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4 **Toxicity evaluation of new agricultural fungicides in primary cultured cortical**
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6 **neurons**
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4 **Abstract**
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6 Fungicides are crucial for food protection as well as for the production of crops of suitable
7 quality and quantity to provide a viable economic return. Like other pesticides, fungicides are
8 widely sprayed on agricultural land, especially in wine-growing areas, from where they can
9 move-off after application. Furthermore, residues of these agrochemicals can remain on crops
10 after harvest and even after some food processing operations, being a major exposure pathway.
11 Although a relatively low toxicity has been claimed for this kind of compounds, information
12 about their neurotoxicity is still scarce.
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15 In the present study, nine fungicides recently approved for agricultural uses in the EU
16 —ametoctradin, boscalid, cyazofamid, dimethomorph, fenhexamid, kresoxim-methyl,
17 mepanipyrim, metrafenone and pyraclostrobin— have been evaluated for their toxicity in
18 primary cultured mouse cortical neurons. Exposure to 0.1–100 μ M for 7 days *in vitro* resulted
19 in a dose-dependent toxicity in the MTT cell viability assay. Strobilurin fungicides kresoxim-
20 methyl (KR) and pyraclostrobin (PY) were the most neurotoxic compounds (lethal
21 concentration 50 were in the low micromolar and nanomolar levels, respectively) causing a
22 rapid raise in intracellular calcium $[Ca^{2+}]_i$ and strong depolarization of mitochondrial
23 membrane potential. KR- and PY-induced cell death was reversed by the calcium channels
24 blockers MK-801 and verapamil, suggesting that calcium entry through NMDA receptors and
25 voltage-operated calcium channels are involved in KR- and PY-induced neurotoxicity. These
26 results highlight the need for further evaluation of their neurotoxic effects *in vivo*.
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51 **Keywords:** cortical neurons; fungicides; neurotoxicity; calcium channels; mitochondrial
52 membrane potential; strobilurin
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4 **1. Introduction**
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6 In recent years, the agricultural industry has experienced strong market and technological
7 competition among the producing countries, resulting in increased production yields while
8 reducing costs for both producers and consumers. In this regard, pesticides are of paramount
9 importance since they allow controlling insect or fungal infestations or growth of weeds, either
10 to handle immediate infestations or to anticipate long-lasting problems (Regueiro et al., 2015).
11 Among them, fungicides are used to manage fungal disease organisms on growing crops, or
12 used as a post-harvest treatment to prevent fungi or molds causing food to rot during storage or
13 transport. Therefore, fungicides have become essential for food protection as well as for
14 maintaining healthy crops, and consistent yields of high quality produce. In fact, fungicides
15 accounted for over 52% of all plant protection products used in the European Union (EU) in
16 2003, with an estimated consumption above 100 kilotons (Eurostat, 2007). Like other
17 pesticides, fungicides are widely sprayed on agricultural land and therefore they can move-off
18 of fields after application, becoming a potential health risk not only for farm workers, but also
19 for residents and children by exposure through the air, soil and water environment (Kamel and
20 Hoppin, 2004). Furthermore, residues of these agrochemicals can remain on crops after harvest
21 and even after some food processing operations (Kaushik et al., 2009).
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42 Because of the growing health concerns about the use of ethylene-bis-dithiocarbamate (EBDC)
43 fungicides, especially maneb and mancozeb (Bjørning-Poulsen et al., 2008; Domico et al.,
44 2006), a number of fungicides showing new modes of action have been released in the recent
45 years. These include compounds which affect cell wall biosynthesis in fungal cells
46 (dimethomorph), sterol biosynthesis in membranes (fenhexamid), amino acid and protein
47 synthesis (mepanipyrim), fungal respiration (ametoctradin, boscalid, cyazofamid, kresoxim-
48 methyl and pyraclostrobin) or even with unknown mode of action (metrafenone) (FRAC,
49 2014). Some of them have been very recently included in EU list of authorized plant protection
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3 products (Table 1) according to Commission Regulation (EC) No 1107/2009 concerning the
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5 “placing of plant protection products on the market” (European Commission, 2009). The main
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7 properties of these new fungicides are their broad spectrum of action, their special penetration
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9 and redistribution properties and their long-term stability, which results in better long-term
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11 efficacy, allowing the use of lower concentrations of active substances (Wightwick et al.,
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13 2010). These fungicides are used to treat and prevent a broad range of fungal diseases such as
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15 late blight and downy mildew on potatoes, eyespot and powdery mildew on cereals, grey
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17 mould on strawberries, grapes, tomatoes and cucumbers, or scab on apples and pears (PPDB,
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19 2014). They have also been proposed as effective agents against the fungal production of
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21 aflatoxins (Sakuda et al., 2014).

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26 Recent studies suggest that exposure to certain agrochemicals, including fungicides such
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28 maneb and mancozeb, may be involved in the development of neurodegenerative disorders
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30 such as Parkinson’s disease (Bjørning-Poulsen et al., 2008; Costello et al., 2009). Although a
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32 relatively low toxicity is claimed for the newly formulated fungicides, which acceptable daily
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34 intakes (ADI) have been established from 0.02 to 10 mg/kg body weight (Table 1), little is
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36 known regarding their contribution to neuronal toxicity. Thus, the aim of the present work was
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38 to investigate the toxic effects of nine recently approved fungicides with different modes of
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40 action on primary cultured cortical neurons.
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45 46 47 **2. Materials and methods**

48 49 **2.1 Materials**

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51 Pregnant NMRI mice (16th gestational day) were obtained from Charles River/Iffa Credo
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53 (Saint Germain-sur-l’Arbreste, France). Plastic multi-well culture plates were purchased from
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55 Nunc (Rockilde, Denmark). Fetal bovine serum (FBS) was from Gibco (Invitrogen, Barcelona,
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57 Spain). Dulbecco’s modified Eagle’s minimum essential medium (DMEM) was obtained from
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3 Biochrom (Berlin, Germany). Isoflurane (FORANE) was from Abbot Laboratories (Madrid,
4 Spain). Fluo-3 acetoxymethyl ester (Fluo-3/AM), rhodamine-123 (Rho-123) and pluronic F-
5 127 were from Invitrogen (Carlsbad, CA, USA). Ametoctradin (AM), boscalid (BO),
6 cyazofamid (CY), dimethomorph (DI), fenhexamid (FE), kresoxim-methyl (KR), mepanipyrim
7 (MY), metrafenone (ME) and pyraclostrobin (PY) were Pestanal Grade standards, of certified
8 purity from 97.9 to 99.9%, from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO),
9 glacial acetic acid, formic acid ($\geq 98\%$), ethanol, methanol, acetonitrile, poly-D-lysine, trypsin,
10 soybean trypsin inhibitor, bovine serum albumin (BSA), DNase, HEPES, (+)-MK-801
11 hydrogen maleate (MK-801), (\pm)-verapamil hydrochloride, antimicyne A, 3-(4,5-
12 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 2',7'-dichlorofluorescein
13 diacetate (DCFH-DA), Triton-X 100 and sodium dodecyl sulfate (SDS) were obtained also
14 from Sigma. D-(+)-glucose monohydrate was purchased from Merck (Darmstadt, Germany).
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33 ***2.2 Instrumentation***

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35 All absorbance measurements were performed on a microplate spectrophotometer Multiskan
36 Spectrum from Thermo Fischer Scientific (San Jose, CA, USA). A microplate
37 spectrofluorometer Spectramax Gemini XS from Molecular Devices (Wokingham, UK) was
38 used for all fluorescence readings.
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47 ***2.3 Neuronal Cultures***

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49 Primary cultures of cortical neurons were prepared from cerebral cortices of 16th gestational
50 day mice fetuses as previously described (Briz et al., 2010; Solà et al., 2011). Pregnant animals
51 were anesthetized with isoflurane, killed by cervical dislocation, and the fetuses were
52 extracted. Cortices were dissected with forceps and mechanically minced, and cells were then
53 dissociated by mild trypsinization 0.02% (w/v) at 37 °C for 10 min followed by trituration in a
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3 DNase solution 0.004% (w/v) containing soybean trypsin inhibitor 0.05% (w/v). The cells
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5 were then suspended in DMEM containing 31 mM glucose, and 0.2 mM glutamine
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7 supplemented with p-aminobenzoate, insulin, penicillin, and 10% fetal bovine serum. The cell
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9 suspension (1.5×10^6 cells per milliliter) was seeded in 6- or 96-well plates precoated with
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11 poly-D-lysine and incubated in a humidified 5% CO₂/95% air atmosphere at 37 °C. A mixture
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13 of 5 μM 5-fluoro-2'-deoxyuridine and 20 μM uridine was added after 1 day *in vitro* (DIV) to
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15 prevent glial proliferation. By using this protocol 94% of the cells are neurons (as identified by
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17 immunostaining with tau antibody) and 2 % of the cells are astrocytes (as identified by
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19 immunostaining with GFAP antibody). Animals were handled in compliance with protocols
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21 approved by the Generalitat de Catalunya Spain, following the EU guidelines.
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29 **2.4 Chemical treatments**

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31 Stock solutions of each fungicide were prepared in DMSO and then stored at -20 °C. Different
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33 working standard solutions were made by appropriate dilution in DMSO and then further
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35 diluted in the culture medium so that the final organic solvent concentration never exceeded
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37 0.5% (v/v), which was not cytotoxic.
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40 For prolonged exposure periods, neurons were treated at 1 DIV and exposed during 7 days. For
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42 short-term exposure experiments, mature cultured neurons were exposed to the fungicides at 7
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44 DIV for periods of time ≤ 24 h. The medium was not changed until the completion of the
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46 experiments.
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51 **2.5 Cell viability**

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53 Following each exposure period, cell viability was first assessed by visual inspection under
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55 phase-contrast microscopy and then by the MTT assay, which is based on the reduction by
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57 viable cells of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a
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3 formazan product via a group of nonspecific mitochondrial dehydrogenases. Briefly, a MTT
4 solution in phosphate buffered saline (PBS) was added to each well to a final concentration of
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6 5 mg/mL and cells were incubated for 3 h at 37 °C. MTT solution was removed and 100
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8 μ L/well of SDS 10% (w/v) in water were added and allowed to dissolve the intracellular
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10 formazan crystals overnight at 37°C in darkness. Absorbance was measured at 570 nm with
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12 background subtraction. Cell viability was expressed as a percentage of the controls (cells
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14 exposed to the DMSO).
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19 Additionally, cell viability was evaluated by the propidium iodide (PI) assay as described by
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21 Rosa *et al.* (Rosa et al., 1997). This fluorescent dye rapidly enters cells with damaged
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23 membranes and binds to nucleic acids, rendering them brightly fluorescent, whereas it is
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25 excluded by living cells (Macklis and Madison, 1990). A PI solution in PBS was added to each
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27 well to a final concentration of 10 μ g/mL and cells were incubated for 1 h at 37 °C.
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31 32 33 **2.6 Mitochondrial membrane potential ($\Delta\Psi_m$)**

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35 Changes in mitochondrial membrane potential were monitored using rhodamine-123 (Rho-
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37 123), a cell permeable cationic dye, which is rapidly sequestered by active mitochondria.
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39 Depolarization of the membrane results in the release of Rho-123 from the mitochondria and
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41 the subsequent increase of fluorescence due to the dequenching of the dye. Cells grown for 7
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43 DIV in 96-well plates were washed twice with Hank's buffered salt solution (HBSS) (1.3 mM
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45 CaCl₂, 5.4 mM KCl, 0.4 mM KH₂PO₄, 0.5 mM MgCl₂, 0.4 mM MgSO₄, 137 mM NaCl, 4.2 mM
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47 NaHCO₃, 0.3 mM Na₂HPO₄, 8 mM HEPES, and 5.5 mM glucose, adjusted to pH 7.4) and
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49 incubated with 10 μ M Rho-123 for 15 min at 37 °C. Cells were washed again to discard the
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51 non-retained Rho-123 and the chemical exposure was performed by adding the fungicide
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53 immediately. After 1 h exposure, fluorescence was determined at 485 nm excitation and 530
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55 nm emission wavelengths. Responses were normalized to maximal fluorescence, obtained after
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3 treatment with a revealing solution of acetic acid/ethanol (1:2, v/v) or after treatment of sister
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5 cells with 15 μ M antimycin (ANT).
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10 ***2.7 Determination of intracellular calcium***

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12 Intracellular calcium levels were measured by using the fluorescent calcium indicator fluo-3
13 acetoxymethyl ester (Fluo-3/AM). This cell-permeable dye is hydrolyzed by intracellular
14 esterases and trapped in cells as Fluo-3, which increases its fluorescence upon binding of Ca^{2+} .
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16 Cells grown for 7 DIV in 96-well plates were washed twice with HBSS and incubated for 1 h
17 at 37 °C with 9 μ M Fluo-3/AM in HBSS containing 0.5% DMSO and 0.08% of the nonionic
18 surfactant pluronic F-127. Excess Fluo-3/AM was rinsed away, and the cells were treated with
19 the studied fungicides in HBSS. Fluorescence was immediately determined at 485 nm
20 excitation and 530 nm emission wavelengths. Results were expressed as the intracellular
21 calcium percentage of the untreated cells (DMSO).
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35 ***2.8 Cellular uptake of fungicides***

36 *2.8.1 Extraction and cleanup*

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38 Cells were grown in 6-well plates and after 7 DIV they were exposed to the selected fungicides
39 (100 μ M AM, 100 μ M CY, 10 μ M KR and 1 μ M PY) for 8 h. At the end of the experiment,
40 culture medium was removed and cells were rinsed three times with HBSS. Cells were then
41 scrapped into 2 mL ultrapure water and disrupted by sonication (5 x 10 s pulses, 30% power)
42 on ice with an ultrasonic homogenizer. Protein content of the cell lysates was determined by
43 the Bradford assay (Bio-Rad Laboratories, München, Germany) using bovine serum albumin
44 (BSA) as protein standard.
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56 Fungicides were extracted from the homogenates by solid-phase extraction (SPE) using
57 polymeric SPE cartridges Strata-X (200 mg, 6 mL) from Phenomenex (Torrance, California,
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3 USA). Briefly, 1.8 mL sample were loaded into the SPE cartridge previously conditioned with
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5 5 mL 2% (v/v) formic acid in methanol followed by 5 mL water. After washing with 2 mL
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7 water, fungicides were eluted with 10 mL 2% (v/v) formic acid in methanol. The eluate was
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9 brought to dryness under a gentle stream of nitrogen, reconstituted in 300 µL of
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11 methanol/water/formic acid (30:69.8:0.2, v/v/v), filtered through a 0.20 µm PTFE syringe filter
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13 and analyzed by HPLC–DAD/ESI-MS/MS.
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17 Recoveries for AM, CY, KR and PY were evaluated at 0.5 and 50 ng/mL. Values ranged from
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19 88% to 99% at both spiked levels. Limits of quantification (LOQ), defined for a signal-to-noise
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21 ratio of 10 (S/N=10), below 0.3 ng/mL were obtained for these four compounds.
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24 The cellular uptake of fungicides was normalized to milligrams of cell protein. The percentage
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26 of cellular uptake was calculated with respect to the amount of fungicides initially present in
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28 the culture medium.
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31 32 33 *2.8.2 HPLC–DAD/ESI-MS/MS analyses*

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35 Analyses were carried out in a Thermo HPLC system (Thermo Fisher Scientific, San Jose, CA,
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37 USA) consisting of a TSP AS3000 autosampler with column oven, a Surveyor LC quaternary
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39 pump, a Surveyor PDA diode array detector (DAD) coupled to a triple stage quadrupole mass
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41 spectrometer TSQ Quantum Access equipped with a electrospray ionization (ESI) source.
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43 Instrument control and data acquisition were performed with Xcalibur 2.0 software also from
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45 Thermo.
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49 Chromatographic separation of the fungicides was performed on a reversed-phase column
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51 Luna C18(2) (150 x 3.0 mm, 3 µm) from Phenomenex, maintained at 30 °C. Mobile phases A
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53 and B were respectively, water/formic acid (99.8:0.2, v/v) and acetonitrile/water/formic acid
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55 (95:4.8:0.2, v/v/v). The following linear gradient was used: hold at 65%A for 1 min, decreased
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57 to 20%A over 6 min, decreased to 5%A over 13 min and hold for 2 min, then returned to initial
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3 conditions over 2 min and re-equilibrated for 7 min. The flow rate and the injection volume
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5 were set at 0.4 mL/min and 20 μ L, respectively.
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8 UV/Vis spectra were recorded from 200 to 600 nm with the DAD, whereas the mass
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10 spectrometer was operated in the positive ESI mode at a spray voltage of 4500 V. Nitrogen
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12 (>99.98%) was employed as sheath gas and auxiliary gas at pressures of 40 and 10 arbitrary
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14 units, respectively. The capillary temperature was set to 270 $^{\circ}$ C. The detection was performed
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16 in the multiple reaction monitoring (MRM) mode using argon (>99.999%) as collision-
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18 induced-dissociation (CID) gas at 1.5 mTorr. Optimized MS/MS ion transitions for each
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20 compound are detailed in Table 2. The linearity of the method was tested using standard
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22 solutions at six concentration levels from 5 ng/mL to 1000 ng/mL for all compounds.
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24 Calibration curves were found to be linear in the studied ranges with determination coefficients
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26 (R^2) \geq 0.9941 and RSD (n=3) \leq 7%.
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31 32 ***2.9 Data Analysis***

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34 Data are expressed as mean \pm standard error of the mean (SEM) of at least three experiments
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36 from independent culture batches, each performed in triplicate; the number of independent
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38 cultures (n) for each experiment is indicated in the corresponding figure caption. Statistical
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40 significance was determined by one-way ANOVA followed by Dunnett's multiple comparison
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42 tests when comparing more than two groups, and by two-way ANOVA followed by
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44 Bonferroni's test when comparing two factors. Dose-response curves were fitted to sigmoidal
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46 curves. All statistical calculations were made using the software package GraphPad Prism
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48 version 4.0 (GraphPad Software, San Diego, CA).
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54 55 **3. Results**

56 57 ***3.1 Effects of short- and long-term exposure on cell viability***

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4 Exposure of cultured cortical neurons to 100 μM of the fungicides cyazofamid (CY),
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6 kresoxim-methyl (KR) and pyraclostrobin (PY) for 24 h resulted in a significant decrease in
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8 cell viability $>20\%$ (the considered Low Effect Level –LOEL) (Fig. 1A, white bars). In
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10 agreement with the general concept that toxicity is dependent on both drug concentration and
11
12 exposure time, the toxicity of the fungicides increased after long-term exposure and cell
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14 viability was significantly reduced ($p < 0.001$) by all the evaluated fungicides (CY, KR, PY and
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16 ametoctradin –AM-, boscalid –BO-, dimethomorph –DI-, fenhexamid –FE-, mepanipyrim –
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18 MY- and metrafenone -ME) (Fig. 1A, black bars). Concentration-dependent behavior was
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20 observed for all fungicides, being PY and KR the most neurotoxic compounds, exhibiting
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22 LC50 values in the nanomolar and low micromolar range, respectively, when the exposure was
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24 continued for several days. Furthermore, all the compounds except KR showed higher toxicity
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26 at the longer exposure period. The results were confirmed by phase-contrast microscopic
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28 visualization and propidium iodide uptake (data not shown). Fig. 1B shows the concentration
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30 response curves for the effects of fungicides on cell viability after short exposure (24 hours *in*
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32 *vitro*) and continuous exposure (7 days *in vitro*). Table 3 shows the concentration that produces
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34 50% of cell cytotoxicity (LC50). CY was tested up to 30 μM in the continuous exposure
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36 because of the formation of crystals after two days of exposure at higher concentrations. For
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38 comparison, we also determined the toxicity of the widely used fungicide maneb in cultured
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40 cortical neurons after continuous exposure for 7 days *in vitro*. LC50 value was $26 \pm 2 \mu\text{M}$
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42 (n=3). Furthermore, for the most toxic compound PY no differences were found when mature
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44 cells were continuously exposed from DIV 7 to DIV 13 (LC50: $0.05 \pm 0.01 \mu\text{M}$, n=6) with
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46 respect to neurons continuously exposed during the first week in culture (LC50: 0.06 ± 0.01
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48 μM , n=5).

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50 Furthermore, in order to determine if there was any relationship between intracellular
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52 concentration and the observed cell death, cellular content was quantified for AM, CY, KR and
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PY (Table 4). AM was evaluated as it did not induce major toxicity in neurons whereas the other three compounds exhibited the lowest LC50 values in both short- and long-term exposures. To avoid significant metabolism of the fungicides, the cells were exposed for a short period (8 hours). CY intracellular content and its percentage of cellular uptake were significantly higher than those of AM ($p < 0.01$). On the other hand, assuming that accumulation of PY and KR was lineal in the low micromolar range (1–10 μM), the intracellular content and percentage of cellular uptake of PY were significantly higher than that of KR ($p < 0.01$). These findings were in relation with the highest toxicity of CY compared to AM and of PY compared to KR.

3.2 Pyraclostrobin (PY) and kresoxim-methyl (KR) rise intracellular calcium $[\text{Ca}^{2+}]_i$ and induce mitochondrial membrane potential ($\Delta\Psi_m$) depolarization

It is well known that when Ca^{2+} homeostasis is disrupted, this ion can behave as a powerful activator of multiple damaging processes. We determined whether fungicides increased cytosolic calcium. Fig. 2A shows that PY and KR increased $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner. Results showed a significant increase ($p < 0.01$) of $[\text{Ca}^{2+}]_i$ when cells were exposed to $\geq 1 \mu\text{M}$ PY ($26.7 \pm 2.8\%$) and to $\geq 10 \mu\text{M}$ KR ($31.7 \pm 4.4\%$) in comparison to control conditions (Fig. 2A). AM, DI, FE, ME and MY (up to $100 \mu\text{M}$) and CY (up to $30 \mu\text{M}$) did not induce by themselves an increase of intracellular calcium (data not shown). The increase of intracellular calcium induced by PY and KR was significantly reduced in a nominal calcium-free medium (Fig. 2B).

The inhibition of mitochondrial complex III is one of the proposed mechanisms of action for several fungicides, among them AM, CY, KR and PY (see Table 1). Furthermore, a rise in intracellular calcium may lead to the loss of mitochondrial potential (Duchen, 2000). Five out of the nine evaluated fungicides —CY, FE, KR, ME and PY— were able to significantly

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4 induce ($p < 0.01$) mitochondrial membrane depolarization when tested up to 100 μM in cultured
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6 cortical neurons (Fig. 3A). PY, KR and CY were the compounds that produced highest
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8 mitochondrial membrane depolarization with EC50 values of $5 \pm 1 \mu\text{M}$, $29 \pm 9 \mu\text{M}$ and 20 ± 5
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10 μM ($n=3$), respectively (Fig. 3B).

11 12 13 14 15 **3.3 Neuroprotection with NMDA receptor antagonist and L-type Ca^{2+} channel blocker**

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17 Both elevated intracellular calcium and reduced mitochondrial membrane potential represent
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19 major pathophysiologic conditions affecting neuronal viability. Furthermore, the depolarization
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21 of mitochondrial membrane may be the result of increasing accumulation of calcium within
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23 mitochondria and, viceversa, an increase of cytosolic calcium may be the consequence of
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25 disrupted integrity of the mitochondria. Therefore, we evaluated whether blocking two main
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27 pathways leading to increased intracellular calcium (the N-methyl-D-aspartate receptor -
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29 NMDAR- and the voltage-operated Ca^{2+} channels -VOCC) would have a protective effect
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31 against fungicides-induced toxicity. Results of cell viability in the presence of 1 μM PY or 10
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33 μM KR together with calcium channel blockers (10 μM of MK-801 for the NMDAR and 50
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35 μM of verapamil for the VOCC) are depicted in Fig. 4. MK-801 significantly protected cells
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37 against fungicide effects ($p < 0.01$) and the neuroprotective effect was still increased by a
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39 combination of MK-801 and verapamil ($p < 0.001$).
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47 **4. Discussion**

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49 All the tested fungicides produced a reduction of cell viability. Whereas four of these
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51 compounds (BO, CY, KR and PY) displayed a toxic effect after a short time of exposure (24
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53 hours), all nine fungicides resulted toxic after 7 days of exposure. Those acting by inhibiting
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55 the mitochondrial respiration complex III (CY, KR and PY but not AM, see Table 1) displayed
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57 the higher levels of toxicity. This complex is one of the four multiprotein complexes within the
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3 inner mitochondrial membrane involved in the passage of electrons in the respiratory chain.
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6 The well-known electron transporter ubiquinone or coenzyme-Q (CoQ) binds to complex III at
7
8 the Q_o (quinol-oxidizing) site when it is reduced and at the Q_i (quinol-reducing) site when it is
9
10 oxidized allowing the electron movement. CY seems to bind at the quinol-reducing site Q_i
11
12 whereas KR and PY seem to bind at the quinol-oxidizing site Q_o (FRAC, 2014; Li et al., 2014).
13
14 The inhibition of any of these sites would inhibit the natural electron transport and finally
15
16 induce decreased ATP generation and mitochondrial membrane depolarization eventually
17
18 leading to increased cytosolic calcium and to cell death (Boutilier, 2001; Chinopoulos and
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20 Adam-Vizi, 2010; Pham et al., 2000; Zhang et al., 1998). It is worth to note that the toxicity of
21
22 PY and KR found in this work was higher than that induced by the widely used fungicide
23
24 maneb in cortical neurons.
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29 In this work we have determined the effect of new fungicides with different mode of action on
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31 cultured cortical neurons. In agreement with their mode of action (respiration inhibition of
32
33 mitochondrial complex III), CY, KR and PY were able to significantly induce mitochondrial
34
35 membrane depolarization in mature neurons. This may be related to the alteration of electron
36
37 transport and alteration of the proton gradient across the mitochondrial inner membrane,
38
39 thereby breaking down the mitochondrial membrane potential (Pham et al., 2000). The affinity
40
41 of these compounds for the porcine mitochondrial complex III (PY > KR > CY) (Li et al.,
42
43 2014; Zhao et al., 2009) has a pattern similar to that shown in this work for the mitochondrial
44
45 membrane depolarization, the increase of cytosolic calcium and the loss of cell viability in
46
47 cultured cortical neurons. These three compounds induced the higher cell death as well as the
48
49 higher level of membrane depolarization and increased cytosolic calcium in comparison to the
50
51 control condition. The fact that the increase in intracellular calcium induced by PY and KR was
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53 abolished in the absence of external calcium suggests an influx of calcium ions through
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55 membrane calcium channels (either voltage- or NMDA receptor-operated). This increase of
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4 cytosolic calcium can lead to a neurotoxic cascade eventually inducing cell death. In this sense,
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6 the blockage of membrane calcium channels by MK-801 (antagonist of the glutamate-NMDA
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8 receptor) and by verapamil (antagonist of voltage-operated Ca^{2+} channels) resulted in
9
10 neuroprotection. When both NMDA and L-type channels were unable to conduct calcium
11
12 ions, there was a neuroprotective effect since cell death was further diminished. In this case, a
13
14 reduced entrance of Ca^{2+} ions may be delaying calcium overload and cell death (Dong et al.,
15
16 2006). Nonetheless, the imbalance of $[\text{Ca}^{2+}]_i$ together with the mitochondrial membrane
17
18 depolarization may be responsible for cell death.
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21
22 On the other hand, FE and ME also induced mitochondrial membrane depolarization. FE
23
24 inhibits sterol biosynthesis and specifically targets 3-keto reductase (Debieu et al., 2001).
25
26 There is no known mode of action for ME, however it likely disturbs the organization of the
27
28 actin cytoskeleton (Opalski et al., 2006). Both inhibition of 3-keto reductase and disruption of
29
30 the actin dynamics may indirectly affect mitochondrial membrane fluidity and function
31
32 (Breitenbach et al., 2005; Vrablik and Watts, 2012). Both CY, FE and ME displayed similar
33
34 LC50 values ($\sim 25 \mu\text{M}$) against cortical neurons viability when they were exposed to the
35
36 fungicides for 7 days *in vitro*, despite the fact that CY was much more potent than FE and ME
37
38 in inducing mitochondrial membrane depolarization (Fig. 3A). This suggests that in addition to
39
40 mitochondrial failure, alteration of lipid biosynthesis and of actin dynamics may play a role on
41
42 the cytotoxic effects of FE and ME, respectively. On the other hand, MY scarcely induced
43
44 cytotoxic effects in cortical neurons (LC50 values around $100 \mu\text{M}$ after seven days of exposure
45
46 *in vitro*). Unlike FE and ME, the acute exposure to MY did not alter mitochondrial membrane
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48 potential in cultured cortical neurons. In relation to this, a similar exposure to MY on cultured
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50 hepatocytes did not reduce the cellular ATP content (Terada et al., 1999).
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57 Finally, neither AM nor BO reduced cell viability by more than 50% even after prolonged
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59 exposure for 7 days *in vitro* to $100 \mu\text{M}$ of each compound. Despite the proposed mechanism of
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4 action for AM is through the inhibition of the mitochondrial complex III, it exerts a unique
5
6 mode of action by binding to the very specific stigmatellin sub-site at the Q_o, being currently
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8 the only fungicide belonging to this category (FRAC, 2014). Thus, the difference in toxicity
9
10 observed between the two representatives of the strobilurin family, KR and PY, and the AM
11
12 could be explained based on their differential binding. Although all three compounds bind to
13
14 the Q_o site of the mitochondrial complex III, strobilurin fungicides bind the heme *b_l* proximal
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16 domain, whereas AM has been reported to act by binding in the same domain as stigmatellin,
17
18 i.e. in the distal pocket close to the Rieske iron-sulfur protein (Crofts et al., 1999; Fisher and
19
20 Meunier, 2008).
21
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24 In the case of BO, this compound inhibits mitochondrial complex II which will not have a
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26 direct effect on the electron flux to the mitochondrial respiratory chain. Instead, the inhibition
27
28 of complex II would disturb the tricarboxylic acid cycle (Nicholls, 2002). Therefore we would
29
30 not expect that BO depolarized the mitochondrial membrane potential like complex III
31
32 inhibitors PY, KR and CY did.
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36 In summary, we have reported that the fungicides PY, KR and CY are incorporated into
37
38 neurons in primary cultures of cortical neurons. In doing so, they target the mitochondria as
39
40 they do as pest controllers, disrupt intracellular calcium homeostasis and induce cell death. It is
41
42 noteworthy that the neuronal toxicity was induced at concentrations lower than those inducing
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44 toxicity in human lymphocytes (Çayır et al., 2014). The higher energy demand of neurons in
45
46 relation to other cells in mammals may make neurons to be critically susceptible to lipophilic
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48 compounds that interfere with the mitochondrial respiratory chain. Among them, PY showed
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50 the highest neurotoxicity with a LC₅₀ in the nanomolar level, above 400-fold higher than the
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52 toxicity observed under the same conditions for maneb, a fungicide which has been recently
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54 related with an increased risk of Parkinson's disease. Thus, these results highlight the need for
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56 further evaluation of its neurotoxic effects in vivo.
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4 Regarding AM, which has been very recently approved in the EU for its use in crop protection
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6 products, it was observed a much lower toxicity (LC50 >100 µM) which is in agreement with
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8 the higher acceptable daily intake established for this fungicide (Table 1).
9

10 11 12 **Acknowledgements**

13
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26 Legends

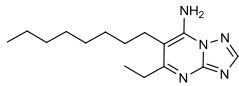
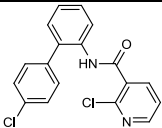
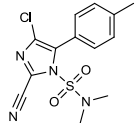
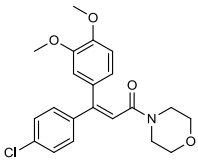
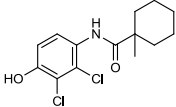
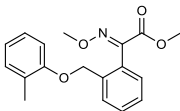
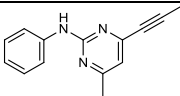
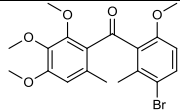
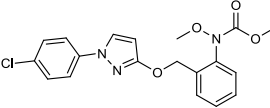
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28 Figure 1.- Effects of fungicides on cell viability measured by the MTT assay in primary
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30 cultures of cortical neurons. A) Cells were acutely exposed to fungicides at day in vitro 7 (DIV
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32 7) for 24 hours (white bars) or continuously exposed from DIV 1 to DIV 8 (black bars). All
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34 fungicides were applied at 100 μ M, except for continuous exposure to CY at 30 μ M due to
35
36 solubility problems. Results are expressed as mean \pm SEM; n = 3-4 cultures. * $p < 0.05$; # p
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38 < 0.001 compared to control cells. B) Concentration-response curves for the effects of
39
40 fungicides on cell viability in cultured cortical neurons. Solid symbols and lines represent
41
42 continuous exposure while open symbols and dashed lines represent acute exposure. Results
43
44 are expressed as mean \pm SEM; n = 3-4 cultures.
45

46 Figure 2.- A) Concentration-response curves for the increase of intracellular calcium induced
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48 by PY (\blacktriangledown ; blue) and KR (\bullet ; red). Results are means \pm SEM of 3-4 independent experiments.
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50 B) The increase of $[Ca^{2+}]_i$ induced by 1 μ M PY (blue bars; diagonal) or 10 μ M KR (red bars;
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52 crossed) was inhibited in the nominal absence of calcium in the medium. Results are mean \pm
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54 SEM of 3-4 independent experiments. ** $p < 0.01$ and *** $p < 0.001$ with respect to control
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56 (black bars) in plain buffer; ## $p < 0.01$ and ### $p < 0.001$ with respect to fungicide treatment in
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58 plain buffer.
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4 Figure 3.- Depolarization of mitochondrial membrane (MM) induced by fungicide exposure.
5 Cells were acutely exposed to fungicides at DIV 7. Values are mean \pm SEM; n = 3 cultures.
6 Responses are normalized to maximum depolarization induced by 15 μ M antimycin (ANT). A)
7 Fungicides were tested at 100 μ M, except KR and PY at 50 μ M. ** p <0.01, *** p <0.001
8 compared to control cells. B) Concentration-response curves for PY (\blacktriangledown ; blue), KR (\bullet ; red)
9 and CY (\blacksquare ; green).
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16 Figure 4.- Effect of calcium channel antagonists on the reduction of cell viability induced by
17 PY and KR. Cells were continuously exposed to vehicle (DMSO) or fungicides from DIV 1 to
18 DIV 8 in the absence (black bars) or in the presence of 50 μ M verapamil (red bars; dotted), 10
19 μ M MK-801 (green bars; horizontal lines) or verapamil plus MK-801 (blue bars; vertical lines)
20 from DIV 1 to DIV 8. Results are expressed as mean \pm SEM of 5-6 independent experiments.
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24 ** p < 0.01 and *** p < 0.001 vs corresponding exposure to fungicide alone.
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Table 1
Structures, characteristics and modes of action of the studied fungicides

Fungicide	Structure	Chemical Group	Log P ^a	Mode of action ^b	Approval year ^c	ADI (mg/kg bw/d)
Ametoctradin (AM)		Triazolo-pyrimidylamine	4.4	Respiration inhibition of complex III: cytochrome bc1(ubiquinone reductase) at Qo site, stigmatellin binding sub-site	2013	10
Boscalid (BO)		Pyridine-carboxamide	2.96	Respiration inhibition of complex II: succinate dehydrogenase inhibitors	2008	0.04
Cyazofamid (CY)		Cyanoimidazole	3.2	Respiration inhibition of complex III: cytochrome bc1(ubiquinone reductase) at Qi site	2003	0.17
Dimethomorph (DI)		Cinnamic acid amide	2.68	Cell wall biosynthesis: cellulose synthase	2007	0.05
Fenhexamid (FE)		Hydroxyanilide	3.51	Sterol biosynthesis in membranes: 3-keto reductase in C4-de-methylation (erg27)	2001	0.2
Kresoxim-methyl (KR)		Strobilurin	3.40	Respiration inhibition of complex III: cytochrome bc1(ubiquinol oxidase) at Qo site (<i>cyt b gene</i>)	1999	0.4
Mepanipyrim (MY)		Anilino-pyrimidine	3.28	Amino Acid and Protein Synthesis: methionine biosynthesis (<i>cgs gene</i>)	2004	0.02
Metrafenone (ME)		Aryl-phenyl-ketone	4.3	Unknown: actin disruption (proposed)	2007	0.25
Pyraclostrobin (PY)		Strobilurin	3.99	Respiration inhibition of complex III: cytochrome bc1(ubiquinol oxidase) at Qo site (<i>cyt b gene</i>)	2004	0.03

^a (Tomlin, 2004); ^b (FRAC, 2014); ^c (European Commission, 2009); Log K_{ow}: octanol-water partition coefficient; ADI: Acceptable Daily Intake

Table 2
Specific MRM conditions for determination of selected fungicides by LC–ESI-MS/MS

Fungicides	t_R (min)	Parent ion	MS/MS transition	CE (eV)
AM	10.83	$[M+H]^+$	276>176 ^a	35
			276>149	36
CY	13.62	$[M+H]^+$	325>108 ^a	15
			325>280	12
KR	13.74	$[M+H]^+$	314>222 ^a	15
			314>235	12
PY	14.51	$[M+H]^+$	388>194 ^a	15
			388>164	16

^aQuantifier transition; CE: collision energy

Table 3.- LC50 values for the cytotoxicity of the fungicides in cortical neurons after exposure for 1 DIV (acute exposure) and 8 DIV (continuous exposure). Values are mean \pm SEM of 3 – 5 experiments. * $p < 0.05$; # $P < 0.001$ with respect to acute exposure

Fungicide	LC50 values (μM)	
	Acute exposure	Continuous exposure
AM	No effect at 100 μM	>100
BO	>100	>100
CY	95 \pm 2	25 \pm 2
DI	No effect at 100 μM	58 \pm 6
FE	No effect at 100 μM	27 \pm 1
KR	2.6 \pm 0.1	4.4 \pm 0.1
ME	No effect at 100 μM	>100
MY	No effect at 100 μM	26 \pm 1
PY	0.15 \pm 0.02	0.06 \pm 0.01

Table 4.- Intracellular levels of selected fungicides in CN after 8 h of exposure at 7 DIV.

Fungicide (MW)	Treatment (μM)	Cellular uptake (ng/mg protein)	% Cellular uptake
AM (275)	100	980.0 ± 1.8 (3.6 nmol/mg prot)	0.73 ± 0.02
CY (325)	100	9115 ± 1526 (28 nmol/mg prot)	6.0 ± 1.0
KR (313)	10	86 ± 3 (0.3 nmol/mg prot)	0.54 ± 0.02
PY (388)	1	37 ± 5 (0.095 nmol/mg prot)	2.14 ± 0.27

Figure 1
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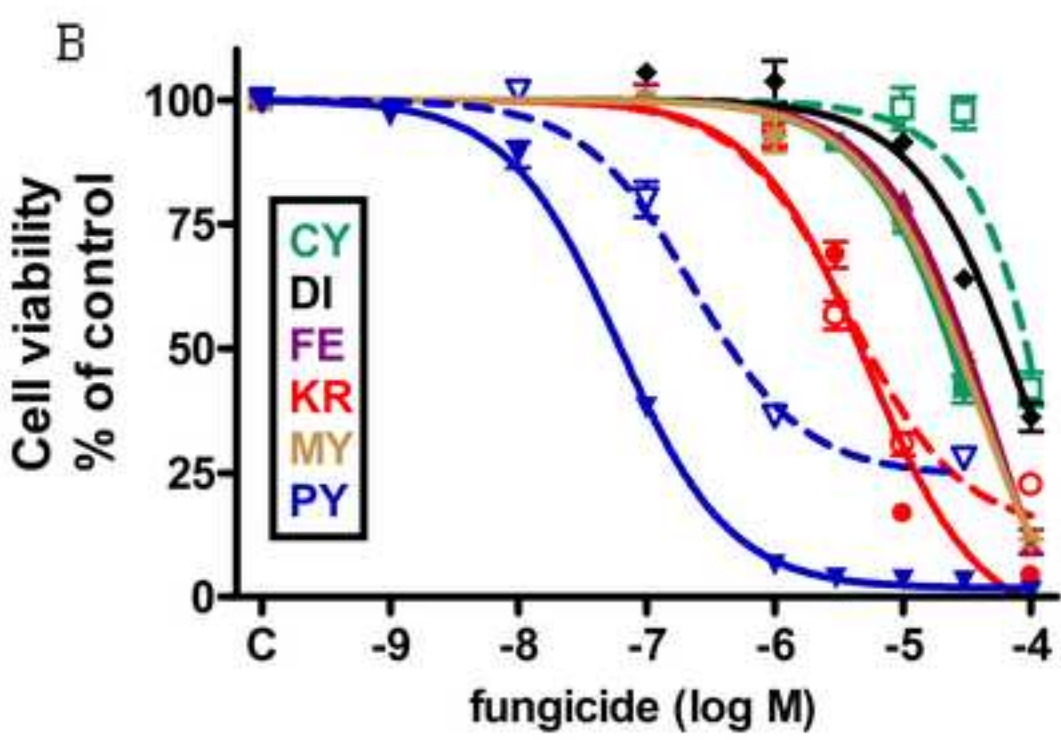
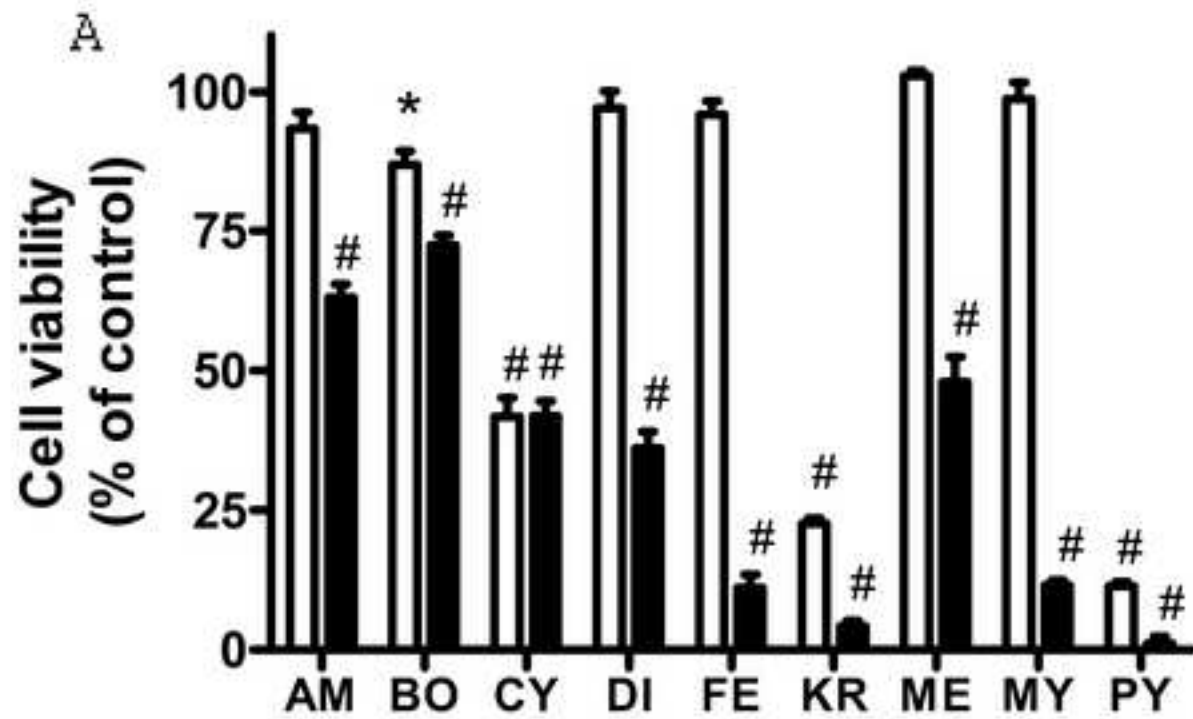


Figure 2
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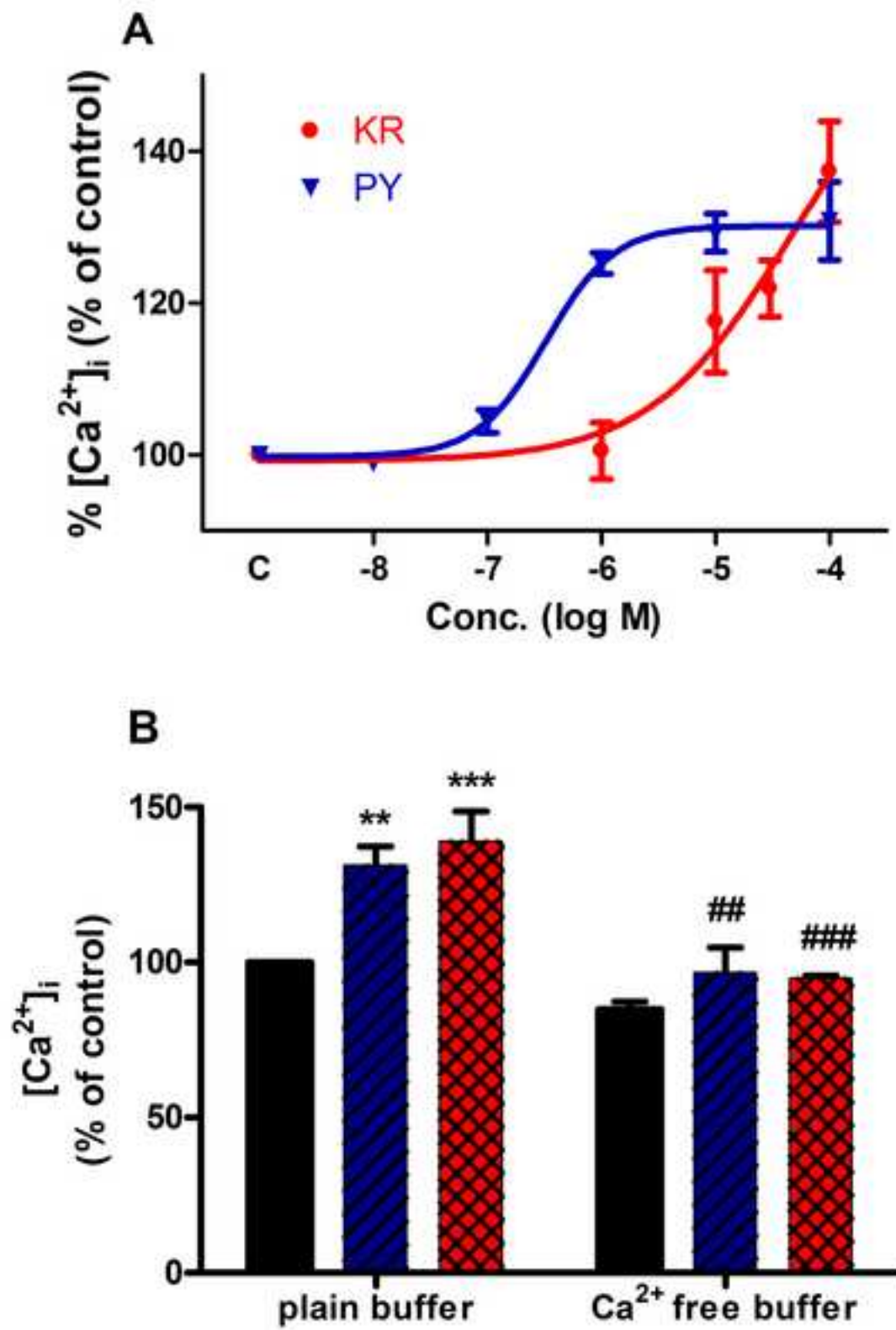


Figure 3
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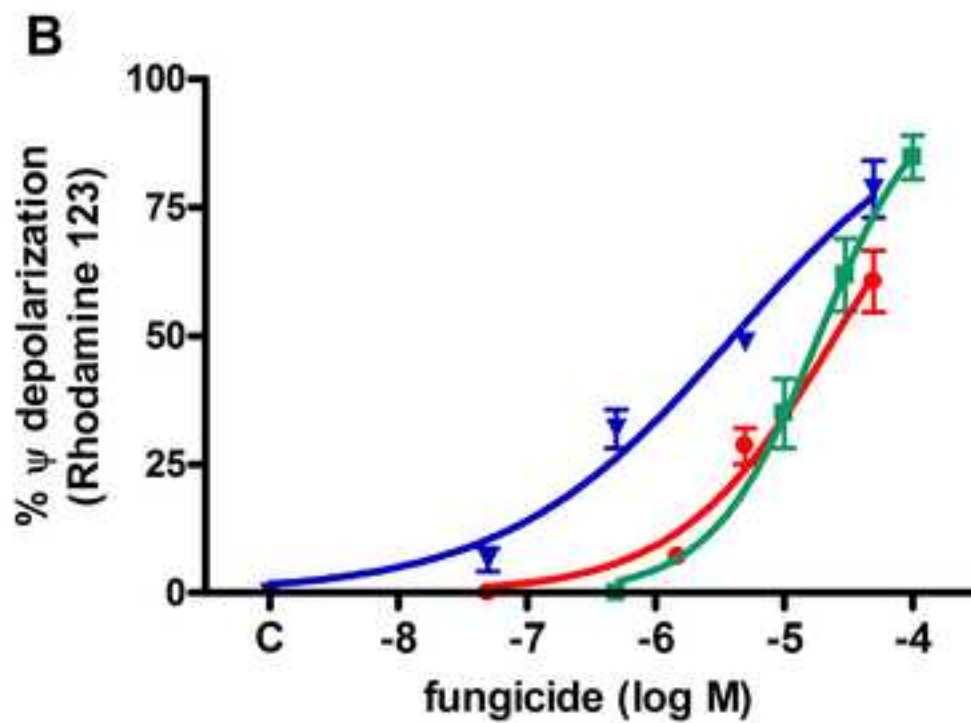
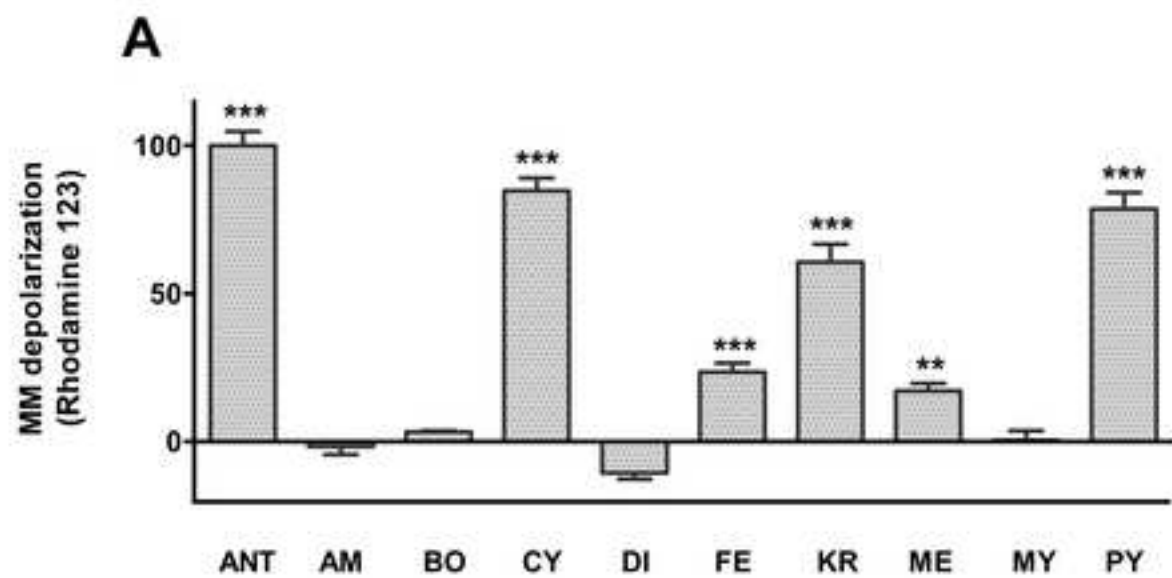


Figure 4
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