Involvement of phospholipase A_2 in the response of *Solanum* species to an elicitor from *Phytophthora infestans*

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A B S T R A C T

Changes in activity of phospholipase A_2 (PLA_2), a key enzyme in lipid metabolism and signal network in defence mechanisms, were investigated in *Solanum* species and *Phytophthora infestans* interaction. We have compared PLA_2 activity in response to an elicitor, a culture filtrate (CF) derived from *P. infestans*, in non-host resistant *Solanum nigrum* var. *gigantea*, field resistant *Solanum tuberosum* cv Bzura and susceptible *S. tuberosum* clone H-8105. To elucidate the contribution of specific forms of PLA_2 to plant defence mechanism reasonably selective PLA_2 inhibitors, haloenol lactone suicide substrate (HELSS) and p-bromophenacyl bromide (BPB), which discriminate between Ca^{+2}-independent PLA_2 ($iPLA_2$) and Ca^{+2}-dependent secretory PLA_2 ($sPLA_2$), were used. The *in vivo* and *in vitro* effects of the inhibitors on PLA_2 activity and on generation of reactive oxygen species (ROS) induced by CF in the studied plants were assayed. We found that PLA_2 activity increased in response to CF treatment, displaying various kinetics and intensity depending on the resistance status of a given genotype. Differences among the genotypes in the effects of each inhibitor on CF-induced PLA_2 activity and on ROS production may reflect the diversity of PLA_2 isoforms in plants. Contrary to BPB, the inhibitory effect of HELSS was observable mainly on CF-induced PLA_2 activity, which suggests that $iPLA_2$ participates in signal transduction in defence reactions. Various effects of the two inhibitors on PLA_2 activity and ROS production suggest different contribution of $sPLA_2$ and $iPLA_2$ to modulation of defence reactions in the interaction between *Solanum* genotypes and *P. infestans*.

Keywords: Phospholipase A_2
Phytophthora infestans
Resistance
Solanum

Abbreviations:

BPB	bromophenacyl bromide
CF	culture filtrate
FA	fatty acid
1,2,-dilinoleoyl PC	1,2,-dilinoleonyl phosphatidylcholine
HELSS	haloenol lactone suicide substrate
HR	hypersensitive response
LA	linoleic acid
LaH	lipid acylhydrolase
LnA	linolenic acid
LOX	lipoxygenase
LPC	lysophosphatidylcholine
LPL	lysophospholipid
PLA	phospholipase A
cPLA	cytosolic Ca^{+2}-dependent phospholipase A
$iPLA_2$	Ca^{+2}-independent phospholipase A_2
$sPLA_2$	secretory phospholipase A_2
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species

Introduction

Recent literature shows that lipid metabolism is implicated in plant response to biotic and abiotic stresses, e.g., Ryu (2004), Muller and Berger (2009). Lipid metabolism is initiated by the release of polyunsaturated fatty acids (PUFAs) from structural membranes by the action of lipid acyl hydrolases (LAHs). Among multiple plant LAHs great interest has been focused on phospholipases A (PLAs), which generate linoleic (LA) and/or linolenic (LnA) acids and lysophospholipids (LPL). These products are themselves biologically active or serve as precursors of other compounds active in a complex signal network. PLAs consist of two types depending on positional specificity: phospholipase A_1 (PLA_1) and phospholipase A_2 (PLA_2), which hydrolyse phospholipids at *sn*-1 or *sn*-2 position, respectively. Based on sequence data and biological properties plant PLA_2s are classified into two groups: the low molecular weight secretory PLA_2 ($sPLA_2$), which is Ca^{+2}-dependent, and the patatin-like PLA, which is homologous in amino acid sequences to animal Ca^{+2}-independent PLA_2 ($iPLA_2$); the patatin-like PLA combines PLA_1 and PLA_2 activity. The plant $sPLA_2$s show significant similarity to animal $sPLA_2$s in the active site and in the calcium- binding loop regions. The plant patatin-like PLAs, $iPLA_2$, function as serine hydrolases and have an active Ser residue in the middle of the consensus sequence GXSXG, which is conserved in animal $iPLA_2$s. The existence of cytosolic plant PLA_2, homologous to animal cytosolic Ca^{+2}-dependent PLA (cPLA), has not yet been well documented (Holk et al. 2002; Lee et al. 2005; Mansfeld et al. 2007). Effective resistance to pathogens is often dependent on the host cell death at the infection site, defined as a hypersensitive response (HR), and on expression of defence genes followed by changes in activity of selected metabolic pathways. La Camera et al. (2005, 2009), using transgenic *Arabidopsis* plants with modulated levels of patatin-like protein (PLP2) infected by pathogens with different lifestyles, observed that PLP2 influenced various forms of cell death in host as well as in the lesion-mimic mutant. Induction of PLA_2 activity in response to pathogen or elicitor treatment of plant tissues has often been reported, e.g., in potato tuber inoculated with *Phytophthora infestans* (Kawakita et al. 1993), in tomato leaves after 15 min incubation with systemin, oligosaccharide

elicitors and chitosan (Narváez-Vásques et al. 1999). It has been found that in elicitor treated tobacco leaves transcripts of genes encoding patatin-like proteins (*NtPat*) and *9-Lox* accumulated with similar profiles (Dhondt et al. 2002). Enhanced PLA_2 activity in potato cells treated with an elicitor from *P. infestans* and increased levels of transcripts of *9-Lox* and *NtPat* in elicited tobacco leaves preceded the biosynthesis of biologically active oxylipins, as demonstrated, respectively, Göbel et al. (2001) and Cacas et al. (2005). Hence, the key role of PLA_2 in defence processes is thought to produce PUFAs, which after oxidation by 9- or 13-lipoxygenase (9-, 13-LOX) are further metabolised into oxylipins. In addition, free PUFAs are important cellular mediators (Scherer et al. 2010). Furthermore, the LPL, another product of PLA_2 activity, has been shown to promote protein kinase activity and H^+- ATPase pumping, thereby affecting the intracellular pH, another important factor in plant defence response (Munnik et al. 1998).

Generation of reactive oxygen species (ROS) due to the action of NADPH oxidase, referred to as an oxidative burst, is one of early events in plant/pathogen interaction. The ROS produced in response to pathogen/elicitor are considered to contribute to HR. They can serve as signals for activation defence reactions or affect cell metabolism directly (Baker and Orlandi 1995; Mur et al. 2008). In contrast to studies on the involvement of PLA_2s in the oxidative burst in mammalian cells (Dana et al. 1994; Levy 2006), reports concerning plant material are scarce. In studies, performed on cultured cells of soybean (Chandra et al. 1996) and tobacco (Piedras et al. 1998) treated with inhibitors of PLA_2, chlorpromazine, p-bromophenacyl bromide (BPB) and quinacrine, the elicitor-induced ROS generation and PLAs activities were modified differently; hence, univocal evidence of a relationship between these activities remains to be addressed.

Elucidation the contribution of specific forms of PLA_2 to plant defence mechanisms remains a challenging task. Generally, there are two basic approaches to this study: using genetically modified material with altered activity of the genes of interest, and, the most straightforward one, employment of specific chemical inhibitors of PLA_2 to asses its implications on a given process. In both cases direct appraisal of the role of specific PLA_2s is rather difficult due to multiple isoforms of PLA_2 and the lack of completely specific inhibitors (Balsinde et al. 1999). One of the PLA_2s

reasonably selective inhibitor is haloenol lactone suicide substrate (HELSS) which binds covalently at or near the active site of $iPLA_2$ and is specific for $iPLA_2$ but not for Ca^{+2}-dependent $sPLA_2$ (Hazen et al. 1991). Holk et al. (2002) reported that HELSS inhibited *in vitro* activity of purified Arabidopsis PLA IVA, which by conserved sequence elements may represent patatin-iPLA gene family. On the other hand, HELSS at high concentrations affects also $cPLA_2$ and can inhibit phosphatidate phosphohydrolase, so that its specificity *in vivo* in intact cells is unclear. Another inhibitor is p-bromophenacyl bromide (BPB), which inactivates $sPLA_2$ by blockage of exposed His or Lys residues (Balsinde et al. 1999; Mansfeld and Ulbrich-Hofman 2007).

Our research interests concern the defence mechanisms in *Solanum* species in response to an elicitor, the culture filtrate (CF) from *Phytophthora infestans*, the pathogenic oomycete that causes late blight, the most destructive potato disease. Interestingly, in this plant /pathogen interaction HR may occur in all forms of resistance: non-host, host (vertical), field (horizontal) as well as in total susceptibility (Vleeshouwers et al. 2000; Tian et al. 2006). In our studies we have compared early metabolic events induced by the elicitor in *Solanum nigrum* var. *gigantea*, *Solanum tuberosum* cv Bzura and clone H-8105, representing, respectively, non-host resistance, field resistance and susceptibility to *P. infestans*. We have found that detached leaves from all examined genotypes treated with CF displayed HR spots, but the timing and intensity of this response varied depending on the resistance status of the plants. The elicitor-induced ROS production, lipid peroxidation and LOX activity in the resistant and the susceptible genotypes differed quantitatively. The relative increase in ROS production was higher in the susceptible H-8105 than in both resistant genotypes. An increase in lipid peroxidation coincided with enhanced LOX activity only in *S. nigrum* (Polkowska-Kowalczyk et al. 2004). Detailed studies on lipid peroxidation revealed that intrinsically elevated lipid metabolism may be correlated with resistance (Polkowska-Kowalczyk et al. 2008). This finding prompted us to undertake studies on the involvement of PLA_2 in defence mechanisms in *Solanum.* In the present work we have demonstrated changes in PLA_2 activity in CF treated leaves of *Solanum* genotypes. The experiments with PLA_2 inhibitors, HELSS or BPB, were performed in order to evaluate the involvement of $iPLA_2$ and $sPLA_2$ in defence strategy. The effects

of the inhibitors on PLA_2 activity and on generation of ROS in the studied interactions are described.

Materials and methods

Plant material and pathogen elicitor

Axenic shoots of *S. nigrum* var. *gigantea*, non-host completely resistant, *S. tuberosum* cv Bzura, polygenically field resistant, and clone H-8105, susceptible to the oomycete pathogen *Phytophthora infestans* (Mont) De Bary, were cultured *in vitro* as described previously (Polkowska-Kowalczyk et al. 2004). The plants grew under controlled conditions: day light fluorescent lamp 150 μmol $m^{-2}s^{-1}$ for 16 h, day/night temperature 22/18^{o}C. The pathogen *P. infestans* (complex race 618 with virulence factors 1, 2, 3, 4, 6, 7, 10 and 11) received from IHAR, Młochów Research Center (Poland) was maintained on rye agar medium at 15^{o}C in the dark. A culture filtrate (CF), which served as an elicitor, was prepared from the pathogen grown in liquid medium. After 6 weeks of growth the medium was separated from the oomycete, dialysed against water for 48 h and lyophilised. The CF residue dissolved in distilled water was quantified as μg glucose equivalents ml^{-1}, as described in detail by Polkowska-Kowalczyk et al. (2004).

Treatment of leaves

The CF was applied in droplets at a concentration of 0.67 μg glucose equivalent g^{-1} FW on the surface of leaves detached from 4-week-old plants; control leaves were treated with distilled water. All leaves were placed on moist filter paper in Petri dishes and kept for 6, 18, 30 and 36 h at 25^{o}C under continuous light, as described in detail by Polkowska-Kowalczyk et al. (2004). At given time intervals the leaves were taken for analysis. In experiments with the PLA_2 inhibitors applied *in vivo*, stock solutions of the inhibitors: HELSS (12 mM in 96% EtOH) and BPB (10 mM in 50 mM TRIS buffer pH 8.5 containing 60% EtOH) were diluted with 50 mM TRIS buffer pH 8.5, giving a final concentration of EtOH within the range of 0.2 - 0.6% in the case of HELSS and in the range of 0.6 - 2.4% in the case of BPB. Detached leaves were immersed with shaking for 1 h in the solutions of inhibitors, whereas the control leaves were kept in similarly diluted solvents of inhibitors.

Afterwards, all leaves were quickly rinsed and placed in Petri dishes on filter paper moistened with distilled water. In some experiments portions of leaves previously preincubated with the inhibitors or its solvents (the control ones) were additionally treated with CF or water (control). All leaves after preincubation were kept for 18 h under conditions described above. Subsequently, the leaves were used for PLA_2 activity assay or for determination of ROS production.

Enzyme extraction

After incubation samples of leaves were ground in extraction medium (1:4, f.wt : v) containing: 0.3 M NaCl, 0.1 M HEPES pH 7.5, 2 mM EDTA, 5 mM DTT, 2 mM SHAM (salicylhydroxamic acid), 1 mM PMSF (phenylmethanesulfonyl fluoride), 50 mM NaF and 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 13000 *g* for 20 min. The obtained supernatant was submitted to fractionation with ammonium sulfate (30 - 60%). After centrifugation the protein precipitate was dissolved in 1 mL of 50 mM TRIS buffer pH 8.5, 1mM PMSF, 5 mM DTT. Then, the enzyme extract was partially purified on Sephadex G-25 column eluted with 50 mM TRIS buffer pH 8.5 containing 1 mM PMSF. All procedures were conducted at 4^oC. The protein content in the obtained extract was determined by the method of Bradford (1976).

Determination of PLA_2 activity

Phospholipase A_2 activity was measured by continuous spectrophotometric method developed for assaying patatin phospholipase activity (Jiménez-Atiénzar et al. 2003). In this method [1,2-dilinoleoyl] PC, prepared as described by Jiménez-Atiénzar et al. (2003), was used as a substrate with LOX serving as the coupling enzyme, which oxidised the linoleic acid released by phospholipase activity. The PLA_2 activity was then followed spectrophotometrically by measuring the increase in absorbance at 234 nm, resulting from formation of hydroperoxides due to the LOX action. In methodical experiments, PLA_2 from hog pancreas (EC 3.1.1.4, Fluka) as well as enzyme extracts obtained from the studied plants were used to optimize the procedure for our material. The established reaction medium contained: 50 mM TRIS buffer pH 8.5, 3 mM $CaCl_2$, 3 mM

deoxycholate, 60 μM [1,2-dilinoleoyl] PC, 400 U LOX (Lipoxidase Type I-B, EC 1.13.11.12; 1,000,000 U/mg, Sigma). Lipoxidase from soybean (Type I-B EC 1.13.11.12, Sigma) and appropriate amounts of exogenous LA (cis-9,cis-12-octadecadienoic acid, Sigma) were used to confirm oxidation of LA by LOX in the reaction medium. Each set of assays was preceded by controls without the enzyme extract and/or lipoxygenase to ascertain the absence of substrate oxidation by lipoxygenase or other compounds in the reaction medium. Inhibitors of PLA_2, HELSS and BPB, added to these samples, did not interfere with the LOX activity, hence appearing applicable to PLA_2 assay. Phospholipase A_2 from hog pancreas (EC 3.1.1.4, Fluka) was used as a standard to check the inhibitory effects of HELSS or BPB at different concentrations. In the case of BPB, PLA_2 as a standard was preincubated with the inhibitor in 50 mM TRIS buffer pH 8.5 and 3 mM $CaCl_2$ for 30 min at 25^{o}C, following which PC, deoxycholate and LOX were added to initiate the enzymatic reaction.

Assay for ROS production

ROS production was evaluated by determining the reduction of nitroblue tetrazolium (NBT) in the medium by $O^{\cdot-}_2$ released from leaf tissues (Doke 1983). The leaves treated with CF or the control ones treated with water were incubated for 1 h in a mixture containing NBT (nitroblue tetrazolium (2,2'-di-p-nitrophenyl-5,5'-diphenyl-[3,3'-dimethoxy-4,4'diphenylene]-ditetrazolium chloride). The mixture was then heated at 85^{o}C, cooled and absorbance at 580 nm was measured as described by Polkowska-Kowalczyk et al. (2004).

Presentation of data

At least two independent series of experiments, each with at least two portions of about 300 mg tissue (8-25 leaves, depending on their size) were performed. The data presented are the mean values ± SD. The significance of difference between mean values was determined by the Student's *t*-test.

Results

PLA_2 activity

In all *Solanum* genotypes PLA_2 activity increased in response to CF treatment but varied with respect to intensity and timing (Fig. 1). In *S. nigrum* leaves, the PLA_2 activity of up to 290% ($p<0.05$) in relation to water treated control was noted at 18 h of CF treatment, decreasing slightly after 30 h of treatment. In elicited Bzura leaves, PLA_2 activity rose at 6 h of treatment to about 160% ($p<0.05$) of the control, and afterwards remained at the elevated level. In contrast, in the susceptible clone H-8105, after an early and transient increase to 170% ($p<0.05$) of the control values, PLA_2 activity decreased to the initial level.

In vivo and in vitro effects of HELSS and BPB on PLA_2 activity

To our knowledge data on the effects of PLA_2 inhibitors on defence processes in plant tissues are scarce. Moreover, Holk et al. (2002) demonstrated that several PLA_2 inhibitors effective *in vitro* were ineffective *in vivo* in auxin-induced hypocotyl elongation growth of *Arabidopsis*. Therefore, experiments were performed with HELSS and BPB applied *in vivo* (leaves incubated in the presence of the PLA_2 inhibitors) and *in vitro* (inhibitors added to enzyme extracts). In experiments *in vivo* the detached leaves were preincubated with HELSS at concentrations of 24, 48, 72 and 96 μM *vs* control (see Material and methods). No symptoms of injury appeared in leaves treated with HELSS at concentrations up to 72 μM or in controls. In leaves of all genotypes HELSS applied *in vivo* did not inhibit PLA_2 activity in a dose dependent manner (Table 1). An inhibitory effect of HELSS was about 10–20% in resistant *S. nigrum* and Bzura, and up to 46% in the susceptible H-8105. When BPB was applied *in vivo* at concentration of 200 μM PLA_2 activity was affected slightly, whereas at concentration of 400 μM leaves were injured. Based on these observations HELSS at the concentration of 72 μM and BPB at 200 μM were used in subsequent *in vivo* assays.

In experiments *in vitro*, when HELSS was added to extracts from leaves pretreated *in vivo* with HELSS or its TRIS buffer solvent (control), changes in PLA_2 activity varied among the genotypes studied. In *S. nigrum* HELSS inhibited PLA_2 activity significantly ($p<0.05$) in a dose dependent

manner, independently of the leaf pretreatment *in vivo* (Fig. 2). In Bzura HELSS applied *in vitro* at all used concentrations caused about 50% ($p<0.05$) inhibition of PLA_2 activity. On the contrary, in the susceptible H-8105, in the presence of 24 or 48 μM HELSS PLA_2 activity decreased about 20% in extracts from the control leaves and those preincubated with HELSS *in vivo* whereas at the highest dose of HELSS, PLA_2 activity declined about 90% ($p<0.05$), regardless of the previous *in vivo* treatment of leaves.

In all genotypes the inhibitory effect of BPB applied *in vitro* to enzyme extracts from leaves pretreated *in vivo* with the TRIS solvent, appeared mostly at 400 μM concentration, leading to reduction of PLA_2 activity from 86 to 67% ($p<0.05$) of control (Fig. 3). In extracts from leaves pretreated *in vivo* with 200 μM BPB, PLA_2 activity was inhibited by BPB in a dose dependent manner in the resistant *S. nigrum* and Bzura. In contrast, in the susceptible H-8105, in extracts from leaves pretreated with BPB or its solvent the activity of PLA_2 increased in the presence of 100 or 200 μM BPB, and decreased to 72% ($p<0.05$) of control only at the concentration of 400 μM.

Effect of HELSS and BPB on PLA_2 activity in CF treated leaves

In order to assign fractions of PLA_2 that responded to CF, and were thereby possibly involved in defence mechanisms, the effects of HELSS and BPB inhibitors, which discriminate between $iPLA_2$ and $sPLA_2$, on CF-induced PLA_2 activity were examined. Detached leaves were preincubated with TRIS solvents of the inhibitors (samples 1) and in 72 μM HELSS or 200 μM BPB (samples 2) for 1 h (Figs. 4 and 5, respectively). All leaves were then rinsed and one portion of leaves from samples 1 and 2 were treated with CF (samples 3 and 4, respectively), whereas the rest of the leaves treated with water served as respective reference for samples 3 and 4. After 18 h of treatment, the activity of PLA_2 was determined in all samples. In the case of HELSS (Fig. 4), comparison of samples 1 and 3 revealed an increase in PLA_2 activity in response to CF treatment only in *S. nigrum* (about 50%, $p<0.05$), whereas in the other genotypes PLA_2 activity practically did not change. Thus, in this experiment, when leaves were preincubated with TRIS solvent, the effect of CF on PLA_2 activity was less pronounced than that in experiments without the PLA_2 inhibitors, when control leaves

remained in water all the time (compare Figs. 1 and 4). Possibly, the change in the pH of the TRIS preincubation medium (pH 8.5) in comparison to water (pH 6) may have affected the response of leaves to CF. Piedras et al. (1998) demonstrated that ROS accumulation in Cf9 tobacco cell suspension challenged with Avr9 depended upon extracellular pH values.

Interestingly, the inhibitory effect of HELSS on PLA_2 activity was pronounced only in CF treated leaves from *S. nigrum* and Bzura (Fig. 4, compare samples 3 and 4); in the absence of CF practically no such effect was observed (compare samples 1 and 2). In *S. nigrum* CF-induced PLA_2 activity (sample 4) decreased about 30% ($p<0.05$) and in Bzura about 20% ($p<0.05$) in relation to that in leaves without preincubation with HELSS (sample 3). In contrast, BPB (Fig. 5) practically did not inhibit PLA_2 activity in control (compare samples 1 and 2) and in CF treated leaves (compare samples 3 and 4) of all genotypes except *S. nigrum.* In the latter the PLA_2 activity even increased slightly.

Effect of HELSS and BPB on ROS production

To ascertain a possible relationship between ROS production and PLA_2 activity, as reported for human cells, we have compared ROS production in response to CF in leaves preincubated with the inhibitors of PLA_2 for 1 h and those without this treatment. When leaves were treated with CF alone ROS production increased in all genotypes studied (Table 2). In leaves preincubated with HELSS before CF treatment, ROS production did not change in comparison with that in leaves without HELSS treatment. When leaves were preincubated with BPB and then treated with CF, ROS production surprisingly increased in *S. nigrum*, to 180% ($p<0.05$) of that in leaves without BPB treatment. On the contrary, in Bzura and H-8105 leaves ROS production decreased by about 40% ($p<0.05$) due to inhibitor treatment.

Discussion

Identification of the function of a specific PLA among multiple isoforms as well as their products in a complex signalling network is a great challenge for researchers. The present results contribute to some degree to the knowledge on the involvement of PLA_2 in plant defence response. Our studies on *Solanum* genotypes revealed: i) an increase in PLA_2 activity in response to CF treatment, ii) various effects of HELSS and BPB applied *in vivo* and *in vitro* on PLA_2 activity, iii) different modification of CF-induced PLA_2 activity by HELSS and BPB, iiii) differences in the effects of HELSS and BPB on CF-induced ROS production. The increase in CF-induced PLA_2 activity varied depending on the resistance status of the genotypes. The highest increase in PLA_2 activity was noted in *S. nigrum*, non-host completely resistant to *P. infestans*, and the lowest one in the susceptible H-8105. This observation is in concert with our previous findings showing that intense lipid metabolism in the early phase of response to the elicitor may be related to resistance (Polkowska-Kowalczyk et al. 2008). An increase in PLA_2 activity in different plant/pathogen interactions was also reported by other authors, e.g., Kawakita et al. (1996), Narváez-Vásquez et al. (1999) and Göbel et al. (2001).

The doses of PLA_2 inhibitors, HELSS and BPB, applied *in vivo* were selected at levels at which they affected PLA_2 activity without symptoms of leaf injury. In all genotypes studied the *in vivo* effects of each inhibitor were ambiguous, but PLA_2 activity decreased markedly when high doses of the inhibitors were added *in vitro* to leaf enzyme extracts. Surprisingly, at low BPB concentrations PLA_2 activity increased in H-8105 leaf extract. Various response of PLA_2 activity to HELSS and BPB, which discriminate between $iPLA_2$ and $sPLA_2$, suggested differences in pools of PLA_2 isoforms and/or their sensitivity to the inhibitors in the studied genotypes. The observed different effectiveness of the inhibitors *in vivo* and *in vitro* may be due to either various accessibility of the inhibitors entering the leaf tissues or to their metabolism in the tissues. It is also conceivable that *in vivo* some fractions of the enzyme might be inactive under surrounding conditions and/or be unavailable to the inhibitors because of their intracellular localization. According to Senda et al. (1996) PLA_2 activity of potato patatin is inactive under acidic conditions in vacuoles and becomes active under basic conditions in the cytosol, where the enzyme is translocated upon pathogen attack.

It has been found that both inhibitors influenced mainly CF-induced PLA_2 activity. The HELSS applied *in vivo* before CF treatment inhibited PLA_2 activity mostly in the non-host *S. nigrum* and the field resistant Bzura. In contrast, when BPB was added, CF-induced PLA_2 activity increased slightly in *S. nigrum* and practically did not change in the other genotypes. The observation that an inhibitory effect of HELSS but not of BPB manifested itself on CF-induced PLA_2 activity, i.e., fraction responding to the elicitor, suggests that $iPLA_2$s are involved in defence signalling, whereas the contribution of $sPLA_2$s remains questionable. Potential function of PLA_2 in plant signal transduction in response to auxin, elicitor or wounding was also suggested by Holk et al. (2002) and Scherer (2002). The stimulation of CF-induced PLA_2 activity in the presence of BPB in *S. nigrum* was rather unexpected. Explanation of this observation is difficult because knowledge on crosstalk among different plant PLA_2 fractions is very limited (Scherer et al. 2010). If one presumes that the observed increase in PLA_2 activity originates mainly from $iPLA_2$ activity (not inhibited by BPB), it is conceivable that it might result from compensation of $sPLA_2$ activity likely lowered due to BPB action. On the other hand, since plant iPLA2 are reported to be upregulated by multiple stresses (Scherer et al. 2010 and ref. therein), in our experiment BPB applied *in vivo* might serve as a toxic factor leading to enhanced activity of $iPLA_2$. Alternatively, it is also conceivable that in *S. nigrum* iPLA2 activity enhanced in the presence of CF might result in high production of LPC. According to Viehweger et al. (2002) the action of LPC might lead to a shift in cellular pH. Lee et al. (2005) reported that plant $sPLA_2$s differ in their pH and Ca^{+2} concentration optima, so that some of them might be inactive in their subcellular locations, requiring a change in pH and Ca^{+2} concentration to achieve maximum activity. Therefore, CF-induced changes in the cellular environment might lead to some activation of $sPLA_2$ and, in consequence, allow to overcome the inhibitory effect imposed by the BPB applied *in vivo*.

Simultaneous inhibition of PLA_2 activity and oxidative burst induced by elicitor or pathogen may imply a relationship between these two responses. Contrary to mammalian cells, studies in plants on the involvement of PLA_2s activities in the generation of ROS by NADPH oxidase in response to elicitation are scarce. Chandra et al. (1996) demonstrated that in soybean cells chlorpromazine, a

nonspecific inhibitor of PLA_2, inhibited both PLA_2 activity and ROS generation induced by extract from the pathogenic fungus *Verticillium dahliae*, which suggested a correlation between these responses. Similarly, Piedras et al. (1998) demonstrated that inhibitors of PLA_2, BPB, quinacrine and chlorpromazine, reduced ROS production in Cf9 tobacco cells treated with Avr9. We have found that in response to CF treatment alone ROS production increased in all genotypes similarly as it has been demonstrated previously (Polkowska-Kowalczyk et al. 2004). The HELSS applied *in vivo* before CF treatment had practically no effect on ROS production, whereas BPB in similar circumstances led to an increase in ROS production in *S. nigrum* leaves but inhibited this process in Bzura and H-8105. These results suggest that ROS production in response to CF may be independent of $iPLA_2$ activation, while the contribution of $sPLA_2$ to this process is plausible. Different effects of the PLA_2 inhibitors on CF-induced PLA_2 activity and ROS production in the non-host *S. nigrum* in comparison with the field resistant Bzura and the susceptible H-8105 may imply various signal transduction pathways in defence mechanisms employed by *S. nigrum* and the other genotypes. On the other hand, considering the obtained results, particularly the data concerning *S. nigrum*, there might be an interplay between $iPLA_2$s and $sPLA_2$s. Perhaps change in $iPLA_2$ activity in the presence of CF and alteration in cellular environment might lead to enhanced $sPLA_2$ activity and possible stimulation of ROS production. Different possibilities of interplay between various forms of PLA_2 have been reported for mammalian cells (Balboa and Balsinde 2006). Kim et al. (2002) showed that $sPLA_2$s, acting on the outer membrane of human neutrophils, release FA and LPC. This in turn, can lead to an increase in cytosolic free Ca^{+2} level and induce phosphorylation of $cPLA_2\alpha$, which then result in $cPLa_2\alpha$-dependent leukotriene biosynthesis. Han et al. (2003) in studies on the effects of expression of genes of Group IIa or V $sPLA_2$ and $cPLA_2\alpha$ on H_2O_2-induced arachidonic acid (AA) release in murine mesangial cells found that both groups of $sPLA_2$ amplified the $cPLA_2\alpha$-mediated response to H_2O_2, resulting in increased release of AA from phospholipids. In the light of the similarities between plant and neutrophile oxidative burst (Piedras et al. 1998) as well as some homologies between plant and animal PLA_2s an assumption of an interplay between plant $iPLA_2$s and $sPLA_2$s seems warranted.

Overall, the results presented here show that in the studied *Solanum* species there occur various fractions of PLA_2, which differ in response to elicitor treatment. The $iPLA_2$s probably participate in signal transduction in defence mechanisms whereas the involvement of $sPLA_2$s is unclear. However, due to a possible interplay among various fractions of PLA_2, all of them might modulate the defence response in interaction between *Solanum* and *P. infestans*. Detailed elucidation of these mechanisms needs further investigation.

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Table 1.
PLA_2 activity ($\Delta A_{234} \cdot min^{-1} \cdot mg^{-1}FW$) in leaves treated *in vivo* with HELSS or BPB

Genotype	**HELSS (µM)**				**BPB (µM)**	
	TRIS (control)	**24**	**48**	**72**	**TRIS (control)**	**200**
S. nigrum var. *gigantea*	1.12±0.13	1.01±0.29	0.88±0.06	0.98±0.11	1.10±0.31	1.06±0.10
Bzura	1.11±0.12	0.96±0.02	0.92±0.04	0.91±0.07	0.91±0.08	0.88±0.16
H-8105	1.24±0.08	0.69±0.01	0.83±0.02	0.90±0.04	0.90±0.05	0.88±0.08

Table 2. Effect of inhibitors of PLA_2 on production of ROS ($\Delta A_{580} \cdot g^{-1}FW \cdot h^{-1}$)

Treatment \ Genotype	*S. nigrum* var. *gigantea*	Bzura	H-8105
TRIS (control)	0.08 ± 0.023	0.36 ± 0.070	0.16 ± 0.013
TRIS + CF	0.25 ± 0.116[a]	1.23 ± 0.299[b]	1.94 ± 0.188[c]
HELSS + CF	0.24 ± 0.092	1.70 ± 0.422	2.00 ± 0.203
BPB + CF	0.45 ± 0.040[a]	0.73 ± 0.076[b]	1.15 ± 0.067[c]

Leaves were incubated in 72 µM HELSS or 200 µM BPB for 1 h before treatment with CF. The values obtained for control leaves differed significantly ($p<0.05$) from those for treated leaves. The same letters indicate significant differences ($p<0.05$) between values obtained from treated leaves. Data represent mean values ± SD ($n \geq 4$).

Legend to Figures

Fig. 1 Changes in phospholipase A_2 (PLA_2) activity in leaves of *Solanum* species treated with culture filtrate (CF) for 6, 18, 30 and 36 h in relation to controls treated with H_2O. The mean activities in the control were: *S. nigrum* - 0.44 $\Delta A_{234} \cdot min^{-1} \cdot mg^{-1}$ protein, Bzura - 0.37 $\Delta A_{234} \cdot min^{-1} \cdot mg^{-1}$ protein, H-8105 - 0.25 $\Delta A_{234} \cdot min^{-1} \cdot mg^{-1}$ protein. The values are mean ± SD ($n \geq 6$). The same letters indicate significant differences ($p<0.05$) between mean values for CF treated leaves and controls in respective genotypes

Fig. 2 Changes in phospholipase A_2 (PLA_2) activity in *Solanum* species in response to HELSS added *in vitro* (vtr) to leaf enzyme extracts obtained from leaves treated *in vivo* (v) with TRIS □ or with 24 μM □, 48 μM ■, 72 μM ■ HELSS for 1 h. Data are means ± SD ($n \geq 4$)

Fig. 3 Changes in phospholipase A_2 (PLA_2) activity in *Solanum* species in response to BPB added *in vitro* (vtr) to leaf enzyme extracts obtained from leaves treated *in vivo* (v) with TRIS □or 200 μM BPB ■ for 1 h. Data are means ± SD ($n \geq 4$)

Fig. 4 Changes in phospholipase A_2 (PLA_2) activity in leaves of *Solanum* species preincubated *in vivo* (v) for 1 h in TRIS or 72 μM HELSS (samples 1 and 2) and then treated for 18 h with culture filtrate (CF): samples 3 and 4 or H_2O: samples 1 and 2. Data are means ± SD ($n \geq 5$). The same letters indicate significant differences ($p<0.05$) between mean values for samples 1 and 3; 3 and 4

Fig. 5 Changes in phospholipase A_2 (PLA_2) activity in leaves from *Solanum* species preincubated *in vivo* (v) for 1 h in TRIS or 200 μM BPB (samples 1 and 2) and then treated for 18 h with culture filtrate (CF): samples 3 and 4 or H_2O: samples 1 and 2. Data are means ± SD ($n \geq 5$). The same letters indicate significant differences ($p<0.05$) between mean values for samples 1 and 3; 3 and 4

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Figure 1

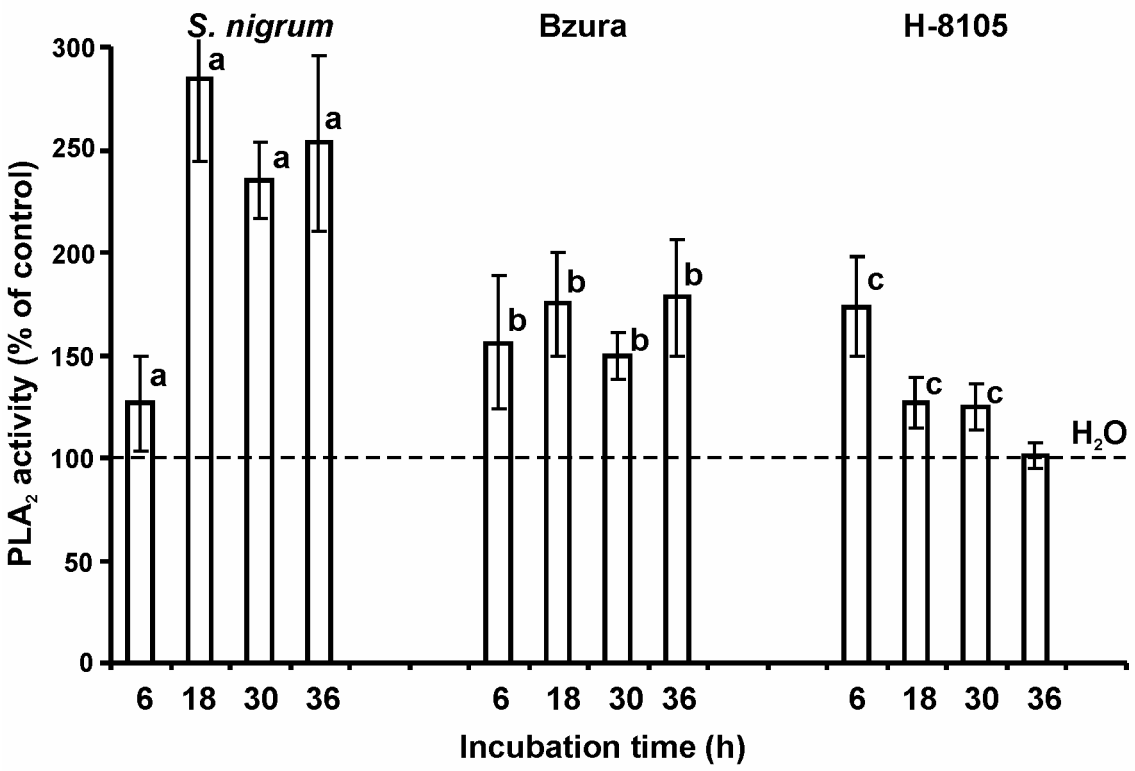

S. nigrum
Bzura
H-8105
PLA2 activity (% of control)
300
250
200
150
100
50
0
a
a
a
b
b
b
c
c
c
H2O
6
18
30
36
Incubation time (h)

Figure 2

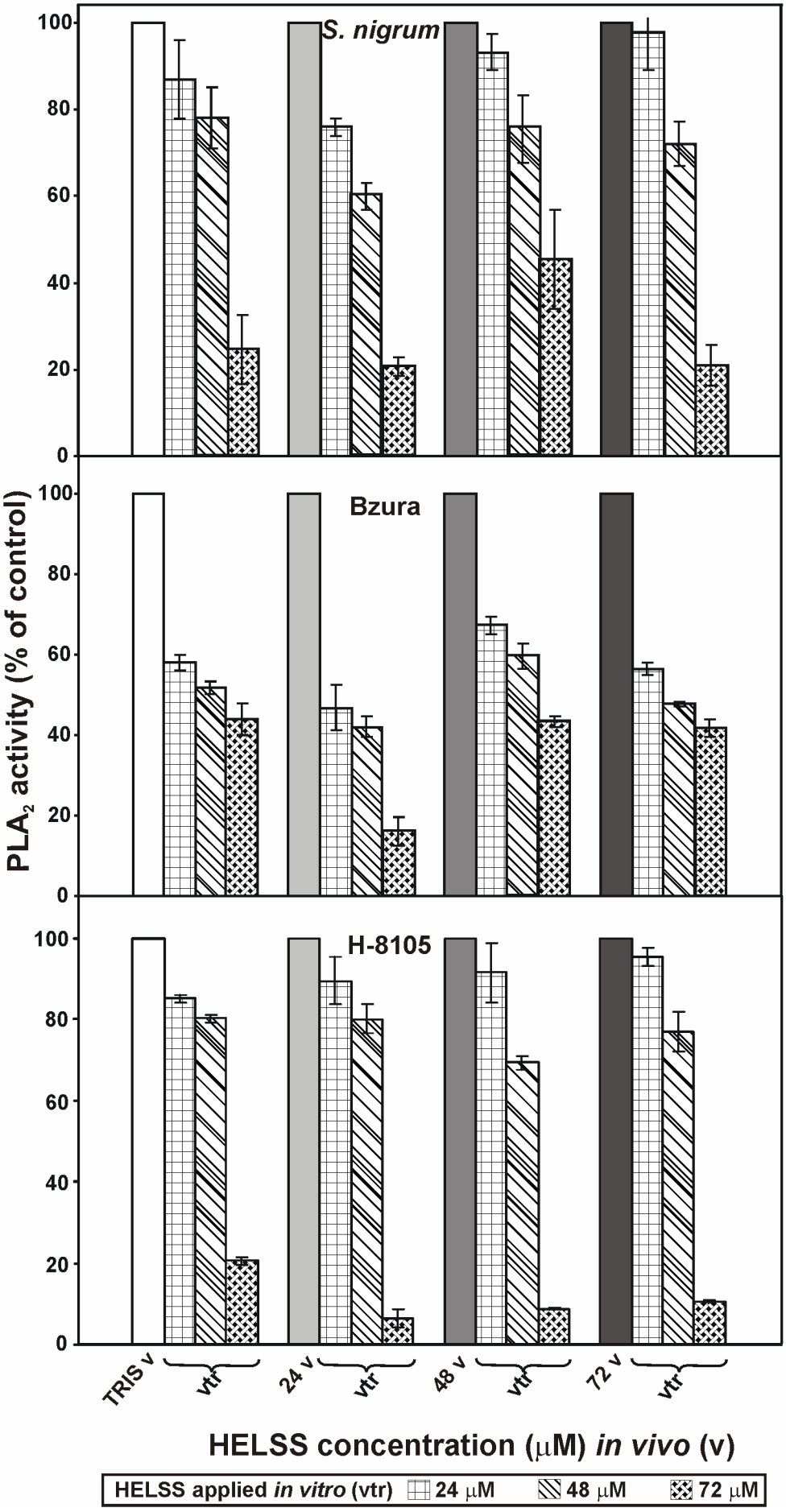

S. nigrum
Bzura
H-8105
PLA2 activity (% of control)
0
20
40
60
80
100
TRIS v
vtr
24 v
vtr
48 v
vtr
72 v
vtr
HELSS concentration (μM) in vivo (v)
HELSS applied in vitro (vtr)
24 μM
48 μM
72 μM

Figure 3

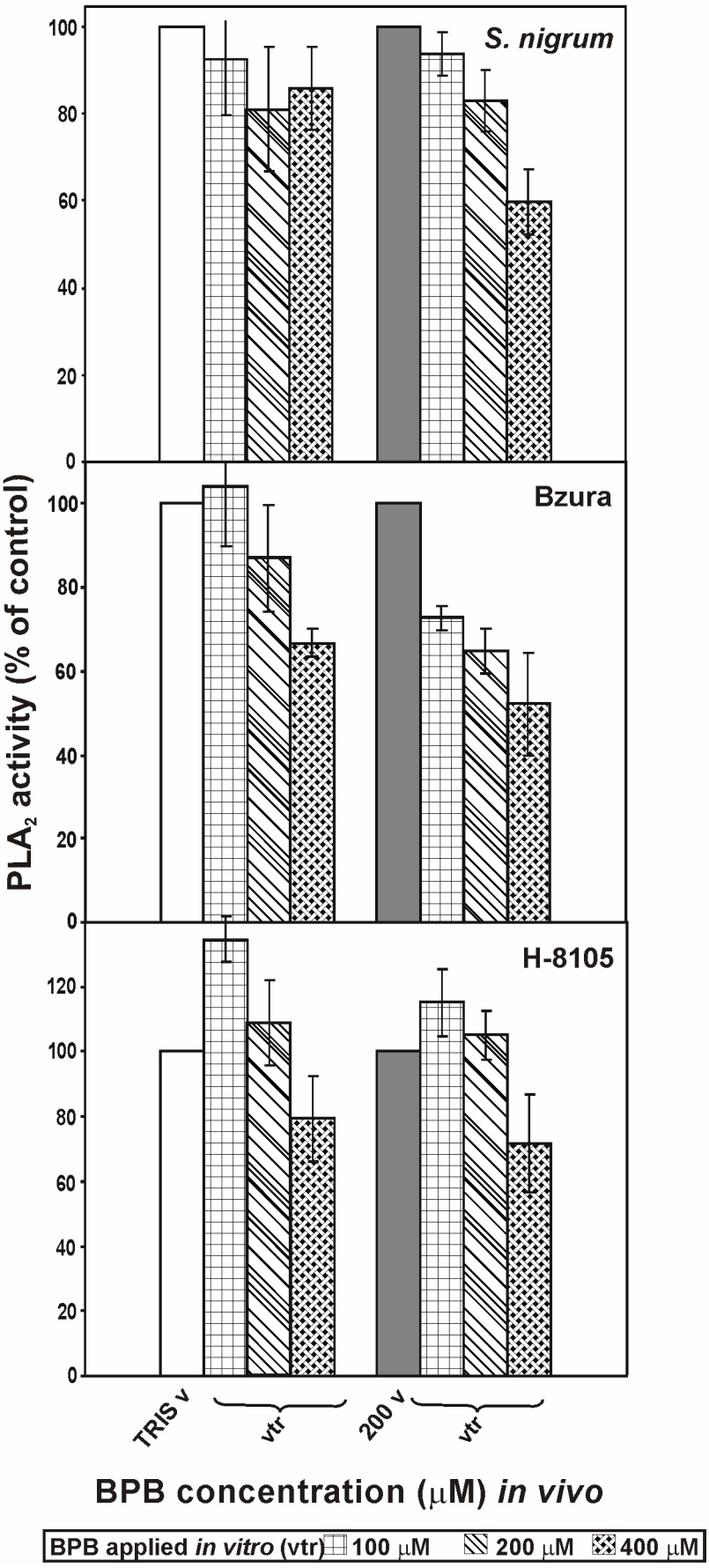

S. nigrum
Bzura
H-8105
PLA2 activity (% of control)
TRIS v
vtr
200 v
vtr
BPB concentration (μM) in vivo
BPB applied in vitro (vtr) 100 μM 200 μM 400 μM

Figure 4

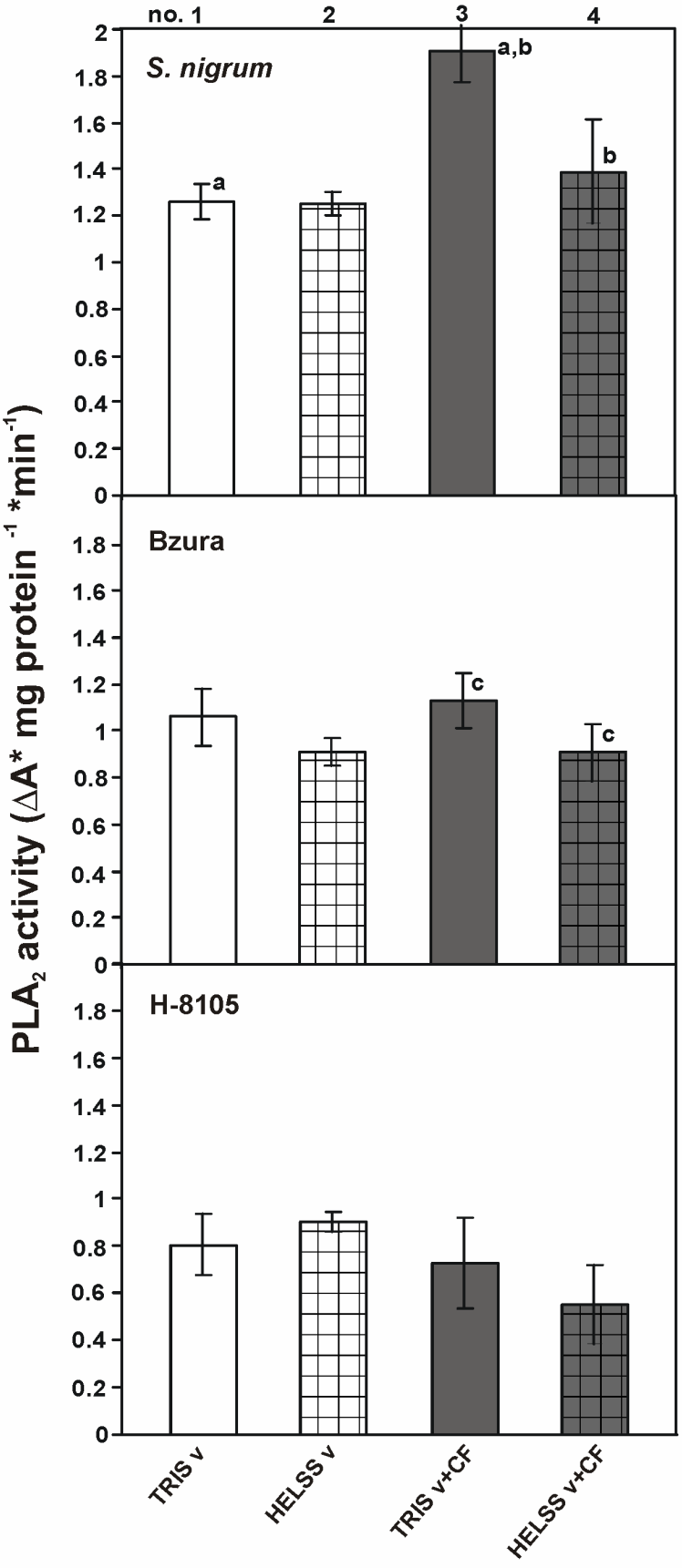
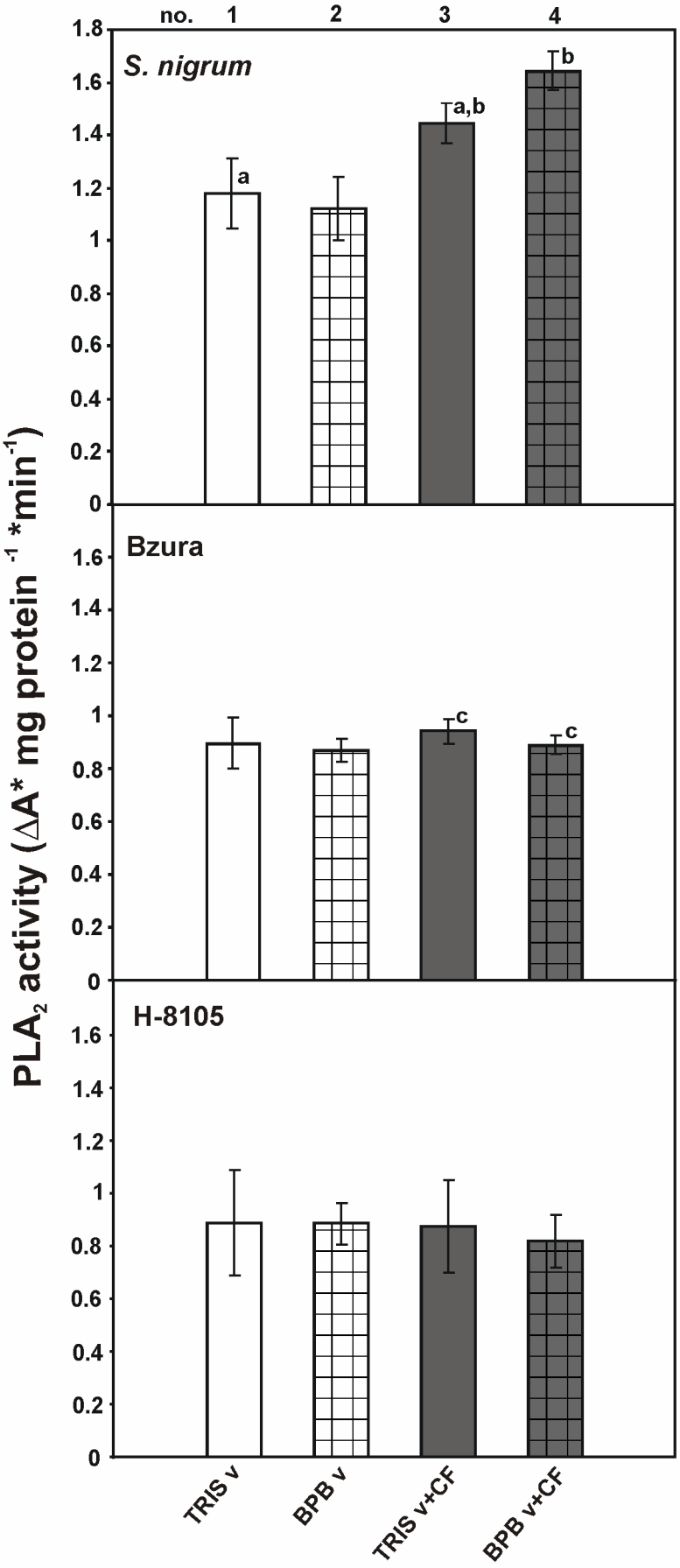

no. 1
2
3
4
S. nigrum
a
a,b
b
Bzura
c
c
H-8105
PLA2 activity (ΔA* mg protein -1 *min-1)
TRIS v
HELSS v
TRIS v+CF
HELSS v+CF

Figure 5


no. 1 2 3 4
S. nigrum
a
a,b
b
Bzura
c
c
H-8105
PLA2 activity (ΔA* mg protein -1 *min-1)
TRIS v
BPB v
TRIS v+CF
BPB v+CF