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Protocol Article

Equipment and protocol for measurement of extracellular electrical signals, gas exchange and turgor pressure in plants



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A B S T R A C T

We present a detailed protocol for measuring extracellular electrical signals in plants using the electrode insertion technique. Using this approach, it is possible to measure long-distance electrical signaling induced by several stimuli, including wounding, current application, irrigation, burning, and others. Additionally, we describe how to associate gas exchange measurements using an infra-red gas analyzer (IRGA, Model Li-6400, Li-Cor) and turgor pressure measurements using a patch clamp pressure probe (ZIM-probe, YARA ZIM-plant Technology) to measure extracellular electrical signals.

- 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.
- The infra-red gas analyzer (IRGA), needs to be grounded because it is an important source of noise for electrophysiological measurements.
- The ZIM-probe did not cause any interference in electrical signal measure.
- Our approach is useful for plant physiologists wishing to implement the technique of measuring electrical signals in plants, in association with other parameters of plant physiology. In addition, our text was written for agricultural and biological scientists who are not electronics specialists.

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A R T I C L E I N F O

Method name: Electrical signals measurement in plants under stress

Keywords: Measurement protocol, Electrophysiology, Gas exchange, ZIM-probe

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Specifications table

Subject Area	Agricultural and Biological Sciences
More specific subject area	Plant Electrophysiology
Protocol name	Extracellular measurement of electrical signals in plants
Reagents/tools	<p>Equipment and software</p> <p>Data acquisition interface (Lab-Trax 4/24T) - World Precision Instruments LabScribe version 3 - iWorx Systems Inc. Infra-red gas analyzer (IRGA) - IRGA, Model Li-6400, Li-Cor Patch clamp pressure probe (ZIM-probe) - YARA ZIM-plant Technology Computer with USB interface.</p> <p>Electronic components</p> <p>Faraday cage with LED lamps that may be linked to a timer to control the photoperiod; Silver wire (0.5 or 0.25 mm diameter) - World Precision Instruments, e.g., cat. No. AGW 2010 and AGT 1010, respectively; Copper wire of 0.2 mm diameter - from local electronics supplier Gold pin - World Precision Instruments, e.g., cat. No. 5482 Gold socket - World Precision Instruments, e.g., cat. No. 5483 BNC cable - from local electronics supplier Male DIN8 connector - from local electronics supplier</p> <p>Reagent</p> <p>3 M KCl solution</p>
Experimental design	<p>The experiments were carried out in the Laboratory of Plant Stress Study (LEPSE) of University of São Paulo, Brazil. The tomato and sunflower plants grew in a greenhouse equipped with evaporative coolers. We transferred 30- to 60-day-old plants from the greenhouse to the laboratory where the measurements of gas exchange, turgor pressure and electrical signals were performed simultaneously. Leaf turgor pressure was measured by using the leaf patch clamp pressure probe 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. In the laboratory, the plants were placed in the faraday cage and the electrical system installed as described below. Only after the electrical measurement is stabilized (around 3 h after inserting the electrodes), the leaf was closed in the IRGA camera to start gas exchange measurements. CO₂ assimilation rate (A, $\mu\text{mol m}^{-2} \text{s}^{-1}$), transpiration ($E$, $\text{mmol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s, $\text{mol m}^{-2} \text{s}^{-1}$) were measured continuously for eight hours, from 9 a.m. to 5 p.m. The equipment (IRGA) was programmed using the 'autolog' function to record measures in time intervals from 10 to 300 s. We analyzed the electrical activity of the plants as the number of signals generated, amplitude, speed and duration of the signals. 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. For post-irrigation records, the plants were subjected to water deficit and after eight days (determined in preliminary tests) re-irrigated. To record variation potentials (PVs), a burning stimulus was applied by placing the flame approximately 3 cm from the leaf for 10 s.</p>
Trial registration	<i>Not applicable</i>
Ethics	<i>Not applicable</i>
Value of the Protocol	<ul style="list-style-type: none"> Record of extracellular electrical signals allow continuous measurements for up to seven days; Our approach is useful for plant physiologists wishing to implement the technique of measuring electrical signals in plants, in association with other parameters of plant physiology; It is a good tool for the study of electrical signaling in plants under biotic and abiotic stress, contributing with the advance about the understanding of the physiological role of the electrical signals in plant response to stress.

Description of Protocol

Background

The differing ion concentrations between the internal and external parts of the plasma membrane of the cell creates an electrical potential difference. According to the commonly accepted convention,

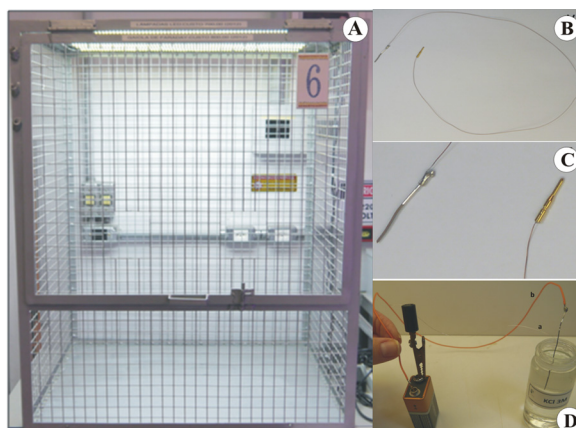


Fig. 1. Faraday cage built by a locksmith at “Luiz de Queiroz” College of Agriculture (ESALQ/USP). Faraday cage dimensions: $90 \times 75 \times 65$ cm; The iron shield spaces are 1 cm^2 (A). Ag/AgCl electrode, 30-cm length (B). Gold pin and silver wire 1.5-cm length with dark end after chloridation (C). Chloridation of a reference electrode used in the soil with 40 cm length and 5 cm of silver wire. Silver wire (a) and (b) reference electrode (D).

the external potential is zero [1]. Usually, the potential difference, also termed voltage, is denoted as V or ΔV and is measured in volts. In plants, electrical activity generates very low voltages, in the order of mV or tens of mV [2]. Using suitable equipment, it is possible to measure electrical potential differences (voltage) or even the electrical current (I) in biological systems [3].

The potential difference measurement principle can be explained by 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 $\Delta V = I/G$. In other words, on extracellular record experiment, the current (I) that flows between parts of a cell through the external resistance (R), producing a potential difference (ΔV). As the impulse propagates, I changes and, therefore, ΔV changes as well [3].

The electrical signal measurement apparatus requires a complete electrical circuit that includes the electrodes and the measuring device (voltmeter, amplifier, analog-to-digital converter and microprocessor-based controller), whereby the current can flow through all components. To ensure good measurements, the equipment should accurately measure the parameter of interest without producing perturbation in the cells or tissues that are being measured. To this end, two requirements must be reached: the electrodes should provide low electrical resistance, while the voltmeter must have a resistance as large as possible [4]. For example: the cell has a resting potential (E) that is measured with a resistance electrode (R_e) that is connected to a voltmeter with infinite resistance (R_{in}), simulating a “perfect voltmeter;” therefore, R_e is in parallel with R_{in} , and $V = ER_{in}/(R_{in} + R_e)$. The larger R_{in} , the closer V is to E ; likewise, high R_e values can be a problem [3]. That is because the input resistance of the data acquisition system should be of the order of $G\Omega$ [5].

Apparatus for electrical signal capturing and recording

Faraday cage with suitable dimensions to accommodate the measuring setup. The Faraday cage can be purchased or built and can be equipped with lamps internally (Fig. 1A) or placed over it, externally. In the first situation, the best option is to use LED lamps that may be linked to a timer to control the photoperiod. The lamps must be grounded.

The 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 enameled copper wire of 0.2 mm diameter (from local electronics supplier) that is soldered at the other end of a gold pin (World Precision Instruments, e.g., cat. No. 5482) (Fig. 1B and C). 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, for herbaceous plants such as tomatoes and sunflower, silver and copper wires of 2–3 cm and 10–15 cm length, respectively, are sufficient. The reference electrode that 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, so as to establish the basic continuity of the electrode circuit. The electrode resistance should be as close to zero Ohms (Ω) as possible. A good measure depends on the quality of the electrodes. That is why it is very important to make a good welding of all the parts that make up the electrode. One suggestion is to remove the enamel that covers the copper wire where the welding will be done. Before being used, the electrodes must be chloridized to coat it with a layer of AgCl, as described below.

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 (gold pin) of this battery, and dip its silver 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 (Fig. 1D). 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.

It is required a data acquisition interface and software (Lab-Trax 4/24T, World Precision Instruments and LabScribe version 3, iWorx Systems Inc.) with four 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 using a BNC cable that 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; for a Lab-Trax 4/24T, four 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 (Fig. 2). Setup is plug-and-play with connection to computer USB interface.

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 [2]. 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; however, 100x gain is sufficient for 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 rates of 40–100 Hz (40–100 samples per second) are typically used [6]. In the first experiments, it is advisable to use high sampling frequencies. After measuring the speed of the recorded signals, lower frequencies can be tested in order to find the one that does not harm the resolution of the signal.

A 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 that will be connected to the stimulator outputs of the equipment. Using 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 (Fig. 3). This approach effectively disconnects the plant from the stimulation system when the pulse is not present [2]. The stimulation electrodes are two Ag/AgCl electrodes connected to the current injection device through the cathode and anode outputs.

Electrodes insertion

The chloridated 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. Because of this, in water-deficit

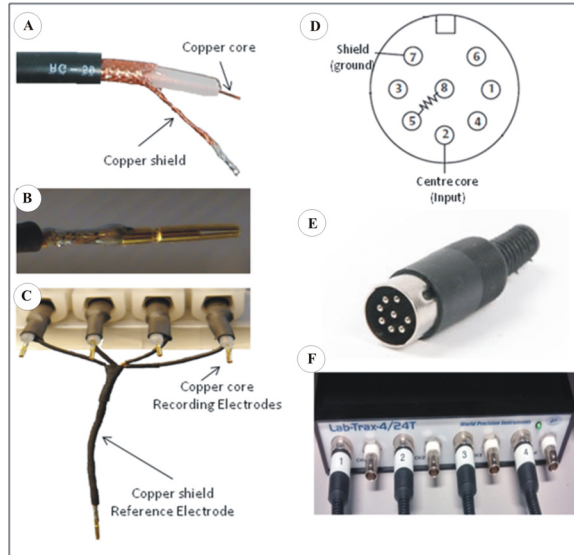


Fig. 2. Electrical connection between the electrodes and the data acquisition interface. The copper core of the BNC cables (A) are connected to gold sockets (B) where the recording electrodes will be placed. The copper shields of the four cables are connected together to a gold socket (C), where the reference electrode will be placed. Following the diagram (D), the copper core is soldered in input 2, and the copper shield is soldered in input 7. In the other end, the BNC cables are connected to the male DIN8 connectors (E). Finally, the four BNC cables are connected to the data acquisition system inputs (F).



Fig. 3. Adjustable voltage source with digital voltmeter and timer, which allows electrically stimulate plants with certain voltage and duration.

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 (Fig. 4). The cathode is always inserted after the anode and the distance between them should be about 1 cm [7]. 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. According to [5] the electrodes must be localized near the excitable cells and on the way of excitation spread when physiological interdependence is being evaluated.

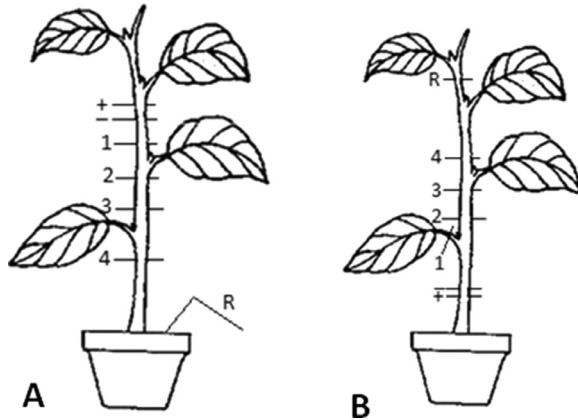


Fig. 4. Examples of electrodes insertion to extracellular electrical signals recording. The reference electrode in the soil (A) and in the shoot apex (B). ± - Stimulation electrodes; 1, 2, 3, and 4 - Recording electrodes; R - reference electrode.

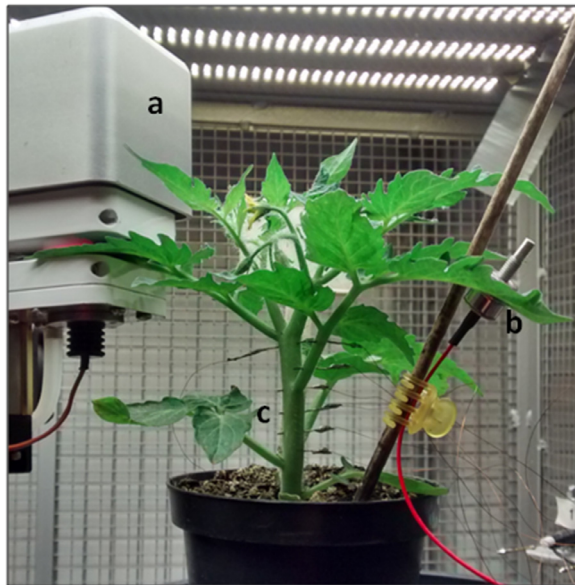


Fig. 5. Gas exchange, turgor pressure and electrical signal measured concurrently in tomato cv. Micro-Tom. a. IRGA chamber; b. Leaf patch clamp pressure probe; c. Ag/AgCl electrodes.

Characterizing electrical signals in plants

Electrical signals evoked by electrical stimulation is the most-often used method to characterize the electrical activity of plant species [13,9,10,11]. 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 [12].

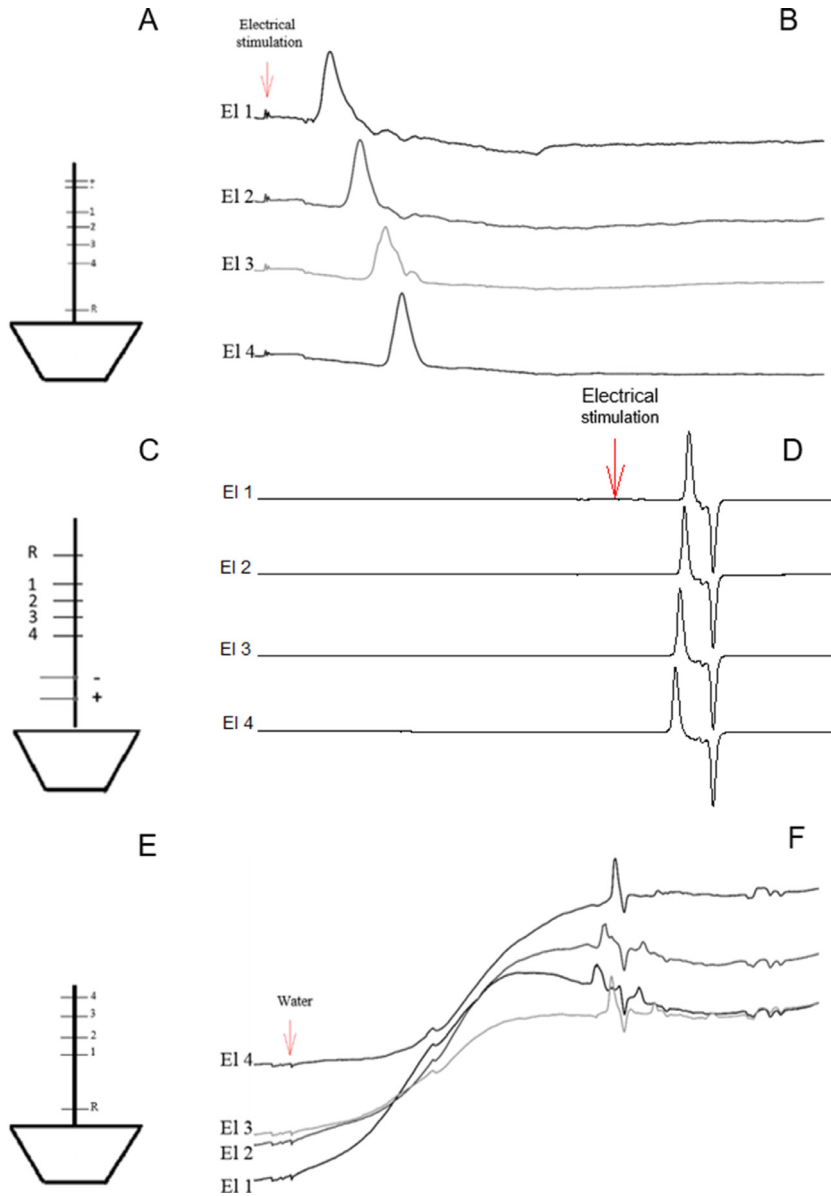


Fig. 6. Diagram of the electrode arrangement (A and E – tomato; C – sunflower). Characteristic traces of action potentials evoked by electrical stimulation (13 V/4 s) in tomato cv. Micro-Tom (B) and (10 V/8 s) in sunflower (C) plant. Red arrow indicates the stimulus artefact. Characteristic traces of variation potential and action potentials generated after irrigation in tomato, MTwt (F).

The excitation threshold and refractory period can be determined by applying stimuli of varying intensities and duration, and with various time intervals between a stimulus and the subsequent 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.

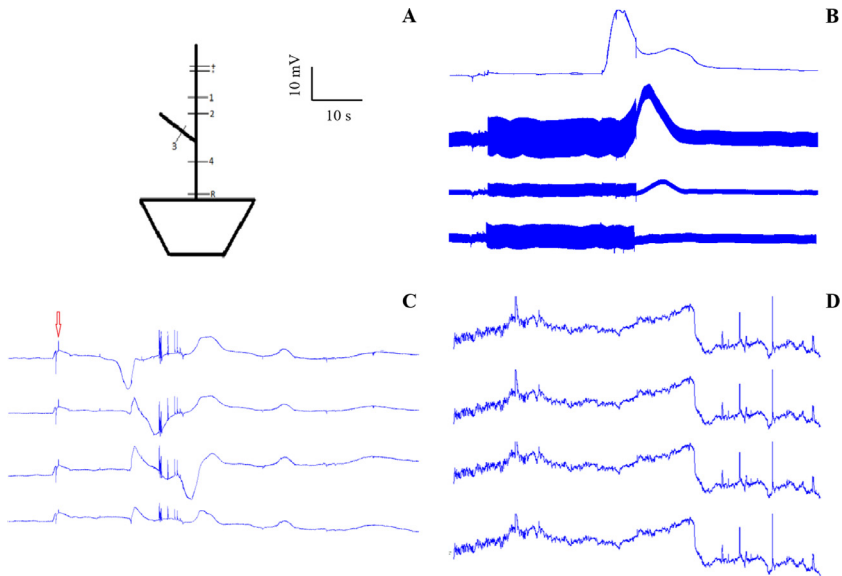


Fig. 7. Diagram of the electrode arrangement (A) and traces illustrating technical problems in an electrical signal registration, evoked by electrical stimulation (15 V/4 s) in tomato plants, cv. Micro-Tom (B). Traces illustrating artifacts caused by clamping the leaf in the IRGA chamber during gas exchange and electrical signal record. Red arrow indicates the time the IRGA turns on (C). Traces illustrating technical problems due to a poorly grounded IRGA during gas exchange and electrical signal record (D).

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 [8], including gas exchange and turgor pressure concurrently (Fig. 5). For gas exchange measurements, using the infra-red gas analyzer (IRGA), it is necessary to ground the equipment properly, because electromagnetic isolation provided by the Faraday cage is hampered by the IRGA chamber that is placed inside the cage. The patch clamp pressure probe (ZIM-probe) was used to turgor pressure measurements. The ZIM-probe does not need to be grounded; however, 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 limbs of healthy and turgid leaves, avoiding the contact with the most prominent veins [14]. In a second step, the electrodes should be inserted in the plant, and finally, connect the IRGA to the measurement leaf.

Results

After an electrical stimulus of 13 V/4 s applied to the stem apex, an action potential was generated in tomato cv. Micro-Tom. Lower intensity and duration stimuli were tested; however, no response or only small depolarizations were observed, that did not propagate, i.e., stimuli lower than 13 V/4 s did not reach the excitation threshold. The AP propagated basipetally along the stem with amplitude, velocity and duration ($t_{1/2}$) of 10 mV, 3.6 cm min^{-1} and 11 s, respectively (Fig. 6B). In sunflower, an AP was recorded after the application of an electrical stimulus of 10 V/4 s. The AP propagated acropetally along the stem with an amplitude, velocity and duration ($t_{1/2}$) of 47 mV, 0.09 cm s^{-1} and 15.7 s, respectively (Fig. 6D). After irrigation stimulus, a wave of depolarization begins in all channels. When the excitation threshold is reached, an action potential is generated and it is registered in all channels, which indicates that the signal has propagated along the stem (Fig. 6F). The electrodes arrangement on the stem can be seen in the Fig. 6A and E in tomato and Fig. 6C in sunflower.

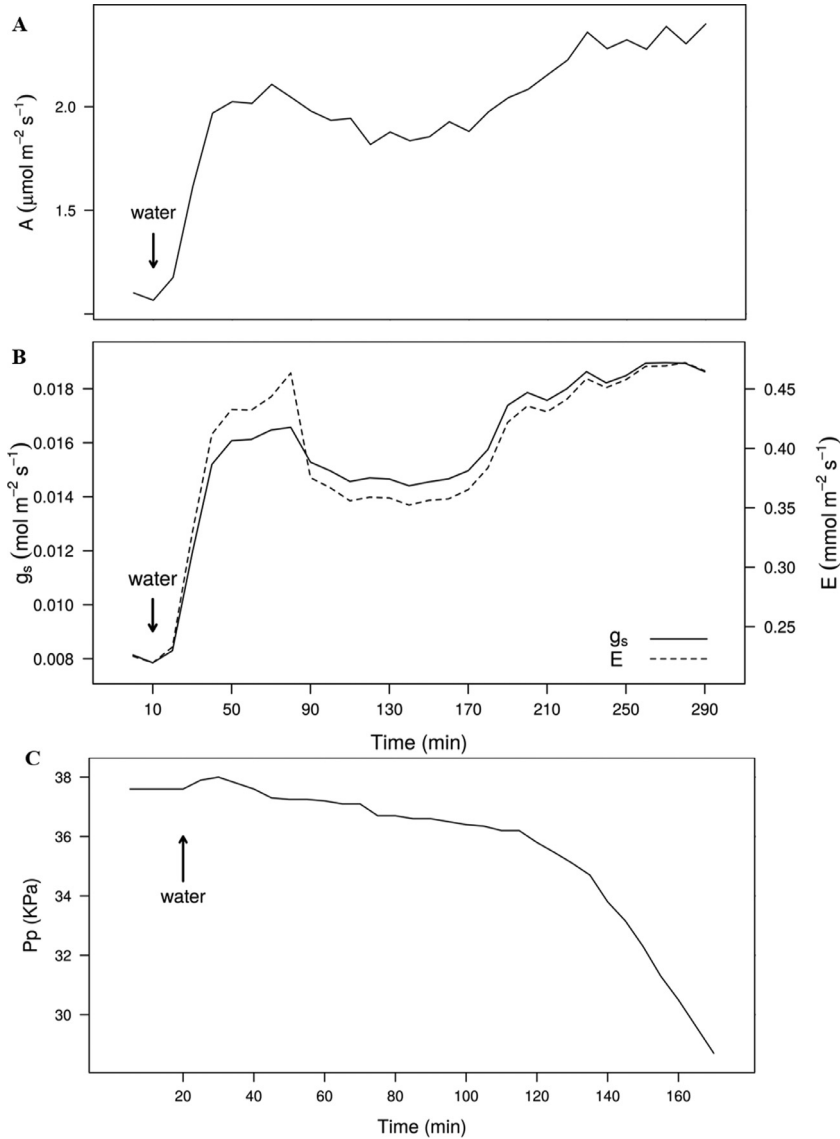


Fig. 8. CO_2 assimilation rate – A (A), stomatal conductance – g_s , transpiration – E (B) and turgor pressure – P_p (A), in tomato cv. Micro-Tom after root watering.

Fig. 7B illustrates a record of a signal after electrical stimulation of 15 V/4 s in tomato plants cv. Micro-Tom. It was possible to notice 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 electrode 4.

The electrical signals measurements associated with gas exchange require proper grounding of IRGA. Otherwise, excessive electrical oscillations and excessive noise can harm the electrical signal record (Fig. 7D). When the IRGA is properly grounded, the artifacts found during the signal record are due to the leaf clamp to the IRGA chamber. The oscillations stopped after three minutes (Fig. 7C). The

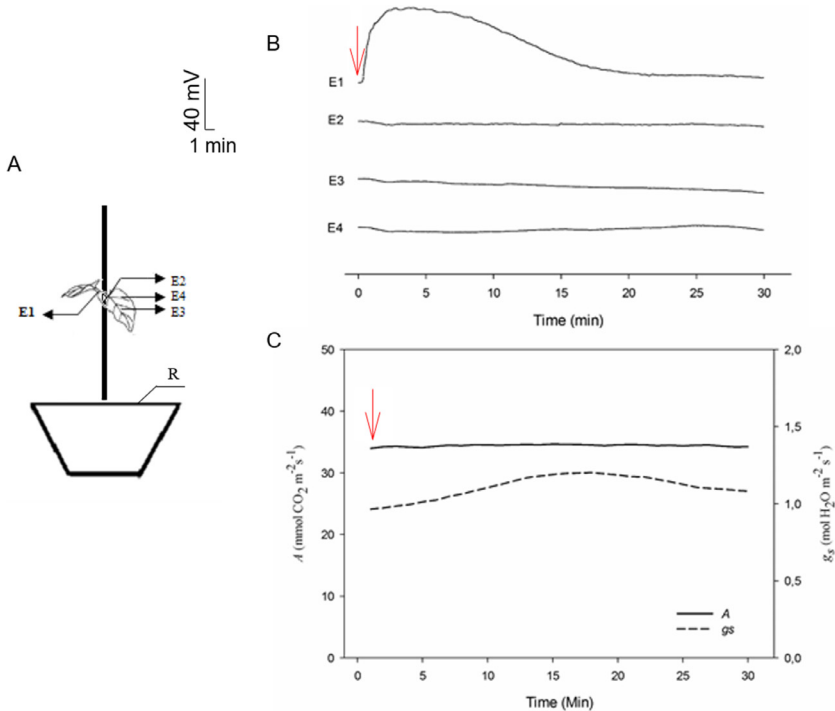


Fig. 9. Diagram of the electrode arrangement (A). Characteristic traces of variation potentials evoked by burning stimulation in sunflower. Red arrow indicates the stimulus artefact (B). CO₂ assimilation rate – A (A), stomatal conductance – gs, measured on the opposite leaf to burning stimulus (C).

ZIM-probe did not cause any interference in electrical signal measure, probably because it works with batteries. The electrodes arrangement on the stem and petiole can be seen in the Fig. 6B and C.

The Fig. 8 represents the responses of gas exchange and turgor pressure to irrigation. The physiological responses occur at different times: 3 min after irrigation, an increase in g_s and E was registered (Fig. 8B), after 5 min the Pp value starts to decrease (Fig. 8C), while the CO₂ assimilation rate only begins to increase after 10 min (Fig. 8A).

Variation potential (VPs) were also measured by continuous monitoring of electrical activity for 60 min after the burning stimulus. Fig. 9B shows a typical VP recorded in E1, inserted in the petiole of the sunflower leaf (Fig. 9A). PV is characterized by rapid depolarization, with 82 mV amplitude, followed by slow repolarization. There were no changes in the CO₂ assimilation rate and stomatal conductance, measured on the opposite leaf, of the same pair, of the stimulated leaf (Fig. 9C).

Troubleshooting

Measurement of plant electrical activity raises a number of challenging issues, including type and position of electrodes, measurement methods, signal conditioning, and it is susceptible to various external interferences. Excessive noise and artifacts are often problems; these may occur in all channels at the same time, either in some channels or only in one. Fig. 7B illustrates a record with noise in channels 2, 3 and 4. The signal propagation can be observed; however, this measure should not be considered in the data analysis because it is not possible to know how much the noise interferes with the signal magnitude. In this case, the solution was re-creating the connection between the BNC cables and pins DIN8. Any connection between the plant and the measuring system must be checked, including electrode chloridation and insertion of the electrode in the plant. Sometimes,

changing the electrode alone 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 adequate moisture.

Insufficient grounding of the measurement setup 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 can also reduce the noise [5]. The IRGA, used to measure gas exchange, poorly grounded is an important source of noise, causing pulses and artefacts propagating rhythmically (Fig. 7D).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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