

Multiple effects of a commercial Roundup® formulation on the soil filamentous fungus *Aspergillus nidulans* at low doses: evidence of an unexpected impact on energetic metabolism

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Abstract Soil microorganisms are highly exposed to glyphosate-based herbicides (GBH), especially to Roundup® which is widely used worldwide. However, studies on the effects of GBH formulations on specific non-rhizosphere soil microbial species are scarce. We evaluated the toxicity of a commercial formulation of Roundup® (R450), containing 450 g/L of glyphosate (GLY), on the soil filamentous fungus *Aspergillus nidulans*, an experimental model microorganism. The median lethal dose (LD₅₀) on solid media was between 90 and 112 mg/L GLY (among adjuvants, which are also included in the Roundup® formulation), which corresponds to a dilution percentage about 100 times lower than that used in agriculture. The LOAEL and NOAEL (lowest- and no-observed-adverse-effect levels) associated to morphology and growth were 33.75 and 31.5 mg/L GLY among adjuvants, respectively. The formulation R450 proved to be much more

active than technical GLY. At the LD₅₀ and lower concentrations, R450 impaired growth, cellular polarity, endocytosis, and mitochondria (average number, total volume and metabolism). In contrast with the depletion of mitochondrial activities reported in animal studies, R450 caused a stimulation of mitochondrial enzyme activities, thus revealing a different mode of action of Roundup® on energetic metabolism. These mitochondrial disruptions were also evident at a low dose corresponding to the NOAEL for macroscopic parameters, indicating that these mitochondrial biomarkers are more sensitive than those for growth and morphological ones. Altogether, our data indicate that GBH toxic effects on soil filamentous fungi, and thus potential impairment of soil ecosystems, may occur at doses far below recommended agricultural application rate.

Keywords Glyphosate · Roundup® · Soil · *Aspergillus nidulans* · Toxicity · Mitochondrial metabolism · Low doses

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Introduction

The use of glyphosate-based herbicides (GBH) is closely associated with intensive farming, so these compounds are released into the environment in large amounts. In addition to the potential risks posed by the consumption of their residues (Mesnage et al. 2015), they could also have deleterious consequences on soil resident microorganisms and consequently on the global health of agricultural soil ecosystems (Carranza et al. 2014; Krzysko-Lupicka and Sudol 2008; Sailaja and Satyaprasad 2006; Zaller et al. 2014).

Roundup® is the major herbicide used worldwide and is being used increasingly because approximately 80 % of commercially grown genetically modified (GM) plants are designed to tolerate it (James 2011). Application of this non selective herbicide to foliage results in the death of most herbaceous plants.

Roundup® is available in various formulations which are composed of mixtures of glyphosate (GLY) (the declared active ingredient in plants), and adjuvants, which are added to increase the stability and allow the penetration of GLY (Cox 2004).

GLY is often described as exhibiting little or no activity in soil due to potential rapid adsorption on soil inorganic and organic particles (Duke and Powles 2008). However, numerous studies show that GLY is available to soil microbial communities (Haney et al. 2000; Wardle and Parkinson 1990). For instance, agricultural soils amended with phosphorus fertilizers contain high levels of unbound GLY because soil sorption sites are occupied by competing phosphate ions (Simonsen et al. 2008). Remaining GLY in the soil solution is thus available not only for potential uptake by plant roots or leaching into groundwater, but also for microorganisms which can ingest it. Moreover, the major GLY breakdown product, aminomethylphosphonic acid (AMPA), is frequently detected in soils subjected to frequent GBH applications (Simonsen et al. 2008). While GBH commercial formulations include various adjuvants, many studies (including regulatory tests) focus only on the active ingredient (GLY). However, the additives cannot be considered to be inert since several studies have shown that these formulations are much more toxic than the active ingredient alone, whether it is for human cells or tissues (Mesnage et al. 2015), earthworm (Piola et al. 2013), oyster (Mottier et al. 2013), water flea (Cuhra et al. 2013), or microorganisms (Braconi et al. 2006; Clair et al. 2012; Lipok et al. 2010; Qiu et al. 2013). However, reports on impacts of GLY, its degradation products and GBH formulation adjuvants on specific microbial species inhabiting non-rhizosphere soil are limited. The assessment of such impacts is particularly crucial in the light of the increasing use of Roundup®-tolerant crops, which involve a much higher spray of this herbicide than in conventional agriculture.

The soil filamentous fungus *Aspergillus nidulans* is an experimental model organism used for decades in basic and industrial microbiology research (Martinelli and Kinghorn 1994). The use of this ascomycete fungus is particularly advantageous since it is not pathogenic to either humans or plants, and it is extremely easy to manipulate, while at the same time avoiding the complexity and accessibility problems found with higher eukaryotes. Growth of *A. nidulans* on simple well-defined media allows a researcher to continuously check physical and chemical conditions, resulting in high reproducibility experiments. This fungus can be maintained by asexual reproduction via mycelial propagation or through the germination of abundant long-lived asexual spores, the conidia, and it can be readily induced to enter the sexual cycle under laboratory conditions. Moreover, *A. nidulans* is a multicellular organism whose life cycle is characterized by different stages of development with the establishment of specialized structures, such as conidiophores (aerial branched hyphae

that bears the conidia) and fruiting bodies, due to functional specialization of cells. It thus offers also the opportunity to study the influence of environmental factors on the various cell differentiation processes that characterize all soil fungi as well as higher eukaryotes. Finally, because it has been extensively studied, *A. nidulans* is a well-characterized organism from a genomic, proteomic, metabolic and physiologic point of view.

In this work, we aimed to investigate the effects of a commercial formulation of Roundup® (R450), containing 450 g/L of GLY, on the survival, growth, morphology and metabolism of *A. nidulans*, used here as a marker of agricultural soil ecosystems health, and in particular non-rhizosphere soil microbial life. Herbicide tolerance is generally regarded as the absence of general toxic effects at the macroscopic level, ignoring potential cellular effects provided that they do not result into visible consequences (growth, morphology, yield). This omission is why we also focused on the cellular effects of Roundup® at a concentration corresponding to a no-observed-adverse-effect level (NOAEL) for these macroscopic parameters.

Materials and methods

Strains, media and general growth conditions

Aspergillus nidulans strains used and/or generated in this study are listed in the Online Resource 1 (Table S1). Standard genetic markers are described in http://www.fgsc.net/Aspergillus/gene_list/index.htm.

Media composition, supplements and basic growth conditions were as described by Cove (1966) using glucose (1 %) or fructose (0.1 %) as carbon sources, and diammonium tartrate or urea (5 mM each) as nitrogen sources. Solid media contained 1.2 or 3 % agar. Plates were incubated at 37 °C, and liquid cultures were carried out at 30 °C in an orbital shaker at 150 rpm.

Chemicals

Glyphosate (N-phosphonomethylglycine, GLY, CAS:1071-83-6), also known as “technical glyphosate”, was purchased from Sigma-Aldrich. The herbicide Roundup® used in this work (R450) was the commercial formulation called “GT plus” (450 g/L of GLY, corresponding to 100 %), available on the market (homologation 2020448, Monsanto, Anvers, Belgium). Solutions of R450 (diluted to 2 %, i.e. 9 g/L of GLY final) or GLY (also 9 g/L) were prepared and serially diluted in minimal medium. The minimal medium was systematically adjusted to pH 6.8 (with NaOH) during its preparation, according to the conventionally used recipe (Cove

1966). After addition of the tested substance, it was re-adjusted (if necessary, depending of the amount of R450 or GLY added) at this same pH, again with NaOH (both R450 and GLY being acidic). The dyes FM[®]4-64 and MitoTracker[®] Red CMXRos were purchased from Molecular Probes Inc (Eugene, Oregon, USA). All chemicals for enzyme assays were purchased from Sigma-Aldrich.

Survival rate, toxicity index (TI) and biomass yield measurements

For survival rate, a rich medium with 3 % agar in a 85 mm diameter Petri dish was first inoculated (at the central point) with the *A. nidulans* strain to be tested, and incubated at 37 °C for 7 days (time required for the mycelium to cover the entire agar surface). Spores from one quarter of the plate were harvested and suspended in 5 mL sterile 0.01 % Tween 80. The conidial suspension was then serially diluted in 10-fold steps, down to 10⁻⁵ (about 10³ spores/mL) in sterile 0.01 % Tween 80. Hundred microlitres of the 10⁻⁵ dilution were plated out onto rich or minimal medium containing increasing concentrations of R450 (0 to 0.5 %) or equivalent amounts of GLY alone. For each R450 or GLY dose, the plating out was performed on six plates. The counting of the number of colonies (i.e. the number of viable spores) was carried out after 2 days incubation at 37 °C.

For toxicity measurements, groups of 12 plates for each R450 dose and medium (rich or minimal) were inoculated at the central point with conidia of the tested strain and incubated at 37 °C. Colony diameters were measured 72 h after inoculation, and TI expressed as percentage reduction with respect to controls (without R450), as originally described by Kappas et al. (1974).

Biomass yields were measured by weighing the mycelia obtained from 100 mL of minimal medium cultures, grown at 30 °C for 15 h in absence or presence of R450 at the indicated concentrations. For each condition, 10 cultures were grown in parallel. Before weighing, mycelia were harvested by filtration through sterile Blutex nylon tissue, washed with water, and wrung dry.

Microscopy imaging analysis

For confocal microscopy, *A. nidulans* strains were inoculated on an agar-rich medium surface, and plates were incubated at 37 °C for 3 days. Confocal live-cell imaging was performed according to the inverted agar method as previously described (Hickey et al. 2005). Fluorescence experiments were done with an inverted confocal laser scanning microscope LSM 510-Meta (Carl Zeiss, Germany) as previously described (Flipphi et al. 2014). Differential interference contrast (DIC) images were obtained simultaneously with the fluorescent channel using 543 nm laser wavelength

and a transmission detector. Morphological and fluorescence intensity description of mitochondrial network (using the Red CMXRos dye) was based on 3D image reconstructions: z-stack images were collected along the z-axis with a 0.32 μm step interval. Images were imported and deconvolved using Autoquant X software (MediaCybernetics, Bethesda, MD). Number and volume of mitochondrion and total fluorescence intensity were obtained with IMARIS software (Bitplane Company, Zurich, Switzerland), using Imaris MeasurementPro module and surface rendering method.

Video-microscopy was carried out as previously described (Flipphi et al. 2014). The exposure time was 33 msec. Time-lapse acquisitions and measurement tools available from the AxioVision 4.7 Zeiss software were used to monitor all the growth parameters (growth rate, germination lag-time and ratio, spore diameter, hyphae width).

Enzyme assays

Crude extracts were prepared from 400 mL of minimal medium cultures, grown at 30 °C for 15 h in absence or presence of R450 at the indicated concentrations. Mycelia were harvested by filtration, washed with cold sterile water, wrung dry, and ground with a pestle in a mortar under liquid N₂ to a fine powder. Crude extracts were made by suspending the ground powder (150 mg/mL) in precooled potassium phosphate buffer (20 mM, pH 7.5) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), shaking by vortexing (several times 1 min with 1 min intervals in ice), and centrifuging for 15 min at 4300×g at 4 °C. The cell debris was discarded and the supernatant was successively centrifuged (at 4 °C) for 30 min at 14,500×g and 20 min at 16,000×g to remove the remaining insoluble material. Protein concentrations were determined spectrophotometrically at 532 nm using the bicinchoninic acid (BCA) protein assay as described by Smith et al. (1985), with bovine serum albumin as the standard.

Enzyme activities for citrate synthase (CS), malate dehydrogenase (MDH), malate synthase (MS), aconitase (ACO) and succinate dehydrogenase (SDH) were determined spectrophotometrically as described by Srere et al. (1963), England and Siegel (1969), Woodward and Merrett (1975), Fansler and Lowenstein (1969), and Singer et al. (1966), respectively.

Statistical analysis

Experiments were repeated at least in duplicate from at least three independent liquid cultures ($n \geq 6$) or carried out from several parallel plates or liquid cultures ($n \geq 6$), as specified in each case and unless otherwise stated. All data were presented as the means ± Standard Errors (SEM). Statistical differences from the control were determined by unpaired Student *t* test

using significance levels at $p < 0.0001$ (****), $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*).

Results

Survival and growth

We assessed the toxicity potential of R450 as well as GLY alone on the soil fungus *A. nidulans* by testing the ability of asexual spores (conidia) to germinate (and thus to form colonies) on solid rich (Fig. 1a) or minimal (Fig. 1b) media with several R450 doses (dilutions from 0.005 to 0.5 %, i.e. containing 22.5 mg/L to 2.25 g/L GLY among adjuvants) or with equivalent concentrations of technical GLY. These doses were below those recommended for agricultural use (dilution of 1 or 2 %, i.e. 4.5–9 g/L GLY). These toxicity tests showed a

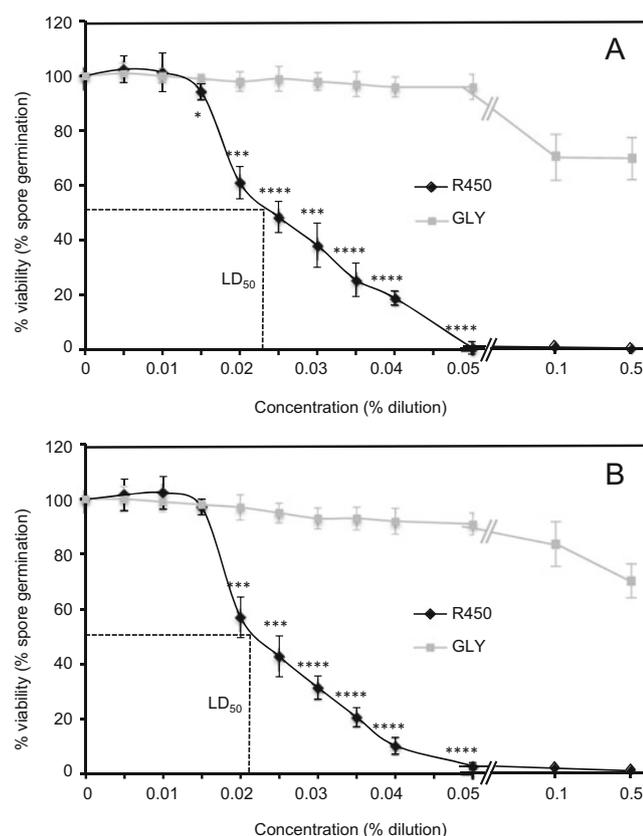


Fig. 1 Effects of R450 and equivalent quantities of GLY on *A. nidulans* viability on rich medium (a) or minimal medium (b). Spores were plated on media containing increasing concentrations of R450 or equivalent concentrations of GLY at the same pH. The results (number of colonies, i.e. number of viable spores) are presented as % comparatively to growth on untreated media. The LD₅₀ is indicated by a dashed line. Standard errors of the mean (SEMs) are indicated in all instances (from eight independent experiments: $n = 8$). Significance of the effects (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) is tested against the control (0 % R450 or GLY)

concentration-dependent response for both R450 formulation and GLY alone, with no significant difference between the two types of media. However, R450 always showed much higher dose-dependent cytotoxicity than its active ingredient, GLY, suggesting an additional effect provoked by the adjuvants. The median lethal dose (LD₅₀) of R450 was between 0.02 and 0.025 % (around 100 mg/L GLY among adjuvants), i.e. a dilution percentage about 100 times lower than the agricultural one. From 0.05 % (0.225 g/L GLY among adjuvants), the mortality was 100 %, whereas GLY alone caused only 30 % mortality at a concentration 10 times higher, (2.25 g/L). As shown in Fig. 2a, growth of *A. nidulans* at LD₅₀ and slightly lower concentrations of R450 (from surviving spores) was dramatically affected. In order to obtain a quantitative estimate of R450 toxicity, we measured the percentage reduction in colony diameter compared to controls, expressed as toxicity index (TI) (Fig. 2b). For the sake of comparisons with other toxic substances (see Discussion), TI was measured after 3 days of incubation at 37 °C, but similar values were obtained after 4, 5 (Fig. 2b) and 6 days. As might be expected, the TI increased with the dose of R450. Besides growth retardation, these tests also revealed an effect of R450 on the mycelial organization, highly visible from 0.02 % (Fig. 2c).

Microscopic analyses (DIC) from mycelia growing on solid rich media in absence or presence of R450 at LD₅₀ (0.025 %) showed a significant morphological difference: the presence of R450 resulted in a high degree of hyphae branching with multiple, random secondary germ tube emergence at the tip, giving a “stump” appearance (Fig. 3). Such a branching alteration, which is symptomatic of a failure to establish or to maintain polarity, was consistent with the mycelial disorganization previously observed (Fig. 2c). Images obtained by DIC at low magnification and giving an overview of the filaments at the edge of the colony perfectly demonstrated this mycelial alteration (Fig. S1 in Online Resource 1). For more details, we used video-microscopy to follow and compare the growth of *A. nidulans* in liquid culture in the absence or presence (0.025 %) of R450 (Online Resources 2 and 3, respectively). All growth parameters are summarized in Table 1. Besides affecting the growth rate of hyphae, which was decreased by about 40 % when R450 was added in the medium, the presence of this pesticide also resulted in a slight increase of the spore diameter as well as of the hyphae width, suggesting either an extended isotropic growth of the conidia before the emergence of germ tubes, or a modification of the wall structure affecting osmoregulation.

Determination of the NOAEL and LOAEL doses

To determine the maximal dose of R450 at which no morphological or growth effect is observable in *A. nidulans*, we conducted a monitoring of the growth parameters both in solid and liquid media, at doses of R450 for which no mortality and

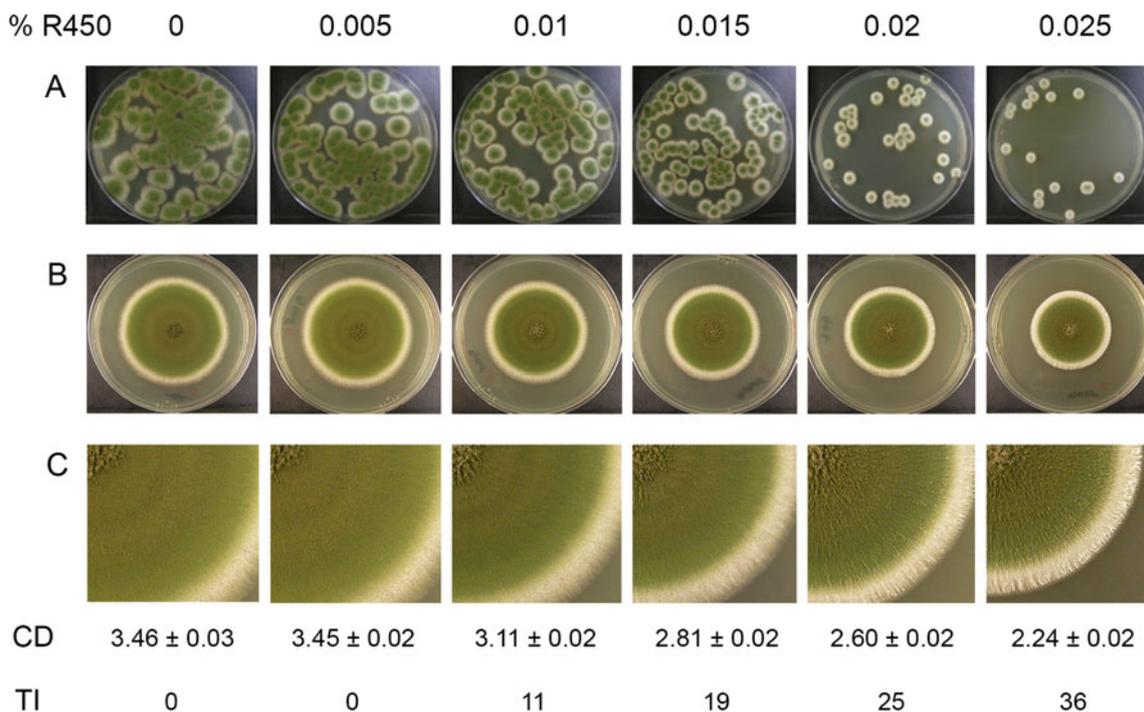


Fig. 2 Effects of R450 on *A. nidulans* growth and morphology. Plates containing rich medium with the indicated R450 concentration were inoculated with spores either in suspension (a) or at the central point (b and c). c Enlarged view of a sector of the colony shown in b to visualize the mycelial organization. The plates were incubated for 2 days (a) or 5 days (b and c) at 37 °C. Plating with spore suspension (a) were used to measure the survival rates as described Fig. 1, while central point inoculations (b and c) were used to measure the colony diameters (CD)

for a quantitative estimate of the toxicity (TI). CDs are given as mean values ± SEM (cm) obtained from 6 to 12 colonies after 3 days incubation at 37 °C (6 ≤ n ≤ 12). On minimal medium in presence of 0.01, 0.015, 0.02 and 0.025 % R450, TI value was 13, 26, 30 and 41 %, respectively. In all instances (on rich and minimal medium with 0.01 to 0.025 % R450), the significance level of statistical differences from the control (0 % R450) was at **** p < 0.0001

no toxicity (TI=0 %) was observed (i.e. inferior to 0.01 %). Since no toxicity was detected at 0.005 % (Fig. 2), we first tested the TI at 0.0075 % (i.e. the median dose between 0.005 % and 0.01 %) both on rich and minimal media. This dose still provoked a slight but clear growth retardation (Fig. S2 in Online Resource 1), with a TI ranging from 6 to 8 %. Moreover, a slight difference in the mycelium colour was detectable on minimal medium after 6 and 7 days of growth. A slightly lower concentration (0.007 %) proved to cause no growth retardation (TI=0 %) and no mycelial difference compared with the control (Fig. S2). The detailed analysis of the growth parameters in liquid culture (using video-microscopy)

and the measure of the biomass yield at 0.007 % (as well as at 0.0075 % as a positive control) showed no significant difference to the control (Table 1). Given that abnormal hyphal branch formation (observed at the microscopic level) (Fig. 3d) had consequences on the mycelial organization visible at the macroscopic level (Fig. 2c), we also carried out microscopic (DIC) analysis to ensure that the hyphal branching in the presence of 0.007 % R450 was the same as that observed in the absence of herbicide. While 0.0075 % of R450 still resulted in a relatively high degree of secondary germ tubes emergence (Fig. 3c), the hyphae tip showed no morphological abnormality at 0.007 % (Fig. 3b). Based

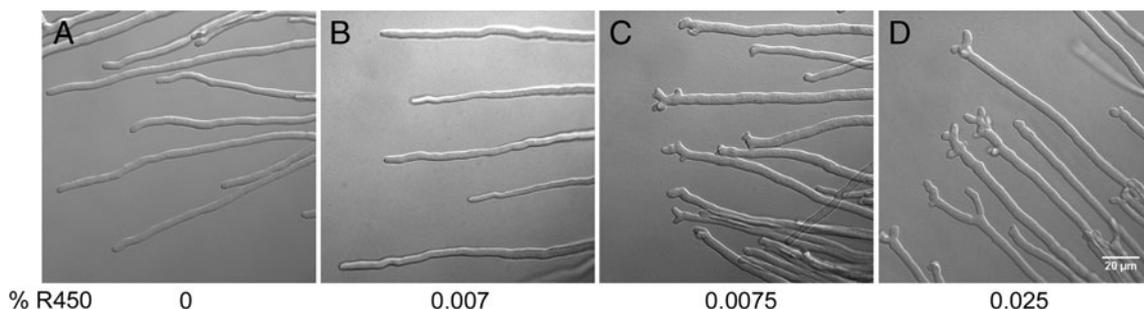


Fig. 3 Effects of R450 on hyphae branching (cell polarity). Images were obtained by differential interference contrast (DIC) microscopy of hyphae apices of the CV125 *A. nidulans* strain grown for three days at 37 °C on solid rich media in the absence or presence of the indicated R450 dose

Table 1 Growth parameters of *A. nidulans* in liquid culture in absence and presence of low doses of R450

% R450	Growth rate ^a ($\mu\text{m}/\text{min}$)	Germination ^b		Spore diameter ^{c, d} (μm)	Hyphae width ^{a, d} (μm)	Biomass yield ^e ($\text{mg}/100 \text{ mL}$)
		lag time (min)	%			
0	0.155 \pm 0.009	220 \pm 10	96 \pm 2	3.51 \pm 0.22	2.59 \pm 0.07	110 \pm 7
0.007	0.150 \pm 0.012	220 \pm 10	96 \pm 3	3.14 \pm 0.38	2.65 \pm 0.09	115 \pm 7
0.0075	0.124 \pm 0.012**	220 \pm 10	96 \pm 2	3.20 \pm 0.45	2.45 \pm 0.07	88 \pm 6**
0.025	0.093 \pm 0.009****	400 \pm 10****	30 \pm 3****f	4.01 \pm 0.17*	3.16 \pm 0.18***	nd ^g

^a Average values \pm SEM were obtained from 10 hyphae in each one of three independent experiments ($n = 30$)

^b Average values \pm SEM were obtained from duplicates (two different Lab-Tek[®] chambers: see Materials and methods) of three independent experiments ($n = 6$)

^c Average values \pm SEM were obtained from 10 spores in each one of three independent experiments ($n = 30$)

^d Spore diameter and hyphae width were measured from the images acquired at $t = 16 \text{ h}$

^e Average values \pm SEM obtained from 10 parallel 100-mL minimal medium liquid cultures ($n = 10$)

^f It should be noted that the LD₅₀ in liquid media was below 0.025 % since only 30 % (instead of 50 %) of spores germinated, suggesting that the fungus was still more sensitive to Roundup in liquid cultures than in solid ones

^g nd not determined

Significant differences versus control (0 % R450) are presented: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

on all these data, we then defined this R450 concentration (containing 31.5 mg/L GLY among adjuvants) as the maximal dose at which there is no observable adverse effect (NOAEL) on growth and morphology. Consequently, the lowest R450 dose resulting in observable adverse effects (LOAEL) on these macroscopic parameters was 0.0075 % (containing 33.75 mg/L GLY among adjuvants).

Functional and metabolic effects of R450

Effects on membrane trafficking

Hyphal polarity in *A. nidulans* has been shown to be functionally linked to membrane trafficking during endocytosis (Lee et al. 2008), as is the case for budding polarity in the yeast *Saccharomyces cerevisiae* (Costa and Ayscough 2005). To determine whether Roundup[®] also affects endocytosis in *A. nidulans*, we stained live cells of the CV125 strain grown in the absence or presence of R450 with FM[®]4-64 (Fischer-Parton et al. 2000) and tracked the uptake of the dye over time. The pesticide doses tested were LD₅₀ (0.025 %), the NOAEL for growth and morphology (0.007 %) as well as an intermediary concentration (0.015 %). Similar to previous observations (Penalva 2005), the dye appeared in several internal structures after 15 min, but only for the control and for the two lowest R450 concentrations (Fig. 4a, images 1–3). In presence of 0.025 % R450, the dye uptake was delayed. It remained mainly in the plasma membrane, and only a tiny fraction entered the cell, resulting into a diffuse labelling within the hyphae (Fig. 4a, image 4). After

60 min, in all four conditions, most of the dye was taken up by cells and localized to endomembranes (Fig. 4a, images 5–8).

Mitochondrial effects

Mitochondrial perturbation has been largely recognized as a contributing factor to various drug-induced toxicities (Pereira et al. 2009; Li et al. 2014). To visualize mitochondria, we used the MitoTracker[®] dye Red CMXRos. As expected, the fluorescence intensity was greater in the apical part of the hyphae than in the distal portion (as defined in Fig. 5a), but the overall intensity was greatly reduced at the LD₅₀ (0.025 %) (Fig. 4b, images 9 and 12). Surprisingly, such a fluorescence intensity drop also applied to the NOAEL (0.007 %) (although to a lesser extent), while the intermediate dose (0.015 %) led to a completely different pattern, with a similar intensity in both portions of the hyphae (Fig. 4b, images 10 and 11).

Since the dye CMXRos accumulates inside the mitochondria, variations in the fluorescence intensity of these organelles might reflect changes in their number or/and volume. Using Imaris software, we measured these two parameters, for each R450 concentration and control, in the two portions of the hyphae (Fig. 5a). The pattern for the total volume of mitochondria was quite superimposable on that for the average number (Fig. 5b, c), suggesting that the average size of mitochondria remained unchanged after exposure to the herbicide. In the distal region, R450 resulted in a dramatic reduction of both number and volume of mitochondria independently of the dose (and without any significant difference between the different doses). However, in the

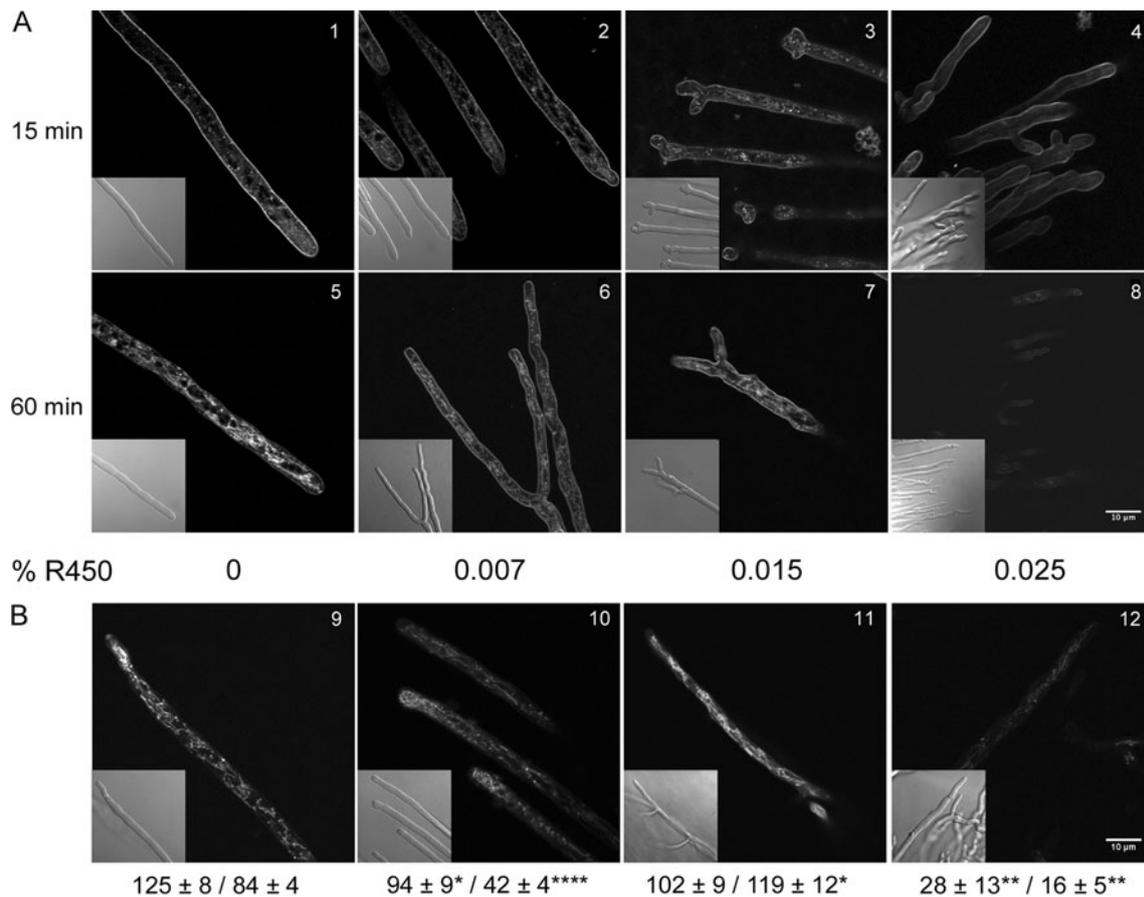


Fig. 4 Effects of R450 on endocytosis monitored with FM4-64 (**a**), and on mitochondrial pattern using the MitoTracker dye Red CMXRos (**b**). *A. nidulans* strain CV125 was grown for three days at 37 °C on rich media in absence or presence of the indicated R450 dose. Insets show the corresponding DIC images. **a** Hyphae were incubated with FM4-64 (4.1 μM in liquid medium) between 5 and 60 min before observations. **b** After 5 min incubation with CMXRos (500 nM in liquid medium), the excess of dye was removed by rinsing the mycelium (with liquid medium). Numbers under the images indicate CMXRos fluorescence

intensities (given as arbitrary units/μm² and obtained with IMARIS software) in the apical/distal parts of the hyphae as they are defined in Fig. 5a. Average values ± SEM were obtained from 10 hyphae on each one of three plates ($n = 30$). Significant differences versus control (0 % R450) are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. N.B. For 11 and 12, the DIC images shown in *inset* seem different from the confocal ones because some hyphae are not in the focal plan (mycelial disorganization characteristic of the R450 effect: see Fig. S1 in Online Resource 1)

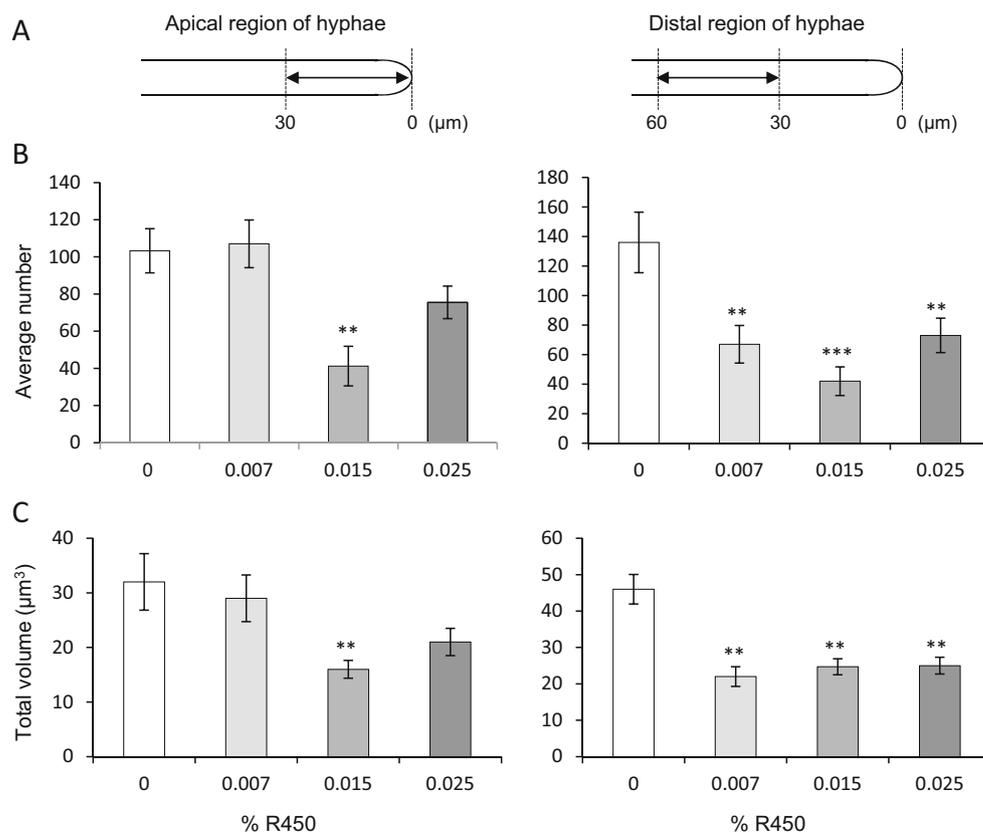
apical region, such a reduction was only observed at the intermediary concentration (0.015 %) (Fig. 5b, c).

CMXRos accumulation being dependent upon membrane potential, it stains active mitochondria. To better assess the functional state of mitochondria, we measured different enzyme activities of the Krebs tricarboxylic acid (TCA) cycle from crude extracts of strain CV125 mycelia harvested after liquid culture. Because of the difficulty of the fungus *A. nidulans* to grow in liquid medium at the LD₅₀ (see Online Resource 3), resulting in a low yield of mycelial biomass under these conditions, activities were measured only at the intermediate (0.015 %) and NOAEL (0.007 %) doses (Table 2). Citrate synthase (CS), malate dehydrogenase (MDH) and aconitase (ACO) activities are shared with the glyoxylate cycle which is located, at least partially, in the peroxisomes. To ascertain the nature of possibly disturbed metabolism (mitochondrial or/and peroxisomal), we also measured the activity for malate synthase

(MS), one of the two glyoxylate pathway key enzymes which have been shown to be peroxisomal (Maggio-Hall and Keller 2004), as well as for succinate dehydrogenase (SDH) which is exclusive to the mitochondrial matrix (Kregiel 2012). While peroxisomal MS activity remained unchanged in absence or presence of R450, the other four activities were increased by 30 to 100 % when R450 was added at 0.015 %, suggesting a stimulation of mitochondrial metabolism. Such a metabolic stimulation also occurred, although to a lesser extent, at the NOAEL dose associated to growth and morphology (Table 2).

In *A. nidulans*, the mutation *cbxC34* was identified as conferring resistance to carboxin, an inhibitor of SDH whose activity in such a mutant grown in the presence of this systemic fungicide is restored to about 50 % (Gunatilleke et al. 1976). In view of the above data obtained with strain CV125, it seemed relevant to analyse the metabolic effects of R450 in this other genetic context. Therefore, the different enzyme

Fig. 5 Effects of R450 on the average number (**b**) and total volume (**c**) of mitochondria in the apical (*left panels*) and distal (*right panels*) parts of the hyphae (**a**). Strain growth and Red CMXRos staining were as described in legend of Fig. 4. Number and volume of mitochondria were obtained using Imaris software and surface rendering method. SEMs are indicated in all instances (from 10 hyphae on each one of three plates; $n = 30$). Significance of the effects (** $p < 0.01$; *** $p < 0.001$) was tested against the control (0 % R450)



activities measured previously were also determined in a strain carrying the mutant allele *cbxC34* (NA1390), in absence and presence of 0.015 % R450 (Table 2). First, in the absence of the herbicide, SDH activity was 2.4 times higher in this mutant than it was in the wild-type strain, while each one of the other four activities was similar in both strains. This

confirmed the “gain-of-function” nature of the *cbxC34* mutation. Second, in the presence of 0.015 % R450, each enzyme activity (including SDH) was similar to that observed in the same condition in the other strain, confirming the stimulation effect of R450 on the mitochondrial metabolism without affecting the peroxisomal one. However, for the mutated strain,

Table 2 Effects of R450 on mitochondrial and peroxisomal enzyme activities of wild-type (+) and SDH mutant (*cbxC34*) *A. nidulans* strains

Strain	% R450	Enzyme specific activities ^a				
		CS	MDH	ACO	SDH	MS
CV125 ^b (+)	0	91 ± 3 (16)	2280 ± 75 (10)	30 ± 3 (3)	28 ± 3 (3)	28 ± 2 (10)
	0.007	141 ± 11 (4)****	3534 ± 103 (4)****	nd ^d	nd	nd
	0.015	178 ± 6 (16)****	4788 ± 63 (10)****	51 ± 3 (3)**	37 ± 2 (3)*	25 ± 3 (10)
NA1390 ^b (<i>cbxC34</i>)	0	84 ± 8 (3)	1984 ± 22 (3)	33 ± 3 (3)	67 ± 6 (3)** ^c	30 ± 3 (3)
	0.015	173 ± 11 (3)**	4266 ± 136 (3)****	53 ± 5 (3)*	42 ± 4 (3)*	33 ± 2 (3)

^a Specific activities are given as nmol of product formed (for CS and ACO) or substrate consumed (for MDH, SDH and MS) per min per mg of total proteins ± SEM, and were obtained from duplicates or triplicates of one to eight independent experiments ($3 \leq n \leq 16$, as indicated in *italics* between brackets). The significant differences presented are versus the same strain in absence of R450 (except for ^c): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

^b Crude extracts were prepared from mycelia of the *A. nidulans* strains grown during 15 h at 30 °C in liquid culture with fructose and urea as carbon and nitrogen sources, respectively, and in absence or presence of R450 at the indicated dose

^c This significance level is versus the wild-type control strain (CV125) grown under the same conditions (0 % R450)

^d nd not determined

the presence of R450 did not result in a further increase, but a reduction (about 40 %), in SDH activity, suggesting a higher sensitivity to the herbicide of the CbxC mutated protein (offset by its overproduction). Consistently, *cbxC34* mutants proved to display a greater ability to adapt to the presence of the herbicide, despite a higher initial sensitivity (Fig. S3 in Online Resource 1), as confirmed by the TI values and the much higher survival rate than the wild-type control in the presence of 0.025 % R450 on minimal medium (Table S2 in Online Resource 1).

Discussion

This study demonstrates that a Roundup® formulation (R450) is toxic to a soil filamentous fungus at low doses, far below the recommended agricultural application rates. Yet it is important to note that the amounts found in soils are much less than those that are sprayed, due to dispersion (Perruzo et al. 2008). However, these soil concentrations are difficult to assess because they depend on many factors: climate (especially rains occurring after application), soil quality and structure, time elapsed from application, and of course the number of applications (Aparicio et al. 2013; Lupi et al. 2015; Perruzo et al. 2008). Moreover, only GLY and its major breakdown product, AMPA, are investigated in GBH-treated soils, and not the other molecules (adjuvants)—which are found not to be inert—present in the formulations.

The comparison of R450 toxicity here studied with that of other substances in *A. nidulans* is confronted with two difficulties: first, the tested substance is most often the active ingredient and not the commercial formulation; second, the concentrations tested do not correspond to a reference dose (such as LD₅₀, which, most often, remains unknown in this organism). Therefore, the only way to establish a kind of toxicity scale (based on the TI values) is to take into account, for the molecule to compare, a concentration corresponding to that of GLY in the R450 dilution used. On this basis, R450 would be 25 to 60 times less toxic than fungicides such as benzimidazoles and thiophanates, respectively, and about 400 times less toxic than benomyl (Kappas et al. 1974). Since these molecules were selected or designed to kill specifically fungi, such toxicity ratios are not surprising. However, comparison with other herbicides shows that R450 would be 10 times less toxic than 2,4-(dichlorophenoxy) acetic acid, and about 10 times more toxic than atrazine and propazine (Kappas 1988).

Many studies have shown the toxic effects of Roundup® at sublethal concentrations in non-target organisms: amphibians (Hedberg and Wallin 2010; Paganelli et al. 2010; Relyea 2005), other aquatic organisms (Bringolf et al. 2007; Cuhra et al. 2013; Jiraungkoorskul et al. 2003; Langiano Vdo and Martinez 2008; Marc et al. 2004; Mottier et al. 2013), earthworm (Piola et al. 2013), human and rat cells or tissues

(Mesnage et al. 2015), and microorganisms (Clair et al. 2012; Lipok et al. 2010; Qiu et al. 2013; Tsui and Chu 2003). In plants, the mode of action of Roundup® is quite clear since GLY disrupts the shikimic acid pathway through inhibition of the 5-enolpyruvyl-shikimate-3-phosphatase synthase (EPSPS) enzyme, blocking the synthesis of essential aromatic amino acids and precursors of other critical aromatic compounds including plant growth regulators (Duke et al. 2003). However, in other organisms that may not have the shikimate pathway, mechanisms responsible for Roundup® toxicity appear multiple and may vary from one organism or cell type to another. For instance, Roundup® has been shown to induce cell cycle dysfunction using the sea urchin early development model (Marc et al. 2004), to impair retinoic acid signalling in *X. laevis* embryos (Paganelli et al. 2010), to inhibit intracellular transport through disassembly of the cytoskeleton in *Xenopus laevis* (Hedberg and Wallin 2010), to inhibit photosynthesis in the cyanobacterium *Microcystis aeruginosa* (Qiu et al. 2013), to induce necrosis and apoptosis and/or to promote endocrine disrupting effects by altering especially cytochrome P450 aromatase in various human cell lines as well as in mature rat testicular cells (Mesnage et al. 2015), and also to target mitochondria in rat brain and liver tissues (Astiz et al. 2009; Peixoto 2005).

Apart from plants, the shikimate pathway is also present in fungi and bacteria (Bentley 1990). In *A. nidulans*, EPSPS is part of a pentafunctional enzyme (encoded by the *aromA* locus) which catalyses the five central steps of the shikimate pathway (Charles et al. 1986), as is also the case for *Neurospora crassa* (Catcheside et al. 1985). However, the existence of the shikimate pathway in fungi does not necessarily mean that Roundup® operates as in plants. EPSPS from different organisms have been divided into two classes according to intrinsic GLY sensitivity. Class I (GLY-sensitive) includes EPSPS from all plants and from some bacteria such as *Escherichia coli* and *Salmonella typhimurium*, while EPSPS found in some other bacterial species including *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Agrobacterium* sp. strain CP4 belong to class II (GLY-tolerant) (Funke et al. 2009). EPSPS of the filamentous fungus *N. crassa* has been shown to be GLY-sensitive (Boocock and Coggins 1983), but it is not known to date whether that of *A. nidulans* belongs to class I or II. However, it has been shown that *aromA* mutants were auxotrophic for three aromatic amino acids: phenylalanine, tryptophan and tyrosine (Roberts 1969). An inhibition of EPSPS could therefore lead to the same auxotrophies. The fact that the survival rates in presence of Roundup®, for the strain CV125 (haploid) were the same on rich medium (which contains these amino acids) and minimal medium (which is devoid of them) suggests therefore that GLY does not inhibit (or only partially inhibits) the *A. nidulans* EPSPS enzyme, and that the multiple cellular effects of R450 reported in this study are likely due to various

targets of GLY and/or adjuvants present in the formulation. For instance, the disruption of hyphal polarity and endocytosis could be a side-effect of the surfactant polyethoxylated tallowamine (POEA), present in Roundup® formulations and necessary for an effective uptake of GLY in plants (Riechers et al. 1994). It is indeed known that ethoxylated adjuvants can insert into cell membranes, disrupting their structure and functions as previously shown in bacteria (Nobels et al. 2011). Furthermore, while technical GLY (CAS n° 1071-83-6) is the regulated molecule as the active ingredient in GBHs (Commission implementing regulation EU n° 540/2011 as regards the list of approved active substances: <http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32011R0540>), glyphosate is generally present under isopropylammonium (IPA) salt in the formulations, as it is specifically the case in R450. However, it has been reported that the IPA-GLY would be more toxic than GLY itself (Lee et al 2009). Therefore, IPA should be considered as a non-inert adjuvant and it cannot be excluded that it is partly responsible for the increased toxicity of the formulation.

Based on the Krebs TCA cycle enzyme activities, mitochondrial metabolism appeared to be stimulated despite the overall decrease in the number and volume of mitochondria. In other words, this metabolic stimulation was even more important when normalized against the total number or the size of mitochondria. Such an effect of R450 in *A. nidulans* might seem surprising since Roundup® was previously shown to damage basic mitochondrial functions in rat liver (Peixoto 2005) including the TCA cycle. Alteration of SDH activity, which is part of the TCA cycle and of the respiratory chain, was even used as a biomarker to evaluate Roundup® cytotoxicity in human cells (Mesnage et al. 2015). However, Peixoto (2005) has shown that simultaneously with the inhibition of SDH (and cytochrome c reductase), Roundup® promotes a collapse of the transmembrane electric potential ($\Delta\psi$). Therefore, it is likely that the stimulation of the mitochondrial enzyme activities in presence of R450 in *A. nidulans* results from a decrease in $\Delta\psi$, i.e. an uncoupling effect that generates an acceleration of respiration and Krebs TCA cycle. Moreover, since CMXRos accumulation is dependent upon membrane potential, such a $\Delta\psi$ collapse could also explain the decrease in mitochondrial fluorescence intensity in the presence of R450 (observed at 0.007 % and severely at 0.025 %). At 0.015 % R450, the supply of protons by the stimulated TCA cycle would compensate for the uncoupling effect, thereby restoring fluorescence intensity at least equivalent to that of the control. While this compensation would be only partial at 0.007 % (TCA cycle activities being stimulated by only a factor 1.5), the $\Delta\psi$ decrease would be too severe at 0.025 % R450. It has been indeed shown that Roundup® promotes the decrease in $\Delta\psi$ in a dose-dependent manner (Peixoto 2005). Then, the stimulation of the bioenergetic metabolism would be a cellular response in *A. nidulans* to offset

the effects of Roundup®. This interpretation is supported by the greater tolerance to R450 of the strain harbouring the gain-of-function *cbxC34* mutation conferring a two to three times higher intrinsic SDH activity.

Another key point of our study is the fact that these mitochondrial disruptions were also evident at the NOAEL dose for growth and morphology. This implies that metabolic effects due to pesticide residues may occur at exposure doses that do not result in any macroscopic expression, regardless of the exposed organism. Such metabolic disturbances could affect compositional quality and might therefore question the safety of food derived from herbicide-tolerant plants (Zobiolo et al. 2010). Thus, our data are likely to challenge the concept of “substantial equivalence” (i.e. close nutritional and compositional similarity between two crop-derived foods) that was used to claim that GM crops are as safe and nutritious as currently consumed plant-derived foods (Aumaitre 2002). Since this concept, that underpins the assessment of all agricultural GM plants in the world, applies at the chain end (i.e. to the food from these plants), it seems logical that it should also take into account the agricultural practices associated to the cultivation of the GM plant if they are different from those used for conventional crops. Precisely, in the case of herbicide-tolerant GM crops, these practices are not the same as for their conventional counterparts since the former are sprayed with the herbicide. Surprisingly, during substantial equivalence studies, either the tested plants are not sprayed with the recommended herbicide (Millstone et al. 1999), or spraying is carried out but the herbicide residues are not measured (Cuhra 2015). However, these residues may add toxic properties to the final plant product either through their inherent toxicity or indirectly, by affecting the plant metabolism. Roundup®-tolerant GM soybeans have been shown to accumulate high residues of GLY and AMPA (Böhn et al. 2014), and our data show that Roundup® exposure at a very low dose may cause cellular and metabolic effects without affecting the macroscopic parameters.

Conclusions

The present study demonstrated that a Roundup® formulation (R450) is toxic to the soil fungus *A. nidulans* at doses far below recommended agricultural application rate. Thus, this herbicide might potentially impair agricultural soil ecosystems. The effects included alteration of growth, defects in cellular polarity, delay in endocytosis and mitochondrial disruptions. The LD₅₀ corresponded to a percentage dilution 100 times lower than the agricultural one. As previously shown for different organisms and cell lines, the Roundup® formulation proved to be much more toxic than GLY alone, indicating once again that the adjuvants are not inert. Contrary to what was previously observed for human cells or tissues, R450

caused a stimulation of mitochondrial enzyme activities, revealing that the mode of action of Roundup® on energetic metabolism can vary from one organism to another, and that the use of SDH activity collapse as a biomarker of cytotoxicity cannot be transposed to all of them. These mitochondrial effects were also evident at an even lower concentration, corresponding to the NOAEL dose for growth and morphology. This suggests that metabolic disturbance (with possible nutritional impact in the case of crops) may occur at doses which do not cause any visible toxic effect (such as agricultural doses for herbicide-tolerant plants). This finding suggests that it would be relevant to consider the putative effects of herbicide residues in the determination process of substantial equivalence for herbicide-tolerant GMOs since interactions of chemical compounds with mitochondrial functions can result in severe impairment of the general metabolism.

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