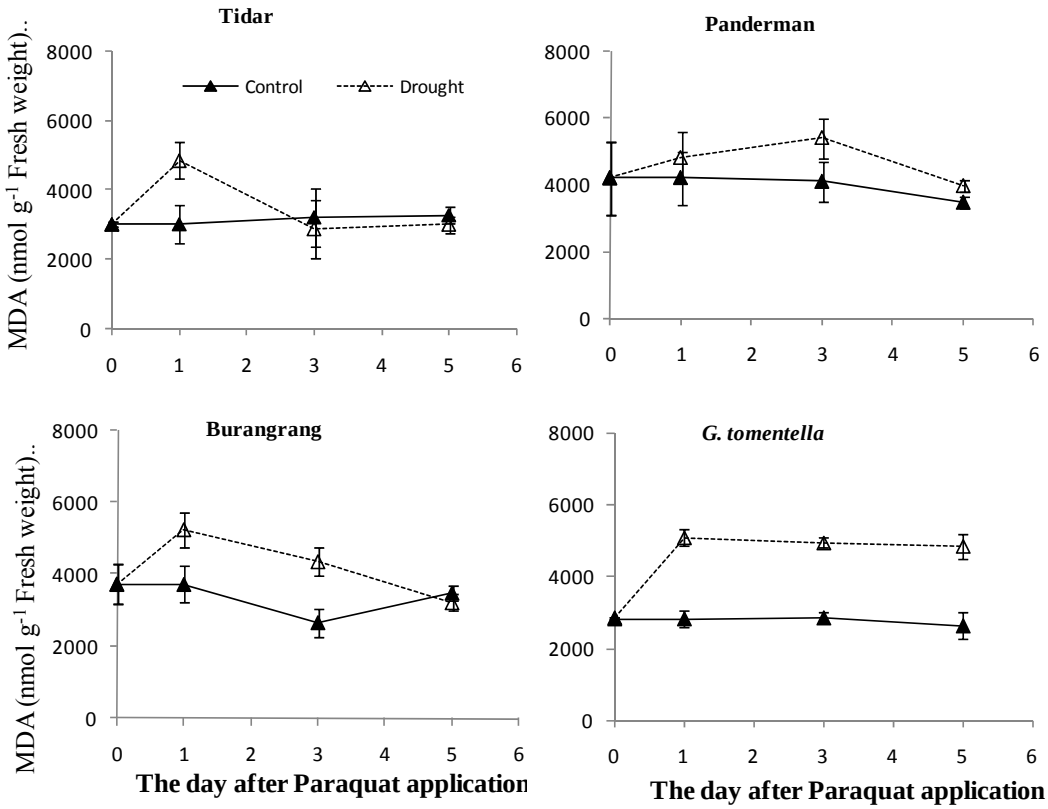

Figure 6. Malondialdehyde (MDA) content of soybean leaves of Tidar, Burangrang, Panderman and wild soybean *G. tomentella* 5 days after Paraquat application (mean ± SE of t-student test at α of 5%, n=3).