Research article

Aluminum directly inhibits alternative oxidase pathway and changes metabolic and redox parameters on *Jatropha curcas* cell culture

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**ABSTRACT**

Aluminum (Al) toxicity has been recognized to be a main limiting factor of crop productivity in acid soil. Al interacts with cell walls disrupting the functions of the plasma membrane and is associated with oxidative damage and mitochondrial dysfunction. *Jatropha curcas* L. (*J. curcas*) is a drought resistant plant, widely distributed around the world, with great economic and medicinal importance. Here we investigated the effects of Al on *J. curcas* mitochondrial function and cell viability, analyzing mitochondrial respiration, phenolic compounds, reducing sugars and cell viability in cultured *J. curcas* cells. The results showed that at 70 μM, Al limited mitochondrial respiration by inhibiting the alternative oxidase (AOX) pathway in the respiratory chain. An increased concentration of reducing sugars and reduced concentration of intracellular phenolic compounds was observed during respiratory inhibition. After inhibition, a time-dependent upregulation of AOX mRNA was observed followed by restoration of respiratory activity and reducing sugar concentrations. Cultured *J. curcas* cells were very resistant to Al-induced cell death. In addition, at 70 μM, Al also appeared as an inhibitor of cell wall invertase. In conclusion, Al tolerance in cultured *J. curcas* cells involves a inhibition of mitochondrial AOX pathway, which seems to start an oxidative burst to induce AOX upregulation, which in turn restores consumption of O₂ and substrates. These data provide new insight into the signaling cascades that modulate the Al tolerance mechanism.

1. Introduction

Aluminum (Al) is the most abundant metal and the third most abundant element of the earth's crust. Al is a major component of the soil (7% of mineral soils) and most exists in a bounded state. Al is known to be toxic to the roots of plants in its soluble form, which occurs when soil becomes acidic. Al accumulation in the root leads to the inhibition of root growth (Blamey et al., 2004; Kochian et al., 2015) due to its interactions with the cell walls, disrupting the functions of the plasma membrane (Horst et al., 2010; Ishikawa and Wagatsuma, 1998; Jones and Kochian, 1997). Al toxicity has been recognized to be a main limiting factor of crop productivity in acid soil (Magalhaes et al., 2018; Uexküll and Mutert, 1995).

Al toxicity in plants is also believed to be associated with oxidative damage and mitochondrial dysfunction (Yamamoto et al., 2002; Boscolo et al., 2003; Panda et al., 2008; Li and Xing, 2011). Mitochondria, referred as "the powerhouse of the cell", are double membrane-bound cytoplasmic organelles that generate most of the cell’s supply of adenosine triphosphate (ATP). In plant mitochondria the electron transport chain (ETC) is composed of five complexes in the inner membrane (IM): complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome c reductase), complex IV (cytochrome c oxidase) and alternative oxidase (AOX), as well as two mobile electron transporters: cytochrome c and ubiquinone. At the matrix, the electrons of NADH (from the tricarboxylic acid cycle or other several dehydrogenases) can enter the ETC through complex I or at the ubiquinone level via alternative NADH-dehydrogenases, and electrons of FADH₂ enter at complex II. For NAD(P)H generated at the matrix or cytoplasm, alternative NAD(P)H dehydrogenases are present in both sides of the IM to transfer electrons to ubiquinone. Electrons from ubiquinone have two pathways to follow up to molecular oxygen (which is reduced to H₂O): through complex III, cytochrome c and complex IV (cytochrome pathway) or by AOX (alternative pathway). Complexes I, III and IV couple their electron transfer reactions with H⁺
translocation across the inner mitochondrial membrane, resulting in a proton gradient which is used by the ATP synthase complex for the phosphorylation of adenosine diphosphate (ADP) (for review (Mackenzie and McIntosh, 1999; Schertl and Braun, 2014)). Mitochondria are also major producers of cellular reactive oxygen species (ROS) (Gonzalez-Flecha and Boveris, 1995), such as superoxide anion (O$_2^-$) generated through electron leaking of the ETC (Yao et al., 2002; Panda et al., 2008). Environmental stresses that increase ROS in plants induce an alternative pathway, leading to proposals that this pathway may reduce ROS damage in plant cells (Purvis and Shewfelt, 1993; Wagner and Moore, 1997; Saha et al., 2016). Additionally, the release of cytochrome c from mitochondria is a primary cellular trigger for programmed cell death (reviewed in (Hirsch et al., 1997)).

Studies using plant cells have shown that Al toxicity severely affects the mitochondrial respiratory functions and alters the redox status, which seems to cause programmed cell death (Keith et al., 1998; Yamamoto et al., 2002; Boscolo et al., 2003; Panda et al., 2008; Li and Xing, 2010). Mitochondria from tobacco cells subjected to Al stress presented an inhibition of mitochondrial respiration, related to the decreased capacity of both alternative- and cytochrome-pathways, membrane potential loss, reduced ATP content, enhancement of ROS production, and release of cytochrome c from mitochondria (Panda et al., 2013). In addition, in Arabidopsis cells Al induced loss of mitochondrial transmembrane potential sensitive to cyclosporine A, suggesting the opening of a mitochondrial permeability transition pore, mitochondrial swelling, generation of mitochondrial ROS, and caspase-3-like activation (Li and Xing, 2011).

**Jatropha curcas** L. (J. curcas) is a drought resistant shrub or tree belonging to the Euphorbiaceae family, distributed in the neotropics from Mexico to Brazil and the entire tropics of Africa and Asia (Grim, 1996). This plant has received extensive attention due to its economic importance, medicinal significance, and for its seed oil as a commercial source of fuel (Datta and Pandey, 1993). Nevertheless, the determination of reducing sugars and phenolic compounds was conducted according to the method described by Kidby and Davidson (1973) and Sinkard and Singleton (1977), respectively. All determinations were performed in triplicate.

**2.5. Cell wall protein quantification and invertase activity**

The 200 μL of the cell wall obtained in each treatment was suspended in 500 μL of milli-Q water. In a 96-well plate, 50 μL of re-suspended sample, 50 μL of milli-Q water and 100 μL of Biuret reagent were pipetted in triplicate. For standard curve, bovine serum albumin was used at concentrations 1, 2, 3, 4 and 5 mg/mL. The blank was prepared by replacing the sample with milli-Q water. The plate was read in a microplate reader at 540 nm. For invertase activity, 10 mg of cell wall protein were transferred to 750 μL of 0.1 M sodium acetate buffer (pH 5) and 250 μL of 25 mg/mL sucrose were added. The mixture was incubated at 40 °C, shaken for 15 h and placed in ice to stop the reaction. Three controls were performed: 1) cell wall in 1 mL buffer; 2) 500 μL buffer plus 500 μL fructose 50 μg/mL; 3) cell wall, 500 μL buffer plus 500 μL fructose. All experiments were made in triplicate.

**2.6. Cell wall permeabilization**

Approximately 5 g of fresh J. curcas cell suspension (from 12 to 15 days) were incubated in 40 mL MS medium pH 5.8, supplemented with 0.1 M sucrose, 0.56 M mannitol, hemi-cellulose (1.25 U/mL) and pectinase (0.125 U/mL) for 1 h at room temperature. The cell suspension was then centrifuged at 1000 × g for 10 min and resuspended in 2.1 mL MES buffer 10 mM pH 5.6, supplemented with 2% sucrose, for oxygen consumption analysis.

**2.7. Oxygen consumption analysis**

The J. curcas cells (1g) were incubated in 2.1 mL air saturated MES...
buffer 10 mM pH 5.6, supplemented with 2% sucrose, and oxygen consumption was monitored in an Oxygraph-2k respirometer (Oroboros, Innsbruk, Austria) equipped with DataLab 5.0 software, at 25 °C and stirring at 50 rpm.

2.8. Analysis of mRNA expression

Total RNA was isolated using TRizol reagent (Invitrogen, Grand Island, NY, USA), solubilized in RNase-free H2O, and quantified by measuring the optical density (OD) at 260 nm (NanoDrop spectrophotometer; Thermo Fisher Scientific, USA). For cDNA synthesis, 1.5 μg of RNA was used. The mRNA transcript levels were quantified using the Eppendorf Realplex4 Mastercycler Instrument (Eppendorf) and SsoFast EvaGreen (BioRad), according to the manufacturer’s instructions. The primer sequences (Sigma-Aldrich) were: AOX Forward 5′ TGAATTAC TTGGTGCGGAGG 3′ and Reverse 5′ TTCCTTAACTCCTTCCATGCC 3′; Actin Forward 5′ ATGGCAGATGCCGAGGATAT 3′ and Reverse 5′ CAG TTGTGGACCACTTGCA 3′. After normalization with β-actin, the relative expression of mRNAs was determined by the ΔΔCt method (Seale et al., 2011).

2.9. Statistic analyses

Data were analyzed using SPSS version 24 (SPSS Inc., Chicago, IL). Fig. 1B is separated into 2 × 2 [traces (before addition) by addition (Al3+ and control)] and mixed-design ANOVAs were used. An independent sample t-test was employed to compare measures between the two groups. The level of statistical significance was set at p < 0.05.

3. Results

3.1. Effects of aluminum on oxygen (O2) consumption and AOX expression

The O2 consumption by J. curcas cell suspension in the absence of light (Fig. 1A and B) shows that the addition of 70 μM Al3+ (after 20 min of monitoring) immediately inhibited 30% cellular respiration when compared to cells respiring at control condition (without Al3+). To elucidate the mechanism of respiratory inhibition, the cell wall of J. curcas cells was permeabilized using hemicellulase and pectinase digestion, and the effect of Al on the cytochrome and alternative pathways of ETC was investigated (Fig. 1C). It can be observed that O2 consumption by J. curcas cells (basal) is partially reduced by potassium cyanide (KCN), a complex IV inhibitor, indicating the inhibition of the cytochrome pathway of ETC. In this condition, the addition of 70 μM Al3+ (black circle) significantly reduced the rate of cellular respiration compared to cells without Al3+ addition (white square). The subsequent addition of benzhydroxamic acid (BHAM), an inhibitor of the AOX complex, promoted an inhibitory effect only in cells respiring in the absence of Al3+ (white square), demonstrating that AOX complex is already inhibited in the presence of Al3+. The residual O2 consumption is probably due to other reactions involving O2 consumption independent of mitochondrial respiration. In addition, extending the monitoring of J. curcas cells respiration for 120 min (Fig. 1D), it can be observed that the inhibition of respiration by 70 μM Al3+ reached 48% respiration in the control condition after 30 min. After 60 min, the inhibition appeared almost reversed and after 120 min, the respiration reached initial values. Similar results were found when J. curcas cells are incubated in the presence of AOX inhibitor BHAM. To elucidate the mechanism of reversed respiratory inhibition, under the same conditions we measured the transcription

![Image](image-url)
levels of AOX. Fig. 2 shows increased (~2 fold) transcription levels of AOX in the presence of Al\(^{3+}\) in since 30 min of incubation, while in the presence of BHAM, the increase reached ~4 fold at 120 min, in a time-dependent manner. Together, these results demonstrate that Al\(^{3+}\) is a potent inhibitor of the mitochondrial AOX in \emph{J. curcas} cells and that AOX inhibition by Al\(^{3+}\) induces the upregulation of AOX as a defense mechanism to maintain mitochondrial respiration.

3.2. Effects of aluminum on intra- and extra-cellular concentrations of phenolic compounds

When the intact \emph{J. curcas} cell suspension was incubated with 70 \textmu\text{M} Al\(^{3+}\) for 60 min, a time-dependent decrease in the concentrations of phenolic compounds at the intracellular compartment compared to control condition was found (Fig. 3), indicating an elevated consumption of these compounds. This can be related to respiratory impairment/AOX inhibition, since reduced rates of electron transport in the respiratory chain stimulate ROS production in plants (Millenaar and Lambers, 2003; Plaxton and Podesta, 2006). Superoxide and H\(_2\)O\(_2\) formed in mitochondria are involved in an oxidative burst induced by Al\(^{3+}\), as shown in \emph{Arabidopsis} (Li and Xing, 2011). No changes in the concentrations of phenolic compounds were found in the EM.

3.3. Effects of aluminum on intra- and extra-cellular concentrations of reducing sugars and invertase activity

Under the conditions described above, the concentration of reducing sugars in \emph{J. curcas} cell suspension is firstly incremented by Al\(^{3+}\) treatment up to 30 min in both intra- and extra-cellular compartments (Fig. 4), which can be associated to partial inhibition of mitochondrial respiration. These concentrations are quickly decreased after 45 and 60 min, which can be associated to the restoration of respiration by

Fig. 2. AOX mRNA expression under incubations of Al\(^{3+}\) (70 \textmu\text{M}) or BHAM (3 mM) for 30, 60 and 120 min in \emph{J. curcas} cell suspension. Mean ± SEM. * statistically different for 30, 60 and 120 min within the group. # statistically different of 120 min within the group (p < 0.05). N = 10.

AOX upregulation. However, the concentration of reducing sugars in the presence of Al\(^{3+}\) after 60 min reached values below control conditions in both intra- and extra-cellular compartments, suggesting an interruption of sucrose hydrolysis, the main precursor of glucose and fructose (reducing sugars) in the culture medium. As in plant cells the concentrations of glucose and fructose are controlled by cell wall invertase (CWI; (Roitsch et al., 2003)), the invertase activity was measured in the presence of sucrose (substrate) and Al\(^{3+}\) in the cell wall fraction of \emph{J. curcas} cells (Fig. 5). It can be observed that from 30 \textmu\text{M}, Al\(^{3+}\) decreases the release of glucose when compared to the control condition, an effect close to that promoted by CWI competitive inhibitor (high fructose concentration). This result shows that Al\(^{3+}\) is also a potent inhibitor of CWI activity in \emph{J. curcas} cells.

3.4. Effects of aluminum on cell viability

The capacity of 70 \textmu\text{M} Al\(^{3+}\) to induce cell death in \emph{J. curcas} cell suspension was compared to the effects of 1 \textmu\text{M} salicylic acid (SA, elicitor, positive control), which activates a special type of programmed cell death and hypersensitive response (HR) related to cell defense (Alvarez, 2000; Moore et al., 2002). In Fig. 6A it can be observed that \emph{J. curcas} cells in the presence of Al\(^{3+}\) presented a time-course unviability very similar to the control condition, which was smaller than the elicitor condition. As a control, under the same conditions \emph{Bauhinia} \emph{forsicata} cells in the presence of Al\(^{3+}\) present a time-course unviability very similar to the elicitor condition, which was higher than the control condition (Fig. 6B). These results demonstrate the high tolerance of \emph{J. curcas} cells to Al\(^{3+}\) toxicity, which can be associated to the mechanisms of AOX inhibition and upregulation mentioned above.

Fig. 3. Concentrations of phenolic compounds after 30, 45 and 60 min of Al\(^{3+}\) (70 \textmu\text{M}) incubation in intracellular (intra) and extracellular (extra) fractions of \emph{J. curcas} cell suspension. Mean ± SEM. * statistically different to Al\(^{3+}\) condition at the same incubation time (p < 0.05). N = 9.

Fig. 4. Concentrations of reducing sugars after 30, 45 and 60 min of Al\(^{3+}\) (70 \textmu\text{M}) incubation in intracellular (intra) and extracellular (extra) fractions of \emph{J. curcas} cell suspension. Mean ± SEM. * statistically different to Al\(^{3+}\) condition at the same incubation time (p < 0.05). N = 6.

Fig. 5. Concentrations of glucose released by the activity of invertase in the cell wall fraction of \emph{J. curcas} cell suspension in the presence of 30, 50 and 70 \textmu\text{M} Al\(^{3+}\). Invertase inhibitors: 50 mg/mL fructose. Mean ± SEM. * statistically different to other conditions (p < 0.05). N = 3.
mitochondrial ROS production and oxidative damage (Yamamoto et al., 2002; Panda et al., 2008; Li and Xing, 2011). Here, besides respiratory inhibition, we also observed an elevated consumption of phenolic compounds in the presence of Al\(^{3+}\), consistent with a state of oxidative burst, as demonstrated in Al-treated protoplasts of *Arabidopsis* (Li and Xing, 2011). It was recently shown that O\(_2\) released from mitochondria is involved in Al-induced upregulation of AOX\(_{1a}\) gene expression in *Arabidopsis* (Liu et al., 2014). In fact, we found upregulation of AOX mRNA in the presence of Al\(^{3+}\) which can be associated to the restored respiration of *J. curcas* cells after 120 min. AOX upregulation during Al stress was demonstrated in *Arabidopsis*, in which the AOX\(_{1a}\) transcript level increased in a time-dependent manner, reaching 4.5-fold at 60 min of Al (0.5 mM) incubation (Li and Xing, 2011). AOX helps to maintain the high electron flux through the respiratory chain, decreasing mitochondrial ROS production (Millenaar and Lambers, 2003; Plaxton and Podesta, 2006). Such flexibility in plant respiration is considered an essential mechanism which makes plants adapt better to stress conditions. Al-induced upregulation of AOX\(_{1a}\) gene expression in turn can prevent Al-induced cell death reducing mitochondrial ROS production, preventing the loss of mitochondrial transmembrane potential, the swelling of the mitochondria and caspase-3-like activation (Liu et al., 2014). In fact here, *J. curcas* cells exposed to at 70 μM Al\(^{3+}\) presented similar viability compared to the control condition and high resistance when compared to the cell death condition, which could be associated to the Al-induction of AOX expression. In experiments using *Arabidopsis* mutants, Li and Xing (2011) demonstrated that protoplasts lacking the AOX\(_{1a}\) gene exhibit lower viability while protoplasts over-expressing the AOX\(_{1a}\) gene exhibit higher viability compared to wild type protoplasts after Al treatment.

In plants, as in plant cell cultures, Al-triggered ROS production seems to be a cause of the loss of growth capability. In pea seedlings, above 5 mM Al affects O\(_2\) consumption, ATP production, and inhibits root elongation (Yamamoto et al., 2002), while in *J. curcas* seedlings even high Al concentrations up to 0.5 mM do not change the fresh weights of cotedledons, hypocotyls and radicles, suggesting normal growth and development, which is disrupted at very high Al concentrations above 1 mM (Ou-yang et al., 2013). Together these studies suggest that *J. curcas* has a mechanism of Al resistance. Generally, in Al-resistant plants these mechanisms include the chelation of cytosolic Al\(^{3+}\) by organic acid anions from tricarboxylic acid (TCA) cycle and subsequent sequestration into the vacuole, the upregulation of genes encoding transporter membrane proteins and pectin methylesterase, the biosynthesis of antioxidant enzymes, and the regulation of pectin content in the cell wall (for a review, (Nunes-Nesi et al., 2014)). Recently, in *Stylosanthes* was revealed an Al\(^{3+}\) signaling cascade to enhance citrate anabolism involving G-proteins, phospholipase C, inositol triphosphate, diacylglycerol, Ca\(^{2+}\) and protein kinases, thereby activating transcription and anion channels in plasma membrane (Jiang et al., 2018). Here, in *J. curcas* cells the AOX pathway inhibition promoted by Al\(^{3+}\) could represent a mechanism to fast increase the availability of organic acids, suggesting an initial event to decrease Al toxicity, followed by AOX upregulation.

5. Conclusion

The results indicate that Al\(^{3+}\) tolerance in cultured *J. curcas* cells involves a direct inhibition of mitochondrial alternative pathway. It leads to oxidative burst which can activate AOX upregulation, which in turn restores O\(_2\) consumption. These data provide new insight into the signaling cascades that modulate the Al tolerance mechanism.

Conflict of interest

The authors declare no conflicts of interest.
Contributions

AHC conducted the cell culture establishment and elicitation assays. TMV conducted the analysis of cell viability, protein quantification, invertease activity and oxygen consumption. AHC and TMV conducted the quantification of reducing sugars and phenolic compounds. CRPD analyzed the mRNA expression. LCA and CGVR designed the experiments, analyzed the results and wrote the paper.

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