



Research article

Root signalling and modulation of stomatal closure in flooded citrus seedlings

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ABSTRACT

In this work, we studied the sequence of responses induced by flooding in citrus plants, with the aim of identifying the signals that lead to stomatal closure. One-year-old seedlings of *Carrizo citrange*, grown in sand under greenhouse conditions, were waterlogged for 35 d and compared with normally watered well-drained plants. Significant decreases in stomatal conductance and transpiration were detected between flooded and control seedlings from a week after the beginning of the experiment. However ABA concentration in leaves only started to increase after three weeks of flooding, suggesting that stomata closed in the absence of a rise in foliar ABA. Therefore, stomatal closure in waterlogged seedlings does not appear to be induced by ABA, at least during the early stages of flood-stress. The low levels of ABA detected in roots and xylem sap from flooded seedlings indicated that it is very unlikely that the ABA increase in the leaves of these plants is due to ABA translocation from roots to shoots. We propose that ABA is produced in old leaves and transported to younger leaves. Flooding had no effect on water potential or the relative water content of leaves.

Soil flooding reduced root hydraulic conductance in citrus seedlings. This effect was already evident after a week of waterlogging, and at the end of the experiment, flood-stressed seedlings reached values of root hydraulic conductance below 12% of that of control plants. This reduction was related to down-regulation of the expression of PIP aquaporins. In addition, whole plant transpiration was reduced by 56% after 35 d under flooding conditions. Flood-stress also decreased the pH of sap extracted from citrus roots. Evidence is presented suggesting that acidosis induced by anoxic stress in roots causes gating of aquaporins, thereby decreasing hydraulic conductance. Additionally, stomatal closure finely balances-out low pH-mediated losses of root hydraulic conductance therefore maintaining stable leaf hydration.

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1. Introduction

Soil flooding is a major abiotic stress that has a negative impact on survival of non-tolerant plant species growing in some natural environments [33]. Also, an excess of water damages many agricultural crops leading to elevated economic losses [53]. However, some species have developed mechanisms of tolerance to anoxia or have acquired some characteristics of adaptation to anaerobic conditions [5]. Plant strategies to grow and survive during long periods of waterlogging include biochemical, anatomical and morphological changes [11].

Waterlogging generally arises due to poor soil drainage combined with excessive rainfall or irrigation. A widespread cause of flooding is the destruction of soil structure by high levels of

exchangeable Na^+ , which causes dispersion of soil aggregates and results in the blocking of soil pores [26]. These effects contribute to impeding air and water movement in soil, leading to depletion of O_2 and accumulation of CO_2 . Alterations in the chemical conditions of the soil under these conditions have been widely reported [47].

The effects of flooding on plants mainly relate to the decline of aerobic root respiration that impairs ATP synthesis, disrupting the metabolism. Moreover, soil inundation induces a variety of physiological dysfunctions that alter plant growth, including hormonal imbalances, altered distribution of carbohydrates, deficient nutrient uptake, early senescence of leaves and injury in organs, which sometimes precede plant death.

Some reviews have been focussed on the signals and sensory mechanisms [13] that trigger plants responses to flooding [36,50].

A common response to flooding appears to involve the reduction of stomatal conductance to prevent water loss, with a subsequent decrease in net CO_2 assimilation by leaves [8,29,37,41,46,60,61]. It has been suggested that abscisic acid (ABA)

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accumulation in leaves may be responsible for stomatal closure in flooded plants [9,30,42,62], although it is doubtful that roots are the main source of ABA under flooding conditions [32,63].

In addition, flooded plants present reduced water flux, at least under high evaporative demand, that seems to be due to the down-regulation of root hydraulic conductance by anoxia [6,15]. This decrease in root hydraulic conductance has also been associated with stomatal closure [2,14,16,17,55].

It is generally accepted that water transport across biological membranes is facilitated by aquaporins. Aquaporins are 26–30 kDa membrane proteins belonging to the major intrinsic proteins (MIPs) family [23] forming water channels that facilitate the passive flow of water through cell membranes and maintaining water content in cells and tissues [40]. There is some evidence that, among aquaporins, the subfamily of plasma membrane intrinsic proteins (PIPs) appear to play a critical role in the control of water transport through root tissues, regulating the transcellular pathway [34]. PIPs are further divided into two evolutionary groups based on homologous sequences: PIP1 and PIP2, each presenting several isoforms [10].

Reduced root hydraulic conductance in flooded plants may be the result of aquaporin gating, which is regulated by the cytosolic pH during anoxic stress [56]. It has also been observed that the pH of the xylem sap of some plant species increases when exposed to environmental stresses [59]. Some studies have detected increases in the pH of xylem sap from tomato plants growing in flooded soil when compared with well-drained plants [19,31]. Coinciding with this, flood-stressed tomato plants showed reduced stomatal conductance that could not be fully attributed to an increase in ABA [20].

Citrus respond to flooding by decreasing stomatal conductance and gas exchange [24,43,49,58]. In addition, increases in ABA content have been detected in leaves of flooded citrus plants [3]. It has also been reported that root hydraulic conductance is reduced by anoxic stress [55]. So, a prolonged period of waterlogging impairs growth and causes leaf damage as well as chlorosis and wilting [3, 4, 55].

The regulatory processes that control stomatal behaviour play a key role in stress tolerance, since, on the one hand, they protect the plant from dehydration and, on the other, allow CO₂ capture. Accordingly, the aim of this work was to identify the signals that lead to anoxia-induced stomatal closure in citrus. We correlate the temporal sequence of plant physico-chemical changes during flooding-stress with variations in stomatal conductance. We study changes in stomatal conductance (*g_s*), transpiration rate (*E*), ABA content, leaf water potential (Ψ_s), leaf relative water content (RWC), root hydraulic conductance (*K_r*), aquaporin activity, water transport and xylem sap pH in response to flooding. Given that there are differences in flooding tolerance among citrus rootstocks [3,24,49,54,55,58], *Carrizo citrange* (a hybrid of *Citrus sinensis* (L) Osb. × *Poncirus trifoliata* (L) Raf), a moderate flooding tolerant genotype, was used to determine the effects of time under waterlogging on plant performance.

2. Materials and methods

2.1. Plant material and growth conditions

Ten-month-old plants of *C. citrange* (CC) were used. Plants were cultured under glasshouse conditions with supplementary light (<50 μmol m⁻² s⁻¹, 400–700 nm) to extend the photoperiod to 16 h. Temperatures ranged between 16 and 18 °C at night and 24–28 °C by day. Relative humidity was maintained at approximately 80%. During the experimental period, photon flux density in the glasshouse reached maximum values of 1250 μmol m⁻² s⁻¹ at 12:00 a.m. in sunny days.

Plants were grown individually in 4 L pots filled with coarse sand. All plants were irrigated twice weekly until the beginning of the experiment with the following nutrient solution: 3 mM Ca(NO₃)₂, 3 mM KNO₃, 2 mM MgSO₄, 2.3 mM H₃PO₄, 17.9 μM Fe-EDDHA, 46.25 μM H₃BO₃, 54.4 μM MnSO₄·H₂O, 7.65 μM ZnSO₄·7H₂O, 0.55 μM MoO₃ and 0.5 μM CuSO₄·5H₂O. Nutrient solution pH was adjusted to 6.5 with 1 M KOH or 1 M H₂SO₄. 1 L of solution per pot was used in each watering event. Excess solution drained out of the pot, thereby avoiding salt accumulation in the sand.

Plants growing as a single shoot were selected for uniformity of size at the beginning of the experimental treatments. Plants were randomly divided into two uniform groups. One group (control plants) was watered normally and well drained (as indicated above); the other group was maintained under continuous substrate waterlogging that was imposed by placing the pots inside plastic containers in which the level of the nutrient solution remained 2 cm above the soil surface. When needed, more solution was added to maintain the water level. An opaque plastic sheet was used to cover the surface of containers to avoid algal proliferation. The plants were randomised over the experimental area and analysed individually. A row of plants, not included in the experiment, was placed around the perimeter.

Treatments were applied for 35 d and samples were collected weekly. Six replicates per treatment were used in each sampling date to measure leaf stomatal conductance (*g_s*), transpiration rate (*E*), whole plant transpiration (*T_p*), total water potential (Ψ_s), relative water content (RWC), root hydraulic conductance (*K_r*) and xylem sap pH. Gas exchange, water relationships, *K_r* and sap pH were determined in independent groups of plants cultured at the same time in similar conditions. Three independent replicates per sample were used for ABA analyses while another three were used for aquaporin determinations.

2.2. Defoliation treatments

Two sets of both flooded and control plants, treated as above, were used for stomatal conductance measurements and ABA analyses. Half plants of each set were defoliated, removing ten mature leaves from the lower part of the stem and leaving the upper part with leaves. Defoliation was done by clipping the leaves from the plants at the petiole base near the stem. The plants were left for 2 d before treatments to allow recovery from clipping injury. Afterwards, young (upper) and mature (lower) leaves from intact plants and young (upper) ones from defoliated ones were collected weekly. Treatments were maintained for 35 d. Additionally, stomatal conductance (*g_s*) was determined in leaves at the end of the experiment (see Section 2.3).

2.3. Gas exchange parameters

Stomatal conductance (*g_s*) and transpiration rate (*E*) of single attached leaves were measured outdoors between 10.00 and 11.30 h on sunny days, which facilitated measurement under relatively stable conditions.

Photosynthetically active radiation (PAR) at the leaf surface was adjusted to a photon flux density of 1000 μmol m⁻² s⁻¹. A closed gas exchange CIRAS-2 (PP-systems, Hitchin, UK) was used for the measurements. Leaf laminae were fully enclosed within a PLC 6 (U) universal leaf autocuvette in a closed circuit model and kept at 25 ± 0.5 °C, with a leaf-to-air vapour deficit of about 1.7 Pa. The air flow rate through the cuvette was 0.5–1.5 L min⁻¹. Ten consecutive measurements were taken at 3 s intervals.

Determinations were performed weekly, using two uniform fully expanded mature leaves from the mid-stem zone of each of

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