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Effects of polyamines on cadmium- and copper-mediated alterations in wheat (*Triticum aestivum L*) and sunflower (*Helianthus annuus L*) seedling membrane fluidity

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ABSTRACT

We investigated if wheat (Wh) and sunflower (Sf) plants watering with 1 mM $CdCl_2$ or $CuCl_2$ for 5–15 d during germination and seedling altered membrane fluidity (MF) of their leaves and roots, and if plant pre-treatment with the polyamines (PAs) putrescine (Put), spermidine (Spd) or spermine (Spm) prevented those alterations. Cd impaired Wh and Sf growth, while Cu only affected Sf growth. Cu and Cd increased MF of leaves of both plant species, while Cd decreased MF of Sf roots. Plant treatment for 15 d with 0.1 mM Put, Spd or Spm did not affect plant growth and had opposed effects on the MF of both plants. Finally, Wh and Sf were pre-treated with PAs for either 5 or 10 days followed by metal treatment until day 15. While Put did not affect membrane MF, Spd and Spm decreased it between 5 and 10 d of plant treatment. Together, experimental results demonstrate that during plant development (a) Cd and Cu have noxious effects on plants membrane biophysical properties that could be partially responsible of their toxicity, and (b) this deleterious effect could be only partially prevented by plant pretreatment with the PAs.

1. Introduction

The continuous discharge of waste generated by mineral, industrial and agricultural activities during the last century has increased dramatically the concentration of heavy metals in the environment. Based on their toxicity and cumulative behavior, heavy metals pose health risks to microorganisms, plants, animals, and humans. Among other routes of exposure, animals and humans incorporate them via the ingestion of contaminated vegetables. Therefore, the avoidance of contamination with heavy metals of edible plants is critical not only for preserving the quality of the cultivars but also of animal and human health.

Copper (Cu) is an essential, redox-active micronutrient that is required for normal plant growth and development [1]. However, high concentrations of Cu are phytotoxic since this metal affects negatively numerous biochemical reactions and physiological processes including photosynthesis [2], carbohydrate metabolism [3], respiration [4], and CO_2 [5] and nitrogen fixation [6], among others, thus disturbing normal plant development. On the other hand, cadmium (Cd) is one of the most aggressive, persistent, and widely spread heavy metal. Cd contaminates soils, water, and the atmosphere, mostly as a by-product of industrial activities. Among others, the use of phosphorus-based fertilizers constitutes the main input of Cd into agricultural soils. Cd and Cu can bind to extra and intracellular macromolecules and affect both biochemical processes and structures. In plants, the uptake of Cd, Cu and other heavy metals occurs through the nutrient transport system. In particular, transmembrane carriers engaged in the incorporation of Ca, Fe, Mg, Cu and Zn in plants are also capable of transporting Cd, leading to its accumulation within cells [7]. Conversely, some of these metals inhibit the uptake of Cd from the rhizosphere solution, hence limiting its accumulation in the roots [8]. The cell wall constitutes the first barrier against the entrance of multivalent

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; Hgl, Hoagland's nutrient solution; MF, membrane fluidity; PA, polyamine; Put, putrescine; ROS, reactive oxygen species; Sf, sunflower; Spd, spermidine; Spm, spermine; Wh, wheat

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metal cations into the symplast due to its capacity to bind substantial amounts of diverse heavy metals [9]. Albeit this mechanism contributes to limit their uptake, heavy metals can induce the rearrangement of carbohydrate and glycoprotein networks in the wall, thus compromising its plasticity and water permeability [10]. The next barrier is the plasma membrane, which regulates Cu and Cd uptake in an active manner [11]. Therefore, the structure and functions of plasma membrane components are also susceptible to alterations upon their interaction with the metals.

Polyamines (PAs), such as putrescine (Put), spermidine (Spd), and spermine (Spm), are polycationic metabolites that form a group of essential growth regulators in plants. Among others, PAs regulate cell elongation and division, root growth, flower formation, embryogenesis, membrane stabilization, and DNA replication [12,13]. Several molecular targets for PAs have been suggested. As unique polyvalent cationic metabolites, the binding of PAs to negative surfaces confers these molecules the capacity to act as molecular chaperones, to stabilize membranes, proteins and nucleic acids, and to modify their conformation and/or assembly [14]. In addition, PAs can scavenge reactive oxygen species (ROS) and other free radicals [15], and stimulate the antioxidant defense system [16]. Due to their strong antioxidant capacity and high tissular concentration, PAs are key in the limitation of the extent of oxidative stress-mediated damage to biological macromolecules [17]. In addition, exogenous PAs have been used to improve plant tolerance to heavy metal-induced stress [18,19]. Moreover, endogenous PAs levels are increased in plants under diverse kinds of stress [20,21]. However, the precise physiological and molecular mechanisms underlying their protective effect in plants remain elusive.

In this study, we aimed to investigate the effects of Cd and Cu on MF of whole leaves and roots from wheat (Wh) and sunflower (Sf) plants during the first stages of plant growth (post-germination and seedling growth), and if PAs could modulate metal-mediated alterations in MF. These plant species were chosen because of their importance in the agricultural economy of Argentina and other countries in Latin America, and because both Cd and Cu affect their growth [22].

2. Materials and methods

2.1. Materials

Wheat (*Triticum aestivum*) and sunflower (*Helianthus annuus L*) seeds were provided by Nidera (Buenos Aires, Argentina). The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was from Invitrogen/Molecular Probes (Eugene, OR, USA). Put, Spd and Spm, $CuCl_2$, $CdCl_2$ and all the other reagents used in this work were from the highest quality available and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant growth conditions

The surface of Wh and Sf seeds was sterilized in 20% (v/v in sterile distilled water) commercial bleach for 10 min, followed by four washes with sterile distilled water. Seeds were sown immediately for germination in vermiculite and grown in a climate-controlled room at 24 ± 2 °C and 50% relative humidity, with 16/8 h light/darkness photoperiod (light intensity ~300 μmol m^{-2} s^{-1}). Between days 0 and 15, plants were irrigated with half strength Hoagland's nutrient solution (Hgl) [23] without (control) or with 1 mM $CdCl_2$ (Cd) or $CuCl_2$ (Cu), or with 0.1 mM Put, Spd or Spm. In parallel, a subset of plants was pretreated for the first 5 or 10 d with 0.1 mM Put, Spd or Spm in Hgl, and subsequently watered with 1 mM Cd or Cu in Hgl until day 15. Plants were harvested and weighted, and leaves and roots were detached. Relative water content in the samples was calculated as the ratio between dry and fresh weights (%).

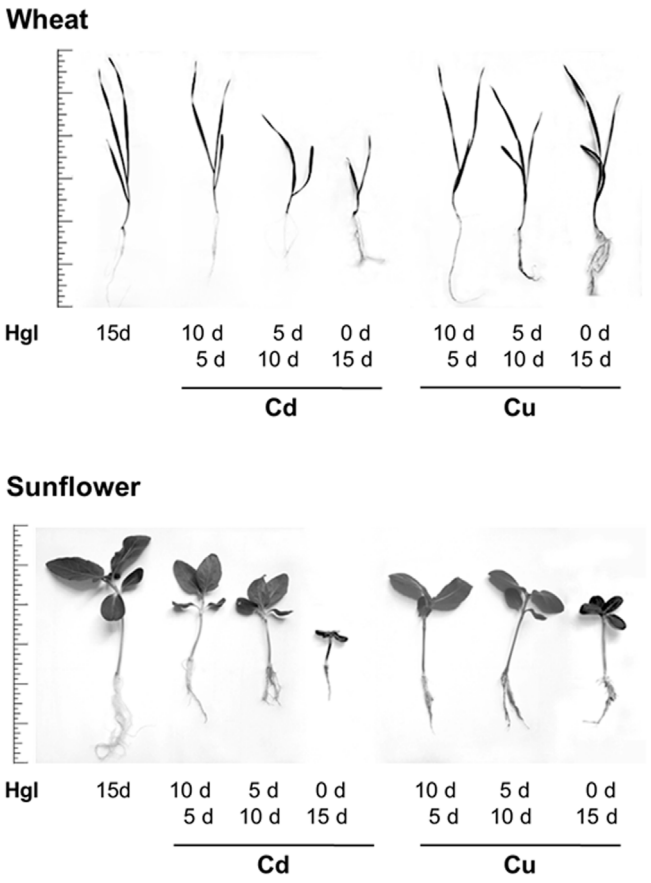

Fig. 1. Effects of Cd or Cu exposure on Wh and Sf plant growth. Wheat and sunflower plants were grown in the following conditions: 15 d in Hoagland's nutrient solution (Hgl; control), 10 d in Hgl followed by 5 d in Hgl supplemented with 1 mM Cd or Cu (5 d), 5 d in Hgl followed by 10 d in Hgl supplemented with 1 mM Cd or Cu (10 d), or 15 d in Hgl containing 1 mM Cd or Cu (15 d). At day 15, plants were harvested and photographed. Images correspond to a representative experiment (n = 4). For comparative purposes, an arbitrary scale is shown at the left of the images.

2.3. Chlorophyll content

Chlorophyll content in Wh and Sf leaves was quantified as described previously [24]. Briefly, chlorophyll was extracted from 0.1 g leaf samples with 3 ml of 96% ethanol. Samples were incubated for 20 min at 55 °C to achieve complete leaf discoloration, and chlorophyll content in the extracts was calculated from their absorbance at 654 nm ($\varepsilon = 39.8\ M^{-1}\ cm^{-1}$).

2.4. Evaluation of membrane fluidity (MF)

Portions of the leaves and roots were carefully cut, and chlorophyll was extracted from the leaves with 96% ethanol at 50 °C until visual discoloration. Samples were infiltrated under vacuum for 3 cycles of 15 s with 40 μM DPH in 0.125 mM Tris-HCl buffer (pH 7.4) and incubated for 3 h at 25 °C to allow the incorporation of the probe into membranes. Samples were mounted onto 11 × 22 mm glass coverslips (Thomas Scientific, Swedesboro, NJ, USA) and placed into a CCH-1 holder (C&L Instruments Inc., Hershey, PA, USA) that held the coverslip at 45° relative to the excitation beam. MF was estimated from DPH steady-state fluorescence anisotropy (λexcitation: 365 nm; λemission: 450 nm) recorded at 24 ± 1 °C in a Kontron SFM-25 spectrofluorometer (Kontron Instruments SPA, Milan, Italy) equipped with temperature control and 10-nm monochromator bandwidth.

Table 1
Effects of Put, Spd or Spm pre-treatment on Cd and Cu-mediated changes in plant tissue relative water content (%).

Plant	Tissue	Situations			Hgl	Put	Spd	Spm
		Hgl or PAs	Metal					
Wheat	leaves	15 d	none		89.9 ± 0.2	92.1 ± 0.1 [a]	91.1 ± 0.1	91.8 ± 0.3
		10 d	Cd	5 d	89.1 ± 0.8	89.8 ± 0.2 [c]	89.2 ± 0.4	89.2 ± 0.3 c
		5 d		10 d	87.3 ± 0.7[a]	90.8 ± 0.5 [b]	89.4 ± 0.6 [b]	88.5 ± 1.0 [c]
		0 d		15 d	83.5 ± 0.3 [a]			
		10 d	Cu	5 d	90.2 ± 0.4	89.9 ± 0.4 [c]	89.2 ± 0.4 [c]	89.2 ± 0.3 [c]
		5 d		10 d	88.8 ± 0.5 [a]	88.9 ± 0.5 [c]	89.5 ± 0.6 [c]	88.5 ± 0.8 [c]
		0 d		15 d	86.7 ± 0.5 [a]			
	roots	15 d	none		95.1 ± 0.3	96.3 ± 0.4	94.5 ± 0.2	95.3 ± 0.3
		10 d	Cd	5 d	94.0 ± 0.6	93.0 ± 0.6 [c]	93.3 ± 0.8	93.2 ± 0.5
		5 d		10 d	91.9 ± 0.8 [a]	91.8 ± 0.5 [c]	91.3 ± 0.9 [c]	91.6 ± 1.0 [c]
		0 d		15 d	91.7 ± 0.3 [a]			
		10 d	Cu	5 d	93.9 ± 0.6	94.0 ± 0.4 [c]	93.6 ± 0.5	93.9 ± 0.4
		5 d		10 d	92.4 ± 0.8 [a]	91.8 ± 0.4 [c]	92.6 ± 0.5	92.8 ± 0.2 [c]
		0 d		15 d	91.3 ± 0.4 [a]			
Sunflower	leaves	15 d	none		91.3 ± 0.4	92.2 ± 0.1	93.1 ± 0.1	92.6 ± 0.3
		10 d	Cd	5 d	91.4 ± 0.4	91.2 ± 0.4	91.2 ± 0.5	90.0 ± 0.7 [c]
		5 d		10 d	89.3 ± 1.0 [a]	89.3 ± 0.5 [c]	87.9 ± 1.4 [c]	90.4 ± 0.4
		0 d		15 d	88.4 ± 0.1 [a]			
		10 d	Cu	5 d	91.1 ± 0.4	91.3 ± 0.4	91.2 ± 0.5	91.1 ± 0.7
		5 d		10 d	90.0 ± 0.7	90.2 ± 0.9 [c]	90.0 ± 1.0 [c]	90.1 ± 1.0 [c]
		0 d		15 d	89.3 ± 0.1 [a]			
	roots	15 d	none		96.8 ± 0.1	96.8 ± 0.5	97.1 ± 0.2	97.2 ± 0.1
		10 d	Cd	5 d	95.0 ± 0.2 [a]	94.8 ± 0.2 [c]	94.8 ± 0.4 [c]	95.1 ± 0.3 [c]
		5 d		10 d	94.0 ± 0.2 [a]	93.3 ± 0.5 [c]	93.6 ± 0.3 [c]	94.3 ± 0.2 [c]
		0 d		15 d	93.7 ± 0.5 [a]			
		10 d	Cu	5 d	94.7 ± 0.1 [a]	94.9 ± 0.2 [c]	95.1 ± 0.5 [c]	95.0 ± 0.3 [c]
		5 d		10 d	94.6 ± 0.4 [a]	93.4 ± 0.7 [c]	94.0 ± 0.6 [c]	94.7 ± 0.2 [c]
		0 d		15 d	93.9 ± 0.5 [a]			

Results are shown as mean ± S.E.M. (n ≥ 4). [a] significantly different from the value measured in the same tissue from plants watered with Hoagland (Hgl) solution alone ($P < 0.05$, one-way ANOVA). [b] significantly different from the value measured in the same tissue from plants treated with the same metal and exposure time ($P < 0.05$, one-way ANOVA). [c] significantly different from the value measured in the same tissue from plants pre-treated with the corresponding PA ($P < 0.05$, one-way ANOVA).

Table 2
Effects of Put, Spd or Spm pre-treatment on Cd and Cu-mediated changes in chlorophyll content (μg/g fresh tissue).

Plant	Situations			Hgl	Put	Spd	Spm
	Hgl or PAs	Metal					
Wheat	15 d	none		1.88 ± 0.08	1.88 ± 0.07	1.93 ± 0.03	1.90 ± 0.02
	10 d	Cd	5 d	1.54 ± 0.04 [a]	1.95 ± 0.04 [b,c]	1.56 ± 0.07 [a,c]	1.70 ± 0.02 [c]
	5 d		10 d	1.43 ± 0.04 [a]	1.39 ± 0.04 [c]	1.34 ± 0.06 [a,c]	1.70 ± 0.10 [b,c]
	0 d		15 d	1.65 ± 0.04 [a]			
	10 d	Cu	5 d	1.69 ± 0.03 [a]	1.97 ± 0.10 [b]	1.86 ± 0.04 [c]	1.66 ± 0.17 [c]
	5 d		10 d	1.61 ± 0.10 [a]	1.93 ± 0.05 [b,c]	1.41 ± 0.05 [a,c]	1.55 ± 0.12 [c]
	0 d		15 d	1.72 ± 0.05			
Sunflower	15 d	none		1.86 ± 0.07	1.82 ± 0.09	1.79 ± 0.08	1.72 ± 0.06
	10 d	Cd	5 d	1.80 ± 0.06	2.06 ± 0.04 [b]	1.83 ± 0.05	1.82 ± 0.09
	5 d		10 d	1.74 ± 0.04	1.87 ± 0.04	1.95 ± 0.06 [b]	1.58 ± 0.08
	0 d		15 d	1.76 ± 0.05			
	10 d	Cu	5 d	2.06 ± 0.07 [a]	2.00 ± 0.06	1.89 ± 0.04 [c]	2.67 ± 0.09 [a,b,c]
	5 d		10 d	2.05 ± 0.06 [a]	2.39 ± 0.10 [b]	1.95 ± 0.04 [c]	2.45 ± 0.10 [a,b,c]
	0 d		15 d	1.99 ± 0.06			

Results are shown as mean ± S.E.M. (n ≥ 4). [a] significantly different from the value measured in the same tissue from plants watered with Hoagland (Hgl) solution alone ($P < 0.05$, one-way ANOVA). [b] significantly different from the value measured in the same tissue from plants treated with the same metal and exposure time ($P < 0.05$, one-way ANOVA). [c] significantly different from the value measured in the same tissue from plants pre-treated with the corresponding PA ($P < 0.05$, one-way ANOVA).

2.5. Statistics

Results are expressed as the mean ± standard error of the media (SEM). Two-way analysis of variance (ANOVA) followed by Holm-Sidak's multiple comparisons test, and correlations were performed using the routines available in GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). A probability (P) value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Effects of PAs on Cd- and Cu-mediated alterations of plant growth during the early stages of plant development

To corroborate that in our experimental model Cd and Cu affected the initial stages of plant development, Wh and Sf plants were exposed to 1 mM of the metals between days 0 and 15 post-sowing. The use of vermiculite as substrate for plant growing allowed us to achieve

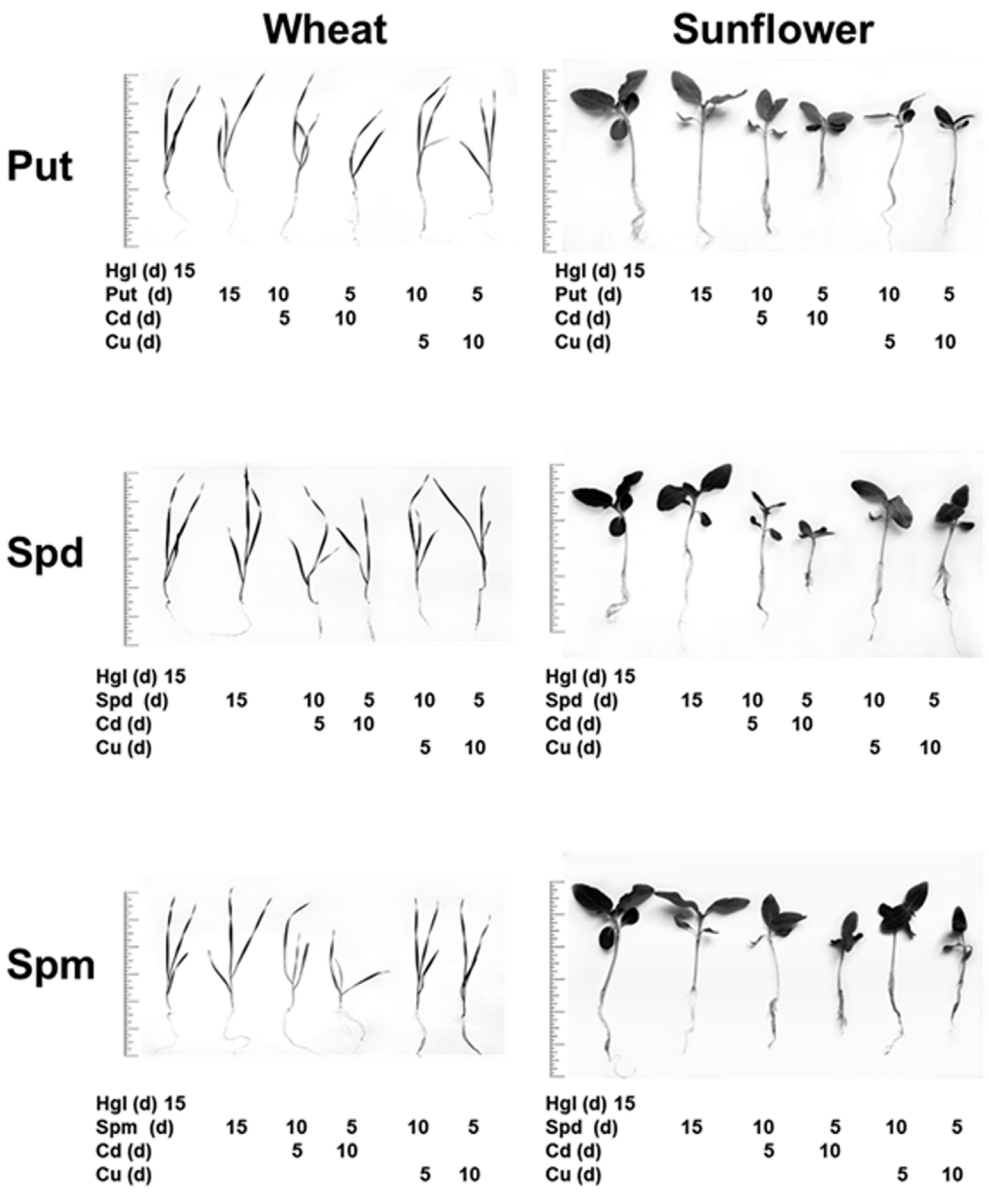

Fig. 2. Effects of PAs pretreatment on Cd- and Cu-mediated impairment of plant growth. Wheat and sunflower plants were grown in the following conditions: 15 d in Hgl (control); 15 d in Hgl containing 0.1 mM putrescine (Put, upper panels), spermidine (Spd, central panels) or spermine (Spm, lower panels); 10 d in Hgl containing 0.1 mM Put, Spd or Spm followed by 5 d in Hgl containing 1 mM Cd or Cu; or 5 d in Hgl containing 0.1 mM Put, Spd or Spm followed by further 10 d in Hgl containing 1 mM Cd or Cu. At day 15, plants were harvested and photographed. Images correspond to a representative experiment (n = 4). For comparative purposes, an arbitrary scale is shown at the left of the images.

theoretical concentrations of free Cd (~38 ppm) and Cu (~13 ppm) available to the plants compatible with those found in moderately contaminated agricultural soils [25]. Based on previous analysis of metal speciation in aqueous solutions and on the composition of Hoagland's nutrient solution, the most abundant species expected to be present in the media were $CdCl_2$, $CdCl^+$ and Cu^{2+} [26,27]. However, the contribution of other metal species to the effects observed could not be discarded *a priori*.

Under the conditions used for the experiments, all seeds germinated but, as expected, both Wh and Sf plants exposed to Cd for 15 d were smaller than their corresponding controls (Fig. 1). Even when the roots from Cd-treated Wh plants were shorter than those of the controls, their topology was preserved. The inhibitory effect of Cd on plant growth was particularly marked in Sf plants, which presented an engrossed primary root compatible with the initiation of seedling growth. In contrast, the exposure of Wh plants to Cu did not affect their development, while the exposure of Sf plants to Cu caused minor but visible developmental impairment. Both Wh and Sf leaves and roots of plants exposed for 15 d to Cd or Cu presented significantly lower water content than the controls (Table 1). The leaves from Cd-treated Wh plants were the most dehydrated of the group, with 7% lower content of water than the controls ($P < 0.05$) at day 15. In addition, these leaves had 12% lower chlorophyll content (Table 2). Despite their smaller size, chlorophyll content in Sf plants exposed to Cd or Cu was comparable to the value measured in the controls.

Put, Spd and Spm are essential regulators of plant growth. Several lines of evidence indicate that PAs protect plants against Cd- or Cu-induced toxicity [12,13,28]. In fact, increased Put, Spd and Spm endogenous contents were found in the shoots of Sf plants exposed to Cd or Cu during the seedling stage [29]. In addition to the endogenous synthesis, PAs can be absorbed from the rhizosphere and distributed. For example, the uptake of Put by tomato and maize seedlings is rapid, reaching the upper portion of the plant after only 30 min of exposure to Put [30]. In the current model, we administered the PAs by watering Wh and Sf plants with Put, Spd or Spm dissolved in Hoagland's nutrient solution. The administration of the PAs for 15 d did not affect the normal growth of plants, as evidenced from their overall morphology (Fig. 2). Supporting that, the relative water content in their leaves and roots (Table 1) as well as their chlorophyll content (Table 2) were comparable to those of the controls.

We next investigated if plant pre-treatment with the PAs for 5 or 10 d could partially or totally prevent the noxious effects of Cd and Cu

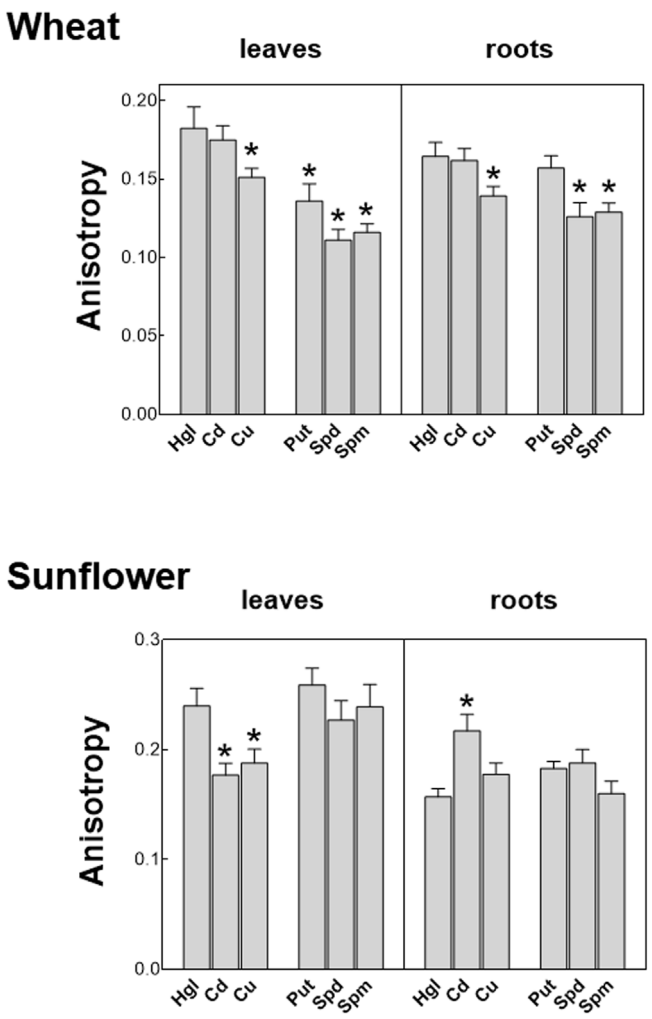

Fig. 3. Effect of 15-d plant treatment with Cd, Cu, or the PAs on MF of whole Wh and Sf leaves and roots. Wheat and sunflower plants were grown for 15 d in Hgl without (control) or with 1 mM Cd or Cu, or 0.1 mM Put, Spd or Spm. MF of whole leaves and roots was evaluated from the fluorescence anisotropy of the probe DPH. Results are shown as the mean ± SEM (n = 6). * denotes a significant difference from the data measured in control (Hgl) plants ($P < 0.05$).

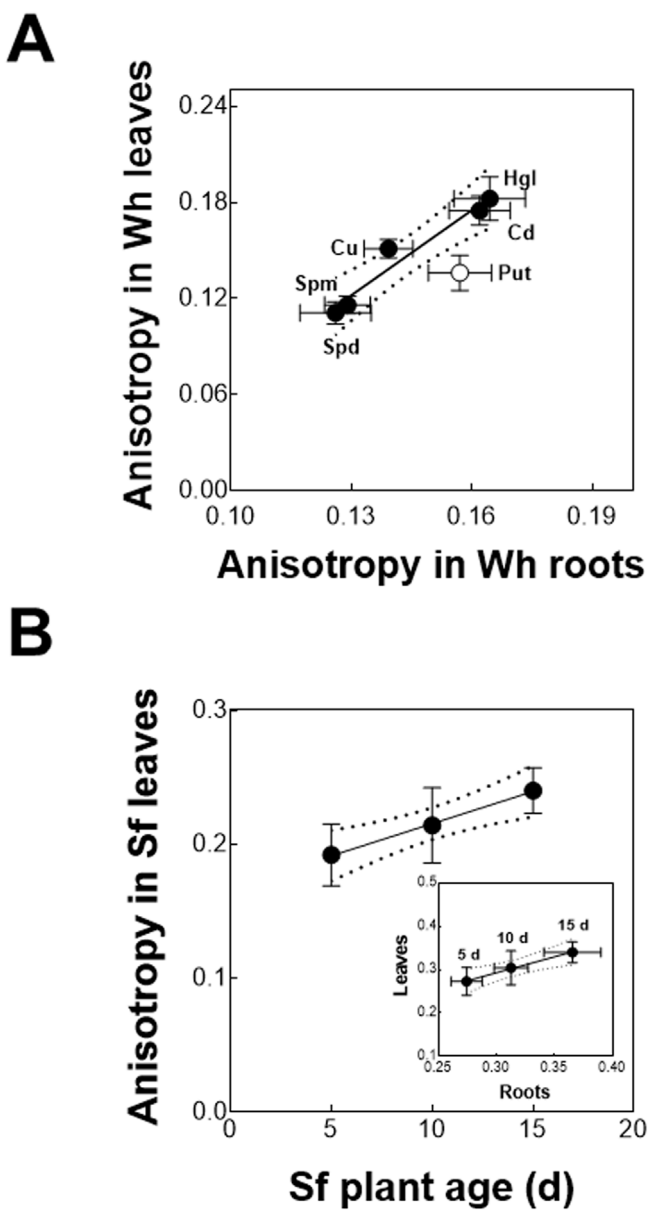

Fig. 4. Correlations. **(A)** Wheat plants were grown for 15 d in Hgl without (control) or with 1 mM Cd or Cu, or 0.1 mM Put, Spd or Spm. MF of whole leaves and roots was measured from the fluorescence anisotropy of DPH. The values recorded for Put-treated plants (open circle) were not included in the correlation analysis. **(B)** Sunflower plants were grown in Hgl for 5, 10 or 15 d and MF of their leaves was measured. *Inset*: correlation between anisotropy values in the leaves and roots of sunflower plants and recorded at the different growth ages investigated. Results are shown as the mean ± SEM (n = 3). Dotted lines denote the 95% confidence band of the best fit-line.

on plant growth. Cd-induced alterations on Wh plant growth were prevented by none of the PAs assessed, and the morphology of Cu-treated Wh plants remained close to that of the controls regardless the presence of the PAs (Fig. 2). Put and Spm caused no noticeable effects on Cd-mediated inhibition of Sf plant growth while Spd aggravated growth impairment caused by Cd. None of the PAs prevented Cu-mediated inhibition of Sf plant growth. Despite their lack of effect on metal-mediated growth impairment, both Put and Spd prevented the dehydration caused by Cd in Wh plants (Table 1). However, the PAs did not alleviate the dehydration caused by the metals in the remaining experimental situations investigated (Table 1). Finally, the effects of the PAs on metal-mediated decrease in chlorophyll content were also variable (Table 2). Put improved chlorophyll content both in Wh and Sf plants exposed to Cd or Cu. In contrast, Spd decreased chlorophyll content in Wh plants exposed to the metals, with no changes being observed in Sf plants submitted to similar conditions. Lastly, Spm increased chlorophyll content (30–45%) in Cu-treated Sf plants, this content being significantly higher than that in control plants ($P < 0.05$).

The cellular functions of PAs are diverse, and sometimes contradictory, and so are their roles in plant stress. Altogether, even when PAs play a major role in promoting plant growth, their effect on plants exposed to the metals was only partial. While PAs alleviated the effects of Cu on Wh plant growth, they had null effects on the remaining situations assessed. Also, PAs failed to avoid metal-mediated plant dehydration. The lack of results observed in our model could be related to the potential ambiguous responses given by PAs. Supporting that, PAs have been assumed to be important in preparing the plant for stress tolerance and to directly help in avoiding the causes of stress, but at the same time, their own catabolic products could be responsible for causing stress damage [31]. Interestingly, although PAs did not prevent chlorosis in Wh plants upon to metal exposure, they prevented it in Sf plants and even raised chlorophyll contents to values beyond the

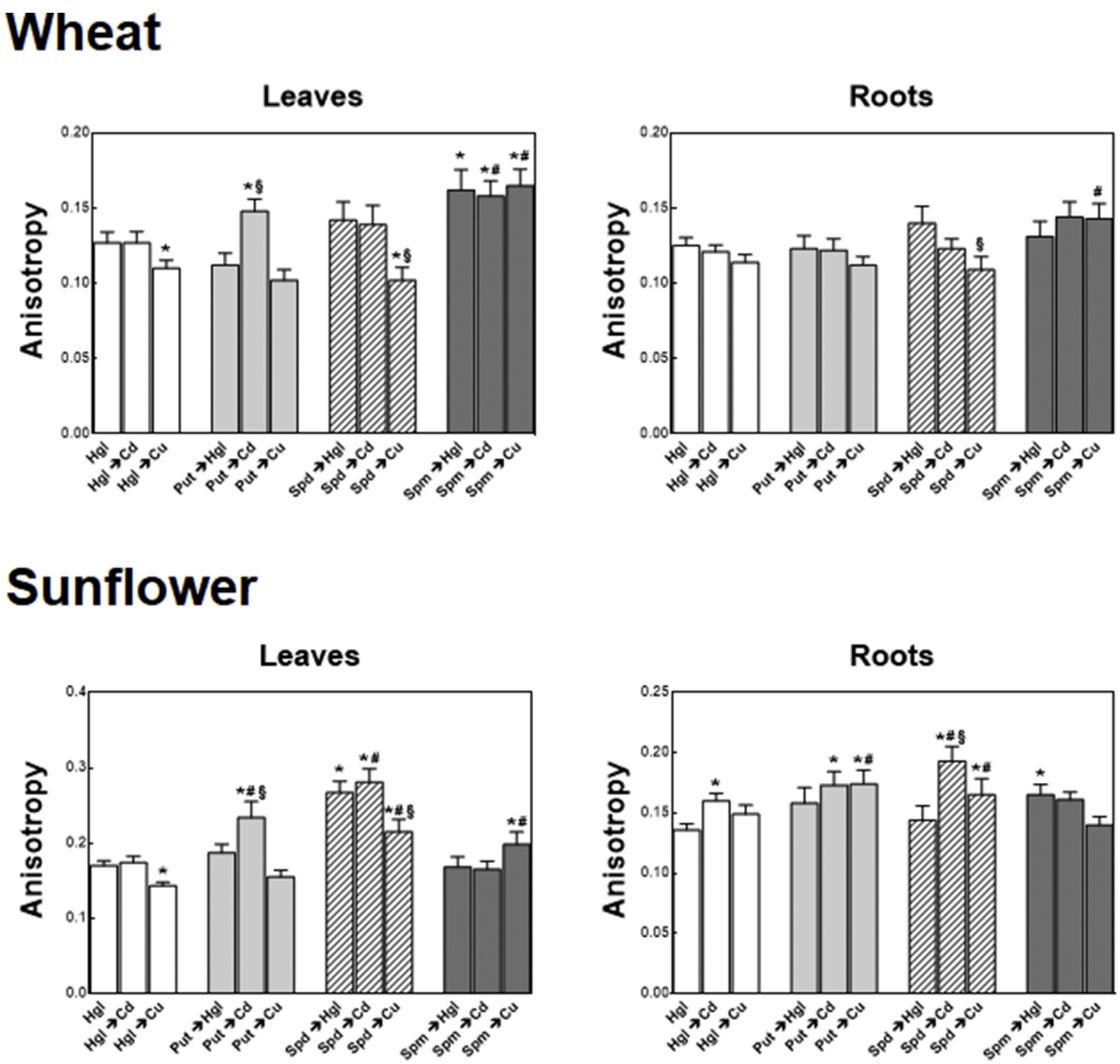

Fig. 5. Effect of 5-d plant pretreatment with PAs on Cd- or Cu-mediated changes in plant MF. Wheat and sunflower plants were grown for 5 d in Hgl without (white bars; control) or with 0.1 mM Put (gray bars), Spd (hatched bars) or Spm (dark gray bars), followed by 10 d treatment with 1 mM Cd or Cu. MF of whole leaves and roots was evaluated from the fluorescence anisotropy of the probe DPH. Results are shown as the mean ± SEM (n = 6). *, # and § denote significant differences from the values measured treated with Hgl alone, with the corresponding metal in the absence of PA, and with the corresponding PA alone, respectively ($P < 0.05$, two-way ANOVA).

controls.

3.2. Effects of PA pretreatment on Cd- and Cu-mediated alterations in MF

The lipid bilayer is the basic structure of cell membrane organization [32]. This structure is highly dynamic, and their components are free to move in the lateral plane of the membrane, and to a minor extent, from one hemi-layer to the other in a process denominated flip-flop. One of the biophysical properties that characterize the membranes is the fluidity, determined by the degree of lipid packing [reviewed in Ref. [33]]. In all living organisms, even minute changes in this property can cause major alterations in membrane-associated processes and trigger signaling cascades involved in the initiation and/or progression of tissular abnormalities. Therefore, the maintenance of cell MF is a requisite for the development and thriving of the organisms [34,35].

Among other reasons, MF can be compromised directly or indirectly by physiological and non-physiological metals, including Fe, Cu, Zn, Se, Cr, Cd, Mn, Hg, Pb, Al and Tl [36–40]. As a general mechanism, metal accumulation affects membrane properties by inducing the generation of ROS that oxidize poly-unsaturated fatty acids. Thus, membranes become enriched in shorter, saturated fatty acids with the consequent decrease in their MF [[33] and references therein]. Supporting that, the exposure of different plant species to either Cu or Cd increases the generation of ROS, leading to cellular damage [12,28,29,41]. Hence, the extent of the damage caused by these heavy metals is partially limited by the enzymatic and non-enzymatic antioxidant defense system, the latter including small molecules such as glycine-betaine and proline [42], and the PAs [12]. In addition, multivalent cations can interact in a direct manner with the constituents of the membranes, causing the rearrangement of the lipids and increasing their packing [43].

In this work, we used DPH as a probe to sense MF of metal-exposed

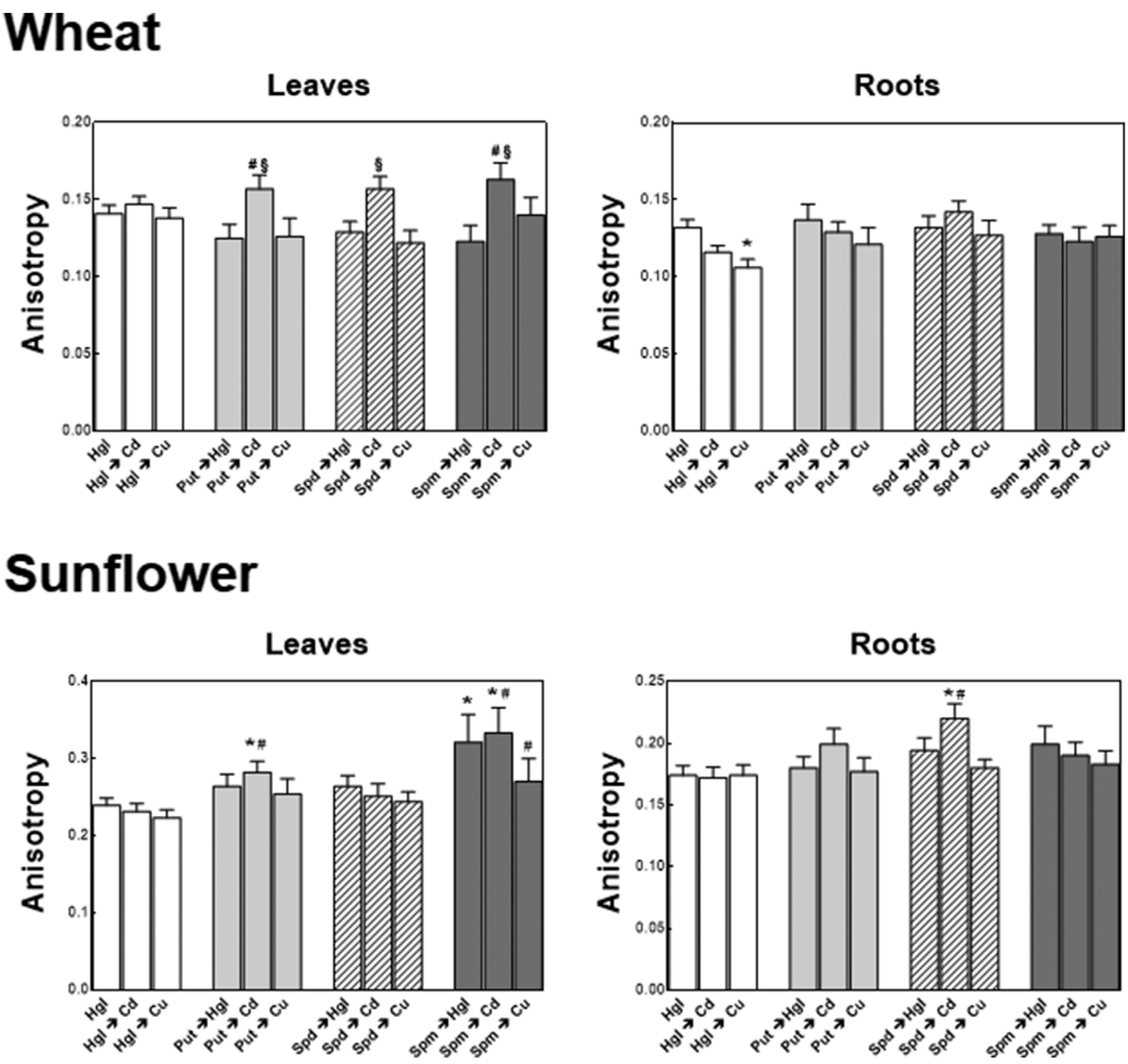

Fig. 6. Effect of 10-d plant pretreatment with PAs on Cd- or Cu-mediated changes in plant MF. Wheat and sunflower plants were grown for 10 d to Hgl without (white bars; control) or with 0.1 mM Put (gray bars), Spd (hatched bars) or Spm (dark gray bars), followed by 5 d treatment with 1 mM Cd or Cu. MF of whole leaves and roots was evaluated from the fluorescence anisotropy of the probe DPH. Results are shown as the mean ± SEM (n = 6). *, # and § denote significant differences from the values measured treated with Hgl alone, with the corresponding metal in the absence of PA, and with the corresponding PA alone, respectively ($P < 0.05$, two-way ANOVA).

plants. DPH is a highly hydrophobic molecule and thus expected to reach the hydrophobic milieu of the membrane. Since the aim of this work was to investigate the fluidity of the membranes in their native environment, with the physiologically-relevant structures well preserved, certain experimental precautions were necessary. First, it was important to consider that vegetal cell wall is an intricate network of cellulose and glycans which, despite being hydrophilic, could partially limit the extent and/or velocity of hydrophobic compounds access to the membrane. Therefore, vacuum infiltration of samples with DPH solution helped the probe to cross the wall, and their subsequent incubation for a prolonged period (3 h) allowed the incorporation of the probe in the membranes, even the inner ones. Second, chlorophyll interferes with DPH fluorescence spectrum (Supp. Fig. 1), and thus, its content in samples must be minimized prior the incorporation of the probe. And third, since the adaxial side of leaves has a thick cuticle that could interfere with fluorescence measurements, the abaxial side was chosen for the analysis. This side is enriched in stomata which play a key role in preserving leaf hydration [44] and CO_2 uptake [45] in metal-exposed plants, their functionality being partially determined by the fluidity of their close environment.

To investigate if, in addition to alter the growth of Wh and Sf plants, Cd and Cu could also affect the biophysical properties of their membranes that would lead to their dysfunction, MF of whole leaves and roots was evaluated. For that, the fluorescence anisotropy of DPH was measured, this parameter being inversely related to MF, with low anisotropy values indicating high MF.

After 15 d of exposure to the metals, the anisotropy values in the leaves and roots of Wh plants exposed to Cd were close to those in controls (Fig. 3), suggesting that this metal did not affect MF. On the other hand, Cu significantly decreased the anisotropy recorded in Wh leaves and roots (17% and 15%, respectively; $P < 0.05$ *vs.* Hgl) indicating that this cation increased Wh MF. Both Cd and Cu decreased

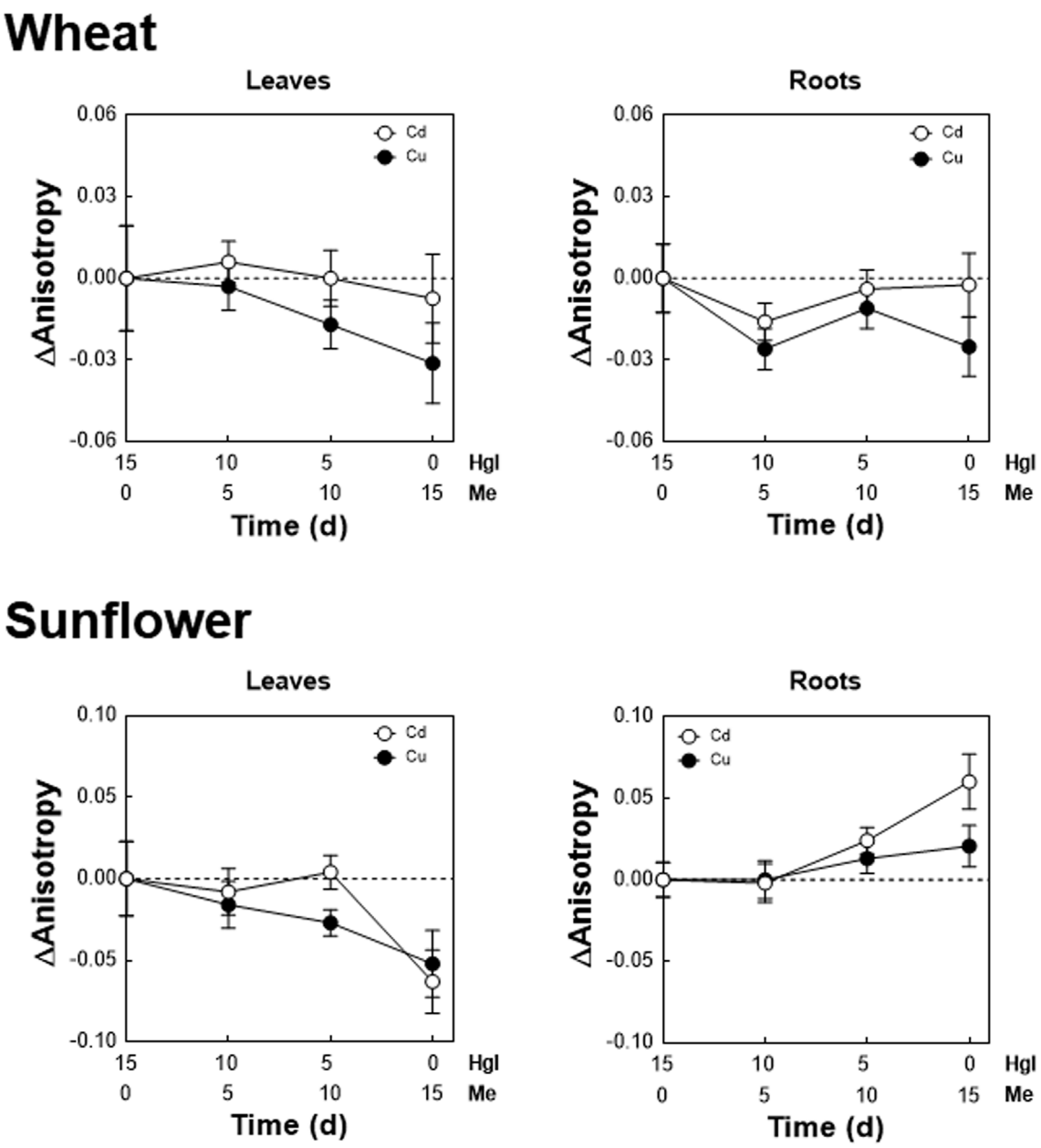

Fig. 7. Kinetics of variations in MF by Cd and Cu. Data taken from Figs. 3, 5 and 6, and expressed as Δanisotropy calculated as: $\Delta Anisotropy = Anisotropy_{Me(xd)} - Anisotropy_{Hgl(15d)}$, where x is the number of days of metal (Me) treatment.

the anisotropy in Sf leaves (25%, $P < 0.005$ *vs.* Hgl). In contrast, Cd increased this parameter in Sf roots (33%, $P < 0.005$ *vs.* Hgl), suggesting that they had decreased MF. Even when 15 d-treatment with the PAs did not affect the growth of Wh or Sf plants, Wh leaves had decreased anisotropy values, ranging from −25 to −36% change respect to the controls ($P < 0.01$). Similar results were observed in the roots of Wh plants exposed to Spd or Spm, with values that were ~23% lower respect to that measured in the controls ($P < 0.05$). None of the PAs affected MF of leaves or roots in Sf plants.

In Wh plants, a positive correlation was found between the effects of Cd, Cu, Spm and Spd on the anisotropy measured in the leaves and roots (r^2: 0.95, $P < 0.005$; Fig. 4A). Interestingly, the effect of Put on Wh MF was higher in the roots than in the leaves, an effect that could be related to different absorption and/or distribution of this PA. On the other hand, positive correlations were found between the plant age of non-treated Sf plants and the anisotropy in their leaves (r^2: 0.99, $P < 0.01$; Fig. 4B), and between the anisotropy values recorded in the leaves and roots (r^2: 0.99, $P < 0.05$; inset to Fig. 4B). By interpolating the value of anisotropy measured in the leaves of Sf plants treated for 15 d with Cd, the developmental age of these plants was estimated in 4.6 d which is compatible with the growth features found in Cd-treated Sf plants (Fig. 1). Based on the linear association observed between MF of Sf leaves and the developmental age of the plants, it is possible to conclude that the morphology of Cd-exposed plants as well as their higher MF corresponded to plants younger than their actual age. Although in those plants MF in the leaves was increased, it was reduced in the roots, an effect that could be caused by local accumulation of the metal that is absorbed and mostly retained in roots. Also, it could be related to a premature lignification of the roots that causes cell wall stiffening, limits nutrient uptake, and ultimately impedes plant growth. Supporting this, it has been demonstrated that Cd induces lignification in soybean, barley, peas, and poplar roots [46–49]. Although Cu also induces root lignification in diverse plant species, in the current experimental model the effects of this metal on root topology and MF were

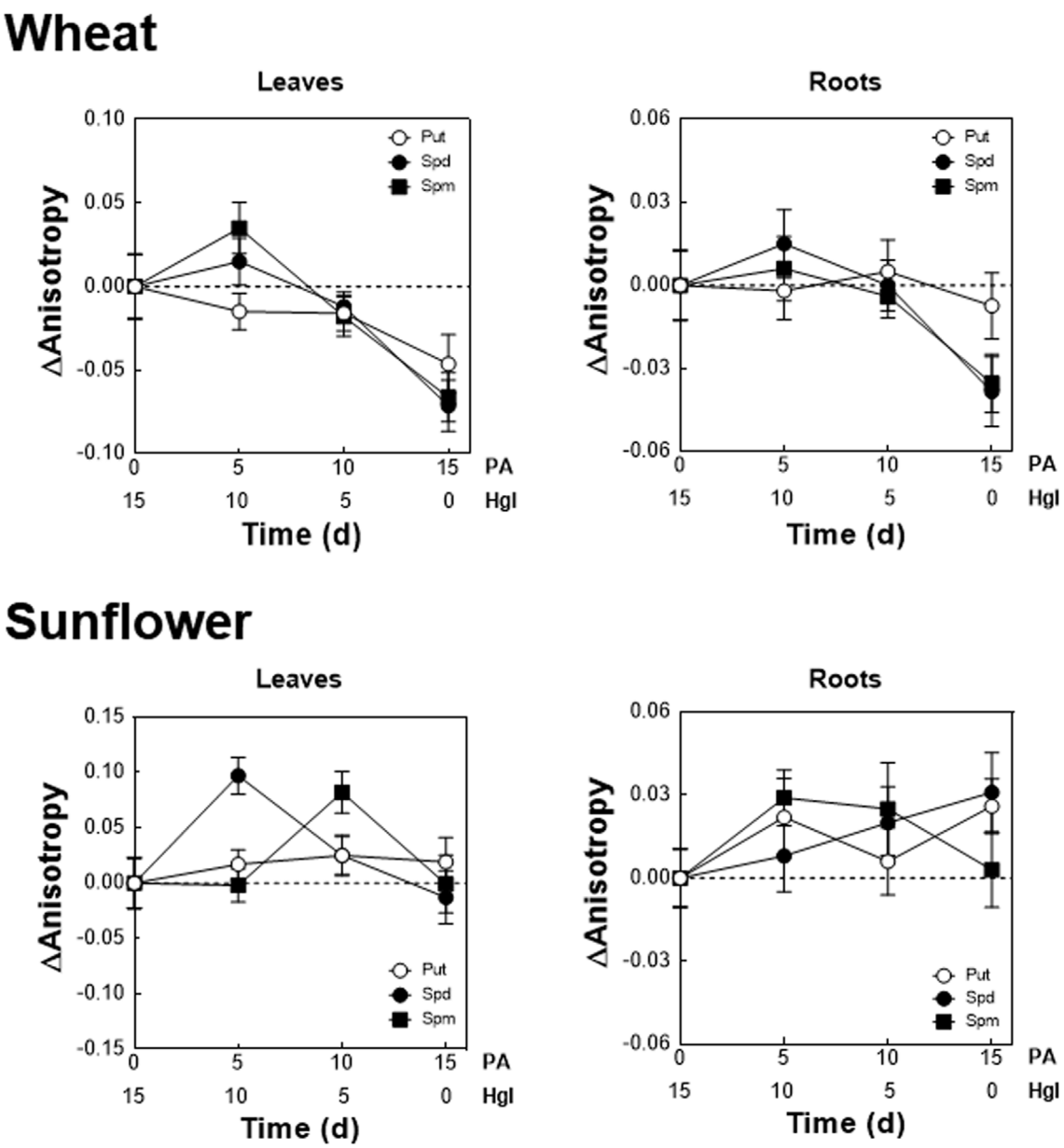

Fig. 8. Kinetics of variations in MF by PAs. Data taken from Figs. 3, 5 and 6, and expressed as Δanisotropy calculated as: $\Delta Anisotropy = Anisotropy_{PA(xd)} - Anisotropy_{Hgl(15d)}$, where x is the number of days of PA treatment.

minimal.

The current knowledge regarding the effects of PAs on membrane biophysical properties is scarce, and rely on a pioneer work of Roberts et al. [50] who demonstrated *in vitro* that PA interaction with liposomes composed of lipids isolated from bean leaf microsomes decreased their fluidity. However, we found no further reports about the effects of PAs on MF of plant membranes in cells of leaves or roots in whole plants. Since 5-d pretreatment with the PAs did not ameliorate the inhibition of plant growth by the metals, we decided to investigate if MF remained affected as well. In the absence of PAs, MF of Wh leaves and roots upon Cd exposure was comparable to those of the controls. In contrast, lower anisotropy values were observed in the leaves of Cu-exposed plants (13%, $P < 0.05$ *vs.* Hgl) (Fig. 5). To notice, in Wh plants pretreated with Put and subsequently exposed to Cd, the anisotropy of the leaves was 16% higher ($P < 0.05$) than the value measured in plants exposed only to Cd. Put and Spd did not prevent the increase in MF caused by Cu. On the other hand, Spm *per se* decreased MF of Wh leaves ($P < 0.05$ *vs*. Hgl) and thus, the subsequent exposure to Cd or Cu resulted in plants with decreased leaf MF comparable to Spm-treated plants. The metals did not affect MF of Wh roots, and the presence of Put also have no effects on this parameter. Since Spd caused a tendency towards higher anisotropy in Wh roots (12%, $P = 0.2$), the value recorded in plants treated with Spd plus Cu was significantly lower (13%, $P < 0.05$) than that in plants treated with Spd alone. Spm did not affect Wh roots MF by itself, but the anisotropy recorded in plants treated with Spm plus Cu was significantly higher than that measured in plants treated only with Cu (25%, $P < 0.05$).

As observed in Wh leaves, Cu increased MF in Sf leaves (17%, $P < 0.05$ *vs*. Hgl; Fig. 5). Similarly, plant treatment with Put plus Cd markedly increased the anisotropy respect to the value measured in plants treated only with Cd (34%, $P < 0.05$). Spd *per se* increased the anisotropy in 57% respect to the controls ($P < 0.01$). Also, and like the observed in the absence of PAs, Cu decreased the anisotropy in 19% ($P < 0.005$) respect to the value measured in the plants treated only with Spd. In contrast to the observed in Wh plants, Spm *per se* did not affect MF of Sf leaves. However, in Spm-treated plants, Cu increased the

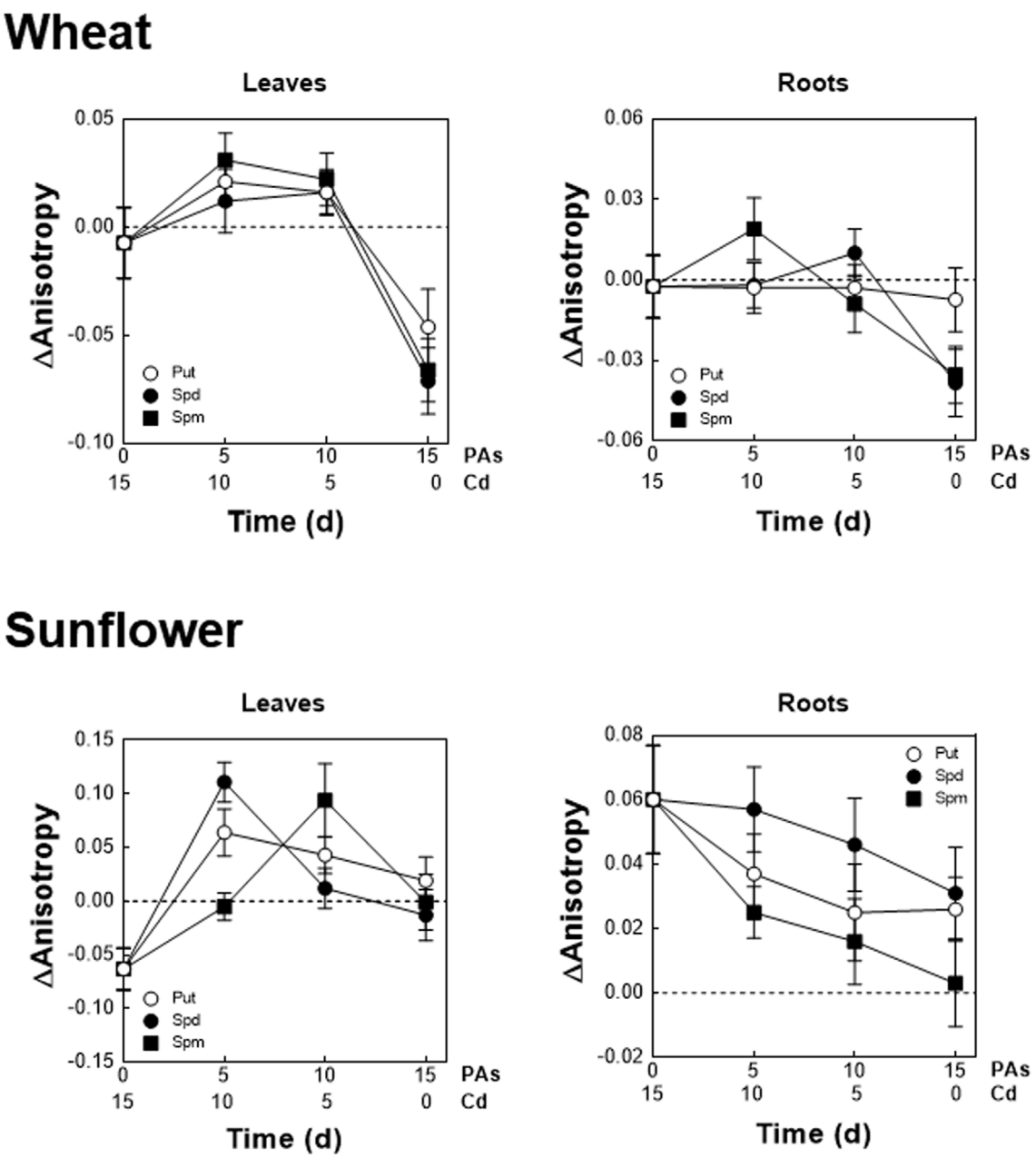

Fig. 9. Kinetics of variations in MF by Cd. Data taken from Figs. 3, 5 and 6, and expressed as Δanisotropy calculated as: $\Delta Anisotropy = Anisotropy_{PA \rightarrow Cd(xd)} - Anisotropy_{Hgl(15d)}$, where x is the number of days of Cd treatment.

anisotropy values in ~17% respect to Spm or to the controls (Hgl), ($P < 0.05$). Remarkably, the value recorded in Sf leaves treated with Spd plus Cu was 38% higher than that measured in plants treated with Cu alone ($P < 0.001$), suggesting that the presence of Spd determines the response of these plants to Cu. In Sf roots, Cd increased the anisotropy in 17% ($P < 0.05$) respect to the value measured in the controls. This cation also increased the anisotropy in plants pretreated with Put (27%, $P < 0.01$ *vs*. Hgl) or Spd (42%, $P < 0.01$ *vs*. Hgl) but without affecting MF in Spm-treated plants. On the other hand, Cu *per se* did not modify MF of Sf roots, although it caused an increase in anisotropy values in the presence of Put (28%, $P < 0.05$ *vs*. Hgl) or Spd (21%, $P < 0.05$ *vs*. Hgl). Spm-treated Sf roots showed increased values of anisotropy respect to the controls (21%, $P < 0.05$) with no additional effects observed for the cations.

Finally, the effects of 10 d-pretreatment with the PAs on metal-mediated alterations on MF were evaluated (Fig. 6). In Wh leaves, the metals had no effects on MF. However, Cd increased the anisotropy values in the presence of Put (25%, $P < 0.05$), Spd (7%, $P < 0.05$) or Spm (32%, $P < 0.01$) respect to the values recorded in plants treated only with Cd. In Wh roots, only Cu decreased the anisotropy value (20%, $P < 0.05$), with no differences observed for the remaining experimental situations. Only Cu affected MF of Sf leaves (7%, $P < 0.05$ *vs*. Hgl). In plants treated with Put or Spm, Cd significantly increased the anisotropy value respect to that measured in plants exposed only to Cd (22% and 44% for Put plus Cd and Spm plus Cd, respectively, $P < 0.05$). It is worth to mention that, since Spm increased this parameter in Sf leaves *per se*, the effect observed in plants treated with Spm plus Cd could not be ascribed to the metal but to the PA. A similar result was obtained in the leaves of Sf plants treated with Spm plus Cu (21%, $P < 0.05$ *vs*. Cu alone). In contrast to that observed in Sf plants pretreated for 5 d with Spm, this PA significantly increased anisotropy values in the leaves when plants were exposed for 10 d to Spm alone or to Spm plus Cd, compared to control values. None of the PAs affected MF of Sf roots. However, in Spd-treated plants a significant increase in

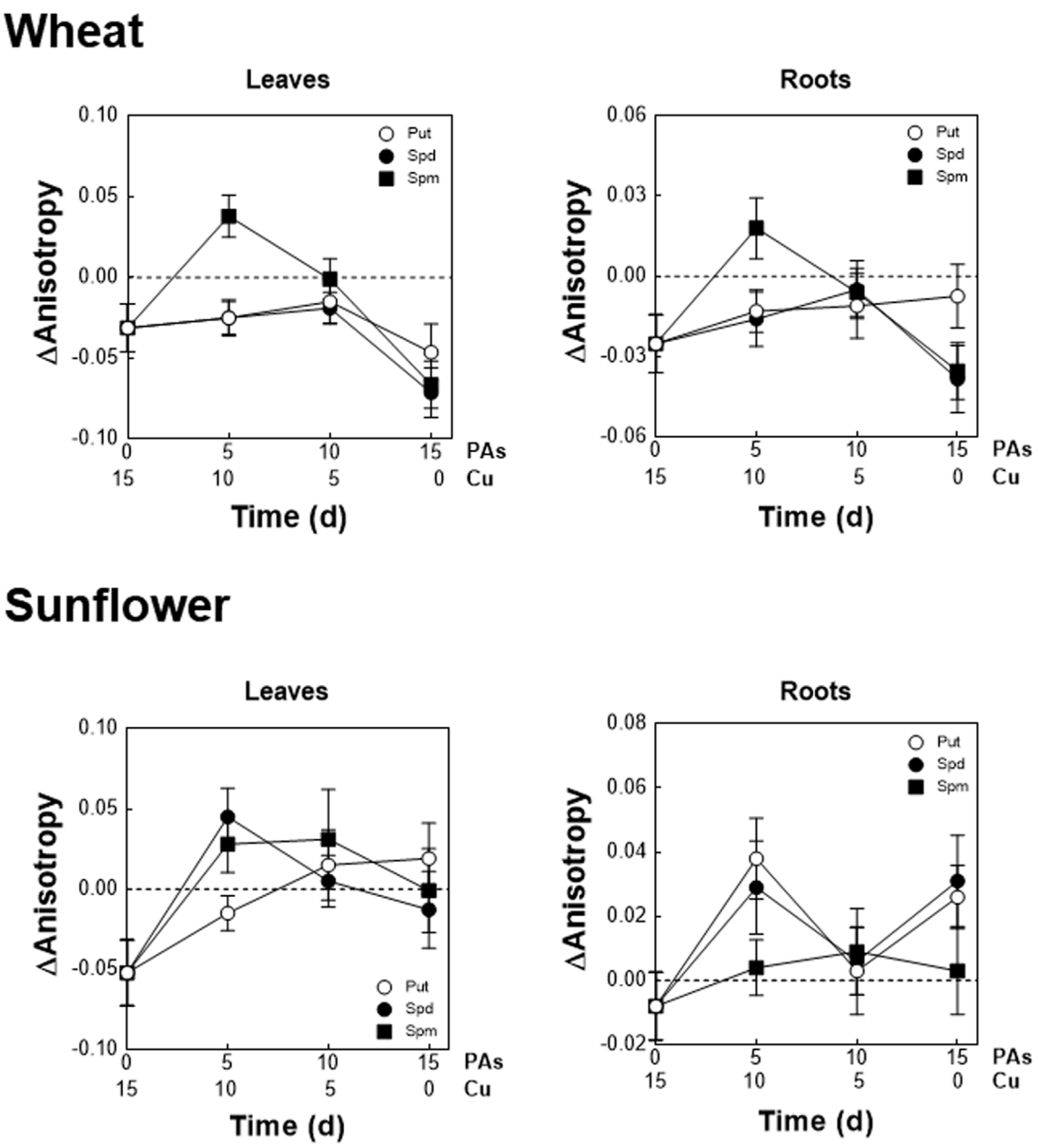

Fig. 10. Kinetics of variations in MF by Cu. Data taken from Figs. 3, 5 and 6, and expressed as Δanisotropy calculated as: $\Delta Anisotropy = Anisotropy_{PA \rightarrow Cu(xd)} - Anisotropy_{Hgl(15d)}$, where x is the number of days of Cu treatment.

the anisotropy value was observed in Cd-exposed plants (28%, $P < 0.005$ *vs*. Hgl). On the other hand, MF measured in Cu-treated plants remained within control values regardless the PA assessed.

Interestingly, the analysis of metal behavior on MF from a kinetic point of view reveals that metals exerted their effect in a manner that depended not only on the length of metal exposure, but also on the stage of plant development at the start of the exposure (Fig. 7). When considering the leaves, Cu –and to a minor extent, Cd– increased their MF as evidenced from the negative Δanisotropy values recorded. On the other hand, MF of the roots were slightly increased in Wh plants and decreased in Sf plants from day 10. Hence, the earlier the onset of seed/plant exposure to heavy metals, the higher the impact on MF. This kind of response is expected, as developing plants require high amounts of nutrients, causing the accumulation of heavy metals along with the uptake of essential ones, and stresses the necessity of soil quality maintenance to obtain healthy crops.

Also, the kinetic analysis of the results suggests a different response of the plant species to the addition of exogenous PAs (Fig. 8). Overall, PAs have fluidifying effect in Wh tissues, but they have the opposite effect in Sf ones. Put increased progressively MF of Wh leaves, as indicated by the negative Δanisotropy values obtained throughout the period assessed. In contrast, Spd and Spm caused an initial decrease in MF followed by a fluidifying effect at prolonged times. A similar pattern of response to PAs was also observed in Wh roots. In contrast, the effect of the PAs in Sf leaves seems to depend on the molecular size and/or the charge of the individual PA. Put, the smallest and less charged one, had an almost negligible effect on MF throughout the period assessed. Spd, the intermediate one, presented an initial rigidifying effect that reached a maximum at day 5 and that returned to baseline values at prolonged times. Finally, Spm –the longest and most charged molecule assessed– had a delayed effect on membrane rigidification, reaching a maximum at day 10 and returning to baseline values at prolonged times. Put and Spd decreased Sf root MF throughout the period investigated, while Spm decreased their MF only in the first 10 days of exposure. The

difference in the kinetics of PA-mediated changes in Sf leaf MF may be caused by differences in the time required to transport the larger PAs to the leaves and to cause their local accumulation. The restauration of MF in Sf leaves found by day 15 could be caused by either the homeoviscous adaptation of the system through alterations in lipid and/or sterols composition, or the metabolization of the PAs to compounds with no effects on MF. Regardless the mechanism involved in the restoration of leaf membrane fluidity, it was absent in Wh plants, as theirs varied continuously throughout the period assessed.

In leaves, the kinetics of MF variations shows that the combination of PAs and Cd resulted in plants with decreased MF (Fig. 9). To notice, in Wh leaves the three PAs had similar kinetic profile on Cd-mediated alteration in MF. In contrast, in Sf leaves, Put and Spd caused maximal membrane rigidification at 5 d of pretreatment while Spm caused the maximal effect at 10 d of pretreatment. On the other hand, the PAs increased MF of Sf roots, minimizing the rigidifying action of Cd. The higher MF observed in this situation could be related to the fact that, even though PAs did not fully alleviate growth impairment in Cd-treated plants, the topology of the roots was less affected than in plants exposed only to Cd. Finally, a different pattern of response was observed in plants exposed to Cu (Fig. 10). Put and Spd had almost no effects on MF of Wh leaves and roots exposed to Cu. In contrast, 5 d of pretreatment with Spm increased the rigidity of the membranes, an effect that was observed in both organs. In Sf plants, where Cu increased MF, the presence of the PAs caused an initial rigidification of the membranes, followed by the restoration of MF to control values by day 10.

The accumulation of Cd and Cu in agricultural environments is related to a general decrease in crop yield. Results obtained in this work indicate that both metals not only impair the growth of these plant species of agronomic interest, but also affect the structural organization and integrity of their organs, leading to chlorosis and necrosis of the tissues. PA supplementation could partially alleviate the alterations produced in cell membranes of Cu-exposed plants by preventing the modifications of plant MF, even though this approach cannot fully avoid the impairment of plant growth and dehydration. On the other hand, PAs were not capable of preventing the noxious effects of Cd on Sf plants, with partial success on preventing its effects on Wh plants. However, the maintenance or even the increase in chlorophyll levels induced by PAs in Sf plants could be an advantage for leaf tissues to avoid the potential oxidative damage produced by the metals. The different response of these two kinds of plants –Wh (monocotyledonous) and Sf (dicotyledonous)– to a same toxic metal, stresses the importance of knowing the molecular mechanisms underlying heavy metal-mediated toxicity for more than one individual vegetal species and remarks the importance of using other models besides *Arabidopsis thaliana* to attain a more complete understanding of the noxious effects of heavy metals on plant physiology.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.abb.2018.07.008.

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