Environmental and Experimental Botany 122 (2016) 109–114

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Phenol modulates lipid kinase activities in *Vicia sativa* plants

Sabrina G. Ibañez*, Ana L. Villasuso, Graciela E. Racagni, Elizabeth Agostini, María I. Medina

Departamento de Biología Molecular, FCEFQyN, Universidad Nacional de Río Cuarto, Argentina

ARTICLE INFO

Article history:
Received 18 August 2015
Received in revised form 14 September 2015
Accepted 16 September 2015
Available online 24 September 2015

Keywords:
Common vetch
Phenol
Phosphatidic acid
Phospholipase D
Phospholipids

ABSTRACT

Vicia sativa, is a leguminous species able to germinate, grow in the presence of phenol and remove this contaminant. However, there are not reports concerning the signals triggered by the pollutant and how plants perceive and transduce this signal in order to adapt to adverse conditions. Phosphatidic acid (PA) has been proposed as a key messenger in plants and it can be generated via phospholipase D (PLD) or via phospholipase C (PLC) coupled to diacylglycerol kinase (DGK). Thus, changes in this minor phospholipid and in enzymes involved in its catabolism were analyzed after treatment with phenol (25 and 100 mg L^{-1}). The results obtained, seem to suggest that the higher concentration could be sensed as a stressful signal, since a rapid (1.5 h) and transient increase in PA, via PLD and a second wave of increase possibly via PLC/DGK was observed after 96 h of exposure with 100 mg L^{-1} of phenol. Besides, a markedly increase in enzymes related with PA metabolism, mainly DGK, phosphatidylinositol kinase (PIK) and PA kinase (PAK), was detected after long term treatment. Thus, this study highlighted the key role of minor phospholipids, especially PA, in the transduction pathway induced by phenol.

1. Introduction

The presence of pollutants in the environment affects the quality of life standards causing severe damages in all living organisms. Phenol is an organic contaminant, extensively found in effluents from a variety of industries due to its multiple applications in the synthesis of resins, perfumes, solvents, and lubricating oils, as well as in the obtention of other chemicals (e.g., plastics, drugs, explosives, pesticides, and detergents) (Iurascu et al., 2009). It has been classified as a priority pollutant by most National Environmental Protection Agency's owing to its potential harm to the environment and mutagenic and also carcinogenic effects to human health at relative low concentrations. Besides, the presence of this toxic compound negatively affects plant metabolism and development. Being plants sessile organisms, plant need – as soon as variations occur – to perceive, transduce and turn on a whole battery of mechanisms in order to adapt to the adverse condition and survive (Vaultier and Jolivet, 2015; Islam et al., 2015).

Lipid signalling has emerged as one of the major signalling networks as adaptive mechanism in response to various environmental cues and adverse growth conditions (Singh et al., 2015). In this sense, membranes are the sites where many signals are perceived by the cell and it is well establish that lipids or lipid-derived molecules are a group of plant messengers that have been described to be involved in stress responses (Meijer and Munnik, 2003; Sun et al., 2013; Ruelland et al., 2015). Among the enzymes that have a role in mediating membrane lipid remodeling it is important to mention the phospholipases that catalyze the initial step of phospholipid breakdown and generate multiple lipid derived second messengers (Singh et al., 2015). Particularly, phosphatidic acid (PA) has emerged as a key messenger in plants, which can be generated by different pathways (Testerink and Munnik, 2005). Phospholipase C (PLC) hydrolyses the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 that is able to release Ca^{2+} from intracellular stores, and diacylglycerol (DAG) (Pokotylo et al., 2014). In plants, DAG is phosphorylated to PA by DAG kinase (DGK). This pathway is referred as the PLC/DGK pathway (Munnik, 2001; Arisz et al., 2009). Phospholipase D (PLD) hydrolyses structural phospholipids, such as phospatidylcholine (PC), generating PA and choline (Munnik, 2001). PLDs have long been considered as the main contributors to PA signalling, but their hegemony is now being challenged by the PLC/DGK pathway (Arisz et al., 2009; Ruelland et al., 2015). Although several PLC and PLD members have been involved in a wide variety of abiotic stress responses in a number of plant species, little is known about their role triggered in signalling events produced by the presence of an organic pollutant (Bargmann and Munnik, 2006; Singh et al., 2015).

* Corresponding author at: Departamento de Biología Molecular, FCEFQyN, UNRC Ruta 36 Km 601, CP 5800 Río Cuarto, Córdoba, Argentina. Fax: +54 358 4676232.
E-mail address: sibanez@exa.unrc.edu.ar (S.G. Ibañez).

http://dx.doi.org/10.1016/j.envexpbot.2015.09.005
0098-8472/

In addition to enzymes involved in PA generation, those responsible for PA shortly removal after its increase may play a role in finishing PA signalling events. Reduction of PA levels after PLD activation may be done by an active PA kinase (PAK) that phosphorylates PA to yield diacylglycerol pyrophosphate (DGPP) or by lipid phosphate phosphatases (LPPs), which dephosphorylate PA to produce DAG (Racagni et al., 2008; Villasuso et al., 2013).

Vicia sativa is a leguminous species with an extensive agricultural use. *Vicia* genus shows several favorable features, like capacity of nitrogen fixation, ability to grow in poor or degraded soils and fast adaptation processes (Pajuelo et al., 2008; Porta et al., 2015). In a previous work, we have demonstrated *V. sativa* ability to germinate and to grow in the presence of phenol. Besides, we have also showed its capability to tolerate and remove the contaminant from aqueous solutions and the response of the antioxidant system against the toxic compound, in order to establish its potential use for phytoremediation purposes. However, little is known about how stress signals are perceived and which are the plant responses triggered by them, being this a poorly explored area of research with a lot of gaps still to be filled (Islam et al., 2015). Therefore, the objectives of this study were to analyze changes in lipid kinase and PLD activities, enzymes that could be involved in lipid metabolism due to the presence of the pollutant.

2. Materials and methods

2.1. Plant materials

The experiments were carried out with seeds and plants of *V. sativa* sp. Nigra. The seeds were surface sterilized as it was previously described by Ibáñez et al. (2012). Glass flasks (250 mL) were filled with 100 mL of autoclaved MS medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose and four disinfected seeds were germinated in each flask. They were incubated in a growing chamber at 25 °C ± 2 °C with a 12-h light/12-h dark regimen on an orbital shaker (100 rpm). Thirty day-old plants were used for phenol treatments described in the section below.

2.2. Sample processing

Two kinds of assays were performed, long term assays (for lipid kinase and PLD activity determinations) and short term assays (only for PLD activity). In the long term assays, *V. sativa* plants were treated with 25 and 100 mg L^{-1} phenol for 96 h after 30 days of growth on MS medium (Ibáñez et al., 2012) or they were treated with 100 mg L^{-1} of the contaminant and harvested after 1.5, 3, 6 and 24 h, for the short ones. Water treatment was used as control in all assays. Phenol concentrations were selected based on our previous studies (Ibáñez et al., 2012). After the assay, aerial part and roots were separated and the later were dried and immediately frozen in liquid nitrogen to be used for further studies.

2.3. Preparation of membranes

Approximately 500 mg of *V. sativa* roots (control and treated with phenol) were thawed and homogeneized in 10 volumes of 50 mM HEPES (pH 7.4) containing: 0.25 M sucrose, 5 mM KCl, 1 mM EDTA and protease inhibitors. This suspension was frozen at −180 °C and thawed three times, homogenized in a glassy Teflon homogenizer and centrifuged at 1000 g for 15 min to remove unbroken cells and cell debris. Then supernatant was centrifuged at 105,000 × *g* for 60 min to obtain the membrane fraction. Membranes were washed and resuspended with 50 mM HEPES, pH 7.4. These membranes were used as a source of PLD and lipid kinases. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.4. Lipid kinase activities determinations

The lipid kinase activities were simultaneously assayed using endogenous lipids as substrates, unless otherwise stated. The membrane fraction isolated (60 μg of proteins), was added to thermally equilibrated 50 mM HEPES buffer (pH 7.4) containing: 0.1 mM EDTA, 0.5 mM DTE, 10 mM $MgCl_2$, 0.2 mM sodium o-vanadate, 1 mM Mg^{2+}-ATP and [γ-^{32}P]ATP, specific activity 450 cpm/pmol and proteases inhibitors. Endogenous lipid phosphorylation was allowed to proceed for 2 min at 30 °C in a final volume of 100 μL. The incubation mixture was subsequently stopped with 1.5 μL of chloroform/methanol (1:2, v/v).

2.5. Phospholipid extraction and separation

Lipids were extracted from reaction assays according to Racagni-Di Palma et al. (2002) and the phospholipids were separated by TLC. The samples were spotted on silica gel plates impregnated with potassium oxalate solution and heated at 110 °C for 60 min just before use. The chromatoplate was developed with chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v). The position of radiolabeled lipids was determined by autoradiography on Kodak film. Spots were scrapped off the plates and quantified using a liquid scintillation counter Beckman LS-6001C (Racagni et al., 1992) or by densitometry of autographs using image analyzer software (ImageJ).

2.6. PLD activity

PLD activity was measured as the production of phosphatidylbutanol (NBD–PtdBt) related to NBD-PA and NBD-PC (1-acyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]dodecanoyl}-*sn*-glycero-3-phosphocholine) levels, determined by TLC, as described below (Ritchie and Gilroy, 1998). NBD-PC was provided by Avanti Polar Lipids and stored at −80 °C in chloroform. Prior to use it was dried under a stream of N_2 and resuspended in chloroform. The standard assay mixture contained 20 mM Mes-NaOH (pH 6.5), 50 mM $CaCl_2$, 0.25 mM SDS, 5 mL fluorescent substrate (NBD-PC, 10–50 μg), 1% (v/v) 1-butanol, 60 μg of proteins, total volume 40 μL. The reaction was initiated by the addition of the substrate and incubated at 30 °C for 30 min with shaking (100 rpm). The reaction was stopped by the addition of 150 μL chloroform: methanol (1:2, v/v), 40 μL chloroform and 40 μL 2 M KCl. Then, the mixture was mixed and centrifuged at 15,000 × *g* for 2 min. The phases were separated and 100 μL chloroform added to the aqueous phase, mixed, and centrifuged at 15,000 × *g* for 2 min, and the lower chloroform phases from each step pooled. Each sample was dried under a stream of N_2 and resuspended in the minimum volume of chloroform:methanol (95:5, v/v) added and spotted onto TLC plates (silica gel G, Fisher Scientific) and developed with 2,2,4-trimethylpentane:acetic acid:H_2O:ethyl acetate (2:3:10:13, v/v). Fluorescence (excitation 460 nm, emission 534 nm) from lipids was measured in a fluorescence spectrophotometer (Image Station 4000 MM PRO-Carestream Molecular Imaging) and quantified with ImageJ.

2.7. Statistical analysis

The results of at least three independent experiments were statistically processed. All dependent variables were subjected to analysis of variance (ANOVA) without transformation. Treatment means of the untransformed data have been presented and were separated by Duncan's multiple range tests at a probability level of $P < 0.05$ using the software STATISTICA (version 6.0).

3. Results and discussion

Since roots are the primary site of contact between plant tissues and contaminants in soil or water, they provide a key assessment of the signalling pathways triggered by the presence of the harmful agent. Thus, we used membrane fractions of *V. sativa* roots to measure lipid kinase and PLD activities.

3.1. Lipid kinase activities

Phenols could act directly in the plasma membrane of cells and probably trigger changes in lipids patterns (Sosa Alderete et al., 2012). Thus, in order to analyze whether the treatment with this organic pollutant can produce changes in minor PLs turnover of *V. sativa* roots, we determined lipid kinase activities. These enzymes belong to the transduction pathway and use [γ-^{32}P]ATP as substrate.

Products of lipid kinases present in membrane fraction of *V. sativa* roots were identified by autoradiography as PA, lysophosphatidic acid (LPA), DGPP, phosphatidylinositol monophosphate (PIP), lysophosphatidylinositol monophosphate (LPIP) and PIP_2 (Fig. 1A). They comprise approximately 9% (PA), 51% (LPA/DGPP), 28% (PIP) and 11% (LPIP) of total lipids determined in control roots, while PIP_2 cannot be quantified due to its low levels. LPA generally represents the minor concentration of DGPP/LPA fraction (Meringer et al., 2012; Racagni et al., 2008). However, in this assays we have not determined the percentage of LPA, which could be produced by phopholipase A or by monoacylglycerol kinase activities. Since more experiments are necessary to solve this point, we only will refer to the minor PLs, DGPP, in sections below. To our knowledge, the results obtained in this study, are the first describing lipid kinase activities in *V. sativa.* In this sense, the values of enzymatic activity reached were approximately of 6.2, 27, 16.8 and 5.7 pmol min^{-1} mg protein^{-1} for DGK, PAK, phosphatidylinositol kinase (PIK) and in the control roots, respectively.

As it is shown in Fig. 1B, after 96 h of treatment with the pollutant we observed a markedly increase in lipid kinase activities, mainly DGK, as well as in PIK, and PAK, in those plants treated with the highest concentration of the contaminant (100 mg L^{-1}). These increments were approximately of 10; 2.4 and 1.8-fold compared to the enzymatic activity DGK, PIK and PAK of control plants harvested after the same time of treatment, respectively. Regarding DGPP increments, it can be related with PA removal, due to this minor PLs could be metabolized in the reaction catalyzed by PAK. Moreover, this enzyme play an important role not only in switching off the PA signal, but also in generating new lipid messengers, such as DGPP (Wang et al., 2006; Racagni et al., 2008; Arisz et al., 2009; Munnik and Vermeer, 2010).

On the other hand, lipid kinase activities of roots treated with 25 mg L^{-1} of the pollutant do not show changes. In the same way, LPIP levels of roots treated with both concentrations of the contaminant were similar to those obtained with control roots. Therefore, these results suggest that phenol concentrations above 25 mg L^{-1} could be sensed by plants as a stress signal, and thus are capable to induce changes in lipid kinase activities. This is in accordance with previous findings, where *V. sativa* seeds germinated with 5 and 25 mg L^{-1} of the pollutant showed higher germination index than control seeds, from the third day until the end of the treatment. This is a common phenomenon, called hormesis, characterized by the stimulation of organism performance in response to low levels of exposure to agents that are harmful and phytotoxic at high levels (Medina et al., 2003 and references therein; Ibáñez et al., 2012). Otherwise, seeds germinated in the presence of 100 mg L^{-1} of the contaminant, do not showed a significant decrease in germination index neither an increase in this parameter along the treatment, compared to control seeds (Ibáñez et al., 2012).

The results obtained in this study, could suggest the participation of minor PLs, like PIP, DGPP and specially PA, in activating the intracelular response mechanisms of *V. sativa* plants to long term treatment with high the pollutant concentrations. Furthermore, they could also be considerer as an evidence of the involvement of these minor PLs as second messengers of the signalling generated after sensing the presence of an organic contaminant, such as phenol.

3.2. PLD activity

Since PA can be synthetized by the action of PLC following a reaction catalyzed by DGK or via PLD pathway (Villasuso et al., 2013; Ruelland et al., 2015), we determined the involvement of this latter enzyme in phenol stress signalling (Fig. 2). To quantify PLD activity, we use the transphosphatidylation reaction, in which the presence of small amounts of a primary alcohol, like 1-butanol, determines the production of novel phosphatidylalcohol (PBut), an

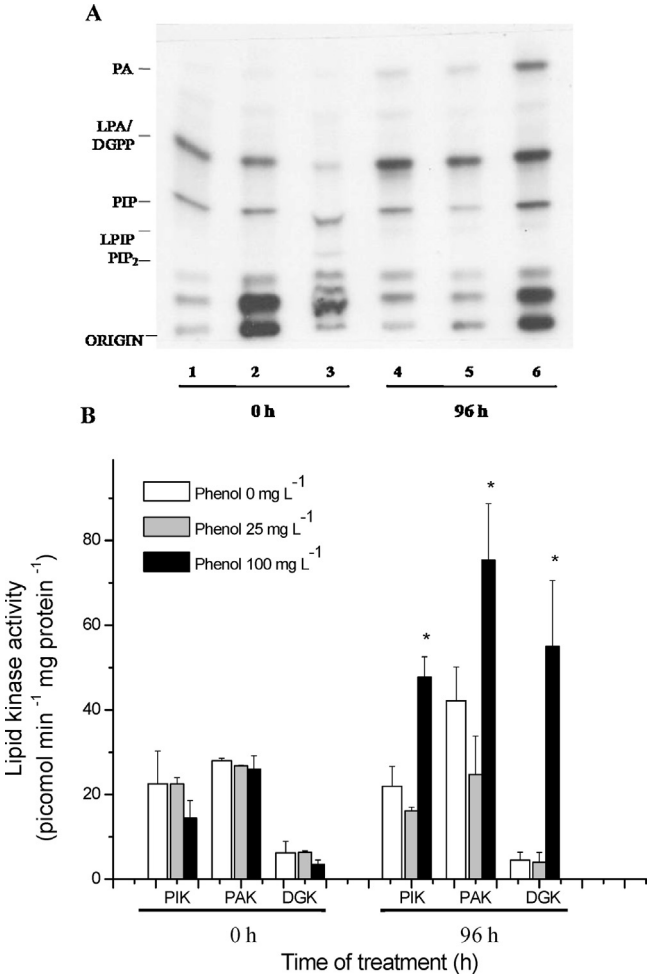

Fig. 1. (A) Representative autoradiograph of lipid kinase products from *V. sativa* roots, treated with 0 (1, 4), 25 (2, 5) and 100 mg L^{-1} of phenol (3, 6) for 0 and 96 h. Lipids were extracted and separated by one-dimensional TLC with chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v). PA: phosphatidic acid; LPA/DGPP: lysophosphatidic acid/ diacylglycerol pyrophosphate; PIP: phosphatidylinositol monophosphate; LPIP: lysophosphatidylinositol monophosphate. (B) The lipid kinase enzymatic activities were determined based on [γ-^{32} P]ATP phosphorylation of *V. sativa* roots membrane fractions. Results are expressed as pmol min^{-1} mg protein $^{-1}$ and shown as mean ± S.E. ($n = 3$). Asterisks (*) represent significant differences according to Duncan's test ($P < 0.05$).

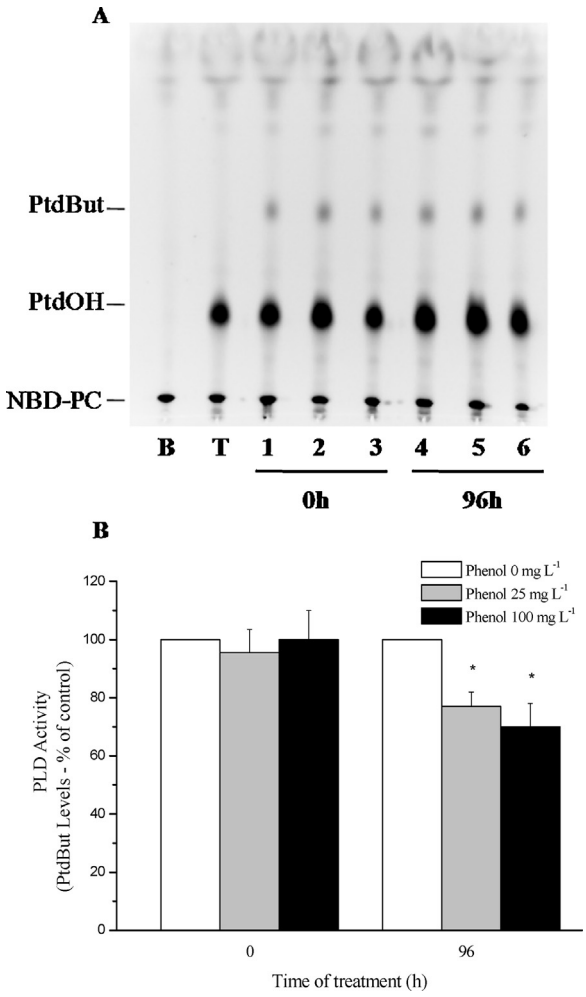

Fig. 2. Effect of long time treatment (96 h) with phenol (25 and 100 mg L^{-1}) on in vivo PLD activity of *V. sativa* roots. (A) Representative TLC, with lipids separated with the solvent containing ethyl acetate. B: control without proteins; T: control without 1-butanol, 1; 4: control roots, 2; 5: roots with 25 mg L^{-1} and 3; 6: roots with 100 mg L^{-1} of phenol. Treatments time are shown below the number of the spots (0 and 96 h). (B) Relative values of PtdBut were obtained using the image analysis software (ImageJ) of mean values ± S.D. of at least three independent experiments ($n = 3$). Roots treated with 0 mg L^{-1} of phenol were considered as representative of 100% activity. Asterisks (*) represent significant differences according to Duncan's test ($P < 0.05$).

inactive compound in the plant cell, instead of PA. Using this unique enzyme property, PA signalling can be uncoupled from PLD activity and the cumulative PLD activity assessed by quantifying the formation of PBut (Ghars et al., 2012).

As it is shown in Fig. 2B, PLD activity decreased 23 and 30% after 96 h of treatment with 25 and 100 mg L^{-1} of phenol, respectively. These data suggest that PLD activity of *V. sativa* plants is negatively regulated with increasing concentrations of the contaminant. Since the enzymatic activity was determined after long term exposure (96 h), we were not able to observe other changes that may have happened at an early response stage. Thus, in order to determine these possible rapid changes, we measure PLD activity after 1.5, 3; 6 and 24 h of treatment using the highest pollutant concentration (Fig. 3).

Results of this study showed an increase of approximately 26% after 1.5 h, reaching a decrease of around 35% of activity after 3 h. At later times (6, 24 h), PLD activity do not show changes respect to control plants. These transient increases in PA production and removal demostrate the complex cross talk of lipid signalling (Wang et al., 2006). Taken together the results obtained in short and in long assays, they showed a biphasic curve with an early increase in PA levels, followed by a decrease, then a period of maintenance in the activity. Finally, a new decrease of activity was observed during the long term harvest. Bojórquez-Quintal et al. (2014) described a biphasic behavior of PLC activity, in coffee roots exposed to aluminum ($AlCl_3$). This effect was dose dependent, since an increase in enzymatic activity was observed at 100 and 500 μM $AlCl_3$ with respect to the control, while 300 μM $AlCl_3$ did not affect root PLC activity. This effect is possibly attributable to the existence of two or more PLC isoforms, as has been suggested in different studies. Regarding PLD isoforms, the fully sequenced model plant *Arabidopsis thaliana* contains twelve PLD family members (three α, two β, three γ, one δ, one ε and two ζ class PLD isoforms) and this diversity has been assorted in higher plant species (Bargmann and Munnik, 2006). Thus, we planned some in silico experiments to find protein homology between *V. sativa* PLD and other classes of PLD from different plant species. Unfortunately, our search in different databases could not identify any sequence noted as PLD from *V. sativa*.

The rapid and transient PA increase obtained in this study, demonstrates and confirms its role in activating signalling events in response to the presence of phenol. Increases in this constituent of the minor portion of membrane lipids under exposure to numerous stresses, have also been described by other authors (Munnik 2001; Bargmann and Munnik, 2006; Wang et al., 2006; Xue et al., 2009; Ghars et al., 2012). Besides, this phospholipid seems to be produced via both pathways previously described, PLC/DGK and PLD, in *V. sativa* roots. In this sense, is important to identify the enzymatic source of PA to understand the cellular role of PA and the cellular regulation of signalling pathways. Accordingly to our results, short time treatments (1.5 h) with phenol (100 mg L^{-1}) may increase PA through PLD pathway while longer treatments (96 h) may increase PA levels through PLC/DGK activity, using DAG produced during membrane lipid hydrolysis catalyzed by phosphatidylinositol-PLC (PI-PLC) or phosphatidylcholine-PLC (PC-PLC) (Singh et al., 2015). However, the determination of these enzymatic activities was not the focus of this study. It is important to mention that PLDs can also act as catabolic enzymes, participating in a slow membrane degradation process (Hong et al., 2008) and that changes in the structure and composition of membranes has been cited among the common phytotoxic effects caused by phenol (Scragg 2006; Coniglio et al., 2008). Therefore, the decrease observed in PLD activity of *V. sativa* roots after 1.5 h of treatment, could contribute to avoid or at least to diminish this deleterious effect caused by the presence of this organic pollutant.

It is noteworthy that PA exerts its biological effects through multiple modes of action: (i) it alters membrane structure, (ii) PA acts as a messenger by specific interactions with protein targets, including activation or inhibition of enzymatic activities, (iii) PA may be converted to other signalling molecules, such as DAG, LPA, DGPP, and free fatty acids, or to be involved in membrane lipid metabolism (Wang et al., 2006; Villasuso et al. and references therein, 2013; Ruelland et al., 2015). Since the produced PA seems to be rapidly metabolized after being generated in *V. sativa* roots after phenol exposure, this could be related with its role as second messenger of the phospholipids derived from its metabolism. On the other hand, regarding PA role as activator of enzymes, we could suggest a relation between this minor PL and peroxidase (Px) activity, which are oxidoreductive enzymes related to the metabolism of several organic contaminants, including phenol (Kumar et al., 2011). In our previous study, we observed an increase in Px activity in *V. sativa* plants treated with 100 mg L^{-1} of phenol. On the other hand, plants treated with lower concentration of the pollutant (25 mg L^{-1}), did not show changes in Px activity compared with control plants. Increased Px levels are thought

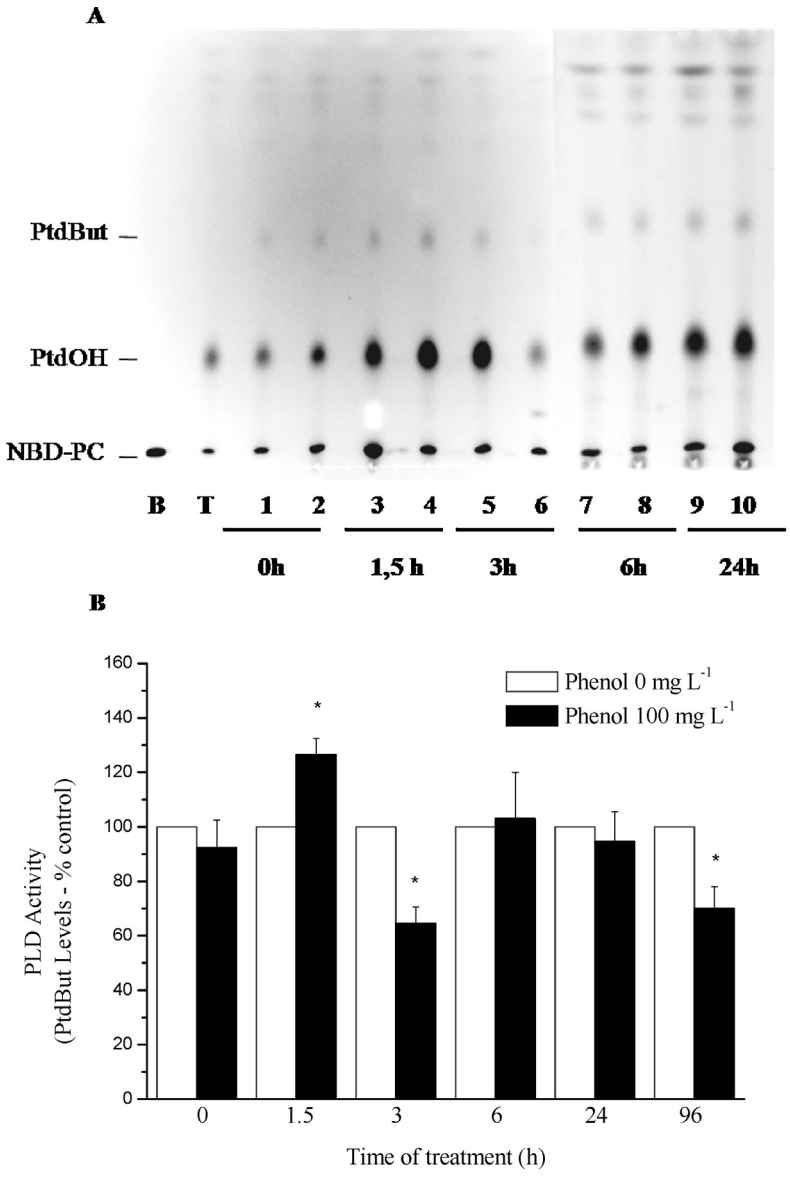

Fig. 3. Effect of short time treatment (1.5; 3; 6 and 24 h) with phenol (100 mg L^{-1}) on in vivo PLD activity of *V. sativa* roots. (A) Representative TLC, with lipids separated with the solvent containing ethyl acetate. B: blank without proteins; T: blank without 1-butanol, 1, 3, 5, 7; 9: control roots, 2, 4, 6, 8; 10: roots treated with 100 mg L^{-1} of phenol. Treatments times are shown below the number of the spots. (B) Relative values of PtdBut were obtained using the image analysis software (ImageJ) of mean values ± S.D. of at least three independent experiments ($n = 3$). Roots treated with 0 mg L^{-1} of phenol were considered as representative of 100% activity. Asterisks (*) represent significant differences according to Duncan's test ($P < 0.05$).

to protect plant cells from free radical oxidation, allowing the plant to adapt to the stressor (Kumar et al., 2009). Moreover, the induction of antioxidant enzymes including Px is an important protective mechanism in minimizing oxidative damage in plants growing in polluted environments (Zhang et al., 2007). Hence, Px could be one of the enzymes target for PA activation under the presence of phenol and thus, this minor PL could be a key messenger involved in protecting *V. sativa* cells against the deleterious effects produced by phenol. Furthermore, these previous results and those obtained in the present study, point out that this legume species is not only capable to sense the presence of the contaminant but also to distinguish stress intensity. This seems to be a key aspect to turn on a quickly response, involving lipid kinases and PLD activities, which will allow the plant to adapt and survive.

4. Conclusion

Even though it is well known that phospholipases and phospholipid derived signal molecules have a key role in stress response mediation, there are no reports concerning the signalling mechanisms triggered by the presence of an organic pollutant, like phenol. Moreover, this study would be the first to describe this effect in roots of a complete plant. Within the context briefly assayed above, the results obtained, indicate that phenol treatment (100 mg L^{-1}) induced markedly increases in lipid kinase activities, mainly DGK, as well as in PIK, and PAK of *V. sativa* roots after long term treatment. Besides, our findings have demostrated PA formation via PLD and also suggested its formation via PLC/DGK, in *V. sativa* response to phenol. However, further investigations are needed to elucidate the complete signalling events that allow

V. sativa adapting to this harmful agent, removing the contaminant and surviving.

Acknowledgments

This work was supported by the grants from SECyT-UNRC, CONICET. SGI have a fellow from CONICET. ALV and EA are members of the Research Career from CONICET (Argentina).

References

Arisz, S.A., Testerink, C., Munnik, T., 2009. Plant PA signaling via diacylglycerol kinase. Biochim. Biophys. Acta 1791, 869–875.
Bargmann, B.O., Munnik, T., 2006. The role of phospholipase D in plant stress responses. Curr. Opin. Plant Biol. 9, 515–522.
Bojórquez-Quintal, E., de, A., Sánchez-Cach, L.A., Ku-González, A., de los Santos-Briones, C., de Fátima Medina-Lara, M., Echevarría-Machado, I., Muñoz-Sánchez, J.A., Hernández Sotomayor, S.M.A., Martínez Estévez, M., 2014. Differential effects of aluminum on in vitro primary root growth, nutrient content and phospholipase C activity in coffee seedlings (*Coffea arabica*). J. Inorg. Biochem. 134, 39–48.
Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Coniglio, M.S., Busto, V.D., González, P.S., Medina, M.I., Milrad, S., Agostini, E., 2008. Application of *Brassica napus* hairy root cultures for phenol removal from aqueous solutions. Chemosphere 72, 1035–1042.
Ghars, M.A., Richard, L., Lefebvre-De Vos, D., Leprince, A.S., Parre, E., Bordenave, M., Abdelly, C., Savouré, A., 2012. Phospholipases C and D modulate proline accumulation in *Thellungiella halophila*/*salsuginea* differently according to the severity of salt or hyperosmotic stress. Plant Cell Physiol. 53, 183–192.
Hong, Y., Zheng, S., Wang, X., 2008. Dual functions of phospholipase D in plant response to drought. Mol. Plant 1, 262–269.
Ibáñez, S.G., Sosa Alderete, L.G., Medina, M.I., Agostini, E., 2012. Phytoremediation of phenol using *Vicia sativa* L. plants and its antioxidative response. Environ. Sci. Pollut. Res. 19, 1555–1562.
Islam, E., Khan, M.T., Irem, S., 2015. Biochemical mechanisms of signaling: perspectives in plants under arsenic stress. Ecotoxicol. Environ. Saf. 114, 126–133.
Iurascu, B., Siminiceanu, I., Vione, D., Vicente, M.A., Gil, A., 2009. Phenol degradation in water through a heterogeneous photo-Fenton process catalyzed by Fe-treated laponite. Water Res. 43, 1313–1322.
Kumar, M., Yadav, V., Tuteja, N., Kumar Johri, A., 2009. Antioxidant enzyme activities in maize plants colonized with Piriformospora indica. Microbiology 155, 780–790.
Kumar, S., Jaggi, M., Taneja, J., Sinha, A.K., 2011. Cloning and characterization of two new class III peroxidase genes from *Catharanthus roseus*. Plant Physiol. Biochem. 49, 404–412.
Medina, V.F., Maestri, E., Marmiroli, M., Dietz, A.C., McCutcheon, S.C., 2003. Plant tolerances to contaminants. In: McCutcheon, S.C., Schnoor, J. (Eds.), Phytoremediation: Transformation and Control of Contaminants. Wiley Intersciencies Series, USA, pp. 189–222.
Meijer, H.J., Munnik, T., 2003. Phospholipid-based signaling in plants. Annu. Rev. Plant Biol. 54, 265–306.
Meringer, M.V., Villasuso, A.L., Pasquaré, S.J., Giusto, N.M., Machado, E.E., Racagni, G. E., 2012. Comparative phytohormone profiles, lipid kinase and lipid phosphatase activities in barley aleurone, coleoptile, and root tissues. Plant Physiol. Biochem. 58, 83–88.
Munnik, T., 2001. Phosphatidic acid: an emerging plant lipid second messenger. Trends Plant Sci. 6, 227–233.
Munnik, T., Vermeer, J.E.M., 2010. Osmotic stress-induced phosphoinositide and inositol phosphate signaling in plants. Plant Cell Environ. 33, 655–669.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15, 473–479.
Pajuelo, E., Rodríguez-Llorente, I.D., Dary, M., Palomares, A.J., 2008. Toxic effects of arsenic on *Sinorhizobium-Medicago sativa* symbiotic interaction. Environ. Pollut. 154, 203–211.
Pokotylo, I., Kolesnikov, Y., Kravets, V., Zachowski, A., Ruelland, E., 2014. Plant phosphoinositide-dependent phospholipases C: variations around a canonical theme. Biochimie 96, 144–157.
Porta, R., Di Pierro, P., Rossi-Marquez, G., Mariniello, L., Kadivar, M., Arabestani, A., 2015. Microstructure and properties of bitter vetch (*Vicia ervilia*) protein films reinforced by microbial transglutaminase. Food Hydrocolloids 50, 102–107.
Racagni, G.E., García, M., Domenech, C., Machado Domenech, E., 1992. Phospholipids in *Trypanosoma cruzi*: phosphoinositide composition and turnover. Lipids 27, 275–278.
Racagni, G.E., Villasuso, L., Pasquaré, S.J., Giusto, N.M., Machado, E., 2008. Diacylglycerol pyrophosphate inhibits the α-amylase secretion stimulated by gibberellic acid in barley aleurone. Physiol. Plant 134, 381–393.
Racagni-Di Palma, G.E., Brito-Argáez, L., Hernández-Sotomayor, S.M.T., 2002. Phosphorylation of signalling phospholipids in *Coffea arabica* L cells. Plant Physiol. Biochem. 40, 899–906.
Ritchie, S., Gilroy, S., 1998. Plant biology abscisic acid signal transduction in the barley aleurone is mediated by phospholipase D activity. Proc. Natl. Acad. Sci. 95, 2697–2702.
Ruelland, E., Kravets, V., Derevyanchuk, M., Martinec, J., Zachowski, A., Pokotylo, I., 2015. Role of phospholipid signalling in plant environmental responses. Environ. Exp. Bot. 114, 129–143.
Scragg, A., 2006. The effect of phenol on the growth of *Chlorella vulgaris* and *Chlorella* VT-1. Enzyme Microb. Technol. 39, 796–799.
Singh, A., Bhatnagar, N., Pandey, A., Pandey, G.K., 2015. Plant phospholipase C family: regulation and functional role in lipid signaling. Cell Calcium 58, 139–146.
Sosa Alderete, L.G., Racagni, G.E., Agostini, E., Medina, M.I., 2012. Phospholipid turnover and phospholipase D activity in tobacco hairy roots exposed to phenol. Environ. Exp. Bot. 77, 141–145.
Sun, Y., Li, L., Macho, A.P., Han, Z., Hu, Z., Zipfel, C., Zhou, J.-M., Chai, J., 2013. Structural basis for induced activation of the *Arabidopsis* FLS2-BAK1 immune complex. Science 342, 624–628.
Testerink, C., Munnik, T., 2005. Phosphatidic acid: a multifunctional stress signaling lipid in plants. Trends Plant Sci. 10, 368–375.
Vaultier, M.N., Jolivet, Y., 2015. Ozone sensing and early signaling in plants: an outline from the cloud. Environ. Exp. Bot. 114, 144–152.
Villasuso, A.L., Di Palma, M.A., Aveldaño, M., Pasquaré, S.J., Racagni, G., Giusto, N.M., Machado, E.E., 2013. Differences in phosphatidic acid signalling and metabolism between ABA and GA treatments of barley aleurone cells. Plant Physiol. Biochem. 65, 1–8.
Wang, X., Devaiah, S.P., Zhang, W., Welti, R., 2006. Signaling functions of phosphatidic acid. Prog. Lipid Res. 45, 250–278.
Xue, H.-W., Chen, X., Mei, Y., 2009. Function and regulation of phospholipid signalling in plants. Biochem. J. 421, 145–156.
Zhang, F., Wang, Y., Lou, Z., Dong, J., 2007. Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two plant seedlings (*Kandelia candel* and *Bruguiera gymnorrhiza*). Chemosphere 67, 44–50.