**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Electrical signaling, gas exchange and turgor pressure in ABA-deficient
tomato (cv. Micro-Tom) under drought**

Francynês da Conceição Oliveira Macedo

Thesis presented to obtain the degree of Doctor in
Science. Area: Plant Physiology and Biochemistry

**Piracicaba
2015**

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DEDICATION

To my mom, Inez Maria de Oliveira,
for being my greatest example of life.

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“The application of statistics to experimental results in plant physiology produce a mean, which is fallaciously assumed to reflect the behavior of all individuals in the population – it is similarly fallaciously to assume that variation from the mean are due to experimental error. If we seek evidence for adaptive and intelligent behavior in plants, we need to pay attention to the complex behaviours of individuals, as did J. C. Bose.”

Anthony Trewavas.

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RESUMO

Sinalização elétrica, trocas gasosas e pressão de turgor em plantas deficientes na produção de ABA (cv. Micro-Tom) sob seca

O presente documento refere-se a pesquisa cujo principal objetivo foi investigar a relação entre sinais hidráulicos, químicos e elétricos em plantas de tomate deficientes da produção de ABA. O documento foi organizado em três capítulos: O primeiro capítulo apresenta um detalhado protocolo de medição extracelular de sinais elétricos em plantas e como associar estas medições a determinação de trocas gasosas usando o Analisador de gás por infravermelho (IRGA) e pressão de turgor usando a sonda de pressão (ZIM-probe). O segundo capítulo refere-se ao registro de potenciais de ação gerados espontaneamente e evocados por estímulo elétrico em tomateiro deficiente na produção de ABA, mutante *sitiens*. O último capítulo apresenta os resultados referentes a medições de pressão de turgor, trocas gasosas e sinais elétricos nos mutantes *notabilis* e *sitiens*, deficientes na produção de ABA re-irrigadas após um período de déficit hídrico. O possível papel dos sinais elétricos na sinalização em plantas em condições de estresse é discutido. As principais conclusões referentes aos capítulos 1, 2 e 3 foram, respectivamente: As plantas mutantes são mais responsivas eletricamente a re-irrigação, após déficit hídrico com que as plantas selvagens. Medições extracelulares de sinais elétricos podem ser realizadas com medidas de trocas gasosas e pressão de turgor utilizando os equipamentos IRGA e ZIM-probe; Sinais elétricos gerados espontaneamente nos mutantes *sitiens* se propagam com amplitude e velocidade maiores do que nas plantas selvagens. *sitiens* é menos responsivo a estímulo elétrico do que plantas selvagens apresentando maior limiar de excitação e período refratário; Os sinais elétricos precedem as alterações nas trocas gasosas pós irrigação em todos os genótipos estudados. A sonda ZIM-probe não se mostrou eficiente para avaliar a pressão de turgor em plantas mutantes sob condições de estresse, mas para as plantas selvagens é uma ferramenta promissora para estudos envolvendo sinalização hidráulica e elétrica.

Palavras-chave: Sinais elétricos; Medidas extracelulares; Ácido abscísico; Estresse

ABSTRACT

Electrical signaling, gas exchange and turgor pressure in ABA-deficient tomato (cv. Micro-Tom) under drought

This document refers to research whose main objective was to investigate the relationship among hydraulic, chemical and electrical signals in poor tomato plants ABA production. The document is organized into three chapters: The first chapter presents a detailed extracellular measurement protocol of electrical signals in plants and how to associate these measurements to determine gas exchange using the Infrared gas analyzer (IRGA) and turgor pressure using the patch clamp pressure probe (ZIM-probe). The second chapter refers to recording of the action potential generated spontaneously and evoked by electrical stimulation in ABA-deficient tomato, mutant *sitiens*. The final chapter presents the results for turgor pressure, gas exchange and electrical signals measurements in mutants *notabilis* and *sitiens* in the re-irrigated after a period of drought. The possible role of electrical signals in the plant signalling under stress conditions is discussed. The main conclusions related to chapters 1, 2 and 3 were: Measurement of extracellular electrical signals can be performed with gas exchange and turgor pressure measurements using IRGA ZIM-probe equipment; Electrical signals generated spontaneously in *sitiens* mutants propagate with amplitude and speed higher than in wild plants. Mutant is less responsive to electrical stimulation showing higher excitation threshold and longer refractory period than wild plants; The mutant plants are more responsive electrically to re-irrigation after drought than wild plants. The electrical signals precede changes in gas exchange in all genotypes, post irrigation. The ZIM-probe was not efficient to evaluate the turgor pressure in mutant plants under stress conditions, but is a promising tool for studies involving hydraulic and electrical signalling in wild plants.

Keywords: Electrical signal; Extracellular measurement; Absciscic acid; Stress

1 INTRODUCTION

Plants need to monitor constantly various environmental parameters such as light, gravity, soil water content, predators, availability of nutrients and others, and quickly transmitting the environment information to adjacent cells or by long distances along the apical-basal axis. To this end, a sophisticated signalling mechanism is needed to integrate the perception, transmission and response (BALUSKA; VOLKMANN; MENZEL, 2005; BRENNER et al., 2006; PELAGIO-FLORES et al., 2011). Environmental changes can cause damage or biotic and abiotic stresses, which limit the growth and development of the plants. Therefore, understanding how plants behave under adverse conditions is of great importance and involves the effort of several areas in plant sciences.

Long distance signalling in water deficit conditions has been subject of many studies, since the lack of water causes severe damage in plants. The stomata are delicate cellular structures that control the CO₂ uptake and water loss, and responds to various stimuli such as light, hormones, CO₂, temperature and humidity. The stomatal closure is the first response of plants to water shortage in the soil, which makes these structures an ideal model for long-distance signaling studies (JIA; ZHANG, 2008). The main root-to-shoot signals candidates under drought stress are hydraulic signals, the phytohormone, abscisic acid (ABA) and pH (DAVIES; ZHANG, 1991; SALIENDRA; SPERRY; COMSTOCK, 1995; WILKINSON, 1999; YAO; MORESHET; ALONI, 2001; SPERRY et al., 2002; COMSTOCK, 2002). More recently, it has also been documented that reactive oxygen species, salicylic acid and methyl jasmonate are also involved in the regulation of stomata (WANG; SONG 2008; HOSSAIN et al., 2011).

Less studied, but with strong evidence of their involvement in signaling to drought are the electrical signals. Trebacz, Dziubinska and Krol (2006) state that the electrical signals are the most important physical phenomena in an organism and they are capable of transmitting information over long distances more quickly than chemical signals such as hormones, for example. Indeed, intracellular electrical signals are one of the most basic way of information transmission in plant cells (FROMM; LAUTNER, 2007). It has been shown that these signals are involved in many vital processes in plants, including photosynthesis (DAVIES, 2004; KOZIOLEK et al., 2004; GALLÉ et al., 2013), respiration (DZIUBINSKA; TREBACZ; ZAWADZKI, 1989; FILEK; KOSCIELNIAK, 1997), gas exchange (FROMM; FEI, 1998; GRAMS et al., 2007) and responses to stresses biotic and abiotic (STANKOVIC; DAVIES, 1996; MAFFEI; BOSSI, 2006; FISAHN et al., 2004).

1.1 Hydraulic signals

Local changes in water tension, turgor or osmotic potential can generate a hydraulic signal, which rapidly propagates through the vascular system of the plant because of the cohesion and tension properties of water (CHRISTMANN; GRILL; HUANG, 2013). Thus, changes in water potential in the roots, caused by lack of water in the soil, or in the leaves caused by transpiration, generates a hydraulic signal, which is accentuated by the endoderm at the root and bundle sheath in leaves (CHRISTMANN et al., 2007). Decreasing in water potential increases tension in xylem vessels, reaching the cells of the vascular parenchyma, where the ABA is biosynthesized (ENDO et al., 2008).

There is evidence supporting the hydraulic signaling as responsible for root-to-shoot communication under drought, showing a strong correlation between declining stomatal conductance and water potential (SPERRY et al., 2002; YAO; MORESHET; ALONI, 2001; FUCHS; LIVINGSTON, 1996; COMSTOCK, 2002; SALIENDRA; SPERRY; COMSTOCK, 1995). Fuchs and Livingston (1996) found that the reduction in stomatal conductance induced in dry soil can be gradually reversed by a root pressurization system. In addition, the conductance of leaves could return to pre-pressurization levels within minutes, since the pressurization was released. Similar findings were made by Saliendra, Sperry and Comstock (1995) in woody plant *Betula occidentalis* and in pepper by Yao, Moreshet and Aloni (2001). According to Christmann, Grill and Huang (2013), sensors yet unidentified realize the hydraulic signal and promotes the conversion of a physical signal into a chemical signal (ABA) which operates in stomatal closure.

Tardieu and Davies (1993) found that a relatively low water potential of *Commelina communis* could significantly promote stomatal closure induced by ABA although less water potential has no direct effect on the opening of the stomata. Although a direct modulation of the sensitivity of the stomata, the hydraulic signals may also modify the concentration or the flow of chemical messengers such as a change in the function of water flow (TARDIEU; DAVIES, 1993). It is very probable that chemical and hydraulic signals work in different plant species and at different stages of stress. It seems that the most likely hydraulic signaling operates in woody plants (YAO; MORESHET; ALONI, 2001; FUCHS; LIVINGSTON, 1996; COMSTOCK, 2002; SALIENDRA; SPERRY; COMSTOCK, 1995).

On the other hand, there are studies showing that plants subjected to drought stress exhibit stomatal closure and inhibition of leaf growth before the reduction of the leaf water potential is measured. Experiments in which the leaf water status was maintained by pressure

equilibration techniques in dry soil (GOLLAN; PASSIOURA; MUNNS, 1986; SCHURR; GOLLAN; SCHULZE, 1992) or 'split root' in dry or watering soil (KHALIL; GRACE, 1993; STOLL; LOVEYS; DRY, 2000), demonstrated that the stomata can be closed independently of the leaf water status.

1.2 Chemical signal: Absciscic acid (ABA)

Among the chemical signals, the plant hormone abscisic acid has excelled, since numerous studies have shown that the lack of water in the soil can induce a dramatic increase in ABA content, both in the root and in the xylem sap, and this increase is closely related with a decrease in stomatal conductance in the leaves (ZHANG; DAVIES, 1989; ZHANG; DAVIES, 1990; ZHANG; DAVIES, 1991; TARDIEU et al., 1996). Remote regulation of stomatal movement by ABA derived from the root under water stress has been extensively revised (WILKINSON, 1999; WILKINSON; DAVIES, 2002; SCHACHTMAN; GOODGER, 2008).

However, there are inconsistent results with the hypothesis of ABA as a main root-to-shoot signal under drought. Tardieu et al. (1996) stated that there is no absolute relationship between stomatal conductance and ABA even in the same species. The action of ABA on the stomata can vary depending on the time of day (TARDIEU et al., 1996), the level of stress (CORREIA; PEREIRA, 1995) or plant individual (SCHURR; GOLLAN; SCHULZ, 1992). According to Shinozaki and Yamaguchi-Shinozaki (2000), drought stress induces ABA-dependent and ABA-independent reactions. Holbrooh et al. (2002) tested the hypothesis that the ABA produced in the roots, controls the stomatal conductance of plants exposed to dry soil, building grafts tomatoes with roots ABA-deficient and wild shoots. The authors considered that the ABA produced by the roots is required for root-to-shoot signaling, and then the response to dry soil may depend on the roots genotype. However, the results showed that the behavior of the stomata depends on the shoot genotype, i.e., the ABA produced in the roots was not necessary for the control of stomatal tomato plants.

Fambrini et al. (1995) had observed similar results, with reciprocal grafts of a sunflower wild type in a mutant deficient in ABA. Christmann et al. (2007) in experiments with *Arabidopsis* showed the necessity of ABA biosynthesis in leaves and not in roots for timely stomatal regulation. These results indicate that other factors, not ABA produced in root caused stomatal closure. Regardless the nature of the signal coming from the roots, the ABA level increases in the leaves of plants under dry soil, even in plants which roots can not

produce ABA. Therefore, the role of ABA in xylem versus its effect in controlling stomatal conductance is not fully elucidated.

1.3 Electrical Signalling in plants under water stress

The electrophysiologist Jagadish Chandra Bose (1850-1937) was the first to consider the importance of electrical signaling in plant cells in coordinating the responses to the environment. He proved that rapid leaf movements in *Mimosa* and *Desmodium* were stimulated by long distance electrical signaling and also showed that plants produce systemic continuous electrical pulses (BOSE, 1926). Thereafter electrical signaling has been studied extensively and it is being shown that these signals are present not only in sensitive plants but are universal in the plant kingdom (DAVIES, 2004).

Various types of electrical signals are transmitted in plants, standing out action potentials (APs) and variation potentials (VPs). The action potential is a bioelectrical response to a stimulus, which can be mechanical, electrical or thermal, able to generate a depolarization of the plasma membrane. When the depolarization reaches the excitation threshold, the AP is generated according to the “all-or-nothing” principle and at this point there is a temporary reversal of the membrane potential (PYATYGIN; OPRITOV, 1990). The AP is spreading quickly among plant tissues and organs, traveling at a relatively high speed and with constant amplitude (DAVIES, 2004; TREBACZ; DZIUBINSKA; KROL, 2006). The VP is characterized by presenting amplitude and shape depending on the stimulus intensity, i.e., not obeying the “all-or-nothing” principle. Moreover, the magnitude and speed of the signal decrease as distance from the stimulation site (STANKOVIC; ZAWADZKI; DAVIES, 1997).

Regarding the involvement of electrical signals and responses to water stress, Fromm and Fei (1998) conducted a study in order to show that changes in gas exchange are also quickly followed by the transmission of an action potential of the roots to the leaves in maize plants of drying soil. The authors concluded that after irrigation the electrical signals preceded changes in gas exchange, indicating that there was no contribution of xylem flow to the quick response of the stomata and the electrical signals can be the primary responses of plants to the changes in soil water content (FROMM; FEI, 1998). Wang et al. (2007) also studied electrical signaling in response to cucumber plants under drought and concluded that the electrical signals, especially action potential, can play an important role in the lack of water in the soil. Oyarce and Gurovich (2011) found similar results in avocado. They observed that changes in membrane potential between the base of the stem and petiole of the leaf in response to the

decrease in the soil water content are associated with decreased stomatal conductance, indicating that the closure of the stomata can be triggered by an electrical signal.

The electrical signals are probably the initial response of plants to an outside stimulus, hence the importance of studying these signals to understand the physiological mechanisms involved in plant responses to stress. In addition, during plant growth, the electrical signals can show different characteristics due to the dim light, high humidity and lack of potassium (YAN et al., 2009). This suggests the potential use of these signals as an early indication of the physiological status of the plants. Therefore, the knowledge and mastery of technique measurements of electrical signals in plants is a promising tool for adjustment and control of plant development and monitoring of stress in the field and in the greenhouse (WANG et al., 2007).

1.4 Statement of the research problem and objectives

The relationship between hydraulic and chemical signals in the regulation of stomata as well as the role of the electric and hydraulic signal under drought have been documented (COMSTOCK, 2002; GRAMS et al., 2007). However, the challenge that remains is to understand how hydraulic, chemical and electrical signals operate in the information transmission from the roots to the leaves in water stress conditions. The use of mutant plants to isolate a factor of interest has proven to be a promising tool for studies of plant physiology. So, we propose to investigate the relation between hydraulic, electrical and chemical signals using tomato plants, cultivar Micro-Tom, deficient in the ABA production. The main question is: what is the effect of the water deficit on gas exchange, turgor pressure and electrical signals in plants whose role of the chemical signal is reduced?

1.4.1 Objectives

General

- ✓ Investigating the relation between hydraulic, electrical and chemical signals in ABA-deficient tomato plants, cultivar Micro-Tom;

Specific

- ✓ Establishing a protocol to measure electrical signals extracellularly, gas exchange and turgor pressure concomitant in ABA-deficient tomato plants;
- ✓ Characterizing electrophysiologically the ABA mutant plants and wild type plants;

- ✓ Relating turgor pressure, gas exchange and electrical signals in ABA-deficient plants under water deficit;

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2. EXTRACELLULAR REGISTRATION OF ELECTRICAL SIGNALS IN PLANTS: IMPLEMENTATION AND TECHNICAL DEVELOPMENT IN BRAZIL

Abstract

A detailed protocol will be presented to measure extracellular electrical signals in plants through the electrode insertion technique. Using this approach it is possible to measure long-distance electrical signaling, induced by several stimuli, such as wounding, current application, irrigation, and others. Additionally, we describe a method to carry out gas exchange measurements using Infra-red gas analyzer (IRGA, Model Li-6400, Li-Cor) and turgor pressure measurements with patch clamp pressure probe (ZIM-probe, YARA ZIM-plant Technology). The principle of the extracellular measurements and the main challenges for installation of extracellular electric signal measurement apparatus are discussed. The extracellular records measure the sum of the electrical activity of a large number of cells and the method requires a complete electrical circuit that includes a measuring device (amplifier and voltmeter) and electrodes that provide a contact between the biological material and the equipment. Associating electrical signal with gas exchange and turgor pressure measurements did not present major methodological challenges. Regarding the use of the Infra-red gas analyzer (IRGA), it is necessary to ground the equipment because it is an important source of noise for electrical physiological measurements. The ZIM-probe did not cause any interference in electrical signal measure and is a good tool for the study of electrical signaling in plants under drought, contributing with the advance about the understanding of the physiological role of the electrical signals.

Keywords: Measuring protocol; Electrophysiology; Gas exchange; ZIM-probe

2.1 Introduction

Bioelectricity refers to the electrical properties of cells, which are derived from the electrical characteristics of the membranes. The transport of ions through the membrane, produces an electrical potential difference between the inside and outside of cells. This potential difference is typically from 30 to 90 mV in most animal cells, but it may be as much as 150 - 200 mV in plant cells (GRADMANN; HOFFSTADT, 1998). Changes in transmembrane potential can create a wave of depolarization, which propagates for long distances and in a short period, resulting in an efficient signalling mechanism among cells, tissues and organs. Electrical signals in both plants and animals are involved in vital physiological processes, but the electrical signalling mechanisms in plants have not yet been fully elucidated, although the first studies involving the electric phenomena in plants date from the 19th century (PICKARD, 1973).

Several physiological effects of electrical signaling in plants have been described in the past three decades, such as respiration (DZIUBINSKA; TREBACZ; ZAWADZKI, 1989), photosynthesis (KOZIOLEK et al., 2003), water uptake (DAVIES; ZAWADZKI; WITTERS,

1991), leaf movements (SIBAOKA, 1969) and responses to biotic and abiotic stresses (FROMM; FEI, 1998; MAFFEI; BOSSI, 2006). The electrical signals seem to act with other plant signals such as hydraulic, mechanical and hormonal, already well documented by the Plant Sciences (GIL et al., 2009; GALLÉ et al., 2015).

Two main types of electrical signals have been recorded in plants: Action potential (AP) and Variation potential (VP). The AP is a temporary reversal of the membrane potential, triggered by any stimulus. Once triggered, AP propagates with constant amplitude and speed obeying the principle of "all or nothing" (PYATYGIN; OPRITOV, 1990). VP, on the other hand, consists of a rapid depolarization and subsequent slow repolarization, and its high persistence over time is a major difference from the AP. The VP is characterized by presenting amplitude and shape dependent of the stimulus intensity. Moreover, the magnitude and speed decrease by distance from the local where the signal was generated (STANKOVIC; ZAWADZKI; DAVIES, 1997; STANKOVIC et al., 1998).

The measurement technique of electrical signals is well established and, in general, includes intracellular and extracellular records (FROMM; LAUTNER, 2007; YAN et al., 2009). The intracellular measurements are based on the use of the microelectrodes which consist in a glass micropipette containing a thin silver wire and filled with 0.1 M KCl. The microelectrode is inserted into a cell with the aid of a micromanipulator, and the reference electrode is placed in contact with the solution surrounding the cell. This method allows precise measurements of the cell resting potential. Because it is invasive, measures must be made in a short period of time (MILLER; WELLS, 2006). It is also possible to measure, intracellularly, the membrane potential of phloem cell making use of the 'aphid technique', as described by Salvador-Recatalà, Tjallingii and Farmer (2014) in their experiments with *Arabidopsis*.

The extracellular recordings measure the sum of the electrical activity of a large number of cells. The main advantage of this technique is to measure electrical potential difference continuously for several days (FROMM; LAUTNER, 2007). There are two types of extracellular potential measurement: using inserted metal electrodes or surface recordings. In the first case, the electrode, which consists of a very thin conductive wire (Ag/AgCl 0,4 – 1,0 mm diameter) is inserted in the shoot, in order to cross the conducting vessels. This method is invasive and can cause injury reactions (FENSOM, 1963). The second method is noninvasive. The electrodes consist in an Ag/AgCl wire in contact with a KCl solution, which becomes viscous with agar, or a conductive aqueous gel is used to provide the appropriate

contact with the plant surface (FROMM; SPANSWICK, 1993; MANCUSO, 1999; MOUSAVI et al., 2014). In both cases, an electrode can either be placed on the distal region of a plant or in the soil to serve as a reference electrode. The electrodes must be connected by cables to a high-input impedance electrometer. After stabilization, the plant may be stimulated (electrical, flaming, cold, heat, cut or other stimuli) at the apex and the electrical response should be registered by electrodes and looked at a computer screen (FROMM; LAUTNER, 2007; MOUSAVI et al., 2014).

In this chapter, a detailed protocol will be presented to measure extracellular electrical signals in plants through the electrode insertion technique. Using this approach it is possible to measure long-distance electrical signaling, induced by several stimuli, such as wounding, current application, irrigation, and others. It is also possible to characterize the electrical signals regarding amplitude, velocity and duration, and relate them with other physiological parameters. In this context, we described the protocol to measure electrical signals in plants associating with gas exchange measurements using Infra-red gas analyzer (IRGA, Model Li-6400, Li-Cor) and turgor pressure measurements with patch clamp pressure probe (ZIM-probe, YARA ZIM-plant Technology).

Extracellular measurements of electrical signals in plants have been developed by study and effort of many plant electrophysiologists worldwide along the years. Nowadays different variants of this method are used by researchers in the area. Here we present the measurement apparatus installed on the Plants Under Stress Studies Laboratory (LEPSE) of University of São Paulo, the first laboratory equipped to conduct plants electrical signals measurements in Brazil. We follow the measurement protocol used by the Biophysics Laboratory at the University Maria Curie-Skłodowska, Poland, which has consolidated experience in plant electrophysiology (PASZEWSKI; ZAWADZKI, 1973; ZAWADZKI; DZIUBINSKA; DAVIES, 1995; STANKOVIC; ZAWADZKI; DAVIES, 1997; DZIUBINSKA; TREBACZ; ZAWADZKI, 2001; DZIUBINSKA, 2003).

2.2 Material and Methods

2.2.1 Apparatus for electrical signal capturing and recording

- Faraday cage with suiTable dimensions to accommodate the measuring setup. Faraday cage can be purchased or built and can be equipped with lamps internally (Figure 1) or placed over it, outwardly. In the first situation, the best option is to use LED lamps,

which may be linked to a timer to control the photoperiod. The lamps must be grounded.

Figure 2.1 - Faraday cage built by the locksmithing at “Luiz de Queiroz” College of Agriculture (ESALQ/USP). Faraday cage dimensions: 90 x 75 x 65cm; The iron shield spaces are 1 cm²

- Ag/AgCl recording electrodes consist of a piece of silver wire (0.5 or 0.25 mm diameter, World Precision Instruments, e.g., cat. No. AGW 2010 and AGT 1010, respectively) soldered to an enamelled copper wire of 0.2 mm diameter (from local electronics supplier), which is soldered at the other end of a gold pin (World Precision Instruments, e.g., cat. No. 5482) (Figure 2.2). The length of the silver wire is variable according to the thickness of the stem or petiole where it will be inserted. The copper wire length is determined by the distance between the plant and the cables of the data acquisition system. In general, to herbaceous plants such as tomatoes and sunflower, silver and copper wires with 2-3 cm and 10-15 cm length, respectively, are enough. The reference electrode which can be inserted at the apex or in the base of the stem or in the ground, has the same composition of the recording electrodes. When it is in the soil, the silver wire should be 4 - 5 cm length. It is necessary to test the resistance of the electrode after finishing the connection of its parts, in order to establish the basic continuity of the electrode circuit. The electrode resistance should be as close to zero (Ohm, Ω) as possible. Before being used, the electrodes must be chloridized to coat it with a layer of AgCl, as described below.

Figure 2.2 – Ag/AgCl electrode 30 cm length. Silver wire 1.5 cm length (A); A gold pin and silver wire with dark end after chloridation (B)

- For chloridation, connect the cathode of a 9-V battery to a silver wire and insert its end in a 3 M KCl solution. Connect the Ag/AgCl electrode to the anode of this battery, and dip its end into the solution for a few tens of seconds. Ag atoms in the silver wire give up their electrons and combine with Cl^- ions in the solution to make insoluble AgCl, which is visible as a dark coating (Figure 2.3). It is very important not to wet the connection between the silver and copper wires, so special care should be taken when immersing the silver wire in KCl solution.

Figure 2.3 – Chloridation of a reference electrode used in the soil with 40 cm length and 5 cm of silver wire. a. silver wire; b. reference electrode

- It is required a data acquisition interface and software (Lab-Trax 4/24T, World Precision Instruments and LabScribe version3, iWorx Systems Inc.) with 4 channels.

Each channel is independent, with its own 24-bit analog-to-digital converter and equipped with the appropriate filters and high-impedance amplification.

- The electrical connection between the electrodes and the data acquisition interface is made via a BNC cable which is connected to a gold socket in one end (World Precision Instruments, e.g., cat. No. 5483) and a male DIN8 connector in the other end. One BNC cable is necessary for each channel, so for a Lab-Trax 4/24T, 4 cables are required. Each cable is connected to the respective channel input, numbered from 1 to 4. Enumerating the cables at both ends facilitates the identification of electrodes inserted in the plant and the record shown on the computer screen. A single reference electrode should be used for all channels (Figure 2.4). Setup is plug-and-play with connection to computer USB interface.

Figure 2.4 – Electrical connection between the electrodes and the data acquisition interface. The copper core of the BNC cables (a) are connected to gold sockets (b) where the recording electrodes will be placed. The copper shields of the 4 cables are connected together to a gold socket (c), where the reference electrode will be placed. In the other end, the BNC cables are connected to the male DIN8 connectors (e). Following the diagram (d), the copper core is soldered in input 2, and the copper shield is soldered in input 7. Finally, the four BNC cables are connected to the data acquisition system inputs (f)

- Signal Conditioning is necessary to improve the signal to noise ratio of the measuring system, once the electrical activity of plants produces a very small voltage (JOVANOVA; VOLKOV, 2012). So, using bioamplifiers and adequate sampling speed are very important. All channels in Lab-Trax 4/24T are equipped with amplifiers and the gain programming resistors can be installed on transducers by rewiring the DIN8 connectors (as demonstrated in the User's Manual). It is possible to apply up to 1000x gain, but 100x gain is enough to extracellular measures. The sampling rate (sampling speed) is determined by the acquisition software and needs to be adapted to the speed of voltage changes. Sampling rate of 40-100 Hz (40-100 sample per second) is typically used (MOUSAVI et al., 2014). In the first experiments it is advisable to use high sampling frequencies. After detecting the speed of the recorded signals, lower frequencies can be tested in order to find the one, which do not harm the resolution of the signal.
- Current injection device is used to stimulate the plants electrically. It is possible to apply a current from the data acquisition, connecting the stimulation electrodes in a BNC cable which will be connected to the stimulator outputs of the equipment. Through the software, the duration and amplitude of the stimulus to be applied may be chosen. Another option is to build a simple electronic device with batteries that allow the application of a certain voltage. This device may be connected to a timer that controls the duration of the stimulus (Figure 2.5). This approach effectively disconnects the plant from the stimulation system when the pulse is not present (JOVANOVA; VOLKOV, 2012). The stimulation electrodes are two Ag/AgCl electrodes connected to the current injection device through the cathode and anode outputs.

Figure 2.5 - Adjustable voltage source with digital voltmeter and timer, which allows electrically stimulate plants with certain voltage and duration

2.2.2 Electrodes insertion

The chlorinated region of the electrodes should be inserted along the stem and petiole to cross the plant organs. The reference electrode can be inserted in the plant or in the soil. In the first case, it is important to place it far from the recording electrodes, in the base or in the apex of the shoot. In the second case, special care should be taken about the soil moisture, which must be always watered to promote optimal electrode contact with the soil solution. Due to it, in water deficit experiments, the reference electrode must be placed in the plant. The stimulation electrodes should be placed at the other end of the reference electrode to create a closed circuit with the measuring system (Figure 2.6). The cathode is always inserted after the anode and the distance between them should be about 1 cm (TREBACZ et al., 1997). After all the electrodes are inserted, the distance between each one, in the plant, must be measured, in order to calculate the velocity of propagation of the signal.

Figure 2.6 – Examples of electrodes insertion to extracellular electrical signals recording. The reference electrode in the soil (A) and in the shoot apex (B). $\pm$ - Stimulation electrodes; 1, 2, 3, and 4 - Recording electrodes; R - reference electrode

According to Dziubinska, Trebacz and Zawadzki (2001) the electrodes must be localized near the excitable cells and on the way of excitation spread when physiological interdependence is being evaluated.

2.2.3 Characterizing electrical signals in plants

Electrical signals evoked by electrical stimulation is the most used method to characterize the electrical activity of plant species (FROMM; SPANSWICK, 1993; STANKOVIC et al., 1998; HERDE et al., 1999; FAVRE; AGOSTI, 2007). Although current application is not a stimulus, in which the plants are naturally exposed, the method provides the researcher control over the causes that are generating the studied phenomenon. Knowing the stimulus origin as well as its intensity and duration, it is possible to determine the excitation threshold, refractory period, amplitude, velocity and duration of signal as well as the propagation direction (PASZEWSKI; ZAWADZKI, 1973).

The excitation threshold and refractory period can be determined applying stimuli of varying intensities and duration, and with different time interval between a stimulus and the next one. The amplitude, speed and duration may be calculated using the tools available in the data acquisition software and the direction of signal propagation may be observed considering the electrodes arrangement in the plant.

2.2.3 Associating electrical signal, gas exchange and turgor pressure measurements

One of the technical advantages of measuring the extracellular electrical signals by electrodes insertion is the possibility to perform measurements of other physiological parameters (FROMM; LAUTNER, 2007), such as gas exchange and turgor pressure concurrently (Figure 2.6). For gas exchange measurements, using the Infra-red gas analyzer (IRGA), it is necessary to ground the equipment properly, since electromagnetic isolation provided by the Faraday cage is hampered by the IRGA chamber, which is placed inside the cage. The patch clamp pressure probe (ZIM-probe) was used to turgor pressure measurements. The ZIM-probe did not need to be grounded, but it should be the first equipment installed in the plant: at least three days before the beginning of the experiments for the measurement of turgor pressure stabilize. The probes should be installed in the limbo of the healthy and turgid leaves, avoiding the contact with the most prominent veins (ZIMMERMANN et al., 2008). In a second step, the electrodes should be inserted in the plant, and finally the IRGA.

Figure 2.7 – Gas exchange, turgor pressure and electrical signal measured concurrently in tomato cv. Micro-Tom. a. IRGA chamber; b. Leaf patch clamp pressure probe; c. Ag/AgCl electrodes

2.2.4 Measuring preparation

In order to obtain reproducible recordings and good results, careful attention has to be given to the plant material and equipment setup.

The plants must be grown under the same environmental conditions, in the same phenological stage and healthy. If the environmental conditions (light, temperature and humidity) between the place where the plants grew and the measurements local are very different, it is necessary to transfer the plants to the measure conditions, at least one day before starting the experiments. Afterward, it is possible to insert the electrodes, however it is important to verify the chlorination of the electrodes before inserting them. After insertion, at least three hours should be waited to start the recording of electrical signals, in order to establish good contact between the electrodes and the plant tissues.

Concerning the measurement apparatus, all connections should be checked, and the channels, physically connected by BNC cables, must be correctly attributed in the software. It is, also, necessary a good electrical insulation against outside interferences.

After starting the recording of the electrical activity of the plants, the Faraday cage should be closed and mechanical disturbances in the plant must be avoided. Even the application of stimuli should be done without direct contact with the plant, unless when it is extremely necessary.

Regarding the gas exchange and turgor pressure measurements associated with the electrical signals registration, some issues should be considered: when turning on and during IRGA warming, the electrical active registration should be discarded, or the record could be stopped during this time, due to the disturbances caused by leaf clamping in the IRGA chamber. It is recommended to start electrical measures a day before the start of gas exchange measurements to ensure the measuring apparatus is working properly. In general, the ZIM-probe did not cause troubles in the electrical recordings. However, in very delicate leaves, the use of a wooden support to hold the probe prevents sudden movements or damage in the leaf, which can cause changes in the electrical activity of the plant.

2.3 Results

After an electrical stimulus of 13V/4s applied to the stem apex, an action potential was generated in tomato cv. Micro-Tom. Lower intensity and duration stimuli were tested, but no response was observed or only small depolarizations, which did not propagate, i.e., lower

stimuli to 13V/4s did not reach the excitation threshold. The AP propagated basipetally along the stem with amplitude, velocity and duration ($\frac{1}{2}$) of 10 mV, 3.6 cm min⁻¹ and 11s, respectively (Figure 2.8).

Figure 2.8 – Diagram of the electrode arrangement (left). Characteristic traces of action potentials evoked by electrical stimulation (13V/4s) in tomato, cv. Micro-Tom. Red arrow indicates the stimulus artefact

Figure 2.9 illustrates a record of a signal after electrical stimulation of 15V/4s in ABA-deficient tomato, *notabilis*, cv. Micro-Tom. It was possible to notice an excessive electrical noise in channels 2, 3 and 4. Even with the technical problems, the signal propagated basipetally along the stem and a small depolarization was registered in the petiole (Electrode 3), but did not reach the electrode 4 (Figure 2.9).

Figure 2.9 – Diagram of the electrode arrangement (left) and traces illustrating technical problems in an electrical signal registration, evoked by electrical stimulation (15V/4s) in ABA – deficient tomato, *notabilis*, cv. Micro-Tom

The electrical signals measures, associated with gas exchange, require proper grounding of IRGA. Otherwise, excessive electrical oscillations and excessive noise can harm the electrical signal record (Figure 2.10). When the IRGA is properly grounded, the artefacts found during the signal record are due to the leaf clamp to the IRGA chamber. The oscillations stopped after three minutes (Figure 2.11). The ZIM-probe did not cause any interference in electrical signal measure, probably because it works with batteries.

Figure 2.10 - Traces illustrating technical problems due to IRGA poorly grounded during gas exchange and electrical signal record

Figure 2.11 - Traces illustrating artefacts caused by clamping the leaf in the IRGA chamber, during gas exchange and electrical signal record. Red arrow indicates the time the IRGA turns on

2.4 Discussion

2.4.1 Principle of the measuring method

The relation between the internal and external potential of the cell, creates a potential difference. According to the commonly accepted convention the external potential is zero (KROL; DZIUBINSKA; TREBACZ, 2010). Usually, potential difference, also termed voltage, is denoted as V or ΔV and measured in *volts*. In plants, electrical activity generates very low voltages, in the order of mV or tens of mV (JOVANOVE; VOLKOV, 2012). Using suiTable equipment, it is possible to measure the electrical potential difference (voltage) or even the electrical current (I) in biological systems (SHERMAN-GOLD, 2012).

Potential difference measurement principle can be explained by the Ohm's law: the potential difference between two points linked by a current path with a conductance (G) and a current (I) is: $\Delta V = IR$ or $\Delta V = I/G$. Meaning, in an extracellular record experiment, the current (I) that flows between parts of a cell through the external resistance (R) produces a potential difference (ΔV). As the impulse propagates, (I) changes and, therefore, (ΔV) changes as well (SHERMAN-GOLD, 2012).

Figure 2.12 - A - In extracellular recording, part of the current (I') that is flowing in the tissue solution, flows through the electrodes with resistance (R) and is measured as the potential difference (ΔV) across the two electrodes (El , recording electrode; El_R , reference electrode) (Adapted from Sherman-Gold, 2012); B- Electrodes inserted in the stem and petiole of tomato cv. Micro-Tom

The measuring electrical signals apparatus requires a complete electrical circuit that includes the electrodes and measuring device (voltmeter, amplifier, analog-to-digital converter and microprocessor-based controller), whereby the current can flow through all components. To ensure good measures, the equipments should accurately measure the parameter of interest without producing perturbation in the cells or tissues which are being measured. To this end, two requirements must be reached: the electrodes should provide a low electrical resistance, while the voltmeter must have a resistance as large as possible (MILLER; WELLS, 2006). For example: the cell has a resting potential (E), which is measured with an electrode of resistance (R_e) that is connected to a voltmeter with a infinite resistance (R_{in}), simulating a “perfect voltmeter”, so R_e is in parallel with R_{in} , therefore: $V = ER_{in}/(R_{in} + R_e)$. The larger R_{in} , the closer V is to E , likewise, high R_e values can be a problem (SHERMAN-GOLD, 2012). That is because, the input resistance of the data acquisition system should be of the order of $G\Omega$ (DZIUBINSKA; TREBACZ; ZAWADZKI, 2001).

2.4.2 Electrical signals characteristics

Plants can be characterized electrophysiologically regarding excitability, excitation threshold and refractory period. Plants which are able to generate and transmit action potentials are considered excitable, according to Krol, Dziubinska and Trebacz (2010). Stimuli of different nature can cause a depolarization in membrane potential of cells, if this depolarization reaches a certain threshold, the AP is generated and propagates with speed and constant amplitude, according to the “all or nothing” principle. The 'threshold' from which the AP is triggered is called the excitation threshold (GRADMANN, 1976). The refractory period is the necessary time for the cell potential return to the rest values after an AP propagation (TREBACZ et al., 1997).

After a stimulating pulse, a change of potential, which coincided with the stimulus, rose. This variation is called stimulus artefact, which is an electronic potential resulting from passive electrical properties (resistance and capacity) of membranes and tissues but did not result from the plant electrical activity (DZIUBINSKA et al., 1983). In Figure 2.8, red arrows indicate artefact that is related to the application of an electrical pulse of 13V/4s. Thereafter, a real action potential was registered in all electrodes inserted in the stem.

According to the all-or-nothing principle, after generation, the AP propagates with constant amplitude and velocity. However, amplitude and the shape of the AP differ greatly in extracellular experiments. This is because each electrode is inserted into a different part of the

organ, sometimes in morphologically differentiated regions such as petioles and stems. As the amplitude of the signal is given by the ion channels activity, metabolic conditions of the plant interfere in the amplitude (KROL; DZIUBINSKA; TREBACZ, 2010). Similarly, the duration of the AP is determined by the efficiency of ion-transporting mechanisms in the plasma membranes. In the other hand, the velocity depends on the transmission pathway properties. Fast signals present short duration (few seconds) while in slow signals the duration is comparatively higher (tens of seconds) (PYATYGIN, 2008). An AP that propagated with 3.6 cm min^{-1} (Figure 2.8) is a slow signal.

2.4.3 Troubleshooting

Measurement of plant electrical activity raises a number of challenging issues, including type and position of electrodes, method of measures, signal conditioning, and it is susceptible to various external interferences.

Excessive noise and artefacts are often problems and they may occur in all channels at the same time, either in some channels or only in one of them. The Figure 2.9 illustrates a record with noise in the channels 2, 3 and 4. The signal propagation can be observed but this measure should not be considered in the data analysis because it is not possible to know how much the noise interferes in the signal magnitude. In this case, the solution was remaking the connection between the BNC cables and pins DIN8. However, any connection between the plant and the measuring system must be checked, including electrodes chlorination and insertion of the electrode in the plant. Sometimes, changing the electrode already solves the problem. The reference electrode may also be a source of error. When it is on the ground, the soil must be maintained with good moisture.

Insufficient grounding of the setup measurement and/or the presence of noise sources close to the Faraday cage can also lead to noise. The correct grounding of the cage may solve the problem. Moving away or grounding the equipment, which may be a source of noise, is also a solution. Placing the data acquisition system in the Faraday cage, reduce the noise too (MOUSAVI et al., 2014). The IRGA, used to measure gas exchange, poorly grounded is an important source of noise, causing pulses and artefacts propagating rhythmically (Figure 2.11).

2.4.4 Extracellular measure of electrical signal and ZIM-probe

The ZIM-probe allows the monitoring of leaf turgor pressure by several consecutive days and these measurements can be related to the water status of the plant (ZIMMERMANN et al., 2008). There are indications that variation potential (VP) is related to hydraulic pressure variation in the xylem. According to Stankovic, Zawadzki and Davies (1997), VP is a local electrical signal that appears because of a hydraulic signal transmitted rapidly in the xylem. Accordingly, the ZIM-probe is a good tool for the study of electrical signals in plants under drought, mainly because it must avoid water potential measurements by invasive methods, since the mechanical damage caused by the collection of plant material samples can generate electrical signals, interfering in the expected results.

2.5 Conclusions

The extracellular measurement is a technique that enables continuous measurements of electrical signals in plants, which can be associated with gas exchange and turgor pressure measures, using the equipments IRGA and ZIM-probe, successfully. It is a promising method for conducting researches related to physiological mechanisms, such as, water uptake, photosynthesis, respiration, stomatal conductance and electrical signals, regarding plants response to the environmental variation, contributing to the advance in the understanding of the physiological role of electrical signals in plants.

The use of the patch clamp pressure probe (ZIM-probe) is particularly important for hydraulic and electrical signals studies, especially concerning variation potential, poorly understood, but it is known about its involvement with the turgor pressure variation in the xylem.

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3 ACTION POTENTIALS IN ABSCISIC ACID-DEFICIENT TOMATO MUTANT (*sitiens*) GENERATED SPONTANEOUSLY AND EVOKED BY ELECTRICAL STIMULATION

Abstract

Action potentials generated spontaneously (SAPs) and evoked by electrical stimulation (APs) in tomato plants (*Solanum lycopersicum* L.) cv. Micro – Tom ABA-deficient mutants (*sitiens* - MT*sit*) and its wild type (MT*wt*) were characterized by continuous monitoring of electrical activity for 66 h and by application of an electrical current supplied extracellularly. MT*sit* generated SAPs which spread along the stem, including petioles with amplitude of 44.5 mV, half-time ($t_{1/2}$) of 33 s and velocity of 5.4 cm min⁻¹. Amplitude and velocity were 43% and 115% higher in MT*sit* than in MT*wt*, respectively. The largest number of SAPs was registered in the early morning in both genotypes. MT*sit* was less responsive to electrical stimuli. The excitation threshold and the refractory period were greater in MT*sit* than in MT*wt*. After current application, APs were generated in the MT*wt* with 21.2 mV amplitude and propagated with 5.6 cm min⁻¹ velocity. Lower intensity stimuli did not trigger AP in these plants. In MT*sit* APs were measured with amplitude of 26.7 mV and spread with velocity of 8.5 cm min⁻¹.

Keywords: ABA; Stress; Electrical signal; Extracellular measure; *Sitiens*

3.1 Introduction

The intracellular and intercellular signalling in plants occurs through signals derived from hydraulic, chemical and electrical sources. Stephen Hales' experiments inaugurated the hydraulic signals research in plants in the early 18th century (HALES, 1961). Afterwards, many studies involving root to shoot long distance signalling demonstrated the involvement of hydraulic signals in water absorption and growth mechanisms as well as in the control of the stomatal conductance (HSIAO, 1973; MALONE, 1993; CHRISTMANN et al., 2007). In the 20th century, after the discovery of plant hormones (AUDUS, 1959), the scientists turned their attention to these substances such as auxin, gibberellins, abscisic acid, ethylene, cytokinins, brassinosteroids, salicylic acid, and jasmonic acid (DAVIES, 2010). With the advent of molecular biology, the understanding of the mechanisms of cell-cell and organ-organ communication by phytohormones, peptides, nucleotides, fatty acids, reactive oxygen species, and others, has been improved; evidencing chemical signals as having a major role in signalling processes (MULLIGAN; CHORY; ECKER, 1997; APEL; HIRT, 2014; KUSHWAH; LAXMI, 2014; SANCHITA; DHAWAN; SHARMA, 2014).

On the other hand, the electrical signalling has received less attention from the plant biologists. The first known recording of the electrical signal in plants was registered by John

Burdon-Sanderson in 1873, on carnivorous plant *Dionaea muscipula* leaves (PICKARD, 1973). The fact that plants are electrically excitable and able to generate and propagate electrical signals is well documented. Two main types of electrical signals are recognized in plants: the action potentials (APs) and the variation potentials (VPs). The AP can be defined as instantaneous change in the electrical potential of the cell membrane. After being triggered, the AP propagates with constant speed and amplitude according to the "all-or-nothing" principle (PYATYGIN; OPRITOV 1990). The VP results from variations in the xylem tension, which alter the turgor pressure of the adjacent cells to the vessels eliciting local electrical potential changes. The amplitude and velocity of the VP decreases with the distance from the site of the stimulus (STANKOVIĆ; ZAWADZKI; DAVIES, 1997).

Not only hydraulic and chemical signals, but also electrical signals act on the perception of environmental stimuli and trigger biochemical and physiological changes such as elongation, growth, water transport, displacement of substances in phloem, reduction of turgor pressure, variation of photosynthesis, respiration, and gene expression, mediating the relation between the plant and its external environment (DZIUBINSKA; TREBACZ; ZAWADZKI, 1989; STANKOVIĆ; DAVIES, 1996; FROMM; LAUTNER, 2007; YAN et al., 2009). It becomes increasingly clear that the long distance signalling involves the integration of many signals. Mechanical and chemical stimuli may cause changes in membrane potential which in some specific situations results in generation of an action potential (KROL; DZIUBINSKA; TREBACZ, 2010). Therefore, it is necessary to investigate the possible correlations among different types of signals in order to set an integrated view of signalling mechanisms in the plant as a whole.

Abscissic acid (ABA) regulates numerous growth and developmental process such as embryo and seed development, germination, seedling establishment, vegetative development as well as general growth, and reproduction. However, ABA roles in plant biotic and abiotic stress response, especially in water relations, are considered the most important physiological effects. ABA level increased substantially in dry conditions and several studies have revealed its actions to the stomata closing, root growth and genes expression involved in responses to stress. All these ABA effects have been extensively reviewed (CUTLER et al., 2010; NILSON; ASSMANN, 2007; JIANG; HARTUNG, 2008). It has long been known that ABA associated with hydraulic signals plays a critical role in root-shoot communication regarding the soil water status (WILKINSON; DAVIES, 2002; KIM, 2014). It has also been observed the involvement of action potentials in that mechanism (FROMM; FEI, 1998; WANG et al., 2007; GIL et al., 2009). The use of mutants proved to be an important tool for plant signalling

studies. Thus, characterizing the generation of action potentials in ABA deficient plants, it is worthwhile to investigate the relationship between electrical signals and ABA.

In the present study, action potentials generated spontaneously and evoked by electrical stimulation were characterized in the tomato, cv. Micro-Tom, mutant *sitiens* which has only approximately 8% of ABA total level of the wild type. The *sitiens* wilted mutant is deficient in functional enzyme activity – aldehyde oxidase (AO) at the final step in ABA biosynthesis, so *sit* is impaired in the conversion of ABA-aldehyde to ABA (TAYLOR et al., 1988). It took a long time for the *sitiens* gene and its mutant alleles to be identified. Harrison et al. (2011) found a new tomato aldehyde oxidase (*TAO*) gene by obtaining an accurate map position and then searching for aldehyde oxidase genes in that genomic region, making use of the tomato whole genome shotgun sequence assembly. This novel *TAO* gene is therefore, named as the *sit* gene. The reduced ABA content is responsible for the lower growth rate of *sitiens* compared to wild type. The mutant shows a high transpiration rate and a low hydraulic conductivity of root, which explains its low leaf water potential and turgor. These factors result in reduced leaf expansion, characterizing their smaller size and their wilting appearance in relation to the wild type (NAGEL; KONINGS; LAMBERS, 1994). Because of its small size, simple genetics, short life cycle, *sitiens* and its wild type are convenient models for plant physiological studies.

Here we present an electrophysiological characterization of those plants, concerning excitability, excitation threshold, refractory period, amplitude, speed, duration and direction of propagation of Spontaneous Action Potentials (SAPs) and APs evoked by electric current application.

3.2 Material and Methods

3.2.1 Plant material and growth conditions

The seeds of tomato (*Solanum lycopersicum* L.) cv. Micro-Tom mutant *sitiens* (MT*sit*) and wild type (MT*wt*) have been provided from the Micro-Tom collection of the Laboratory of Hormonal Control of Plant Development of University of São Paulo, Brazil.

The plants grew in a room with no windows, where the temperature was kept at 22 – 25°C, humidity of 40 – 60%, and a photoperiod of 14 h light (white fluorescent lights providing 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR at the plant level). Mutant plants were grown in the same room in a covered glass boxes where the humidity was around 70-90%.

3.2.2 Electrical activity recording

The experiments were carried out in a room with the same conditions of temperature and humidity as the growth room, but illuminated with a fluorescent light of $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ (PAR) at the level of the plant and photoperiod of 15 h light.

50- to 60-day-old plants were placed in a Faraday cage to ensure electromagnetic isolation. Four measuring electrodes (0.2 mm diameter silver wire) inserted along the stem or petiole were used with different arrangements. The reference electrode was inserted into the stem or placed in the ground. The measurements were initiated three hours after the insertion of electrodes in the plants: time required for the voltage to be stabilized in all electrodes. The input resistance of each channel was 100 M Ω . The electrodes were connected to a data acquisition system with 4 channels, which was connected to a computer and controlled by the software “RealView”.

Spontaneous Action Potentials (SAPs) were measured by continuous monitoring of electrical activity for 66 h. During this time the laboratory was closed to eliminate any external influence and conditions were kept constant, except for the automatically switching lights off and on.

In order to record action potentials (AP) evoked by electrical stimulation, an electric current was supplied extracellularly through Ag/AgCl electrodes. Different voltages were used to determine the excitation threshold.

3.2.3 Statistical Analyses

All analyses were carried out using statistical software R (R Core Team, 2014).

A linear mixed model was fit to SAP amplitude, duration t ($1/2$) and velocity with a random intercept per plant and genotype fixed effect. Significance of the genotype effect was assessed using F tests (BOLKER et al., 2009).

Analysis of variance models were fit to electric variables (amplitude, duration and velocity) with the fixed effect of genotype in the linear predictor.

Goodness-of-fit was assessed using half-normal plots with stimulation envelopes (DEMÉTRIO; HINDE; MORAL, 2014).

3.3 Results

3.3.1 Spontaneous action potentials (SAPs)

Spontaneous action potentials (SAPs) refer to the action potentials generated in the absence of any intentional external stimulus. Their appearance was first described in sunflower by Zawadzki, Dziubinska and Davies (1995). The results presented here refer to continuous measurements of the electrical activity of ten plants of both genotypes, performed over a period of 66 h (day/night). SAPs were recorded in 50% of mutant plants while in wild type this number was 70%. However, the total number of SAPs was equal in both genotypes (Table 3.1).

Table 3.1 – Number of examined plants, number of plants that generated SAPs, total number of SAPs and excitability recorded during 66 h of continuous measurements of electrical activity in MTwt and MTsit

Genotypes	No. of plants	No. of plants/SAP	Total no. of SAPs	Excitability (%)
MTwt	10	7	19	70
MTsit	10	5	19	50

According to Krol, Dziubinska and Trebacz (2010), excitability means the ability to generate and transmit APs. The cells whose the membrane potential varies, but AP does not occur, are not considered excitable. Thus, we considered excitable plants that generated characteristic action potentials, spontaneously or in response to electrical stimulation. SAPs generated in MTsit and MTwt differ statistically concerning the half-time ($t_{1/2}$) which was 187% higher in MTwt compared to MTsit. A marginal statistical difference can be considered regarding the amplitude and propagation velocity. The amplitude, and propagation velocity (measured between two electrodes located consecutively in the plant) were 43% and 108% higher in mutant plants than in wild type plants, respectively (Table 3.2).

Table 3.2 – Amplitude, half-time ($t_{1/2}$) measured at half of its height (amplitude) and propagation velocity of SAP generated in MTwt and MTsit during 66h of continuous registration

Genotypes	Amplitude (mV)	$t_{1/2}$ (s)	Propagation Velocity (cm min ⁻¹)
MTwt	31.17 (± 1.9) b	94.78 (± 12.8) a	2.5 (± 0.6) a
MTsit	44.55 (± 4.4) a	33.07 (± 2.8) b	5.39 (± 1.01) a
<i>P</i>	0.06**	0.02*	0.07**

*significant statistical difference. ** marginally significant statistical difference ($p < 0,05$); n= 10; Mean ($\pm$ std. error)

SAPs recorded in MTwt (Figure 3.1B) and in MTsit (Figure 3.2A and B) propagated through the stem and petioles in different directions.

Figure 3.1 – Characteristic traces of SAP generated in tomato MTwt. Diagram of the electrode arrangement (A). Signal recorded at all electrodes (B). Distances between the electrodes El 1 and El 2, El 2 and El 3, El 3 and El 4 were: 1.0 cm, 1.0 cm, 1.0 cm, respectively

The direction of propagation of SAP is difficult to be defined because it is impossible to know exactly where the signal began. It is possible to make an inference observing the electrode where the signal was first registered. However, inferences must be made carefully. In Figure 3.1B, a recording of spontaneous action potentials in wild type plant can be observed. First, the signal with reversed polarity was registered simultaneously by all four electrodes indicating that the reference electrode detected the AP in the roots through the soil. Then, the signal was recorded sequentially by the four electrodes located along the stem which indicates AP spreading from the roots to the apex. In Figure 3.2A and B the SAP

spreaded over the stem and petioles. In both presented recordings it originated in an apical part of the plant which was concluded on the basis of the sequence of SAP appearance (El.1, El.2, El.3, and El.4). In Figure 3.2A it is, also, possible to see how hard it is to know the beginning of the SAP. The signal is recorded almost simultaneously at electrodes located in different parts of the plant (El 1 and El 2, inserted in the petiole and stem, respectively, Figure 3.2A).

Note that in the experiment shown in Figure 3.2B the reference electrode was inserted in the apical part of the stem.

Figure 3.2 – Characteristic traces of SAP generated in tomato *MTsit*. SAP registered with the reference electrode placed in the soil (A) or inserted into the stem apex (B). El 1 and El 2, El 2 and El 3, El 3 and El 4 were: 1.0 cm, 1.5 cm, 1.2 cm (A) and 0.8 cm, 1.0 cm, 0.8 cm (B), respectively

In Figure 3.3 frequency of SAPs generation during a 24-hour period is presented. The highest number of SAPs occurred in early morning, for both genotypes, whereas in *MTsit* the highest number of SAPs occurred between 04 and 08 a.m. In case of wild type plants, the highest numbers of the SAPs were registered from 06 to 08 a.m.

Figure 3.3 – Frequency of SAPs generation in *MTsit* and *MTwt* in a 24-hour period

3.3.2 Action potentials (AP) evoked by electrical stimulation

The minimum electrical stimulus required to trigger action potentials in *MTwt* was 8V/4s. Stimuli of lower intensities were tested but did not lead to generation of action potentials. Electrical stimulation of 4V/5s, for example, caused a local depolarization which did not spread. Regarding *MTsit*, only the application of an electrical current of 9V/9s triggered action potentials, indicating that the threshold of excitation of the mutant was higher than in the wild type. Furthermore, the excitability of mutant plants was only 20% compared to 70% in *MTwt* and the refractory period was longer in *MTsit* - 5 h than in *MTwt* - 4 h (Table 3.3).

Table 3.3 – Number of tested plants, stimulus intensity, number of plants that generated APs, total number of APs, excitability and refractory period measured after electrical stimulation in *MTwt* and *MTsit*

Genotypes	No. of plants	Stimulus intensity/duration	No. of plant/AP	Total no. of APs	Excitability (%)	Refractory period (h)
MTwt	10	8V/4s	8	11	70	4
MTsit	10	9V/9s	2	5	20	5

Stimuli of higher or equal intensity at intervals of one, two, three and four hours were tested, but no change in the membrane potential or only slight depolarizations were observed.

Action potentials evoked by electrical stimulation exhibited higher amplitude and velocity of propagation in *MTsit* than *MTwt* (Table 3.4). An important question that arises from our results is why the APs in mutant plants propagate with higher amplitude and velocity than in wild type plants.

Table 3.4 – Amplitude, half-time ($t_{1/2}$) of the AP measured at half of its amplitude, and velocity of propagation of AP evoked by electrical stimulation in tomato *MTwt* and *MTsit*

Genotypes	Amplitude (mV)	$t_{1/2}$ (s)	Velocity (cm min ⁻¹)
<i>MTwt</i>	21.2 (± 2.43) a	19.0 (± 1.93) a	5.6 (± 0.51) b
<i>MTsit</i>	26.7 (± 4.77) a	19.0 (± 1.16) a	8.5 (± 0.12) a
<i>p</i>	0.27	0.99	0.01*

* significant statistical difference at 5% probability level. n= 10; Mean ($\pm$ std. error)

APs evoked by electrical stimuli showed uniform shape and propagated basipetally and acropetally through the stem and petioles, in both wild type and mutant plants (Fig. 3.4A and B). However, the APs seem not to reach the leaves or hypocotyls region (data not shown). In the mutant plants, a slight downward peak, after an upward peak was recorded, this indicates that the AP has reached the roots. Stimulation was given at the apex of the stem and the reference electrode was in the ground (Fig. 3.4B).

Figure 3.4 – Diagram of the electrode arrangement and characteristic traces of action potentials evoked by electrical stimulation in tomato, *MTwt* (A) and *MTsit* (B). Distances between the cathode (-) and electrodes El 1, El 2, El 3, El 4 were: 0.7 cm, 1.7 cm, 3.6 cm, 4.6 cm (A) and 2.5 cm, 1.5 cm, 0.5 cm, 1.3 cm (B), respectively

In Figure 3.5 a diagram is shown which demonstrates a route of propagation of action potential evoked by electrical stimulation. After electrical stimulation (vertical dashed line) the signal was first recorded at electrode 3 (vertical line a), then it was recorded almost simultaneously in the electrodes 2 and 4 (vertical line b), and finally measured in electrode 1 (vertical line c) (Figure 3.5B).

Figure 3.5 – Possible route of transmission of the action potential in tomato, *MTsit*, indicated by the red arrow (A). Particular action potential measured after electrical stimulation. Vertical lines show the time in which the events occur: black arrow - time of the stimulus, a, b and c - time in which the signal is recorded on each electrode (B). S - stimulus, El - electrode. Distances between the cathode (-) and electrodes El 1, El 2, El 3, El 4 were: 2.5 cm, 1.3 cm, 0.5 cm, 1.3 cm, respectively

The recordings shown in Figure 3.5 indicate a possible route of transmission of AP in this specific situation. The AP originated near the cathode and propagated by the stem basipetally. Upon reaching the connection between the stem and petiole, the signal then bifurcated, following the architecture of conducting vessels. The signal first reached electrode 3, which was located closer to the stimulus, then electrodes 2 (in the petiole) and 4 (stem) simultaneously, since the distance between these electrodes and the cathode was equal. The electrode 1 was inserted the longest distances from the stimulus and, therefore, was the last to record the signal.

3.4 Discussion

The causes of the SAPs generation are unknown, but their frequency or rhythm show some clues about their physiological significance. The highest number of action potentials generated spontaneously in the early morning can be related to the circadian cycle of the plants, as a photoperiodic response. The accumulation of SAPs at a specific time during a daily cycle of light/dark was also reported by Wagner et al. (2006) who observed a relationship between the generation of APs and the induction of flowering and vegetative

growth. The authors concluded that hydraulic changes at the shoot lead to early flowering stage which are mediated by a specific hydro-electrochemical communication among leaves, shoot apex and root system. These experiments have been established to disturb the photoperiod, inducing the flowering by specific 'timing' of electrical stimulation via surface electrodes (WAGNER et al., 2006).

The environmental variation should also be considered when observing the generation of SAPs. In the growth room the light was turned on at 6 a.m., thus the highest number of SAPs, at this time, could mean a photoperiodic "remembered" response (GOODRICH; TWEEDIE, 2002; BRUCE et al., 2007; THELLIER; LÜTTGE, 2013). In the case of mutant plants, another important factor to be considered is the humidity. ABA deficient plants are extremely sensitive to water deficit (Figure 3.6), and the water vapour pressure deficit may be more important than the lack of water in the soil, because the evaporative demand will be higher. Continuous measurements of the electrical activity of the plants over a period of five days in which the plants were gradually exposed to water deficit, showed that the mutant plants exhibit electrical responses which increased in time, i.e., when the stress became more severe, more APs were generated, compared to the wild type (appendices).

Figure 3.6 – Tomato plants cv. Micro-Tom 30 days old grown in greenhouse, under the same environmental conditions. ABA-deficient mutant *siliens* (A) and wild type (B)

SAPs are generated when the membrane potential exceeds the threshold of excitation. In turn, the excitation threshold is achieved when appropriate number of ion channels is activated by a particular stimulus and triggers an AP that propagates with constant amplitude and velocity, i.e., according to the “all-or-nothing” principle. Stimuli of low intensity can cause variations in membrane potential of the cell but do not trigger an AP (KROL; DZIUBINSKA; TREBACZ, 2010). The excitation threshold, as well as the excitability, may vary among species, organs, tissues or cells, since it depends on the activity of ion channels, their phosphorylation/dephosphorylation status and, therefore, the metabolic conditions of each level of organization (KROL; DZIUBINSKA; TREBACZ, 2010). Ion channel activity and/or the number of active ion channels are subjected to circadian control (STOLARZ et al., 2010) which may explain diurnal variation in SAP generation. The mutant plants were in a naturally unfavourable conditions and the generation and propagation of APs are energy-costly processes, thus the plants seem to use them only when the damage caused by stress is really severe, 'not compensating'.

The refractory period is the minimum time required so that the resting potential is re-established after the propagation of a signal (TREBACZ et al., 1997). It is also necessary time to restore the ion homeostasis in excitable cells. *MTsit* plants required longer refractory periods than the *MTwt* plants. Considering plant excitability, we must take into account the metabolic condition (energy) of these plants and not only the content of ABA itself.

The propagation velocity of SAPs was higher in *MTsit* than in *MTwt*. The characteristics that defines the APs depends on the activity of ion channels in cell membranes (GRADMANN, 1976; TREBACZ; SIMONIS; SCHÖNKNECHT, 1994; OPRITOV; PYATYGIN; VODENEEV, 2002), thus a detailed study of ion activity in these plants, may help to elucidate the results presented here.

According to Herde et al. (1999) the presence of a minimum amount of ABA is essential for the generation of electrical signals, especially APs in tomato. In their studies with mutant *sitiens* Herde et al. (1999) did not record APs after electrical stimulation. Only after damaging stimuli such as burning or cutting. In the present study APs evoked by electrical stimulation in *MTsit* were recorded, but in fact the plants were less responsive to this type of stimulus.

The routes of propagation of AP in plants have been extensively studied and evidence points to the phloem tissue as the propagation pathway for long distances (FROMM; BAUER, 1994; MANCUSO, 1999; DZIUBINSKA; TREBACZ; ZAWADZKI, 2001). Thus, the architecture of the vascular system determines how far the AP spreads (KROL;

DZIUBINSKA; TREBACZ, 2010). In some areas, such as the base of the stalk in *Biophytum sensitivum* (SIBAOKA, 1973), or the leaves of *Helianthus annuus* (DZIUBINSKA; TREBACZ; ZAWADZKI, 2001), for instance, the AP does not propagate. But this feature also depends on the species in question. In *Helianthus* we often observed AP propagation to the roots which may have important physiological impact connected to phloem unloading (FROMM; ESCHRICH, 1988). No differences were observed in propagation route between *sitiens* and wild type plants. This can be explained by the fact that there is no anatomical differences in the vascular system of these plants, although the stem and petiole diameter of *sitiens* is thinner, due the lower turgor, characteristic of mutant plants (NEILL; HORGAN, 1985; NAGEL; KONINGS; LAMBERS, 1994).

3.5 Conclusions

3.5.1 Spontaneous action potentials

- The largest number of SAPs occurs in the early morning;
- MT*sit* generate SAPs that propagate with amplitude and speed 43% and 115% higher than in MT*wt*, respectively;
- SAPs propagate through the stem, including petioles. However it is difficult to determine the direction of propagation because we do not know where a SAP exactly starts;

3.5.2 The plant response to electrical stimulus

- MT*sit* are less responsive to electrical stimuli: the excitation threshold and the refractory period are higher than MT*wt*.
- After stimulation of 8V/4s an AP is generated in the MT*wt* which propagates with amplitude of 21.2 mV and speed of 5.6 cm min⁻¹. Stimuli of lower intensity did not trigger AP in these plants.
- In MT*sit* APs were measured after stimulation of 9V/9s that spreaded with amplitude of 38 mV and speed of 11.5 cm min⁻¹.
- APs generated by electrical stimulation propagate acropetally and basipetally along the stem, including petioles.

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4 GAS EXCHANGE, TURGOR PRESSURE AND ELECTRICAL SIGNALS IN ABA-DEFICIENT TOMATO AFTER IRRIGATION DURING WATER DEFICIT

Abstract

In order to investigate the relation between hydraulic, electrical and chemical signals in plants, turgor pressure, gas exchange and electrical signals concurrently using the Infra-red gas analyzer (IRGA, Model Li-6400, Li-Cor), patch clamp pressure probe (ZIM-probe, YARA ZIM-plant Technology) and extracellular electrical signal measurements were carried out in ABA-deficient tomato plants, *notabilis* and *sitiens*, cv. Micro-Tom, and its wild type, under water deficit. 30- to 60-day-old plants were transferred from the greenhouse to the laboratory where the measurements were performed. The irrigation in mutant plants was ceased after being transferred to the laboratory and on the second day, without water, they were watered. the wild plants were watered after eight days under water deficit. An action potential was registered after irrigation in 20% of the wild type plants, 80% of the mutant *notabilis* and 60% of the mutant *sitiens*. The duration between stimulus (irrigation) and the signal registered in the first electrode was 219 % higher in MTwt than in the MTsit. MTnot responded faster than MTsit, but the values of both did not differ statistically. The genotypes did not differ statistically regarding amplitude, duration ½ and velocity of the action potential propagation. Regarding gas exchange, the water deficit emphasizes the ABA role on stomatal conductance (g_s), and consequently on transpiration (E) and CO₂ assimilation rate (A). Under water deficit the g_s in MTwt was 66 % and 87 % lower than in MTnot and MTsit, respectively. Accordingly, the E and A are higher in mutant plants, but the water use efficiency is lower. The ZIM-probe was not efficient to evaluate the turgor pressure in plants deficient in the production of ABA under stress. However, for wild plants is a promising tool for hydraulic and electrical signaling studies.

Keywords: Drought stress; Action potential; ZIM-probe; *Notabilis*; *Sitiens*

4.1 Introduction

There is evidence for the involvement of the hydraulic, chemical and electrical signals in the root-to-shoot signaling under drought (JIA; ZHANG, 2009). However, the mechanisms of interaction among these signals, whether acting together or individually, locally or over long distances, or what conditions determine the action of one or another way, are still unanswered questions.

The relation between hydraulic and chemical signals in the water deficit response is more investigate (COMSTOCK, 2002). The lack of water in the soil increases the water tension in the xylem changing the pH of the medium, which in turn triggers the release or production of Absciscic acid (ABA) (DAVIES; ZHANG, 1991), which act on stomatal closure (WILKINSON; DAVIES, 2002). Remote regulation of stomatal movement by ABA derived from the root under water stress has been extensively revised (WILKINSON, 1999; WILKINSON; DAVIES, 2002; REN et al., 2007; JIANG; HARTUNG, 2008). However, a number of findings are inconsistent with the hypothesis ABA as single root-to-shoot signal, since the stomatal closure was not related to ABA comes from the roots (TREJO; DAVIES; RUIZ, 1993; MUNNS; CRAMER, 1996; HOLBROOH et al., 2002).

Regarding the involvement of electrical signals and responses to water stress, Fromm and Fei (1998) conducted a study in order to show that changes in gas exchange are also quickly followed by the transmission of an action potential of the roots to the leaves in maize plants of drying soil. The authors concluded that after irrigation the electrical signals preceded changes in gas exchange, indicating that there was no contribution of xylem flow to the quick response of the stomata and the electrical signals can be the primary responses of plants to the changes in soil water content (FROMM; FEI, 1998). Wang et al. (2007) also studied electrical signaling in response to cucumber plants under drought and concluded that the electrical signals, especially action potential, can play an important role in the lack of water in the soil. Oyarce and Gurovich (2011) found similar results in avocado. They observed that changes in membrane potential between the base of the stem and petiole of the leaf in response to the decrease in the soil water content are associated with decreased stomatal conductance, indicating that the closure of the stomata can be triggered by an electrical signal.

In order to investigate the relation between hydraulic, electrical and chemical signals in plants we measured turgor pressure, gas exchange and electrical signals concurrently using the Infra-red gas analyzer (IRGA, Model Li-6400, Li-Cor), patch clamp pressure probe (ZIM-probe, YARA ZIM-plant Technology) and extracellular electrical measurements in ABA-deficient tomato plants under water deficit.

4.2 Material and Methods

4.2.1 Plant material and growth conditions

The seeds of tomato (*Solanum lycopersicum* L.) cv. Micro-Tom mutant *sitiens* (MT_{sit}), *notabilis* (MT_{not}) and wild type (MT_{wt}) have been provided from the Micro-Tom collection of the Hormonal Control and Plant Development Laboratory of São Paulo University, Brazil.

The plants grew in a greenhouse equipped with evaporative coolers. The temperature and the relative humidity ranged from 30 to 35°C and 40 to 60% during the day and 25 to 28°C and 80 to 90% overnight, respectively. The photoperiod was of 14h light (natural lights of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR at the plant level). During the growth, the plants were irrigated four times daily, during 10 minutes, at 9 a.m, 11 a.m, 1 p.m. and 3 p.m.

4.2.2 Imposition of deficit water and re-watering

The experiments were carried out in the Plants under stress studies Laboratory (LEPSE) of São Paulo University, Brazil. The temperature and the relative humidity in the room, where they were measured, ranged from 27 to 30°C and 35 to 50%, respectively.

30- to 60-day-old plants were transferred from the greenhouse to the laboratory where the measurements of gas exchange, pressure turgor and electrical signals were performed simultaneously. The irrigation in mutant plants was ceased after being transferred to the laboratory and on the second day, without water, they were watered. In the case of wild plants, the irrigation was stopped five days before being transferred to the laboratory and the plants were watered again after eight days under water deficit. The water deficit time, which the genotypes were submitted, was determined in preliminary tests.

4.2.3 Leaf Water Potential (Ψ) Measurements

The leaves water potential was measured by thermocouple psychrometry based on Peltier effect. A microvoltmeter (PSYPRO Water Potential System, WESCOR, INC) was used coupled to Wescor C52 chambers.

Five plants of each genotype were subjected to water stress and the water potential was measured daily during this period. Leaf discs were cut and placed in the chamber at 8

a.m. Since then, waiting up the settling time required for the water balance between the air chamber and the sample were achieved. Only after this time the measurement was performed. Plants which were used to determine the water potential were not the same in other experiments, since the thermocouple psychrometry method is destructive and causes mechanical damage to the leaves that could interfere in the electrical signals generation, which may result in mistakes.

4.2.4 Turgor pressure measurements

Leaf turgor pressure was measured by using the leaf patch clamp pressure probe (ZIM-probe, Yara ZIM Plant Technology) which enables real-time recording of the turgor variation. It is a non-destructive or invasive method, so the measurements can be performed for several days without interruption.

To ensure a perfect stabilization of the measures, the probe was installed in plants, even in the greenhouse at least two days before the plants are transferred to the laboratory.

4.2.5 Gas exchange measurements

The gas exchange measurements were performed through Infra-red gas analyzer (IRGA, Model Li-6400, Li-Cor). Initially it was made Light response curve and A/C_i curve to estimate the maximum photosynthetic rate, depending on the availability of light and internal CO_2 concentration for each genotype, under ideal conditions of hydration.

CO_2 assimilation rate (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$), transpiration (E , $\text{mmol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s , $\text{mol m}^{-2} \text{s}^{-1}$), intercellular CO_2 concentration (C_i , $\mu\text{mol mol}^{-1}$) and vapor pressure deficit between the leaf and the air ($VPDL$, KPa) were measured continuously for eight hours, from 9 a.m. to 5 a.m. The equipment was programmed by 'autolog' function to record measures in a time interval from 10 to 300s. Measurements were performed in the last two days of water deficit. The water use efficiency: A/E (WUE, $\mu\text{mol mmol}^{-1}$) was also calculated.

4.2.6 Electrical activity recording

Plants were placed in a Faraday cage to ensure electromagnetic isolation. The cage was equipped with LED lamps, providing plants $290 \mu\text{mol m}^{-2} \text{s}^{-1}$ (PAR). The photoperiod was of 14h light.

Four measuring Ag/AgCl electrodes (0.25 or 0.5 mm diameter silver wire) inserted along the stem or petioles were used with different arrangements. The reference electrode was inserted into the stem or placed in the ground. The measurements were initiated three hours after the insertion of electrodes in the plants: required time so that the voltage could be stabilized in all of the electrodes. The input resistance of each channel was 100 M Ω . The electrodes were connected to a data acquisition system (LAB-TRAX-4/24T, World Precision Instruments) with 4 channels and amplifier, which was connected to a computer and controlled by “LabScribe iWorx” software.

4.2.7 Statistical Analyses

All analyses were carried out using statistical software R (R Core Team, 2014). Different correlation structures were considered and the model that best fit the data was chosen by likelihood ratio test. A linear mixed model was fit to the water potential over time data and A/C_i curve data with a different intercept and slope per genotype and including a random intercept and slope per each plant. Generalized additive mixed models were fit to the light response curve, A , E , g_s , C_i , $VPDL$ and WUE over time data with a different smoothing function with a P-spline basis for each genotype and for each plant. To light response curve, a random intercept per plant was included. Genotype differences were tested using likelihood-ratio tests for nested models.

Regarding the electrical signal data, a bernoulli generalized linear model was fit to the binary variable, representing whether a plant emitted an electric signal or not and multiple comparisons were performed using likelihood-ratio tests for nested models. The amplitude, duration $\frac{1}{2}$, duration between stimuli and the first electrode, and velocity variables were analyzed, using analysis of variance models, and multiple comparisons were performed using Tukey's test ($p = 0.05$).

4.3 Results

4.3.1 Turgor Pressure under drought and after irrigation

The magnetic probe proved to be efficient to monitor the variation of the turgor pressure of plants in the greenhouse, in well-irrigated condition in all genotypes. However, under stress conditions, the contact between the probe and the leaves of the mutant plants was lost, because the typical wilting of these plants is aggravated by the lack of water in the soil. In wild type plants, it was possible to see the diurnal variation of the turgor pressure without and during stress, as well as the turgor recovery in the first moment after re-watering. A clear drought stress signal in wild type plants can be observed in Figure 4.1: on the first night after the stress start, the P_p began to increase comparing to the value from the last night (without stress). In the second night, there was a very strong increase in P_p and the inversion of the curve. The immediate irrigation effect is the decrease of the P_p value, which represent the increase in turgor pressure.

Figure 4.1 – Typical leaf P_p values in tomato cv. Micro-Tom wild type, with corresponding changes in environment temperature (T) and relative humidity (RH). Black line represents the measuring in the greenhouse under well water conditions. Green line represents the measure in the laboratory under deficit water and the arrow signals the irrigation moment

In the mutant plants, the immediate reaction to stress could be observed on the first night after irrigation the stopped, with a sharp increase in P_p values followed by the inversion of the curve and leaf death within few hours (Figure 4.2 B and D). Figure 4.2 A illustrates the immediate leaf death in *MTnot* after stress. In Figure 4.2 C the turgor pressure variation in a *MTsit* plant is shown. The inversion of the curve is the immediate response to deficit water and the irrigation made the turgor-curve return to half-inverse shape.

Figure 4.2 - Typical leaf P_p values in ABA-deficient tomato, *MTnot* (A and B) and *MTsit* (C and D), with corresponding changes in environment temperature (T) and relative humidity (RH). Black line represents the measuring in the greenhouse under well water conditions. Green line represents the measure in the laboratory under deficit water and the arrow signals the irrigation moment

4.3.3 Gas Exchange during drought and after irrigation

They genotypes did not differ statistically as CO_2 assimilation, stomatal conductance, transpiration and water use efficient in optimum hydration conditions. However, when subjected to water deficit, wild plants differ from the ABA-deficient mutants regarding all variables (Table 4.1). Plants did not show recovery after re-watering considering the average

values for each genotype. Measurements were taken every 10 min after irrigation (appendices); the results presented in the Table 4.1 refer to measurement made two hours and thirty minutes after re-irrigation.

The water deficit emphasizes the ABA role on stomatal conductance, and consequently on transpiration and CO₂ assimilation rate. Under water deficit the g_s in MT*wt* was 66 % and 87 % lower than in MT*not* and MT*sit*, respectively. Accordingly, the E and A are higher in mutant plants, but the WUE is lower.

Table 4.1 – CO₂ assimilation rate (A), Stomatal conductance (g_s), Transpiration (E) and Water use efficient (WUE) in tomato cv. Micro-Tom, wild type (MT*wt*) and ABA-deficient mutants (MT*not* and MT*sit*), without water deficit, under water deficit and after re-irrigation

Without water deficit				
Genotype	A ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	g_s ($\text{mol m}^{-2} \text{s}^{-1}$)	E($\text{mmol m}^{-2} \text{s}^{-1}$)	WUE($\mu\text{mol mmol}^{-1}$)
MT <i>wt</i>	14.12 $\pm$ 1.72 a	0.34 $\pm$ 0.07 a	5.41 $\pm$ 1.00 a	3.01 $\pm$ 0.65 a
MT <i>not</i>	13.05 $\pm$ 2.38 a	0.41 $\pm$ 0.09 a	6.03 $\pm$ 1.18 a	2.29 $\pm$ 0.48 a
MT <i>sit</i>	13.43 $\pm$ 1.71 a	0.31 $\pm$ 0.04 a	5.98 $\pm$ 0.51 a	2.28 $\pm$ 0.28 a
F _{2,12}	0.08	0.59	0.13	0.72
<i>p</i>	0.9269	0.5720	0.8773	0.5083
Under water deficit				
Genotype	A ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	g_s ($\text{mol m}^{-2} \text{s}^{-1}$)	E($\text{mmol m}^{-2} \text{s}^{-1}$)	WUE($\mu\text{mol mmol}^{-1}$)
MT <i>wt</i>	3.16 $\pm$ 1.41 b	0.03 $\pm$ 0.01 b	0.68 $\pm$ 0.30 b	4.77 $\pm$ 0.47 a
MT <i>not</i>	9.65 $\pm$ 1.07 a	0.18 $\pm$ 0.03 a	3.74 $\pm$ 0.54 a	2.78 $\pm$ 0.35 b
MT <i>sit</i>	8.19 $\pm$ 1.05 a	0.16 $\pm$ 0.03 a	3.43 $\pm$ 0.43 a	2.39 $\pm$ 0.15 b
F _{2,15}	8.20	11.96	14.85	13.43
<i>p</i>	0.0039*	0.0008*	0.0003*	0.0005*
After re-irrigation				
Genotype	A ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	g_s ($\text{mol m}^{-2} \text{s}^{-1}$)	E($\text{mmol m}^{-2} \text{s}^{-1}$)	WUE($\mu\text{mol mmol}^{-1}$)
MT <i>wt</i>	3.66 $\pm$ 1.26 b	0.03 $\pm$ 0.01 b	0.72 $\pm$ 0.22 b	4.79 $\pm$ 0.45 a
MT <i>not</i>	9.97 $\pm$ 1.10 a	0.19 $\pm$ 0.02 a	4.20 $\pm$ 0.41 a	2.50 $\pm$ 0.31 b
MT <i>sit</i>	8.00 $\pm$ 1.04 a	0.15 $\pm$ 0.03 a	3.31 $\pm$ 0.51 a	2.47 $\pm$ 0.17 b
F _{2,15}	8.04	17,41	20,61	16,08
<i>p</i>	0.0042*	0.0001*	< 0.0001*	0.0002*

* significant statistical difference at 5% probability level. n= 6; Mean ($\pm$ std. error)

The consequence of poor water use efficiency can be observed in the morphology of mutant plants, which even assimilating CO₂ in same proportion as wild plants, in well watered condition, did not grow any more (Figure 4.3).

Figure 4.3 – Tomato plants cv. Micro-Tom, aged 30 days, grown in greenhouse under the same environmental conditions. Wild type (MT_{wt}) and ABA-deficient mutant *notabilis* (MT_{not}) and *sitiens* (MT_{sit})

4.4.4 Electrical Signal generated after irrigation

An action potential was registered after irrigation in 20% of the wild type plants, 80% of the mutant *notabilis* and 60% of the mutant *sitiens*. Electrical signals were evoked by irrigation, in mutant plants after two days under water deficit, while the wild plants responded electrically to irrigation just after eight days without receiving water. Under drought, plants which are deficient in the ABA production not only generate more electrical signal, but also generate it more quickly than the wild type plants. The duration between stimulus (irrigation) and the signal registered in the first electrode was 219 % higher in MT_{wt} than in the MT_{sit}. MT_{not} responded faster than MT_{sit}, but the values of both did not differ statistically (Table 4.2).

Table 4.2 – Duration between stimulus (irrigation) and signal registration by the first electrode (response) (DSR), Amplitude, Duration $\frac{1}{2}$ and Velocity in tomato cv Micro-Tom, wild type (MT_{wt}) and ABA-deficient mutants (MT_{not} and MT_{sit}) after irrigation

Genotype	DSR (s)	Amplitude (mV)	Duration $\frac{1}{2}$ (s)	Velocity (cm min ⁻¹)
MT _{wt}	317.28 $\pm$ 126.21 a	12.68 $\pm$ 5.87 a	48.23 $\pm$ 38.83 a	4.11 $\pm$ 1.94 a
MT _{not}	95.67 $\pm$ 21.59 b	35.37 $\pm$ 5.26 a	17.24 $\pm$ 2.79 a	7.41 $\pm$ 1.26 a
MT _{sit}	99.41 $\pm$ 13.62 b	36.97 $\pm$ 10.75 a	30.19 $\pm$ 4.32 a	9.33 $\pm$ 2.42 a
F _{2,12}	8.18	1.45	2.65	1.11
P	0.0057*	0.2731	0.1117	0.3610

* significant statistical difference at 5% probability level. n= 10; Mean ($\pm$ std. error)

The genotypes did not differ statistically regarding amplitude, duration $\frac{1}{2}$ and velocity of the action potential propagation, although the signal amplitude and speed are higher in mutant plants. The mutant plants did not differ statistically concerning any of the evaluated factors. However, MT_{not} generated more signals, which arose faster than MT_{sit}. On the other hand, action potential propagated in MT_{sit} with amplitude, duration $\frac{1}{2}$ and velocity higher than MT_{not} (Table 4.2). The difference in generation characteristics and propagation of electrical signals between mutant and wild plants is clear. The question is whether the differences between *notabilis* and *sitiens*, although small, is related to the ABA content, since MT_{not} has about 47%, while MT_{sit} has only 8% of ABA.

As well as action potentials, variation potentials were also recorded after irrigation in all genotypes (Table 4.3). The VP propagated with average amplitude (measured in the highest peak) of 24.63 mV, 27.81 mV and 41.33 mV in MT_{wt}, MT_{not} and MT_{sit}, respectively. The amplitude decreased along the time.

Table 4.3 – Number of evaluated plants (NEP), Number of plants that generated electrical signal (NPES) and kind of electrical signal registered and propagation sequence (KELP) in tomato cv. Micro – Tom wild type and ABA-deficient, *notabilis* and *sitiens*

Genotype	NEP	NPES	KELP
MT _{wt}	10	3	1 – AP
			1 – VP and AP
			1 – VP
MT _{not}	10	8	3– AP and VP
			5 – AP
MT _{sit}	10	6	1– VP
			3 – AP and VP
			2 – AP

The Figure 4.4 illustrated an AP in MT_{wt} (Figure 4.4 C), and an AP followed by a VP in MT_{not} and in MT_{sit} (Figure 4.4 D and E), which propagated from the base to apex along the stem (MT_{wt} and MT_{not}) and petiole (MT_{sit}). The VP presented high amplitude in the petiole (Figure 4.4 E).

Figure 4.4 - Diagram of the electrode arrangement (A – *MTwt* and *MTnot*; B – *MTsit*) and characteristic traces of action potentials generated after irrigation in tomato, *MTwt* (C), *MTnot* (D) and *MTsit* (E). Distances between the electrodes EI 1 and EI 2, EI 2 and EI 3, EI 3 and EI 4, EI 4 and R, were: 1.5 cm, 1.0 cm, 0.8 cm, 2.8 cm (C); 0.6 cm, 1.2 cm, 0.8 cm, 1.2 cm (D); and 1.2 cm, 1.9 cm, 1.4 cm, 2.9 cm (E), respectively

In general, an AP was followed by a VP, but a VP followed by an AP was recorded in *MTwt* and *MTnot* (Figure 4.5 A and B, respectively). Indeed, a VP arose after irrigation and during its propagation an AP was recorded (Figure 4.5 A). In Figure 4.5 B it is possible to see

a peak of the reference electrode, which was in the base of the stem, followed by a VP, when an AP is spread. Single VPs were also recorded in *MTwt* and *MTsit*.

Figure 4.5 - Diagram of the electrode arrangement (left) and characteristic traces of variation potential and action potentials generated after irrigation in tomato, *MTwt* (A) and *MTnot* (B). Distances between the electrodes EI 1 and EI 2, EI 2 and EI 3, EI 3 and EI 4, EI 4 and R, were: 0.7 cm, 1.2 cm, 0.7 cm, 1.8 cm (A); and 1.0 cm, 0.6 cm, 1.3 cm, 1.2 cm (B), respectively

4.3.5 Correlating gas exchange, turgor pressure and electrical signals

Wild plants responded to irrigation after eight days under water deficit increasing turgor pressure, stomatal conductance, transpiration and CO₂ assimilation. The generation of electrical signals was recorded in only 20% of the evaluated wild plants. The Figure 4.6 represents the responses of gas exchange and turgor pressure to irrigation in a wild plant, which did not generate electrical signal. The physiological responses occur at different times: 3 minutes after irrigation, an increase in g_s and E was registered (Figure 4.6 B), after 5

minutes the Pp value starts to decrease (Figure 4.6 A), while the CO_2 assimilation rate only begins to increase after 10 minutes (Figure 4.6 C).

Figure 4.6 - Correlations between turgor pressure – Pp (A), stomatal conductance – g_s (B), transpiration – E (B) and CO_2 assimilation rate – A (C) in tomato cv. Micro-Tom wild type (MTwt) after root watering

In a wild type plant, an action potential was recorded 107 seconds after re-watering (Figure 4.7 A). The AP spread along the stem acropetally with amplitude of 16 mV and speed of 3.6 cm min^{-1} . g_s and E began to increase 6 minutes after watering the roots (Figure 4.7 B) and A after 7 minutes (Figure 4.7 C).

Figure 4.7 - Correlations between electrical signal (A), stomatal conductance – g_s (B), transpiration - E (B) and CO₂ assimilation rate – A (C) in wild type tomato (*MT_{wtr}*) after root watering

The correlation between gas exchange and electrical signal after re-watering in mutant plants is shown in Figures 4.8 and 4.9, *notabilis* and *sitiens*, respectively. The mutant plants respond readily to re-irrigation with the generation of an action potential, which arose 48 and 81 seconds after stimuli, and propagated with amplitude of 35 and 59 mV, and velocity of 15 and 5 cm min⁻¹, in MT*not* (Figure 4.8A) and MT*sit* (Figure 4.9 A), respectively. The recovery of the gas exchange in the mutant plants are slower than in wild plants. In general, the ABA-deficient plants showed a reduction in g_s , E and A, as can be observed in Figure 4.8 B and C. However, fluctuations without a clear pattern of decline or increase were also recorded (Figure 4.9 B and C).

Stomatal conductance, transpiration and CO₂ assimilation rate are higher in mutant plants than in wild type. This indicates that two days of drought, despite the aspect-withered of mutant plants, is not a severe stress level. It seems that the mutant plants use electrical signaling at an early stage of stress. However, for the mutant as well as wild plants, the electrical signal foregoing gas exchanges in the leaves.

Figure 4.8 – Correlations between electrical signal (A), stomatal conductance – g_s (B), transpiration – E (B) and CO_2 assimilation rate – A (C) in ABA-deficient tomato *notabilis* (MT*not*) after root watering

Figure 4.9 – Correlations between electrical signal (A), stomatal conductance – g_s (B), transpiration – E (B) and CO₂ assimilation rate – A (C) in ABA-deficient tomato *sitiens* (MT*sit*) after root watering

4. 4 Discussion

The leaf patch clamp pressure probe (ZIM-probe) is a very good tool to measure continuously turgor pressure changes of plant leaves over long periods of time, providing information in real time about the water status of the plant (ZIMMERMANN et al., 2013). Because it is non-invasive equipment, the probe proved to be a great device for monitoring drought in association with electrical signaling measurements. According to Zimmermann et al. (2008), the ZIM-probe determine the leaf turgor pressure measuring the pressure transfer through the leaf patch, i.e. by measuring the output pressure, P_p , upon application of the constantly kept, magnetic clamp pressure, P_{clamp} (up to 450 Kpa). The pressure P_p is inversely coupled with leaf turgor pressure, P_c as expected since P_c is opposing P_{clamp} . This means that P_p is small when P_c is large and vice versa large, when P_c is small (ZIMMERMANN et al., 2013). Thus, during the day when the turgor pressure is low, P_p value is high and at night the relationship is inverse.

The information that a plant is under drought stress reflects in an increase in P_p value (BADER et al., 2014). Some plants react to water deficit by showing inverse curves, i.e. P_p signal increases in the night and decreases in the day (KANT et al., 2014). Similarly to what was observed in wild type tomato plants (Figure 4.1). In mutant plants evaluated, the stress caused an increase in the P_p value, but then the probe signal was lost due to leaf death (Figure 4.2 A, B and D). It was observed the beginning of recovery of turgor pressure after re-irrigation in wild plants and in one mutant plant (Figure 4.2 C). However, measures for longer periods (several days) allow to better analyze of the data generated by the ZIM-probe.

Monitoring the turgor pressure in the mutant plants under stress conditions was difficult due to large loss of water through transpiration caused by low ABA content that these plants present, about 47% in *MTnot* and 8% in *MTsit*. The leaf lose water to the atmosphere quickly and wilt sharply causing the loss of the probe signal. Nevertheless, the probe was successfully used in concomitant measures electrical signal and made the necessary methodological adjustments is a promising tool for the study of electrical and hydraulic signals in plants.

ABA level increased substantially in dry conditions and its actions in stomata closing is clear (WILKINSON; DAVIES, 2002). Plants deficient in the ABA production exhibit high stomatal conductance and transpiration. The CO_2 assimilation is high but the water use efficiency is low (Table 4.1). In dry soil the differences in gas exchange between mutant and wild plants is evidenced, emphasizing the importance of ABA in controlling water loss by

transpiration. The re-irrigation did not cause a rapid recovery in gas exchange variables. However, a quick electrical signal was recorded after re-watering in wild and mutant plants. The electrical responses differ between mutant and wild plants regarding the number of plants that generated electrical signals and how fast the signal rose after irrigation. ABA-deficient plants stand on these questions. But for all genotypes, electrical signals are the first response to root watering as well as documented by Froom and Fei (1998) and Grams et al. (2007) in maize and Gil et al. (2009) in avocado. Grams et al. (2007) observed that electrical and hydraulic signals between root and shoot turned out to have distinct roles on photosynthesis and stomatal aperture in intact maize plants. The hydraulic signal initiated the hydropassive decrease in stomatal aperture, while electric signals provoked the subsequent physiological control of net CO₂ uptake upon re-irrigation under drought stress.

The most important question that arises from the results presented here is why plants deficient in ABA production generate more electrical signals than wild plants. The ABA content influences the generation of electrical signals as postulated by Herde et al. (1999), or stress intensifies the electrical response in plants.

The closing mechanism of the stomata involves the action of Ca²⁺, Cl⁻ and K⁺ ions. ABA triggers the opening of Ca²⁺ channels, resulting in the increase of the concentration of this ion within the guard cell. The cytosolic Ca²⁺ triggers the opening of Cl⁻ channels and malato⁻, which leave the cell causing membrane depolarization which opens the efflux of K⁺ channels. As a result the output of ions, water is lost from the cell occurs and the osmotically lock ostiole (TAIZ; ZEIGER, 2006). The movement of ions that occurs in guard cells, activated by ABA is very similar to the potential generating mechanism of action in higher plants proposed by Opritov, Pyatygin and Vodeneev (2002), according to which, when the excitation threshold is reached is the opening of Ca²⁺ channels. The increase in cytosolic Ca²⁺ leads to opening Cl⁻ channels, which occurs simultaneously with the inactivation of H⁺ pump, leading to depolarization and a momentary reversal of the polarity of the membrane. Repolarization is initially caused by outward K⁺ efflux and by the reactivation of active transport mediated by H⁺-ATPase, causing the value of the membrane potential returns to the resting value. Clearly, the question arises whether the ABA to bind to the receptor in the membrane of guard cells, triggers an action potential, which leads to stomatal closure.

Zimmermann and Felle (2009) argue that the spread of AP is a non-specific component of the signaling pathway that require additional messengers to become specific. This additional role is performed by ions (Ca²⁺, H⁺), sugars, amino acids, nucleotide and hormones. It can be concluded that although AP transmission is faster systemic signal it is

only part of the effector response (ZIMMERMANN et al., 2009). The long distance signaling mechanism in plants is complex and therefore it is urgent conducting studies directed to this topic.

4. 5 Conclusions

- The ZIM-probe was not efficient to evaluate the turgor pressure in plants deficient in the production of ABA under stress. However, for wild plants is a promising tool for hydraulic and electrical signaling studies;
- The genotypes did not differ statistically as CO₂ assimilation, stomatal conductance, transpiration and water use efficient in optimum hydration conditions. However, when subjected to water deficit, wild plants differ from the ABA-deficient mutants regarding all variables. Plants did not show recovery after re-watering considering the average values for each genotype;
- Mutant plants are more responsive to irrigation stimulus after a few days of stress than the wild plants. Mutant plants generate more signals, which arise quickly after re-irrigation than wild plants;
- Mutants and wild plants not differ statistically regarding the amplitude, duration and speed of the electrical signal generated after re-watering;

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5 GENERAL DISCUSSION

In order to advance in the understanding of long-distance signaling mechanisms in plants under water deficit, we propose to investigate possible relationships among hydraulic, chemical and electrical signals in tomato plants deficient in ABA production and its wild type, in dry soil conditions and after re-irrigation.

First, it was necessary to master the technique of extracellular measuring electrical signals in plants and associate these measures with methods used to determine gas exchange and turgor pressure. Subsequently, an electrophysiological characterization of ABA mutant plants was made to understand the generation of electrical signals in the studied plant material. Finally, association among hydraulic, chemical and electrical signals was investigated.

This research has shown that electrical signals are the first response to irrigation after a drought period in tomato and plants with reduced ABA content are more responsive electrically. We conclude that the electrical signals are an important component of the root-to-shoot signaling in stress conditions and that the decrease in endogenous ABA content intensifies the electrical signal action. The next question to be answered is what is the role of electrical signals in the stress signaling and why plants in unfavorable conditions make use of this signal as strongly.

This work made important contributions to the understanding of electrical signaling in plants. In addition, the implementation of the extracellular electrical signals measurement technique in Brazil, enter the country in the context of research in plant Electrophysiology, presenting a new approach to the study of plants under stress.

APPENDICES

Figure 5.1 – Number of SAPs recorded in tomato cv. Micro-Tom, wild type (MTwt) and *sitiens* (MTsit) during five consecutive days under water deficit

Figure 5.2 – CO₂ assimilation rate (A) in ABA-deficient tomato (NOT and SIT) and in wild type, MT after re-irrigation. Red arrow signaling irrigation time

Figure 5.3 – Stomatal conductance (g_s) in ABA-deficient tomato (NOT and SIT) and in wild type, MT after re-irrigation. Red arrow signaling irrigation time

Figure 5.3 – Transpiration (E) in ABA-deficient tomato (NOT and SIT) and in wild type, MT after re-irrigation. Red arrow signaling irrigation time

Figure 5.4 – Water use efficiency (WUE) in ABA-deficient tomato (NOT and SIT) and in wild type, MT after re-irrigation. Red arrow signaling irrigation time

Figure 5.5 – Water potential (Ψ) in ABA-deficient tomato (NOT and SIT) and in wild type, MT, during five days of gradual drought