**Running title**

**Responses of *Hordeum vulgare* to a short-term potassium deprivation**

# Implication of phospholipase D in response of *Hordeum vulgare* root to a short-term potassium deprivation

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**Summary**

To verify a possible implication of lipids and some other compounds such as hydrogen peroxide ($H_2O_2$) and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) in the response of *Hordeum vulgare* to an early potassium deprivation, plants were grown in hydroponics for 30 days with a modified Hewitt nutrient solution containing 3 mM $K^+$ and then incubated for increasing time ranging from 2 h up to 36 h in the same medium deprived of $K^+$. In contrast to leaves, root $K^+$ concentration showed its greatest decrease after 6 h of treatment. The main lipids of the control barley roots were phospholipids (PL), representing more than 50% of the total lipids. PL did not change with treatment whereas free sterols (FS) amounts decreased following $K^+$ deprivation, showing an about 17% reduction after 36 h. As regards the individual PL, 30 h $K^+$ deprivation caused a reduction in phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) levels whereas phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidic acid (PA) increased. The maximum PA accumulation as well as the highest phospholipase D (PLD) activation, estimated by an accumulation of phosphatidylbutanol (PtBut), were observed after 24 h of $K^+$ starvation. At the root level, after 6 h of incubation in –K solution, $H_2O_2$ level showed the maximum value. At the same time G3PDH activity reached the minimum. On the basis of a concomitant stimulation of PLD activity and, consequently, PA accumulation, enhancement of $H_2O_2$ production, and inhibition of G3PDH activity we can suggest a possible involvement of these three compounds in an early response to $K^+$ deprivation.

***Key words:*** Glyceraldehyde 3-phospate dehydrogenase, *Hordeum vulgare*, lipids, phospholipase D, potassium deprivation

**Abbreviations**

ASG, acylated steryl glycosides; CER, cerebrosides; DGDG, digalactosyldiacylglycerols; FS, free sterols; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; GL, glycolipids; $H_2O_2$, hydrogen

peroxide; MGDG, monogalactosyldiacylglycerols; PA, phosphatidic acid; PtBut, phosphatidylbutanol, PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipids; PLD, phospholipase D; PS, phosphatidylserine.

**Introduction**

In their natural environments, plants are subjected to multiple abiotic stresses including drought, salinity, heavy metals, and nutrient deficiencies. The latter constraint affects seriously growth and metabolism of plants on agricultural soils around the world (Wang, 2002). Among the essential mineral nutrients, $K^+$, as macronutrient, is needed for growth and metabolic process of plants. $K^+$ plays an important role in a wide range of functions: photosynthesis, enzyme activation, protein synthesis and osmoregulation (Marschner, 1995). $K^+$ deficiency usually causes numerous physiological disorders, depressed plant growth and development and reduced crop yield and quality. In a natural environment $K^+$ concentrations vary widely in time and space and low-$K^+$ conditions are often transient. Therefore, plants, unable to move, must be able to adjust their uptake systems rapidly to transient shortages in $K^+$ supply (Grieth et al., 2005).

Although much is known about $K^+$ uptake systems, little is known about the mechanisms by which plants sense and respond to variations in the $K^+$ concentrations (Shin and Schachtman, 2004).

In response to $K^+$ deprivation, recent studies indicate the involvement of some compounds considered as signalling molecules such as hydrogen peroxide ($H_2O_2$) (Hafsi et al., 2008), ethylene (Shin and Schachtmann, 2004) and phytohormones such as auxin, and jasmonic acid (Armengaud et al., 2004).

Recently, it has been demonstrated that phospholipase D (PLD, EC 3.1.4.4), besides its catabolic function, has critical roles in cell signalling cascades (Meijer and Munnik, 2003; Navari-Izzo et al., 2006; Russo et al., 2007; Sgherri et al., 2007). This enzyme hydrolyzes structural phospholipids at the terminal phosphate diester bond, leading to the formation of phosphatidic acid (PA) and a free

head group such as choline, in the case of phosphatidylcholine (PC, Zhang et al., 2003). PA is considered a second messenger in response to multiple environmental stresses, i.e. hyperosmotic stress (Munnik et al., 2000), symbiotic interactions (Den Hartog et al., 2001), copper excess (Navari-Izzo et al., 2006; Sgherri er al., 2007), and phosphate deprivation (Russo et al., 2007). In *vivo* and in *vitro* studies in suspension cultured rice cells, Yamaguchi et al. (2004) demonstrated that PLD is activated by $H_2O_2$ and this activation involves a protein tyrosine kinase. It has also been suggested that $H_2O_2$ directly modulates the reactive cysteine residues of protein tyrosine kinase (Meng et al., 2002) and glyceraldehydes-3-phosphate dehydrogenase (G3PDH; EC 1.2.1.12) inhibiting their enzymatic activities (Schuppe-Koistinen et al., 1994). In animal cells (PC12 cells), Kim et al. (2003) identified G3PDH as a $H_2O_2$-dependent positive regulator of PLD2 and found that the interaction between G3PDH and PLD2 was highly dependent on oxidative modulation of catalytic cysteine in G3PDH. Subsequently, these authors suggested that $H_2O_2$, besides its role in the inactivation of the dehydrogenase activity of G3PDH on its catalytic site, may also endow G3PDH with the ability to bind PLD2 and the resulting association is involved in the regulation of PLD2 activity by $H_2O_2$. Recently, in the halophyte *Hordeum maritimum* subjected to a short potassium deprivation, a clear relationship among $H_2O_2$ production, G3PDH inhibition and PLD activation has been also demonstrated (Hafsi et al., 2008).

In this context, the present work focuses on changes in PLD and G3PDH activities, $H_2O_2$ production, and on alterations of root lipids in *H. vulgare* following short periods of $K^+$ starvation. The finding of a link among these compounds should be a useful tool for the further dissection of signalling pathways involved in the perception of $K^+$ changes at cellular and tissue level.

## Materials and methods

### *Plant Material*

Seeds of *Hordeum vulgare* (var. Manel) were obtained from the National Institute of Agronomic Research of Tunis. Seeds were disinfected for 2 min with NaClO (approximately 2% of active

chlorine), abundantly rinsed in distilled water and imbibed for 16 h with running tap water. The seeds were then placed on a floating layer of clay in plastic pots filled with 5 L of tap water. Three days after sowing, the pots were filled with 5 L of modified Hewitt nutrient solution (1966). The nutrient solution contained the following macronutrients: 1.5 mM $MgSO_4$, 3.5 mM $Ca(NO_3)_2$, 5.4 mM $NaNO_3$, 2 mM $NH_4H_2PO_4$, and 3 mM KCl. The micronutrients (ppm) were: Mn (0.5), Cu (0.04), Zn (0.05), B (0.5), Mo (0.02) (Arnon and Hoagland, 1940) and Fe (3) as Fe-tartaric acid complex. Nutrient solutions were renewed twice per week. Cultivation was carried out in a growth chamber with a day/night temperatures of 21°C/16°C, a 16 h photoperiod, a photon flux density of 400 µmol $m^{-2}$ $s^{-1}$ and 70-75% relative humidity. Light was provided by fluorescent tubes (Osram L 140W/20) and incandescent lamps (Philips 25W).

After a period of 30 d, roots of one set of intact plants were washed with distilled water and plants transferred to the same growth medium but deprived of $K^+$ for increasing periods of time (2, 6, 24, 30, and 36 h). The other set of plants was kept in the +K solution as a control. For PLD determination the plants were incubated in the presence of 0.2% *n*-butanol.

***Extraction and separation of lipids***

Lipids were extracted from fresh root tissues by addition of boiling isopropanol followed by chloroform:methanol (2:1, v/v) containing butylhydroxytoluol (50 µg $ml^{-1}$) as an antioxidant. The solvent mixture was then washed with 0.88% KCl to separate the chloroform phase. The upper water phase was re-extracted with chloroform and the chloroform phases combined and dried under a stream of $N_2$. Lipid extracts dissolved in chloroform:acetic acid (100:1, v/v) were transferred to SEP-PAK (Waters, Milford, MA, USA) according to Navari-Izzo et al. (2006) and sequentially eluted with 20 ml of chloroform:acetic acid (100:1, v/v) for neutral lipids, 10 ml of acetone and 10 ml acetone:acetic acid (100:1, v/v) for glycolipids and 7.5 ml of methanol:chloroform:water (100:50:40, v/v/v) for phospholipids (PL). Chloroform (2.25 ml) and water (3 ml) were added successively to the eluate containing the PL to obtain a phase separation and to facilitate their

recovery. The separation of individual lipids was performed by TLC (Silica Gel 60, 0.25 mm thickness; Merck, Damstadt, Germany) with the following solvent mixture: petroleum ether-$Et_2O$-HOAc (80:35:1, v/v/v) for neutral lipids (free sterols); $CHCl_3$-MeOH-$H_2O$ (65:25:4, v/v/v) for glycolipids (steryl glycosides and cerebrosides); $CHCl_3$-MeOH-HOAc-$H_2O$ (85:15:10:3.5) for PL. After development, the bands were located with iodine vapour. Individual lipids were identified by co-chromatography with authentic standards. Total lipids derived from the sum of the moles recovered for free sterols (FS), glycolipids (GL) and PL. FS were extracted with n-hexane and quantitatively assayed as reported by Navari-Izzo et al. (1993) using cholesterol as standard. PL and GL were quantified assaying their phosphorus and glucose contents, respectively (Navari-Izzo et al., 1993).

***Phospholipase D activity***

PLD (EC 3.1.4.4) activity was measured as the in *vivo* production of phosphatidylbutanol (PtBut) essentially as described by de Vrije and Munnik (1997). Following the extraction and separation of the PL fraction from lipids of whole roots and leaves, PtBut was isolated from the rest of the PL developing the TLC plates in the organic upper phase of a solvent mixture composed by ethyl acetate:iso-octane:acetic acid:water (v/v/v/v). After development, bands were located with iodine vapors and PtBut identified by co-chromatography with an authentic standard (Avanti Polar Lipids, Alabaster, AL). Quantitative analysis of PtBut was performed as reported by Navari-Izzo et al. (2006) using $KH_2PO_4$ as a standard.

***$H_2O_2$ determination***

$H_2O_2$ contents were evaluated following the method of Sgherri et al. (1994), with 1 g of tissue and a standard curve in the 1-15 nmol $H_2O_2$ range at 0-4° and using ice-cold solutions. According to the previous authors, this method is very sensitive and reproducible, and it excludes the interference of other peroxides except for a small amount of lipid peroxide.

***$NADP^+$-dependent glyceraldeyde-3-phosphate dehydrogenase (G3PDH)***

The extraction of G3PDH was performed in 100 mM Tris-HCl (pH 8.1), containing 0.1 mM $Na_2$-EDTA, 1 mM diethyldithiocarbamic acid (DIECA) and 4% (w/v) polyclar AT. The assay mixture consisted of 100 mM Tris-HCl (pH 8.1), containing 5 mM $MgCl_2$, 2 mM ATP, 1 mM 3-phosphoglyceric acid, 0.06 U $ml^{-1}$ 3-phosphoglyceric phosphokinase and 70 μM NADPH. $NADP^+$-dependent G3PDH activity was determined using a coupled reaction, by monitoring the reduction in absorbance following NADPH oxidation at 340 nm (Navari-Izzo et al., 1997).

***Potassium content***

Aliquots of roots and shoots were ground to a fine powder and then digested with concentrated $HNO_3$ and $K^+$ content was determined by atomic absorption spectrophotometry (Izzo et al., 1991).

**Statistical analysis**

The results are the means from two replicates of three independent experiments ($n$=3). All data are reported as mean values ± standard errors (SE). The significance of differences among mean values was determined by one-way ANOVA. Comparisons among means were performed using Duncan's multiple-range test. Reported means in figures accompanied by different letters are significantly different at $P \leq 0.01$.

**Results**

During the whole treatment, control plants did not show significant differences in both mineral and biochemical parameters in comparison with plants collected at the beginning of the experiment. For this reason the data reported as control (0 h) are the mean of the values obtained during the whole experimental period.

In roots of *H. vulgare* after 6 h of $K^+$ deprivation a reduction in $K^+$ concentration of about 41% in comparison with +K roots was observed. After this period, a slight increase in $K^+$ concentrations occurred and after 36 h of $K^+$ deprivation the value of $K^+$ in the roots reached the same level as in the control roots (Fig.1). On the contrary the leaves did not show significant reduction in $K^+$ concentration during the whole experimental period in comparison with the control (Fig.1).

The main lipids of the control barley roots were PL, representing more than 50% of the total lipids, followed by FS (17%), cerebrosides (CER, 14%) and lesser amounts of GL (MGDG and DGDG) and acylated steryl glycosides (ASG). PL amounts did not change with the treatment whereas FS amounts decreased following $K^+$ deprivation, with a reduction of 17% after 36 h; in contrast, GL, cerebrosides and ASG increased (Fig. 2). As regards the individual PL, 30 h of $K^+$ deprivation caused a reduction in phopsphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) levels whereas phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidic acid (PA) increased (Fig. 3). As a consequence of PC and PE changes, the PC to PE molar ratio decreased by 50% at 30 h of $K^+$ deprivation in comparison to the control.

The accumulation of PA started after 2 h of $K^+$ deprivation and continued till 24 h, when it reached a value 3.3 fold higher than the control (Fig. 4). Thereafter, PA level decreased remaining anyway higher than in the control at the end of the experiment (36 h). PtBut showed the same trend of PA (Figs, 4, 5); indeed, PtBut accumulation began after 2 h and increased approximately more than 2-fold within 24 h, decreasing thereafter (Fig 5). In the leaves no PtBut formation was observed (data not shown).

In roots, $H_2O_2$ level increased showing a maximum after 6 h of $K^+$ deprivation (1.2 fold increase) in comparison with +K roots. Thereafter, $H_2O_2$ level decreased reaching after 30 h values lower than in the control. The leaves showed an opposite trend decreasing till 6 h and increasing thereafter reaching the control value at the end of experiment (Fig. 6).

In roots and leaves subjected to $K^+$ deprivation, G3PDH activity showed a similar trend but the activity was higher in roots than in leaves for the whole period of treatment. The G3PDH activity

decreased with time reaching the minimum value after 6 h of treatment (35% and 59% of reduction in roots and leaves in comparison with the control, respectively). Thereafter G3PDH activity increased again (Fig.7).

**Discussion**

The early decrease in root $K^+$ concentrations (Fig.1) might be due to its translocation to leaves, in which $K^+$ concentrations are relatively stable for the whole period of $K^+$ starvation (Hafsi et al., 2008).

The removal of $K^+$ from the growth medium led to a decrease in the membrane phospholipids PC, PS and PI. This may reflect the induction of different phospholipases ($PLD_s$) responsible for the hydrolysis of some membrane phospholipids producing water-soluble free head groups (e.g. choline) and PA (Cummings et al., 2002; Meijer and Munnik, 2003). In *H. vulgare*, differently from *H. maritimum* which showed a first activation of PLD after 2 h of $K^+$ deprivation and a second stimulation after 24 h of treatment (Hafsi et al., 2008), PLD activity peaked after 24 h of $K^+$ starvation (3.5-fold PtBut accumulation in comparison with the control) (Fig. 5). PtBut is a relative measure of PLD activity due to the unique ability of this enzyme to transfer its phosphatidyl group to an alcohol forming phosphatidylalcohol (Munnik, 2001; Navari-Izzo et al., 2006). In agreement with Navari-Izzo et al. (2006) and Russo et al. (2007) on the basis of the concomitant increase in PLD activity and the decrease in some membrane PL and, in particular, in the PC content (Fig. 3), we can hypothesize that PC could have served as substrate for PA formation. Following $K^+$ deprivation the decrease in PC, with the simultaneous increase in PE (Fig. 3), suggests that PC could have been converted into PE by polar head group exchange or hydrolysed forming PA. Under severe stress condition, PC hydrolysis results in the formation of DAG for DGDG synthesis, but in *H. vulgare* we detected neither increase in DAG nor in DGDG (data not shown), as a consequence, under $K^+$ deficiency PLD activity inducing PA accumulation may reflect the induction of different phospholipases involved in signalling the lowered $K^+$ status. $K^+$ limitation was quickly sensed by

the root system since PA accumulation was measured as early as 2 h after the transfer of plants in –K medium. The higher amount of PA in comparison with PtBut (12.4 and 5.9 mol% of the total PL, respectively) could derive, besides via PLD, via the PLC/DAG kinase pathway as suggested by Testerink and Munnik (2005) and Russo et al. (2007) or as intermediate in the biosynthesis of GL and PL.

The early and transient increase in $H_2O_2$ in roots (Fig. 6) supports the idea that this molecule could play a signalling role in response to a short-term $K^+$ starvation. Shin and Schachtman (2004) and Shin et al. (2005) showed that $H_2O_2$ is rapidly accumulated in response to a short-term potassium deprivation triggering the expression of certain genes. In addition, the previous authors demonstrated that changes in the kinetics of $K^+$ uptake are due to $H_2O_2$, whose production is localized in a specific region of the roots that has been shown to be active in $K^+$ uptake and translocation. Under the same growth conditions of *H. vulgare*, we have found in the halophyte *H. maritimum*, a great and fast production of $H_2O_2$ after 6 h of $K^+$ starvation in both roots and leaves (Hafsi et al., 2008). In *Arabidopsis* roots most of gene expression was induced after 6 h of $K^+$ deprivation (Shin et al., 2005), and in *H. maritimum* roots the concomitant increase and decrease in $H_2O_2$ level and G3PDH activity, respectively, was sensed after 6 h when the maximum $K^+$ deprivation occurred (Hafsi et al., 2008). Six h of $K^+$ deprivation could represent a crucial time to sense the nutrient deprivation (Shin et al., 2005) and to start an early response at least at root level in *H. vulgare* too (Figs 6-7). The eventually PLD activation (Figs. 4-5) might be, in part, the result of G3PDH inhibition by $H_2O_2$.

Indeed, $H_2O_2$ is the most stable of reactive oxygen species and is capable of rapid diffusion across cell membranes and several studies demonstrated that $H_2O_2$ is implicated in PLD activation: PLD in endothelial cells (Natarajan et al., 1993) and PLDδ in *Arabidopsis* (Zhang et al., 2003), are activated by $H_2O_2$. The mechanism by which PLD is activated by $H_2O_2$ requires further research. In *vivo* and in *vitro* studies in suspension cultured rice cells, PLD is activated by $H_2O_2$ and this activation involves a protein tyrosine kinase (Yamaguchi et al., 2004). Recently it has been

indirectly suggested that G protein- regulated events are likely involved in PLD signalling (Navari-Izzo et al., 2006) even though a recent report indicates that, at least in plants, mastoparan also has the ability to activate MAPK signalling without requiring the involvement of a canonical heterotrimeric G protein (Miles et al., 2004).

It has been demonstrated that in *Arabidopsis* cell extracts, the activity of G3PDH is inhibited by $H_2O_2$ and Hancock et al. (2005) suggested that G3PDH may be a direct target of $H_2O_2$. Furthermore, in animal cells (PC12 cells) the activity of PLD2 was dependent on its interaction with G3PDH (Kim et al., 2003). These authors suggested that $H_2O_2$, besides its role in the inhibition of the dehydrogenase activity of G3PDH, causes structural changes of this enzyme on its catalytic cysteine residue which facilitate its association with PLD2, and as a consequence, the regulation of PLD2 activity by $H_2O_2$.

Besides the evidences *in vitro* and in animal cells (Kim et al., 2003), the present data, together those obtained in *Arabidopsis* (Shin et al., 2005) and *H. maritimum* (Hafsi et al., 2008), support the hypothesis that a link among G3PDH activity, $H_2O_2$ level and PLD activation could play a role also in roots as a part of the signalling machinery activated following a short-time $K^+$ starvation. However, other experiments will be needed to investigate more in deeply the mechanisms by which these three compounds are interconnected and implicated in the response to a short-term $K^+$ deprivation.

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Legends to Figures

**Figure 1**

Potassium concentrations in roots and leaves of *Hordeum vulgare* seedlings incubated in a solution without $K^+$ for increasing periods of time (0-36 h). Results are the means of three replicates each analysed twice ± SE ($n$ = 3). Significant differences ($P \leq 0.01$) among treatments are accompanied by different letters.

**Figure 2**

Total lipid composition of roots of *H. vulgare* seedlings incubated in a solution without $K^+$ for increasing periods of time (0-36 h). Statistical analysis was as in Figure 1. PL, phospholipids; GL, glycolipids; CER, cerebrosides; FS, free sterols; ASG, acylated steryl glycosides. Single lipid class is expressed as mole % of the total moles of lipids.

**Figure 3**

Phospholipid composition of roots of *H. vulgare* seedlings incubated in a solution without $K^+$ for increasing periods of time (0-36 h). Statistical analysis was as in Figure 1. PL, phospholipids; PA, phosphatidic acid; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine. The single PL is expressed as mole % of the total moles of PL.

**Figure 4**

Phosphatidic acid (PA) in roots of *H. vulgare* seedlings incubated in a solution without $K^+$ for increasing periods of time (0-36 h). Contents are expressed as fold stimulation in comparison with the control (horizontal line). Results are the means of three replicates each analysed twice ± SE ($n$ = 3).

**Figure 5**

Accumulation of phosphatidylbutanol (PtBut) in roots of *H. vulgare* seedlings incubated in a solution without $K^+$ for increasing periods of time (0-36 h). At each time point PtBut is expressed as

fold stimulation in comparison with the control (horizontal line, 1.97 mol % of total PL). Results are the means of three replicates each analysed twice ± SE ($n$ = 3).

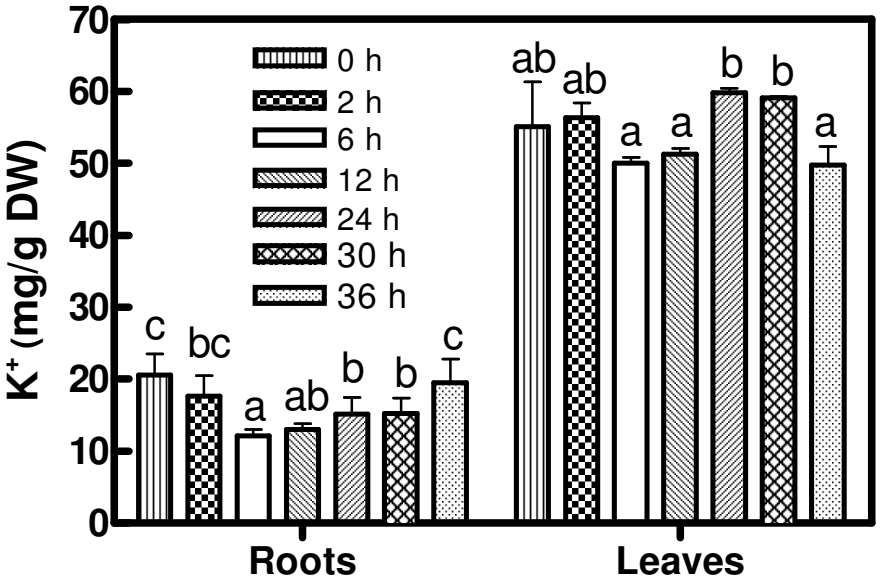
**Figure 6**

Hydrogen peroxide ($H_2O_2$) contents in roots and leaves of *H. vulgare* seedlings incubated in a solution without $K^+$ for increasing periods of time (0-36 h). Statistical analysis was as in Figure 1.

**Figure 7**

Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) activity in roots and leaves of *H. vulgare* seedlings incubated in a solution without potassium for increasing periods of time (0-36 h). Statistical analysis was as in Figure 1.

**Figure 1**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b
a
Roots
Leaves

**Figure 2**

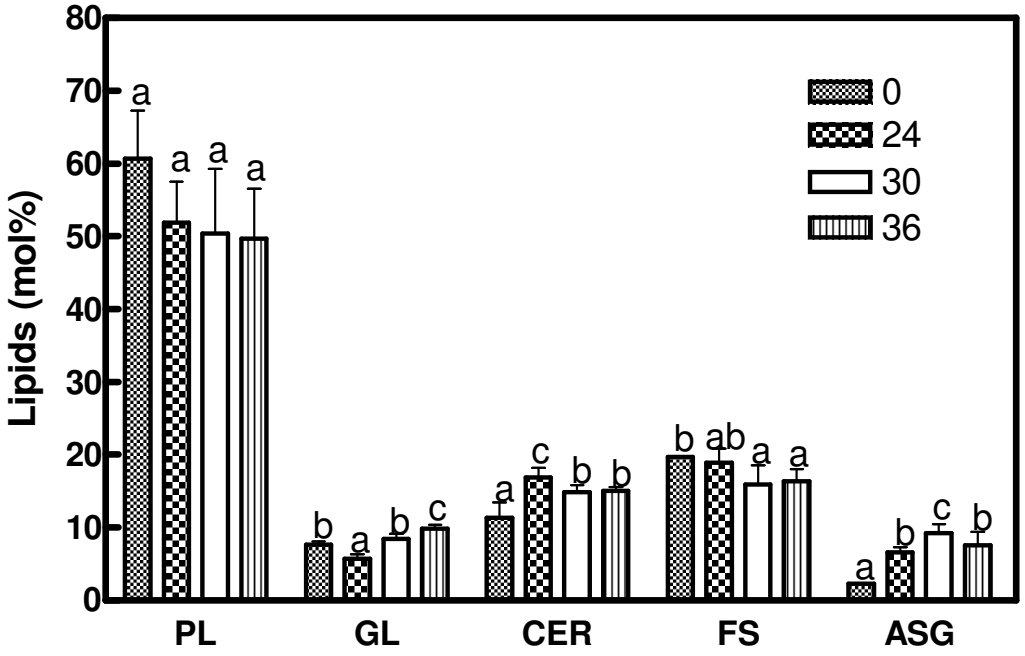

Lipids (mol%)
80
70
60
50
40
30
20
10
0
0
24
30
36
a
a
a
a
b
a
b
c
a
c
b
b
b
ab
a
a
a
b
c
b
PL
GL
CER
FS
ASG

**Figure 3**

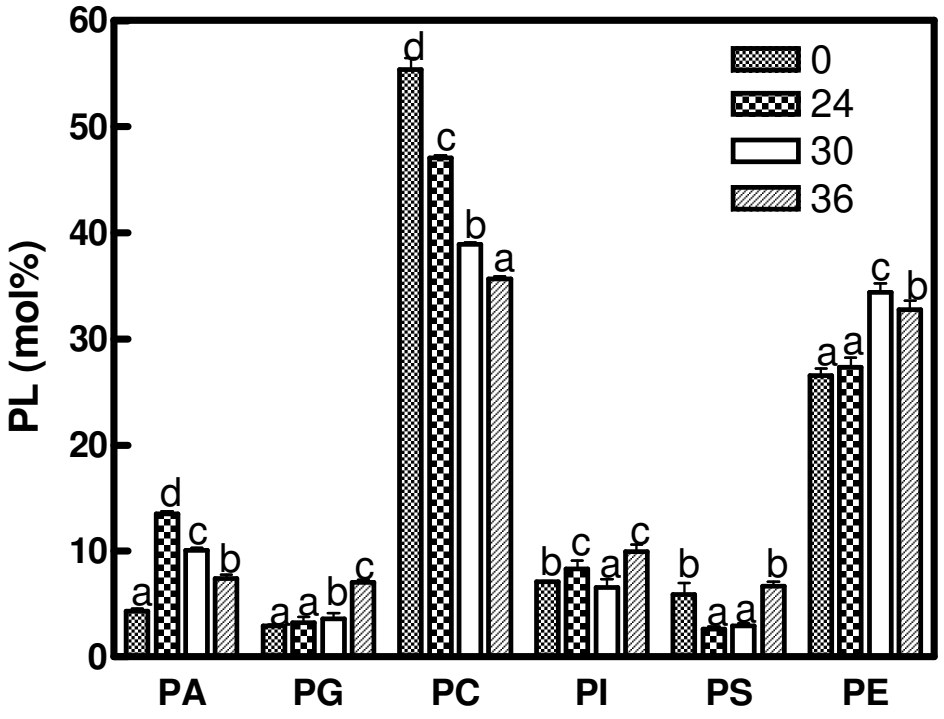

PL (mol%)
60
50
40
30
20
10
0
0
24
30
36
a
d
c
b
a
a
b
c
d
c
b
a
b
c
a
c
b
a
a
b
a
a
c
b
PA
PG
PC
PI
PS
PE

**Figure 4**

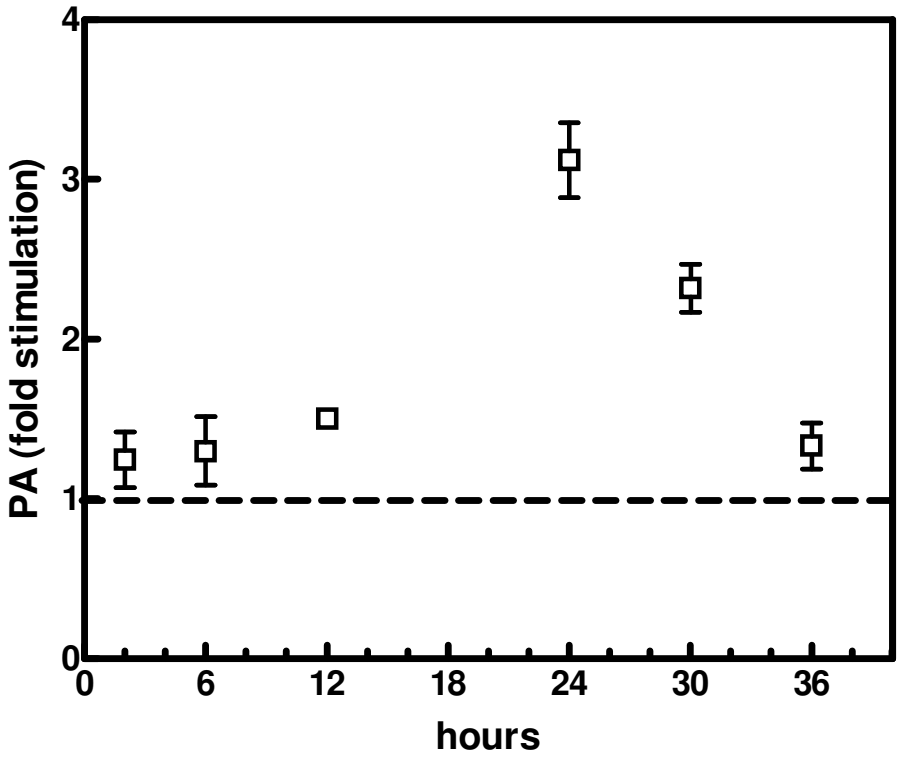


**Figure 5**

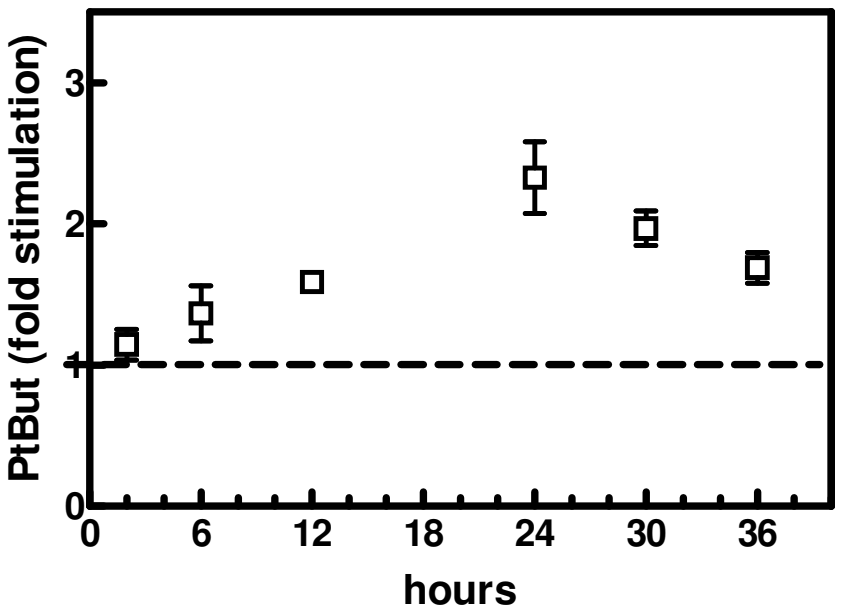


**Figure 6**

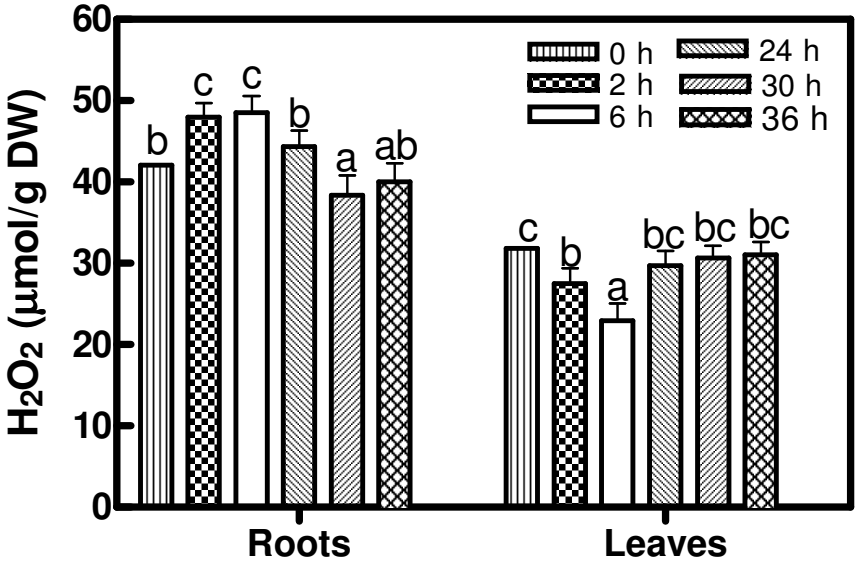
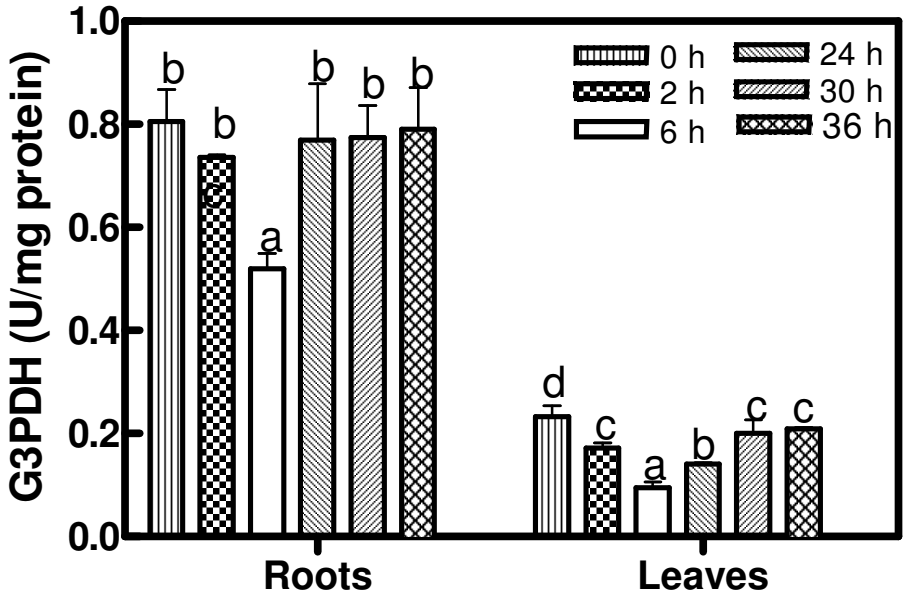

60
50
40
30
20
10
0
H2O2 (µmol/g DW)
0 h
2 h
6 h
24 h
30 h
36 h
b
c
c
b
a
ab
c
b
a
bc
bc
bc
Roots
Leaves

**Figure 7**


1.0
0.8
0.6
0.4
0.2
0.0
G3PDH (U/mg protein)
0 h
2 h
6 h
24 h
30 h
36 h
b
b
a
b
b
b
d
c
a
b
c
c
Roots
Leaves