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ARTICLE ADDENDUM

Effects of exogenously-applied L-ascorbic acid on root expansive growth and viability of the border-like cells

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ABSTRACT

Functions of exogenous L-ascorbic acid in plant roots are poorly understood. Recent study by Makavitskaya et al. (doi.org/10.1093/jxb/ery056) has demonstrated that exogenous ascorbate can be released from roots in response to salt stress, and can trigger elevation in the cytosolic free Ca^{2+}. Here, we report that exogenous ascorbate significantly modifies root elongation in *Arabidopsis thaliana*. Using a medium exchange technique, we have shown that 10–100 μM ascorbate induces small but significant increase in root elongation while higher levels cause its dramatic decrease. Root border cells of *Pisum sativum* have been losing viability twice faster in the presence of ascorbate that under control conditions, as tested by the confocal microscopy and a combined staining with propidium iodide and fluorescein diacetate.

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L-ascorbic acid (ascorbate) is the major plant antioxidant and one of the most abundant carbohydrates in plants.[1-4] Although ascorbate has been long recognised as a major antioxidant in plants, many evidence were accumulated suggesting that this substance may also play regulatory and signalling roles. Our recent data demonstrate that exogenous L-ascorbic acid induces transient elevation in the cytosolic free Ca^{2+} in *Arabidopsis thaliana* roots,[5] thus linking ascorbate to Ca^{2+} signaling; a central signalling phenomenon in plants.[6]

Calcium signals and a polar increase in the cytosolic free Ca^{2+} are responsible for a multitude of physiological processes, such as processing of environmental signals, cell elongation, tissue differentiation, gravitropic reactions, adjustment of respiration and photosynthesis.[6] Accordingly, we hypothesize that ascorbate, which is ubiquitous and abundant in plants, acts as an extracellular signaling and regulatory molecule having high importance for a number of physiological functions.

Root cell elongation is one of the key processes that are regulated by both ROS and cytosolic calcium.[6–9] ROS are produced locally in growing parts of the cell, such as tips of root hairs[7] or the frontal part of elongation zone cells.[8] This leads to the local ROS-induced activation of Ca^{2+}-permeable ion channels stimulating Ca^{2+}-dependent exocytosis, incorporation of vesicles into the plasma membrane and increase of cell size.[7,9,10] Moreover, cell wall needs to be softened locally to allow membrane expansion; this is achieved via cleavage of cell wall polymers by hydroxyl radicals ($HO^{\bullet}$), which are generated by Haber-Weiss cycle catalysed the cell wall-bound transition metals.[11–14] The role of exogenous ascorbate here is obvious: hydroxyl production in Haber-Weiss cycle constantly requires electrons for transition metal reduction from ascorbate. Ascorbate is far more superior reducing agent for Cu^{2+} and Fe^{3+} than any other abundant cell metabolites.[13] Thus it is anticipated that ascorbate supply should modify the elongation growth.

Here, we report a statistically significant stimulation of root elongation after transferring roots on ascorbate-containing media in 10 to 100 μM concentration range (Figure 1A). For example, 30 μM ascorbate increased root elongation rate from 9.93 ± 0.08 mm d^{-1} (control: ascorbate-free) to 10.38 ± 0.07 mm d^{-1} ($n = 170$; $p < 0.0001$; ANOVA). Higher levels of ascorbate (over 0.3 mM) inhibited root elongation (Figure 1), with a 30-fold decrease observed at highest 3 mM ascorbate treatment. Both the root diameter and a length of the elongation root zone also increased slightly in 10–100 μM and then decreased in 0.3–3 mM ascorbate concentration range (Figure 1A, B).

A number of hypotheses can be proposed for interpretation of these data. Ascorbate can potentially be oxidized to dehydroascorbate, which is transported back by cells. Hypothetically, increased dehydroascorbate concentration can affect reduced glutathione pool, which has been shown

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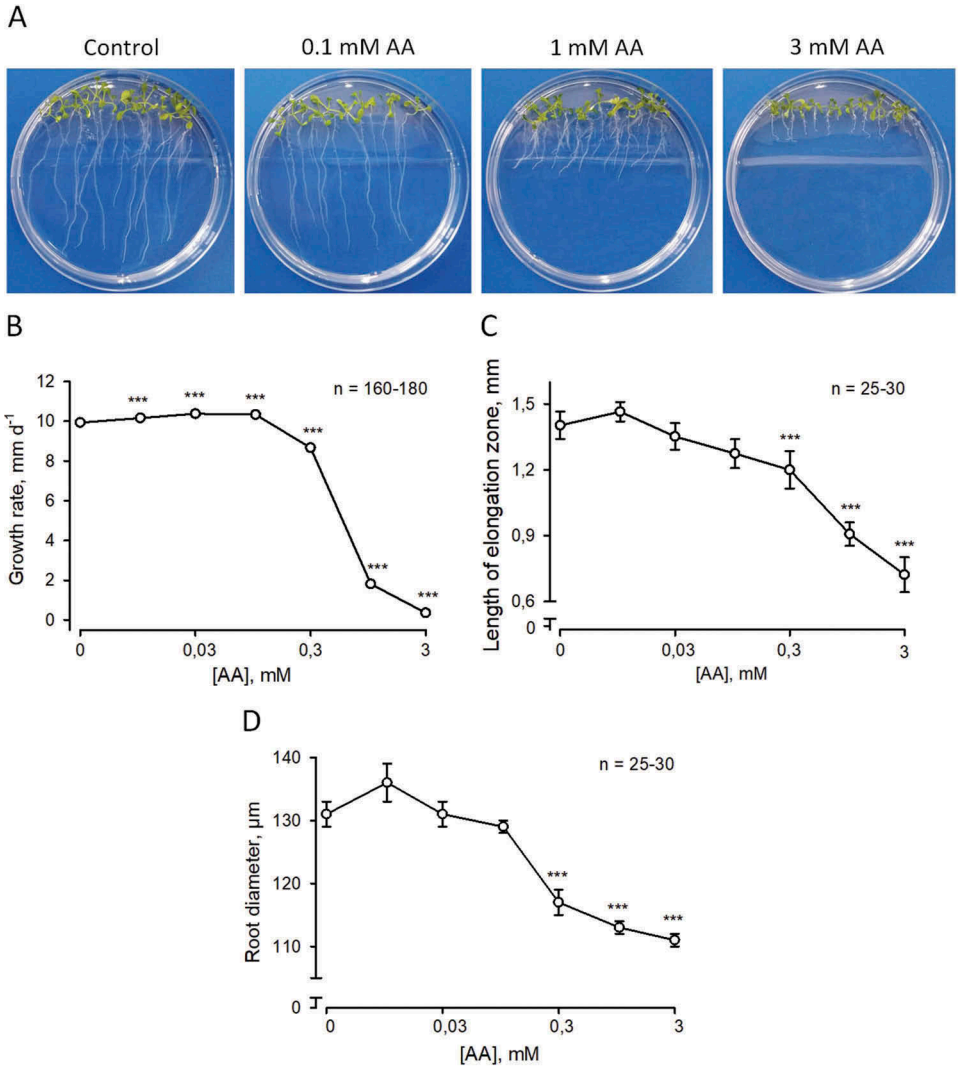

Figure 1. Effect of ascorbate on *Arabidopsis thaliana* L. root growth. (A) Typical nine day-old plants cultivated vertically on Murashige and Skoog medium (original concentrations; Duchefa, #M0221) with ascorbate (concentrations are indicated in the figure). During the first four days, plants were growing without ascorbate, then medium below root tips was removed using razor blade and carefully replaced with the warm block of ascorbate-containing medium. This technique allows to avoid disturbance of plants. Root growth rate (B), length of root elongation zone (C) and root diameter (D) at different ascorbate levels in the medium. All parameters were measured at fifth day after exchange of media. Mean values (± SE) were plotted against the tested ascorbate concentrations (0.01 to 3 mM range). Plant cultivation techniques were as described by Makavitskaya et al.[5]

to be important for maintaining root elongation.[15] The obtained results can also be interpreted from a viewpoint where the moderate polar $HO^{\bullet}$ production and a local Ca^{2+} influx are necessary for root elongation. The physiological levels of ascorbate (10–100 µM) promote these processes (Figure 1). It can be hypothesized that higher ascorbate concentrations can over-produce $HO^{\bullet}$. This over-production is non-polar and so it cannot stimulate elongation growth.[7] Supporting this Foreman et al.[7] reported that addition of $HO^{\bullet}$-generating mixture containing 1 mM ascorbate and transition metal copper stopped polar expansion of root hairs leading to a formation of bubble-like outgrowings.

Our recent work also showed that 1 mM ascorbate increased numbers of cells with symptoms of the programmed cell death (PCD) so it can be toxic.[5] Toxicity of 0.5–2 mM ascorbate has also been reported for animal cells where it depended on medium composition.[16,17] It was hypothesized that, in the presence of ascorbate, transition metal such as copper and iron (in medium) catalyze hydroxyl radical production, which cause toxicity.[16,17] Our previous study demonstrated that hydroxyl radicals are generated in the presence of transition metals and 1 mM ascorbate and that this can induce cell death in *Arabidopsis thaliana* roots in 1–2 days.[18] The medium salines used for *Arabidopsis thaliana* growth test here were from Murashige and Skoog (original concentrations; #M0221; Duchefa, Netherlands), which includes 0.1 µM Cu^{2+} ($CuSO_4 \bullet 5H_2O$) and chelated 100 µM Fe^{3+} (FeNaEDTA). So some moderate catalysis of $HO^{\bullet}$ was hypothetically possible.

Another explanation of the ascorbate-induced inhibition of root elongation is that the elevated ascorbate levels lead to $HO^{\bullet}$-scavenging effect.[13] Ascorbate can also be a scavenger of 'secondary' radical production in $HO^{\bullet}$-induced reactions.[13] $HO^{\bullet}$-producing capacity of ascorbate relies on transition metals, and if ascorbate level grows up without equivalent increase in the transition metal concentration, ascorbate starts

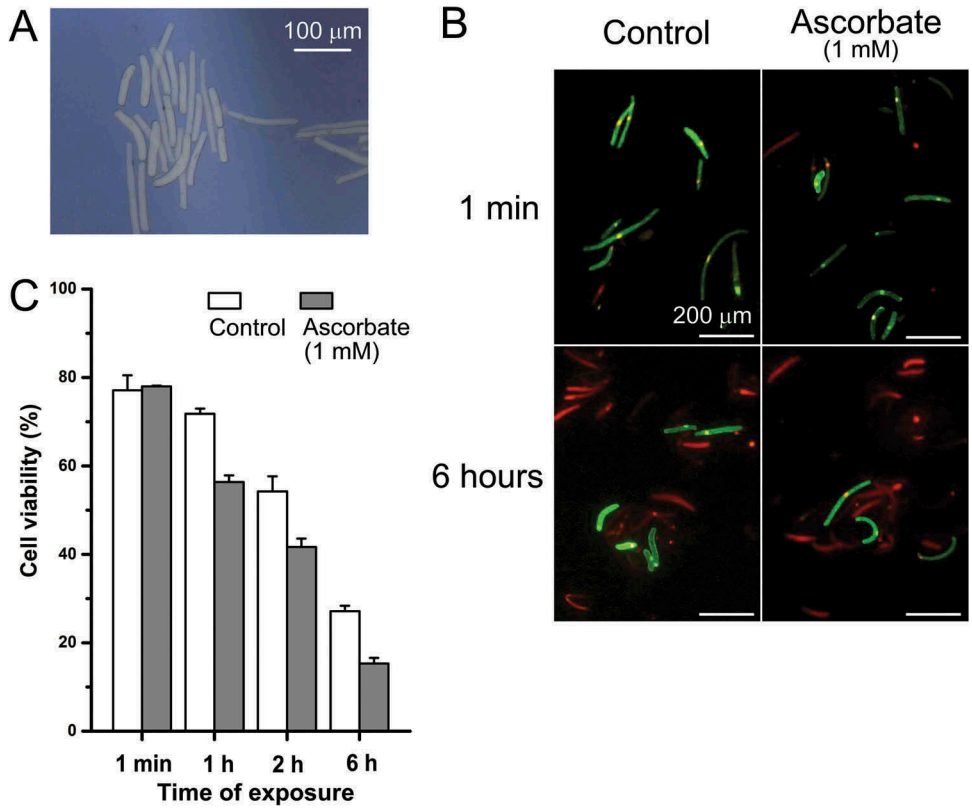

Figure 2. Effect of exogenous L-ascorbic acid on viability of pea root border cells. (A) Typical root border cells of *Pisum sativum* L. (bright field). (B) Epi-fluorescent images (laser scanning confocal microscopy) of propidium iodide (PI; dead cells) and fluorescein diacetate (FDA; viable cells) staining. (C) Mean numbers (± SE) of viable cells (four independent trials) at different times of treatment by ascorbate. Seeds of pea were germinated under the mist culture as described elsewhere.[22–24] Root border cells were harvested and purified as described previously.[22–24] Pellets with root border cells were resuspended in a solution containing 0.3 mM $CaCl_2$, 0.3 mM KCl, pH 6.0 (2 mM Mes), and then were treated with 1 mM L-ascorbic acid. At 1 min, 1 h, 2 h and 6 h, 90 μL of solution containing root border cells was mixed with 10 μL of FDA/1 μL PI solution (FDA from Fluka; PI from Sigma), and cell viability was determined as described elsewhere.[20]

scavenge radicals that produced by the ascorbate-dependent Haber-Weiss cycle (hypothetically, at concentrations above 0.1 mM).

The effect of ascorbate on root growth is fundamental to plant physiology. Ascorbate concentration inside root cells can reach millimoles per liter.[19–21] According to Smirnoff,[4] the apoplastic ascorbate level is lower (about 0.1 mM) that corresponds to stimulatory concentration for root growth found here (Figure 1). An increase in the apoplastic ascorbate concentration can occur in the case of stress effects that cause cell collapse. Dying cells can release ascorbate causing its local elevation up to 1–10 mM (cytosolic level). This can trigger root growth inhibition. Ascorbate can also be released without cell collapse, in a process mediated by anion channels[5] following membrane depolarization by NaCl.

Root border cells are outer layers of cells and, hence, the first target for stresses.[22-24] These cells normally live for a short period of time (only a few days). When dying, they release metabolites to the rhizosphere; those potentially act as signals[22,23] Hypothetically, border cells can be involved in sensing new environment, interacting with neighboring roots and other organisms. Here we used border cells from pea roots to see if their viability will be affected by the presence of ascorbate. In the presence of ascorbate, border cells died twice faster than in control conditions (combined propidium iodide and fluorescein diacetate test; Figure 2). This suggests that a release of ascorbate from inner root layers can be a signal for elimination of root border cells. In the viability test with border cells, medium contained only 0.3 mM $CaCl_2$, 0.3 mM KCl, pH 6.0 (2 mM Mes) prepared using deionized water of Type I (18 mOhm). However pea seeds can contain significant quantity of transition metals, such as copper and iron in seeds. This means that the mechanism of $HO^{\bullet}$-induced toxicity is not excluded for explaining of ascorbate effects on root border cells.

In conclusion, reported data show new insights into ascorbate functions in plants. This includes slight stimulation at physiological levels of 10–100 μM while strong inhibition by 0.3–3 mM. 1 mM ascorbate decreased viability of root border cells. This can potentially be a mechanism of their elimination because ascorbate can be released by inner cell layers via anion channels.[5]

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