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Exposure to chlorpyrifos induces morphometric, biochemical and lipidomic alterations in green beans (*Phaseolus vulgaris*)

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ABSTRACT

Chlorpyrifos (CPF) is a worldwide used pesticide that raises concerns from the environmental and human health perspectives. The presence of pesticides such as CPF in edible vegetables has been already reported, but little is known about the effects induced by this pesticide stress on the morphology, oxidative response and lipid composition of treated plants. In this work, green bean plants (*Phaseolus vulgaris*) were exposed to increasing concentrations of CPF and the different plant parts (roots, stem bases, stem, leaves, pods and beans) were subjected to different analyses. First, morphometric parameters and the oxidative response caused by CPF were explored. In a second phase of the study, an untargeted lipidomic analysis of the different tissue extracts was performed and MALDI-TOF mass spectrometry images of pods and beans were recorded and analysed to illustrate the spatial distribution of the changes observed. As a result of CPF treatment, plants showed a significant decrease in their height, leaf length, and pod number. The biochemical analysis showed lipid peroxidation and the activation of antioxidant mechanisms in roots, stem and leaves. Regarding the lipidomic results, changes in lipid levels were observed, mainly in leaves, pods and seeds. The main changes observed were a reduction of photosynthetic pigments and lipids in leaves and a decrease of triacylglycerols levels in pods and seeds. This last point was confirmed by the analysis of mass spectrometry images of the pods. These observations suggest that CPF would affect the yield of green bean crops as well as the nutritional value of pods and beans. This work represents a step forward in the knowledge of the effects of CPF, one of the most used pesticides worldwide, in plants.

1. Introduction

Chlorpyrifos (CPF, [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate]) is a chlorinated organophosphate insecticide used worldwide to protect a variety of food and feed crops such as fruits, grain, cotton, nuts and vegetables from a broad spectrum of insects, mites and nematodes (John and Shaike, 2015). Although CPF effectively controls a variety of pests in crops, the extensive use of this pesticide has led to public concerns on environmental contamination, food safety and human health (John and Shaike, 2015). From the environmental point of view, a wide range of water and terrestrial ecosystems might be contaminated with CPF and their degradation products (John and Shaike, 2015). CPF has been shown to act as a toxic compound in the aquatic environment where CPF is known to induce oxidative stress. CPF causes a general disruption of neurotransmitters metabolism and muscle exhaustion in zebrafish (Gómez-Canela et al., 2017) and it is also able to reduce the abundance of freshwater arthropods (Zafar et al., 2011). In humans, CPF is known to have cholinergic neurotoxicity and the exposure to CPF has been related to deficits in motor function in infants (Burke et al., 2017).

Many investigations have been directed to the study of CPF uptake and its phytotoxicity in different plant species. For instance, Martínez Vidal et al. (1998) studied the levels of CPF residues in both tomatoes and green beans grown in different types of greenhouses and Zhang et al. (2011) reported different levels of CPF that affected the growth of cabbages. In other studies, Parween et al. (2013, 2012) showed that CPF causes a reduction on growth parameters and chlorophyll content in mung bean (*Vigna radiata*) and also demonstrated that CPF treatment induced oxidative stress responses in these plants. The main defence of plants against oxidative stress, in this case produced by a pesticide, is the induction of an antioxidant response. The antioxidant system of plants is very complex and involves many components. The components addressed in this study were chosen since they are directly or indirectly related to the cell lipids. Lipid peroxidation (LPO) results from the interaction of ROS with PUFA. LPO products are toxic to cells (Cardoso et al., 2017) and can be converted by glutathione S-transferases (GSTs)

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and glutathione peroxidase (GPx) to alcohols (Regoli and Giuliani, 2014), which are less toxic. Dehydroascorbate reductase (DHAR) catalyses the conversion of dehydroascorbate to ascorbate. Ascorbate is an important antioxidant in plants, being involved in the regeneration of oxidized tocopherols, the main antioxidants in cell membranes, which arrest the propagation of LPO (Munne-Bosch, 2005). The determination of these enzymatic activities reflects not only the degree of toxicity but also the ability of the plant to tolerate the oxidative stress imposed by CPF.

There are few reports in the literature that describe the effects of CPF treatment on plant lipid composition. Lipids are a crucial class of bioconstituents of all living cells with very important structural functions in cell membranes and signalling. A process as fundamental for plants as photosynthesis occurs in membranes, which constitute the environment of many biochemical reactions. Lipids that form the membranes and those involved in cell signalling are very diverse and are not static; on the contrary, lipids are constantly being produced, degraded and recycled (Allen, 2016; Tenenboim et al., 2016). Lipid biosynthesis and dynamics are tightly regulated and can rapidly be activated upon biotic and abiotic stress signals. Regarding the impact of pesticides in the lipid composition of plants, lipid peroxidation (LPO) is a common event reported in many crop plants under the exposure to a variety of pesticides (Dubey et al., 2015).

The lipid composition of plants has also relevance from the economic and nutritional points of view. Many studies of plant engineering are focused in the development of genetic strategies that result in specific lipid composition of vegetables and fruits, providing desirable lipid formulas for lubricants, nutraceuticals, natural insecticides, biodiesel, and jet fuels (Horn and Benning, 2016). From the human health perspective, plant oils are commonly considered to be healthier than animal fats, as they contain relatively high amounts of unsaturated fatty acids. Changes in the fatty acid composition of seed storage triacylglycerols can vary the economic value of plant-derived oils. Green bean *(Phaseolus vulgaris)* is an important food legume worldwide and a significant source of fibre, proteins, lipids, antioxidants and vitamins (Campos-Vega et al., 2010). Green beans have been extensively investigated as a functional food with health properties which have been linked to the antioxidant nature of flavonoids. Concerning lipids, green beans are a source of unsaturated fatty acids (61% of total fatty acids) such as oleic (18:1), linoleic (18:2) and linolenic (18:3), being the latter the most abundant, accounting for 43% of the fatty acids (Grela and Günter, 1995). Lipidomics is a field within metabolomics that aims to simultaneously analyse as many lipid compounds as possible in a given sample (Tenenboim et al., 2016). Whereas target lipidomic studies focus in the analysis and quantification of a specific list of lipids, the untargeted analysis uses all the data generated by the analytic instrument and extracts the relevant changes of lipid levels in the different conditions used, by the means of advanced chemometric analytical tools. One of the main advantages of the untargeted methodology is that it enables the discovery of molecules that may be unexpectedly involved in a specific biological process.

In this work, we have explored morphometric, biochemical and lipidomic changes experienced by green bean plants exposed to increasing doses of CPF. Additionally to some growth parameters and the plant response to oxidative stress generated by CPF, an untargeted lipidomic study was carried out by using the Regions of Interest and Multivariate Curve Resolution Alternating Least Squares (ROIMCR) strategy on LC-MS data of plant extracts. The same analytic strategy was implemented to obtain mass spectrometry images of green bean pods and seeds slices using MALDI-TOF, which showed spatial distribution of some of these lipid changes.

2. Materials and methods

2.1. Green bean plant culture and CPF treatment

Seeds of green bean plant (*Phaseolus vulgaris*) were obtained from the local market. Selected seeds were germinated on wet cotton inside transparent plastic glasses at room temperature and sunlight. After 5 days of germination, each seedling was transferred to a plastic pot. The pots had a volume of 150 cm^3 and contained 25 g of substrate (dry weight). One kilogram of substrate mixture contained 550 g of peat substrate, 447 g of vermiculite, 1.5 g of controlled release fertilizer Ferticote-3 and 1.5 g of calcium carbonate. After transference, seedlings were watered with Milli-Q water. After 2 more days, plants were watered with 10 ml of Milli-Q water containing 0 (control), 0.02%, 0.06% and 0.08% (w/v) of CPF (Sigma), which from now will be referred as control, CPF1, CPF2 and CPF3, respectively. These doses (2, 4 and 8 mg of CPF/plant) were chosen taking into account the doses used in previous phytotoxicity studies concerning CPF (Parween et al., 2012, 2013; Zhang et al., 2011) and the doses recommended for its use in agriculture. For each CPF dose, five plant replicates were prepared. Plants were watered every 3 days and left to grown for 45 days at 25 °C next to the laboratory window under normal light/dark cycles. During this time plastic sticks nailed into the ground were used as a support for the plants to grow up. Then, plants were harvested, washed and different morphometric parameters (height, roots length, number of nodes, number and length of leaves, number and length of pods) were measured. The different parts of the plant were separated and ground under liquid nitrogen to a fine powder and transferred into tubes. Then the powders were lyophilized to dryness and further stored at − 80 °C.

2.2. Lipid extraction, LC-MS and data analysis

To start the extraction procedure, 1 ml of tert-butyl methyl ether/methanol (1:1) was added to 5 mg of plant powder. Then, this mixture was fortified with internal standards of lipids (1,2,3-17:0 triacylglycerol, 1,3-17:0 D5 diacylglyceride, 17:0 cholesteryl ester, 17:1 lyso phosphatidylethanolamine, 17:1 lyso phosphoglyceride, 17:1 lyso phosphatidylserine), 200 pmol each, and vortexed vigorously. Samples were then sonicated for 10 min and 500 µl of H_2O/methanol (3:1) were added. After vortexing, samples were centrifuged at 10,000*g* for 10 min and the upper phase was transferred to a new tube and evaporated under nitrogen. Residues were resuspended in 150 µl methanol, and centrifuged at 10,000*g* for 5 min. Then, 130 µl of the supernatants were transferred to a conic HPLC vials for injection. LC-MS analysis consisted of a Waters Acquity UPLC system connected to a Waters LCT Premier orthogonal accelerated time of flight mass spectrometer (Waters), operated in both positive and negative electrospray ionization mode. Full scan spectra from 50 to 1500 Da were acquired, and individual spectra were summed to produce data points each of 0.2 s. Mass accuracy and reproducibility were maintained by using an independent reference spray via the LockSpray interference. The analytical column was a 100 × 2.1-mm inner diameter, 1.7 mm C8 Kinetex (Phenomenex). The two mobile phases were phase A: MeOH, 1 mM ammonium formate and phase B: H_2O, 2 mM ammonium formate, both contained 0.2% formic acid. The column was held at 30 °C.

The detailed data analysis procedure is available as Supplementary methods.

2.3. Biochemical analysis

For lipid peroxidation assessment, lyophilized samples were extracted by adding 10% trichloroacetic acid (TCA) (1:2 v/w) and sonicating during 15 s at 0.5 cycles s^{-1}. LPO was measured by quantification of thiobarbituric acid reactive substances (TBARS), according to the protocol described by Buege and Aust (1978), based on the reaction of lipid peroxidation products such as malondialdehyde (MDA), with 2-