Plant Electrophysiology

Alexander G. Volkov (Ed.)

Plant Electrophysiology

Theory and Methods

With 142 Figures, 26 in Color, and 4 Tables

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Preface

Plants continually gather information about their environment. Environmental changes elicit various biological responses. The cells, tissues, and organs of plants possess the ability to become excited under the influence of environmental factors. Plants synchronize their normal biological functions with their responses to the environment. The synchronization of internal functions, based on external events, is linked with the phenomenon of excitability in plant cells. The conduction of bioelectrochemical excitation is a fundamental property of living organisms.

The conduction of bioelectrochemical excitation is a rapid method of long distance signal transmission between plant tissues and organs. Plants promptly respond to changes in luminous intensity, osmotic pressure, temperature, cutting, mechanical stimulation, water availability, wounding, and chemical compounds such as herbicides, plant growth stimulants, salts, and water potential. Once initiated, electrical impulses can propagate to adjacent excitable cells. The bioelectrochemical system in plants not only regulates stress responses, but photosynthetic processes as well. The generation of electrical gradients is a fundamental aspect of signal transduction.

This book consists of a historical introduction to plant electrophysiology, and two parts. The first one deals with the methods in plant electrophysiology. Seven chapters present methods of measuring the membrane potentials, ion fluxes, transmembrane ion gradients, ion-selective microelectrode measurements, patch-clamp technique, magnetic measurements, new solid state microsensors and electrochemical sensors. The second part deals with experimental results and theoretical interpretation. All chapters are comprehensively referenced throughout.

Green plants are a unique canvas for studying signal transduction. Plant electrophysiology is the foundation for discovering and improving biosensors for monitoring the environment; detecting effects of pollutants, pesticides, and defoliants; monitoring climate changes; plant-insect interactions; agriculture; and directing and fast controlling of conditions influencing the harvest.

I am grateful to my colleagues for their valuable contribution to this book. We thank the authors for the time they spent on this project and for teaching us about their work. I would like to thank our Acquisition Editor, Dr. Christina Eckey, and our Production Editor, Ursula Gramm, for their friendly and courteous assistance.

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PART I METHODS OF PLANT ELECTROPHYSIOLOGY

1 Historical Introduction to Plant Electrophysiology

Rainer Stahlberg

It is hardly conceivable that reflex responses, memory and brain activity were once explained without consideration of the electrical activity in nerves and muscles. One must remember that electricity was only known then either as lightning or as the repelling/attracting charges that certain substances (such as amber, the Greek word for which is electron) accumulate when rubbed against wool or other textiles. Among the first people who thought about electrical phenomena and their possible biological consequences were de Sauvages (1706–1767), S. Hales (1677–1761), J.A. Nollet (1700–1770) and most importantly the prior Pierre Bertholon de St Lazare (1742–1791), who proposed to improve agriculture with a novel electroculture of crops (Bertholon 1783). This idea was repeatedly revived, e.g. by Lemstrom (1902), who attempted to demonstrate stimulating effects of natural electrostatic fields by growing plants outside and under Faraday cages. Effects of electrical fields on plants and animals continue to be a flourishing field of serious study and some controversy (see Chapter 11).

The birth of the larger field of experimental electrophysiology, however, is inseparably intertwined with the discovery of useable forms of electricity itself. The well-known common starting point was Luigi Galvani's discovery of "animal electricity" or his observing the contraction of isolated frog legs suspended between copper hooks and the iron grit of his balcony (Galvani 1791). Aside from stimulating dubious medical treatments such as "galvanism" and "mesmerism", this momentous event established electrophysiology as a major discipline of biology (Galvani's work was continued by the studies of A. Matteucci, E. Du Bois-Reymond and many others, see below) and stimulated A. Volta to develop the first practical batteries (the existence of batteries in ancient Egypt has been suggested, but cannot be reliably confirmed). These portable sources of electricity were called galvanic elements. Based on the different redox potentials of metals and non-metals, they provided reliable sources of various fixed voltages. This invention not only laid the foundations of electricity as a novel discipline of the physical sciences but also turned electricity into useable reality that would later serve as the basis for at least two industrial revolutions. Electrical currents, voltages, resistances and fields

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could now be experimentally studied and applied to wires and wire networks as well as to animals and plants. The physical understanding of batteries itself also served well as a model to explain some fundamental phenomena of electrophysiology such as the stunning of prey by electrically hunting fishes from the new world (Du Bois-Reymond 1848). As reflected in this book, electrophysiology became to encompass not only the development of methods and instruments for the actual measurement of electrical signals but also the study of physiological effects deriving from electric and electromagnetic currents and fields.

It soon became clear that the role of the electric current in the contraction of frog legs was not to provide the energy for the movement, but to simulate a stimulus that existed naturally in the form of directionally transmitted electrical potentials. Frog legs had just been first and serendipitous current-recording devices to indicate the flow of electrical current in the moment they touched the iron grit of the balcony and their violent jerks were supposedly visible enough to scare Mrs. Galvani, the observant wife of the great scientist. In follow-up studies both Matteucci and Du Bois-Reymond then recognized that wounding of nerve strands generated the appearance of a large voltage difference (called wound potential) between the wounded (internal) and intact (external) site of nerves. This wound potential was the first, crude measurement of what later became known and understood as membrane or resting potential of nerve and other cells. Importantly, this potential could be measured and it was soon found that electrical or mechanical stimulation of the nerve reduced its size (in today's terms: these stimuli caused a depolarization). To describe the phenomenon, novel terms such as action potential (AP) and action current were created (Du Bois-Reymond 1848). After plasmolysis experiments in plant cells suggested that all living cells are surrounded by semi-permeable membranes (Pfeffer 1873, 1906, 1921), it did not take long until W. Nernst (1889) and J. Bernstein (1912) proposed an updated understanding of existing potentials and AP-mediated excitations on the basis of the existence and collapse of K^+ ion gradients across the plasma membrane. It was also recognized that nerves propagate such excitations instantly or with very high speed. In 1850, H. von Helmholtz succeeded in actually measuring this speed in the *Nervus ischiadicus* of frogs and Hermann (1868) developed the "Strömchen" theory to explain the speed and efficiency of AP propagation in nerves in analogy with a leaky wire cable. Until about 1930, this seemed to be all that was to know about nervous signals. However, clever experiments showed surprisingly that signaling between nerve cells through their dendritic connections does not occur by way of a continuation of the electrical action current but by the release of chemical signals diffusing through an intercellular cleft. Following the anatomical work of S. Ramon y Cajal, the biochemical studies of O. Loewi and the terminology of Sir Charles Sherrington, the phenomenon of synaptic transmission was recognized and this meant a gigantic step towards the understanding of nervous integration (Eccles 1964). With these events, the full range of modern electrophysiology

was established and the following examples are added to remind us that this progress was not confined to the academic field but inspired many practical improvements in medical and psychological diagnosis. In 1895, electrocardiography (EEC) was tested and introduced by W. Einthoven and in 1934 H. Berger developed a related method for brain responses in the form of electro-encephalography (EEG; Grey Walter 1954; Brazier 1962). The discovery of piezo-electricity in bones led the way to novel electro-therapeutic treatments for accelerated healing of fractures (Basset 1965). The realization that diaphoretic and alternative changes in skin resistance closely relate the emotional state of individuals turned into another important tool of diagnosis for psychological tests and criminal investigations; the lie detector.

1.1 Intracellular recording of membrane potentials and other improvements

For many years, the application of external electrodes to the surface of plant and animal organs was the only available technique for measuring potentials. The only way to deduce the internal potential of cells was through measuring "wound potentials" in the manner described above (Beutner 1920). Rather than relying on such indirect methods, the membrane theory (Bernstein 1912) made it desirable to measure directly the value of cell membrane potentials. This was facilitated by the introduction of microelectrodes (KCl-filled glass micropipettes with a tip diameter small enough to be inserted into living cells; Montenegro et al. 1991) to record intracellular, i.e. real, membrane potentials (V_m). This technique was first adopted for giant cells from axons of cephalopods such as *Loligo* and *Sepia* (see Keynes 1958) and charophytic algae such as *Chara* and *Nitella*. Early attempts to insert microelectrodes into charophytic cells resulted in long-term damage and were reflected in very low V_m values around −30 mV (Brooks and Gelfan 1928). Improved talent, glass needles, incubation procedures and micromanipulators led to a rapid (i.e. within 1–4 min) return of the initially depolarized V_m of *Nitella* cells to values between −100 and −170 mV (Umrath 1930, 1932; Osterhout 1936). Aside from making the first reliable measurements of V_m values in plant cells, the work of Umrath and Osterhout shows the first intracellular recordings of plant APs as well. When this new technique was complemented with precise electronic amplifiers and voltage clamp circuits in the 1940 s, it permitted measurement of ion currents instead of voltages, and with it monitoring of the activity of ion channels. The smart application of these techniques led to a new, highly detailed understanding of the ionic species and mechanisms involved in V_m changes, especially APs (Hodgkin et al. 1949). Now it could be seen that the depolarization during an AP went beyond zero and well into the range of positive voltages, indicating that other ions in addition to K^+ must participate in the AP. Voltage clamp was introduced to demonstrate the contribution of

various ion currents involved in the AP in nerve cells (Hodgkin et al. 1949; Hille 1992) as well as *Chara* cells (Lunevsky et al. 1983; Wayne 1994). Whereas the depolarizing spike in animal nerve cells is driven by an increased influx of Na^+ ions, plant APs were found to involve influx of Ca^{2+} and/or efflux of Cl^- ions (Sibaoka 1969, 1991). To this day, charophytic algae have served as important models and stepping-stones on the way to the investigation of higher plant cells (see Chapter 16).

Parallel voltage (V) and current (I) measurements allowed I-V-curves to be plotted and so permitted to differentiate between the action of an ion channel (ohmic or parallel changes in I and V) or ion pump (non-ohmic relation between V and I changes; Higinbotham 1973). These new recording techniques led to the recognition of another important difference between plant and animal cells. Whereas most animal cells in their resting stage are very close to the Nernst potential for K^+ ions (as first suggested by Nernst 1889), plant cells can obtain much higher values due to the operation of an electrogenic H^+-ATPase-driven pumps (up to a record V_m value of –296 mV reported by R. Spanswick in *Elodea canadensis*; Higinbotham 1973; see also Chapter 10). As a next step to improve recording possibilities, the patch clamp technique was invented. By going from single cells to isolated membrane patches, one can record the current of as small a unit as a single channel (Neher and Sakmann 1976). Developed for animal cells, this technique was rapidly adopted for plant cells as well (e.g. Hedrich and Schroeder 1989).

1.2 Plant action potentials

The first known recording of a plant AP was done on leaves of the Venus flytrap (*Dionea muscipula* Ellis) in 1873 by the medical physiologist Sir John Burdon-Sanderson in England. This event was organized by C. Darwin, who had found *Dionea* a "most animal-like plant" that showed analogy to the animal nerve reflex (Darwin 1875,1896). Burdon-Sanderson measured the voltage difference between adaxial and abaxial surfaces of a *Dionea* leaf half while he stimulated the other half mechanically by touching the hairs (Burdon-Sanderson 1873, 1899). Ever since then, the trap closure in *Dionea* has been considered as a model case that shows comparable roles of APs in plants and nerve–muscle preparations of animals (e.g. Simons 1992). However, this was and is not a generally accepted view. Reminding his readers that Burdon-Sanderson measured the APs in leaves that were prevented from closure by a plaster harness, Stern (1924), in a first consolidating monograph on plant electrophysiology, concluded that APs had no proven direct connection with the closure movement and that APs produced before and after trap closure do not seem to differ (see similar results by Hodick and Sievers 1988). However, Stern noted that while in resting *Dionea* leaves the upper site is positive relative to the lower one, this relation gets inverted with stimulation.

Other objects of investigation were sensitive plants in the genus *Mimosa,* where the folding movement of the leaflets actually makes the propagating wave of excitation visible. After the wounding of a leaflet action spikes were found to arise in parallel with the visible leaflet movements (Kunkel 1878; Haberlandt 1890; Biedermann 1895; Bose 1906, 1926). However, it was Dutrochet and Pfeffer (1873, 1906) who found that an experimental interruption of the vascular bundles by incision prevented the excitation from propagating beyond the cut. While they concluded that the stimulus moved through the woody or hadrome part of the bundles (in modern terms the xylem), Haberlandt cut or steam-killed the external, non-woody part of the vascular bundles (the leptom, i.e. in modern terms the phloem) and emphasized that not the xylem but the phloem strands were the pathways to conduct the excitation signals in plants. "The effects of incision show that stimuli are actually propagated in this system of highly turgescent tubes and that the mode of transmission is a hydrodynamic one" (Haberlandt 1914). However, this hypothesis was difficult to prove (Tinz-Fruchtmeyer and Gradmann 1990) and up to this day we do not know much about pressure propagation in the phloem except that pressure gradients are considered vital and the driving force of mass flow and net solute transport (Lee 1981; van Bel 2003).

It was namely for that reason that Ricca (1916) and Snow (1924) suggested an alternative mechanism in which an excitation substance is released into the xylem and moved by the transpiration flow is the ultimate cause for the propagating excitation. The most convincing experiment in favor of a chemical substance was to cut through a *Mimosa* stem and then reconnect the two pieces with a water-filled tube. Flame-stimulation of leaves connected to the lower part of the stem frequently caused an excitation response in the upper shoot. It is often forgotten, however, that other researcher could not confirm these results (e.g. Koketsu 1923; Bose 1925, 1926). Observing both leaflet movement and electrical signals, Bose (1926) finally proposed that vascular bundles act analogous to nerves by enabling the propagation of an excitation that moved from cell to cell.

Ignoring Haberlandt's and Bose's results, Houwinck (1935) proposed that wound excitation in Mimosa can be propagated by a chemical wound signal (called Ricca's factor) in the xylem which then could be translated into an AP via the mediation of a new type of electric signal, which he called variation potential. One cannot help noticing that the conversion of a chemical into an electrical signal is a process with striking parallels to post-synaptic events in animals. Houwinck's idea circumvented the existing controversy by including both chemical and electrical signals in the transmission mechanism for the excitation signal in *Mimosa*. In spite of Houwinck's diplomatic proposal, the conflict between chemical and electrical propagation persists to this day Cheeseman and Pickard 1977; Schildknecht 1984). A recent modification in the controversy is the recognition that massive wounding causes a large and propagating pressure increase at the wound site. These wound-induced increases in xylem pressure cannot only temporarily reverse the direction of

the transpiration-driven xylem flow (Malone 1996) but are also sufficient cause for a large depolarization in the form of a slow wave potential (Stahlberg and Cosgrove 1996, 1997). Accordingly, the hydrodynamic propagation of electrical signals proposed by Kunkel (1878) and Haberlandt (1914) has been found to occur less in the phloem (Tinz-Fruchtmeyer and Gradmann 1990) than in the xylem, where it provides the major mechanism for the propagation of a propagating signal called slow wave potentials (Stahlberg et al. 2006).

The majority of recent studies in *Mimosa* and other plant species confirmed Haberlandt's suggestion of the phloem being the pathway of excitation. APs have their largest amplitude near and in the phloem and there again in the sieve cells (Sibaoka 1969; Opritov 1978; Fromm and Eschrich 1988; Fromm and Bauer 1994; Rhodes et el. 1996; Dziubinska et al. 2001). Other studies found that AP-like signals propagate with equal rate and amplitude through all cells of the vascular bundle (Herde et al. 1998). Bose (1907, 1913, 1926) went one huge step ahead when he started studies with isolated vascular bundles (e.g. in the fern *Adiantum)*. Comparing the amplitudes, he found the response to heat in the isolated vascular bundles to be much stronger than in the intact stem. Bose found a series of interesting results; among them an increase in amplitude of heat-induced spikes by repeated stimulation (tetanisation) and by incubation of the strands in 0.5% solution of sodium carbonate and other salts. This daring advance has yet to be repeated and confirmed by other labs. Since the recorded behavior of the isolated vascular strands was comparable to that of isolated frog nerves, Bose felt justified in referring to them as plant nerves.

1.3 "Plants have no nerves!?"

Although Burdon-Sanderson described APs in in *Dionea* plants as early as 1873 and Bose described APs in *Mimosa* as early as 1906, the scientific community was slow to respond with experimental and theoretical follow-up. This lack of enthusiasm was at least in part conditioned by the reiterated belief that plants have no nerves and muscles, that the APs were not involved in activities of primary relevance for plant life such as, e.g. photosynthesis. And yet for some, the existence of APs in *Dionea* and *Mimosa* plus the discovery of plant mechanoreceptors not only in *Dionea*, but also at tendrils and surfaces of common plants (Haberlandt 1890, 1906) was sufficient stimulation to look for structures that could facilitate the rapid propagation of signals. Around 1900, several researchers started took a closer look at plasma strands that run across the lumen of many plant cells, continue over several cells and might possibly serve as excitation-conducting structures similar as nerves. Strands were shown to occur and likely to be involved in the traumatotropic responses of several plant roots (Nemec 1901), but were also seen in the leaves of insectivorous butterworts of the genus *Pinguicula* where they

connect the mucous glue-containing hair tips with the more basal peptidase-producing glands (France 1909, pictured in France 1911). Haberlandt reinvestigated these views and suggested later that the only potential nerve-like structures of plants were the vascular bundles, and in particular the phloem (Haberlandt 1914; but see also recent re-evaluation by Baluska and Hlavacka 2005).

From then on and often to this day papers and textbooks reiterate the statement that "plants have no nerves". This unproductive expression ignores the work of Darwin, Pfeffer, Haberlandt and Bose, together with the result that nerves and vascular bundles share the analog function of conducting electrical signals. Similar anatomical and functional differences were never seen as an obstacle to stating that both plants and animals consist of cells. The mechanistic similarity of excitations in plant and nerve cells were elegantly demonstrated by direct comparison of action potentials in *Nitella* and the giant axon of squids (Cole and Curtis 1938, 1939). Today, the consideration of nerve-like structures in plants involves an increasing number of further f aspects of comparison. We know that many plants can efficiently propagate action potentials and hydraulo-electric signals in the form of slow wave potentials (variation potentials) and that the long-distance propagation of these signals proceeds in the vascular bundles. We also know that plants like *Dionea* can propagate APs with high efficiency and speed without the use of vascular bundles because their cells are electrically coupled through plasmodesmata. Other analogies with neurobiology include vesicle-operated intercellular clefts in axial root tissues (the so-called plant synapses; Baluska et al. 2005) as well as the existence and operation of substances like neurotransmitters and synaptotagmins in plant cells (e.g. Wipf et al. 2002). Such similarities were recently the focus of studies presented at the First Symposium on Neurobiology of Plants in 2005 (Baluska et al 2006).

For a long time, plants were thought to be living organisms whose limited ability to move and respond was appropriately matched by limited abilities of sensing (Trewawas 2003). Exceptions to this rule were made only for plants with rapid and/or purposeful movements such as *Mimosa pudica (*also called *the* sensitive plant*)*, *Drosera (sundews), Dionea muscipula* (flytraps) and tendrils of climbing plants. These sensitive plants attracted the attention of outstanding pioneer researchers such as Burdon-Sanderson (1873, 1899), Pfeffer (1873), Haberlandt (1890, 1906, 1914), Darwin (1896) and Bose (1926). They found them not only to be equipped with various mechanoreceptors that exceeded the sensitivity of a human fingerbut also to trigger action potentials (APs) that implemented these movements.

Although at the time a hardly noticed event, the discovery that normal plants such as pumpkins had propagating APs just as the esoteric "sensitive" plants (Gunar and Sinykhin 1962, 1963; Karmanov et al. 1972) was a scientific breakthrough with important consequences. First, it corrected the long-held belief that normal plants are less sensitive and responsive than so-called "sensitive plants." Second, it led to a new, eagerly pursued belief that such

widely distributed electric signals were not random fluctuations but indeed carried important messages with a broader relevance than the established induction of organ movements in "sensitive plants." In different laboratories around the world, this anticipation became the driving force for a renewed quest for the meaning of the electrical signals (Pickard 1973; Pyatygin 2003).

The ensuing studies made considerable progress in linking electrical signals with respiration and photosynthesis (Gunar and Sinykhin 1963; Koziolek et al. 2003), pollination (Sinykhin and Britikov 1967; Spanjers 1981), phloem transport (Opritov 1978; Fromm and Eschrich 1988; Fromm and Bauer 1994) and the rapid, plant-wide deployment of plant defenses (Wildon et al. 1992; Malone et al. 1994; Herde et al. 1995, 1996; Volkov and Haak 1995; Stankovic and Davies 1996, 1998; Volkov 2000).

1.4 The photoelectric response of green leaves

From the view of many botanists, it was probably equally or more important to decipher the mechanism of action potentials as it was to find the particularities in electric behavior that derive from photosynthetic activity in green plant cells. The first to address this question was Haake (1892). Using leaves of various species, he established that relative to the midvein, the mesophyll had a positive voltage in the dark that turned negative under illumination (in modern understanding and assuming that the midvein potential did not change, this result can be interpreted as a light-induced hyperpolarization of the mesophyll). The further steps in deciphering of the photoelectric response have been described by Higinbotham (1973), Rybin (1977) and also by Lüttge and Higinbotham (1979). Jeschke (1970) and Spanswick (1974) found that illumination of *Elodea* and *Nitella* cells caused them to hyperpolarize by 50–130 mV (in *Elodea canadensis* up to a record V_m value of –296 mV) due to the increased activity of the P-type H^+ ATPase.

For the photoelectric response of higher land plants, it was most revealing to compare green and chlorophyll-free cells within the same variegated leaf. Such a comparison identified a rapid light-induced depolarization as the major photosynthetic contribution to the photoelectric response of mesophyll cells from leaves of higher plants (Stahlberg et al. 2000). The depolarization is associated with and can be simulated by the reduction of inter- and intracellular levels of carbon dioxide (Stahlberg et al. 2001). It is inhibited by the electron-transport blocker DCMU (3–3′-4′-dichloropphenyl-1,1-dimethylurea) and may involve K^+, Ca^{2+} and/or Cl^- currents (Spalding et al. 1992; Elzenga et al. 1995; see also Chapter 10). This transient depolarization response differs from the light-induced hyperpolarizations reported as the major photosynthetic light responses in *Elodea* and *Nitella* cells. A delayed hyperpolarization associated with the P-type H^+ ATPase is also present in leaf cells of higher land plants. It occurs in response to photosynthetic and other factors in a way that

remains unresolved to this day (Stahlberg and Van Volkenburgh 1999). Plants also generate other, non-photosynthetic types of intracellular and intercellular electrical events in response to light. Recently, it was found that the irradiation of soybean plants at 450±50 nm induced APs and that their suppression by ion channels blockers inhibited the phototropic response of these plants (see Chapter 19).

By studying the particularities of photosynthesis, plant transporters, plant membrane potentials, action potentials, slow wave potentials and their coupled responses, electrophysiological studies contributed much to the understanding of the living world and one of its central questions: the defining similarities and differences between animals and plants. Details of these and other contributions can be found in the following chapters.

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