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Original article

Root-level exposure reveals multiple physiological toxicity of triazine xenobiotics in *Arabidopsis thaliana*

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ABSTRACT

Herbicides are pollutants of great concern due to environmental ubiquity resulting from extensive use in modern agriculture and persistence in soil and water. Studies at various spatial scales have also highlighted frequent occurrences of major herbicide breakdown products in the environment. Analysis of plant behavior toward such molecules and their metabolites under conditions of transient or persistent soil pollution is important for toxicity evaluation in the context of environmental risk assessment. In order to understand the mechanisms underlying the action of such environmental contaminants, the model plant *Arabidopsis thaliana,* which has been shown to be highly responsive to pesticides and other xenobiotics, was confronted with varying levels of the widely-used herbicide atrazine and of two of its metabolites, desethylatrazine and hydroxyatrazine, which are both frequently detected in water streams of agriculturally-intensive areas. After 24 h of exposure to varying concentrations covering the range of triazine concentrations detected in the environment, root-level contaminations of atrazine, desethylatrazine and hydroxyatrazine were found to affect early growth and development in various dose-dependent and differential manners. Moreover, these differential effects of atrazine, desethylatrazine and hydroxyatrazine pointed to the involvement of distinct mechanisms directly affecting respiration and root development. The consequences of the identification of additional targets, in addition to the canonical photosystem II target, are discussed in relation with the ecotoxicological assessment of environmental xenobiotic contamination.

1. Introduction

Conventional agriculture uses large amounts of pesticides to enable large-scale crop protection, increase of crop yields and economic viability of agriculture. However, pesticides, including herbicides, also induce chemical stresses that can affect crop growth and development. In general, phytosanitary products, through drift, run-off and leaching, significantly contribute to soil and water pollutions that can affect natural plant communities (Dévier et al., 2011; Helander et al., 2012). Given their dissemination, persistence and accumulation in the environment, pesticides behave as Persistent Organic Pollutants (POPs), which are a cause of concern because of their physico-chemical properties that favor their cellular uptake, consequent intracellular transport, storage in the tissue of organisms, and bioaccumulation through the food chain (Jones and de Voogt, 1999; Nadal et al., 2015). Environmental compartments act as transfer intermediates or as sinks for agricultural POPs, starting from soils surrounding application sites in agriculturally intensive areas, moving through field margins and ending, *via* airborne or soil processes, in ground waters and streams (Lohmann et al., 2007). These agricultural POPs are indeed detected as micropollutants in surface or ground waters in concentrations ranging from ng/L to μg/L (Luo et al., 2014; Vandermaesen et al., 2016).

Under such conditions of environmental contamination, many different organisms are exposed to variable concentrations of such agricultural POPs, from terrestrial non-target plants to aquatic micro- and macro-flora and fauna (Coutris et al., 2011; Graymore

Abbreviations: ATZ, atrazine; d, day; DEA, desethylatrazine; F_0, minimum fluorescence; F_m, maximum fluorescence in dark conditions; F'_m, maximum fluorescence in light conditions; F_v, variable fluorescence; h, hour; HA, hydroxyatrazine; L, length of the primary root; NPQ, non-photochemical quenching; POPs, persistent organic pollutants; ppm, parts per million; PSII, photosystem II; PSI, photosystem I; ROS, reactive oxygen species; RRG, relative root growth.

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et al., 2001). Given the diversity of pesticide applications, agricultural soil, field margin soil and surface water are commonly contaminated with mixtures of pesticides at various levels, from residual to very high. Besides the chemical complexity of such mixtures, their impact on organisms, and particularly on plants, depends on contamination pathways (spray drift, run-off, leaching), on the plant organ that is initially exposed, as well as on the frequency and duration of the exposure. Plant exposure in field margins often occurs at the root system level. It can also be transient and dynamic, as the composition and the single-component concentrations in contaminating mixtures vary as a consequence of application rate, raining events and geomorphological properties of the field.

Understanding the mechanisms underlying such complex environmental impact requires well-defined experimental procedures focusing on model xenobiotics and model organisms. Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] (ATZ) is one of the most widely applied herbicides worldwide, especially in the USA, Brazil, China and India (Pathak and Dikshit, 2012). The persistence of atrazine in soils and waters has been demonstrated by numerous studies. Its presence is detected in the environment (Table 1) several years after end of use occurs, as in the European Union where it has been banned in 2004. Because of its intensive use, its persistence in the environment and its toxicological effects, atrazine is a typical POP at the global level (Loos et al., 2010). Atrazine pollution can hinder the growth of succeeding crops and poses significant threats to non-target organism and ecosystem health (Sun et al., 2016). Moreover, abiotic chemical reactions, microorganism-mediated processes or plant metabolism reactions lead to the production of several atrazine degradation products, such as desethylatrazine (DEA) and hydroxyatrazine (HA) (Barriuso and Houot, 1996; Blumhorst and Weber, 1994; Tan et al., 2015). Although these compounds are also detected in the environment (Table 1), few dedicated ecotoxicological studies are available (Ralston-Hooper et al., 2009). In plants, atrazine is known to inactivate the D1 subunit of Photosystem II (PSII), thus leading to interruption of the photosynthetic electron transport chain, photosynthesis inhibition and production of Reactive Oxygen Species (ROS) (Rutherford and Krieger-Liszkay, 2001). Conversely, plants can tolerate herbicide and chemical stress through the induction of antioxidative, repair, or detoxification pathways (Ramel et al., 2007; Sulmon et al., 2006; Tan et al., 2015). Moreover, atrazine degradation products, at low environmental concentrations, can alter plant metabolism and gene expression in the absence of any visible effect on photosynthesis and growth (Serra et al., 2013).

It is therefore essential to understand the processes involved in plant responses to herbicide-related pollution under conditions that take into account the characteristics of environmental exposure: (i) variety of herbicide-related pollutants including degradation products, (ii) range of contamination, from low to high levels, (iii) root system primary exposure, (iv) transient dynamics. For that purpose, we chose to study a coherent chemical series consisting of ATZ, DEA and HA, which, respectively, differ from the parent molecule by the absence of an ethyl group and of chlorine (Fig. 1). Plant responses to increasing doses of ATZ, DEA and HA applied at the root level were comparatively analyzed in seedlings of the model plant *Arabidopsis thaliana*, which has been shown to be highly responsive to pesticides and other xenobiotics (Ramel et al., 2007, 2009, 2012). Plants were exposed during 24 h to concentrations covering the range of triazine contamination detected in the environment, and especially in field margins of agriculturally-intensive areas (Table 1). Analysis of physiological traits aimed to characterize early responses induced by low doses of active ingredient and theoretically non-active metabolites. We found that root-level contaminations of ATZ, DEA and HA could affect early growth and development in a dose-dependent and differential manner. Moreover, the differential effects of ATZ, DEA and HA pointed to the involvement of distinct mechanisms that remain to be characterized and that may contribute to contrasting consequences on environmental impact.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of *A. thaliana* (Columbia ecotype, Col-0) were surface sterilized in sodium hypochlorite (2.63%):ethanol (1:1, v/v), rinsed in absolute ethanol, and dried overnight. Germination and growth were carried out under axenic conditions in square Petri dishes. Growth medium consisted of 0.8% (w/v) agar in 1X Hoagland basal salt mix (Caisson laboratories, Smithfield, UT, USA) adjusted to pH 6. After seeds were sown, Petri dishes were placed in the dark at 4 °C for 48 h in order to break dormancy and homogenize germination, and were then transferred to a control growth chamber at 22 °C/20 °C under a 16 h light (6000 lx)/8 h dark regime. Alternatively, seedlings were transferred on vermiculite, an inert and hydroponic culture substrate, and grown for a month with 1X Hoagland watering.

2.2. Xenobiotic treatments

ATZ, DEA and HA were purchased from Sigma (St. Louis, MO, USA) as analytical standards and used to prepare 10 mM stock solutions. They were added axenically at various concentrations to melted agar-Hoagland medium or to liquid 1X Hoagland watering solution. After growth on xenobiotic-free medium, seedlings were transferred at the end of the night period to fresh agar-Hoagland medium in the absence or presence of various concentrations of xenobiotics. Alternatively, young plants grown on vermiculite were exposed to xenobiotics by watering with xenobiotic-containing Hoagland solution.

2.3. Growth parameters

Length of the primary root (L) was measured on vertical plates and Relative Root Growth (RRG) was calculated as $[(L_X - L_0)/L_0]$ with X as days after transfer (T_0). Fresh weight of whole plantlets was determined for individual biomass analysis after measuring all the other parameters.

2.4. Chlorophyll fluorescence

Maximum PSII efficiency (F_v/F_m) and Stern–Volmer Non-Photochemical Quenching (NPQ) capacity were measured using a PAM chlorophyll fluorometer system (Heinz Walz, Effeltrich, Germany) in saturating pulse mode. After 30 min of dark adaptation, minimum fluorescence (F_0) of leaves was determined under weak red light, a subsequent saturating pulse of red light was applied, and maximum fluorescence was then measured (F_m). The photochemical yield of open PSII reaction centers, commonly known as PSII efficiency, was calculated as $F_v/F_m = (F_m - F_0)/F_m$. Alternatively, plants were dark-adapted for 30 min, maximum fluorescence (F_m) of leaves was then measured under dark conditions after the saturating pulse was applied, and a further 1 min exposure to actinic light was applied to measure maximum fluorescence under light conditions (F'_m). NPQ was calculated as $NPQ = (F_m - F'_m)/F'_m$ (Bilger and Björkman, 1990).

2.5. CO_2 exchange

After 20 days of growth on xenobiotic-free agar-Hoagland medium under 16 h-light photoperiod, seedlings were transferred