









# Restricted O<sub>2</sub> consumption in pea roots induced by hexanoic acid is linked to depletion of Krebs cycle substrates

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## Abstract

Plant roots are exposed to hypoxia in waterlogged soils, and they are further challenged by specific phytotoxins produced by microorganisms in such conditions. One such toxin is hexanoic acid (HxA), which, at toxic levels, causes a strong decline in root O<sub>2</sub> consumption. However, the mechanism underlying this process is still unknown. We treated pea (*Pisum sativum* L.) roots with 20 mM HxA at pH 5.0 and 6.0 for a short time (1 h) and measured leakage of key electrolytes such as metal cations, malate, citrate and nonstructural carbohydrates (NSC). After treatment, mitochondria were isolated to assess their functionality evaluated as electrical potential and O<sub>2</sub> consumption rate. HxA treatment resulted in root tissue extrusion of K<sup>+</sup>, malate, citrate and NSC, but only the leakage of the organic acids and NSC increased at pH 5.0, concomitantly with the inhibition of O<sub>2</sub> consumption. The activity of mitochondria isolated from treated roots was almost unaffected, showing just a slight decrease in oxygen consumption after treatment at pH 5.0. Similar results were obtained by treating the pea roots with another organic acid with a short carbon chain, that is, butyric acid. Based on these results, we propose a model in which HxA, in its undissociated form prevalent at acidic pH, stimulates the efflux of citrate, malate and NSC, which would, in turn, cause starvation of mitochondrial respiratory substrates of the Krebs cycle and a consequent decline in O<sub>2</sub> consumption. Cation extrusion would be a compensatory mechanism in order to restore plasma membrane potential.

## 1 | INTRODUCTION

Low-molecular-weight monocarboxylic acids (LMWA) can accumulate to high levels in waterlogged soils (Armstrong & Armstrong, 1999; Strobel, 2001), and at such concentrations, these acids become toxic to plants (Shabala, 2011). However, plants have evolved defense

mechanisms against this chemical toxicity. For example, it has recently been shown that subtoxic concentrations of LMWA can serve an important role as triggers for the formation of an inducible root barrier to radial O<sub>2</sub> loss (ROL) (Colmer et al., 2019). In *Oryza sativa* (rice), the ROL barrier was induced by acetic, propionic, butyric (BtA) and hexanoic (HxA) acids at concentrations that did not impact root respiration

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and only slightly reduced root extension rates, whereas in *Hordeum marinum* the concentrations that resulted in barrier formation also significantly reduced root extension rates, except in the case of acetic acid (Kotula et al., 2014).

The toxicity of the undissociated forms of acetic, propionic, butyric and hexanoic acids has recently been demonstrated for excised root tips of rice (Colmer et al., 2019). The dose–response relationship between root tissue respiration and concentration of the undissociated acids showed that root tip respiration was reduced by 50% in the range of 1.2–2.7 mM, with BtA and propionic acid being the most toxic ones (Colmer et al., 2019). The specific mode of action on the respiratory system is unknown, but there are strong hints that the toxicity may be related to a nonspecific membrane depolarization, resulting in loss of electrolytes in general and  $K^+$  in particular (Lee, 1977; Pang et al., 2007).

Plants can excrete inorganic electrolytes and assimilate C from their roots (Badri & Vivanco, 2009; Canarini et al., 2019), including soluble carbohydrates, amino and organic acids and secondary metabolites. It has been suggested that this phenomenon is driven by carbon surplus accumulated when plant growth is limited, in particular during nutrient limitation (Prescott et al., 2020).

Root exudation of sugars has been related to the establishment of interactions with beneficial microorganisms, manipulation by pathogenic microorganisms and parasitic plants and in response to abiotic stresses, such as mineral or water deficiency (Hennion et al., 2018). It has been suggested that, even if not secreted as major components in the rhizosphere, sugars could act as general chemoattractants for microorganisms, especially glucose and fructose (Badri et al., 2013). Although little is known about the mechanism and the transporters involved in these processes, it has been proposed that members of the SWEET (Sugar Will Eventually be Exported Transporters) family could be responsible for sugar export at the root plasma membrane (Canarini et al., 2019, and references therein). Interestingly, the extrusion of simple sugars from roots has been reported as a response to P starvation (Carvalhais et al., 2011).

Among the electrolytes, roots can excrete organic acids, mainly citrate and malate (Wu et al., 2018), and such excretion has often been associated with tolerance to stresses. Organic acids secreted in the rhizosphere are involved in phosphate solubilization, obtained from nearly insoluble Ca-, Al- and Fe-Pi salts (Koyama et al., 1988), in the acquisition of iron in Strategy I plants (Kobashi & Nishizawa, 2012), in the detoxification in acidic soils by chelating aluminum (Kochian et al., 2004) and copper (Murphy et al., 1999), and in the establishment and regulation of relationships with the soil microbial community (Rudrappa et al., 2008). The best characterized carriers present in the plasma membrane that are involved in citrate and malate efflux belong to the ALMT (aluminum-activated malate transporters) family (Roberts, 2006; Sasaki et al., 2004; Sharma et al., 2016). Their most studied role regards the interactions of excreted organic acids with  $Al^{3+}$  in the soil, leading to insoluble (with malate) or complexed (with citrate) forms, representing an efficient strategy to overcome such metal toxicity (Kar et al., 2021; Sharma et al., 2016; Wu et al., 2018).

Loss of organic electrolytes can interfere with respiration, directly linked to the Krebs cycle (tricarboxylic acid (TCA) cycle), which

converts pyruvate and cytosolic malate to  $CO_2$  and leads to the production of  $FADH_2$  and NADH. These coenzymes fuel oxidative phosphorylation, allowing most of the ATP production of the cell (Ferne et al., 2004). Many organic acids, and TCA cycle intermediates as well, are further involved in some physiological and metabolic processes in plant cells and organs, including the biosynthesis of amino acids and fatty acids (Igamberdiev, 2020; Igamberdiev & Eprintsev, 2016; Medeiros et al., 2021). Therefore, the exchange of TCA cycle intermediates between mitochondria and cytosol is necessarily very efficient in sustaining the multiple metabolic needs of the cell. The mitochondrial transport of organic acids has been characterized, and many carriers have been identified (Toleco et al., 2020). In particular, carriers have been described for shuttling of dicarboxylates and tricarboxylates, among which are malate, succinate, 2-oxoglutarate, oxaloacetate, fumarate citrate and isocitrate. Three main mitochondrial carriers that have been described in plants are classified as dicarboxylate carriers (DICs), dicarboxylate/tricarboxylate carriers (DTC) and succinate/fumarate carriers (SFC) (Toleco et al., 2020).

In this work, we focused our research on the effects of HxA on pea roots to identify the mechanisms involved in its phytotoxicity. Since this organic acid induces a dramatic decrease in  $O_2$  consumption, we tested the hypotheses that its toxicity was mainly due to (1) leakage/extrusion of metabolites, (2) a direct effect on root mitochondria metabolism or (3) an interplay between these two possibilities. We tested the hypotheses by evaluating key physiological parameters at both tissue ( $O_2$  consumption and leakage of electrolytes and solutes) and mitochondrial level (formation of transmembrane electrical potential and  $O_2$  consumption).

## 2 | MATERIALS AND METHODS

### 2.1 | Plant materials, growth conditions and treatments

Seeds of *Pisum sativum* (L.) cultivar “Meraviglia d'Italia” (Ingegnoli, Italy) were sown in river sand. Etiolated seedlings were maintained in darkness at 25°C and 60% relative humidity and daily watered with distilled water. The primary roots from 5 to 7-day-old seedlings (approximately 2 g for each sample) were excised and incubated in 40 mL of 20 mM MES-KOH at either pH 5.0 or 6.0 (controls) and in 20 mM MES-KOH at either pH 5.0 or 6.0 with either 20 mM HxA or 20 mM BtA. The roots were incubated in Falcon tubes at 25°C for 1 h. Following the incubation, the roots were rinsed three times in distilled water before being transferred to 20 mL glass vials filled with distilled water. The glass vials were stored at 5°C for 20 h, and the solutions were used for the experiments. The roots were weighed (fresh mass [FM]) and microwaved for 3 min at 600 W to stop further enzymatic activities, dried at 55°C for 24 h and weighed again. Finally, the roots were ground into fine powder with mortar and pestle and stored dry in sealed tubes at room temperature.

## 2.2 | O<sub>2</sub> consumption of root tissues

Rates of O<sub>2</sub> consumption by excised root segments were measured following the procedure described in Colmer et al. (2019) using a MicroResp system (Unisense A/S, Denmark). Root segments were inserted into a 4-mL glass vial containing 20 mM MES-KOH at either pH 5.0 or 6.0. Each vial was fitted with a stir bar and a mesh to separate the stir bar and tissue, and the stirring rate was set to 600 rpm (stirrer controller unit MR2-St-Co, Unisense A/S, Denmark). The sealed vials were placed in a rack and submerged into a constant temperature bath (25°C), and left to stabilize for 15 min. O<sub>2</sub> was measured in each vial using an O<sub>2</sub> optode (OP-MR, Unisense A/S) every 10–20 min, during which the pO<sub>2</sub> within the solution-filled vials declined from approximately 275–235 μmol L<sup>-1</sup> O<sub>2</sub> (ca. 22–18 kPa); air equilibrium of O<sub>2</sub> at 25°C is 257.9 μmol L<sup>-1</sup>. Vials with incubation medium, but without tissues, served as blanks. O<sub>2</sub> consumption rates were calculated as the difference in O<sub>2</sub> concentration (μmol L<sup>-1</sup> O<sub>2</sub>) between two time points (Figure S1, Supporting Information) multiplied by the vial volume using Rate (SensorTrace Suite version 3.1.50, Unisense A/S) and finally divided by the FM of the tissue to obtain the O<sub>2</sub> consumption rate in nmol O<sub>2</sub> g<sup>-1</sup> FM s<sup>-1</sup>. O<sub>2</sub> consumption was measured for 30 mm root segments (10–40 mm zone behind the tip, representing the recently “expanded tissues”) with short-term (1 h) exposures to HxA. Two primary roots of 60–80 mm length were excised from 5 to 7-day-old seedlings and incubated for 1 h at 25°C in 35 mL 20 mM MES-KOH without (controls) or with increasing concentrations of HxA up to 20 mM total concentration. Following the 1-h incubation, 30 mm long root segments were excised and inserted into the 4 mL glass vial of the MicroResp system containing 20 mM MES-KOH. The rate of O<sub>2</sub> consumption by these tissues was measured typically within 45 min. O<sub>2</sub> consumption was also measured for segments of roots that had been exposed to HxA for 4 h and then measured using the MicroResp system as described above.

## 2.3 | Detection of electrolytes and solutes in roots and in root exudates

The electrical conductivity of the solutions (EC<sub>1</sub>) was measured at 25°C using an EC meter (CO300, VWR, Denmark). The vials were then autoclaved for 2 h, chilled to 25°C and EC was again measured (EC<sub>2</sub>). The relative leakage of electrolytes from the roots was calculated as EC<sub>1</sub> divided by EC<sub>2</sub> and thus expressed in relative units.

The concentrations of K<sup>+</sup>, Ca<sup>2+</sup>, Na<sup>+</sup> and Mg<sup>2+</sup> in the solutions were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 2000 DV, Perkin-Elmer Instruments Inc., Waltham, MA, USA, CT) equipped with an autosampler.

Malate concentration in the roots and in the solutions was measured as NADH formation (Bergmeyer, 1974) using the Megazyme L-Malic acid assay kit (Bray Business Park, Bray, Co., Wicklow, Ireland). The increase in absorbance was detected at 340 nm by a multiplate reader (Perkin-Elmer, Victor 3). The amount of malate in

the roots was detected after resuspension of the root powder (20 ± 2 mg) in 500 μL of 50 mM Tris-HCl (pH 7.5). The malate concentration in the samples was calculated using a calibration curve obtained with the standard solution provided with the enzymatic kit.

Citrate concentration in the roots and in the solutions was measured as NADH oxidation (Bergmeyer, 1974) using the Megazyme Citric acid assay kit (Bray Business Park, Bray, Co.). The decrease in absorbance was detected at 340 nm by a multiplate reader (Perkin-Elmer, Victor 3). The amount of citrate in the roots was detected after resuspension of the root powder as described above for malate detection. The citrate concentration in the samples was calculated using a calibration curve obtained with the standard solution provided with the enzymatic kit.

## 2.4 | Detection of nonstructural carbohydrates

The detection of nonstructural carbohydrates (NSC) in the roots and in the solutions was performed following the protocols proposed by Quentin et al. (2015) and Landhäusser et al. (2018), modified for small samples. To obtain the crystallized soluble NSC, each root sample (20 ± 2 mg) was put in 0.5 mL of 80% (vol/vol) ethanol, incubated at 80°C for 30 min, centrifuged at 15,000g for 3 min (Mikro 120, Hettich zentrifugen, Tuttlingen, Germany). The supernatants were transferred to a fresh tube, and the pellets were resuspended in 0.3 mL and again incubated at 80°C for 30 min and centrifuged at 15,000g for 3 min. This procedure was repeated, and the collected supernatants (approximately 1.1 mL each) were incubated at 55°C until the complete evaporation of ethanol and then resuspended in 500 μL of 50 mM Tris-HCl (pH 7.5).

The pellets, obtained after ethanol precipitation and containing root starch, were resuspended in 500 μL of 10 mM Tris-HCl (pH 6.9). The gelatinization of starch was performed by incubation at 100°C for 1 h. Starch hydrolysis was performed in the presence of 100 U α-amylase at 55°C overnight. After the addition of 500 μL of 25 mM Na-acetate (pH 4.6) and 25 U of amyloglucosidase, the reaction was performed at 70°C overnight. Finally, the reaction was stopped by boiling the samples for 5 min.

The soluble NSC content was measured in both roots and their exudates by the anthrone method (Yemm & Willis, 1954). The increase in absorbance at 620 nm was detected by a multiplate reader (Perkin Elmer Victor 3) and converted into g glucose g<sup>-1</sup> FM, using glucose as a standard.

Starch quantification was performed, after starch hydrolysis, as NADPH formation, according to Bergmeyer (1974). The samples (5 μL) were resuspended in 300 μL of 50 mM Tris-HCl, 2 M MgCl<sub>2</sub>, 50 mM NADP<sup>+</sup>, 0.4 M NaATP, 0.3 U Hexokinase and 0.5 U of Glucose-6-phosphate dehydrogenase. The reaction was carried out at 32°C for 20 min. The increase in absorbance of NADPH was detected at 340 nm by a microplate reader (Perkin Elmer Victor 3) and then converted in mg starch g<sup>-1</sup> FM, using potato starch, subjected to the same hydrolysis procedure as the samples, as a standard.

## 2.5 | Detection of root viability

Root viability was evaluated following the method described by Watanabe and Lam (2008). Briefly, after the treatment for 1 h in the presence or absence of 20 mM HxA, either at pH 5.0 or 6.0, the root tips were washed three times in distilled water, blotted with towel paper and incubated in PBS (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl and 137 mM NaCl, pH 7.4) and 2.5 μg mL<sup>-1</sup> fluorescein diacetate (FDA) for 10 min, at room temperature. The roots were then washed three times with PBS and observed under a fluorescence microscope (Leica Fluovert, filter set with excitation at 450–490 nm, dichroic 510 nm). The living cells were identified by their fluorescence after the cleavage of the acetyl moiety from the FDA by endogenous esterase activity.

## 2.6 | Isolation of mitochondria

After the root treatments described above, mitochondria were isolated, as described by De Col et al. (2018). Each bulk of roots was homogenized twice in a mortar at 4°C in 60 plus 50 mL of extraction buffer (0.3 M sucrose, 20 mM HEPES-Tris [pH 7.6], 1 mM EGTA, 1 mM DTE, 0.6% [wt/vol], PVPP, 0.3% [wt/vol] BSA). The homogenate was filtered through six layers of gauze and centrifuged at 2500 g, SS34 Sorvall rotor) for 4 min at 6°C; the supernatant was then re-centrifuged at 28,000 g for 5 min. The pellet was re-suspended and homogenized, using 60 mL of the extraction buffer, without PVPP, and centrifuged at 2500 g for 4 min. The supernatant was finally centrifuged at 28,000 g for 5 min. The resulting pellet was resuspended in 0.6 mL (final volume) of 0.25 M sucrose, 10 mM MOPS-Tris (pH 7.4), 10 μM EGTA-Tris and 0.1% (wt/vol) fatty acid-free BSA (resuspension buffer). The mitochondrial suspension was kept on ice and immediately used for protein determination, mitochondrial transmembrane electrical potential and O<sub>2</sub> consumption measurements; an aliquot of 1 mg of mitochondrial protein from each treatment was stored at -20°C for the assay of cytochrome c oxidase activity.

## 2.7 | Mitochondrial membrane potential measurement

The transmembrane electrical potential ( $\Delta\Psi$ ) was estimated by fluorescence quenching of safranin O using a PerkinElmer LS50B spectrofluorometer, as described by Casolo et al. (2005). The wavelengths were set to 495 and 586 nm (2.5 nm slit width) for excitation and emission, respectively. The assay was performed at 25°C in 0.25 M sucrose, 10 mM MOPS-Tris (pH 7.4), 10 μM EGTA-Tris, 1 mM KPi, 0.1% (wt/vol) fatty acid-free BSA, 5 μM safranin O and 0.2 mg of mitochondrial protein, in a final volume of 2 mL. The reactions were started with either 5 mM succinate or 10 mM malate plus 10 mM glutamate. Evaluation of the substrate-dependent electrical potential was obtained from the fluorescence difference before and after the complete  $\Delta\Psi$  collapse, caused by the addition of 5 μM FCCP.

## 2.8 | O<sub>2</sub> consumption by isolated mitochondria

Rates of O<sub>2</sub> consumption by mitochondria were measured in 0.25 M sucrose, 10 mM MOPS-Tris (pH 7.4), 10 μM EGTA-Tris, 1 mM KPi, 0.1% (wt/vol) fatty acid-free BSA, 5 μM safranin O and 0.2 mg of mitochondrial protein, in a final volume of 2 mL. The solutions were placed in vials and mixed at a stirring rate of approximately 100 rpm. Vials were sealed with Parafilm M to restrict O<sub>2</sub> entry. The temperature was set to 25°C using an immersion heater and monitored using a temperature microsensor (OPTO-TEMP, Unisense A/S) inserted into the vial. The vials were positioned in a chamber connected to a water bath through a circulation pump. O<sub>2</sub> was measured using a needle-equipped O<sub>2</sub> optode (OP-MR, Unisense A/S, Denmark), recording data every 1 s, during which the O<sub>2</sub> concentration within the solution declined from approximately 265–211 μmol L<sup>-1</sup> O<sub>2</sub>. The method was benchmarked with data collected with the MicroResp system, positioning vials in a rack submerged in a constant temperature bath. No significant difference was found between the two approaches (data not shown). The reactions were started with the addition of 5 mM succinate or 10 mM malate plus 10 mM glutamate, and O<sub>2</sub> consumption was recorded for at least 5 min using Logger (SensorTrace Suite 3.1.50, Unisense A/S). O<sub>2</sub> consumption rates were calculated by the linear decline in O<sub>2</sub> concentration over 100 s at least 1 min after the substrate addition.

## 2.9 | Determination of cytochrome c oxidase activity

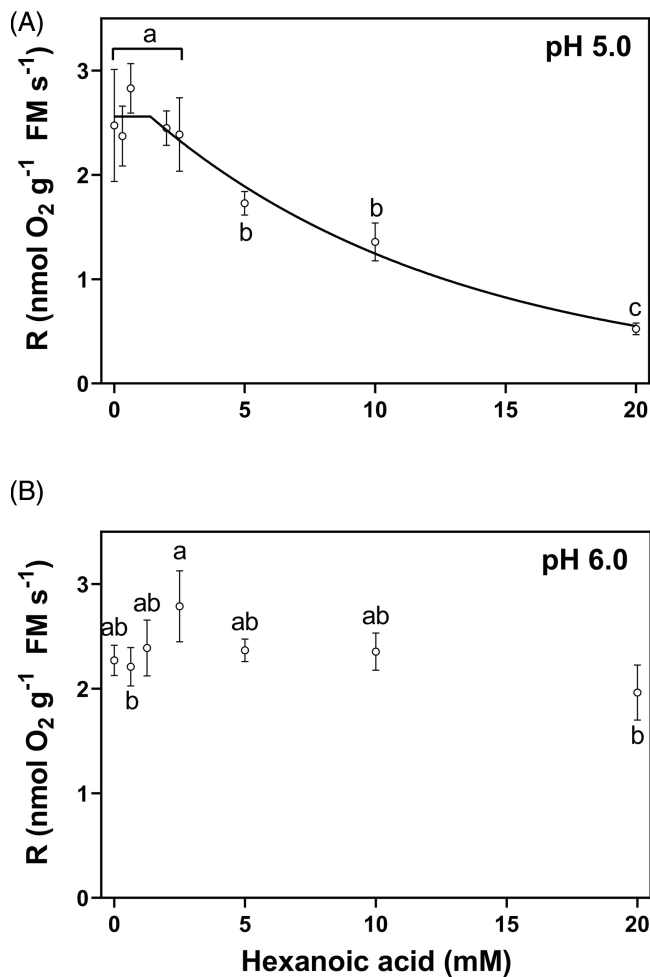
Cytochrome c oxidase activity was assayed in isolated pea root mitochondria as described by Moore and Proudlove (1983), with minor modifications. The assay was performed in 10 mM HEPES-Tris (pH 7.2), 0.3 M sucrose, 5 mM MgSO<sub>4</sub>, 0.01% (wt/vol) Triton X-100, and 50 μM reduced cytochrome c. The reactions were started by the addition of 100 μg mitochondria, and the decrease in absorbance was detected at 550 nm by a diode spectrophotometer 8453 (Agilent, Santa Clara, CA, USA).

## 2.10 | Protein quantification

The protein concentration was estimated according to the method of Bradford (1976), using BSA as a standard.

## 2.11 | Data analysis

Statistical analyses were performed using R Studio software (R Core Team, 2020). The effects of HxA, BtA or DTE were tested using two-way nested ANOVA. The test was performed using linear mixed models provided in the “nlme” package (Pinheiro et al., 2018) and including the date of the analysis as a random effect. The ANOVA test was then run on the linear mixed model using the “anova.lme” function (“nlme”



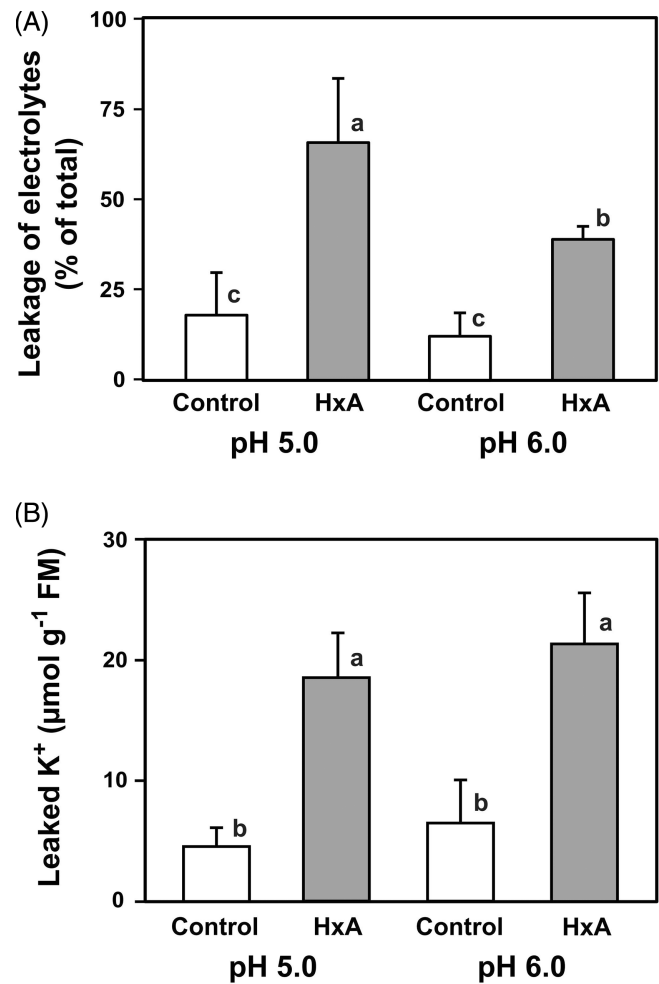
**FIGURE 1** O<sub>2</sub> consumption rate (R) of intact root tissues of *P. sativum* with increasing concentration of HxA at pH 5.0 (panel A) or 6.0 (panel B). Intact roots were first incubated for 1 h in different concentrations of HxA, ranging from zero to 20 mM. O<sub>2</sub> consumption was subsequently measured in the absence of HxA in 4 mL cuvettes on 30 mm segments of the primary root of 5–7-day-old seedlings germinated in darkness at 25°C. Data are means ± SD ( $n = 4–8$ ). Different letters indicate significant differences ( $p < 0.05$ ; ANOVA)

package). Assumptions of models were verified using the diagnostic plots of model residuals and the Shapiro–Wilk test ( $p > 0.05$ ). The least mean square test for multiple comparisons was applied using the “lsmeans” (Lenth & Lenth, 2018) and “multcomp” (Hothorn et al., 2008) packages as a post hoc test on model results ( $p < 0.05$ ), including the Tukey-adjusted comparisons as  $p$ -value adjustment term.

### 3 | RESULTS

#### 3.1 | O<sub>2</sub> consumption of intact root tissues

Some LMWA, such as acetic, propionic, butyric and hexanoic acids, are known phytotoxins in certain anaerobic waterlogged soils

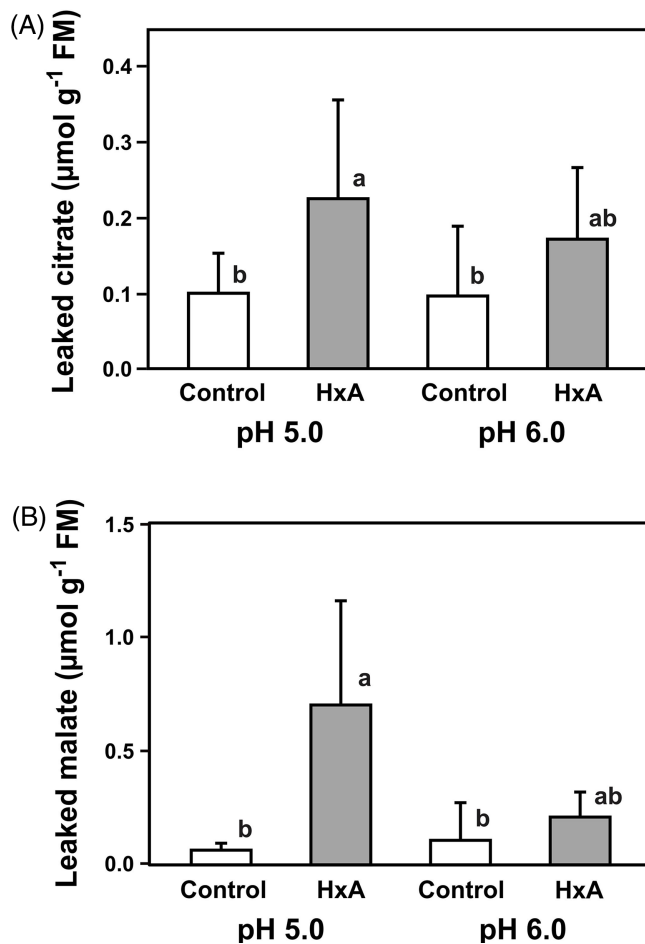


**FIGURE 2** Leakage of total electrolyte (panel A) and K<sup>+</sup> (panel B) measured on excised primary roots of *P. sativum* following 1 h incubation without (Control) or with 20 mM HxA at either pH 5.0 or 6.0. EC was measured after 20 h. Data are means ± SD ( $n = 6$ ) and letters indicate significant differences ( $p < 0.05$ ; two-way nested ANOVA)

(Armstrong & Armstrong, 1999; Shabala, 2011), and thus we tested the influence of HxA on root O<sub>2</sub> consumption during short-term exposure. First, the influence of HxA on O<sub>2</sub> consumption was established for intact primary roots.

Hexanoic acid had a dose-dependent inhibitory effect on root tissue O<sub>2</sub> consumption at pH 5.0 but not at pH 6.0 after 1 h of incubation (Figure 1). Following a short plateau (R<sub>0</sub>) where low concentrations of HxA had no significant effect on O<sub>2</sub> consumption, tissue O<sub>2</sub> consumption dramatically declined with increasing concentration of HxA. The EC<sub>50</sub>, where the O<sub>2</sub> consumption had decreased by 50% relative to R<sub>0</sub>, was 7.2 mM (Figure 1A), but even at the highest concentration used (20 mM), there was still some residual O<sub>2</sub> consumption of the tissue. In the control, without HxA in the incubation medium, tissue O<sub>2</sub> consumption was not significantly different at pH 6.0 compared with pH 5.0 (results not shown). Moreover, a follow-up experiment showed that the residual O<sub>2</sub> consumption was also present after 4 h of exposure to HxA (Figure S2). These findings





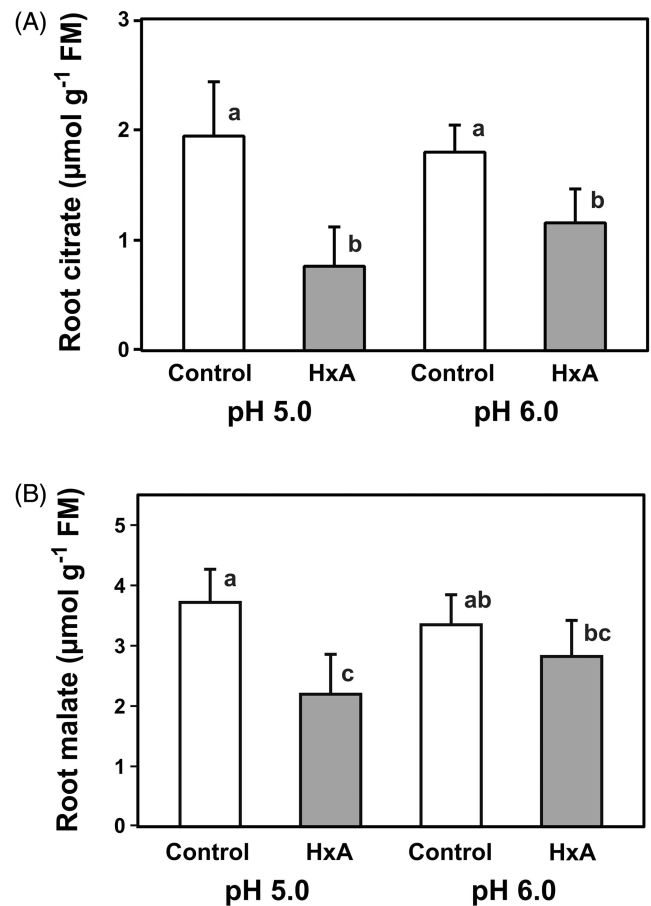
**FIGURE 3** Efflux of citrate (panel A) and malate (panel B) in primary roots of *P. sativum* after 1 h incubation without (Control) or with 20 mM HxA at either pH 5.0 or 6.0. Data are means  $\pm$  SD ( $n = 6$ ) and letters indicate significant differences ( $p < 0.05$ ; two-way nested ANOVA)

strongly suggest that the toxicity of HxA, as measured by a significant decline in tissue  $O_2$  consumption, is caused by its undissociated form but never completely restricts  $O_2$  consumption, regardless of concentration and exposure time.

### 3.2 | Leakage of electrolytes and solutes from intact root tissues

LMWA have been shown to cause leaky cell membranes (Shabala, 2011), and thus we assessed electrolyte leakage from intact root tissues that had been exposed to 20 mM HxA for 1 h. Electrolyte leakage was substantially higher from roots that had been exposed to HxA compared with controls and at pH 5.0 compared to pH 6.0 (Figure 2A), supporting the notion that these acids cause increased permeability of the plasmalemma (Shabala, 2011). Two-way nested ANOVA showed a significant effect of both HxA treatment and pH (Table S1).

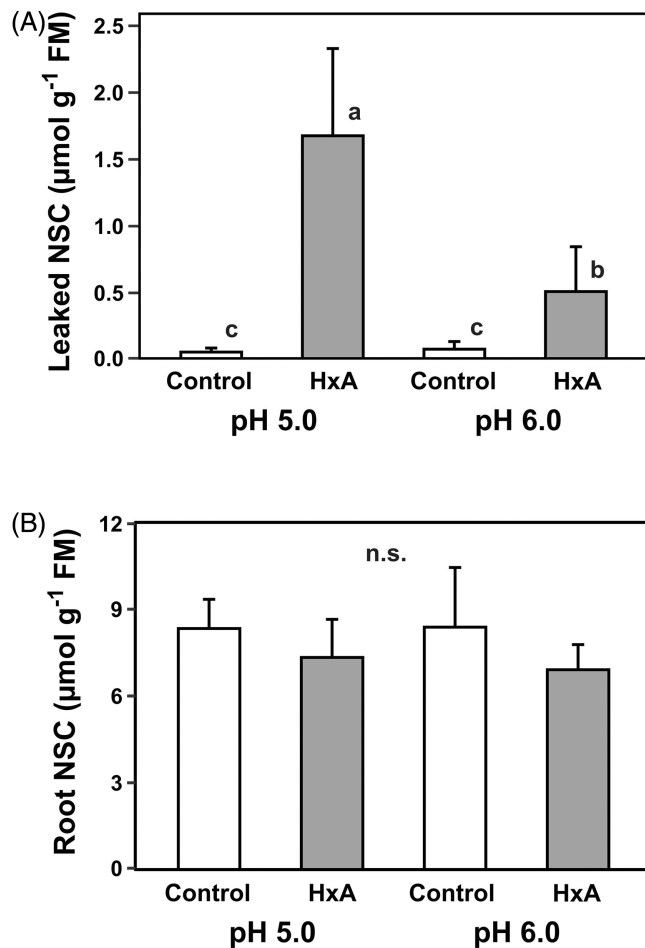
To further unravel the main compounds involved in the phenomenon, the leakage of  $K^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$  and  $Na^+$  was measured.



**FIGURE 4** Root concentration of citrate (panel A) and malate (panel B) in primary roots of *P. sativum* after 1 h incubation without (Control) or with 20 mM HxA at either pH 5.0 or 6.0. Data are means  $\pm$  SD ( $n = 6$ ) and letters indicate significant differences ( $p < 0.05$ ; two-way nested ANOVA)

Treatment with HxA increased  $K^+$  leakage, and also a pH effect was observed (higher  $K^+$  release at pH 6.0); however, conversely, with respect to root  $O_2$  consumption,  $K^+$  leakage was not affected by the interaction between them (Figure 2B and Table S2). Similarly,  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Na^+$  leakage was affected by HxA treatment, but not by pH or by their interaction (Figure S3A–C).

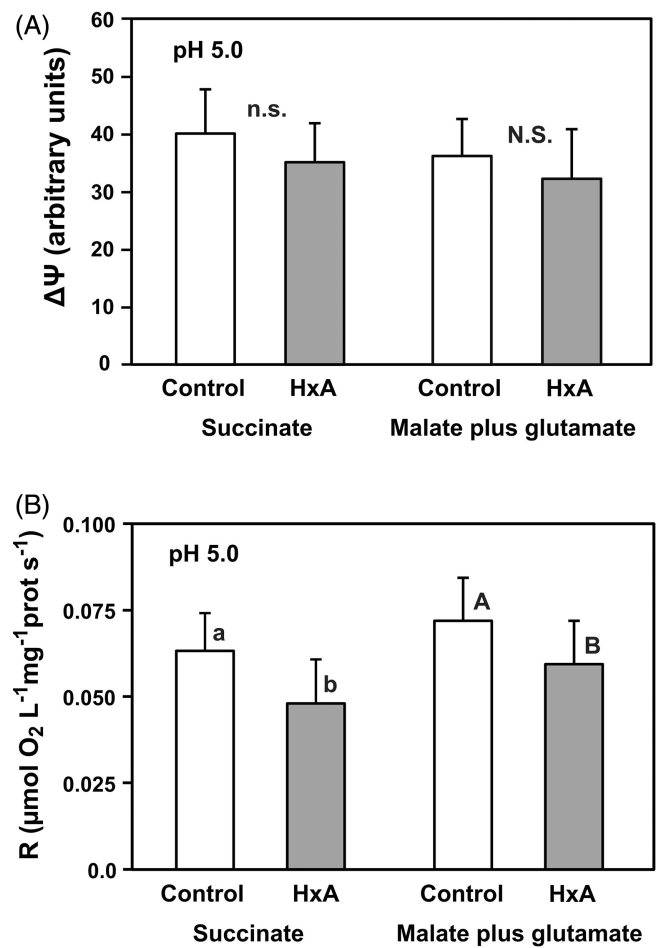
Roots can secrete many compounds in the rhizosphere under normal as well as under stress conditions (Canarini et al., 2019), mainly organic acids, among which citrate and malate are the most studied. Interestingly, in the presence of 20 mM HxA, the leakage of citrate and malate showed a pattern similar to the leakage of total electrolytes (Figure 3A,B and Table S3). In particular, the amounts of citrate and malate leaked from roots after HxA treatment were approximately 1.5- and 3.5-fold higher at pH 5.0 than at pH 6.0, respectively. The concentration of citrate and malate was also evaluated in intact roots after treatments with HxA at both pH 5.0 and 6.0. The decrease in root tissue citrate (Figure 4A) and malate (Figure 4B) concentration was consistent with the pattern observed by the release of such organic acids, showing a more pronounced effect of HxA at pH 5.0



**FIGURE 5** Efflux (panel A) and root concentration (panel B) of nonstructural carbohydrates (NSC) in *P. sativum* after 1 h incubation without (Control) or with 20 mM HxA at either pH 5.0 or 6.0. Data are means  $\pm$  SD ( $n = 6$ ) and letters indicate significant differences ( $p < 0.05$ ; two-way nested ANOVA)

with respect to pH 6.0. A very similar pattern was induced when pea roots were treated with BtA (Figures S4 and S5).

The efflux of sugars from roots is another important issue regulating the relationships between roots and the soil environment, including the interactions with soil microorganisms (Canarini et al., 2019; Hennion et al., 2018). Furthermore, glucose and fructose fuel glycolysis and mitochondrial respiration, so their evaluation is essential to establish a potential carbon starvation. The release of soluble nonstructural carbohydrates (NSC) showed a dramatic increase after root treatment with HxA (Figure 5A), with a pattern similar to those described above for total electrolytes, citrate and malate, with the higher amount of soluble NSC released after treatment at pH 5.0. However, in intact roots, the concentration of NSC was just slightly decreased by the HxA treatments at both pH levels (Figure 5B), and no significant differences were obtained for root starch, which was around  $0.2 \text{ mg g}^{-1} \text{ FM}$  in all root samples (results not shown). Again, BtA treatments resulted in very similar behavior with respect to those obtained in the presence of HxA (Figure S6).



**FIGURE 6** Evaluation of electrical potential ( $\Delta\Psi$ ) formation (panel A) and O<sub>2</sub> consumption rate (R, panel B) by mitochondria isolated from *P. sativum* roots. Pea roots were incubated at pH 5.0 for 1 h without (Control) or with 20 mM HxA. Succinate or malate plus glutamate were used as respiratory substrates. Data are means  $\pm$  SD ( $n = 4$ ) and different letters indicate significant differences ( $p < 0.05$ , *t*-test). Lower- and upper-case letters refer to separate *t*-tests applied to data

### 3.3 | Impact of root treatment with HxA on isolated mitochondria

To test if there was a direct toxic effect of HxA treatment on mitochondria or if the root leakage of electrolytes and solutes, induced by HxA, can influence the mitochondrial activities, we isolated mitochondria from the primary roots after 1 h incubation in the absence (controls) or presence of 20 mM HxA at both pH 5.0 and 6.0. The activities evaluated were: (1) the formation of transmembrane electrical potential ( $\Delta\Psi$ , negative inside the mitochondrial matrix), induced by succinate or malate plus glutamate as respiratory substrates; (2) the O<sub>2</sub> consumption, induced by succinate or malate plus glutamate and (3) the activity of cytochrome *c* oxidase.

Figure 6A shows the  $\Delta\Psi$  formation, evaluated as the decrease in safranin O fluorescence after energization with the substrates by mitochondria isolated from pea roots following incubation without

(control) or with (treatment) HxA at pH 5.0. When either succinate or malate plus glutamate was utilized, no significant difference was observed in  $\Delta\Psi$  formation by mitochondria isolated from treated roots at pH 5.0 (Table S6).

Mitochondrial respiration was slightly affected when the roots were treated with HxA at pH 5.0 (Figure 6B). In detail, mitochondria obtained from roots incubated with HxA showed a decrease in  $O_2$  consumption induced by both succinate and malate plus glutamate. Statistical analyses confirmed the significant effect of HxA treatment on  $O_2$  consumption induced by both succinate and malate plus glutamate (Table S6). The effect of HxA was negligible with respect to both succinate and malate plus glutamate-dependent  $\Delta\Psi$  formation and  $O_2$  consumption in mitochondria isolated from roots incubated at pH 6.0 (results not shown).

To test if the effect of HxA on mitochondrial activities was mediated by an induction of oxidative stress, pea roots were incubated at pH 5.0 for 1 h with or without HxA and in the absence or in the presence of 10 mM DTE. This antioxidant did not induce a recovery of  $O_2$  consumption in intact roots subjected to HxA (results not shown). Nevertheless,  $\Delta\Psi$  formation in isolated mitochondria was affected by the treatments (Figure S7), especially when succinate, rather than malate plus glutamate, was utilized as substrate. In the absence of HxA, mitochondria did not respond to DTE (results not shown). Finally, the cytochrome *c* activity of mitochondria isolated from roots treated with or without HxA at pH 5.0 did not exhibit significant differences (results not shown).

## 4 | DISCUSSION

Metabolites produced in fermentation processes by soil bacteria can accumulate at toxic levels in waterlogged soils. This is particularly important in acidic soils rich in labile organic matter where anaerobic metabolism results in the accumulation of LMWA (Shabala, 2011), among which, HxA plays a crucial role, being highly toxic to plant roots (Armstrong & Armstrong, 1999). In organic soils, high local concentrations of HxA (and BtA) in close proximity to roots could be achieved since the diffusion rate is extremely low in liquid solutions. These conditions would be exacerbated by acidification of the apoplast caused by an increase in proton pumping induced in root cells as a consequence of ion leakage and acid load (Guern et al., 1986). In the present study, we found that the respiration of pea root tissues was adversely affected by HxA already at low concentrations (5 mM), being detrimental at high concentrations (20 mM). The treatment resulted in a substantial decrease in  $O_2$  consumption, and it was linked to the leakage of electrolytes and soluble carbohydrates at the tissue level. Our dose–response experiments confirmed the toxic nature of HxA (and BtA), showing that tissue  $O_2$  consumption was greatly reduced with increasing concentration of this phytotoxin (Figure 1).

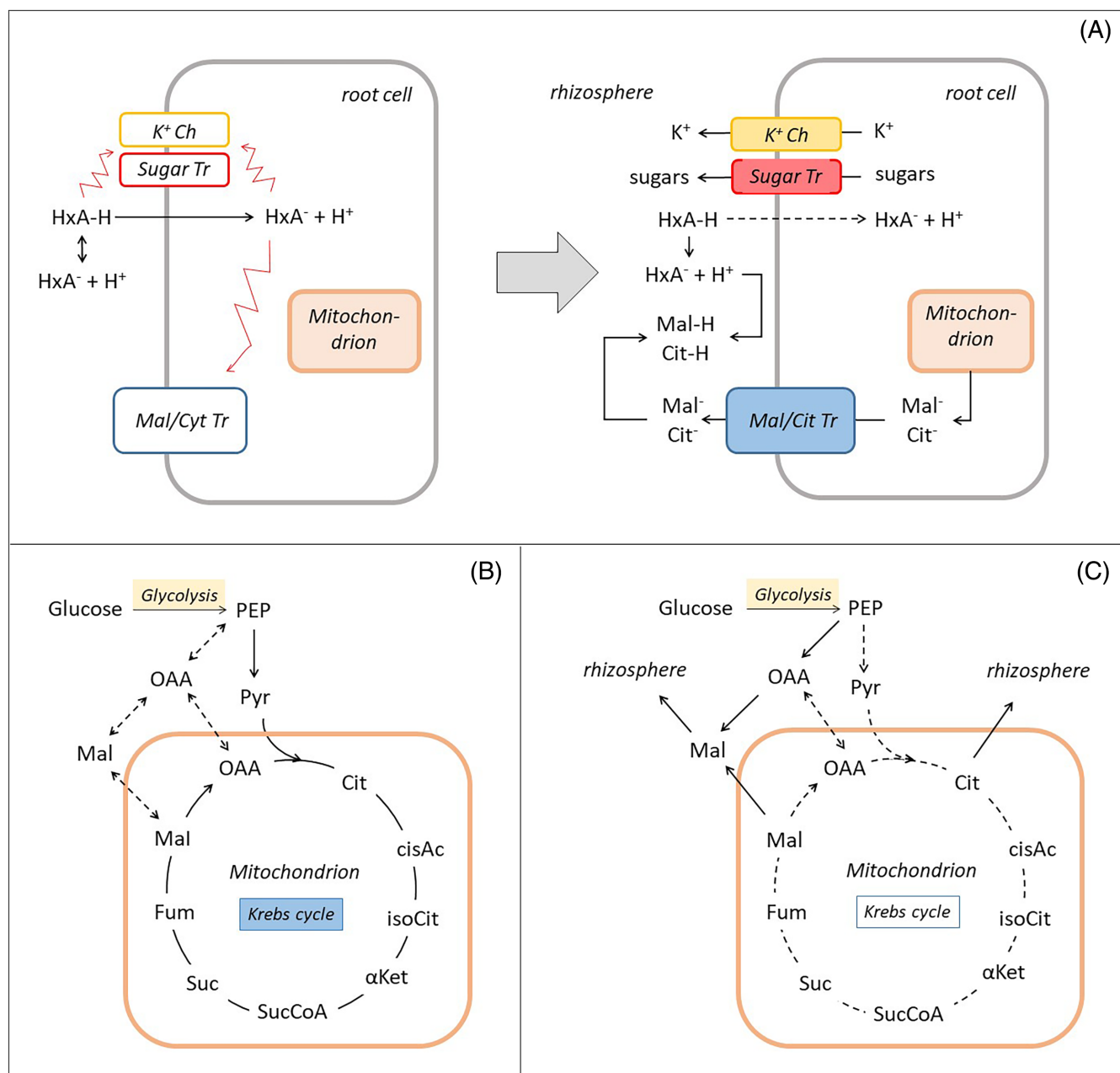
HxA has been involved in acute and subchronic phytotoxicity in seed germination, seedling elongation and plant growth in garden cress *Lepidium sativum* (Himanen et al., 2012). When incubated at low concentrations and for a long time, HxA induced the formation of a

ROL barrier and the decline in respiration in rice roots (Colmer et al., 2019). In the present study, the decline of  $O_2$  consumption was induced by HxA within 1 h of exposure (Figure 1), showing that the lower  $O_2$  consumption is not due to ROL barrier formation, which would otherwise have reduced radial  $O_2$  diffusion from the external medium into the root tissue (Jiménez et al., 2021). Below, we discuss these findings in the context of the involvement of mitochondria in an attempt to unravel the mode of action of HxA.

Previous studies have focused on the effects of organic acids on root growth (Armstrong & Armstrong, 1999; Kotula et al., 2014). Recently, Colmer et al. (2019) showed that respiration declined when roots were exposed to HxA and other organic acids, including BtA. Indeed, the adverse effect of HxA on root respiration is strongly dependent on pH (Figures 1 and 2), confirming the growth responses of rice to acetic and propionic acids, HxA and BtA, which showed greater declines at low pH (Colmer et al., 2019). Moreover, our study showed that total leakage of electrolytes from root tissues exposed to HxA was significantly higher at pH 5.0 when compared to pH 6.0 (Figure 2A). In roots of *Hordeum marinum*, HxA caused a decline in growth,  $K^+$  efflux and the induction of barrier formation to ROL via deposition of suberin in the root hypodermis/exodermis (Kotula et al., 2014). Efflux of electrolytes have been reported, for example, for nitrate and UV-absorbing materials (Lee, 1977), but a detailed explanation of the mechanism of action of low-molecular-weight monocarboxylic acids on roots is still missing. Basically, it has been proposed that these acids may induce changes in membrane structure and permeability (Lee, 1977). However, we did not observe any change in root cell viability (results not shown) that would be expected if the plasma membrane were damaged by HxA and BtA. Moreover, lipophilic weak acids have been reported to be able to cross artificial bilayer vesicles (Li et al., 2011) and the cell membrane in their undissociated form (Pang et al., 2007; Pesci & Beffagna, 1985). Indeed, considering the  $pK_a$  of HxA 4.88, the percentage of undissociated form is substantial at acidic pH: 20 mM total concentration of HxA at pH 5.0 results in around 8.6 mM of its undissociated form (HxA-H); at pH 6.0, it falls to 1.4 mM, and at neutral pH, it drops down to 0.15 mM. These values are very similar to those calculated for BtA, which possesses a comparable  $pK_a$  (4.82). Therefore, at low pH, the influx of HxA (and other organic acids) could be substantial in its neutral form, and the following events are expected to take place (Guern et al., 1986; Mathieu et al., 1986; Pesci & Beffagna, 1985): (1) decrease of cytosolic pH, (2) subsequent activation of the plasma membrane  $H^+$ -ATPase and the vacuolar proton pumps (Reid et al., 1989) and (3) activation of malate decarboxylation, with both activations (2 and 3) being instrumental in contrast the cytosol acidification. This sequence of events would, conversely to our observations, imply cell hyperpolarization, stimulation of  $K^+$  uptake (Marré et al., 1983) and increased respiration.

In our experimental conditions, we observed that respiration was inhibited, despite mitochondria isolated from HxA-treated roots were still functional. These pieces of evidence allow us to speculate that HxA and BtA have direct and/or indirect effects other than the simple acidification of the root cells, part of which also happens at pH 6.0. Hence, a more complex scenario is needed to explain HxA and BtA





**FIGURE 7** Hypothetical model of hexanoic acid action in pea root at pH 5.0. At low pH, hexanoic acid penetrates the root cell in its undissociated form ( $HxA-H$ ) and stimulates  $K^+$ , citrate, malate and NSC extrusion by an unknown mechanism; citrate and malate efflux might represent an attempt to detoxify  $HxA$ -treated cells by subtracting protons from  $HxA-H$  and therefore decreasing its entrance in the root cell, since the permeability of  $HxA^-$  is low (panel A). Citrate/malate cycle in nontreated root cell (panel B). Citrate/malate cycle in  $HxA$ -treated root cell (panel C). The leakage of citrate and malate from the cell depletes the cytosolic and mitochondrial pools, inducing a decrease in Krebs cycle and, consequently, mitochondrial respiration.  $HxA$ , hexanoic acid; Cit, citrate; cisAc, Cisaconitate; isoCit, isocitrate;  $\alpha$ Ket,  $\alpha$ -ketoglutarate; SucCoA, succinyl-CoA; Suc, succinate; Fum, fumarate; Mal, malate; OAA, oxaloacetate; PYR, pyruvate; PEP, phosphoenolpyruvate;  $K^+$  Ch, potassium channel; Sugar Tr, sugar transporter; Cit/Mal Tr, dicarboxylate/tricarboxylate transporter. Solid and dotted lines indicate complete or decreased activity of reactions or transport processes, respectively

toxicity to plant roots:  $HxA$  and  $BtA$  stimulated citrate, malate and NSC release from cells, which induced  $K^+$  efflux that could be required to compensate for the organic acids-dependent depolarization. However, dramatic  $K^+$  leakage occurred also at pH 6.0 (Figure 2B), while citrate, malate and NSC extrusion were significantly

lower compared to that observed at pH 5.0 (Figures 3, 5A and 5B–5C). These observations suggest that  $K^+$  leakage, even if appreciable at both pHs, is unlikely to be linked to the inhibition of  $O_2$  consumption. Citrate, malate and soluble NSC extrusion, on the contrary, might explain the decrease in respiration because phosphoenolpyruvate

(PEP) would be mainly used to replenish the intracellular citrate and malate by the activity of cytosolic PEP carboxylase rather than fueling the Krebs cycle. Eventually, mitochondrial respiration would suffer from substrate starvation, with a decline in  $O_2$  consumption but without a dramatic impairment of mitochondrial functionality. The proposed model is also consistent with a slight decrease in mitochondrial  $O_2$  consumption observed due to oxalacetate restriction in the matrix of mitochondria purified from treated roots and the maintenance of a minimal level of  $O_2$  consumption by the roots, even at very high concentrations of HxA after 4 h of exposure (Figure S2).

Citrate and malate extrusion from roots is a well-known strategy that plants use in response to aluminum detoxification (Kollist et al., 2011) and phosphorus starvation (Dong et al., 2004; Hoffland et al., 1992). Based on these results, we propose a model in which citrate and malate might also be involved in the limitation of the effect that HxA (and BtA) could induce by penetrating into the root cells in their undissociated form at low pH (Figure 7). Citrate and malate are both exported from roots to rhizosphere through specific organic acid anion channels as double charged anion (Kollist et al., 2011). In particular, the class of the aluminum-activated malate-transporters (ALMT) that play a role in different malate-mediated functions (Palmer et al., 2016) could be involved. However, also other less specific carriers could be involved, such as members of the SLow Anion channel Homologs (SLAH) family (Negi et al., 2008). Considering that  $pK_{a1}$  and  $pK_{a2}$  of citric acid are 3.1 and 4.8, and  $pK_{a1}$  of malic acid is 3.4, respectively, such organic acids can take up protons from the soil solution. In the acidic microenvironment of the soil space explored by roots, this would lead to a rise of pH, promoting the subsequent transition of either HxA ( $pK_a = 4.88$ ) or BtA ( $pK_a = 4.82$ ) from protonated to deprotonated form, restricting their entrance into the roots and therefore reducing their effects.

The effect of HxA (and BtA) at different pHs was also evident from the increase in extrusion of soluble NSC from roots after the treatments, especially at pH 5.0 (Figure 5A). A similar mechanism observed for organic acid release would be responsible for soluble NSC efflux, although the carriers/transporters involved in this process are still elusive (Canarini et al., 2019; Hennion et al., 2018). We suggest that the effect of HxA (Figure 5B), but also that of BtA (Figure S6B), on soluble NSC concentration in roots was negligible. Indeed, the sugar pool in parenchymatic cells of roots is quite high and can sustain the small amount of soluble NSC that is secreted. The starch content was not significantly affected by treatments, probably because the treatment with either HxA or BtA was quite short (1 h) and even if starch hydrolysis were induced, its degradation rate would be limited and eventually not sufficient to replenish the sugar pool. These observations exclude that the energetic metabolism of roots is jeopardized.

Direct effects of HxA on mitochondria seem unlikely since this organic acid when added to isolated mitochondria, did not significantly affect  $\Delta\Psi$  formation, nor did  $O_2$  consumption decline (results not shown). Indeed, once into the cytosol, at neutral pH, the majority of HxA is in its dissociated form (more than 99% as  $HxA^-$  at pH 7.2), which would be unable to cross the mitochondrial

inner membrane, being impermeable because of its charge and repelled by the negative charge of the mitochondrial matrix.

Nevertheless, we suggest that HxA could also exert effects on root mitochondria by inducing oxidative stress by ROS production, leading to alteration of mitochondrial metabolism. It is well-known that plant mitochondria are involved in ROS production, but those organelles could also be considered as sensors, acting as receptors and amplifiers of ROS produced in either apoplast or cytosol (Noctor & Foyer, 2016). Mitochondrial Complexes I, II and III are recognized as the sites of ROS production, especially when the components are highly reduced, for example, when phosphorylation is a limiting factor (Huang et al., 2016; Møller, 2001). In particular, Complex II, corresponding to succinate dehydrogenase (SDH), has been identified as potentially responsible for ROS production by single-electron reduction of  $O_2$  at FAD- and ubiquinone-binding sites (Huang & Millar, 2013). In mitochondria isolated from pea roots, SDH activity decreased when treated with HxA at pH 5.0 but not at pH 6.0, evaluated both as  $O_2$  consumption (Figure 6B). In our experiments, the possible involvement of ROS, or ROS-derived molecules, in HxA toxicity seems to be limited to the effects on isolated mitochondria, giving only a partial contribution to the inhibition of  $O_2$  consumption in intact roots: indeed, incubation of pea roots with the antioxidant DTE did neither influence  $O_2$  consumption nor did it modify electrolyte leakage (results not shown). On the contrary, mitochondria isolated from roots incubated with HxA and DTE showed a partial recovery of  $\Delta\Psi$  when succinate was used as substrate (Figure S7) and a decrease in the  $O_2$  consumption rate (results not shown). Hence, Complex II seems to be the main target of the oxidative process induced by HxA, while neither Complex I nor cytochrome *c* oxidase were affected.

In conclusion, our study demonstrates that the negative effect of HxA on pea root  $O_2$  consumption was linked to a nonspecific response, leading to leakage of solutes from pea roots. We showed that HxA inhibition of  $O_2$  consumption in pea roots was exerted mainly at pH 5.0 when the concentration of its neutral form (HxA-H) is appreciable and capable of crossing the plasma membrane (Figure 7). Therefore, we propose that the entrance of HxA-H, and its dissociation at neutral cytosolic pH, induces the efflux of cellular citrate, malate and soluble NSC (Figures 3 and 5) and the concomitant leakage of other solutes such as  $K^+$  and  $Mg^{2+}$  (Figures 2B and S3B). In particular, citrate and malate extrusion from treated roots would represent a strategy to protect the plant from the detrimental effects of HxA. The metabolism of root cells is consequently affected, and in particular mitochondrial respiration, since the TCA cycle is limited by the lack of metabolites and cellular solutes, which declined after 1 h of HxA exposure. Since the metabolic activities of isolated mitochondria were only marginally influenced by HxA treatments, we conclude that the oxidative metabolism is mainly inhibited by starvation of TCA cycle substrates and only slightly affected by the induction of oxidative stress.

## AUTHOR CONTRIBUTIONS

Valentino Casolo, Marco Zancani and Ole Pedersen planned and designed the experiments. All authors performed various subexperiments. Elisa Pellegrini executed the statistical analyses. All authors contributed to result interpretation and discussion. Valentino Casolo,

Marco Zancani and Ole Pedersen wrote the paper. All authors were involved in manuscript editing.

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## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as all new created data is already contained within this article (and in the supplementary material).

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#### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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