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Deciphering growth inhibition mechanisms in *Vicia faba* L. under saline-alkaline salt stress in relation to apoplastic pH, nutrients, metabolites, and gene transcription

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Chapter 1: General Introduction

Plants face many hostile environmental conditions which are commonly known as abiotic stresses (Vandenbroucke and Metzloff, 2013) and are the primary causes of crop loss worldwide. Stress caused by abiotic factors alters plant metabolism leading to adverse effects on the growth, development and productivity of plants. Salinization is one of the most important causes of crop productivity reduction in many areas of the world (Lovelli *et al.*, 2012; Mühling and Läuchli, 2002; Munns, 2011). Salinity is getting severer as a result of land clearing and unsustainable irrigation practices and through pressures for bringing marginal land into production (Munns and Gilliam, 2015). Salinity is a compound stress consisting of both the excess salts and alkaline stresses, the latter seems to be much severer than the former one (Brand *et al.*, 2002). Globally, about 831 million hectares of land are saline-alkaline (Jin *et al.*, 2006; Wang *et al.*, 2008), of which approximately 397 million hectares (47%) are saline, and 434 million hectares (53 %) are saline-alkaline (Kawanabe and Zhu, 1991).

The presence of excessive amount of soluble salts in soils leads to the suppression of plant growth and is one of the world's oldest and most widely distributed environmental challenges (Fig. 1). The accumulation of salts over long periods of time in arid and semiarid zones is the main natural cause of salinity (Munns and Tester, 2008). Apart from the natural salinity, irrigation of cultivated lands with brackish water under higher soil evaporation and plant transpiration rates is significant reason behind the concentration of the salts in the root zone (FAO, 2008); Fig. 2).

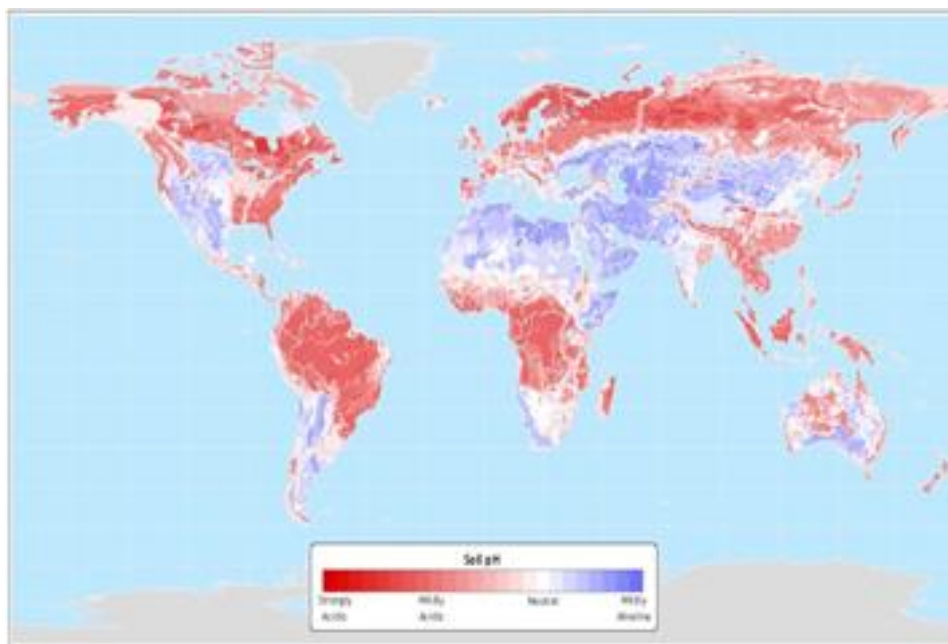


Figure 1. Distribution and extent of saline-alkaline soil around the world according to the pH scale (<http://nelson.wisc.edu/soil pH>).

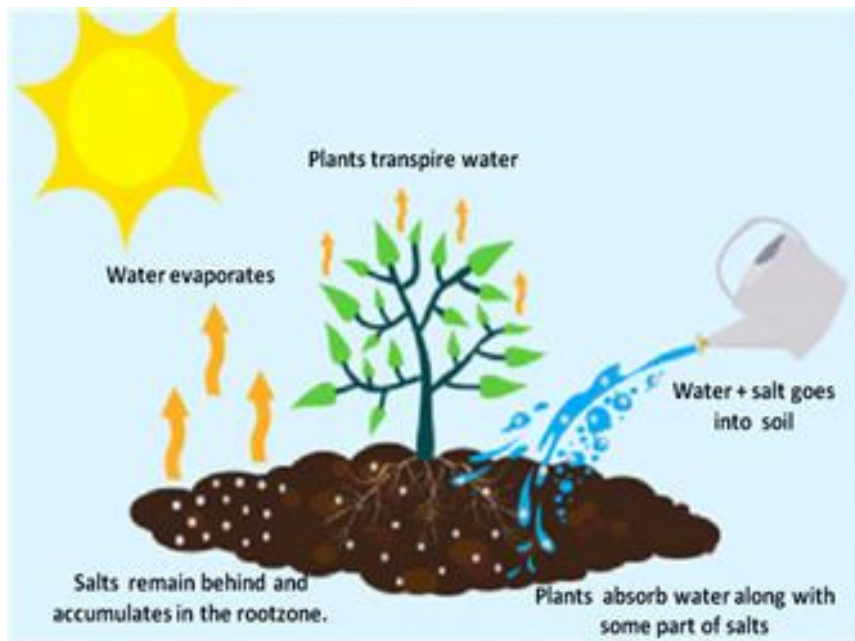


Figure 2. A diagrammatic illustration of salt accumulation in soil.

1.1 Plant responses to soil salinity

1.1.1 Salt stress

Salinity stress is a very complex problem and consists of two main components viz. osmotic stress and ion toxicity (Demir and Kocaçalışkan, 2002; Munns and Tester, 2008). Plant growth response to these salts, especially NaCl, in two phases (Munns and Tester, 2008) (Fig. 3). The osmotic stress phase causes water deficit in the rhizosphere and leads to inhibition of the growth of young leaves. Second, the subsequent accumulation of excess salts in leaves, primarily of Na⁺, leads to ionic stress phase that leads accelerated senescence of mature leaves. The plants' response to soil salinity is also a very complex process and includes alterations in metabolism, physiology, and morphology (Lee and Van Iersel, 2008; Poljakoff-Mayber and Gale, 1975). Elevated levels of salts affect plants' growth by imposing water stress, ion toxicity, nutritional disorders, oxidative damage, membrane disorganization, reduction of cell division and expansion, and genotoxicity (Hasegawa *et al.*, 2000; Munns, 2002; Zhu *et al.*, 2007). Furthermore, plants physiological processes such as respiration rate, photosynthetic efficiency and leaf net CO₂ assimilation rate are severely affected by salinity (Ashraf and Shahbaz, 2003).

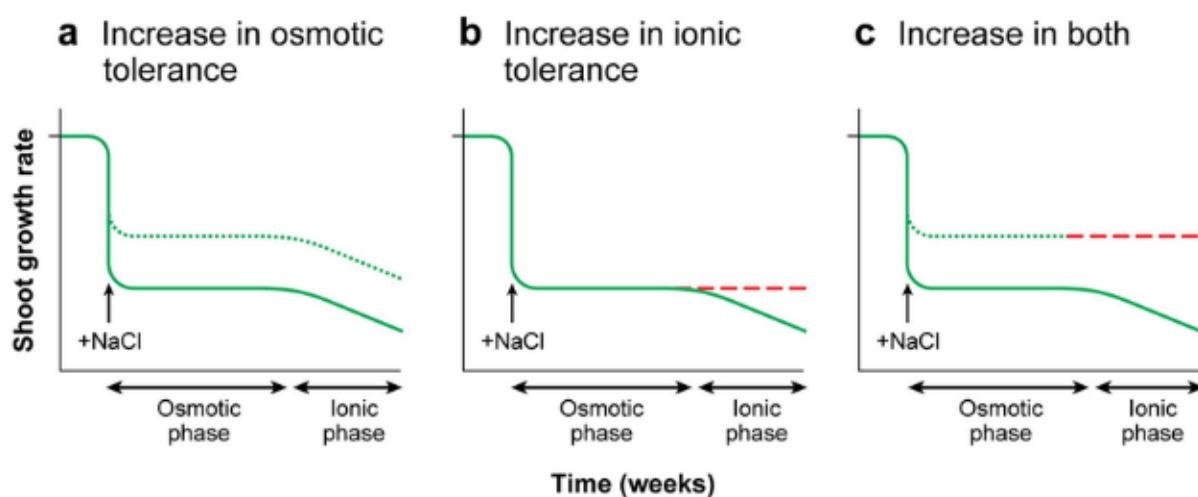


Figure 3. The growth response to salinity stress during the osmotic stress phase and ion toxicity stress phase (Munns and Tester, 2008). a) The solid green line represents the change in the growth rate after the addition of NaCl while the broken green line represents the hypothetical response of a plant with an increased tolerance to the osmotic component of salinity stress; (b) The broken red line represents the response of a plant with an increased tolerance to the ionic component of salinity stress; (c) The green-and-red line represents the response of a plant with increased tolerance to both the osmotic and ionic components of salinity stress.

The response of plants suffering from stress varies not only with degree of stress but also depends on plant species, genotype and crop growth stage. For example, if maize plants have short-term exposure to salt stress, it will influence plant growth mainly due to osmotic reasons (Sumer *et al.*, 2004), while toxic concentration of Na^+ will not be reached (Fortmeier and Schubert, 1995). As maize is moderately sensitive to salt stress, shoot growth is inhibited in the first phase of salt stress (El Sayed, 2011; Pitann *et al.*, 2009; Szalai and Janda, 2009; Wakeel *et al.*, 2011). De Costa *et al.* (2007) observed stunted maize growth with dark green leaves without showing any toxicity symptoms under salt stress. In order to achieve optimal growth, lowering of apoplastic pH is needed, which is referred to as acid-growth theory (Hager, 2003). The acidification of cell apoplast is very important for cell wall extensibility as it activates cell-wall-loosening enzymes such as expansins (Cosgrove, 2005). According to Zörb *et al.* (2005), during the first phase of salt stress, inefficient plasma membrane H^+ -pumping by ATPase reduces cell wall acidification resulting in impaired growth of maize seedlings, which may be due to changes in gene expression. Pitann *et al.* (2009) and Wakeel *et al.* (2011) observed that the maize hybrid SR03 can better maintain the H^+ pumping and decreased apoplastic pH than its parent hybrid Pioneer 3906. This differential behavior was explained by the fact that SR03 was better able to maintain cell wall acidification but still showed stunted growth, the factors other than apoplastic pH which are possibly responsible for limiting cell wall extensibility (Geilfus *et al.*, 2010; Pitann *et al.*, 2009). However, for better growth of maize, maintaining low pH under salt stress is not a necessary parameter

(Hatzig *et al.*, 2010). In this context, it was also shown that alterations of the cell wall composition, and here especially the synthesis of cell wall sugars and pectin, can explain at least partly, why plant growth is reduced under salt stress (Uddin *et al.*, 2014).

1.1.2 Alkaline or alkali salt stress

Although salinity stress seems to be well described in the literature (Mühling and Läuchli, 2002; Munns and Tester, 2008; Shahid *et al.*, 2013), the existing studies have in common that only the effects of the neutral salt (NaCl) were mainly investigated. There exists a little information on alkaline stress (Guo *et al.*, 2009; Shi and Sheng, 2005), which in most cases is focused only on limed soils or alkaline growth substrates (Brand *et al.*, 2002; Nuttall *et al.*, 2003), but not Na-salt born alkaline conditions. On the other hand, it is well recognized that alkaline conditions have negative effects on nutrient availability. While the neutral salts such as NaCl (sodium chloride) or Na₂SO₄ (sodium sulfate) impose salt ion stress, alkaline stress is mainly caused by the alkaline salts (Na₂CO₃ or NaHCO₃) in the soil (Yang *et al.*, 2008).

When talking about salinity and/or sodicity, salt stress and alkaline stress are co-occurring in nature, with alkalization causing severe problems in large areas (Kawanabe and Zhu, 1991). Alkaline soils are usually categorized by low availability of plant nutrients, high concentrations of HCO₃⁻ and CO₃²⁻, and high pH (Marschner *et al.*, 2008; Misra and Tyler, 1999). By definition, alkalinity is the concentration of soluble alkali metals with the ability to neutralize acids (Bailey, 1996). Bicarbonate (HCO₃⁻) and carbonate (CO₃²⁻) are the principal contributors to alkalinity, whereas hydroxide, borate, ammonia, organic bases, phosphates, and silicates are considered minor contributors (Petersen, 1996). The presence of HCO₃⁻ and CO₃²⁻ and other alkaline salts impart to the buffer capacity of the soil and cause an increase in soil pH, resulting in the precipitation and adsorption of the major plant macro- and micronutrients (Cartmill *et al.*, 2008). Alhendawi *et al.* (1997) found that irrigation of plants with water having high alkaline salts resulted in frequent chlorosis of young leaves. Furthermore, alkaline stress involves a combination of osmotic stresses, ion-induced injury and high pH (Chen *et al.*, 2011; Munns, 2002; Yang *et al.*, 2008). The high pH caused by alkaline salts may rigorously affect soil structure, interferes with ion uptake and intracellular ion balance in plants (Yang *et al.*, 2009), limits plant growth (Shi and Sheng, 2005; Yang *et al.*, 2009) and photosynthesis. It can restrict absorption of inorganic anion such as Cl⁻, NO₃⁻ and H₂PO₄⁻, which greatly affect the selective absorption of K⁺ (Yang *et al.*, 2009). Moreover, under salt stress, Na⁺ which is present in very high concentration competes with K⁺ at soil root interface, negatively affect its uptake, and thus impair ionic balance to a great extent. Similar to neutral salt stress, sodium contents in root cells have also been found to increase with the high level of alkalinity whereas potassium decreases (Yang *et al.*, 2009; Guo *et al.*, 2009; Munns and Tester, 2008). Under high pH conditions, availability of micronutrients, except for molybdenum, is also restricted (Marschner *et al.*, 2008). In general, under salt stress Na⁺ replaces K⁺ when taken up into root

cells of the plants. Under alkaline stress, it is not known whether it is because of pH factor or Na^+ and if alkaline stress acts differently on other cations (Ca^{2+} and Mg^{2+}). In contrast to alone neutral salt stress, combined salt-alkaline stress leads to a significant decrease in Ca^{2+} , Mg^{2+} , Fe^{2+} , Cu^{2+} , Zn^{2+} and Mn^{2+} (Liu *et al.*, 2012), while sole alkaline stress results increased Ca^{2+} , Mg^{2+} , Fe^{2+} concentration (Guo *et al.*, 2015; Yang *et al.*, 2009). Paz *et al.* (2012) reported that Zn^{2+} was reduced slightly under alkaline stress, while Cu^{2+} and Mn^{2+} remained unaffected. Like the cationic nutrients, anions (H_2PO_4^- , NO_3^-) are also affected by soil pH and Na-uptake. Guo *et al.* (2009) found decreased N and P content in wheat shoot due to a pH-related inhibition of uptake mechanisms.

1.2 Plant adaptations

It is reasonable to visualize those plants growing in saline environments have developed a variety of strategies at the whole plant as well as at the cell level that let them defeat the salinity stress (Fig. 4). If there are more components involved under alkaline stress and how plants actually behave and adapt physiologically to alkaline stress (high pH) or cannot do anything against it, is still in question. The plant adaptations to salinity stress are discussed as follows:

1.2.1 Osmotic and ionic balancing for salinity tolerance

Salinity stress tolerance is very complicated at the whole plant as well as at cellular level (Ashraf and Harris, 2004). In the osmotic phase, the effect dominating the growth response is not specific to salinity stress but is indeed associated with water deficit. Strategies employed by the plants include improved water-use efficiency, osmotic adjustment, the transformation of morphological or developmental patterns to conserve water, and the advancement of the flowering stage (Colmer *et al.*, 2006; Munns, 2011). The leaf development in plants growing in saline soil is not accompanied by an increase in leaf water relationships within a period of days (Munns, 2002). Thus, irrespective of the plant water status, the regulation of phytohormones (such as ABA) (Geilfus *et al.*, 2018) and photosynthate seem a primary management strategy to sustain an optimum growth rate under drought or saline stress (Munns, 2011). With regard to ion toxicity stress, the first adaptation takes place in the root tissues. Roots can exclude at least 95% of salt ions back into the soil solution, or they restrict their translocation to the shoots in order to slow down salt accumulation to toxic ranges (Munns, 2011). There are three mechanisms, which are used by plant cells to avoid toxic increase of Na in the cytosol (Zhu, 2001). The primary mechanism is the controlled entry of Na into plant cells by selective ion uptake. Second, excess of Na inside the cell can be stored in cell vacuoles and this vacuolar compartmentalization is a well-organized approach for plant cells to treat with salt stress since the stored Na also helps in osmotic adjustment. Due to this reason, halophytes are well adapted to high salinity stress and they are recognized for bulk accumulation of Na in the cell vacuole (Flowers *et al.*, 1977). The third strategy to cope with high salts is that- Na in the cytosol may be exported or sent back-to apoplastic spaces via SOS pathway, and has a pivotal regulatory function in salt tolerance (Zhu, 2001). The presence of Na^+/H^+ antiporters on the

plasma membrane is prerequisite to accomplish this task (Blumwald, 2000). In addition, excess Na is also excreted through salt bladders or salt glands by some types of plants, such as recretohalophyte (Zhu, 2001). Moreover, plants also vary in their capacity to tolerate high pH soils.

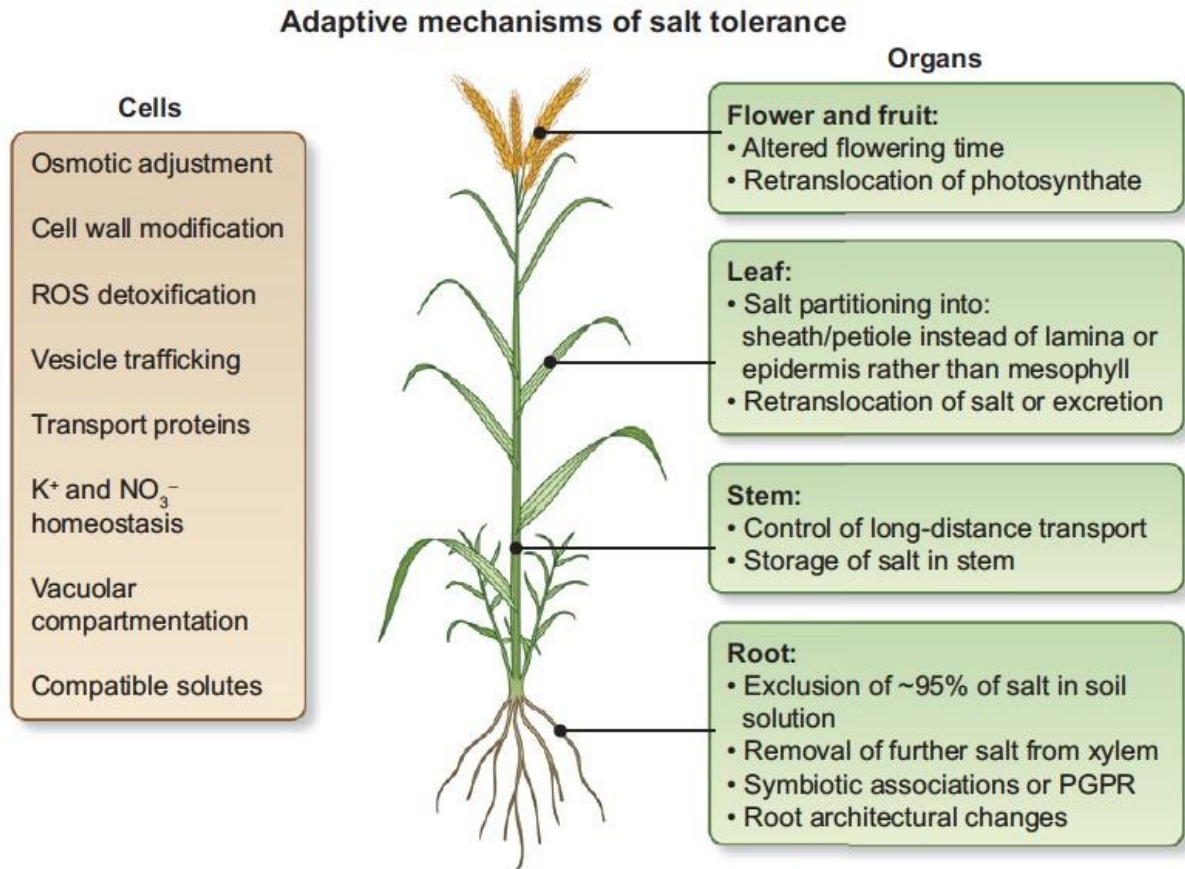


Figure 4. Adaptive mechanisms for salt tolerance in plants (Munns and Gilliam, 2015).

In quite alkaline soils, some plant roots can exude high amounts of acids into the soil solution. In response, this lowers the pH instantly around the roots and increases nutrient uptake. In extremely alkaline soils (pH > 8.0), even these plants experience nutrient deficiencies. In an effort to defeat the special toxic effects caused by salinity, plants employ a variety of other guard mechanisms, for instance, the production of compatible osmolytes (i.e., amino acids, sugars, and alcohols). The produced osmolytes try to maintain the osmotic pressure within the plant cell (Sahi *et al.*, 2006), thereby adjusting water uptake, water balance and photosynthesis. In addition, these metabolites also play a significant function in membrane and protein protection against reactive oxygen species. It is also concluded in the previous research that there is also over or low expression of definite proteins in response to salt stress (Moons *et al.*, 1997) that eradicates ROS production and, heat-shock proteins help sustain the proper folding of proteins (Tiroli-Cepeda and Ramos, 2010). To acclimatize the stress, the H^+ ATPase transporter offers an important role to adapt the soil alkalization by mediating proton secretions (Yang *et al.*, 2010).

1.2.2 Role of ion transporter for salinity adaptation

Ion transporters and their localization in key cell types underpin plant salinity tolerance (Osakabe *et al.*, 2014). Ion transporter-like PM H⁺-ATPase, SOS1, Chloride channel (CLC1), potassium transporter (AKT2/3, KUP/HAK), and aquaporin protein are key factors involved in salinity resistance in plants. Proton pumps (PM H⁺-ATPase) play an essential role in cellular ion transport and sodium sequestration. P-type ATPases (PM H⁺-ATPase) localize at the plasma membrane maintain a negative membrane potential. During salt stress, the positive charge of sodium ions depolarizes the membrane potential. Consequently, the H⁺-ATPases become more active to counteract this depolarization, decreasing sodium import and potassium export (Shani *et al.*, 2017). Moreover, P-ATPases fuel the exclusion of sodium from the root, a process mediated by the sodium/proton antiporter SOS1/NHX7 (Gaxiola *et al.*, 2007). Similarly, the tonoplast-localized H⁺-ATPases build up the proton-motive force necessary for sodium sequestration in the vacuole. These functions of H⁺-ATPases show how they influence the transport of other ions through their effect on membrane polarity and pH of intercellular compartments. In addition to Na⁺, Cl⁻ compartmentation is also important for salt tolerance, as elevated levels of Cl⁻ in the cytosol maybe harmful, and some plants are particularly more sensitive to Cl⁻ toxicity than the others. Since the vacuole is moderately positive charged with reference to the cytoplasm, part of the vacuolar Cl⁻ sequestration could proceed through ion channels, and several voltage-gated anion channels of the CLC family have been detected in the tonoplast of various species. In rice, CLC1 was significantly upregulated in response to salinity and was involved in salinity tolerance via vacuolar sequestration of chloride (Diédhiou and Golldack, 2006).

Current progress in molecular genetics and plant electrophysiology indicates that plants' ability to maintain a high cytosolic K⁺/Na⁺ ratio may be crucial to salt tolerance. In plants, HAK/KUP/KT family transporters play a crucial role in K⁺ acquisition and translocation, growth and development, and response to stresses. Expression patterns and physiological roles of some HAK/KUP/KTs from cluster I were characterized in both Arabidopsis and rice (Grabov, 2007; Very and Sentenac, 2003; Wang and Wu, 2013). In Arabidopsis, it was found that AtHAK5 played a key role in high-affinity K⁺ uptake under K⁺ concentration even below 10 μM (Gierth *et al.*, 2005; Qi *et al.*, 2008). In rice, OsHAK1, OsHAK5 and OsHAK21 were significantly induced in roots exposed to K⁺ deficiency and salt stress, and were shown to be involved in the maintenance of K⁺ homeostasis and salt tolerance (Chen *et al.*, 2015; Shen *et al.*, 2015; Yang *et al.*, 2014). Similarly, AKT2/3 (Shaker-type) potassium channels also play key role in K⁺ homeostasis and salt tolerance, are located at the plasma membrane and mediate most of the K⁺-selective voltage-gated currents at both hyperpolarized and depolarized membrane potentials (Very and Sentenac, 2003). The function of AKT2/AKT3 (which enables bi-directional K⁺ transport) is attributed to phloem loading and/or unloading (Marten *et al.*, 1999; Shabala and Cuin, 2008) and K⁺ uptake into leaf mesophyll cells (Dennison *et al.*, 2001). It has been shown that AKT2/3 K⁺ channel is upregulated during salinity (Marten *et al.*, 1999), suggesting its involvements in the

recirculation of K^+ through the phloem. Furthermore, the involvement of aquaporin under salinity resistance is well known. Aquaporin proteins are a large multigenic family that regulates a large proportion of water transport across membranes, are rapidly influenced both transcriptionally and post-translationally by salt (Chaumont and Tyerman, 2014). Overexpression of a PM intrinsic protein (PIP) in soybean increased shoot Na^+ exclusion and increased seed yield from a saline field (Zhou *et al.*, 2014). Hence, ion transport is the fundamental factor determining salinity tolerance in plants.

1.2.3 Role of phytohormones for salinity adaptation

Phytohormones play an essential role in plant growth and development. To cope with salinity stress during different developmental stages of a plant, phytohormones like abscisic acid (ABA), indole acetic acid (IAA), gibberellic acid, jasmonic acid, salicylic acid, brassinosteroids and ethylene are induced or their levels are modulated (Ryu and Cho, 2015). Abscisic acid (ABA) plays an important role in salt stress tolerance because of its rapid accumulation in response to stress. ABA has key roles in stomatal closure and regulation of root growth under abiotic stress, and guard cells and various root tissues are the hotspots for their functions (Geng *et al.*, 2013; Kim *et al.*, 2010). Salinity induces the expression of ABA biosynthetic genes (e.g., 9-cis-epoxycarotenoid dioxygenases (NCEDs), ABA DEFICIENTs (ABAs), and aldehyde oxidase (AAO3) in specific vascular tissues (Barrero *et al.*, 2006; Endo *et al.*, 2008; Ruiz-Sola *et al.*, 2014). The perception of ABA by pyrabactin resistance-like abscisic acid receptor (PYR/PYL) leads to inactivation of PP2C/ABI1, which induces the phosphorylation activity of subclass III SnRKs (SnRK2.2, SnRK2.3 and SnRK2.6). Various downstream phosphorylation targets of SnRK2 kinases mediate processes including ion transport, ROS production, gene transcription, and closing of stomata are modulated by ABA, and participated in induction of salt resistance mechanisms.

1.3 Alleviation of salinity by ABA priming

Plants evolved mitigation mechanisms at various organizational levels to resist salt stress. Salt stress activates many mechanisms, such as stomatal regulation, various morphological modifications, osmolyte accumulation, sodium exclusion by roots, and vacuolar partitioning of excess ions that contribute to plant resistance. Hence, to pre-emptively strengthen the salt resistance in plants, it is vital to activate the resistance mechanisms before the onset of salt stress.

Priming represents a sustainable, fast, cost-effective, and environmental-friendly procedure for crop production under stressed environments (Tanou *et al.*, 2012). The priming process is referred to as pre-treatment of plants, seeds, or roots, with mild stressor agents to activate resistance responses in plants to future stress exposure (Tanou *et al.*, 2012). Priming induces physicochemical and transcriptional adaptations, which allow plants to react faster and more robustly after they encounter biotic or abiotic stress (Savvides *et al.*, 2016). So far, numerous priming agents, such as salicylic acid, β -amino butyric

acid, kinetin, or abscisic acid (ABA), have been used to enhance resistance against drought, cold, or salt stress (Savvides *et al.*, 2016).

ABA is considered a stress hormone, and its rapid accumulation in plants under various abiotic stresses shows its involvement in stress resistance of plants (Geilfus *et al.*, 2018; Zörb *et al.*, 2013). Long-term root application of ABA to stressed plants has shown to improve drought resistance in rice (Teng *et al.*, 2014), salt-resistance in potato (Etehadnia *et al.*, 2008), and common bean (Khadri *et al.*, 2007). These studies indicated that the improvement in growth of salt-stressed plants induced by exogenously applied ABA was related to decreased sodium (Na^+)-to-potassium (K^+) ratio and chloride (Cl^-) accumulation, and increased accumulation of proline or soluble sugars. In this way, ABA priming was demonstrated as a potential tool in mitigating salt stress in crops.

1.4 Objectives

Vicia faba L. plant was selected in this study to investigate the mechanisms underlying saline-alkaline stress. It is a moderately sensitive plant to salt stress (Lauchli, 1984) and most likely also responds to alkaline stress. Up to now, the mechanisms which lead to growth reduction under alkaline salt stress are not well understood because the dissection of the involved stress components viz. high pH, sodium and bicarbonate was lacking. Hence, for the first time, this thesis experimentally separated these stresses and assessed their effect on growth and various physiological, metabolic, and transcriptional measures.

This study provided a broad analysis of the impact of immediate (150 min) and short term (4 day) effects of alone high pH and alkaline salt stress treatment on plant growth related factors such as gas exchange, leaf water content, apoplastic pH, metabolite accumulation, ion relations, oxidative damage, and changes in the expression of certain key genes. Furthermore, this thesis provided an inclusive analysis of one time ABA priming to induce long term (8 day) salinity resistance in plants.

Hence, present study aimed to answer the following research questions:

- 1). Does alkaline pH or osmotic stress induced by alkali salt elicit fast physiological, metabolic and transcriptional changes for early plant adaptation during the first few hours after the onset of stress treatment?
- 2). Is high pH, high sodium or high bicarbonate primary responsible for early growth reduction under alkaline salt stress?
- 3) Is one-time ABA priming induced long term transcription changes in ion transporter and salt stress signaling genes responsible for improvement in ion relation and osmolyte composition to mitigate salt induced growth reduction?

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Chapter 2

Alkali salt stress causes fast apoplastic alkalization and shifts in ion and metabolite composition and transcription of genes during the early adaptive response of *Vicia faba* L

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Alkali salt stress causes fast leaf apoplastic alkalinization together with shifts in ion and metabolite composition and transcription of key genes during the early adaptive response of *Vicia faba* L

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ABSTRACT

The mechanisms by which plants respond to alkali salt stress are still obscure, and the relevance of alkaline pH under combined alkali salt stress. Early stress responses can indicate mechanisms leading to damage and plant resistance. The apoplast contains essential determinants for plant growth, specifically early apoplastic pH fluctuations are induced by many stressors and hypothesized to be involved in stress signalling. Hence, this study aims to identify fast responses specific to alkaline pH and alkali salt stress by exposing the root of hydroponically grown *Vicia faba* L. plants to 150 min of either 50 mM NaHCO₃ (pH 9) treatment or alkaline pH 9 alone. Apoplastic pH was monitored in real-time by ratiometric fluorescence microscopy simultaneously with SWIR transmission-based measurements of leaf water content (LWC). Moreover, we examined the effect of these stresses on apoplastic, symplastic and xylem ion and metabolite composition together with transcriptions of certain stress-responsive genes. Physiological and transcriptional changes were observed in response to NaHCO₃ but not to alkaline pH alone. NaHCO₃ elicited a transient reduction in LWC, followed by a transient alkalinization of the apoplast and stomatal closure. Simultaneously, organic acids and sugars accumulated. Fast upregulation of stress-responsive genes showed the significance of gene regulation for early plant adaptation to alkali salt stress.

1. Introduction

Soil salinization and alkalization co-occur in nature. Salt stress can be defined as the excess of neutral salts (NaCl, Na₂SO₄) and alkali stress as the excess of alkaline salts (NaHCO₃, Na₂CO₃) in the rooting medium that negatively affects plant growth. While salt stress in soil generally involves osmotic stress and ion-induced plant tissue injury, an additional detrimental effect of alkaline pH is associated with alkali salt stress [2–4]. Thus, alkali stress is more severe than salt stress alone [5]. Due to high concentrations of carbonates (CO₃²⁻) and bicarbonates (HCO₃⁻), alkaline soils are usually characterized by alkaline pH and low availability of plant nutrients [6,7]. Globally, about 831 million hectares of land are saline-alkaline [8,9], of which approximately 397 million hectares (47%) are saline, and 434 million hectares (53%) are saline-alkaline [10].

Bicarbonate (HCO₃⁻) and carbonate (CO₃²⁻) are the principal contributors to soil alkalinity, whereas hydroxide, borate, ammonia, organic bases, phosphates, and silicates are considered minor contributors [11]. The presence of HCO₃⁻ and CO₃²⁻ and other alkaline salts impart to the buffer capacity of the soil and cause an increase in soil pH, resulting in the precipitation and adsorption of the major plant macro- and micronutrients such as calcium, magnesium, iron and zinc [12,13]. Plants irrigated with water having high alkaline salt concentrations always show chlorosis (due to iron deficiency) of young leaves [14,15], resulting in reduced root growth and uptake or utilization of nutrients. Additionally, higher exchangeable sodium (exchangeable sodium percentage > 15) in the presence of alkaline pH (pH > 8.3) will replace Ca²⁺ and Mg²⁺ on the soil exchange sites will lead to dispersion of the soil particles [16]. Consequently, soil structure and physical properties are negatively affected, causing an increase in swelling and

Abbreviation: ABA, Abscisic acid; CDS, Coding sequence; IC, Isocratic ion chromatography; ICP-MS, Inductively coupled plasma mass spectroscopy; LED, Light emitting diode; LWC, Leaf water content; *NCED*, 9-cis-epoxycarotenoid dioxygenase; *PEPCase*, Phosphoenolpyruvate carboxylase; *PIP*, Plasma membrane intrinsic protein; PM, Plasma membrane; qRT-PCR, Quantitative reverse transcription-PCR; SWIR, Short wave infrared.

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dispersion of soil. Alkaline pH causes precipitation of Ca^{2+} and Mg^{2+} in the soil solution and also interferes with proton-coupled ion uptake, thus disturbing the intracellular ion balance in plants [3]. All this limits photosynthesis and plant growth [3,17]. The plants in alkaline soils have to survive physiological drought (due to osmotic stress) and ion toxicity (excess sodium accumulation) and also have to struggle to maintain intracellular ion balance and adjust external pH through the root exudation of organic acids [18,19]. Additionally, our recent study revealed that under mid-term (4 days) alkali salt stress, severe growth reduction is primarily caused by excess bicarbonate concentration, rather than by high pH alone or by high pH combined with sodium stress [1].

Although a substantial amount of information is available about mid-to-long-term saline-alkaline stress responses, covering aspects of alkaline pH, osmotic and ionic stress, nutrient availability, organic acids, osmolytes, or sugar content in affected plants [3,17,20], early responses occurring immediately after the onset of alkali salt stress are largely unknown. Since quick transitions from non-alkaline soil conditions to alkaline salt stress are uncommon in nature, the investigation of short-term stress responses can be useful to understand mechanisms of damage as well as to identify and understand stress tolerance mechanisms in plants. Secondly, the different deleterious effects of alkali salt stress on growth reduction are not well understood because the dissection of the involved stress components viz. alkaline pH and salt effect, is lacking.

Plant growth in general is explained by the acid-growth theory [21, 22] which states that cell wall extensibility is increased by acidification of the apoplast. It is assumed that auxin mediates the acidification of the apoplast to a pH lower than 5.0 [23], thus stimulating cell elongation [24]. A lowered apoplastic pH is presumed to activate wall loosening expansins, thus enhancing cell growth [25]. Inhibition of cell elongation is one primary effect of drought and salt stress [4]. Water stress is accompanied by a decrease of proton extrusion to the apoplast resulting in an increased apoplastic pH and growth inhibition [26]. Apoplastic alkalization is often seen as a general response to environmental stressors like drought and salinity [27,28]. Being the extracellular compartment of plant cells, the apoplast is known for its role in abiotic and biotic stress signal perception [29,30]. The transient apoplastic alkalization has been discussed as a general stress factor [28,29,31]. It is believed to be a root-to-shoot signal, transmitting information regarding an (ongoing) stress event to distant plant organs. Geilfus et al. [32] reported the involvement of fast pH transients in the salt stress responses of *Vicia faba*: Shortly after imposing salt stress, a transient rise in leaf apoplastic pH increased abscisic acid (ABA) concentrations in guard cells, which initiated stomatal closure. Fast apoplastic pH transients to NaCl stress thus have physiological relevance in salt stress resistance mechanisms.

Nevertheless, it remains obscure if apoplastic pH transients also occur under short term alkali salt stress, and if so, which stress component of the alkali stress, i.e. alkaline pH vs the composite osmotic and, ionic- component elicit(s) early apoplastic pH responses. Moreover, specific resistance mechanisms of the early alkali salt stress response, which may be prompted by pH transients, need to be identified. Intuitively, one could assume that the alkaline pH of the rooting medium might propagate to the plant apoplast, viz. alkalize it. This could be relevant for acid growth and other key processes such as ion uptake. Hence, the present study focuses on an experimental separation of alkaline pH from combined alkali salt stress effects to determine whether alkaline pH is the decisive component of the composite alkali salt stress for the early apoplastic pH responses. *Vicia faba* L. plant was selected in this study to investigate the mechanisms underlying fast response (150 min) of alkali salt stress. It is a moderately sensitive plant to salt stress [33] and most likely responds to alkaline stress [34].

Moreover, identifying early stress responses and ascribing them either to alkaline pH alone at the roots makes effects or the combined alkali salt stress. We also examined the ion and metabolite composition

in the leaf apoplast, the xylem and the leaf symplast under these conditions. Transcriptional stress responses were examined in certain key stress-responsive genes such as an ABA biosynthesis-related gene 9-cis-epoxycarotenoid dioxygenase (*NCED*), an aquaporin gene (*PIP*), plasma membrane proton transporters (PM H^+ -ATPase isogenes *vha4*, *vha5*), and a carbon metabolism-related gene phosphoenolpyruvate carboxylase (*PEPCase*), which are involved in the early adaptive response of the plant to stress [1,13,35–38]. We hypothesized that alkali salt stress has a specific response of apoplastic pH and a set of damage and resistance mechanisms not elicited by alkaline growth medium pH alone.

2. Material and methods

2.1. Plant cultivation and experimental design

Vicia faba L. (cv. Sirocco: NPZ GmbH, Hohenlieth, Germany) was cultivated under hydroponic culture condition in a controlled climatic chamber (14/10 h day/night; 20/15 °C; 50/60% humidity). Seeds were surface-sterilized with 0.5% sodium perchlorate for 3 min and then washed thoroughly with deionized water. Afterwards, seeds were soaked in aerated 0.5 mM CaSO_4 solution overnight and sown in moist sterilized quartz sand for germination. After one week, three uniform-sized seedlings were transferred to 4.5 L plastic pots containing a quarter strength of the nutrient solution [39]. To avoid any osmotic shock reaction, the nutrient solution's concentration was gradually increased to $\frac{1}{2}$, $\frac{3}{4}$, and full-strength on the second, third, and fourth day after transplantation, respectively. The nutrient solution was changed after every 2 days to replenish the depleted nutrients. After 30 days of cultivation, plants were divided into three experimental groups. The first group of plants was treated with a 50 mM concentration of NaHCO_3 salt (EC $3.8 \pm 0.2 \text{ dS m}^{-1}$) over 150 min which had a pH of 9 ± 0.2 , the second group with alkaline pH alone in the nutrient solution (2 mM CAPSO buffer, pH 9 ± 0.2 , EC $1.43 \pm 0.2 \text{ dS m}^{-1}$) over 150 min and, last, the third group that served as a control (pH 6.2, EC $0.63 \pm 0.2 \text{ dS m}^{-1}$) without any stress treatment. Plants from each group were used for real-time *In-planta* leaf apoplastic pH ($n = 4$) and water content measurement ($n = 4$). Another fraction of each group was used to collect and determine apoplastic, symplastic, and xylem sap minerals and compound ($n = 12$). Finally, another fraction of each group was used to determine certain key gene transcripts ($n = 12$).

2.2. In planta real-time monitoring of leaf water content and apoplastic pH

For real-time co-monitoring of leaf water content (LWC) and apoplastic pH, intact and plant-attached leaves were mounted in a humidity-controlled cuvette equipped with an LWC sensor based on short wave infrared (SWIR) transmission as described by [40]. The leaf was attached to the glass bottom of a cuvette on top of an inverted microscope (iMIC, Thermo Fisher, Germany) which allows ratiometric fluorescence microscopy. White light illumination was provided by an LED (PPFD of $250 \mu\text{mol m}^{-2} \text{ s}^{-1}$). Humidity control (0.72 kPa; 70% relative humidity at 20 °C) was achieved by streaming a constant flow of humid air into the cuvette and removing varying amounts of humidity with a cold trap arranged in a bypass flow which was driven by a variable flow pump (Walz, Germany). An LWC-sensor based on a SWIR transmission ratio continuously measured LWC near the pH measurement site. Values were recorded by a data-logger (CR23x, Campbell Scientific). For in situ measurement of apoplastic pH, 30 μM solution of the ratiometric fluorescent pH indicator dye Oregon Green (OG) 488-dextran (Invitrogen GmbH, Darmstadt, Germany) was infiltrated into the leaf apoplast of intact leaves using a needleless syringe [39]. Fluorescence images were acquired as a time series with an iMIC inverted microscope Thermo Fisher, Germany) at 440 nm (pH insensitive) and 490 nm (pH sensitive). Emission was collected at 535/25 nm

for both channels. The fluorescence ratio F490/F440 was obtained as a measurement of pH on a pixel-by-pixel basis. Ratio data were converted into apoplastic pH values by using an *in vivo* calibration [39]. The Boltzmann fit was used to fit a sigmoidal curve to the calibration data.

2.3. Osmolality measurement

To determine osmolality, leaves were shock-frozen in liquid nitrogen for a few seconds and then thawed at room temperature. Subsequently, the thawed leaves were centrifuged at 327 g for 5 min, and collected fluid was regarded as cell sap. Subsequently, the osmotic potential of cell sap was measured on a freezing point depression osmometer O30 (Genotec GmbH, Berlin, Germany).

2.4. Stomatal conductance

Stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) was measured via a portable gas exchange measurement system (LI 6400XT, Li-COR Biosciences Inc., USA). For measurements, intact, fully expanded fifth leaves were placed across a 2×3 cm leaf chamber. The conditions for the measurements inside the leaf chamber were maintained identical to the external conditions of the climatic chamber. The light was provided by a LED red light source built into the top of the leaf chamber ($250 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$), and the CO_2 concentration was controlled by Li-Cor LI-6400 CO_2 injection system ($400 \mu\text{mol CO}_2 \text{ mol}^{-1}$).

2.5. Collection and determination of apoplastic, symplastic washing fluid and xylem sap

The fully expanded leaves were selected for apoplastic washing fluid (AWF) collection and symplastic fluid isolation based on the infiltration-centrifugation technique [41,42]. Briefly, leaves were washed with Mili-Q water, and then intact leaves were gently placed into 100 ml plastic syringes which were filled with 70 ml Mili-Q water. By pulling the plungers of syringes, the leaves were infiltrated with an estimated 20 kPa. Afterwards, leaves were blotted with tissue paper and centrifuged at 71 g for 5 min at 4°C to separate AWF. Residual leaf tissues were shock frozen in liquid nitrogen for several seconds and then thawed at room temperature. Subsequently, the thawed leaves were centrifuged at 327 g for 5 min, and the collected fluid was regarded as symplastic fluid. Both AWF and symplastic fluid were stored at -80°C .

Xylem sap collection was performed according to the method given by Nakamura et al. [43]. Briefly, the shoot was decapitated approximately 2–3 cm above ground level. To avoid contamination with phloem sap or broken cells, the cut section of plant shoot was washed with Mili-Q water, and a silicon tube was fitted to the stump to avoid contamination with phloem sap. Xylem sap collected at the initial 3 min after cutting the shoot was not used in the analysis. For each experiment, xylem exudate samples were collected for 3 h between 11.00 and 14.00 h. Collected samples were frozen at -80°C until subsequent analysis.

For the determination of macronutrient and Na^+ concentration in apoplastic, symplastic and xylem sap, inductively coupled plasma mass spectroscopy (ICP-MS; Agilent 7700, Agilent Technologies Inc., USA) was used as described by Wu et al. [44]. Anions and sugar were determined by using ion chromatography (DX5000; Dionex, Germany) as described by Shahzad et al. [42].

2.6. RNA isolation and RT-qPCR analysis

To analyze the mRNA transcript level of certain key genes (*vha4*, *vha5*, *Vf PIP*, *Vf PEPcase*, *VfFam NCED*), total coding RNA of leaves was extracted using TRIzol (Invitrogen) according to manufacturer's instructions. The concentration and purity of RNA were determined by a NanoVue Plus Spectrophotometer (GE Healthcare Life Science), and integrity was checked by gel electrophoresis. Total coding RNA ($1 \mu\text{g}$)

isolated was reverse transcribed following the manufacturer's instructions (Verso cDNA synthesis kit, Thermo Fisher Scientific, USA), including DNase I treatment. The quality of synthesized cDNA (1/10 dilution) was examined by standard PCR with the most stable reference gene ubiquitin-conjugating enzyme (*Vf CYP*) [35,45,46]. Quantitative RT-PCR was performed by PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) with primers shown in Supplementary Table S1 on CFX96 Real-Time System (Bio-Rad Laboratories GmbH, München, Germany). Each reaction ($20 \mu\text{l}$) contains 100 nM of each primer, $2 \mu\text{l}$ of diluted cDNA templates, and other reaction components. After an initial denaturation step (95°C for 5 min), RT-qPCR was carried out over 40 cycles (95°C for 15 s, 60°C for 30 s, 72°C for 30 s), followed by a melt curve stage (95°C for 15 s, 60°C for 1 min, 95°C for 15 s, 24°C for 15 s). Three biological replicates and three technical replicates were used for each treatment. Transcript levels of genes were normalized with endogenous control (*Vf CYP*), and the expression changes of target mRNAs were determined using the $2^{-\Delta\Delta\text{Ct}}$ method [47]. For gene validation, the RT-qPCR amplicon was confirmed by sanger sequencing (Supplementary Table S2).

2.7. Statistical analysis

Data were statistically analyzed following one-way ANOVA, which was performed using SPSS software (version 17.0). Significant differences among the means were determined by Tukey test at $p \leq 0.05$.

3. Result

3.1. Early effect of alkaline pH alone and NaHCO_3 stress on stomatal conductance

Upon one-time root application of 50 mM NaHCO_3 salt, the stomatal conductance rate started to decrease with a delay of 50–55 min and showed a gradual decline over 150 min of observation (Fig. 1). At the end of the observation, the stomatal conductance rate was half of its initial value. On the other hand, both control and alkaline pH treatment in the rooting medium exhibited a steady rate of stomatal conductance for the 150 min of observation (Fig. 1).

3.2. Early effect of alkaline pH alone and NaHCO_3 stress on leaf water content (LWC), apoplastic pH and osmolality

Using real-time co-measurement of LWC and apoplastic pH, we observed that root application of 50 mM NaHCO_3 salt led to a transient change in LWC and apoplastic pH, whereas alkaline pH and control treatments never altered these two variables (Fig. 2A, B). Upon root application of 50 mM NaHCO_3 , the examined leaf showed a rapid and continuous decline in LWC by 1.3% of its initial value. This decline lasted for 25–30 min. After that, LWC began to recover, and it took another ~70 min to reach and subsequently overshoot its initial value (Fig. 2C left Y-axis). On the contrary, apoplastic pH responded with a delay of 25–30 min and showed a transient alkalization by a rise in pH unit of 0.5 (4.8–5.3). This transient alkalization persisted for 55–60 min (Fig. 2C right Y-axis). After that, apoplastic pH started to drop and reached its initial acidified value.

Observing that root application of 50 mM NaHCO_3 induced transient change in LWC, we found that after 150 min of treatment, leaf osmolality was increased by 26% compared to the control treatment. However, no significant difference between alkaline pH and control treatments was recorded (Fig. 3).

3.3. Early effects of alkaline pH alone and NaHCO_3 stress on the mineral composition of leaf apoplastic, symplastic washing fluid and xylem sap

As expected, the plant treated with 50 mM NaHCO_3 treatment showed a higher concentration of sodium (Na^+) in apoplastic,

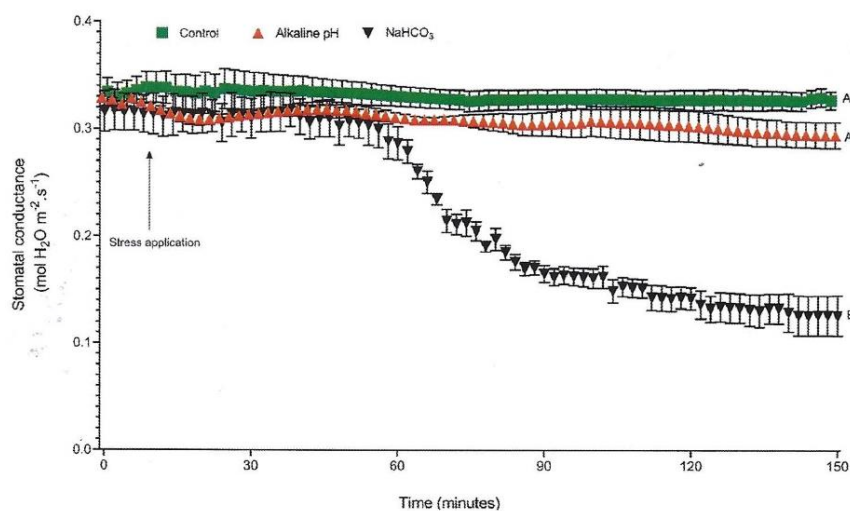


Fig. 1. Stomatal conductance measurements of the fifth leaf of thirty days old *Vicia faba* as influenced during the first 150 min after initiating alkali salt stress (50 mM NaHCO_3 , pH 9 ± 0.2) (black color) and alkaline pH stress (pH 9 ± 0.2) (red color) as plotted over time. Control treated (pH 6 ± 0.2) (green color) plants were not treated with either alkali salt or alkaline pH. Readings were taken every 2 min. Mean \pm SE of four independent replications. Different letters indicate statistical significance ($p \leq 0.05$) between control, alkaline pH and NaHCO_3 treated plants. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

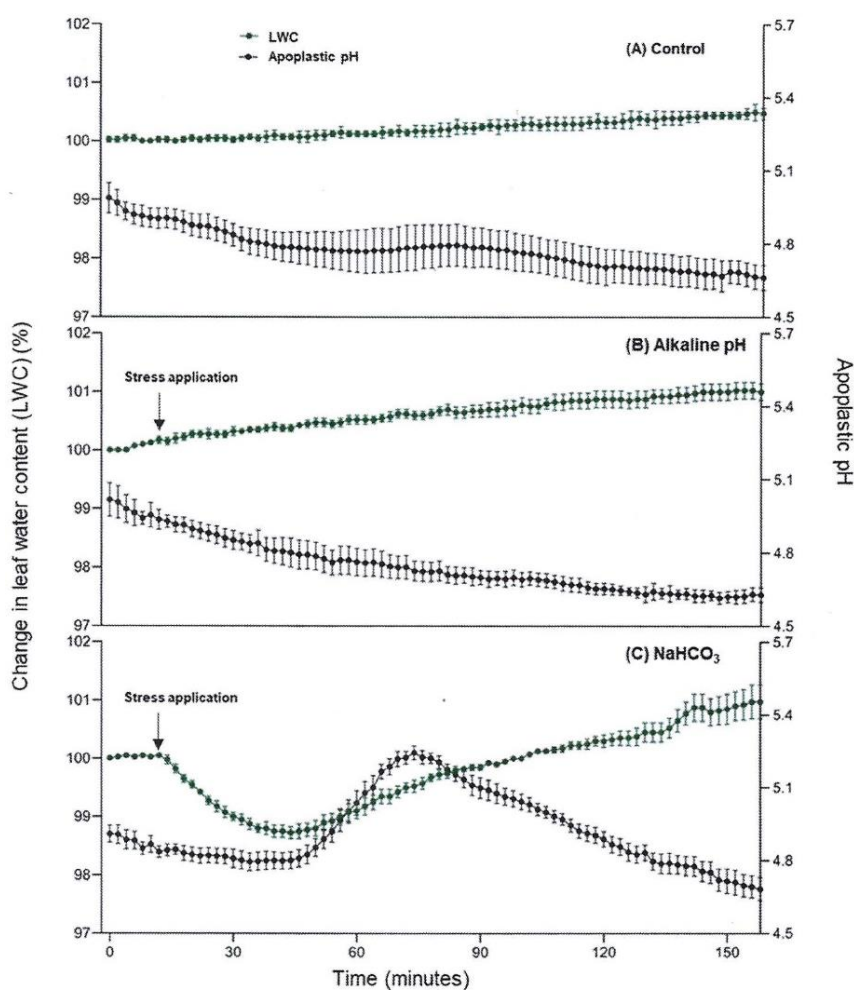


Fig. 2. Course of real-time co-measurement leaf water content (% change) (left Y-axis, green color) and apoplastic pH (right Y-axis, black color) of the fifth leaf of thirty days old *Vicia faba* over 150 min – Upon one-time root application of treatment transient change in LWC (green color) and apoplastic pH (black color) was recorded in every two minutes. (2 A) Control (pH 6 ± 0.2), (2 B) alkaline pH (pH 9 ± 0.2), (2 C) NaHCO_3 (pH 9 ± 0.2). Average kinetics data (\pm SE) of four equivalent plants' measurements achieved from independent experiments ($n = 4$ biological replicates). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

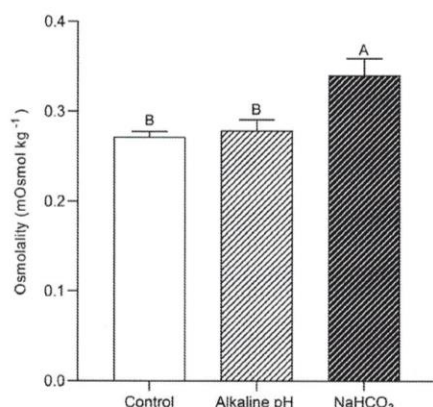


Fig. 3. Thirty days old *Vicia faba* leaves sap osmolality as influenced during the first 150 min under control (pH 6 ± 0.2), alkaline pH (pH 9 ± 0.2), and NaHCO₃ (50 mM, pH 9 ± 0.2) stress treatment. The data ± SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

symplastic washing fluid, and xylem sap (Fig. 4A, B, C), with increasing order of symplastic washing fluid (5.8 fold) > xylem sap (4 fold) > apoplastic washing fluid (2.8 fold) compared to control treatment. On the other hand, alkaline pH treatment showed no significant difference in Na⁺ concentration with control treatment.

Under 50 mM NaHCO₃ stress treatment, potassium (K⁺) concentration in leaf apoplastic and symplastic washing fluid showed an inverse relationship. In leaf apoplastic washing fluid, K⁺ concentration was increased by 2.7-fold (Fig. 4D), while symplast washing fluid showed a decline in K⁺ concentration by a factor of 0.23 (Fig. 4E) than the control treatment. Apoplastic and symplastic washing fluids of alkaline pH

treatment showed no significant difference in K⁺ concentration with the control plant (Fig. 4D, E). The potassium concentration of xylem sap remained unchanged among all three treatments (Fig. 4F).

The concentration of anions, such as nitrate (NO₃⁻) and sulfate (SO₄²⁻), was differentially affected under the three treatments (Fig. 5). Under 50 mM NaHCO₃ stress, both apoplastic washing fluid and xylem sap showed a decline in nitrate concentration by 75% (Fig. 5A) and 60% (Fig. 5C), respectively, when compared to the control treatment. Under alkaline pH treatment, nitrate concentration was only reduced in xylem sap by 23% (Fig. 5C), while apoplastic washing fluid showed no significant difference with the control plant (Fig. 5A). However, the nitrate concentration of symplastic washing fluid remained unchanged among all three treatments (Fig. 5B). Under 50 mM NaHCO₃ stress, the sulfate concentration of apoplastic washing fluid was increased by 1.8-fold (Fig. 5D), whilst xylem sap showed a decline in sulfate concentration by a factor of 0.55 (Fig. 5F), compared to the control treatment. The alkaline pH treatment had no significant difference in sulfate concentration of apoplastic and xylem sap with the control plant (Fig. 5D, F). Likewise, sulfate concentration of symplastic washing fluid revealed no significant difference between all three treatments (Fig. 5E).

3.4. Early effect of alkaline pH alone and NaHCO₃ stress on metabolite composition of leaf apoplastic, symplastic washing fluid and xylem sap

It was observed that only under 50 mM NaHCO₃ stress, organic acids (malate and citrate) concentrations were increased in apoplastic, symplastic washing fluid and xylem sap (Fig. 6). Malate concentration was found to be higher in the apoplast washing fluid (2.6 fold) (Fig. 6A), while symplastic washing fluid and xylem sap showed a two-fold increment compared to the control treatment (Fig. 6B, C). Likewise, citrate concentration was also increased in the following increasing order; apoplastic washing fluid (6.7 fold) (Fig. 6D) > xylem sap (4.8 fold) (Fig. 6F) > symplastic washing fluid (2.7 fold) (Fig. 6E) than the control treatment. With the alkaline pH treatment, the concentrations of

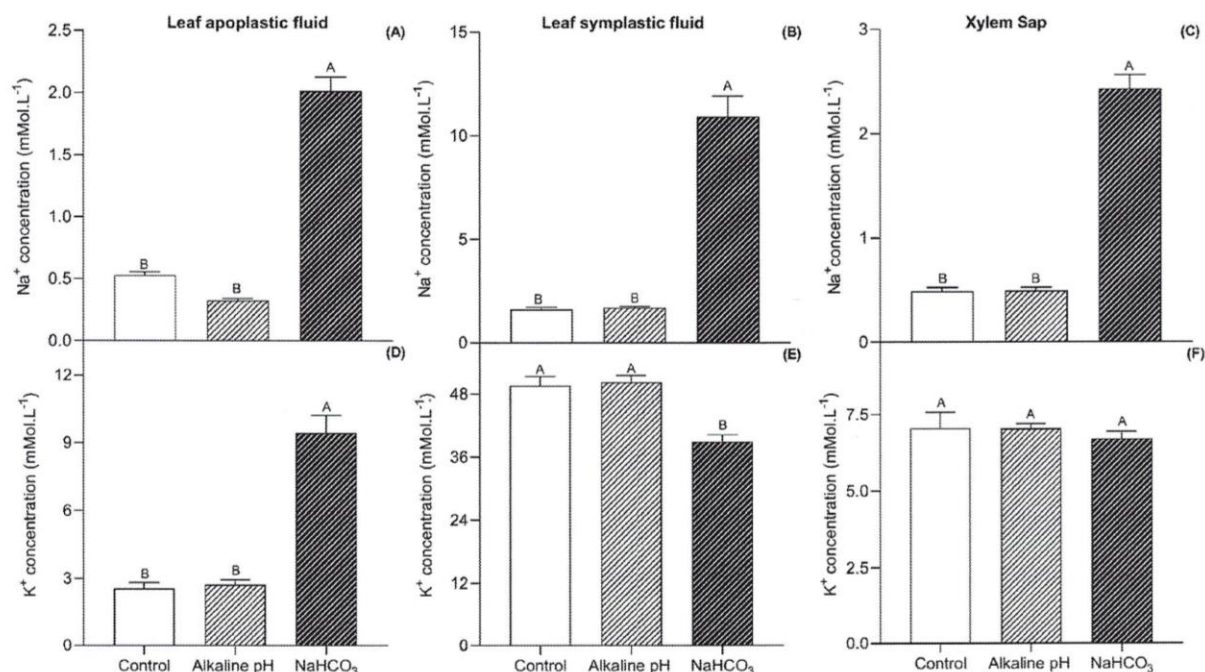


Fig. 4. Concentration of Na⁺ (apoplastic fluid; A, symplastic fluid; B, Xylem sap; C), K⁺ (apoplastic fluid; D, symplastic fluid; E, Xylem sap; F) in thirty days old *Vicia faba* leaves as influenced during the first 150 min under control (pH 6 ± 0.2), alkaline pH (pH 9 ± 0.2), and NaHCO₃ (50 mM, pH 9 ± 0.2) stress treatment. The data ± SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

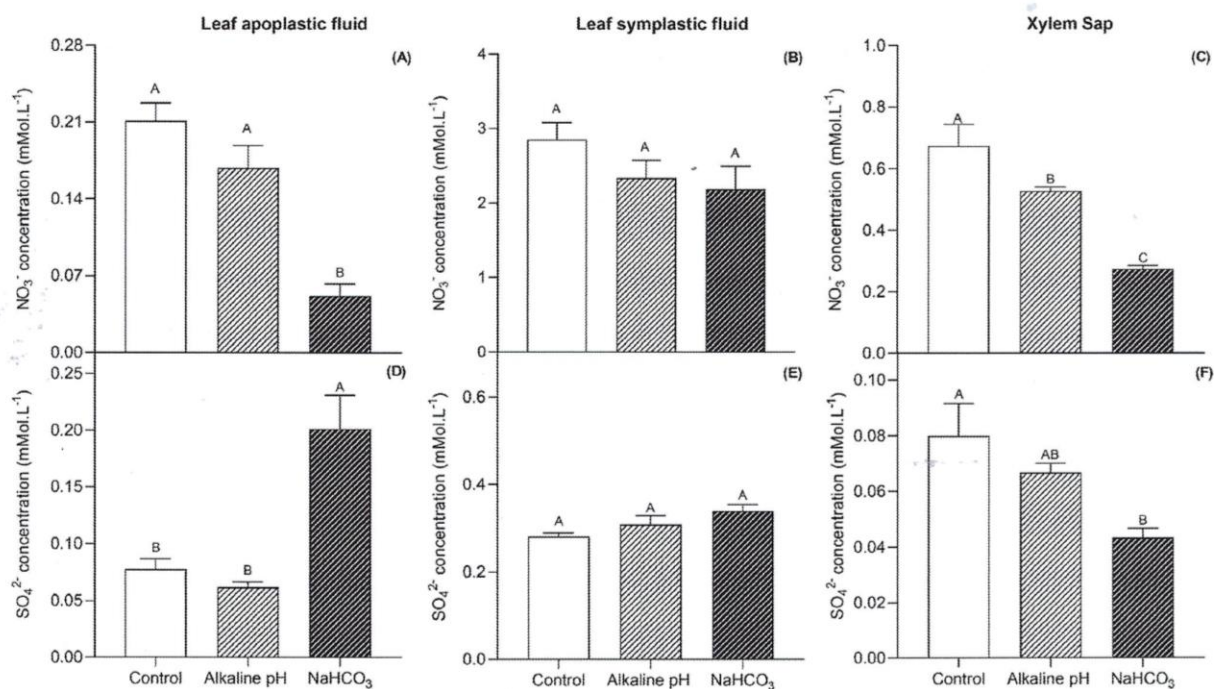


Fig. 5. Concentration of NO_3^- (apoplastic fluid; A, symplastic fluid; B, Xylem sap; C), SO_4^{2-} (apoplastic fluid; D, symplastic fluid; E, Xylem sap; F) in thirty days old *Vicia faba* leaves as influenced during the first 150 min under control (pH 6 ± 0.2), alkaline pH (pH 9 ± 0.2), and NaHCO_3 (50 mM, pH 9 ± 0.2) stress treatment. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

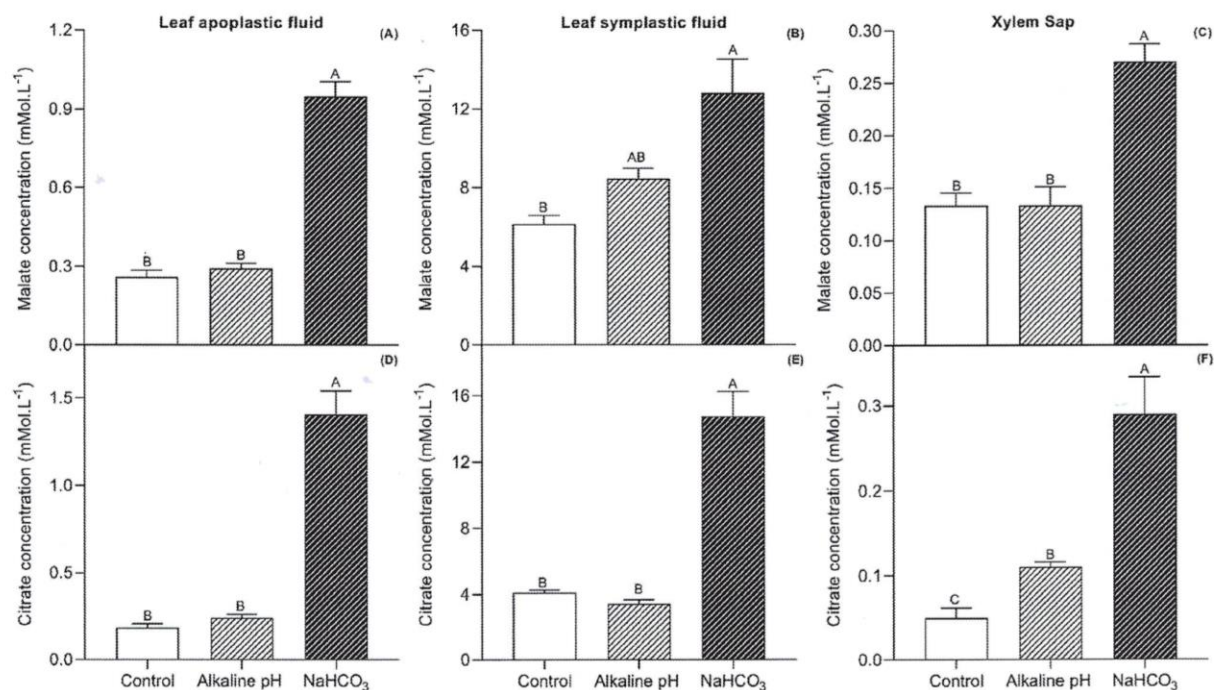


Fig. 6. Concentration of malate (apoplastic fluid; A, symplastic fluid; B, Xylem sap; C), and citrate (apoplastic fluid; D, symplastic fluid; E, Xylem sap; F) in thirty days old *Vicia faba* leaves as influenced during the first 150 min under control (pH 6 ± 0.2), alkaline pH (pH 9 ± 0.2), and NaHCO_3 (50 mM, pH 9 ± 0.2) stress treatment. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

malate and citrate in both apoplastic and symplastic washing fluid, and xylem sap remained unchanged (Fig. 6A, B, C, D, E), except for citrate, which was increased by two-fold in the xylem sap (Fig. 6F) compared to the control treatment.

Besides organic acid, higher sugar accumulation (glucose, fructose, sucrose) was detected under 50 mM NaHCO_3 treatment in apoplastic, symplastic washing fluid and xylem sap (Fig. 7). In apoplastic washing fluid and symplastic washing fluid, monosaccharides (glucose, fructose) concentrations were equally increased by one-fold and two-fold (Fig. 7A, B, D, E), respectively, compared with the control treatment. Monosaccharides in xylem sap did not differ significantly from control (Fig. 7C, F). Also, sucrose concentration was increased in the order of apoplastic washing fluid (3 fold) followed by symplastic washing fluid (1.5 fold) and finally xylem sap (1 fold) than the control treatment (Fig. 7G, H, I). Moreover, sugar concentrations remained relatively unchanged under alkaline pH treatment in apoplastic, symplastic washing fluid and xylem sap (Fig. 7A, B, C, E, F), except that fructose concentrations in apoplastic washing fluid, were raised by 75% compared to the control treatment (Fig. 7D).

3.5. Early effect of alkaline pH alone and NaHCO_3 on the relative mRNA abundance of certain key genes

To address the question of whether observed changes in stomatal conductance, leaf water content, apoplastic pH, subcellular ion and metabolite pattern correlate with the transcriptional response of genes such as ABA biosynthesis-related gene 89-cis-epoxycarotenoid dioxygenase (*NCED*), aquaporin gene plasma membrane intrinsic protein (*PIP*), membrane ion transporters PM H^+ -ATPase isogenes (*vha4*, *vha5*), and carbon metabolism-related gene phosphoenolpyruvate carboxylase (*PEPCase*) were quantified. Under NaHCO_3 treatment, the relative mRNA abundance of all analyzed genes was significantly elevated in comparison to the control; *NCED* (11 fold) (Fig. 8A), *PIP* (3 fold) (Fig. 8B), *vha4* (2.8 fold) (Fig. 8C), *vha5* (2.3 fold) (Fig. 8D), *PEPCase* (4.5 fold) (Fig. 8E). However, the relative mRNA abundance of these genes remained relatively unchanged under alkaline pH treatment compared to control treatment (Fig. 8).

4. Discussion

In our previous work Sagervanshi et al. [1], we observed that rather than alkaline pH alone pH or alkaline pH plus Na^+ , excess bicarbonate, by inducing over-accumulation of organic acids (through dark fixation)

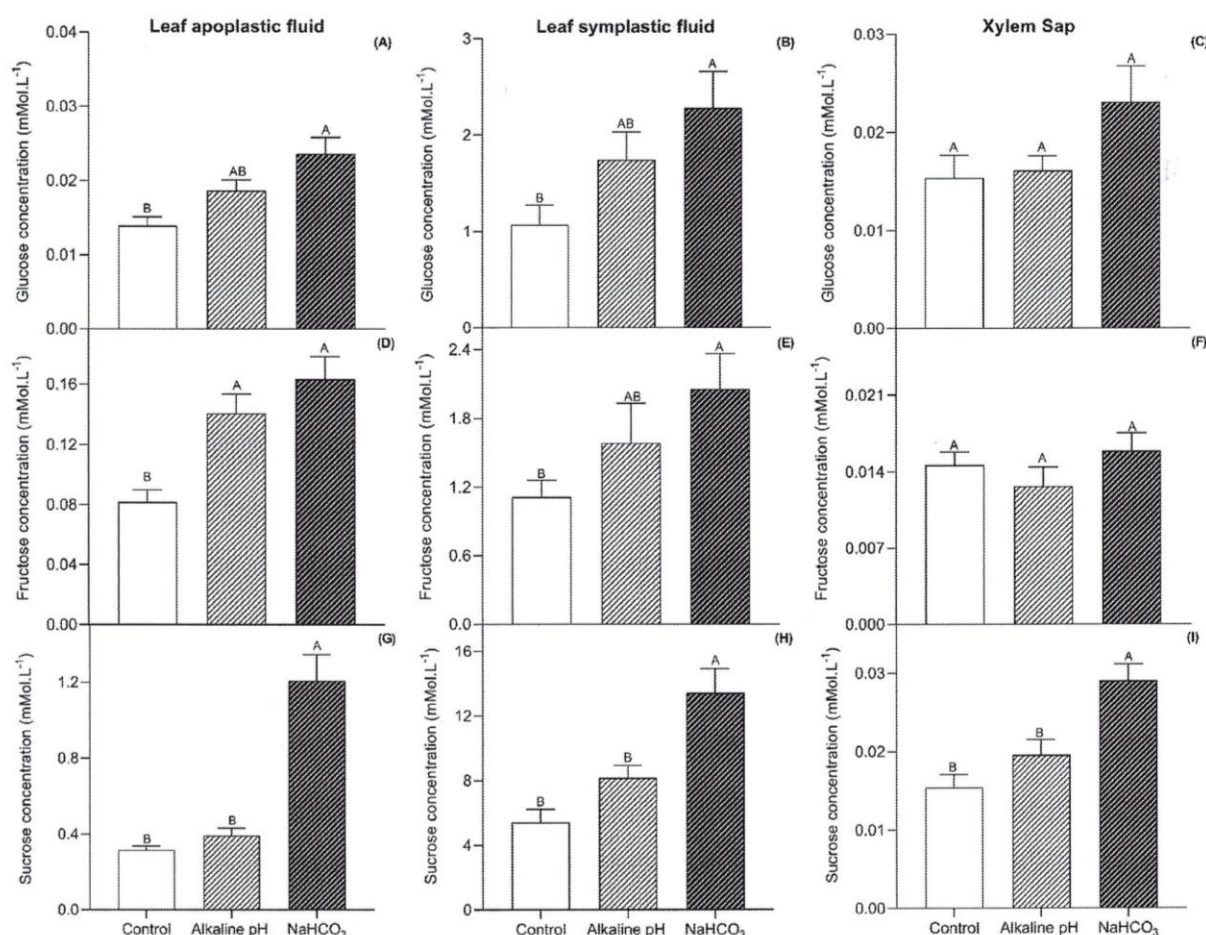
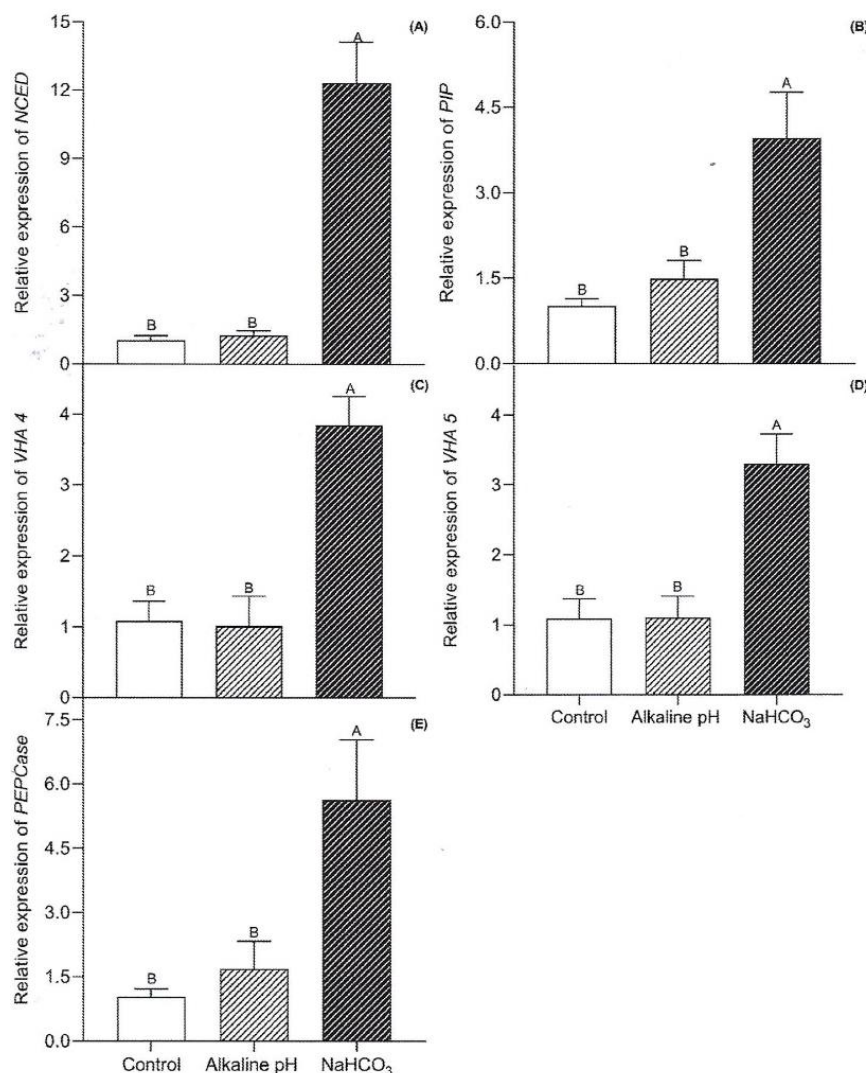


Fig. 7. Concentration of Glucose (apoplastic fluid; A, symplastic fluid; B, Xylem sap; C), fructose (apoplastic fluid; D, symplastic fluid; E, Xylem sap; F), and sucrose (apoplastic fluid; G, symplastic fluid; H, Xylem sap; I) in thirty days old *Vicia faba* leaves as influenced during the first 150 min under control (pH 6 ± 0.2), alkaline pH (pH 9 ± 0.2), and NaHCO_3 (50 mM, pH 9 ± 0.2) stress treatment. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).



(B) Fig. 8. Relative gene expressions of 9-cis-epoxycarotenoid dioxygenase (*NCED*; A), plasma membrane intrinsic protein (*PIP*; B), PM H⁺-ATPase isoform (*VHA4*; C, *VHA5*; D) and phosphoenolpyruvate carboxylase (*PEPCase*; E) in thirty days old *Vicia faba* leaves as influenced during the first 150 min under control (pH 6 ± 0.2), alkaline pH (pH 9 ± 0.2), and NaHCO₃ (50 mM, pH 9 ± 0.2) stress treatment. The data ± SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments (p ≤ 0.05).

in plant roots, was found to be the main growth-limiting factor under four days of alkali salt stress. These results motivated us to investigate rapid responses that evolve within the first 150 min upon stress treatment. However, such fast adjustments might be relevant for an initial stress perception and subsequent systemic information propagation from stressed to non-stressed parts of the plants.

Hence, we are dissecting here the early 150 min responses to root application of alkaline pH alone and/or NaHCO₃ salt stress on leaf apoplast, symplast and xylem ionic and metabolic milieu for the first time. The experimental treatment “NaHCO₃ salt stress” is composed of osmotic, ionic and alkaline pH stress at the roots. The experimental treatment “alkaline pH” lacks the ionic and the osmotic stress component. Thus, filtering for effects that occur only in the group “NaHCO₃ salt stress” but not in the control group or in the “alkaline pH” group will identify effects that ensue specifically in response to alkali salt stress.

Another novelty of our study is that we have co-measured real-time leaf water content along with apoplastic pH over 150 min at given experimental conditions. Our results showed that roots exposed with an alkaline rooting medium did not induce any significant fast change at leaf apoplast, symplast and xylem ionic and metabolic milieu. In

contrast, NaHCO₃ triggered several physiochemical and transcriptional changes during early 150 min one-time root application.

4.1. Early effect of NaHCO₃ stress on stomatal conductance

Our results reveal that only NaHCO₃ treated plants displayed a decrease in stomatal conductance after root application of NaHCO₃. In contrast, alkaline pH-treatment alone and control treatment showed nearly stable stomatal conductance for up to 150 min (Fig. 1). The NaHCO₃ induced lower stomatal conductance may be related to: 1) higher accumulation of apoplastic SO₄²⁻ (Fig. 5D), which incorporated into cysteine, caused ABA production and stomatal closure [48,49] 2) higher concentration of malate in the apoplast (Fig. 6A), which triggered a depolarization of guard cells via activation of anion channel, resulting in stomatal closure [50] 3) increased apoplastic sucrose concentrations (Fig. 7G), which could osmotically, cause stomatal closure [51] 4) transient alkalization of leaf apoplast (Fig. 2C right Y axis) triggered upregulation of ABA biosynthesis gene *NCED* (Fig. 8A), elicit stomatal closure (Fig. 1) [35] 5) osmotic stress from the sodium and bicarbonate ions.

4.2. The fast response of leaf water content (LWC) and apoplastic pH

Alkali salt (NaHCO_3) induced a transient decrease in LWC by 1.3% over a period of ~30 min and recovered by another ~60 min (Fig. 2C left Y-axis). On the contrary, apoplastic pH showed a transient rise of 0.5 pH units with a delay of 30 min, lasting for over ~70 min (Fig. 2C right Y-axis). The rapid transient decrease in LWC is the response to be expected from root exposure to a solution of higher osmotic potential, causing a hydraulically transmitted reduction in leaf turgor and water content [4]. The subsequent recovery of LWC can be attributed to stomatal closure (Fig. 1) and the increased biosynthesis of organic anions (Fig. 6) and sugars (Fig. 7), which reduced the osmotic potential (Fig. 3) and increased LWC. The osmometer results confirmed that only the NaHCO_3 treatment had induced a higher osmolality (Fig. 3). The delayed stomatal closure (45–50 min delay) (Fig. 1) after salt addition may also have supported the gradual recovery of LWC (Fig. 2C left Y-axis). Moreover, increased expression of *PIP* (aquaporin) (Fig. 8B) may also be related to the recovery of LWC under stress conditions, as increased aquaporin abundance can enable plants to adjust variable water deficit conditions [52].

Wegner and Zimmermann [53] reported that the NaHCO_3 treatment-induced xylem sap alkalization in roots of intact maize plants but without describing a mechanism. It is unclear whether this effect measured in roots will extend to the leaf apoplast. Apparently, bicarbonate ion can passively uptake, translocated from root to leaves, by long-distant transported via xylem vessels to the leaves where, after transformation by carbonic anhydrase, the resulting CO_2 can be assimilated along with the atmospheric CO_2 [54–57].

Keeping that in mind, we are newly reporting that the one-time root application of NaHCO_3 induces transient alkalization of leaf apoplastic pH (Fig. 2C right Y-axis). A higher accumulation of malate and citrate in the apoplast (Fig. 6) might have contributed to a transient rise in leaf apoplastic pH (Fig. 2 right Y-axis). In this context, Geilfus [27] suggested that ABA indirectly alkalizes the apoplast by inducing the efflux of organic acids anions such as malate into the apoplast. After arrival in the acidic apoplast, the divalent and negatively charged malate associates with protons, possibly resulting in a decline of the free proton concentration in the apoplastic fluid, hence alkalizing leaf apoplast. We expected that similar mechanisms may have occurred to induce transient alkalization of leaf apoplast under NaHCO_3 , as the ABA biosynthesis gene *NCED* mRNA abundance (Fig. 8A) was also upregulated and observed to be positively correlated with a higher organic acid accumulation (Fig. 6).

Another novelty from our results is that we suggested the time-delayed inverse relationship between LWC and apoplastic pH (Fig. 2C). Our results showed that the transient changes in LWC precede the transient change in apoplastic pH. Our data indicated that transient decline in LWC may have occurred due to salt-induced osmotic shock, which was recovered later by accumulating osmolytes like organic acids and sugars (Figs. 6, 7) via proton-coupled co-transport mechanisms, supported simultaneously alkalization of leaf apoplast (Fig. 2C right Y-axis). This assumption was in line with the finding of Ma et al. [58] who suggested activation of the proton (H^+) coupled solute (sugar and other metabolites) co-transport mechanisms required for osmoberalancing under water deficit conditions. The higher abundance of *PM H⁺-ATPases* as indicated by an increased expression of their isogenes (Fig. 8C, D) may have strengthened the electrochemical proton gradient for proton-coupled membrane transport of solutes and ions, in turn increasing osmotic potential, resulted in recovered LWC and reacidified apoplastic pH (Fig. 2C). Likewise, Cushman [59] suggested that plants utilize *PM H⁺-ATPases* for osmoregulation via primary extrusion or sequestration of protons to generate H^+ electrochemical gradients, which drive secondary ion and nutrient transport processes via H^+ -symport/antiport systems. Hence for osmoregulation, an inverse relationship between LWC and apoplastic pH can be established (Fig. 2C).

4.3. Mineral-relationship of the leaves subcellular washing fluid and xylem sap influenced under alkali salt stress

Knowing that one-time root application of stress treatment, especially NaHCO_3 treatment, induces a significant change in leaf water content and apoplastic pH, the question arose whether these changes in LWC and apoplastic pH are correlated with changes in ionic and metabolite relations in leaf subcellular washing fluid and xylem sap. Hence, we isolated and analyzed apoplastic washing fluid, symplastic washing fluid and xylem sap from the same experimental set-up. We observed that plants treated with 50 mM NaHCO_3 showed a higher concentration of sodium [Na^+] in both subcellular washing fluid and xylem sap (Fig. 4A, B, C). This effect is certainly caused by the presence of sodium salt (NaHCO_3 , 50 mM) at the growth medium [60,61]. The increase of [Na^+], which was found in this study, nicely corresponds with the resulting [NaHCO_3] obtained for [K^+] (Fig. 4E, F, G). Potassium concentration [K^+] was higher in apoplastic washing fluid (Fig. 4E) and simultaneously lower in symplastic washing fluid (Fig. 4F). This suggests a sodium-potassium competitive effect for ion uptake at plasma membrane level [61]. Since Na^+ uptake is driven via K^+ transporters (HKT family), uptake competition is owed to the similar chemical characteristics of K^+ and Na^+ , having, e.g., an equal ion radius, resulting in identical transport characteristics of these two ions and thus leading to uncontrolled uptake of Na^+ [62].

Anions (NO_3^- , SO_4^{2-}) concentration was significantly influenced under 50 mM NaHCO_3 stress treatment (Fig. 5). It was observed that apoplastic washing fluid (Fig. 5A) and xylem sap (Fig. 5C) had lower amounts of [NO_3^-]. Since bicarbonate is transported radially into the root stele [53], a presence of high NaHCO_3 salt in the rooting medium can lead to transport competition between HCO_3^- and other anions (NO_3^- , SO_4^{2-}) (Fig. 5), thus hindering overall uptake [54]. Furthermore, Alkaline pH conditions induced by NaHCO_3 salt in the root medium might also have disturbed proton/anion co-transport mechanisms, resulting in reduced uptake of anions (Fig. 5) [11]. Interestingly, we observed that sulfate (SO_4^{2-}) concentration was increased in apoplastic washing fluid (Fig. 5D); this might be attributed to the initial water deficit caused by NaHCO_3 treatment (Fig. 2). Malcheska et al. [49] found that water deficit caused an increased xylem sap [SO_4^{2-}] and leaf apoplastic [SO_4^{2-}], which upregulated the ABA biosynthesis *NCED* gene expression in guard cells and closed stomata.

The co-occurrence of higher apoplastic [SO_4^{2-}] (Fig. 5D), *NCED* expression (Fig. 8A) and reduced stomatal conductance in plants treated with 50 mM NaHCO_3 could suggest that sulfate related signalling plays a similar role under bicarbonate stress, via sulfate triggered cysteine biosynthesis led in ABA production and stomatal closure [48].

4.4. NaHCO_3 induces a fast change in metabolite relations

Plants treated with 50 mM NaHCO_3 showed a peculiar increase in organic anion (malate, citrate) concentrations in symplast, apoplastic washing fluid and xylem sap (Fig. 6). The increased organic acids concentration may be associated with bicarbonate induced cytoplasmic alkalisation [63,64], which activates *PEPCase*. The synthesis of malate and other organic acids stabilizes the pH of the cytoplasm [65]. In our study, we also observed a higher mRNA abundance of *PEPCase* (Fig. 8E) which possibly contributed to the increased organic acid accumulation (Fig. 6) E. An increased organic acids concentration in the apoplast (Fig. 6A, D) and xylem sap (Fig. 6C, F) might have driven by symplastic organic acid synthesis (Fig. 6B, E) [66].

Soluble sugar (glucose, fructose, sucrose) concentrations were increased under NaHCO_3 stress in both symplast, apoplastic washing fluid and xylem sap (Fig. 7), increased cytoplasmic sugar concentrations due to osmotic adjustment should also cause an increase in the apoplast due to the changed transport balance [67]. Geilfus et al. [68] found that biotic stress induced alkalization impaired sugar-proton-cotransport and led to accumulation of phloem derived sucrose in the leaf

apoplast. Hence, in our study, increased sucrose concentration in apoplast might also be related to transient alkalinization of leaf apoplast (Fig. 2 C right Y-axis). The increased cytoplasmic sugar concentration together with the other osmolytes contributed to the observed increase in osmolality (Fig. 3), which partly explains the recovery of LWC (Fig. 2C left Y-axis).

4.5. NaHCO_3 stress influences transcription of key genes

Knowing that root application of NaHCO_3 induces several changes in physiological, ionic and metabolite relations in leaves within 150 min, in order to investigate corresponding changes at the transcription level, certain key genes transcripts were analyzed. We observed that the ABA biosynthesis gene *NCED* was highly upregulated (11-fold) under NaHCO_3 stress than control and alkaline pH treatment (Fig. 8A). This upregulation of *NCED* followed the transient alkalinization, which started 45 min after treatment, and the initial decline in LWC (Fig. 2C). This agrees with the findings of Qin and Zeevaart [69] and Thompson et al. [70] who observed a fast (within only 15–30 min) upregulation of the *NCED* gene Arabidopsis under water deficit conditions which lasted for several hours. Upregulation of *NCED* may have also been involved in ABA-dependent stomatal closure (Fig. 1). Moreover, recently, Geilfus et al. [35] found that transient alkalinization of the leaf apoplast elicits an increased mRNA abundance of the *Vp14* gene (orthologs of *NCED*) in maize leaves. A similar mechanism may have occurred in NaHCO_3 treated plants, where the transient alkalinization of the leaf apoplast (Fig. 2C, right Y-axis) could have induced transcription of *NCED* (Fig. 8A).

Root application of NaHCO_3 induces a transient rapid reduction in LWC followed by recovery after about 40 min of overshooting the initial LWC (Fig. 2C left Y-axis). These water fluxes across the cell membranes imply a role of plasma membrane-bound water channel (aquaporin, *PIP*), the higher mRNA abundance of *PIP* (Fig. 8B) could have been induced by higher ABA levels following increased *NCED* expression (Fig. 8A) because it has been shown that ABA can upregulate *PIP*-type aquaporins in Arabidopsis and maize [37,71,72]. But it could also be directly induced by water deficit (Fig. 2C left Y-axis), resulting in higher hydraulic conductivity of the plant [73].

An increase in mRNA abundance of *PM H⁺-ATPase isogenes* (*VHA4*, *VHA5*) (Fig. 8C, D) might be related to NaCl treatment [74]. Since sodium extrusion by Na^+/H^+ antiporters is powered by the electrochemical H^+ gradient, the operation of the plasma membrane H^+ -ATPase will increase the sodium extrusion [62]. Furthermore, Pertl et al. [75] reported a role of *PM H⁺-ATPase* in osmoregulation in *Lilium* pollen grains, whereupon hyperosmotic shock, the turgor pressure of cell decreased and elicited a higher activity of *PM H⁺-ATPase*, which promoted water uptake to readjust the turgor pressure. Thus, we assume that similar mechanisms may have occurred after salt (NaHCO_3) application to root, as it caused a transient drop in LWC and subsequent recovery after some time (Fig. 2 left Y-axis) which can be attributed to higher mRNA abundance of *PM H⁺-ATPase isogenes* (Fig. 8C, D). Similarly, the carbon metabolism gene *PEPCase* was only induced by NaHCO_3 treatment (Fig. 8E). The increased mRNA abundance of *PEPCase* could have helped to fix excess HCO_3^- in the organic acid pool [1, 54].

Taken together, we conclude that NaHCO_3 treated plants generally show an increase in the expression of the observed genes, which might be involved in the early adaptive response to NaHCO_3 stress.

5. Conclusion

This study was designed as a novel take to experimentally separate and investigate the early (150 min) effects of the alkali salt stress components, i.e., alkaline pH and osmotic salt stress phases of NaHCO_3 salt on *Vicia faba* leaves at the apoplastic, symplastic and xylem level. As hypothesized, early root application of alkaline pH alone did not induce

stomatal, leaf water, apoplastic pH, metabolic and transcriptional responses in the leaves. In contrast, early root application of alkaline salt stress caused several physiological and transcriptional changes. A transient drop-in leaf water content was followed by a transient alkalinization of the leaf apoplast. Re-acidification of the apoplast indicates that a proton gradient driven osmoregulation led to the recovery of LWC. This recovery was also driven by metabolite synthesis (malate, citrate, soluble sugar) and stomatal closure. These processes correspond with the fast upregulation of genes for ABA biosynthesis (*NCED*), aquaporin (*PIP*), ion transporter (*PM H⁺-ATPase isogenes*), and carbon metabolism (*PEPCase*), which all are involved in the early adaptive response of the plant to NaHCO_3 stress.

CRedit authorship contribution statement

Amit Sagervanshi and Karl H. Mühling conceptualized and designed the experiments. Amit Sagervanshi performed experiments and analyzed data. Amit Sagervanshi wrote the initial draft of the manuscript. Christoph Martin Geilfus and Hartmut Kaiser participated in data analysis, writing, and editing the paper. All the authors have read, edited, and approved the final manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

The material used in this study is available for non-commercial research purposes upon reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111253.

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Supplementary data

Table-S1 List of primer pairs used for RT qPCR analysis

Primer name	Genbank accession number		Sequence of forward (f) and reverse (r) primers (5'-3')	Annealing temp (°C)	Product size (bp)
<i>VfVHA4</i>	AJ310523	F	GGCTTGGGATCTTGTCATTGAG	60	194
		R	CTCTCCTTTTAGCTTCTTCGGC		
<i>VfVHA5</i>	AJ310524	F	GGCAAAGAAGAGAGAGAAGCAC	60	196
		R	CTGATTCGACGTGACCCTTTAG		
<i>VfNCED_fam*</i>	AB080193.1	F	GACATGTTTCGAGAGCGCG	60	192
	NM_001254322.2	R	CAATGGGTTGGCGCCGTTAC		
	AB771415.1				
<i>VfPIP1</i>	AY667436.1	F	GCTTGGTCTTTCGGTGGC	60	173
		R	CCAGCACCACAGATAGCACC		
<i>VfPEPCase</i>	AJ011303.1	F	CTCAGCCTCCAGATACCATTC	60	156
		R	TGGGAGATATTGGAGGATGC		
<i>VfCYP</i>	L32095	F	TGCCGATGTCACTCCCAGAA	60	199
		R	CAGCGAACTTGGAACCGTATA		

*Family specific conserved primer.

Table-S2 Results from Sanger sequencing of RT-qPCR amplicon

Sequencing of the real-time qRT-PCR products confirmed specificity of the primer pair. Column 1 indicates the name of the primer pair. The corresponding DNA sequencing result is presented in column 2. The sequences were aligned against NCBI's standard nucleotide collection (nr/nt) databases. The search was limited to *fabaceae* (taxid:3803). Best blast hit is presented in column 3 together with the respective Gensbank accession number. Column 4 shows the resulting description of the Blast hits. Column 5-6 shows parameters for statistical quality of the hit.

Primer pair used for RT-qPCR	Sanger sequencing summary of RT-qPCR amplicon	Significant alignments of sequences(NCBI blastn result on nucleotide collection)			
		Genbank accession number	Description	Max score/ Total score	Query coverage (%)/ Max ident (%)
<i>VfPIP</i>	TGTTGCTCTGTTGAC CGCAGCGCTGGATCT CTGGGGGTCACATAA ACCCAGCAGTGACAT TCGGTTTGTCTTGG CGAGGAAACTGTCTC TAACAAGAGCAGTG TTCTACATCGTGATG CAGGTTCTCGGTGCT ATCTGTGGTGCTGG	AY667436.1	<i>Vicia faba</i> plasma membrane intrinsic protein (PIP, Aquaporin) mRNA	233/233	98/95
<i>VfPEPCase</i>	GCTACAAACTGTA GTCAAGGCGAAGTT ATTGAGCAATCATT GGTGAGCAGCACTTG TGTTTCAGAACGCTA CAACGTTTCACTGCA GCTACACTAGAGCAT GGAATGCATCCTCCA ATATCTCCCAA	AJ011303.1	<i>Vicia faba</i> Phosphoenolp yruvate carboxylase mRNA predominantly expressed in leaf tissue	211/211	93/98
<i>VfVHA4</i>	CGATGCATTATACAG GGAAAAGGACTTTG GGAAGGAACAACGT GAGCTTCAATGGGCA CATGCGCAAAGAAC GCTGCACGGATTACA ACCACCAGACACCA AGATGTTACCGAGC GAACTCATGTCACCG	AJ310523.1	<i>Vicia Faba</i> isoform of PM H ⁺ -ATPase mRNA	297/297	89/100

	AACTCAATCAAATGG CCGAAGAAGCTAAA AGGAGAG				
<i>VfVHA5</i>	GAGGATAACTGTCTC GTGCGACTCTCCTGG TCTTCAATCACCCGA AACCAACCAACCTTTT CAATGACAAGAATA GCTACAGGGAGCTTT CTGAGATTGCTGAGC AAGCCAAAAGACGC GCTGAGGTTGCACGG CTTAGGGAGCTTCAC ACTCTAAAGGGTCAC GTCGAATCAG	AJ310524.1	<i>Vicia Faba</i> isoform of PM H ⁺ -ATPase mRNA	291/291	89/99
<i>VfNCED_fam*</i>	TAAAAAACCCCTGCG CTCGTAGTCGTGTGA TTTTCCCGGTAACGC TGAGCGAATGAACG ACTGGTTGTTCCGGG ACAGGAGCAAAGTT TCCGGAGATTTGAAT TCTCGGGTCCGAAGT TTGGGAAGTGGATG TTGGAGCTCGCGTGA GAGCAACGCGCTCTC GAACATGTCA	AB080193.1 NM_001254 322.2 AB771415.1	<i>Vicia faba</i> family specific(<i>fabea</i> <i>ce</i>) <i>NCED</i> mRNA (9-cis- epoxycaroteno id dioxygenase, ABA biosynthesis gene)	198/198	82/91

*Family specific conserved primer.

Chapter 3

Early growth reduction in *Vicia faba* L. under alkali salt stress is mainly caused by excess bicarbonate and related to citrate and malate overaccumulation

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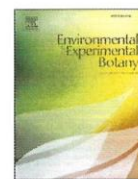
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ABSTRACT

Rather than high pH or Na^+ , excess bicarbonate, by inducing over-accumulation of organic acids (through dark fixation) in plant roots, was hypothesized to be the main growth-limiting factor under alkali salt stress. Thus, the effect of four treatments viz. control (pH 6.0), high pH (9.0), high pH (9.0) plus sodium sulfate (25 mM) and sodium bicarbonate (50 mM, pH 9.0) on growth, gas exchange and leaf water content, organic acid accumulation, ion relations, oxidative damage and changes in the expression of key genes was investigated in *Vicia faba*. The bicarbonate treatment showed the highest growth reduction, *PEPCase* gene expression and accumulation of citrate and malate in roots. Bicarbonate treated plants preferentially accumulated Fe^{2+} and Ca^{2+} in roots and reduced root exudation of citrate and malate. Moreover, these plants experienced K^+ and Ca^{2+} deficiencies, which caused a massive increase in H_2O_2 concentration in leaves and roots, leading to severe oxidative damage. Bicarbonate treated plants showed reduced levels of *PM H⁺-ATPase*, *SOS1*, *CS*, and *MDH* gene expression in roots. The cascade of events confirms the hypothesized central role of organic acid accumulation in bicarbonate induced stress, making bicarbonate more detrimental to plant growth than high pH or its combination with sodium salt.

1. Introduction

Soil salinity is one of the most widely occurring abiotic stresses that negatively affects growth and yield of crop plants around the globe. Salt-affected soils constitute about 7% of the total land area or 33 % of the potential agricultural land area of the world (Gupta and Abrol, 1990; Munns and Tester, 2008). Soil salinity may be constituted by neutral salts such as sodium chloride (NaCl) and sodium sulfate (Na_2SO_4) or alkaline salts including sodium carbonate (Na_2CO_3) or sodium bicarbonate (NaHCO_3) (Yang et al., 2007). Usually, salt stress and alkaline stress co-occur in nature, with alkalization causing more severe problems than salinity alone (Kawanabe and Zhu, 1991). To soil alkalinity, bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) are the principal contributors, whereas hydroxide, borate, ammonia, organic bases, phosphates, and silicates are considered minor contributors (Petersen, 1996). The excess amounts of HCO_3^- and CO_3^{2-} and other alkaline salts increase

soil pH with a high buffer capacity, resulting in the precipitation and unavailability of various macro- and micronutrients to plants (Cartmill et al., 2008; Pérez-Martín et al., 2021). Alkaline stress is basically a combination of various stresses such as osmotic stress, ion-induced injury, nutrient deficiency, and high pH (Chen et al., 2011; Munns, 2002; Yang et al., 2008).

Although mechanisms underlying the negative effects of salinity on plant growth have been studied abundantly, most of these studies have focused on neutral (NaCl or Na_2SO_4) salinity (Mühling and Lächli, 2002; Munns and Tester, 2008; Shahid et al., 2013). As compared to neutral salt stress, little information is available regarding the effect of alkaline stress induced by Na_2CO_3 or NaHCO_3 salts on the growth of glycophytes (Guo et al., 2009; Shi and Wang, 2005). In the existing few studies, only limed soils or alkaline growth substrates were considered (Brand et al., 2002; Nuttall et al., 2003), but Na-salt born alkaline conditions have not been studied. A few reports have shown that severe

Abbreviations: CDS, coding sequence; H_2O_2 , hydrogen peroxide; IC, isocratic ion chromatography; ICP-MS, inductively coupled plasma mass spectrometry; LED, light emitting diode; LWC, leaf water content; MDA, malondialdehyde; MSI, membrane stability index; NIR, near-infrared; PM, plasma membrane; qRT-PCR, quantitative reverse transcription-PCR; ROS, reactive oxygen species.

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adverse effects of alkali salts on plant growth are related to disturbance in photosynthesis and ionic homeostasis and metabolisms (Guo et al., 2016; Guo et al., 2015). However, these studies did not dissect whether the reported adverse effects were associated with high pH, bicarbonate, or Na^+ toxicity. Hajiboland et al. (2003) compared the effect of high pH (8.0) and 10 mM HCO_3^- on growth of rice, wheat, and rye, but the study could not point out clear cut differences between the two growth conditions. The possible reason could be the level of HCO_3^- used for the study, which was not stressful for all the studied plant species. In a later study, the same research group found higher accumulations of organic acids in roots of the plants grown with 10 mM HCO_3^- (Hajiboland et al., 2005). The plant species which were able to maintain organic acids at a lower level through efficient root exudation were able to grow better than those showing lesser root exudation. Accordingly, the authors speculated that over-accumulation of organic acids under HCO_3^- could be a main growth-limiting factor for plants. However, the information on how over-accumulation of organic acids under bicarbonate salinity could inhibit plant growth still remains obscure. Moreover, these studies did not include the combined effect of neutral Na salt and high pH that was required to isolate the effect of Na associated with HCO_3^- .

Thus, to reveal growth inhibition mechanisms underlying HCO_3^- -induced over accumulation of organic acids, and to dissect early adaptive response of plants to high pH alone, high pH plus neutral Na salt and NaHCO_3^- , a pot study was conducted on *Vicia faba* under controlled environment. We examined the changes in ionic relationships, osmotic adjustment, oxidative profile, and metabolite accumulation at tissue levels. We also investigated the mRNA expression level of specific key genes, whose products reinforce plants to withstand adverse condition induced by alkaline or Na stress, such as ion transporters (*PM H⁺-ATPase*, *SOS1*) (Munns and Tester, 2008; Palmgren, 2001) and carbon metabolism genes (*PEPCase*, *MDH*, *CS*).

We hypothesized that HCO_3^- induces a higher accumulation of organic acids in plant tissues than high pH alone or high pH plus neutral Na^+ salt due to HCO_3^- fixation and impaired root exudation of organic acids. Bicarbonate induced over accumulation of organic acids will create a nutrient imbalance in plant tissues through nutrient specific complexation and restricted translocation.

2. Materials and methods

2.1. Plant cultivation and treatment application

Seeds of *Vicia faba* (cv. Fuego: NPZ GmbH, Hohenlieth, Germany) were soaked in an aerated 0.5 mM CaSO_4 solution for 24 h and then sown into quartz sand for germination. After one week, healthy and uniform-sized seedlings were transferred to plastic pots containing 5 L of a quarter strength nutrient solution (Geilfus and Mühling, 2013). Four seedlings were cultivated in each pot in a controlled climatic chamber (14/10 h day/night; 20/15 °C; 50/60 % humidity). To avoid osmotic shock, the concentration of nutrient solution was progressively increased to one-half, three-quarters and full strength on 2nd, 3rd, and 4th day of transplantation, respectively. The nutrient solution was renewed after every two days to replenish the exhausted nutrients. Twenty eight days old plants (fifth leave stage) were subjected to the following four treatments: control (only nutrient solution, pH 6.0 ± 0.2), high pH (2.5 mM CAPSO buffer, pH 9 ± 0.2), high pH (9 ± 0.2) + neutral salt (Na) (25 mM Na_2SO_4) and alkali salt (50 mM NaHCO_3 , pH 9 ± 0.2). The level of NaHCO_3 (50 mM) used in this study was selected based on a preliminary experiment in which four levels of NaHCO_3 viz. 25 mM (pH 8.2), 50 mM (pH 9.0) and 75 mM (pH 9.8) were tested. We observed that 50 mM of NaHCO_3 was a suitable concentration to achieve a reasonably high pH (9.0) in the growth medium, at which plants were also growing well for the experimental period. Moreover, literature shows that salinity levels between 50–150 mM NaCl do not lead to ion toxicity in cytosol resulting in lethal affects for glycophytes, like *Vicia faba* which is moderately salt sensitive plant (Nampei et al., 2021). After 4 days of

growth under stress treatments, plants were harvested and separated into leaf and root samples. Leaf and root samples were thoroughly washed with deionized water to remove the adhering ions from the surface, dried at 60 °C for 72 h and their dry weights were recorded. The dried materials were ground to a fine powder and used for mineral analysis. Fresh plant materials were used to analyze the mRNA level of certain genes. For this purpose, the second batch of experiment was grown at the same time under identical conditions. After washing with deionized water, fresh plant materials were shock-frozen in liquid N_2 and stored at -80 °C until further analysis.

2.2. Measurement of gas exchange attributes

Just before harvesting, the intact youngest fully expanded leaves were chosen to measure gas exchange parameters. Photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and stomatal conductance ($\text{mol. H}_2\text{O m}^{-2} \text{ s}^{-1}$) were measured with an open flow portable photosynthesis system (LI 6400XT, Li-COR Biosciences Inc., USA). For measurements, leaves were placed across a 2×3 cm leaf chamber. The conditions for the measurements inside the leaf chamber were maintained identical to the external condition of the climatic growth chamber. The light was provided by a LED red light source built into the top of the leaf chamber ($250 \mu\text{mol m}^{-2} \text{ s}^{-1}$), and the CO_2 concentration was controlled by Li-Cor LI-6400 CO_2 injection system ($400 \mu\text{mol CO}_2 \text{ mol}^{-1}$) as identical to outside condition.

2.3. Ratiometric non-invasive measurement of leaf water content (LWC)

Non-invasive *in planta* measurement of leaf water content was started one day before the onset of stress treatments and continued till the day of harvest. A circular area of 2–3 cm diameter in the middle of the 5th leaf was selected for daily measurements. For the quantification of leaf water content, a ratiometric near-infrared (NIR) transmission setup was employed. The measurement uses the ratio in absorption at 1450 nm (strong absorption by liquid water) and 1050 nm (non-absorbing wavelength), which varies with leaf water content (Sagervanshi et al., 2021). The custom-made water sensor (developed by H. Kaiser) device had a dual infrared LED emitter mounted on top of a leaf clip by a 45 ° angle and a photodiode collecting transmitted light below the leaf. A data logger was used to control the device and recording of data. For calibration of the water sensor IR absorption ratio and gravimetrically determined LWC was recorded on several detached leaves. *Vicia faba* leaves were placed in deionized water at 4 °C overnight to achieve full saturation. On the following day, NIR transmission ratio was determined at three-time intervals (0, 5, 10 min) during air drying of these leaves while simultaneously recording leaf weight. The linear relation between NIR transmission ratio and gravimetric water content was used to convert the sensor data into water content (g m^{-2}).

2.4. Determination of mineral concentration

For the determination of mineral concentrations, oven-dried and finely ground leaf and root samples (200 ± 10 mg), were digested with 10 mL of 69 % HNO_3 (ROTIPURAN Supra for ICP, 69 %) in a closed-vessels microwave digestion system (MARS 6 Xpress, CEM Corporation, Matthews, USA), adjusted to the following conditions: 2 min at 100 °C, 1 min at 120 °C, 20 min at 180 °C and 20 min cooling time (Jezek et al., 2015). The digested samples were diluted with Milli-Q water (18.2 M Ω cm conductivity) to 100 mL and stored at 4 °C until further analysis. Concentrations of sodium (Na), macronutrients (potassium, K; calcium, Ca) and micronutrients (iron, Fe; manganese, Mn; zinc, Zn) were measured by inductively coupled plasma mass spectroscopy (ICP-MS; Agilent 7700, Agilent Technologies Inc., USA).

2.5. Determination of anions and organic compounds

Water-soluble sugars, free organic and inorganic anions were

extracted by hot water following the procedure of Cataldi et al. (2000) with some minor modifications. Briefly, dried and powdered leaf and root samples (~20 mg) were dissolved and boiled for 5 min in 1.5 mL of sterile deionized water, mixed thoroughly by vortexing and immediately placed on an ice-water bath for 30 min. The mixtures were then centrifuged and the supernatants were collected. For the precipitation of proteins, the supernatants were mixed with chloroform, centrifuged and the resultant supernatants were cleaned by passing through strata C-18 columns (Phenomenex, Torrance, CA, USA). The anions and sugars concentrations, in cleaned supernatants, were determined by isocratic ion chromatography (IC-5000 Capillary Reagent-Free IC System, Thermo Scientific, USA).

2.6. Collection and determination of root exudates

On the day of harvest, the plants were removed from the nutrient solutions, and roots were thoroughly washed with Milli-Q water to remove the traces of nutrient solution. Roots of four plants from each treatment, were transferred to aluminium foil wrapped glass containers containing continuously aerating 500 mL of Milli-Q water and placed in the climatic chamber under light conditions at 20 °C for a period of 6 h to collect root exudates. After collection, one drop of 0.02 % sodium azide was added into each sample to avoid microbial degradation of organic matter. Subsequently, each sample was filtered through a 0.45- μ m membrane filter and concentrated to 5 mL by using a rotary evaporator (Büchi, R-114, Germany) at 40 °C with 0.1 kPa. The concentrated samples were filtered through a 0.22- μ m membrane filter again and then stored at -20 °C until analysis of organic acids (Wu et al., 2016). Organic acid was determined by isocratic ion chromatography (IC-5000 Capillary Reagent-Free IC System, Thermo Scientific, USA).

2.7. Determination of malondialdehyde (MDA) and H₂O₂ content

The lipid peroxidation, in terms of MDA content, was measured following the procedure of (Wu et al., 2018). Briefly, 0.2-g fresh materials were homogenized in 2 mL 0.1 % (w/v) trichloroacetic acid (TCA) in an ice bath, and the homogenates were centrifuged at 12,000g for 10 min at 4 °C. Aliquots (1 mL) of the supernatants were mixed with 1 mL 0.1 % (w/v) thiobarbituric acid [dissolved in 20 % (w/v) TCA], heated in boiling water bath for 30 min, and immediately cooled in ice bath to stop the reaction. Subsequently, the samples were centrifuged at 10,000g for 5 min at 4 °C, and the absorbance was recorded at 532 nm and 600 nm by a spectrophotometer. The MDA content was calculated using the to extinction coefficient 155 mM⁻¹ cm⁻¹ (Velikova et al., 2000).

H₂O₂ content was measured according to (Velikova et al., 2000). The extraction procedure was the same as described for MDA content. To 0.5 mL aliquot of the supernatant, 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI were added and incubated for 15 min. The absorbance of the supernatant was measured at 390 nm by a spectrophotometer. The absorbance values were converted to H₂O₂ content using a series of H₂O₂ standards.

2.8. Determination of membrane stability index (MSI)

The membrane stability index (MSI) was measured in terms of leakage of electrolytes from leaf and root tissues after heating of samples in boiling water (Wu et al., 2015). Fresh leaf and root samples were washed with Milli-Q water to remove surface adhering ions. The samples were weighed (0.2 g) in duplicates in glass tubes glass tubes, added with 20 mL of Milli-Q water and incubated for 10 min. During incubation, the samples were shaken every 2 min. One set of the tubes with leaves or roots was heated in water bath at 40 °C for 30 min (C1), while the other set was heated at 100 °C for 15 min (C2). After the solutions had cooled to room temperature, their electrical conductivities were recorded (DDS-307 conductivity meter, Shanghai, China). The MSI was calculated using the following formula:

$$\text{MSI (\%)} = (1 - C1/C2) \times 100 \%$$

2.9. Primer design and Sanger sequencing

For analyzing the mRNA transcript level of Phosphoenolpyruvate carboxylase (PEPCase) and ion transporter genes plasma membrane proton ATPase [PM H⁺-ATPase *isogenes* (*vha4*, *vha5*)], primers were designed from sequences available on the NCBI database for *Vicia faba*. Since genome sequences of salt overly sensitive1 (*SOS1*) (Sagervanshi et al., 2021), malate dehydrogenase (*MDH*), and citrate synthase (*CS*) are not annotated for *Vicia faba*, *Fabaceae* family specific primers were designed based on conserved regions in close relatives: The coding sequence (CDS) of genes of interest from *Glycine max*, *Vigna radiata*, *Cicer arietinum*, and *Medicago truncatula* were retrieved from the NCBI database (<https://www.ncbi.nlm.nih.gov/nucleotide/>). After that, multiple CDS sequences were aligned using the CLC workbench tool (<https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/>), and the homologous conserved region was chosen for primer design. A pair of primers was designed from the conserved aligned sequence for each gene by manual selection of oligonucleotide sequences on the CLC workbench tool. Characteristics of screened primers were checked and evaluated *in silico* by the online tool oligo calc (<http://biotools.nubic.northwestern.edu/OligoCalc.html>) and multiple primer analyzer tools provided by Thermo Fisher. All primer pairs were purchased from Eurofins Genomics (Ebersberg, Germany). The details of the primers are listed in Supplementary Table S1. All the primers were tested and validated by PCR amplification to confirm, (1) if they work for *Vicia faba* and (2) are they specific to the target cDNA. The thermal-cycling was performed as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55–60 °C for 30 s, and extension at 72 °C for 30 s and final extension for 5 min at 72 °C. PCR products obtained were run on agarose gels to ensure the correct length of the amplicon. Furthermore, amplification of the correct gene was confirmed by the sequencing of the amplicon (Sanger sequencing, Institut für Klinische Molekularbiologie, Kiel) and comparison with the BLASTN nucleotide collection database (Supplementary Table S2).

2.10. Gene expression analysis

Total RNA of leaves and roots were extracted using TRIzol (Invitrogen) according to the manufacture's protocol. The concentration and purity of RNA were determined by NanoVue Plus spectrophotometer (GE Healthcare Life Science, USA), and integrity was checked by gel electrophoresis. The coding RNA (one μ g) was reverse transcribed following the manufacturer's instructions (Verso cDNA synthesis kit, Thermo Fisher Scientific, USA), including DNase I treatment. The quality of synthesized cDNA (1/10 dilution) was checked by standard PCR with the most stable housekeeping gene *Vf Cyclophilin* (*CYP*) (Gutierrez et al., 2011; Sagervanshi et al., 2021). Quantitative RT-PCR was conducted by PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) with primers shown in Table S1 on CFX96 Real-Time System (Bio-Rad Laboratories GmbH, München, Germany). For each reaction, the total volume was 20 μ l, containing 100 nM of each primer and 2 μ l of diluted cDNA templates. After an initial denaturation step (95 °C for 5 min), RT-qPCR was carried out over 40 cycles (95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s), followed by a melt curve stage (95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, 24 °C for 15 s). Three biological replicates and three technical replicates were used for each treatment. Transcript levels of gene were normalized with endogenous control (*VfCYP*), and changes in the expression of target mRNAs were determined using the 2^{- $\Delta\Delta$ Ct} method (Livak and Schmittgen, 2001).



Fig. 1. Plant height and root morphology of 28 days old *Vicia faba* L. plants grown under control, high pH, high pH + Na and NaHCO_3 stress treatments for four days.

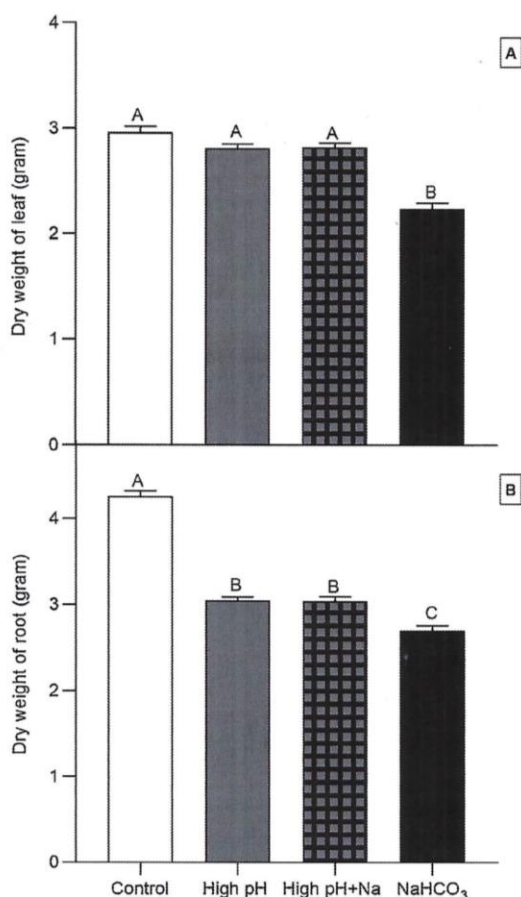


Fig. 2. Leaf (A) and root (B) dry matters of *Vicia faba* L. grown under control, high pH, high pH + Na and NaHCO_3 stress treatment for four days. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatment's treatments ($p \leq 0.05$).

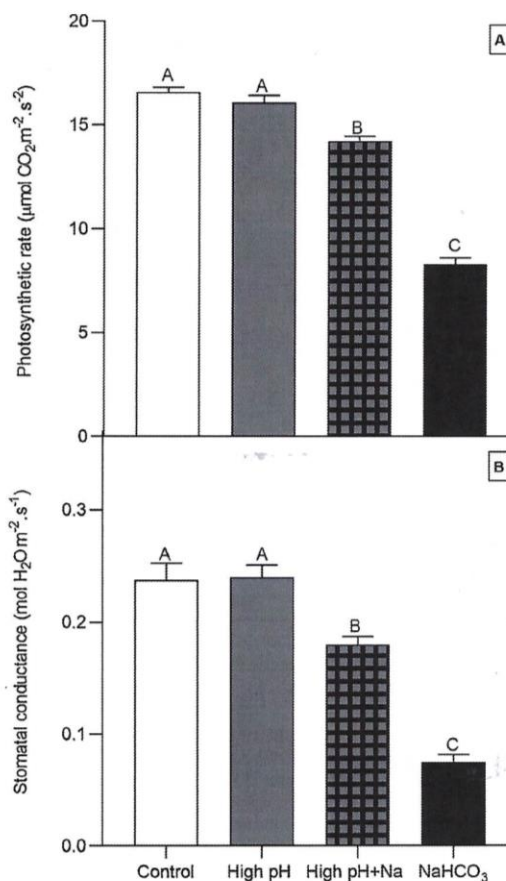


Fig. 3. Photosynthetic rate (A) and stomatal conductance (B) of *Vicia faba* L. grown under control, high pH, high pH + Na and NaHCO_3 stress treatments for four days. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

2.11. Statistical analysis

The data were statistically analyzed following one-way ANOVA, which was performed using SPSS software (version 17.0). Significant differences among the means were determined by Tukey's HSD test at $P \leq 0.05$.

3. Results

3.1. Growth and gas exchange

Plant height and root growth were strongly inhibited under four days of 50 mM NaHCO_3 stress treatment (alkali salt) compared to control, High pH, and High pH + Na (High pH+25 mM Na_2SO_4) treatments (Fig. 1). The plants grown with NaHCO_3 showed substantial root damage with large parts of the fine roots appearing black and void of root hairs (Fig. S1). In NaHCO_3 treated plants root and leaf dry biomass were reduced by 35 % and 17 %, respectively compared to control plants (Fig. 2). High pH and High pH + Na treatments also reduced root dry matter, i.e. by 23 % and 28 % compared to control (Fig. 2A); however, these treatments did not have significant adverse effects on leaf dry matter (Fig. 2B).

NaHCO_3 treated plants showed a decline in photosynthetic rate by 50 % and in stomatal conductance by 70 % compared to control plants

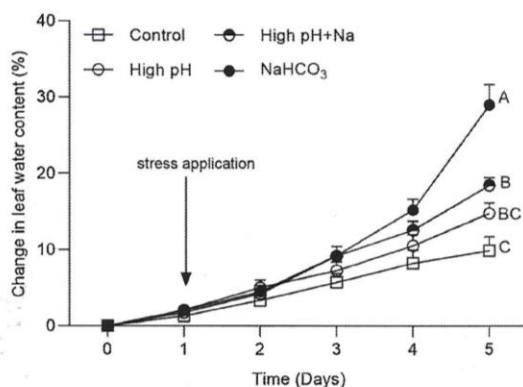


Fig. 4. Daily change (%) in leaf water content (g cm^{-2}) of *Vicia faba* L grown under control, high pH, high pH + Na and NaHCO_3 stress treatments for four days. The data \pm SE are means of four independent pot replicates. Different letters on data points at the 5th day of measurement represent significant differences between the treatments ($p < 0.05$).

(Fig. 3). High pH + Na treated plants showed only 15 % and 20 % reduction in photosynthetic rate and stomatal conductance, respectively as compared to control plants. However, high pH treatment neither

affected photosynthetic rate nor stomatal conductance (Fig. 3).

3.2. Leaf water content (LWC)

The daily measurement of LWC started one day before the onset of treatment application and lasted till the day of harvesting (5 days of consecutive measurement). In general, plants under all the treatments showed a progressive increase in LWC during the experiment (Fig. 4). On 5th day of measurement, LWC of high-pH treatment did not differ from that of control. Interestingly, in NaHCO_3 treated plants LWC sharply started to increase from day 4th onward and showed 2.5-fold higher increment than control plants on the 5th day of measurement. In contrast, the High pH + Na treatment only showed a 0.90-fold extra increment in LWC over the control on the 5th day of measurement (4th day after treatment application).

3.3. Elemental concentration of minerals in leaves and roots

As expected, High pH + Na and NaHCO_3 treated plants showed 10- and 11-fold, respectively higher Na^+ concentration in leaves than control plants (Fig. 5A). Similarly, High pH + Na and NaHCO_3 treated plants had 3.5- and 10-fold, respectively higher Na concentration in roots than control plants (Fig. 5B). Potassium concentration in leaves and roots of NaHCO_3 treated plants decreased by 33 % and 63 %, respectively compared to control plants (Fig. 5C, D). However, under High pH + Na

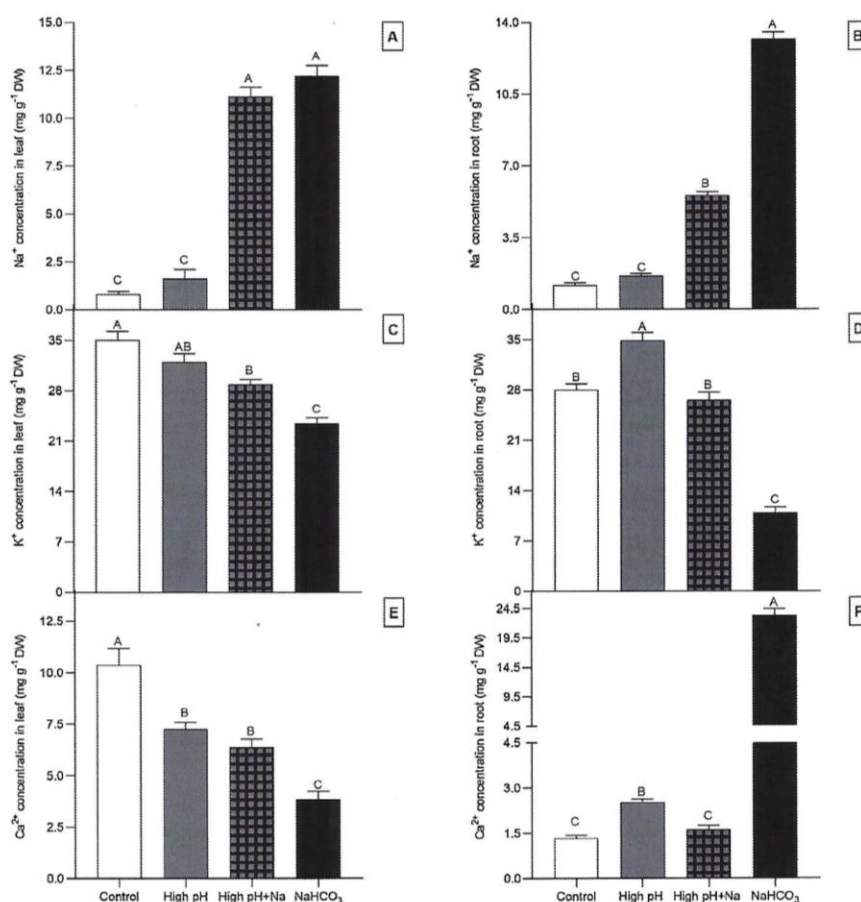


Fig. 5. Concentration of Na^+ (A, leaf; B, root), K^+ (C, leaf; D, root), and Ca^{2+} (E, leaf; F, root) in *Vicia faba* L grown under high pH, high pH + Na and NaHCO_3 stress treatment for four days. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p < 0.05$).

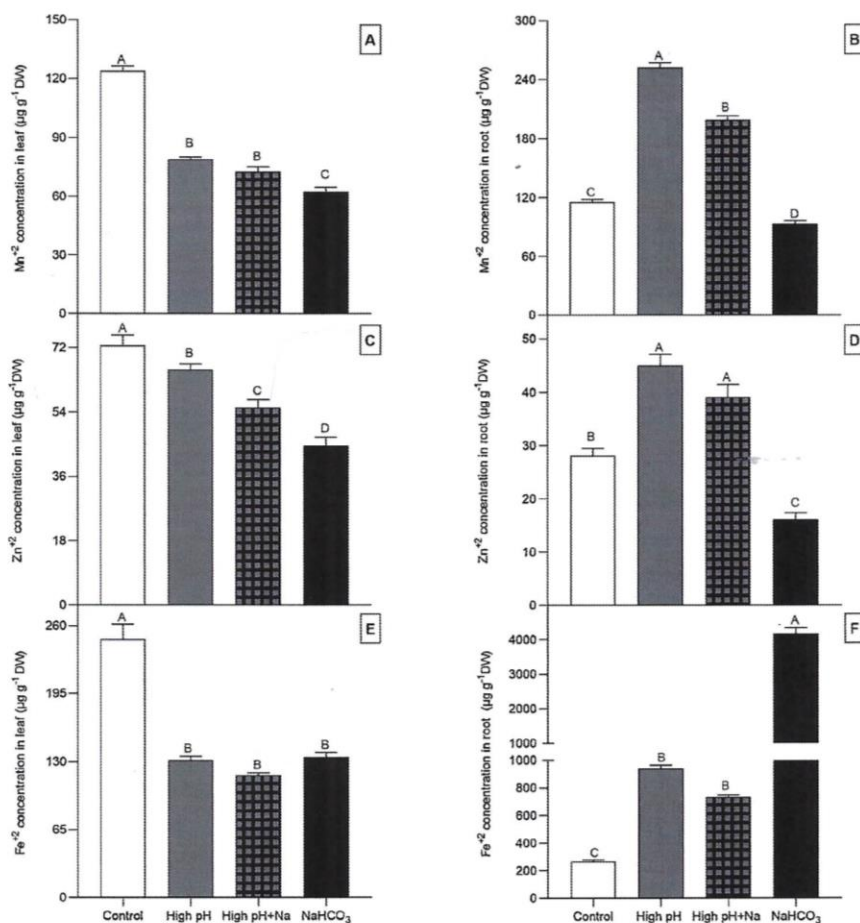


Fig. 6. Concentration of Mn²⁺ (A, leaf; B, root), Zn²⁺ (C, leaf; D, root) and Fe²⁺ (E, leaf; F, root) in *Vicia faba* L. grown under control, high pH, high pH + Na and NaHCO₃ stress treatments for four days. The data ± SE are means of four independent pot replicates. Different letters on data bars represent significant differences between the treatments ($p \leq 0.05$).

treatment, K⁺ concentration in leaves decreased by 17 %, but remained unaffected in roots compared to the control (Fig. 5C, D). High pH treatment did not affect K⁺ concentration in leaves, but increased it by 21 % in roots than control (Fig. 5C, D). Compared to control, all the stress treatments decreased Ca²⁺ concentration in leaves and it followed the order: High pH (29 %) < High pH + Na (38 %) < NaHCO₃ (63 %) (Fig. 5E). Contrarily, in roots, NaHCO₃ increased Ca²⁺ concentration by 16-fold, High pH treatment by 0.90-fold and High pH + Na did not affect as compared to the control (Fig. 5F). Overall, of all treatments NaHCO₃ lead to the strongest changes in ion composition with decreased K⁺ concentrations in the whole plant and decreased (Ca²⁺) in leaves contrasts which a massive increase in root (Ca²⁺). Micronutrients (Mn²⁺, Zn²⁺, Fe²⁺) concentration were differentially affected by the stress treatments in leaves and roots (Fig. 6). All the stress treatments substantially decreased the concentrations of the above-mentioned micronutrients in leaves. Mn²⁺ and Zn²⁺ concentrations decreased by the stress treatments in the following similar order: High pH (Mn 35 %, Zn 8%) < High pH + Na (Mn 40 %, Zn 24 %) < NaHCO₃ (Mn 50 %, Zn 38 %), with higher decline in Mn concentration than Zn concentration under each treatment (Fig. 6A, C). The Fe²⁺ concentration was equally decreased by the stress treatments, and it averaged to be 44 % compared to control treatment. However, in roots, Mn²⁺, Fe²⁺, and Zn²⁺ concentrations increased under High pH treatment by 100, 60, 250 %, respectively and under High pH + Na treatment by 73, 40 and 170 %,

respectively than control (Fig. 6B, D, F). Contrarily, NaHCO₃ decreased Mn²⁺ (20 %) and Zn²⁺ (42 %) concentration in roots, but increased that of Fe²⁺ by 14-fold than control.

Nitrate concentration in leaves was decreased below levels of control by High pH, High pH + Na and NaHCO₃ treatments by 25 %, 66 %, 75 %, respectively (Fig. 7A). In roots, only NaHCO₃ had a negative effect on NO₃⁻ concentration, being 31 % lower than control (Fig. 7B). In leaves, sulfate concentration was increased under High pH + Na and NaHCO₃ treatments by 8- and 1-fold, respectively, than the control treatment (Fig. 7C). In roots, only NaHCO₃ treatment reduced sulfate concentration (by 30 %) (Fig. 7D). Phosphate concentration in leaves was decreased by 56 % under High pH + Na treatment, while both High pH and NaHCO₃ treatments resulted in 32 % decline compared to the control treatment (Fig. 7E). In roots, only the Na salt treatments showed a decrease in phosphate concentration, i.e. High pH + Na reduced PO₄³⁻ concentration by 18 % and NaHCO₃ by 35 % as compared to the control treatment (Fig. 7F).

3.4. Accumulation of organic compounds

Concentrations of organic anions (Malate, citrate) and soluble sugars (glucose, fructose, sucrose) in leaves and roots were significantly affected by the stress treatments. In leaves, malate concentration was increased by 0.28- and 1.4-fold under High pH and NaHCO₃ treatment,

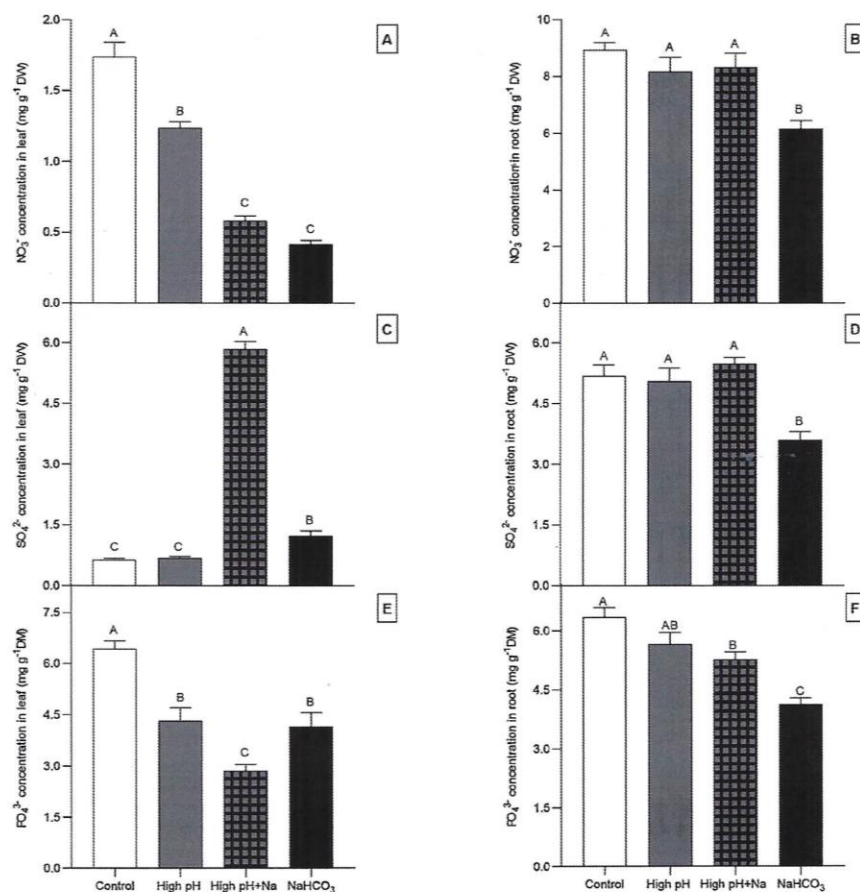


Fig. 7. Concentration of NO_3^- (A, leaf; B, root), SO_4^{2-} (C, leaf; D, root) and PO_4^{3-} (E, leaf; F, root) in *Vicia faba* L. grown under control, high pH, high pH + Na and NaHCO_3 stress treatments for four days. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

respectively, whereas High pH + Na treatment had no effect as compared to control (Fig. 8A). In roots, malate concentration was increased in the following increasing order of treatments: High pH + Na (1-fold) < High pH (1.7-fold) < NaHCO_3 (3.7-fold) than control (Fig. 8B). NaHCO_3 treatment led to a vast increase in citrate concentration in leaves (2.7 fold) and roots (7-fold) (Fig. 8C, D). In comparison, High pH and High pH + Na treatment exhibited an about 2-fold increase in citrate concentration in both the tissues.

Both High pH and NaHCO_3 treatments equally increased leaves glucose concentration, by about 2.3-fold, while High pH + Na treatment showed an increase of 1.3-fold compared to the control treatment (Fig. 9A). Root glucose concentration was increased by 0.45-fold under both High pH and High pH + Na treatment, whereas NaHCO_3 treatment decreased it by 0.43-fold as compared to control treatment (Fig. 9B). In leaves, fructose concentration was increased in the following increasing order of the treatments: High pH + Na (1.4-fold) < High pH (3.3-fold) < NaHCO_3 (5.5-fold) (Fig. 9C). In contrast, root fructose concentration was decreased by 44% in NaHCO_3 treatment, and 23% both in High pH and High pH + Na treatments as compared to control (Fig. 9D). Sucrose concentration was strikingly increased by NaHCO_3 both in leaves (2.5-fold) and roots (1.5-fold), whereas the corresponding increases under High pH and High pH + Na treatments were 0.30- and 0.50-fold compared to control (Fig. 9E, F).

3.5. Root organic acid exudation

High pH and High pH + Na treatments showed statistically equal increase in malate and citrate exudation, i.e. by 1.7- and 1.2-fold, respectively, compared to control (Fig. 10). On the contrary, NaHCO_3 treatment decreased malate and citrate exudation by 0.52- and 0.65-fold, respectively than the control treatment (Fig. 10).

3.6. Accumulation of H_2O_2 , MDA, and MSI indexes

Leaves and roots of NaHCO_3 treated plants showed a massive increase in H_2O_2 concentration, i.e. 9.7- and 27-fold, respectively, higher than the control treatment (Fig. 11A, B). However, High pH and High pH + Na treated plants showed less and equal increases in H_2O_2 concentration, ~1.5-fold higher in roots and 5.5-fold higher in leaves, than control plants (Fig. 11C, D). In agreement with the highest concentration of H_2O_2 , MDA concentration was also the highest under NaHCO_3 treatment, i.e. 6.5-fold higher in leaves and 3.5-fold higher in roots than to control. High pH and High pH + Na treatments also showed equal increase in leaves and roots MDA content and it valued around 4- and 1.7-fold of control, respectively.

The substantially increased concentrations of H_2O_2 and MDA in plant tissues under NaHCO_3 treatment coincided with a significantly lower MSI index (leaves, 25%; roots, 35%) than the control treatment (Fig. 11E, F). The effect of High pH and High pH + Na treatments on MSI

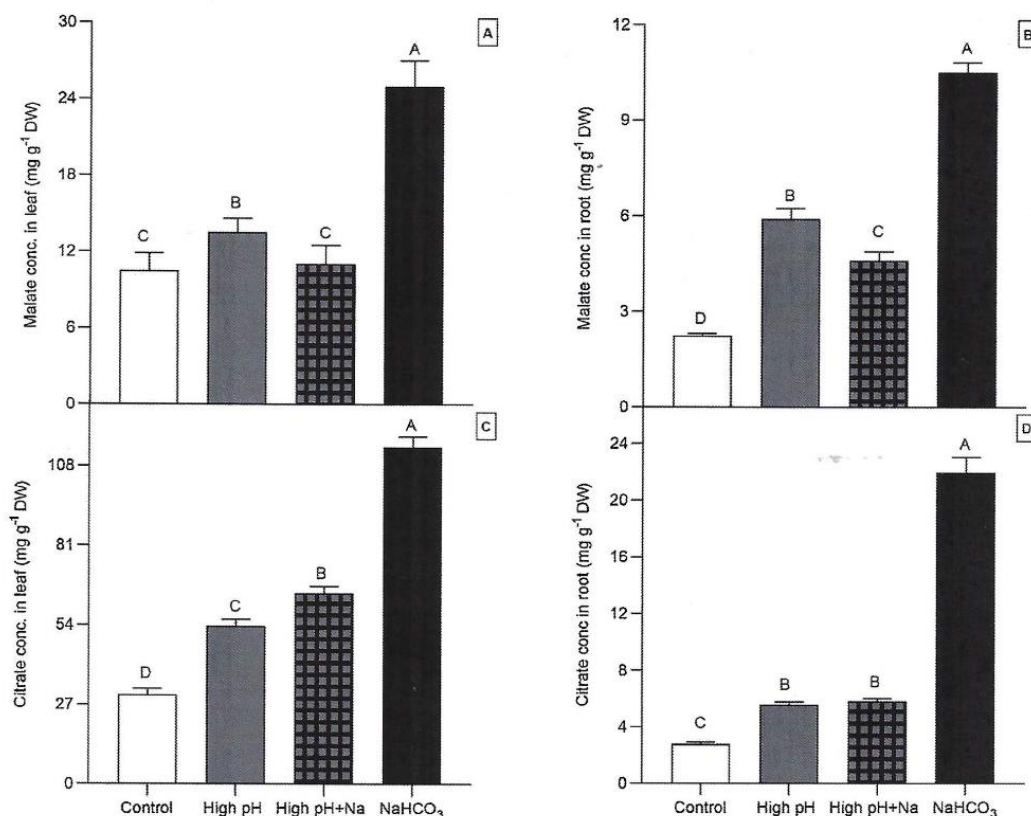


Fig. 8. Concentration (conc.) of malate (A, leaf; B, root) and citrate (C, leaf; D, root) in *Vicia faba* L. grown under control, high pH, high pH + Na and NaHCO₃ stress treatments for four days. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

was non-significant.

3.7. Relative mRNA expression of the genes involved in membrane ion transport and carbon metabolisms

The stress treatments differentially altered the relative expression of mRNA of genes related to membrane ion transport (*PM ATPase isoforms*, *SOS1*) and carbon metabolisms [*Phosphoenolpyruvate carboxylase (PEP-Case)*, *Citrate synthase (CS)*, *Malate dehydrogenase (MDH)*] in leaves and roots. The mRNA expression of the *VHA5* isoform was affected only in roots; compared to control it was upregulated 1.5- and 1.25-fold, respectively under High pH and High pH + Na treatments, whereas downregulated by 0.63-fold under NaHCO₃ treatment (Fig. 12). The mRNA expression of *VHA5* isoform showed an equal upregulation only under High pH and High pH + Na treatment, i.e. by 1-fold in leaves and 2.3-fold in roots (Fig. 12). Only High pH and High pH + Na treatments, and only in roots, *SOS1* expression was upregulated by 0.8- and 2-fold, respectively.

In leaves, *PEPCase* mRNA expression was upregulated by 3-fold under NaHCO₃, 1-fold under High pH + Na and 0.73-fold under High pH treatment relative to control (Fig. 13A). In roots, only NaHCO₃ treated roots showed upregulation (4-fold) of *PEPCase* mRNA expression (Fig. 13B). NaHCO₃ treated plant also showed a strong upregulation of mRNA expression of *MDH* (5.6-fold) and *CS* (9.5-fold) in leaves (Fig. 13C, E). A 2.5-fold higher relative mRNA expression of *CS* was recorded in leaves of High pH + Na treated plants compared to control plants (Fig. 13E). In roots, 1.6-fold higher upregulation of *CS* was observed under both High pH, and High pH + Na treatments (Fig. 13F).

4. Discussion

Unlike neutral salt (NaCl and Na₂SO₄) stress, alkali salt stress (NaHCO₃ and Na₂CO₃) is considered a more severe constraint for plant growth. However, it awaits clarification whether bicarbonate or high pH under alkali salt stress are the primary growth-limiting factors for plants. Hence, the present study demonstrated for the first time that growth of *Vicia faba* is impaired more by NaHCO₃ stress, than alone high pH (pH~9), and high pH + Na salt.

4.1. Growth related responses

The plants subjected to NaHCO₃ stress showed the severest root injury (Fig. 1, Supplementary Figs. S1, S2) and a substantially higher reduction in the root and leaf dry matters (Fig. 2) than that of high pH and high pH + Na treated plants. This substantial growth inhibition under NaHCO₃ treatment was related to a decline in photosynthetic rate (Fig. 3A) (Wu and Xing, 2012), and a relatively higher accumulation of H₂O₂ (Fig. 11A) (Zhang et al., 2017) and organic acids (Hajiboland et al., 2003). Possibly, the over accumulation of H₂O₂ under NaHCO₃ treatment (Fig. 11A) led to stomatal closure via the ABA signaling pathway (Zhang et al., 2001), which dropped internal CO₂ concentration to <80 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ (Supplementary Fig. S3) and eventually photosynthetic rate was reduced to one-half (Fig. 3A). Moreover, root growth inhibition in NaHCO₃ treatment coincided with the lowest PM H⁺-ATPase expression (Fig. 12B, D), which was incapable of apoplastic acidification required for acid-induced growth (Cosgrove, 2005). Following four days of growth under alkaline conditions, all

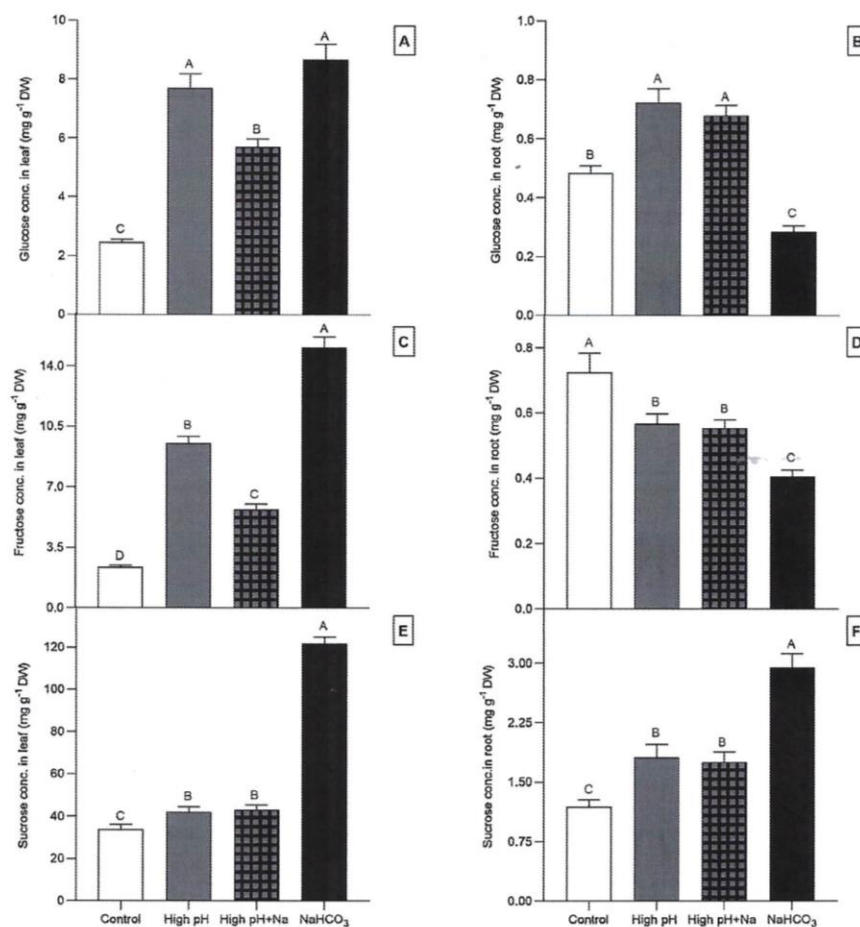


Fig. 9. Concentration (conc.) of glucose (A, leaf; B, root), fructose (C, leaf; D, root) and sucrose (E, leaf; F, root) in *Vicia faba* L. grown under control, high pH, high pH + Na and NaHCO₃ stress treatments for four days. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

three-treatments showed an increase in LWC compare to control, but the magnitude of increase was the highest (2.5-fold) in NaHCO₃ treated plants. This might be linked to the lowest stomatal conductance in this treatment which reduced water loss (Fig. 3B), and the highest accumulation of organic acids (Fig. 8) and soluble sugars (Fig. 9) which reduced the osmotic potential (Supplementary Fig. S4) (Meyer et al., 2010; Talbot and Zeiger, 1993). Since plants were grown in aerated hydroponic culture system, irrespective of damage (Wang et al., 2016), water flow in the roots would have been passive (Balliu et al., 2021; Blok et al., 2017). Additionally, higher accumulation of osmolytes (sugar and organic acid) reduced osmotic potential, which might be related to enhanced water uptake in NaHCO₃ treated plants (Basu et al., 2007; Steudle, 2000).

4.2. Ionic uptake and homeostasis

The plants showed a higher accumulation of Na⁺ in roots under NaHCO₃ treatment than high pH + Na treatment (Fig. 5B). A concomitant lower mRNA expression of *SOS1* (Na⁺/H⁺ antiporter) and *PM H⁺-ATPase* in NaHCO₃ treatment implies that plants might have failed to exclude Na⁺ efficiently (Sagervanshi et al., 2021). This resulted in a stronger competitive effect of Na⁺ on K⁺ uptake, which was reflected as a strong reduction in K⁺ concentration in roots - reaching the critical deficiency level of 10 mg g⁻¹ (Plank, 1989), or lower boundary of the

range required for optimum growth of plants (20–50 mg g⁻¹ DM of the vegetative tissues; (Marschner, 2011)). Other way around, lower K⁺ concentration in NaHCO₃ treated plants could have also arisen from a lower PM H⁺-ATPase activity as lower proton gradient across the plasma membrane can limit proton dependent K⁺ uptake (Li et al., 2018; Maathuis et al., 1997). Impaired and damaged roots under NaHCO₃ treatment might have also hampered K⁺ acquisition. On the other hand, the high pH treatment showed a higher K⁺ concentration, especially in roots, compared to control and both sodium salt treatments. Under extracellular alkaline conditions, plants could use a K⁺/H⁺ antiporter to accumulate K⁺ in cytosol and release H⁺ in the rhizosphere for intracellular pH homeostasis and to avoid the alkalinity induced damage (Jia et al., 2017). Thus, we assume that in the high pH treatment, a higher PM H⁺-ATPase activity (indicated by higher transcript expression of isogenes) builds up a higher proton gradient to elicit similar mechanisms for intracellular pH homeostasis, thereby plants accumulated higher quantities of K⁺.

The Ca²⁺ concentration in leaves was decreased under all the treatments, with a higher decrease under NaHCO₃ treatment (3.8 mg g⁻¹) and reached lower than critical deficiency levels of Ca²⁺ for optimum growth (5.0–30.0 mg Ca²⁺ g⁻¹ DM; (Mengel and Kirkby, 1987)) (Fig. 5A). As Ca²⁺ translocation is driven by the xylem stream, the decrease in [Ca²⁺] could be related to lower stomatal conductance (Fig. 3B) which reduced the transpiration rate (Tanner and Beever,

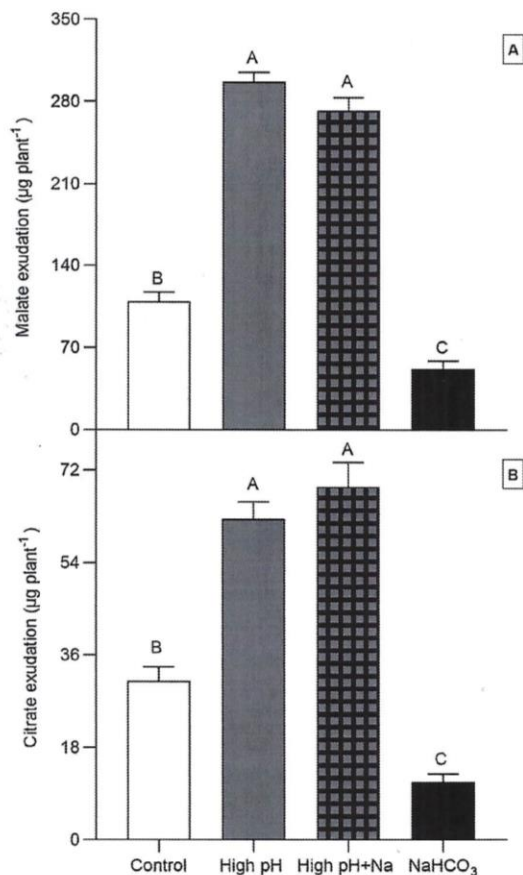


Fig. 10. Root exudation of malate (A) and citrate (B) by *Vicia faba* L. grown under control, high pH, high pH + Na and NaHCO₃ stress treatment for four days. The data ± SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

2001). Furthermore, lower [Ca²⁺] in leaves could have resulted from a damaged root apex and/or regions of lateral root initiation (Figs. 11F, S1 and 2D), the regions which are mainly responsible for Ca²⁺ transport to the shoots (White, 2001). Contrarily, in roots, NaHCO₃ treatment showed a 16-fold increase in [Ca²⁺], an effect that is explained by the chelation and deposition of Ca²⁺ in roots by the over accumulated organic acids, especially citrate which increased in concentration by 7-fold (Fig. 8D). Ryan et al. (2001) found that tricarboxylates (e.g. citrate) chelate Ca²⁺ and Fe²⁺ more strongly than dicarboxylates (e.g. malate). Calcium ions can cross-link carboxyl groups of organic acids, forming a stiff gel-like matrix (Cosgrove, 2005; Grignon and Sentenac, 1991). Kinzel (1989) reported that in calcareous soils, high HCO₃⁻ concentrations in the rhizosphere and correspondingly high uptake rates of both bicarbonate and calcium may result in the formation of CaCO₃ precipitates in the plant tissues.

In leaves, all the three high pH treatments decreased Mn²⁺, Zn²⁺ and Fe²⁺ concentrations while in roots only the NaHCO₃ treatment decreased Mn²⁺ and Zn²⁺ concentrations (Fig. 6). Similar to Ca²⁺, Fe²⁺ concentration in roots increased by 14-fold under NaHCO₃ treatment (Fig. 6F), and chelation by organic acids and deposition of those chelates in roots also appears to be a possible mechanism for Fe²⁺ over-accumulation in root. Spiro et al. (1967) and Bienfait and Scheffers (1992) reported negative effects of organic acids in Fe transport to shoots. At high pH, ferric citrate may form large, non-diffusible

polymers in roots, which plants cannot transport to the aerial organs (Kosegarten and Koyro, 2001; Zribi and Gharsalli, 2002), thus they are deprived of required Fe²⁺. The deposition of insoluble organic acid-Ca²⁺/Fe²⁺ complexes in roots might have also hampered the uptake of Mn²⁺ and Zn²⁺ and consequently the plants suffered from these nutrient disorders, resulting in growth impairment. In line with their normal root growth and/or higher PM ATPase transcript expression (Fig. 12B, D), high pH and high pH + Na treated plants showed increased concentrations of Fe²⁺, Mn²⁺ and Zn²⁺ in roots, the first two elements would have contributed to the detoxification of ROSs (Fig. 11B) while Zn²⁺ is known to improve membrane integrity (Tufail et al., 2018).

The lower or higher accumulation of anions (NO₃⁻, SO₄²⁻, and PO₄³⁻) in roots under NaHCO₃ and high pH or high pH + Na treatments, respectively also seems related with condition of root system (Supplementary Figs. S1, S2) and PM H⁺-ATPase activity indicated by higher mRNA abundance of isogenes (Fig. 12B, D), as their uptake (H⁺ Co-transport) is also facilitated via proton gradient. Furthermore, the decline in NO₃⁻ might have also originated from deficiency of K⁺, which facilitates the uptake and transport of NO₃⁻ by serving as an accompanying counter cation (Blevins et al., 1978). Overall, it is inferred that the competitive effect offered by excess Na, decreased PM H⁺ ATPase activity, damaged root system and change in metabolite accumulation and their root exudation contribute disturbance in ionic uptake and homeostasis under NaHCO₃ treatment.

4.3. Accumulation of metabolites and their root exudation

The most interesting and novel finding of this study is that plants synthesized and accumulated substantially higher amounts of organic acids and soluble sugars under all high pH treatments. The accumulation of organic acid is a specific physiological response to alkaline stress, and accumulated organic acids play an important role in the maintenance of pH and charge balance under stress (De Cheng et al., 2002). However, the accumulation of organic acids (malate and citrate) under NaHCO₃ treatment was higher than the other high pH treatments (Fig. 8). A high activity of carbon metabolism enzymes viz PEPCase, Citrate synthase and Malate dehydrogenase, as indicated by the higher mRNA abundance of their genes in this treatment (Fig. 13), might have enhanced dark fixation of bicarbonate into organic acids, resulting in their increased accumulation (Lee and Woolhouse, 1969; Poschenrieder et al., 2018). Moreover, the damaged root system (Canarini et al., 2019), lower PM H⁺-ATPase activity and K⁺ deficiency (Carvalho et al., 2011) might have restricted exudation of organic acids (Fig. 10A, B), which further increased their accumulation in roots. Over accumulation of organic acids, through chelation and precipitation of Ca²⁺ and Fe²⁺ in roots and thereby disturbing nutrient balance, especially of K⁺ (as explained above), seem to be the key plant growth limiting mechanism under bicarbonate stress. Lee and Woolhouse (1969) found that root growth of calcicole grass was inhibited by higher malate accumulation due to dark fixation of bicarbonate into malate.

On the other hand, plants under high pH and high pH + Na treatment, thanks to an intact root system (Supplementary Figs. S1, S2) and higher PM H⁺-ATPase activity (Fig. 12A, B, C, D), showed a higher exudation of organic acids (Fig. 10A, B), and thus withstood the adverse high pH condition by avoiding excess accumulation of these acids in root and leaf tissues.

The over accumulation of organic acids in roots and leaves was highly correlated with the over accumulation of soluble sugars in leaves, which are the precursors for the biosynthesis of organic acids (Bennet-Clark, 1937). NaHCO₃ treatment showed a comparatively higher rise in the level of soluble sugars in leaves, probably due to an impaired root system, which disturbed the source-sink relationship. Accumulation of soluble sugars in leaves in response to a decreased sink demand has been related to the down-regulation of photosynthesis (Nebauer et al., 2011), as has been found in our study. Guo et al. (2015) found that changes in sugar levels in plants are not passive responses to stress, but

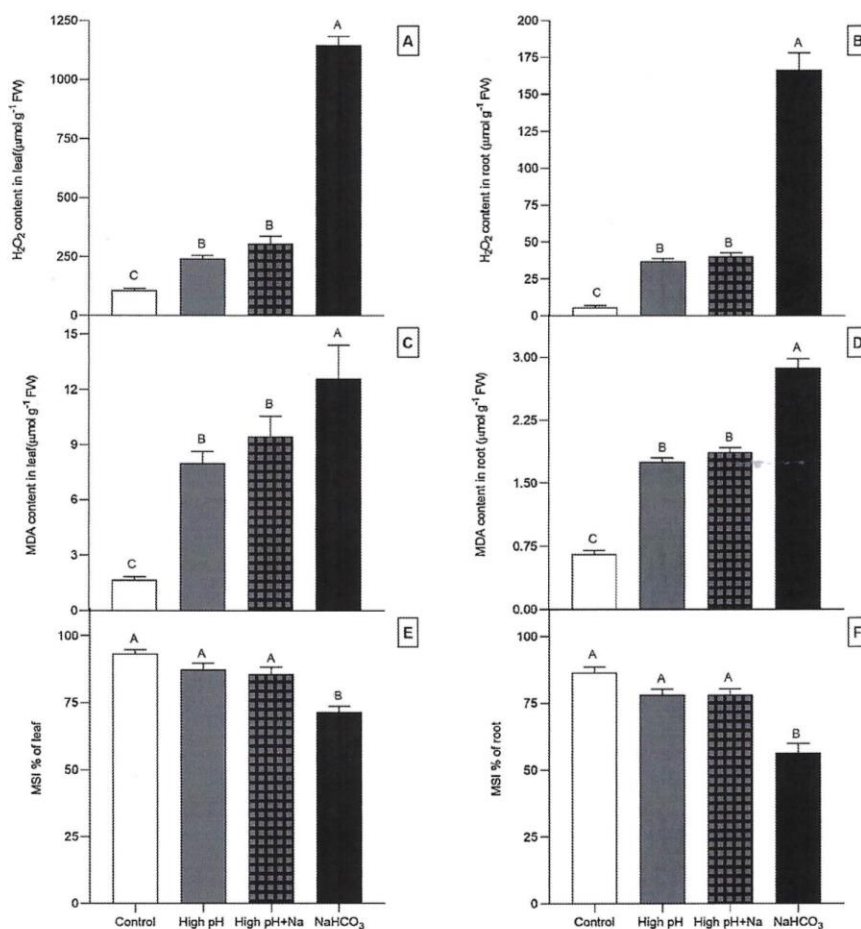


Fig. 11. H₂O₂ concentration (A, leaf; B, root), MDA content (C, leaf; D, root) and MSI (E, leaf; F, root) of *Vicia faba* L. grown under control, high pH, high pH + Na and NaHCO₃ stress treatment for four days. The data ± SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

rather a consequence of active metabolic processes of adaption to high-pH and alkali stress. Since sugars act as osmolytes and ROS scavengers (Keunen et al., 2013), their level in the treatments corresponded well to ROS accumulation in this study. Thus, increased production of soluble sugars to act as osmolytes and ROS scavengers in bicarbonate-stressed plants drive their conversion to organic acids and increase their accumulation in plant tissues, which then leads to a cascade of changes negatively affecting plant growth.

4.4. Oxidative stress response

Our results revealed that plants under all high pH treatments showed an increased accumulation of H₂O₂ (Fig. 11A, B), being highest under NaHCO₃ which can be explained by severe nutritional disorders (Munns and Tester, 2008). Hernandez et al. (2012) reported that K⁺ deficiency induced a higher production of H₂O₂ in *Solanum lycopersicum* L. roots. Secondly, a higher accumulation of organic acids could have induced H₂O₂ production by activation of NADPH oxidases and inhibition of PM H⁺-ATPase (Bissoli et al., 2020). High levels of H₂O₂ under NaHCO₃ treatment might have triggered lipid peroxidation of the plasma membrane, producing more MDA as a by-product (Fig. 11C, D). This is also confirmed by the lower membrane stability index of NaHCO₃ treated plants (Fig. 11E, F).

4.5. Transcription of ion transporters and carbon metabolism genes

Knowing that ionic and metabolic relations were influenced by all the three high pH conditions, especially under NaHCO₃ treatment, the question arose whether the changes in ionic and metabolic relations functionally correlate with the changes in the expression level of ion channels and carbon metabolism genes. Thus, we investigated the expression of ion transporters such as PM H⁺-ATPase isogenes (*VHA 4*, *VHA 5*) and *SOS1* (Na⁺/H⁺ antiporter) and carbon metabolism genes viz. *phosphoenolpyruvate carboxylase (PEPCase)*, *citrate synthase (CS)* and *malate dehydrogenase (MDH)* in root and leaf tissues. We observed that the expression of PM H⁺-ATPase isogenes was upregulated by high pH and high + Na treatment, whereas the NaHCO₃ treatment showed a lower expression. A moderate accumulation of H₂O₂, as has been observed in high pH and high + Na treatments, is known to increase the activity and expression of PM H⁺-ATPase (Huang et al., 2019) and *SOS1*. This helps plants to acclimatize to saline growth conditions by improving nutrient uptake and translocation and excluding Na⁺ by generating a H⁺ gradient across the membranes (Palmgren, 2001). Higher PM H⁺-ATPase might have also supported apoplastic acidification for acid-induced growth (Cosgrove, 2005) and effective root exudation of organic acids (Guo et al., 2013; Zeng et al., 2016). On the other hand, NaHCO₃ treated plants showed a lower expression of PM H⁺-ATPase. This might be due to a higher Ca²⁺ concentration in roots which acts as a negative regulator

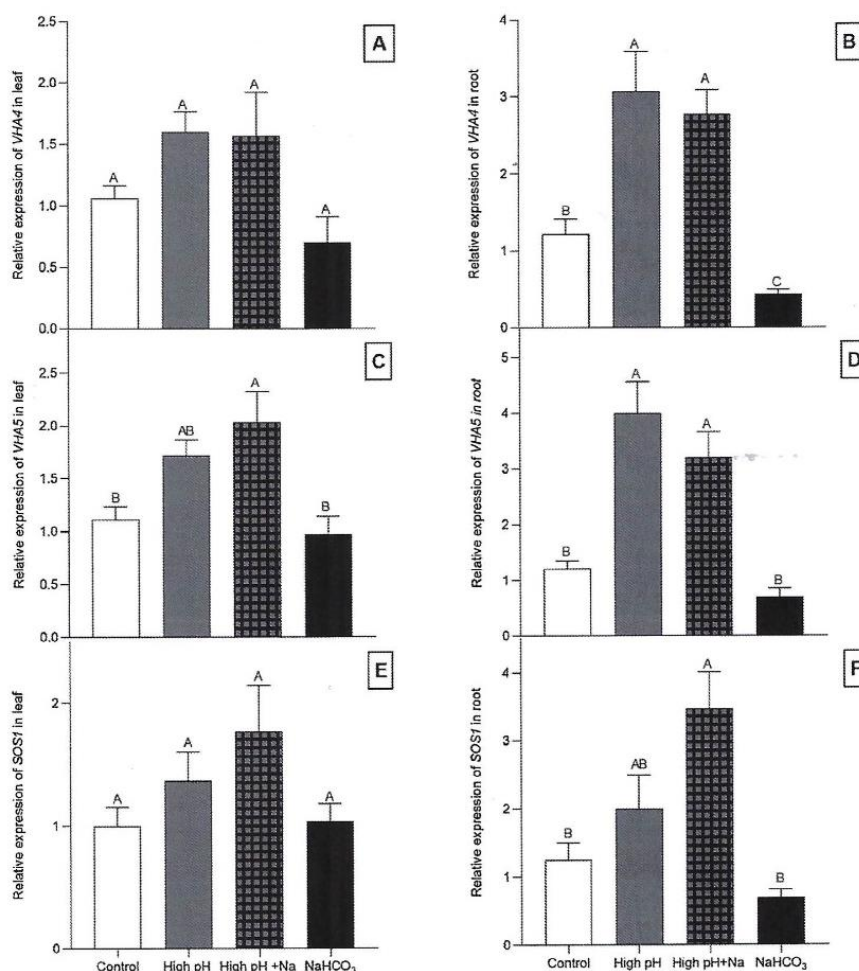


Fig. 12. The relative expression of plasma membrane H^+ ATPase isoforms *VHA4* (A, Leaf; B, Root), *VHA5* (C, leaf, D, root) and *SOS1* gene (E, leaf; F, root) in *Vicia faba* L. grown under control, high pH, high pH + Na and $NaHCO_3$ stress treatment for four days. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

of PM H^+ -ATPase activity (Kinoshita et al., 1995) and impaired normal growth. Moreover, under $NaHCO_3$ treatment, the mRNA abundance of *SOS1* was slightly reduced, and it might be related to lower PM H^+ -ATPase expression, because a concomitant higher PM H^+ -ATPase activity is a prerequisite for transport by *SOS1* (Munns and Tester, 2008; Sun et al., 2009).

Our results show that organic acid accumulation was increased under all three high pH treatments. Accordingly, we observed that *PEPCase* transcript expression was higher and positively correlated with the higher organic acid accumulation under $NaHCO_3$ treatment. Abadía et al. (2002) reported that cytoplasmic alkalisation associated with bicarbonate uptake could activate *PEPCase* and thus increased the synthesis of organic acids to maintain the pH of the root cell cytoplasm. Miller et al. (2010) found that an increase in *PEPCase* activity may feed the TCA cycle via malate, also increasing the MDH activity. The increase in *PEPCase* activity in plant is accompanied by large increases in MDH and CS enzymatic activities, supporting the significance of *PEPCase* in carbon fixation (López-Millán et al., 2000). Thus, we assume that an increase in transcription of these genes (Fig. 13) might have increased the enzyme activity, leading to higher organic acid concentrations.

High pH and high pH + Na showed an increased transcription of all these three carbon metabolism genes (Fig. 13), which in turn post-

translationally might have increased their abundance, resulting in higher synthesis and accumulation of the organic acids. Higher exudation of organic acids from roots in high pH and high pH + Na treatment is associated with enhanced activities of *PEPCase*, malate dehydrogenase, and citrate synthase (Ryan et al., 2001).

5. Conclusions

Compared to high pH and high pH + Na, the excess bicarbonate ($NaHCO_3$) treatment had the most adverse effects on *Vicia faba*, especially the root growth, photosynthesis, and stomatal conductance. Taken together, the results suggest that high bicarbonate concentration led to following events: Bicarbonate fixation into organic acids in excess to their exudation by roots, resulted in an overaccumulation of organic acids in the roots. The organic acids formed insoluble complexes with Fe^{2+} and Ca^{2+} in the roots which further reduced the root exudation of these organic acids and impaired nutrient uptake. Eventually, $NaHCO_3$ treated plants experienced K^+ and Ca^{2+} deficiencies. These nutrient disorders generated a massive rise in H_2O_2 concentration, resulting in disintegrated plasma membranes, root tissue damage and growth impairment. Although high pH and high pH + Na treatment also showed an accumulation of organic acids, the plants maintained their normal

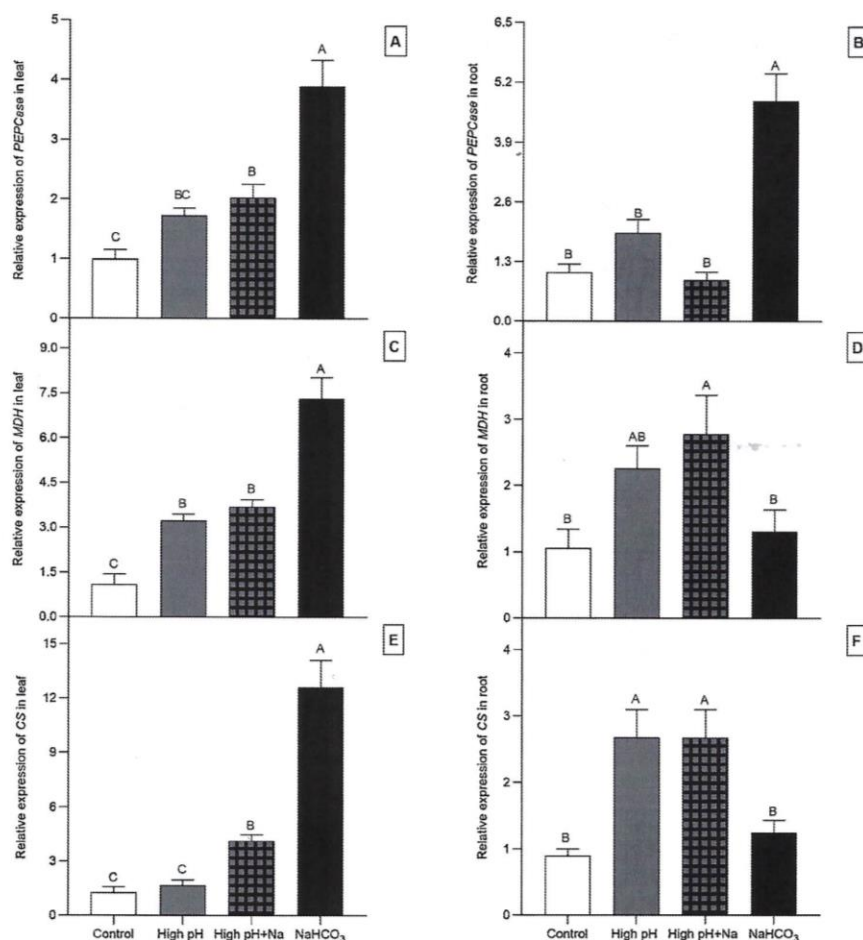


Fig. 13. Relative expression of *phosphoenolpyruvate carboxylase (PEPCase)* (A, leaf; B, root), *malate dehydrogenase (MDH)* (C, leaf; D, root), *Citrate synthase (CS)* (E, leaf; F, root) in *Vicia faba* L. grown under control, high pH, high pH + Na and NaHCO₃ stress treatment for four days. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

exudation, preventing overaccumulation in roots and later adverse growth effects. High pH and high pH + Na treated plants were able to maintain a sufficient level of PM H⁺-ATPase, SOS1, CS, and MDH expression in roots which enhanced their resistance to bicarbonate alkaline stress. This chain of events demonstrated by our results gives an explanation why high pH + bicarbonate stress is more detrimental to plant growth than high pH alone or in combination with sulfate.

Authors' statement

Amit Sagervanshi and Karl H. Mühling conceptualized and designed the experiments. Amit Sagervanshi performed experiments and analyzed data. Amit Sagervanshi and Asif Naeem wrote the initial draft of manuscript. Asif Naeem, Hartmut Kaiser and Britta Pitann participated in data analysis, writing, and editing the paper. All the authors have read, edited, and approved the final manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance Environmental and Experimental Botany.

Data availability

The material used in this study is available for non-commercial

research purposes upon reasonable request.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.envexpbot.2021.104636>.

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Supplementary data

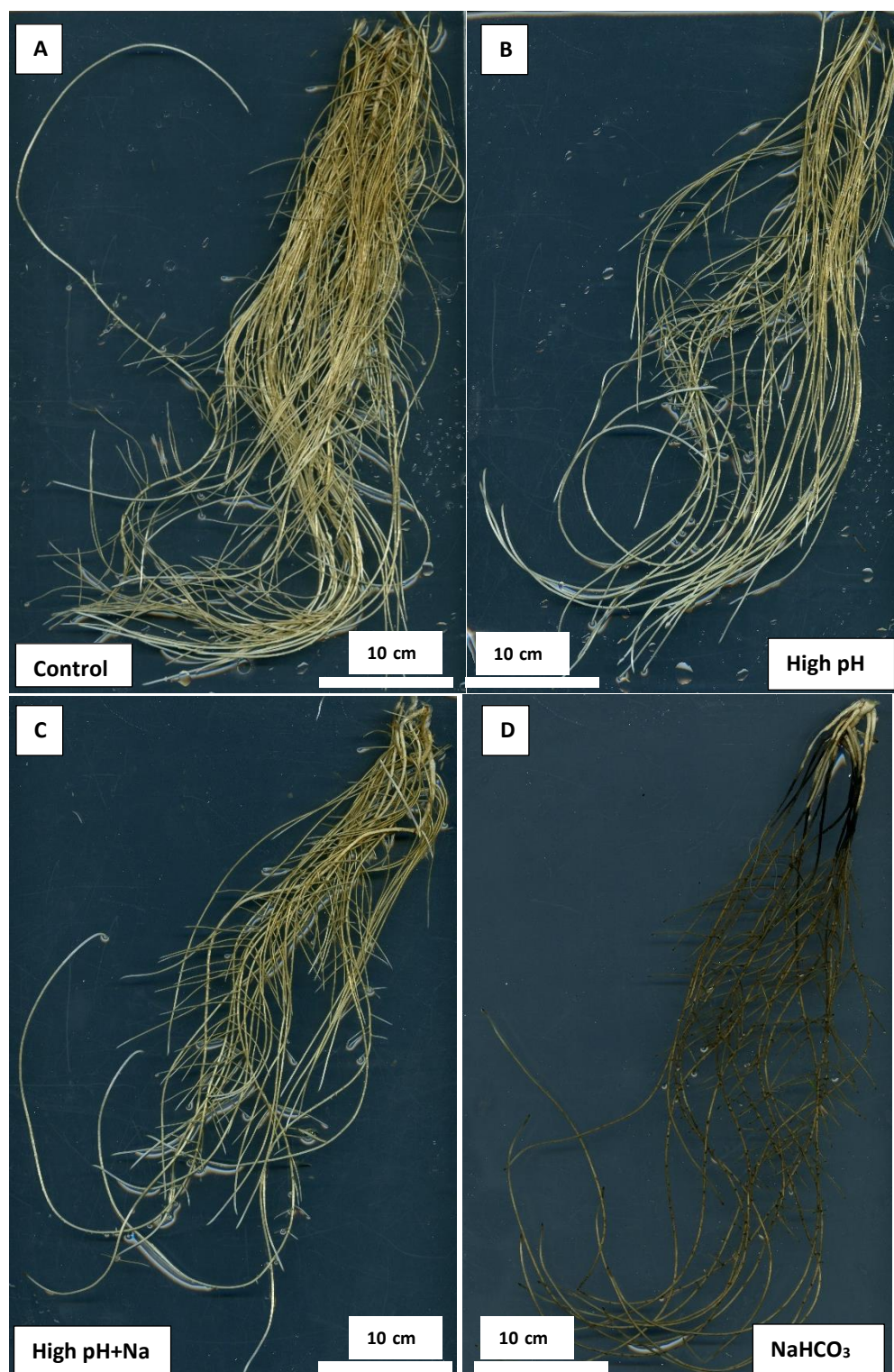


Fig. S1. Root morphology of *Vicia faba* L grown under (a) control, (b) high pH (2.5 mM CAPSO), (c) high pH+Na (2.5 mM CAPSO+25 mM Na₂SO₄) and (d) 50 mM NaHCO₃ stress treatment for four days. The scanned root image revealed that NaHCO₃ stress treatment induce root die blackening.

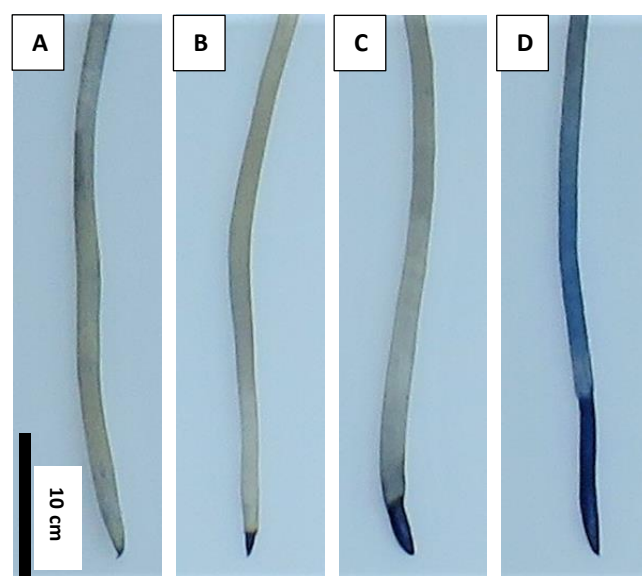


Fig. S2. Evan's blue staining (cell viability) of root tips of *Vicia faba* L grown under (a) control, (b) high pH (2.5 mM CAPSO), (c) high pH+Na (2.5 mM CAPSO+25 mM Na₂SO₄) and (d) 50 mM NaHCO₃ stress treatment for four days. The root tip image of NaHCO₃ stress treatment showed strongest Evan's blue stain, which means higher root cell death.

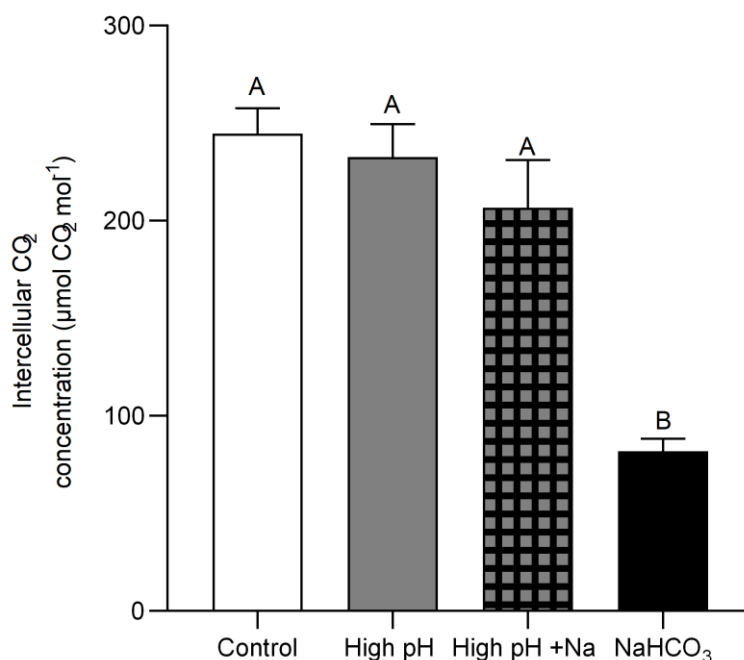


Fig. S3 Intercellular CO₂ concentration (C_i) of leaves of *Vicia faba* L. grown under control, high pH (2.5 mM CAPSO), high pH+Na (2.5 mM CAPSO+25 mM Na₂SO₄) and 50 mM NaHCO₃ stress treatment for four days. The data ± SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

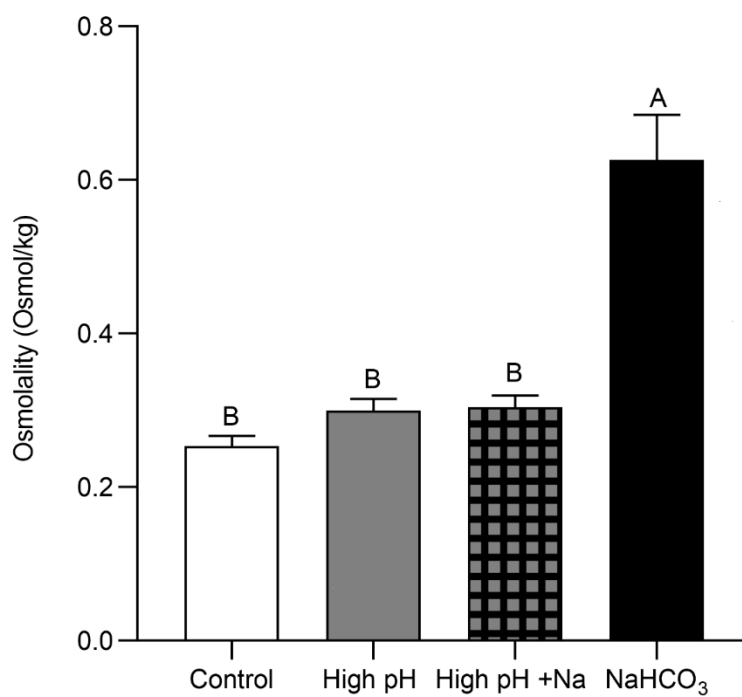


Fig. S4. Osmolality of leaves of *Vicia faba* L. grown under control, high pH, high pH+Na and NaHCO₃ stress treatments for four days. The data \pm SE are means of four independent pot replicates. Different letters on columns represent significant differences between the treatments ($p \leq 0.05$).

Table S1. List of primer used for RT qPCR analysis

Primer name	Genbank accession number		Sequence of forward (f) and reverse (r) primers (5'-3')	Annealing temp (°C)	Product size (bp)
<i>VfPEPCase1</i>	AJ011302.1	F	GGAGATTTTCAGGGATGAGAGC	60	156
		R	CTGAGTCGGATGAGCAGTAAG		
<i>VfPEPCase2</i>	AJ011303.1	F	CTCAGCCTCCAGATACCATTC	60	156
		R	TGGGAGATATTGGAGGATGC		
<i>VfVHA4</i>	AJ310523	F	GGCTTGGGATCTTGTTCATTGAG	60	194
		R	CTCTCCTTTTAGCTTCTTCGGC		
<i>VfVHA5</i>	AJ310524	F	GGCAAAGAAGAGAGAGAAGCAC	60	196
		R	CTGATTCGACGTGACCCTTTAG		
<i>VfSOS1_fam*</i>	MG680455.1	F	GTTCGTGGACCACTTGTAAG	60	177
	XM_028387494.1	R	GCTCCCATGCGTAAAAGTTG		
	JQ287499.1				
	MF405370.1				
<i>VfCS_fam</i>	NM_001354206.1	F	TGGTGGGAATGAGAGGAATGACAGC	60	168
	XM_020368503.2	R	GTCAATAGAAGCCACAGTACAGCC		
	XM_028082598.1				
<i>VfMDH_fam</i>	NM_001289254.1	F	TCTCCCTCTACGATATCGCC	60	166
	XM_020368058.2	R	ATTCCAGGCTTTCTGGGCAC		
	XM_004509982.3				
<i>VfCYP</i>	L32095	F	TGCCGATGTCACTCCAGAA	60	199
		R	CAGCGAACTTGGAACCGTATA		

*Family specific conserved primer

Table S2. Results from Sanger sequencing of RT-qPCR amplicon

Primer pair used for RT-qPCR	Sanger sequencing summary of RT-qPCR amplicon	Significant alignments of sequences (NCBI blastn result on nucleotide collection)			
		Genbank accession number	Description	Max score/ Total score	Query coverage (%) / Max ident (%)
<i>VfPEPCas e1</i>	AAACCAAGTCTGG TTTTTCACGCATC AAAAACTTCCTGA GGAGATTTCTTCA TGTTAAACACAAG TCTCTTGAGAGTT TCTTCGATGTCCG ACTCCGTAGTCGC ATTGCTCTCATCC CTG AAATCTCC	AJ011302. 1	<i>Vicia faba</i> Phosphoenolpyruvate carboxylase (PEPCase 1 isoform) mRNA predominantly expressed in root tissue	215/215	95/98
<i>VfPEPCas e2</i>	GCTACAAACTGTA AAGTCAAGGCGA AGTTATTGAGCAA TCATTTGGTGAGC AGCACTTGTGTTT CAGAAC GCTACAACGTTTC ACTGCAGCTACAC TAGAGCATGGAAT GCATCCTCCAATA TCTCCCAA	AJ011303. 1	<i>Vicia faba</i> Phosphoenolpyruvate carboxylase (PEPCase 1 isoform) mRNA predominantly expressed in leaf tissue	211/211	93/98
<i>VfVHA4</i>	CGATGCATTATAC AGGGAAAAGGAC TTTGGGAAGGAAC AACGTGAGCTTCA ATGGGCACATGCG CAAAGAACGCTGC ACGGATTACAACC ACCAGACACCAA GATGTTACCGAG CGAACTCATGTCA CCGAACTCAATCA AATGGCCGAAGA AGCTAAAAGGAG AG	AJ310523. 1	<i>Vicia Faba</i> isoform of PM H ⁺ -ATPase (Sagervanshi et al., 2021)	297/297	89/100
<i>VfVHA5</i>	GAGGATAACTGTC TCGTGCGACTCTC CTGGTCTTCAATC ACCCGAAACAACC AACCTTTTCAATG ACAAGAATAGCTA CAGGGAGCTTTCT GAGATTGCTGAGC AAGCCAAAAGAC GCGCTGAGGTTGC ACGGCTTAGGGAG	AJ310524. 1	<i>Vicia Faba</i> isoform of PM H ⁺ -ATPase (Sagervanshi et al., 2021)	291/291	89/99

	CTTCACACTCTAA AGGGTCACGTCGA ATCAG				
<i>VfCS_fam*</i>	CTACCTCATGAAT GAGGCACCGCCTG TTGACACCGGACG AGGGAATTCGCTT TAGGGGCA TGACAATTCCTGA CTGCCAGAAAACA CTTCCAGGTGCTT TTCCTGGTGGGGA GCCATTGC CTGAGGCTGTACT GTGGCTTCTATTG ACACA	NM_0013 54206.1 XM_0203 68503.2 XM_0280 82598.1	<i>Vicia faba</i> family specific(<i>fabaceae</i>) mitochondrial citrate synthase mRNA	193/193	83/94
<i>VfSOS1_fa m</i>	TAAATTCAATTGA AATTGCGTAGGCT TGACCCTTTATAA AGAAGGTGCCAA ATCGAATGGTATT TGGTTAATTTCTA ATGGAGTGGTTAA GTTAGAAAGCAA GACGATTCAAAGC AAGCACCCATTTT ATCCAACTTTTAC GCATGGGAGCA	JQ287499. 1 MF405370 .1 XM02036 6788.2	<i>Fabaeace</i> family specific primer for sodium/hydrogen antiporter (Sagervanshi et al., 2021)	115/115	93/86
<i>VfMdh_fa m</i>	AACTCTCGTGATG CCTAGAGGCAGG GACATATGACGAA AAGAGATTGTTTG GGGTTACAA CCCTTGACGTGGT TAGGGCAAAAAC TTCTATGCTGGGA AAGCCAATGTTCC A	NM_0012 89254.1 XM_0203 68058.2 XM_0045 09982.3	<i>Vicia faba</i> family specific(<i>fabaceae</i>) mitochondrial malate dehydrogenase mRNA	154/154	84/96

*Family specific conserved primer

Sequencing of the real-time qRT-PCR products confirmed specificity of the primer pair. Column 1 indicates the name of the primer pair. The corresponding DNA sequencing result is presented in column 2. The sequences were aligned against NCBI's standard nucleotide collection (nr/nt) databases. The search was limited to *fabaceae* (taxid:3803). Best blast hit is presented in column 3 together with the respective Gensbank accession number. Column 4 shows the resulting description of the Blast hits. Column 5-6 shows parameters for statistical quality of the hit.

Chapter 4

One-time ABA priming induces long-term salinity resistance in *Vicia faba* L: changes in key transcripts, metabolites and ionic relations

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One-time abscisic acid priming induces long-term salinity resistance in *Vicia faba*: Changes in key transcripts, metabolites, and ionic relations

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Abstract

Abscisic acid (ABA) priming is known to enhance plant growth and survival under salinity. However, the mechanisms mediating this long-term acclimatization to salt stress are still obscure. Specifically, the long-term transcriptional changes and their effects on ion relations were never investigated. This motivated us to study the long-term (8 days) effect of one-time 24 h root priming treatment with 10 μ M ABA on transcription levels of relevant regulated key genes, osmotically relevant metabolites, and ionic concentrations in *Vicia faba* grown under 50 mM NaCl salinity. The novelty of this study is that we could demonstrate long-term effects of a one-time ABA application. ABA-priming was found to prevent the salt-induced decline in root and shoot dry matter, improved photosynthesis, and inhibited terminal wilting of plants. It substantially increased the mRNA level of AAPK and 14-3-3 ABA inducible kinases and ion transporters (*PM H⁺-ATPase*, *VFK1*, *KUP7*, *SOS1*, and *CLC1*). These ABA-induced transcriptional changes went along with altered tissue ion patterns. Primed plants accumulated less Na⁺ and Cl⁻ but more K⁺, Ca²⁺, Zn²⁺, Fe²⁺, Mn²⁺, NO₃⁻, and SO₄²⁻. Priming changed the composition pattern of organic osmolytes under salinity, with glucose and fructose being dominant in unprimed, whereas sucrose was dominant in the primed plants. We conclude that one-time ABA priming mitigates salt stress in *Vicia faba* by persistently changing transcription patterns of key genes, stabilizing the ionic and osmotic balance, and improving photosynthesis and growth.

1 | INTRODUCTION

Salinity is characterized by excessive soluble salts in the growth medium, which impose osmotic and ionic stress in plants (Munns & Tester, 2008). Key processes of photosynthesis, respiration, and transpiration are affected, thus limiting shoot and root growth (Munns & Tester, 2008). Field bean (*Vicia faba*) grown with an excess of sodium chloride (NaCl) has been reported to show severe growth retardation due to a high

accumulation of Na⁺ that interferes with K⁺ uptake (Slabu et al., 2009). It possibly disrupts the regulation of cytoplasmic enzymes and stomatal conductance (Isayenkov & Maathuis, 2019). Moreover, in *Vicia faba* grown under NaCl stress, higher accumulation of chloride (Cl⁻) in the chloroplast is thought to impair the photosynthetic machinery and thus to induce chloroplast degeneration leading to chlorosis or necrosis of the leaf tissue (Geilfus, 2018). To resist salt stress, plants have evolved mitigating mechanisms at different organizational levels. Many of these

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mechanisms, for example, stomatal regulation, various morphological modifications, osmolyte accumulation, exclusion of salt ions by roots, and vacuolar partitioning of excess ions, are activated by salt stress, increasing resistance following the stress event. Hence, to pre-emptively strengthen the salt resistance in plants, it is vital to activate the resistance mechanisms before the onset of salt stress.

Priming represents a sustainable, fast, cost-effective, and environmental-friendly procedure for crop production under stressed environments (Tanou et al., 2012). The priming process is referred to as pre-treatment of plants, seeds, or roots, with mild stressor agents to activate resistance responses in plants to future stress exposure (Tanou et al., 2012). Priming induces physicochemical and transcriptional adaptations, which allow plants to react faster and more robustly after they encounter biotic or abiotic stress (Savvides et al., 2016). So far, numerous priming agents, such as salicylic acid, β -amino butyric acid, kinetin, or abscisic acid (ABA), have been used to enhance resistance against drought, cold, or salt stress (Savvides et al., 2016).

ABA is considered a stress hormone, and its rapid accumulation in plants under various abiotic stresses shows its involvement in stress resistance of plants (Geifus et al., 2018; Zörb et al., 2013). Long-term

root application of ABA to stressed plants has shown to improve drought resistance in rice (Teng et al., 2014), salt-resistance in potato (Etehadnia et al., 2008), and common bean (Khadri et al., 2007). These studies indicated that the improvement in growth of salt-stressed plants induced by exogenously applied ABA was related to decreased sodium (Na^+)-to-potassium (K^+) ratio and chloride (Cl^-) accumulation, and increased accumulation of proline or soluble sugars. Finkelstein (2013) reported that ABA regulates transcription of 1–10% of the *Arabidopsis* genome. Thus, we expect that ABA priming induces salt stress resistance in plants by changing the expression patterns of genes relevant to salt ion exclusion or inclusion. Previous studies on the effect of ABA priming on salinity resistance did not examine the expression of genes that are involved in salt resistance together with critical ionic load in the tissues. Thus, we hypothesized that a one-time 24 h ABA priming event induces long-term salt resistance via: (1) long-term effects on transcription of genes involved in salt stress signaling and ion regulation, (2) stabilizing ionic and osmotic relations, and (3) enhancing photosynthesis and plant growth. Therefore, we studied the long-term effects of short-term ABA priming on the mRNA levels of ABA-inducible kinases (ABA activated protein

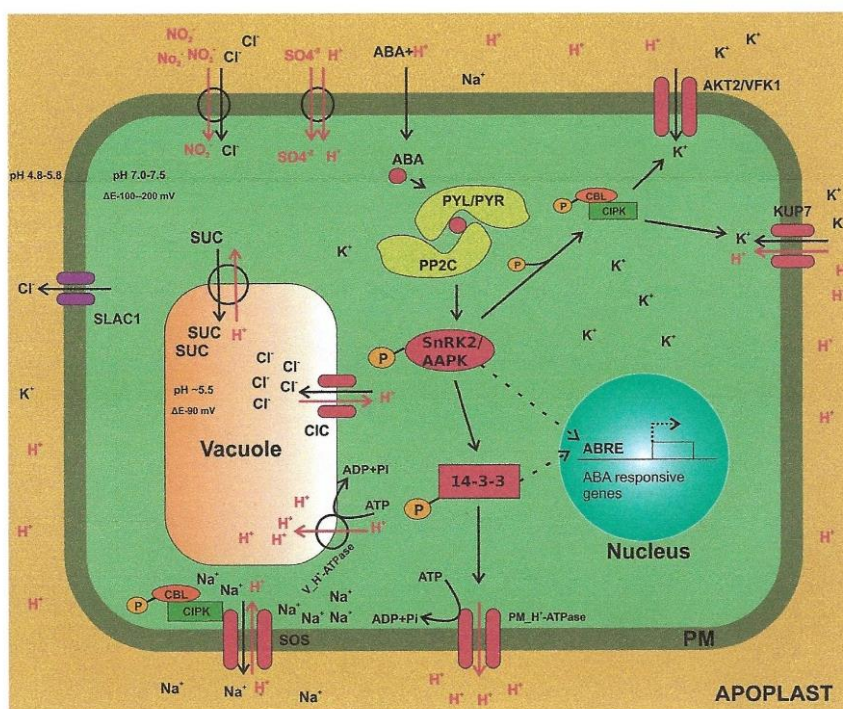


FIGURE 1 Schematic illustration of the current knowledge about transporters, ion channels, regulatory proteins, and signaling molecules are involved in ion regulation under salt stress and being affected by ABA priming. The increased expression after ABA priming is highlighted in red color. ABA receptors in the cytoplasm and nucleus activate ABA-dependent signaling cascades via primary enhanced expression and activation of AAPK/SnRK2 (ABA dependent kinase), which may subsequently phosphorylates other signaling kinases like 14-3-3, CIPK, CBL, CDPK, MAPK, etc. These components, along with SnRK2, may phosphorylate ABA-responsive elements like ABRE, ABF2 (transcription factors), which may further activate expression of stress response genes, and ion transporters for stress adaptation. Interaction of 14-3-3 with the plasma membrane (PM) H^+ -ATPase may increase the proton-pumping activity and creates an electrochemical H^+ gradient across the PM, which may lead to membrane hyperpolarization, energizes secondary transport and activates voltage-dependent ion channels like CLC1, SLAC1, AKT2/3 for ion transport. The salt exclusion transporter SOS1 and the potassium transporter HAK/KUP is activated via phosphorylation by CIPK and CBL and by protonation. ABA priming is suggested to enable an increased activation of these interacting mechanisms helping plants to acclimate to salt stress

kinase [AAPK and 14-3-3], which are important regulators of both ABA responsive genes and activators of transport proteins involved in ion regulation. The expression of important membrane ion transporter genes (viz. plasma membrane [PM] proton ATPase, potassium transporter, sodium antiporter and chloride channel) was also studied (for a visual explanation of the studied molecular mechanisms, see Figure 1). The priming-induced changes in ion transport expression were associated with ion relations and osmolyte composition under salinity stress. Based on this, we tested whether these changes were associated with a stabilized leaf water content, improved photosynthesis, and plant growth under salinity stress.

2 | MATERIAL AND METHODS

2.1 | Plant cultivation, ABA priming, and salinity treatment

Vicia faba L. (cv. Sirocco: NPZ GmbH, Hohenlieth, Germany) was cultivated under hydroponic culture condition in a controlled climatic chamber (14/10 h day/night; 20/15°C; 50/60% humidity). Seeds were surface sterilized with 0.5% sodium perchlorate for 3 min and then washed thoroughly with deionized water. Afterward, seeds were soaked in aerated 0.5 mM CaSO₄ solution overnight and sown in moist sterilized quartz sand for germination. After 1 week, uniform-sized seedlings were transferred to 2.5-L plastic pots containing a quarter strength of the nutrient solution (Geilfus & Mühling, 2013). To avoid any osmotic shock reaction, the concentration of the nutrient solution was progressively increased to 1/2, 3/4, and full-strength on second, third, and fourth day after transplantation, respectively. The nutrient solution was changed after every 2 days to replenish the depleted nutrients. Unprimed and ABA-primed plants were grown without (0 mM) and with 50 mM sodium chloride (NaCl). Thus, the experiment had a two-factor factorial design in a completely randomized arrangement with four independent pot replicates for each treatment. Before induction of salinity treatment, 2 weeks old plants were pre-treated with ABA by adding the hormone to the nutrient solution (for the timing of treatments, see Figure 5).

For ABA priming, plants growing in nutrient solutions were supplied with 10 μM of ABA for 24 h, while the unprimed plants did not receive an ABA pre-treatment. Fifteen-day (fourth leaf stage) old unprimed and ABA-primed plants were subjected to 0 and 50 mM NaCl salinity in the nutrient solution. The salinity level of 50 mM was increased progressively for 2 days to avoid sudden osmotic shock. Thereafter, the nutrient solution, without (control treatment) and with the required concentration of NaCl salt (50 mM NaCl treatment), was changed after every day to replenish the depleted ions. After 8 days of growth under non-saline or saline conditions, plants were harvested, and roots and leaf samples were separately collected. Leaf and root samples were thoroughly washed with deionized water to remove the adhering ions from the surface, dried at 60°C for 72 h, and the dry weights were recorded. The dried materials were ground to a fine powder and used for mineral analysis. Fresh plant materials were used to analyze the abundance of certain transcripts. For this,

the second batch of plants was grown at the same time under identical conditions. After washing with deionized water, fresh plant materials were shock-frozen in liquid N₂ and stored at -80°C until analysis.

2.2 | Gas exchange measurements

Gas exchange measurements were carried out on intact, fully expanded third-youngest leaves before harvesting. Photosynthetic rate (μmol CO₂ m⁻² s⁻¹) and stomatal conductance (mol H₂O m⁻² s⁻¹) were measured with an open flow portable photosynthesis system (LI 6400XT, Li-COR Biosciences Inc.). For measurements, leaves were placed across a 2 × 3 cm leaf chamber. The conditions for the measurements inside the leaf chamber were maintained identical to the external conditions of the climatic chamber. The light was provided by a LED red light source built into the top of the leaf chamber (250 μmol quanta m⁻² s⁻¹), and the CO₂ concentration was controlled by Li-COR LI-6400 CO₂ injection system (400 μmol CO₂ mol⁻¹) as identical to outside conditions.

2.3 | Non-invasive leaf water content (LWC) measurement

Non-invasive *in planta* measurement of leaf water content was started 2 days before the onset of ABA priming and continued till the day of harvest. A circular area of 2–3 cm diameter in the middle of the fourth leaf was selected for daily measurements. For the quantification of leaf water content, a ratiometric near-infrared (NIR) transmission setup was employed. The measurement uses the ratio in absorption at 1450 nm (strong absorption by liquid water) and 1050 nm (non-absorbing wavelength), which vary with leaf water content (Zhang et al., 2019). The custom-made water sensor (developed by H. Kaiser) device had a dual infrared LED emitter mounted on top of a leaf clip by a 45° angle and a photodiode collecting transmitted light below the leaf. A data logger was used to control the device and record data. For calibration of the water sensor, several detached *Vicia faba* leaves were placed in deionized water at 4°C for overnight. On the following day, NIR transmission ratio was determined at three-time intervals (0, 5, 10 min) during air drying of these leaves. Simultaneously, water content was measured by the gravimetric method. The linear relation between NIR transmission ratio and gravimetric water content was used to convert the sensor data into water content (g m⁻²).

2.4 | Mineral analysis of plant tissue

For mineral analysis, oven-dried and finely ground leaf and root samples (200 mg) were digested with 10 ml of 69% HNO₃ (ROTIPURAN Supra for ICP, 69%) in a closed-vessels microwave digestion system (MARS 6 Xpress, CEM Corporation) adjusted to the following conditions: 2 min at 100°C, 1 min at 120°C, 20 min at 180°C and 20 min cooling time. Afterward, the digested samples were diluted with

Milli-Q water (18.2 MΩ cm conductivity) to 100 ml and stored at 4°C until further analysis. Concentrations of Na and macronutrients (K and Ca) and micronutrients (iron, Fe; manganese, Mn; zinc, Zn) were measured by inductively coupled plasma mass spectroscopy (ICP-MS; Agilent 7700, Agilent Technologies Inc.) as described by Jezek et al. (2015).

2.5 | Determination of free sugars and inorganic anions

Water-soluble sugars and free inorganic anions were extracted by hot water following the procedure of Cataldi et al. (2000) with minor modifications. Dried and powdered leaf and root samples (~20 mg) were dissolved and boiled for 5 min in 1.5 ml of sterile deionized water, mixed thoroughly by vortexing and immediately placed on an ice-water bath for 30 min. Then mixtures were centrifuged, and the supernatant was collected. For the precipitation of proteins, the supernatants were mixed with chloroform and centrifuged. Again, the supernatant was collected and cleaned by passing through strata C-18 columns (Phenomenex, Torrance). Afterward, both the anions and sugars were determined by isocratic ion chromatography (IC-5000 Capillary Reagent- Free IC System, Thermo Scientific).

2.6 | Primer design and Sanger sequencing

For analyzing the mRNA transcript level of ABA inducible kinases AAPK and 14-3-3, and ion transporter genes PM H⁺-ATPase *isogenes* (*vha2*, *vha4*, *vha5*), and *Vicia faba* *potassium channel1* (*VFK1*), primers were designed from sequences available on NCBI database for *Vicia faba*. Since genome sequences of salt overly sensitive1 (*SOS1*), *potassium uptake permease 7* (*KUP7*), and *chloride channel1* (*CLC1*) are not annotated for *Vicia faba*, *Fabaceae* family-specific primers were designed based on conserved regions: The coding sequences (CDSs) of genes of interest from *Glycine max*, *Vigna radiata*, *Cicer arietinum*, and *Medicago truncatula* were retrieved from the NCBI database (<https://www.ncbi.nlm.nih.gov/nucleotide/>). Thereafter, multiple CDS sequences were aligned by using the CLC workbench tool (<https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/>), and the homologous conserved region was chosen for primer design. A pair of primers were designed from the conserved aligned sequence for each gene by manual selection of oligonucleotide sequences on the CLC workbench tool. Characteristics of selected primers were checked and evaluated in silico by the online tool oligo calc (<http://biotools.nubic.northwestern.edu/OligoCalc.html>) and multiple primer analyzer tools provided by Thermo-Fisher. All primer oligos were purchased from Eurofins Genomics (Ebersberg, Germany). The details of the primers are listed in Table S1. All the primers were tested and validated by PCR amplification to ensure that (1) they work in *Vicia faba* and (2) there are specific to the targeted cDNA. The thermal-cycling program was performed as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s,

annealing at 55–60°C for 30 s, and extension at 72°C for 30 s and final extension for 5 min at 72°C. PCR products obtained were run along with 1 kb DNA ladder (Gene ruler, Thermo Fisher Scientific) on 1% TBE agarose gels to ensure the correct length of the amplicon. Furthermore, amplification of the correct gene was confirmed by the sequencing of the amplicon (Sanger sequencing, Institut für Klinische Molekularbiologie) and comparison with the BLASTN nucleotide collection database (Table S2).

2.7 | Gene expression analysis

Total RNA of leaves and roots were extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. The concentration and purity of RNA were determined by a NanoVue Plus Spectrophotometer (GE Healthcare Life Science), and integrity was checked by gel electrophoresis. Total coding RNA (1 μg) isolated was reverse transcribed following the manufacturer's instructions (Verso cDNA synthesis kit, Thermo Fisher Scientific, USA), including DNase I treatment. The quality of synthesized cDNA (1/10 dilution) was checked by standard PCR with the most stable housekeeping gene *Vf Cyclophilin* (*CYP*) (Gutierrez et al., 2011; Neuhaus et al., 2013). Quantitative RT-PCR was conducted by PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) with primers shown in Table S1 on CFX96 Real-Time System (Bio-Rad Laboratories GmbH, München, Germany). Each reaction (20 μl) contains 100 nM of each primer, 2 μl of diluted cDNA templates, and other reaction components. After an initial denaturation step (95°C for 5 min), RT-qPCR was carried out over 40 cycles (95°C for 15 s, 60°C for 30 s, 72°C for 30 s), followed by a melt curve stage (95°C for 15 s, 60°C for 1 min, 95°C for 15 s, 24°C for 15 s). Three biological replicates and three technical replicates were used for each treatment. Transcript levels of gene were normalized with endogenous control (*Vf CYP*), and the expression changes of target mRNAs were determined using the 2^{-ΔΔCt} method (Livak & Schmittgen, 2001).

2.8 | Statistical analysis

Data were statistically analyzed following two-way ANOVA, which was performed using SPSS software (version 17.0). Significant differences among the means were determined by Tukey's HSD test at $P \leq 0.05$.

3 | RESULTS

3.1 | Growth, dry matter, and gas exchange attributes

Under saline condition, plant height and root growth were severely stunted in unprimed plants, while this effect was not observed for ABA-primed plants (Figure 2). Leaf dry matter of unprimed plants

decreased by 33% under saline condition as compared to non-saline condition (Figure 3A). However, ABA-primed plants did not show a decrease in leaf dry matter accumulation under saline condition. Similarly, reduction in root dry matter of unprimed plants under salinity was 21% as compared to non-saline condition. In contrast, salinity did not affect the root dry matter accumulation of ABA-primed plants (Figure 3B).

Under non-saline conditions, the photosynthetic rate of unprimed and ABA-primed plants was similar. Under saline condition, photosynthetic rate of unprimed plants declined by 45%, while that of ABA primed plants decreased by only 10% (Figure 4A). ABA priming under non-saline condition decreased stomatal conductance, measured after 4 h of light, by 27% as compared to unprimed plants (Figure 4B). However, under saline condition, ABA primed plants had a 53% higher stomatal conductance than unprimed plants.

3.2 | Leaf water content

In general, leaf water content (LWC) showed an increasing trend during the 8 days of observation, possibly due to ongoing maturation (Figure 5). ABA priming caused a significant extra increment of ca. 10% compared to the unprimed treatments. Under salinity, LWC in the unprimed plants increased at a high rate to a maximum on day 5, and onward to that rapidly decreased till the end of the experiment when visual wilting was observed. This decline was most likely caused by visual dieback of the fine roots, effectively impeding water supply to the shoot. The ABA-primed plants under salinity neither showed the large initial increase nor the later rapid decline in LWC; instead,

they responded mainly parallel to the unprimed and primed non-saline treatments.

3.3 | Concentration of minerals in leaves and roots

Under saline conditions, Na^+ concentration increased 11- and 18-fold, respectively, in leaves and roots compared to the values recorded under non-saline conditions (Figure 6A,B). ABA priming under saline condition decreased leaf and root Na^+ concentration by a factor of 0.60 and 0.44, respectively. Potassium concentration in leaves and roots of unprimed plants showed a 17 and 30% decrease, respectively, under saline condition compared to non-saline condition (Figure 6C,D). ABA-primed plants did not show this salinity-induced decline in K^+ concentration in both the tissues. Salinity decreased Ca^{2+} concentration in leaves of both unprimed and ABA-primed plants by 28%, compared to non-saline unprimed plants (Figure 6E). Similarly, the root Ca^{2+} concentration declined by 25% under salinity in unprimed plants, in contrast to primed plants, which sustained their Ca^{2+} concentration (Figure 6F).

Salinity, ABA priming, and their interaction in some cases (Figure 7) significantly affected micronutrient (Zn^{2+} , Fe^{2+} , and Mn^{2+}) concentrations in leaves but not in roots. Under non-saline condition, unprimed and ABA-primed plants had similar Mn^{2+} concentration in both tissues, while Zn^{2+} and Fe^{2+} concentration in leaves were 16% and 32% higher in ABA-primed plants (Figure 7A,C,E,G). Salinity generally decreased micronutrient concentrations in leaves, but more so in unprimed than ABA-primed plants. Consequently, in leaves, ABA priming restored Zn^{2+} and Mn^{2+} (Figure 7A,E) and improved Fe^{2+}

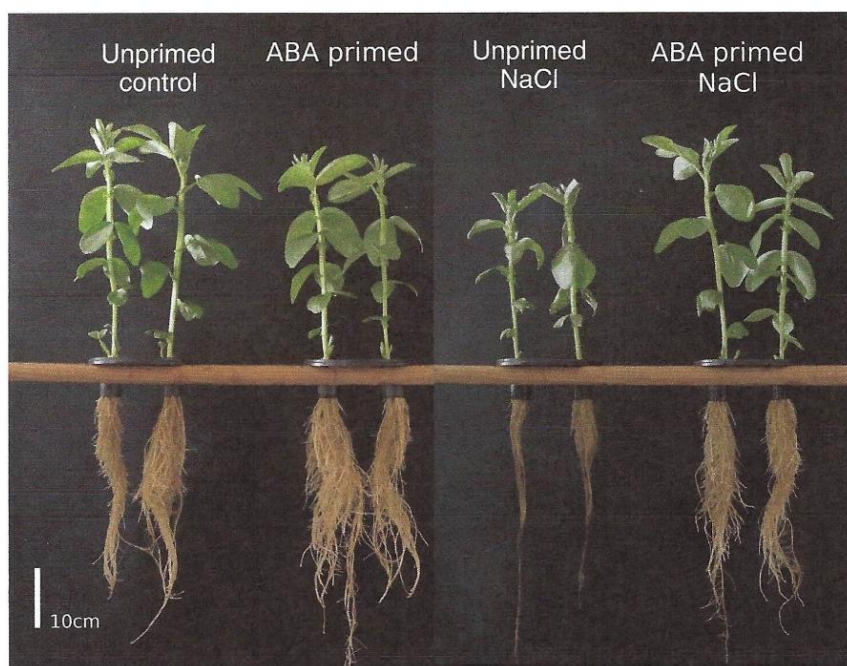


FIGURE 2 Effect of ABA priming on plant height and root morphology of *Vicia faba* grown without or with 50 mM NaCl salinity. The 24-h lasting ABA priming with 10 μM ABA at the roots was done 1 day before application of salt stress and 8 days before pictures were taken

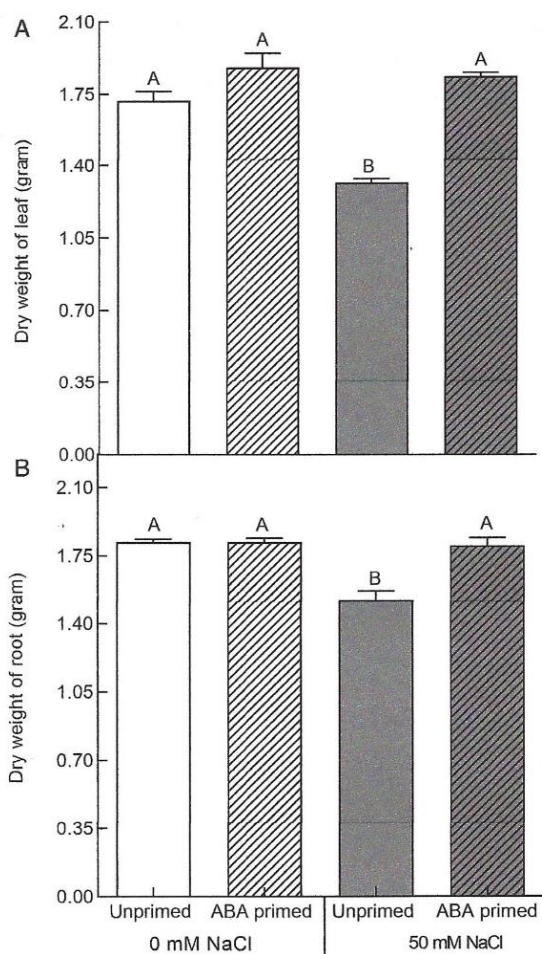


FIGURE 3 Effect of ABA priming on the leaf (A) and root (B) dry matter of *Vicia faba* L. grown without or with 50 mM NaCl salinity. The data \pm SE are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

concentration by 82%, as compared to unprimed plants under saline condition (Figure 7C).

Salinity increased Cl^- concentration in leaves and roots of unprimed plants by 4.6- and 5.1-fold, respectively (Figure 8A,B). ABA-priming decreased Cl^- concentration in leaves and roots by a factor of 0.60 and 0.53, respectively, compared to the unprimed plants (Figure 8A,B). Salinity decreased nitrate (NO_3^-) and sulfate (SO_4^{2-}) concentrations in leaves by 80 and 37%, respectively, in unprimed plants (Figure 7C,E). ABA priming doubled the NO_3^- and SO_4^{2-} concentrations in leaves as compared to unprimed plants under saline condition (Figure 8C,E). Under salinity, NO_3^- concentration in roots decreased by 69% in unprimed plants as compared to non-saline unprimed. In comparison, ABA priming improved NO_3^- concentration in roots by 150% as compared to saline unprimed plants (Figure 8D).

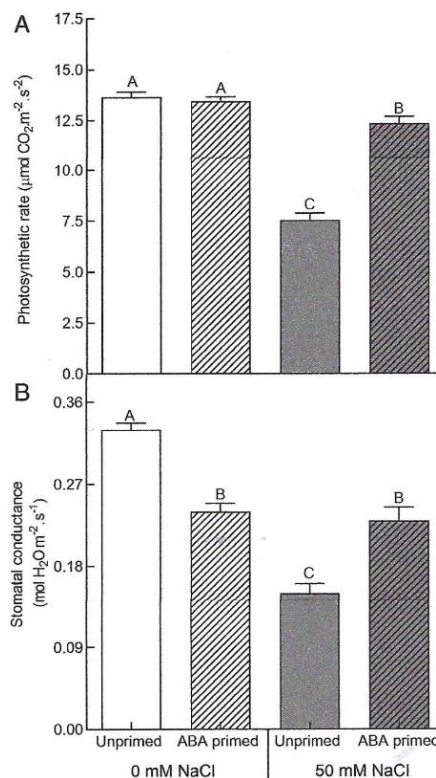


FIGURE 4 Effect of ABA priming on photosynthetic rate (A), and stomatal conductance (B) of *Vicia faba* L. grown without or with 50 mM NaCl salinity. The data \pm SE are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

Concentrations of SO_4^{2-} in roots were neither affected by salinity nor by ABA priming under saline condition (Figure 8F).

3.4 | Accumulation of organic osmolytes by leaves and roots

In leaves, the salinity response of unprimed plant was dominated by three- and twofold increase in glucose and fructose concentrations, respectively (Figure 9A,C). The sucrose concentration, however, remained unchanged. Priming inverted this pattern: it reduced the fructose and glucose concentrations back to the level of non-saline conditions and led to a ca. twofold increase in sucrose concentration, effectively shifting the dominant sugars in osmotic adjustment from fructose and glucose to sucrose (Figure 9A,C,E). A similar shift to sucrose as prevalent sugar was observed in the roots, where salinity halved the sucrose concentration in roots of unprimed plants and priming led to a slight increase under salinity. At the same time, the salinity-induced increase in glucose and fructose concentrations was a little (fructose) or absent in ABA-primed plants (Figure 9B,D,F).

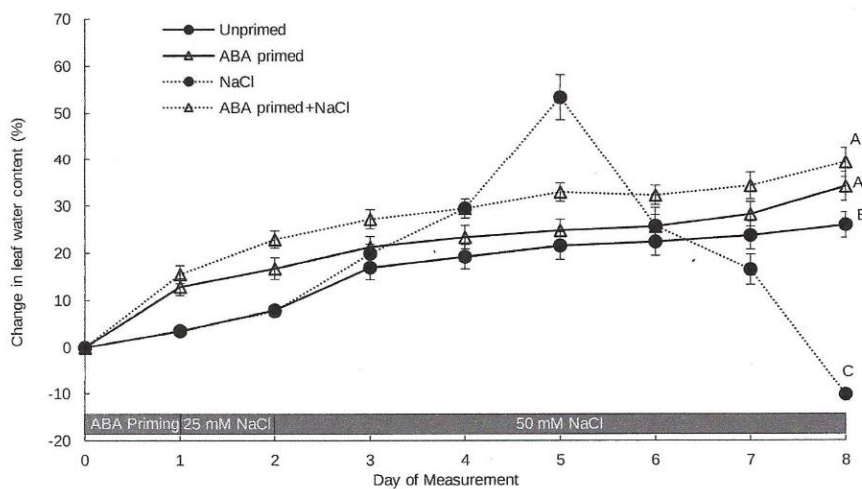


FIGURE 5 Change (%) in leaf water content relative to the initial LWC of *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data \pm SE are means of seven independent pot replicates. The different letters at the eighth day of measurement show significant differences at $P \leq 0.05$

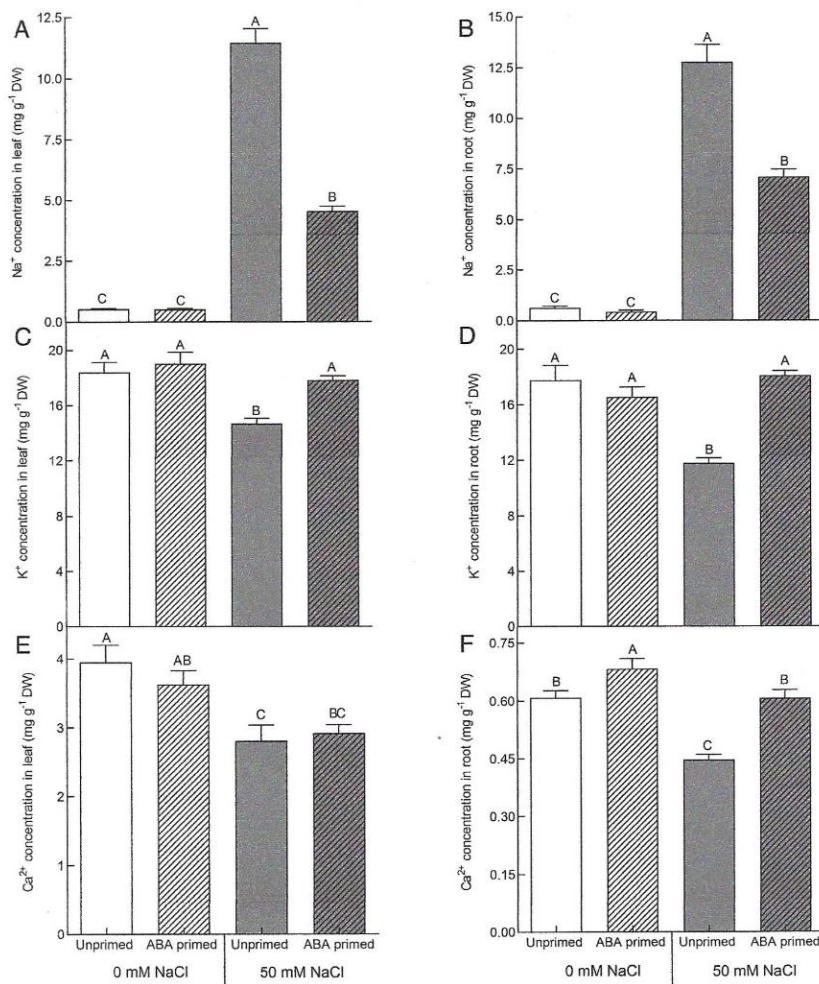
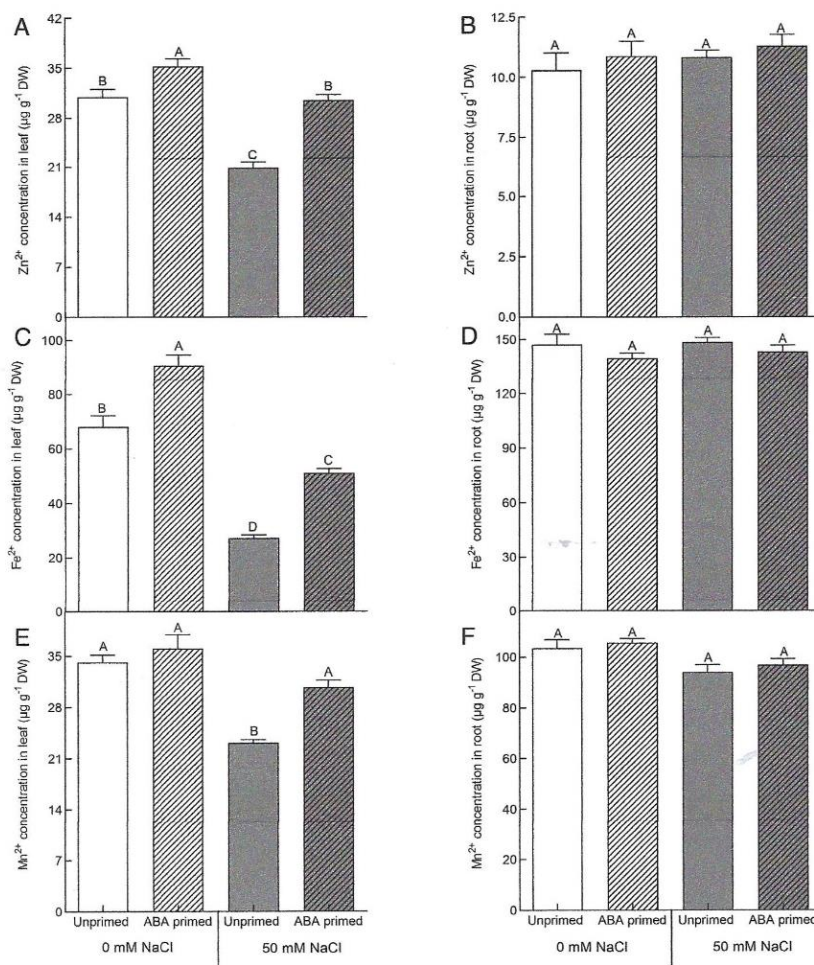


FIGURE 6 Effect of ABA priming on the concentration of Na⁺ (A, leaf; B, root), K⁺ (C, leaf; D, root), and Ca²⁺ (E, leaf; F, root) by *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data \pm SE are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

FIGURE 7 Effect of ABA priming on the concentration of Zn^{2+} (A, leaf; B, root), Fe^{2+} (C, leaf; D, root), and Mn^{2+} (E, leaf; F, root) by *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data \pm SE are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$



3.5 | Relative transcript abundance of ABA-inducible kinase and membrane transporters genes

Under saline conditions, ABA-primed leaves and roots showed significantly higher *AAPK* transcripts levels than unprimed plants (Figure 10A,B). Irrespective of ABA priming and salinity, 14-3-3 transcript remained unchanged in leaves (Figure 10C). However, in roots, ABA priming increased the mRNA expression of 14-3-3 by a factor of 3.2 under non-saline and 2.8 under saline condition compared to unprimed plants (Figure 10D).

The mRNA expression of membrane ion transporters *PM H⁺-ATPase isogenes* (*vha2*, *vha5*), *KUP7*, *VFK1*, *SOS1*, and *CLC1* was decreased under saline condition (Figures 11 and 12). In leaves under saline condition, ABA priming clearly increased transcript levels, restoring those of *vha2* and *vha5* to non-saline levels and doubling the mRNA expression of *vha4* compared to unprimed plants (Figure 11A, C,E). Similarly, in roots, ABA-primed plants showed significantly higher mRNA expression of all three isogene transcripts than unprimed plants (Figure 11B,D,F). Under salt stress, the relative mRNA

expression of *KUP7* was only affected in leaves, where unprimed showed suppression of transcript, while ABA-primed restored it to the level of non-saline treatment (Figure 12A,B). Moreover, 1.8-fold higher mRNA expression of *KUP7* in roots of ABA-primed versus unprimed plants was observed under non-saline condition only (Figure 12B). Additionally, *VFK1* mRNA abundance significantly increased by both salinity and ABA priming treatments in leaves (Figure 12C), 2.8-fold upregulation in *VFK1* was recorded in ABA-primed plants as compared to unprimed plants under non-saline condition. In roots, only ABA-primed plants showed significant upregulation compared to unprimed plants under saline condition (Figure 12D).

In leaves, *SOS1* mRNA expression was upregulated by salinity but not ABA priming (Figure 12E,F). In roots, ABA priming upregulated the mRNA expression of *SOS1* by 1.0- and 1.5-fold under saline and non-saline condition, respectively, than the unprimed counterparts. The relative mRNA expression of the *CLC1* was upregulated in ABA-primed plants in both tissues, but more prominently in the roots, 2.82- and 6.5-fold as compared to unprimed plants under non-saline and saline conditions, respectively.

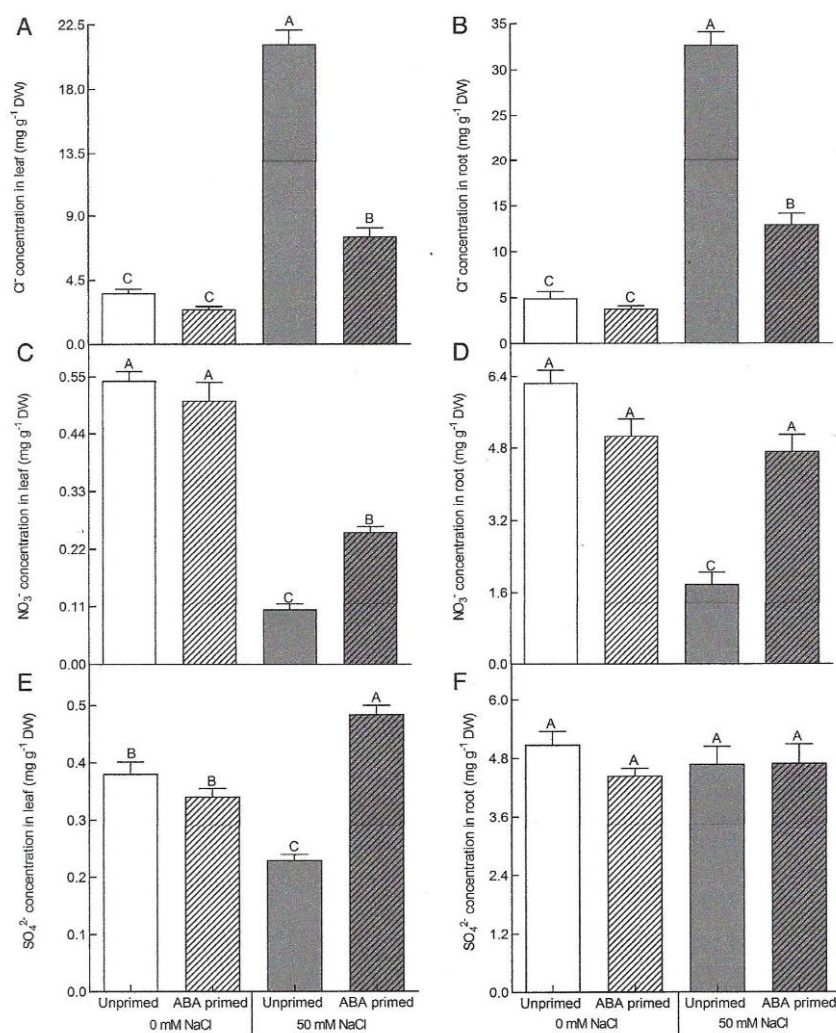


FIGURE 8 Effect of ABA priming on the concentration of Cl⁻ (A, leaf; B, root), NO₃⁻ (C, leaf; D, root), and SO₄²⁻ (E, leaf; F, root) by *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data ± SE are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

4 | DISCUSSION

Short-term ABA application substantially improved plant growth and excess salt ion exclusion for a long time (8 days). As a high turnover plant hormone, ABA is too short-lived (Ren et al., 2007; Yang & Zeevaart, 2006) to have any direct effect on plants 1 week after its application. Therefore, one can assume that the ABA-induced changes reflect the transformation into a more salt-resistant transcriptional and physiological state, a process usually referred to as “priming.”

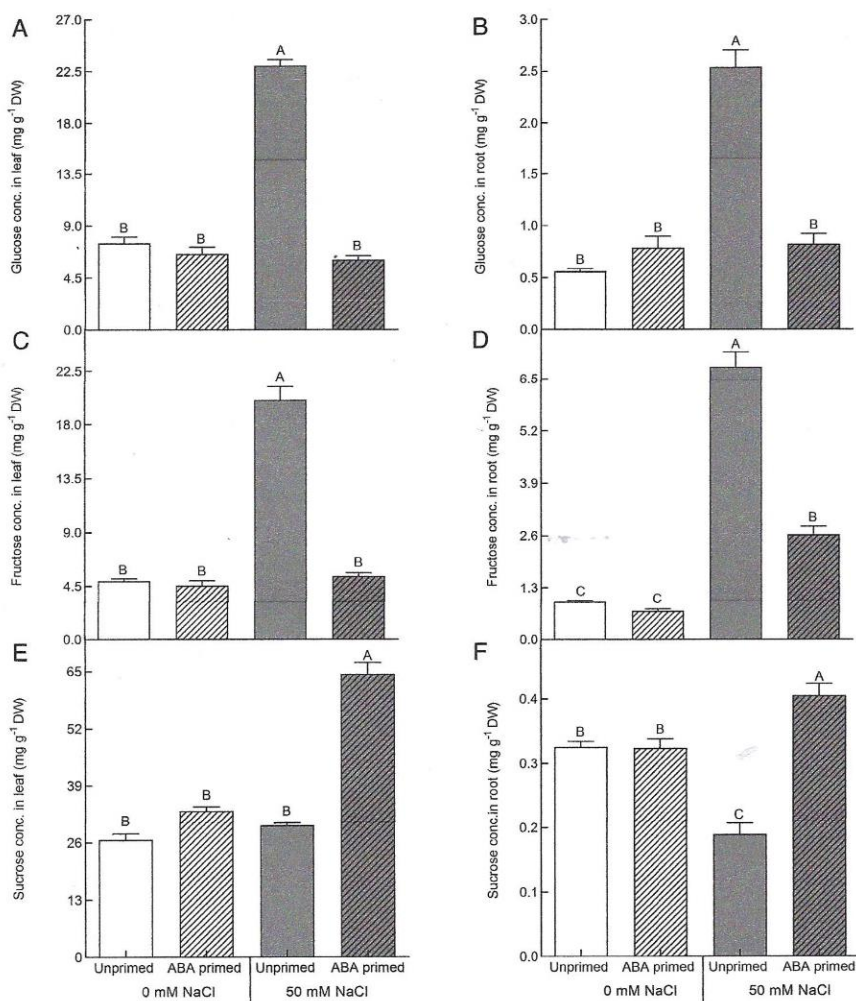
4.1 | ABA priming prevents salt-induced growth reduction

In our study, unprimed plants showed significant growth retardation under salt stress, which was mitigated by ABA priming (Figure 2). Higher dry matter accumulation by ABA-primed plants may be explained by attenuation of the inhibitory effects Na⁺ and Cl⁻ on

photosynthesis (Figure 4A) due to their substantially lower accumulation in primed as compared to unprimed plants (Figures 6A,B and 8A, B). Improved plant growth may also be linked to higher PM H⁺-ATPase expression (Figure 11), which may facilitate (1) re-translocation of salt ions (Shabala et al., 2020), (2) apoplastic acidification for acid-induced growth (Cosgrove, 2005), or (3) higher auxin transport under water stress (Xu et al., 2013).

After 8 days of growth under saline condition, leaf water content (LWC) of unprimed plants decreased by 10% as compared to its initial value, whereas ABA-primed plants maintained a steady increase in LWC (Figure 5). The decrease in LWC of unprimed plants under saline condition can be explained by salinity induced root damage (Figures 2 and S1), which impaired the uptake of water, changed the pattern of osmolyte accumulation (Figure 9) and induced stomatal closure (Figure 4B). In contrast, the roots of ABA-primed plants had a normal length (Figure S1), and thus the shoot did not show a decline in LWC under saline condition (Figure S1). Higher sucrose concentrations in ABA-primed plants (Figure 9E) might also have contributed to higher

FIGURE 9 Effect of ABA priming on the concentration (conc.) of glucose (A, leaf; B, root), fructose (C, leaf; D, root), and sucrose (E, leaf; F, root) in *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data \pm se are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$



LWC because of decreased osmotic potential. Moreover, ABA-primed plants accumulated more K^+ (Figure 6C,D) that helped in osmotic adjustments.

4.2 | ABA priming modulates ion concentrations under salt stress

As expected, plants that grew under saline conditions accumulated high quantities of Na^+ and Cl^- in roots and leaves; however, one-time ABA priming effectively reduced Na^+ and Cl^- accumulation (Figures 6A,B and 8A,B). The lower accumulation of Na^+ in ABA-primed plants might be explained by the higher expression of *SOS1* (Na^+/H^+ antiporter) gene (Figure 12E,F), whose product efficiently excludes Na^+ as has been shown for some glycophytes such as *Arabidopsis* (Shi et al., 2002), white clover (Li et al., 2017), and black locust (Chen et al., 2017). The driving force for Na^+/H^+ antiport at plasma membrane is provided by the activity of the PM H^+ -ATPases whose

transcripts were also upregulated in primed plants under salt stress (Figure 11).

At the same time, higher K^+ concentration in ABA-primed plants (Figure 6C,D) indicates that Na^+ did not repress K^+ uptake. The enhanced expression of K^+ transporter (*KUP7*) and K^+ channel (*VFK1*) after ABA pre-treatment in roots under salt stress (Figure 12A,D) may have contributed to the increased K^+ uptake (Figure 6C,D). Higher transcript abundance of three isogenes of PM H^+ -ATPase in ABA-primed plants observed in this study could also have contributed to higher K^+ accumulation, via hyperpolarization-activated opening of K^+ channel (Maathuis et al., 1997), and providing the driving force for H^+/K^+ cotransport (*KUP7*) and K^+ channel transport (*VFK1*) (Li et al., 2018; Maathuis et al., 1997). In ABA-primed plants, the enhanced accumulation of K^+ could have been one of the key mechanisms that helped avoid salt damage, as K^+ is a key osmoticum for osmotic adjustment and activator of various enzymes (Munns & Tester, 2008). Salinity significantly reduced Ca^{2+} in unprimed plants (Figure 6E,F), whereas ABA priming restored Ca^{2+} concentration in

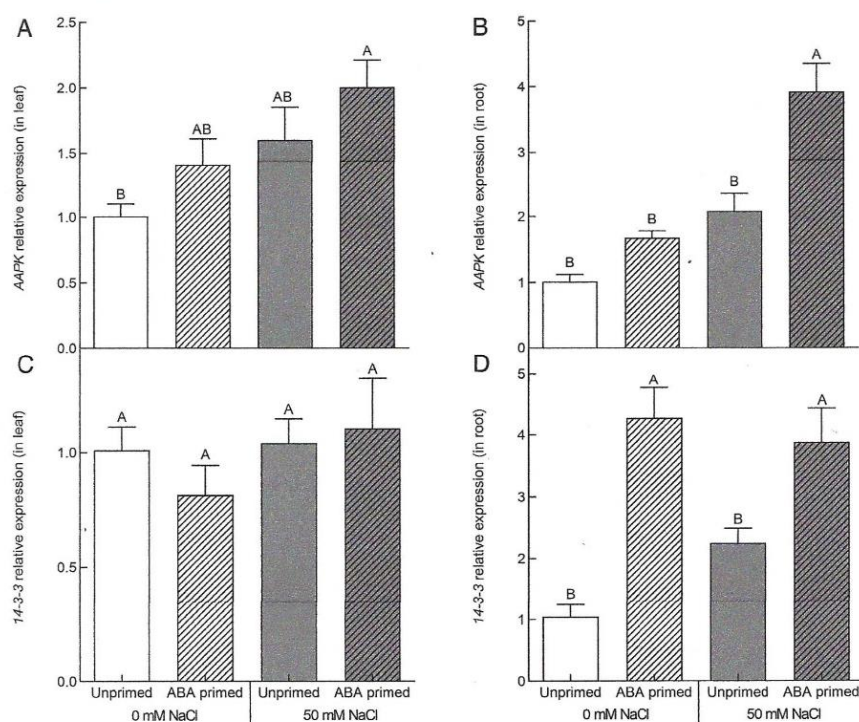


FIGURE 10 Effect of ABA priming on relative expression of ABA inducible kinase gene in *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data \pm SE are means of three independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

roots (Figure 6F). Owing to the involvement of Ca^{2+} in various cell wall functions (Manishankar et al., 2018), its deficiency can be a problem under salinity, which could be mitigated by ABA priming. Another novelty of the present research is that ABA priming was also found to have beneficial or restorative effects on the concentration of micronutrients (Zn^{2+} , Fe^{2+} , Mn^{2+}) in both root and leaves. This effect may be explained by an upregulation of PM H^+ -ATPase expression that is important for the establishment of the proton gradient across the plasma membrane and thus activating secondary transport of these micronutrients. The uptake of Fe^{2+} , Mn^{2+} , and Zn^{2+} is driven by this proton gradient (Gupta et al., 2016; Socha & Gueriot, 2014). Actually, Fe^{2+} and Zn^{2+} tissue concentration of unprimed plants under salinity fell below the threshold for a deficiency, which is about $30 \mu\text{g g}^{-1}$ for Fe^{2+} and $25 \mu\text{g g}^{-1}$ for Zn^{2+} for beans (Kosegarten et al., 1998; Rafique et al., 2015). This may have contributed to the limitation of photosynthesis (Figure 4A). Additionally, as these micronutrients (Zn^{2+} , Fe^{2+} , Mn^{2+}) serve as co-factors for various key metabolic enzymes, their lack in the unprimed salt-stressed plants may have contributed to growth retardation.

4.3 | ABA priming modulates anion contents

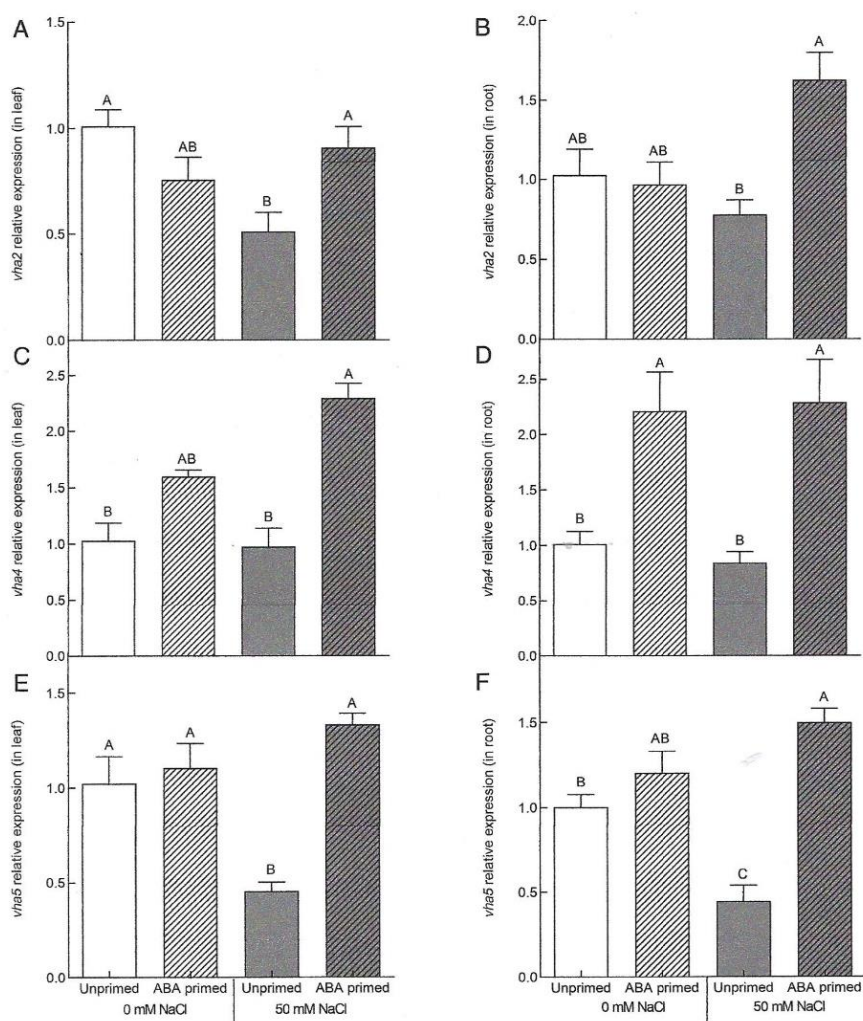
Chloride concentration under salt stress was much lower in primed than in unprimed plants (Figure 8A,B), indicating that ABA priming also induced Cl^- exclusion mechanisms as was found by Qiu et al. (2016) in *Arabidopsis*. The enhanced expression of *CLC1* in primed plants may have contributed to enhance the sequestration and compartmentation of Cl^- in the roots (Figure 12G,H). The limited root

to shoot translocation of Cl^- due to the upregulation of *CLCs* has been confirmed by Wang et al. (2015) in transgenic *Arabidopsis* plants and by Wei et al. (2016) in soybean. Conversely, with increased Cl^- contents in unprimed plants under salinity, in our study, the uptake of NO_3^- and SO_4^{2-} was strongly reduced (Figure 8C–F). This might be due to the antagonistic interaction of Cl^- with these anions. Basically, Cl^- accumulation takes place at the expense SO_4^{2-} and, especially NO_3^- (Zhang et al., 2020) due to the sharing of the same transporters (Geilfus, 2018). ABA priming significantly increased the content of these anions under saline conditions (Figure 8). Together with a decreased Cl^- accumulation, this indicates that priming reduced the antagonistic effect of Cl^- (Qiu et al., 2016). Other ABA-induced mechanisms might also have contributed to increased SO_4^{2-} content. For example, Cao et al. (2014) reported that ABA application increased the transcript level of a $\text{SO}_4^{2-}/\text{H}^+$ co-transporter (*SULTR3*) and other S-metabolism-related genes, causing a higher SO_4^{2-} concentration.

4.4 | ABA priming modulates sizes of sugar pools

Sugar accumulation has diverse roles in salinity stress response. Sugars provide osmoprotection, ROS scavenging, energy, and act as signaling molecules (Halford et al., 2011). In the present study, we have found a peculiar change in sugar accumulation patterns between unprimed and primed plants under salinity. Under salt stress, unprimed plants showed a higher accumulation of hexoses (glucose and fructose, Figure 9A,D), while ABA priming enhanced the accumulation of sucrose (Figure 9E,F). Accumulation of hexoses has been found

FIGURE 11 Effect of ABA priming on relative expression of plasma membrane H⁺-ATPase isoforms in *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data \pm SE are means of three independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$



to induce enzymatic and transcriptional feedback inhibition of photosynthesis (Martínez-Carraseo et al., 1993; Paul & Pellny, 2003; Sehar et al., 2019), possibly being one cause for the lower photosynthetic rate of unprimed plant under salt stress (Figure 3A). Fivefold higher sucrose level in ABA-primed plants was observed, compared to the unprimed plants, under (Figure 9C,D). This could be attributed to a higher K⁺/Na⁺ ratio (Figure 6A–D), which removes Na⁺ induced allosteric deactivation of various metabolic enzymes involved in sucrose synthesis (Hasanuzzaman et al., 2018) under salt stress. Sucrose is the only phloem mobile sugar; a high sucrose concentration may thus also improve the source-sink relations by providing carbon assimilates for growth and sufficient energy for ion homeostasis (Rosa et al., 2009).

4.5 | ABA priming modulates gene expression of ABA inducible kinases and membrane transporters

In the present study, we found a long-lasting effect of ABA priming on the expression of genes governing salt-resistance in plants, even

though the ABA-priming application was done 8 days ago. Salt stress in unprimed plants had little or no effect on the expression of ABA-activated protein kinase (AAPK) in root and leaf tissues; however, ABA priming increased expression levels under salinity (Figure 10A,B). AAPK activates various downstream signal components such as CIPK, CDPK, 14-3-3, and other ABA-responsive elements via phosphorylation (Figure 1). Kulik et al. (2011) found that overexpression of *SnRK2.6*, an orthologue of AAPK, in transgenic *Arabidopsis* plants induced resistance to multiple stresses via higher expression of ABA-dependent stress-responsive genes, secondary root development, and improved osmotic potential. Hence, higher expression of AAPK in ABA-primed plants in this study could have induced salinity resistance through the same mechanisms. *SnRK2.6* has also been shown to be upregulated in pepper plants by exogenous application of ABA and salt treatment (Ruggiero et al., 2019). Moreover, elicitation of 14-3-3 by AAPK, in turn, facilitates the upregulation of ABA-responsive genes in an ABA-dependent manner (Takahashi et al., 2007; Yan et al., 2004) (Figure 1). Under salinity stress, we also observed a higher level of 14-3-3 mRNA transcript in roots of ABA-primed plants compared to

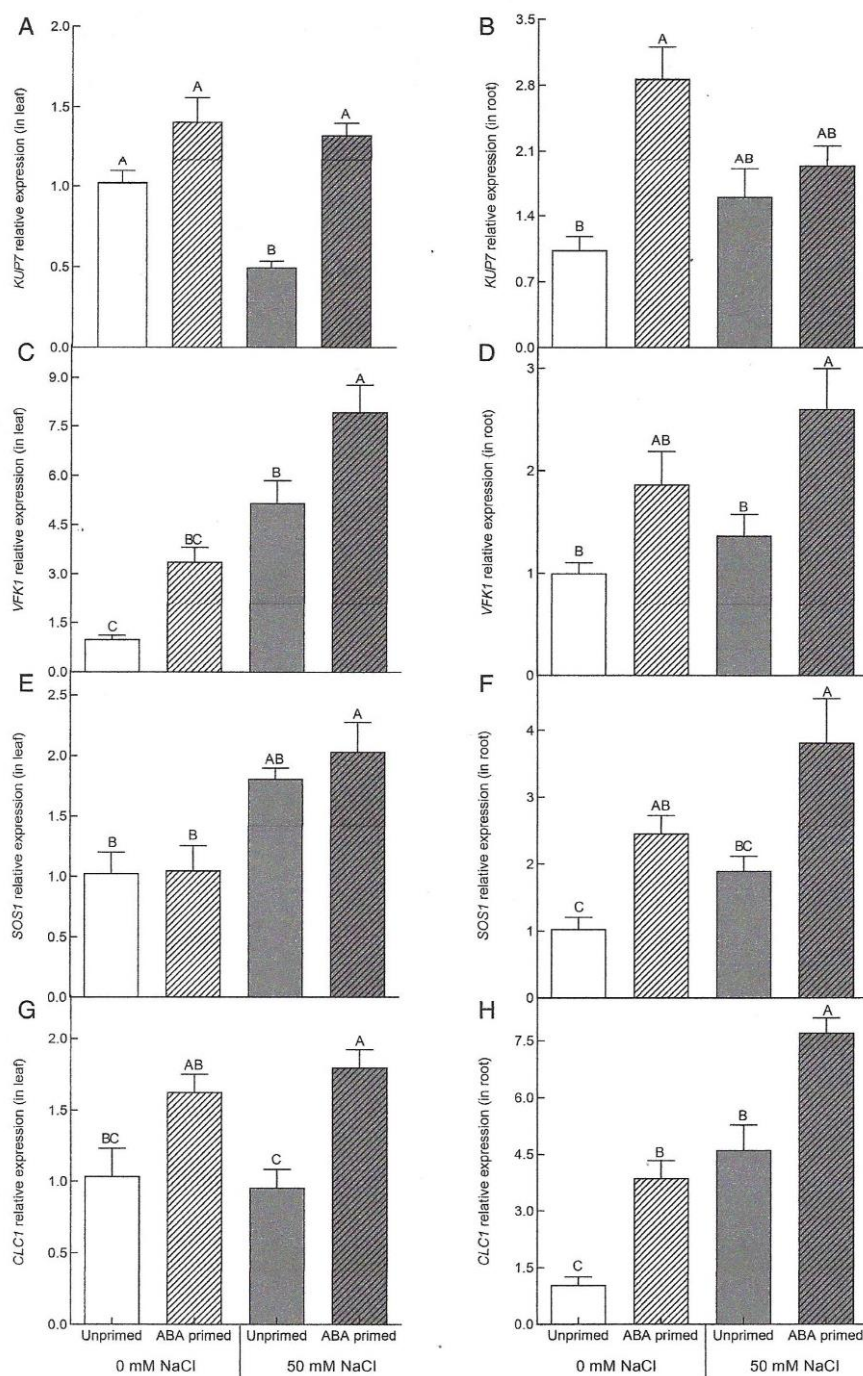


FIGURE 12 Effect of ABA priming on the relative expression of membrane ion channel and transporter in *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data \pm SE are means of three independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

unprimed plants (Figure 10C,D). A previous study demonstrated that 14-3-3 expression was upregulated by exogenous application of ABA and salt in rice (Yashvardhini et al., 2018). Overexpression of 14-3-3 in transgenic cotton showed improved drought resistance by regulating metabolic processes and PM H^+ -ATPase activity (Yan et al., 2004) (Figure 1). Our results thus suggest that ABA priming has a long-term effect on the expression of central ABA-dependent kinases.

Knowing that ABA priming reduces the effects of salinity on the ion composition, the question whether these changes in ionic relations functionally correlate with the changes in expression level of ion channels arose. Hence, we analyzed the expression patterns of different ion transporter genes in both root and leaf tissues. The expressions of three different isogenes of PM H^+ -ATPase were upregulated by ABA priming under both normal and saline growth conditions

(Figure 11). Post-translationally, PM H⁺-ATPases could have been activated by the increased levels of 14-3-3 and sucrose in ABA-primed plants (Figure 1), as both are activators of PM H⁺-ATPases (Falhof et al., 2016; Okumura et al., 2016). Similarly, expressions of KUP7 transporter and K⁺ channel VFK1 were upregulated in leaf and root tissues of ABA-primed plants under both non-saline and saline conditions (Figure 12A-D). Thus, the higher concentration of ions, particularly K⁺, as observed in ABA-primed plants under salt stress (Figure 6C,D), could be attributed to the above transporter's upregulation. KUP7 is located at the plasma membrane and is regarded as a high-affinity K⁺/H⁺ co-transporter for K⁺ uptake. ABA-dependent SnRK2.6 kinases activate it, and its K⁺/H⁺ co-transport is energized by PM H⁺-ATPase activity (Han et al., 2016; Li et al., 2018) (Figure 1). The observed upregulation by ABA priming of both AAPK and PM H⁺-ATPases could activate and drive K⁺ uptake by the more abundant KUP7. VFK1 is a voltage-gated inward rectifying plasma membrane K⁺ channel protein, an orthologue of *Arabidopsis* potassium channel 2/3 (AKT2/3), which plays a dual role in phloem cell of leaves and roots by loading K⁺ from source tissues and unloading K⁺ into sink organs (Dreyer & Uozumi, 2011; Gajdanowicz et al., 2011). ABA-induced hyperpolarization of the root plasma membrane was found to activate AKT2/3 (Roberts & Snowman, 2000). Thus, the higher expression of VFK1 in ABA-primed roots and leaves could have contributed to the increased K⁺ concentrations.

Our results show that SOS1 transporter expression was upregulated in roots of ABA-primed plants (Figure 12E,F). SOS1 is the plasma membrane Na⁺/H⁺ antiporter, located in parenchyma cells at the xylem/symplast boundary in leaf, stem, and root cells. It plays a crucial role in Na⁺ exclusion and governs its long-distance translocation (Munns & Tester, 2008; Shi et al., 2002). SOS1 has been found to be upregulated via ABA-dependent CIPKs activation (Sripinyowanich et al., 2013; Zhao et al., 2019) or by ABA-responsive transcription factors via the ABA signaling pathway (Osakabe et al., 2014). Hence, the observed concomitant higher expression of PM H⁺-ATPase and SOS1 in ABA-primed plants might have increased the K⁺/Na⁺ ratio by expelling Na⁺ from the cytoplasm.

Chloride exclusion and sequestration are important mechanisms in salt resistance. The vacuole sequestration depends on Cl⁻/H⁺ antiport by CLCs, which are voltage-gated vacuolar Cl⁻ channels. Gene expression of CLC1, an isoform of the CLCs (Diédhiou & Gollack, 2006), was upregulated by ABA priming in leaves and roots, and also by salinity (Figure 12G,H), possibly being a reason for the priming induced salt resistance. This suggestion is supported by an enhanced salt resistance after overexpression of CLC1 in transgenic *Arabidopsis* (Wang et al., 2015) and soybean plants (Wei et al., 2016).

Taken together, we conclude that ABA priming generally increases the expression of the observed genes under salinity, leading to increased activity of kinases involved in stress signaling and important ion transport proteins, ultimately causing the observed improvement of salt resistance mechanisms in plants (Figure 1).

5 | CONCLUSION

This is the first study that thoroughly documents the long-term (8 days) benefits of a one-time 24-h ABA priming application. Long-

lasting upregulation of genes for ABA inducible kinase AAPK, 14-3-3 protein, PM-ATPases, and ion transporters under saline conditions was the consequence of ABA priming. This may have helped ABA-primed plants to adapt to salt stress by limiting Na⁺ and Cl⁻ uptake and translocation and favoring K⁺, Ca²⁺, Zn²⁺, Fe²⁺, Mn²⁺, NO₃⁻, and SO₄²⁻ accumulation. The observed lower Na⁺ and Cl⁻ accumulation in leaves of ABA-primed plants is likely to have mitigated the salt-induced impairment of photosynthesis and may have improved plant growth under salinity. Furthermore, priming enhanced root growth and changed osmolyte composition by favoring sucrose and K⁺ over hexoses, which could explain the stabilized leaf water content. Together, these results clearly show, for the first time, that short-term ABA priming generates long-lasting changes in interacting cellular processes on different organizational levels, which enhance salt resistance in plants. These results are essential for the understanding of mechanisms involved in priming by previous stresses. It could also lead to improved agricultural practices by inducing readiness in crops to resist salt, drought, and other environmental stresses.

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AUTHOR CONTRIBUTIONS

Amit Sagervanishi, Karl H. Mühling, and Hartmut Kaiser conceptualized and designed the experiments. Amit Sagervanishi performed experiments, analyzed data, and wrote initial draft of manuscript. Asif Naeem, Christoph-Martin Geilfus, and Hartmut Kaiser participated in data analysis, writing, and editing the paper. All the authors have read, edited, and approved the final manuscript.

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SUPPORTING INFORMATION

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Chapter 5. General discussion

Understanding growth response of *Vicia faba* to saline-alkaline stress is important not only for a better understanding of plant resistance mechanisms but also for the screening and breeding of the crop plants for better stress adaptations.

The primary objective of this study was to identify and characterize mechanisms underlying the plant growth response under saline-alkaline conditions. Compared to earlier studies under salinity stress, the immediate and short-term effects of individual stress components viz. high pH, sodium and bicarbonate, of alkali salt on plant growth physiology were dissected. For this purpose, growth-related attributes, gas exchange, leaf water content, mineral and metabolite relation, and related transcripts of *Vicia faba* were compared between high pH and alkali salt stress (NaHCO₃) conditions. Furthermore, we also evaluated the potential of ABA priming to alleviate salt stress-related growth inhibition. To accomplish this, short term ABA-primed and unprimed plants were grown under saline stress conditions. Similar to previous experiments, growth-related attributes, gas exchange, leaf water content, mineral and metabolite relation, and related transcripts were measured. These aspects are discussed in detail in the following sections.

5.1 Apoplastic pH appears to be involved in early adaptaion of *Vicia faba* to alkali salt stress

A substantial amount of information on mid- and long-term responses of plants to high pH, osmotic and ionic stress and mainly focused on the nutrient availability, organic acids, osmolytes, or sugar content in affected plants (Guo *et al.*, 2016; Guo *et al.*, 2015; Yang *et al.*, 2007; Yang *et al.*, 2008), while the immediate response that occurs minutes to hours after the onset of alkali salt stress (NaHCO₃) is largely unknown. Secondly, the different deleterious effects of alkali salt stress on growth reduction are not well understood because the dissection of the involved stress components viz. alkaline pH and salt effect, is lacking.

Fast apoplastic pH transients to NaCl stress, showed a physiological relevance in salt stress resistance mechanisms. Nevertheless, it remains obscure if apoplastic pH transients also occur under short term alkali salt stress, and if so, which stress component of the alkali stress, i.e. the osmotic-, the ionic- or the soluble alkali metal-component, elicit(s) early apoplastic pH responses. Moreover, specific resistance mechanisms of the early alkaline stress response, which may be elicited by pH transients need to be identified.

Hence, the present study aimed to experimentally identify that either alkaline pH alone or alkali salt stress elicits early (150 min) apoplastic pH responses. Thus, hydroponically grown *Vicia faba* L. plants were exposed to 150 min of either alkaline pH alone or 50 mM NaHCO₃ stress treatment. Fluorescence microscopy-based ratiometric in-planta real-time monitoring of apoplastic pH were performed

simultaneously with NIR transmission based measurements of leaf water content (LWC). Moreover, we also examined the effect of these stresses on apoplastic, symplastic and xylem ion and metabolite composition, and quantified the mRNA abundance of certain key stress-responsive genes such as *NCED*, *PIP*, *PM H⁺-ATPase*, *PEPCase*. Results revealed that NaHCO_3 and not the alone alkaline pH treatment induced several physiological and transcription changes. Just after the addition of NaHCO_3 to rooting medium, fast transient decrease in LWC (Chapter 1, Fig. 2 left Y-axis), followed by a transient alkalization of leaf apoplastic pH (Chapter 1, Fig. 2 right Y-axis) and stomatal closure (Chapter 1, Fig. 1) was observed. The quick transient decrease in LWC is the response to be expected from root exposure to a solution of higher osmotic potential causing a hydraulically transmitted reduction in leaf turgor and water content (Munns and Tester, 2008). The subsequent recovery of LWC can be attributed to stomatal closure (Chapter 1, Fig. 1) and the increased biosynthesis of organic anions (Chapter 1, Fig. 6) and sugars (Chapter 1, Fig. 7), which reduced the osmotic potential (Chapter 1, Fig. 3) and increased LWC. The osmometer results confirmed that only the NaHCO_3 treatment induced a higher osmolality (Chapter 1, Fig. 3). The delayed stomatal closure (45-50 minutes delay) (Fig. 1) after salt addition might also have supported the gradual recovery of LWC (Fig. 2C, left Y-axis). The transient alkalization of leaf apoplastic pH (Fig. 2C, right Y-axis) might be related to a higher accumulation of malate and citrate in the apoplast (Chapter 1, Fig. 6). In this context, Geilfus (2017) suggested that ABA indirectly alkalizes the apoplast by inducing the efflux of organic acids anions such as malate from from symplast into the apoplast, which scavenge protons. Furthermore, proton based co-transport for maintaining osmoregulation in leaf might be another factor for transient change in apoplastic pH (Cushman, 2001).

Knowing that one-time root application of stress treatment, especially NaHCO_3 treatment, induces a significant change in leaf water content and apoplastic pH, the question arose whether this change in LWC and apoplastic pH are correlated with changes in ionic and metabolite relations in leaf apoplast, symplast and xylem. We observed that plants treated with NaHCO_3 showed a higher concentration of sodium $[\text{Na}^+]$ in all three compartments (Chapter 1, Fig. 4A, B, C). This effect is certainly caused by the presence of sodium salt (NaHCO_3 , 50 mM) at the growth medium (Kader and Lindberg, 2005; Munns and Tester, 2008). The increase of $[\text{Na}^+]$, which was found in this study, nicely corresponds with the decreased in $[\text{K}^+]$ (Chapter 1, Fig. 4E, F, G). Potassium concentration was higher in apoplastic (Fig. 4E) and simultaneously lower in symplastic washing fluid (Chapter 1, Fig. 4F). This suggests a sodium-potassium competitive effect for ion uptake at plasma membrane level (Munns and Tester, 2008). Anions (NO_3^- , SO_4^{2-}) concentrations were significantly influenced under NaHCO_3 . Since HCO_3^- is transported radially into the root stele (Wegner and Zimmermann, 2004), its presence in high concentration could have imparted competitive effect on the uptake of other anions (NO_3^- , SO_4^{2-}) (Chapter 1, Fig. 5), and thus hindering their overall uptake.

A peculiar quick increase in organic anion concentration (malate, citrate) (Chapter 1, Fig. 6) in all three compartments (apoplast, symplast, xylem) might be associated with bicarbonate induced cytoplasmic alkalization (Landsberg, 1986; Landsberg, 1981), which activates PEPCase, resulting in increased organic acid concentration and thus maintaining cytoplasmic pH equilibrium (Davies, 1973). Since, a higher mRNA abundance of *PEPCase* was also observed (Chapter 1, Fig. 9C), increased organic acid accumulation (Chapter 1, Fig. 6) is nicely explained by the increased expression of *PEPCase* (Chapter 1, Fig. 9C). Increased organic acids concentration (Chapter 1, Fig. 6) might be related to higher accumulation of soluble sugars (glucose, fructose, sucrose) (Chapter 1, Fig. 7) which are the precursors of organic acids (Bennet-Clark, 1937). Moreover, increased concentration of organic acid anions and soluble sugars in leaf apoplast might have also relevance to stress signaling. Fast upregulation of stress responsive gene such as *NCED*, *PIP*, *PM H⁺-ATPase*, *PEPCase* (Chapter 1, Fig. 8, 9) are collaboratively involved in early recovery and osmoregulation of plant for adaptation to alkali salt stress.

5.2 High bicarbonate, neither high pH nor sodium, inhibits growth of *Vicia faba* under alkali salt stress

Plant growth is reduced by salt stress in growth mediums due to osmotic stress and ion injury (Munns and Tester, 2008); alkali stress exerts the same stress factors with the additional effect of high pH. Alkali stress is dominated by the presence of alkali salts (Na_2CO_3 or NaHCO_3) in the soil (Yang *et al.*, 2007), and is usually categorized by low availability of plant nutrients, high concentrations of HCO_3^- and CO_3^{2-} , and high pH (Kawanabe and Zhu, 1991; Marschner, 2011).

Numerous studies have shown that alkali salt stress causes severe growth reduction in glycophytes. However, all these studies solely used alkali salt and did not separate the individual effects of each component alkali salt stress including high pH, sodium and bicarbonate. Hence, we experimentally separated these stresses for the first time and assessed their effect on growth and various physiological, metabolic, and transcriptional measures.

This was done by observing the effects of four treatments viz. control (pH = 6.0), high pH (9.0), high pH (9.0) plus sodium sulfate (25 mM) and sodium bicarbonate (50 mM, pH 9.0) on growth, gas exchange, leaf water content, ion relations, organic acid accumulation, oxidative damage, and changes in the expression of key genes in *Vicia faba* over 4 days of cultivation under stress condition. The growth of *Vicia faba* was impaired more by NaHCO_3 stress, than alone high pH, and high pH+Na salt. The plants subjected to NaHCO_3 stress showed the severest root injury (Chapter 2, Fig. 1, and Fig. S1) and a substantially higher reduction in the root and leaf dry matters (Chapter 2, Fig. 2) than that of high pH and high pH+Na treated plants. This substantial growth inhibition under NaHCO_3 treatment was related to a decline in photosynthetic rate (Chapter 2, Fig. 3A) (Wu and Xing, 2012) and a profound accumulation of H_2O_2 (Chapter 2, Fig. 11A) (Zhang *et al.*, 2017) and organic acids

(Hajiboland *et al.*, 2003). Moreover, root growth inhibition in NaHCO_3 treatment coincided with the lowest *PM H⁺-ATPase* expression (Chapter 2, Fig. 12B, D), which was incapable of apoplastic acidification required for acid-induced growth (Cosgrove, 2005). Following four days of growth under alkaline conditions, all three treatments showed an increase in leaf water content (LWC) compare to control, but the magnitude of increase was the highest (2.5-fold) in NaHCO_3 treated plants. This might be associated with the lowest stomatal conductance in this treatment which reduced water loss (Chapter 2, Fig. 3B), and the highest accumulation of organic acids (Chapter 2, Fig. 8) and soluble sugars (Chapter 2, Fig. 9) which reduced the osmotic potential (Chapter 2, suppl. Fig. 3) (Meyer *et al.*, 2010; Talbott and Zeiger, 1993). As expected, plants grown under high pH+Na salt and NaHCO_3 treatment showed a higher accumulation of Na^+ , but a higher accumulation of Na^+ in roots was observed under NaHCO_3 treatment than high pH+Na treatment (Chapter 2, Fig. 5B). A concomitant lower mRNA expression of *SOS1* (*Na⁺/H⁺ antiporter*) and *PM H⁺-ATPase* in NaHCO_3 treatment implies that plants might have failed to excluded Na^+ efficiently (Sagervanshi *et al.*, 2020). This resulted in a stronger competitive effect of Na^+ on K^+ uptake, which was reflected as a strong reduction in K^+ concentration in roots (Chapter 2, Fig. 5D) - reaching the critical deficiency level of 10 mg g^{-1} (Plank, 1989), or lower boundary of the range required for optimum growth of plants ($20\text{-}50 \text{ mg g}^{-1}$ DM of the vegetative tissues; Marschner, 2011). Additionally, Ca^{2+} concentration in leaves was decreased under all the treatments, with a relatively higher decrease under NaHCO_3 treatment (3.8 mg g^{-1}) and reached lower than critical deficiency levels of Ca^{2+} for optimum growth ($5.0\text{-}30.0 \text{ mg Ca}^{2+} \text{ g}^{-1}$ DM; (Mengel and Kirkby, 1987) (Chapter 2, Fig. 5A). As Ca^{2+} translocation is driven by the xylem stream, the decrease in Ca^{2+} concentration could be related to lower stomatal conductance (Chapter 2, Fig. 3B) through reduced transpiration rate (Tanner and Beevers, 2001).

Micronutrients (Mn^{2+} , Zn^{2+}) and anions (NO_3^- , SO_4^{2-} , PO_4^{3-}) uptake was solely hinder under NaHCO_3 treatment, and might be related with Ca^{2+} , Fe^{2+} concentration in roots, which 14-fold increased under NaHCO_3 treatment (Chapter 2, Fig. 6F). Chelation by organic acids and deposition of those chelates in roots also appears to be a possible mechanism for reduced the uptake of Mn^{2+} and Zn^{2+} and also for lower root exudation (Chapter 2, Fig 10). Other related reasons could be damaged root system (Chapter 2, Fig. S1, S2,), and lower *PM H⁺-ATPase* activity, indicated by higher mRNA abundance of isogenes (Chapter 2, Fig. 12B, D), as cation uptake (H^+ Co-transport) is also facilitated via a proton gradient (Marschner, 2011).

A peculiar increment in organic acids and soluble sugars concentration was observed under all high pH treatments (Chapter 2, Fig. 8, and Fig. 9). The accumulation of organic acids (malate and citrate) under NaHCO_3 treatment might be associated with higher *PEPCase* expression and lower root exudation. On the other hand, plants under high pH and high pH+Na treatment, thanks to an intact root system and higher *PM H⁺-ATPase* activity, showed a higher exudation of organic acids (Chapter 2, Fig. 10A, B), and thus withstood the adverse high pH condition by avoiding excess accumulation of these acids in

root and leaf tissues. While, accumulation of soluble sugars in leaves in response to a decreased sink demand has been related to the down-regulation of photosynthesis (Nebauer *et al.*, 2011), as has been found in our study.

Plant grown under alkali salt stress (NaHCO_3) showed nutrient disorder caused by organic acid-metal (Fe^{2+} , Ca^{2+}) complexation leading to ROS accumulation and oxidative damage to roots (Chapter 2, Fig. 11). These plants showed decreased mRNA abundances of ion transporter (PM H^+ -ATPase isogenes, *SOS1*) and carbon metabolism (*MDH*, *CS*) gene (Chapter 2, Fig. 12, Fig. 13) resulting in growth reduction. On the other hand, high pH and high pH+Na treated plants were able to maintain a sufficient level of ROS and *PM H⁺-ATPase*, *SOS1*, *CS*, and *MDH* expression in roots which enhanced their resistance to bicarbonate alkaline stress. This chain of events demonstrated by our results gives an explanation why high pH+bicarbonate stress is more detrimental to plant growth than high pH alone or in combination with sulfate.

5.3 One-time ABA priming induces long term salinity resistance in *Vicia faba*

ABA is considered a stress hormone, and its rapid accumulation in plants under various abiotic stresses shows its involvement in the stress resistance of plants (Geilfus *et al.*, 2018; Zörb *et al.*, 2013). Several studies have shown that pretreatment (priming) of ABA improves drought resistance in rice (Teng *et al.*, 2014), salt resistance in potato (Etehadnia *et al.*, 2008), and common bean (Khadri *et al.*, 2007). However, the mechanisms mediating this long-term acclimatization to salt stress were still obscure. Specifically, the long-term transcriptional changes and their effects on ion relations were never investigated. Thus, we hypothesized that a one-time 24 h ABA priming induces long-term salt resistance in *Vicia faba* via: (1) long-term effects on transcription of genes involved in salt stress signaling and ion regulation, (2) stabilizing ionic and osmotic relations, and (3) enhancing photosynthesis and plant growth.

This was done by observing the effects of four treatments viz. unprimed (only nutrient solution), ABA primed (nutrient solution + 10 μM ABA), unprimed (nutrient solution + 50 mM NaCl), ABA primed (nutrient solution + 10 μM ABA+ 50 mM NaCl) over 8 days of cultivation under stress condition. For ABA priming, plants growing in nutrient solutions were supplied with 10 μM of ABA for 24 h, while the unprimed plants did not receive ABA pretreatment. It was observed that short-term (24 h) ABA application substantially improved plant growth and excluded the uptake of salt ions for a long time (8 days).

The unprimed plants showed significant growth retardation under salt stress, which was mitigated by ABA priming (Chapter 3, Fig. 2). Improved plant growth may also be linked to higher PM H^+ -ATPase expression (Chapter 3, Fig. 11), which might have facilitated (1) re-translocation of salt ions (Shabala *et al.*, 2020), (2) apoplastic acidification for acid-induced growth (Cosgrove, 2005), or (3) higher auxin transport under water stress (Xu *et al.*, 2013). After 8 days of growth under saline condition, leaf water

content (LWC) of unprimed plants decreased by 10% (Chapter 3, Fig. 5) as compared to its initial value, whereas ABA-primed plants maintained a steady increase in LWC (Chapter 3, Fig. 5). The decrease in LWC of unprimed plants under saline condition can be explained by salinity induced root damage (Chapter 3, Figs 2 and S1), which impaired the uptake of water, changed the pattern of osmolyte accumulation (Chapter 3, Fig. 9) and induced stomatal closure (Chapter 3, Fig. 4B).

Additionally, ABA priming modulated ion and metabolite concentration under salt stress. Under saline conditions, one-time ABA priming effectively reduced Na^+ and Cl^- accumulation in plants, while unprimed plants accumulated high quantities of Na^+ and Cl^- (Chapter 3, Figs 6A, B and 8A, B). The lower accumulation of Na^+ in ABA-primed plants might be explained by the higher expression of *SOS1* (Na^+/H^+ antiporter) gene (Fig. 12E, F), whose product efficiently excludes Na^+ as has been shown for some glycophytes. The enhanced expression of *CLC1* in primed plants may have contributed to limit uptake and translocation of Cl^- . Together with a decreased Na^+ and Cl^- accumulation, this indicates that priming reduced the antagonistic effect of these ions, resulting in efficient accumulation of K^+ , Ca^{2+} , Zn^{2+} , Fe^{2+} , Mn^{2+} , NO_3^- , and SO_4^{2-} in plants (Chapter 3, Fig. 6 and Fig. 8). Furthermore, we observed an atypical change in sugar accumulation patterns between unprimed and primed plants under salinity. Under salt stress, unprimed plants showed a higher accumulation of hexoses (glucose and fructose, Chapter 3, Fig. 9A, D), while ABA priming enhanced the accumulation of sucrose (Chapter 3, Fig. 9E, F). Accumulation of hexoses has been found to induce enzymatic and transcriptional feedback inhibition of photosynthesis (Martínez-Carraseo *et al.*, 1993; Paul and Pellny, 2003; Sehar *et al.*, 2019), possibly being one cause for the lower photosynthetic rate of unprimed plant under salt stress (Chapter 3, Fig. 3A). Whereas, high sucrose concentration might thus also improved the source-sink relations by providing carbon assimilates for growth and sufficient energy for ion homeostasis (Rosa *et al.*, 2009).

Knowing that ABA priming significantly improved and restored plant growth, water content, ion and metabolite relation, we further correlated these changes with transcript levels of certain salt stress signalling (*AAPK*, *14-3-3*) and ion transporter (*PM H⁺-ATPase*, *SOS1*, *VFK1*, *KUP7*, *CLC1*) gene (Chapter 3, Fig. 10, Fig. 11). It was found that ABA priming upregulated ABA inducible kinase *AAPK*, *14-3-3* protein, *PM H⁺-ATPases*, and ion transporters under saline conditions on a long-term basis, while unprimed plant failed to maintain efficient transcript level of these genes, resulting in impaired plant growth. Taken together, we conclude that one-time ABA priming mitigates salt stress in *Vicia faba* by persistently changing transcription patterns of key genes, stabilizing the ionic and osmotic balance, and improving photosynthesis and growth.

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Summary

Soil salinization and alkalization frequently co-occur, causing severe problems in crop production by reducing vegetative growth and yield. The plant responses to alkali stress are not fully understood because of the fact that the effects of individual components of alkali salt stress (high pH, sodium and bicarbonate) on plants growth were never dissected. Hence, for the first time, we experimentally separated the individual components of alkali salt stress and investigated their effect on the growth and various physiological, metabolic, and transcriptional responses of *Vicia faba*. Moreover, the mechanisms underlying survival and long-term growth improvement in short-term ABA-primed plants under saline conditions were identified.

The first study figured out if the early (150 min) apoplastic pH response in *Vicia faba* is related to alkaline pH, alkali salt stress or both of these stress components of alkali salt. The apoplastic ionic milieu contains essential determinants for early stress signal perception and transmission, cell expansion and hence has a pivotal role in plant growth under stress condition. Hydroponically grown *Vicia faba* L. plants were exposed to either high pH alone or 50 mM NaHCO₃ stress treatment (also at high pH) for 150 min. It was observed that only the NaHCO₃ and not the alkaline pH induced several physiological and transcriptional changes. In NaHCO₃ treated plants, simultaneous NIR transmission-based measurement of leaf water content (LWC) and fluorescence microscopy-based ratiometric *in planta* real-time monitoring of apoplastic pH showed transient reduction in LWC and alkalization of the leaf apoplast, . These changes finally led to stomatal closure in NaHCO₃ plants. These events also coincided with an accumulation of organic acids and sugars in the apoplast, symplast and xylem fluids that helped in osmoregulation. Additionally, upregulation of key stress-responsive genes such as *NCED*, *PIP*, PM H⁺-ATPase, *PEPCase* showed the significance of gene regulation for early plant adaptation to alkali salt stress.

In the second experiment, the hypothesis that not the high pH or Na⁺, rather excess bicarbonate, by inducing over-accumulation of organic acids (through dark fixation) in plant roots, is the main growth-limiting factor under short-term alkali salt stress, was investigated. Thus, the effect of four treatments viz. control (pH 6), high pH (9), high pH (9) plus sodium sulfate (25 mM) and sodium bicarbonate (50 mM, pH 9) on growth, gas exchange and leaf water content, organic acid accumulation, ion relations, oxidative damage and changes in the expression of key genes was investigated in *Vicia faba*. The bicarbonate treatment showed the highest growth reduction, *PEPCase* gene expression and accumulation of citrate and malate in roots. Bicarbonate treated plants preferentially accumulated Fe²⁺ and Ca²⁺ in roots and showed reduced root exudation of citrate and malate. Moreover, these plants experienced K⁺ and Ca²⁺ deficiencies, which caused a massive increase in H₂O₂ concentration in leaves and roots, leading to severe oxidative damage. Bicarbonate treated plants showed reduced levels of *PM*

H⁺-ATPase, *SOS1*, *CS*, and *MDH* gene expression in roots. The cascade of events confirms the hypothesized central role of organic acid accumulation in bicarbonate induced stress, making bicarbonate more detrimental to plant growth than high pH or its combination with sodium salt.

In the third experiment, the long-term (8d) effect of one-time 24h root priming treatment with 10 μ M in abscisic acid (ABA) on growth response and survival, the transcriptional changes, and their effects on ion relations in *Vicia faba* grown under 50 mM NaCl salinity were investigated. ABA priming prevented the salt-induced decline in root and shoot dry matter, photosynthesis and terminal wilting observed in the unprimed plants. It substantially increased the mRNA level of *AAPK* and *I4-3-3* ABA inducible kinases and ion transporters (*PM H⁺ ATPase*, *VFK1*, *KUP7*, *SOS1* and *CLCI*). These ABA-induced transcriptional changes went along with altered tissue ion patterns: Primed plants accumulated lesser Na⁺ and Cl⁻ but more K⁺, Ca²⁺, Zn²⁺, Fe²⁺, Mn²⁺, NO₃⁻ and SO₄²⁻. Priming changed the composition of organic osmolytes under salinity, with glucose and fructose being dominant in unprimed in contrast to sucrose in the primed plants. It was concluded that one-time ABA priming mitigates salt stress in *Vicia faba* by persistently changing transcription patterns, stabilizing the ionic and osmotic balance, and improving photosynthesis and growth.

Overall, this study provides a key insight into the earlier and short-term plant responses to alkali stress components, and the mechanisms mediating growth, sustenance and survival in ABA-primed *Vicia faba* plants under salinity stress.

Zusammenfassung

Bodenversalzung und Alkalisierung treten häufig gemeinsam auf und verursachen durch Reduzierung des Wachstums und Ertrages schwerwiegende Probleme im Pflanzenbau. Die Reaktionen der Pflanzen auf Alkali-Salzstress sind noch nicht vollständig geklärt, da die Auswirkungen der einzelnen Komponenten (hoher pH-Wert, Natrium und Hydrogenkarbonat) auf das Pflanzenwachstum bisher nicht ausreichend untersucht wurden. Daher wurden in dieser Studie zum ersten Mal die einzelnen Komponenten von Alkali-Salzstress experimentell getrennt und ihre Auswirkungen auf das Wachstum, verschiedene physiologische und metabolische Reaktionen und die Genexpression von *Vicia faba* L. untersucht. Darüber hinaus wurden Mechanismen identifiziert, die dem Überleben und der langfristigen Wachstumsverbesserung unter Salzstress nach kurzzeitigem ABA-Priming zugrunde liegen.

In der ersten Studie wurde untersucht, ob die frühe (150 Minuten) apoplastische pH-Reaktion von *Vicia faba* mit dem alkalischen pH-Wert der Nährlösung, dem Alkalisalzstress oder beiden Stresskomponenten des Alkali-Salzstress zusammenhängt. Das apoplastische Ionenmilieu enthält wesentliche Determinanten für die frühe Wahrnehmung und Weiterleitung von Stresssignalen und die Zellexpansion und spielt daher eine zentrale Rolle für das Pflanzenwachstum unter Stressbedingungen. In Nährlösung gewachsene *Vicia faba*-Pflanzen wurden 150 Minuten lang entweder nur einem hohen pH-Wert oder einer 50 mM NaHCO₃-Stressbehandlung (bei ebenfalls hohem pH Wert) ausgesetzt. Es wurde beobachtet, dass nur NaHCO₃ und nicht der alkalische pH-Wert alleine verschiedene physiologische Veränderungen und Änderungen der Genexpression auslöste. Bei den mit NaHCO₃ behandelten Pflanzen zeigte die gleichzeitige Messung des Blatt-Wassergehalts (LWC) mittels NIR-Transmission und die auf Fluoreszenzmikroskopie basierende ratiometrische Messung des apoplastischen pH-Werts eine vorübergehende Verringerung des LWC und eine Alkalisierung des apoplastischen pH-Werts im Blatt. Diese Veränderungen führten schließlich zum Schließen der Stomata in NaHCO₃-Pflanzen. Diese Ereignisse fielen auch mit einer Akkumulation von organischen Säuren und Zuckern im Apoplasten, Symplasten und in der Xylemflüssigkeit zusammen, die zur Osmoregulation beitragen. Darüber hinaus zeigte die Hochregulierung wichtiger stressempfindlicher Gene wie NCED, PIP, PM H⁺-ATPase und PEPCase die Bedeutung der Genregulation für die frühe Anpassung der Pflanzen an Alkalisalzstress.

Im zweiten Versuch wurde die Hypothese untersucht, dass nicht der hohe pH-Wert oder Na⁺, sondern die hohe Hydrogenkarbonat Konzentration, durch eine Akkumulation organischer Säuren (durch Dunkelfixierung) in den Pflanzenwurzeln, der wichtigste wachstumsbegrenzende Faktor bei kurzfristigem Alkalisalzstress ist. Daher wurde die Wirkung von vier Behandlungen, nämlich Kontrolle (pH 6), hoher pH-Wert (9), hoher pH-Wert (9) plus Natriumsulfat (25 mM) und Natriumhydrogenkarbonat (50 mM, pH 9) auf Wachstum, Gasaustausch und Wassergehalt der Blätter,

Akkumulation organischer Säuren, Ionenkonzentrationen, oxidative Schäden und Änderungen der Expression von Schlüsselgenen bei *Vicia faba* untersucht. Die Hydrogenkarbonat-Behandlung zeigte die stärkste Reduktion des Wachstums, der PEPCase-Genexpression und der Akkumulation von Citrat und Malat in den Wurzeln. Mit Hydrogenkarbonat behandelte Pflanzen akkumulierten insbesondere Fe^{2+} und Ca^{2+} in den Wurzeln und zeigten eine verringerte Wurzelabscheidung von Citrat und Malat. Darüber hinaus kam es bei diesen Pflanzen zu einem K^+ - und Ca^{2+} -Mangel, der einen massiven Anstieg der H_2O_2 -Konzentration in Blättern und Wurzeln verursachte, was zu schweren oxidativen Schäden führte. Mit Hydrogenkarbonat behandelte Pflanzen zeigten eine verringerte Expression von PM H^+ -ATPase, SOS1, CS und MDH-Genen in den Wurzeln. Diese Kaskade von Ereignissen bestätigt die angenommene zentrale Rolle der Akkumulation organischer Säuren bei Hydrogenkarbonat-induziertem Stress, wodurch Hydrogenkarbonat für das Pflanzenwachstum schädlicher ist als ein hoher pH-Wert oder seine Kombination mit Natriumsalz.

Im dritten Experiment wurde die Langzeitwirkung (8 Tage) einer einmaligen 24-stündigen Wurzelpriming-Behandlung mit 10 μM Abscisinsäure (ABA) auf die Wachstumsreaktion und das Überleben, Transkriptionsänderungen und ihre Auswirkungen auf die Ionenbeziehungen in *Vicia faba* Pflanzen untersucht, die unter 50 mM NaCl-Salzgehalt gewachsen waren. Das ABA-Priming verhinderte den durch Salzstress bedingten Rückgang der Trockenmasse von Wurzeln und Sprossen, der Photosynthese und des Welkens im Endstadium, der bei den nicht geprimten Pflanzen beobachtet wurde. Es erhöhte den mRNA-Gehalt von AAPK und 14-3-3 ABA-induzierbaren Kinasen und Ionen transportern (PM H^+ ATPase, VFK1, KUP7, SOS1 und CLC1) erheblich. Diese ABA-induzierten Transkriptionsänderungen gingen mit veränderten Ionengehalten in den pflanzlichen Geweben einher: Geprimte Pflanzen akkumulierten weniger Na^+ und Cl^- , aber mehr K^+ , Ca^{2+} , Zn^{2+} , Fe^{2+} , Mn^{2+} , NO_3^- und SO_4^{2-} . Durch das Priming änderte sich die Zusammensetzung der organischen Osmolyte bei Salzstress, wobei Glucose und Fructose in den nicht geprimten Pflanzen dominierten, während Saccharose in den geprimten Pflanzen vorherrschten. Daraus wurde gefolgert, dass ein einmaliges ABA-Priming den Salzstress in *Vicia faba* abschwächt, indem es die Transkriptionsmuster nachhaltig verändert, das ionische und osmotische Gleichgewicht stabilisiert und die Photosynthese und das Wachstum verbessert.

Insgesamt bietet diese Studie einen wichtigen Einblick in die frühen und kurzfristigen Reaktionen auf Alkalistress-Komponenten sowie in die Mechanismen, die das Wachstum und Überleben in ABA-geprimten *Vicia faba*-Pflanzen unter Salzstress ermöglichen.

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