Bioelectrical responses of selected horticultural plants upon application of stimuli and certain stresses

by

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I. INTRODUCTION

The electrical nature of living plant tissues has been the subject of various investigations (5, 9, 20, 25, 27, 28). These have involved the effects of applied electrical potentials and the measurement of self-generated plant potentials. An example of the first effect would be electrical resistance measurements of plant tissues, which could be on either a micro or macro scale. A prominant example of self-generated potentials would be the bioelectric signals that arise in metabolizing leaves when exposed to appropriate illumination, as demonstrated by Waller (32) and Glass (14).

Early attempts by such researchers as Merrill (23), Dexter (12), Swingle (31), and later Koostra (19) to evaluate the viability of dormant plants or seeds by testing the electrical conductivity of solutions leached from such plant material, were indirect electrical measurements. More recently, additional interest has arisen in plant responses to environmental pollution. Although physical manifestations of environmental effects usually appear later in growth patterns, the forces that engender these changes were present much sooner, as pointed out by the investigations of Goldsmith (15) and Bose (6), and should be perceivable in significant plant parameters.

Various possible plant variables that might serve as indicators were enumerated by Benner and Sherwood (3) from which a) impedance of plant stems and b) plant leaf biopotentials

were chosen for investigation. Since Lund et al. (20) concluded that electric energy generated by cells originated in living protoplasm and not from differences in concentrations of ions in solutions of cell sap medium, there should be discernible electrical patterns which express injury or indicate a plant's capability for future growth. This was strengthened by Newman's (24) findings that an electric potential seemed to accompany auxin translocation.

With recent technical advances in electronics and biomedical instrumentation, it has become increasingly feasible to look for electrical phenomena produced by living plants, preferably in vivo, while the plants go about their normal metabolic activities. Techniques that would cause a minimum of disturbance to the plant's growth regime should be used.

Measurable differences or changes in a plant may be accentuated when the plant is under stress. A medical analogy would be the Master 2-step, a standardized exercise for suspected cardiac patients, while they undergo an electrocardiogram (ECG). For plants, the relative response to some stimulus such as an electrical charge or selective illumination either before, during, or after some stress such as heat, cold, dryness, anesthetics or pollutants might help indicate the state of vigor of the plant, and/or make a given plant more responsive to another stress.

The purpose of this research was to examine the feasibility of some plant monitoring techniques. Such techniques

should be valuable in several respects. Included would be evaluations of plant vitality and potential performance by electronic means that would ideally be nondestructive or noninjurious to the plant. The techniques should also enable better evaluation of various parts of a plant and respective responses to environment, whether natural climatic factors or man-made introductions. This could involve more qualitative assessments of disease, injury, or pollution damage.

Such instrumented plants might additionally serve as detectors and accumulators or integrators of known environmental pollutants. They could also forewarn of unsuspected or unanticipated toxic products of the increasingly complex processes of modern society.

From the multiplicity of possible techniques, two were selected for study. Plant tissue impedance and biopotential measurements were selected for examination as alternate techniques whose results might complement and supplement one another.

II. REVIEW OF THE LITERATURE

Controversy has accompanied various efforts over the past century to induce beneficial currents in plants by means of electrostatic and/or electromagnetic potential fields. Wheaton (33) reviewed in 1970 the inconclusive results of using either atmospheric electricity, electrostatically-modified growth environments, or locally-generated soil-current flows. Controlled experiments of each type were run by Dorchester (13) in the hope of enhancing growth or yield. Although his results failed to show statistical significance, the general conclusion was reached by Dorchester, Lund et al. (20) and other researchers that while some small electrical stimulation may be beneficial it is easy to exceed a plant's tolerance, thus resulting in detrimental effects.

Bose (6) ran many tests on Mimosa pudica where the reactions to electrical or mechanical stimuli were easily perceived by movements of the leaves, leaflets and/or pulvinus. He definitely established that mechanical stimuli resulted in the self-generation of electrical potentials within the plant. Waller (32), Glass (14), and Black and Forsyth (4) elaborated on the electrical signals generated by plant parts when exposed to photo-stimulation. Evidence for the existence of ambient electrodynamic fields without specific stimuli was presented by Burr and Northrup (9). The source of the presence of these fields was investigated by Blinks (5), Lund et al.

(20), and others. Briggs et al. (7) and Dainty (11) worked out detailed hypotheses involving electrochemical theory of ion movement and ion pumps. Lund and Kenyon (21) studied the relationship between continuous bioelectric currents and cell respiration. Lund et al. (20) concluded that the electrical energy generated by cells originated in the living protoplasm, rather than from differences in concentrations of ions in intracellular or extracellular solutions. The metabolic activities of the living cells resulted in electrical phenomena that differ from dead cells.

All of the researchers dealing with endogenous electrical plant signals were confronted by problems of signal coupling to the plants. Some research was done with excised plant parts, such as Avena coleoptiles and Elodea leaf parts by Lund et al. (20) and Glass (14), respectively; others used intact plants in vivo. With either the use of electrodes piercing the plant tissues or surface contact on plant parts, polarization of the electrodes would produce increasing deterioration of the plant signals. Most researchers avoided this problem by using non-polarizing electrodes. Early examples were pairs of saturated zinc sulfate ($ZnSO_4$) electrodes or zinc sulfate-zinc amalgam combinations. In 1925, Waller (32) used a pair of $ZnSO_4$ "U-tubes" with a sodium chloride (NaCl) bridge to the portions of plants, since $ZnSO_4$ would be toxic if in direct contact with the plant tissues. Glass (14) used a tap-water bridge to isolate his saturated $ZnSO_4$ electrodes from excised Elodea

leaves; in some tests he employed snug hollow glass rings for the water contact to plant parts. Later, Lund (20) used similar hollow glass rings or other water bridges to $ZnSO_4$ electrodes.

Calomel was another popular nonpolarizing electrode, as described in detail by Ives and Janz (16). Bures relied upon it for his various tests, either with a liquid-holding wick to touch plant tissues or a physiological saline (NaCl) solution interface (8). In 1966, Sinyukhin and Rutkovskii (30) isolated calomel electrodes from plant tissues by interposing glass tubes filled with 3% agar and 3 M KCl. These tubes were connected to the plant by camel's hair brushes soaked in water.

When silver wire is coated with a thin layer of silver Chloride (AgCl), it can serve as a nonpolarizing electrode. Bures, Petran and Zacher (8) used a silver-silver chloride electrode with a Ringer solution in agar as a contact to muscle fibers in animals, but it could be adapted to plants. Black and Forsyth (4) used two "chloridized" silver probes with one inserted into the soil of potted tomato plants and the other in the plant stem. In 1972, Pickard (26) reported on electrical signal connections to plants with "freshly chlorided silver wire electrodes" centered in "small glass pipettes containing 0.1 M potassium chloride (KCl) solution." A cotton thread extending from the glass tip served as a wick to touch the plant and was made conductive by soaking in 1% agar made with 0.1 M KCl. This constituted a substantiation of similar

electrodes developed independently at Iowa State University by the writer in 1970-71 and are subsequently described in this thesis.

Platinum wire electrodes were used by Koostra (19) for insertion into seeds to measure the resistance of seed tissues. The use of platinum minimized the polarization problem. Another way of circumventing polarization when a potential is applied to tissues to measure their resistance or conductance is to use an alternating (a-c) rather than a constant polarity (d-c) voltage. Adaptations of this technique were used by Sinyukhin and Rutkovskii (30) in 1966 as a stimulus to elicit a biopotential response, and by Benner and Sherwood (3) in 1970 to compare plant stem impedances after certain stresses had been applied. In the latter case, the rapidity of the polarity reversal (i.e. the frequency of the a-c) was also a parameter and may be the first usage of sweep-frequency tests in measuring plant impedance.

Direct connection to plants has been made by Sibaoka (29) using metal micro-electrodes inserted directly into cell protoplasma to obtain flux mapping contours. Backster (1) measured the GSR ("galvanic skin response") between top and bottom of plant leaves using 2 x 3 cm flat stainless steel plates on either side, separated by agar-impregnated gauze. Since Backster was primarily interested in psychic responses, possible polarization of his electrodes may not have been considered. Karlsson (17, 18) inserted 0.4 mm gold leads into

plant petioles, since gold has a reduced tendency to polarize.

Additional interpretation of plant biopotentials was attempted by Sinyukhin and Rutkovskii (30) in 1966 when they found correlation between the age of the plant material (Tilia cordata Mill.) and the shape of the biopotential response curves after stimulation by electrical pulses. The two year old linden seedlings had one response pattern during spring vegetative growth, another pattern while blooming, and negligible response during fall senescence. Differences in response curves were also found between healthy and sickly transplants in both the first and second years.

Wheaton (33) and Dorchester (13) reported on both electrostatic and electrokinetic ambient fields applied to growing plants in attempts to alter growth patterns. In these cases, the instrumentation was not sufficient to monitor internal plant signals, and overall growth and yield measurements were too variable to be conclusive.

III. METHODS AND RESULTS

Guidelines

Some guidelines for operational tests were established to facilitate validity and reproducibility of results. Insofar as possible, nondestructive tests of the plants were utilized. When living plants were tested *in vivo* (e.g. in pots), the plants could be reused, tests could be repeated, and effects of intentional changes noted on the same specimens. Nondestructive tests would also be appropriate for later field operations on any commercial scale.

Test Plant Regime

Choice of plant material to be tested was influenced by plant varieties available in the horticulture greenhouses, by the likelihood that the varieties used would continue to be available, with sufficiently common field usage that they could readily survive in public areas, and by their relative freedom from special considerations such as special or unusual temperature, moisture, light or growing medium requirements.

The test plants were started from seeds and grown in a 1:1:1 peat:perlite:soil mixture in the greenhouses. At the time of testing, plants were in 3 or 4-inch pots. The greenhouse regime provided them with adequate daily watering, normal ambient light and temperature, and necessary pest control. Test specimen plants were obtained from the greenhouse at the start of each day, and returned at the end of the day. For any

given variety, plants were selected for uniform size and vigor. Each pot was numbered for subsequent identification.

A. Plant Impedance Tests

1. Methods

Plants were favored that were of a convenient size for handling and for attaching the electrodes. For the varieties tested, the tomato plants (Lycopersicon esculentum 'Rutgers' and L. esculentum 'Supersonic') were juvenile specimens prior to visible flower buds, and about 20 cm tall with stems between 6 and 9 mm in diameter; snapdragon (Anthirrinum majus 'Bright Butterflies') specimens were approximately 25 cm tall with flower buds showing, and stems between 5 and 7 mm in diameter and easily penetrated by pins; pepper plants (Capsicum annuum 'Canape') were without flower buds and about 18 cm tall with 4 mm diameter stems; broccoli (Brassica oleracea Italica 'Calabrese') were also juvenile without flower buds, and 20 to 24 cm tall with 5 to 9 mm stems; zinnia (Zinnia elegans 'Canary Yellow') were 20 to 25 cm tall with flower buds starting to show, and with 6 to 8 mm stems that were less conductive (electrically) than the other plants, so that a 1 cm spacing of the electrodes gave better readings. Most of the plants tested were Rutgers tomato and Canary Yellow zinnia, and their results were chosen for the summary graphs of Figures 2 and 3.

The signals were applied to the plant through a pair of stainless steel pins. They were mounted on a rigid block

which maintained spacing and limited the depth of penetration of the pins into the vascular tissues of the plant stems. Spacings of both 1 cm and 2 cm between pins were tried. The closer spacing was considered more appropriate for the more fibrous and mature stems where the stem conductivity could be expected to be poor. Depth of penetration into stems was set at approximately 3 mm.

Checks of the stainless steel pin electrodes were made to insure cleanliness before they were pressed firmly into the stem of the plant. Tests were made regarding depth of penetration and it was found that once the pin electrodes were deep enough to support their weight and that of their spacing member, further penetration did not appreciably affect results; therefore, depth was not a critical factor.

In some cases, the stainless steel pins were left in the stem of the plant being tested, with the leads disconnected after the initial test. Later, the leads were reconnected for subsequent trials.

Alternative procedures involved removing the pin electrodes during the treatment and either reinserting them in new stem positions or in the previous puncture-holes for later determinations. From test results, it appeared that reinsertion of the electrodes in the same plant stem holes resulted in no greater variation than the day-to-day variations of the same plant with electrodes left in place, or reinsertion of electrodes into a fresh portion of a plant stem, <u>provided</u> that

sufficient time had not elapsed for layers of calloused, suberized, or dead tissue to build up around the old holes sufficient to adversely affect the tissue conductivity. Consequently, electrodes were left inserted in the plant if the test period was only a few days, but were removed and reinserted in fresh areas in the case of longer intervals. This avoided major corrosion of the "stainless steel" that tended to occur with electrodes left in plant tissues for extended periods.

Plant impedance tests were run in an air-conditioned laboratory, using the circuit of Figure 1. During a test period, the air temperature of the room changed very little, and humidity and light levels remained essentially constant.

Additional tests were made of the effect on impedance with frequency for three different diameters of stainless steel wire electrodes in the same plant and with the same spacing, as well as the different spacings between electrodes for a given electrode diameter. To avoid introducing another variable in the series of tests, a uniform pin diameter of 7.62 mm was used on all tests; the electrode spacings previously indicated were kept consistent for given plant species.

Polarization of the electrodes was circumvented by using alternating voltages for plant conductance measurements. A sinusoidal waveform was obtained from a Hewlett-Packard Model 200C sweep frequency oscillator, whose output voltage was constant within $\pm$ 1 db from 20 Hertz (Hz) to 600,000 Hz. Such

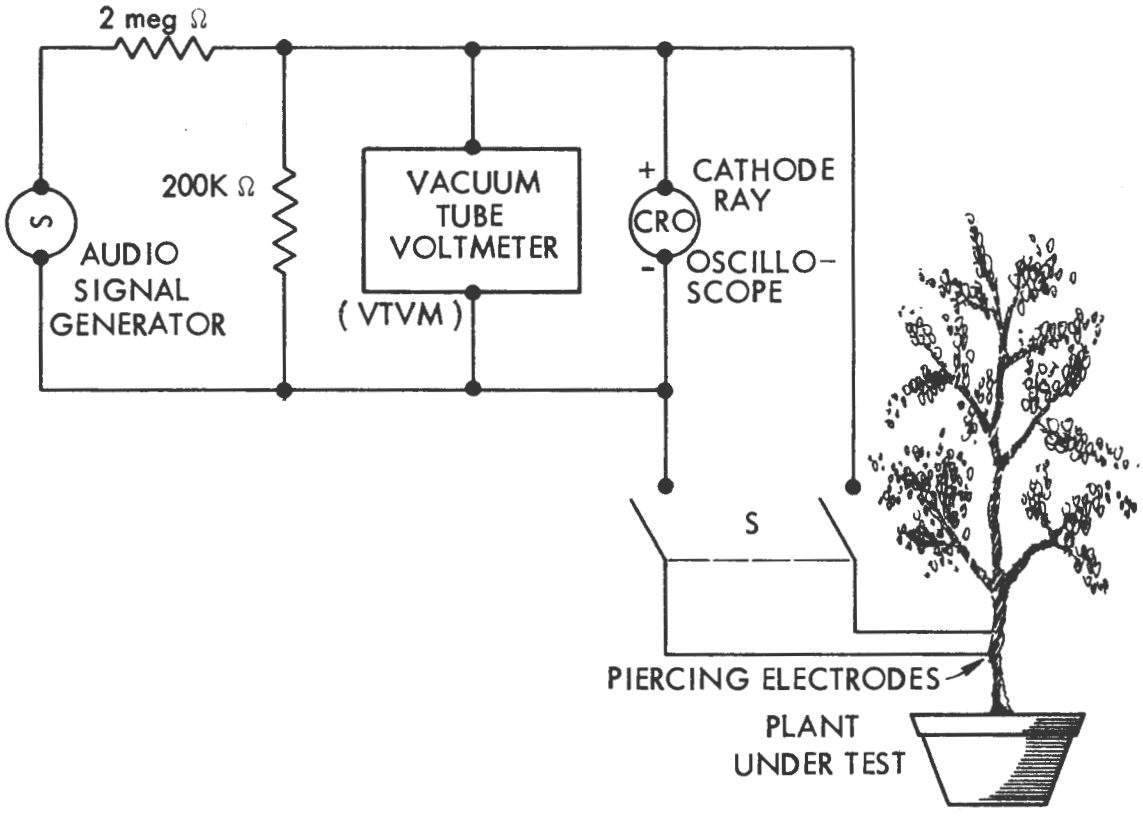

Figure 1. Circuit of equipment used in plant impedance tests

a waveform was considered to be a compatible form of alternating voltage signal for the application. A 1.0-volt (r.m.s.) signal was established as a safe level to be applied to the plant since the tissue resistance path had been found to be of the order of 100,000 ohms. Application of Ohm's law (I=E/R) yielded a maximum current flow in the range of 10 microamperes, a value that various researchers (e.g., Black and Forsyth (4), Dorchester (13), and Lund et al. (20)) had previously determined to be well below a value harmful to the plants.

The voltage levels were read with a vacuum tube voltmeter (VTVM), an instrument having a 10 megohm input impedance, thereby resulting in negligible loading of the circuitry. The 1.5 volt (full scale) range of the RCA Voltohmyst (Model WV-97A) was used, permitting readings to the nearest 0.01 volt.

As indicated in the schematic diagram of Figure 1, a cathode ray oscilloscope (CRO) was also connected in the circuit to monitor the signal waveform. Since this CRO had a 2 megohm input impedance, it had negligible effect in the circuit. As a monitor, the CRO permitted a continuous check on the signal output of the signal generator for any possible waveform distortion by the plant, and also served as a warning of circulating ground currents and/or stray pickup (mostly 60 Hz or its harmonics). A five-inch DuMont 304 oscilloscope served these functions.

A 2 megohm resistance (Figure 1) in series with the oscillator helped isolate it from the plant load, and a

200,000 ohm parallel resistor maintained some fixed load, regardless of whether the 2-pole switch S was open, or was closed to connect to the plant. The VTVM indicated the voltage drop across the plant; this was converted to the corresponding resistance with a current flow of 10 microamperes through the plant.

2. Results

Of the various varieties of plants mentioned previously, only some tomato (L. esculentum 'Rutgers') and zinnia (Z. elegans 'Canary Yellow') plants were tested sufficiently to warrant presentation here. For Figures 2 and 3, each line represents a different plant. For a given plant, successive trials gave essentially identical results. However, other plants of the same variety had individual variations within an overall range. In general, there was somewhat of a decrease of impedance with increase in frequency of applied signal. For tomatoes (Figure 2), this started between 3 and 30 KHz, whereas zinnia (Figure 3) impedances decreased very little until after 30 KHz. The curves for zinnia had a minimum point near 200 KHz, while tomato curves had minima varying between 150 KHz to 500 KHz. For both types of plants, the impedance dropped as much as 20% of the low frequency (1 KHz) value (close to 10 megohms), at the minimum point. There was not a radical differentiation between species of plants by this method.

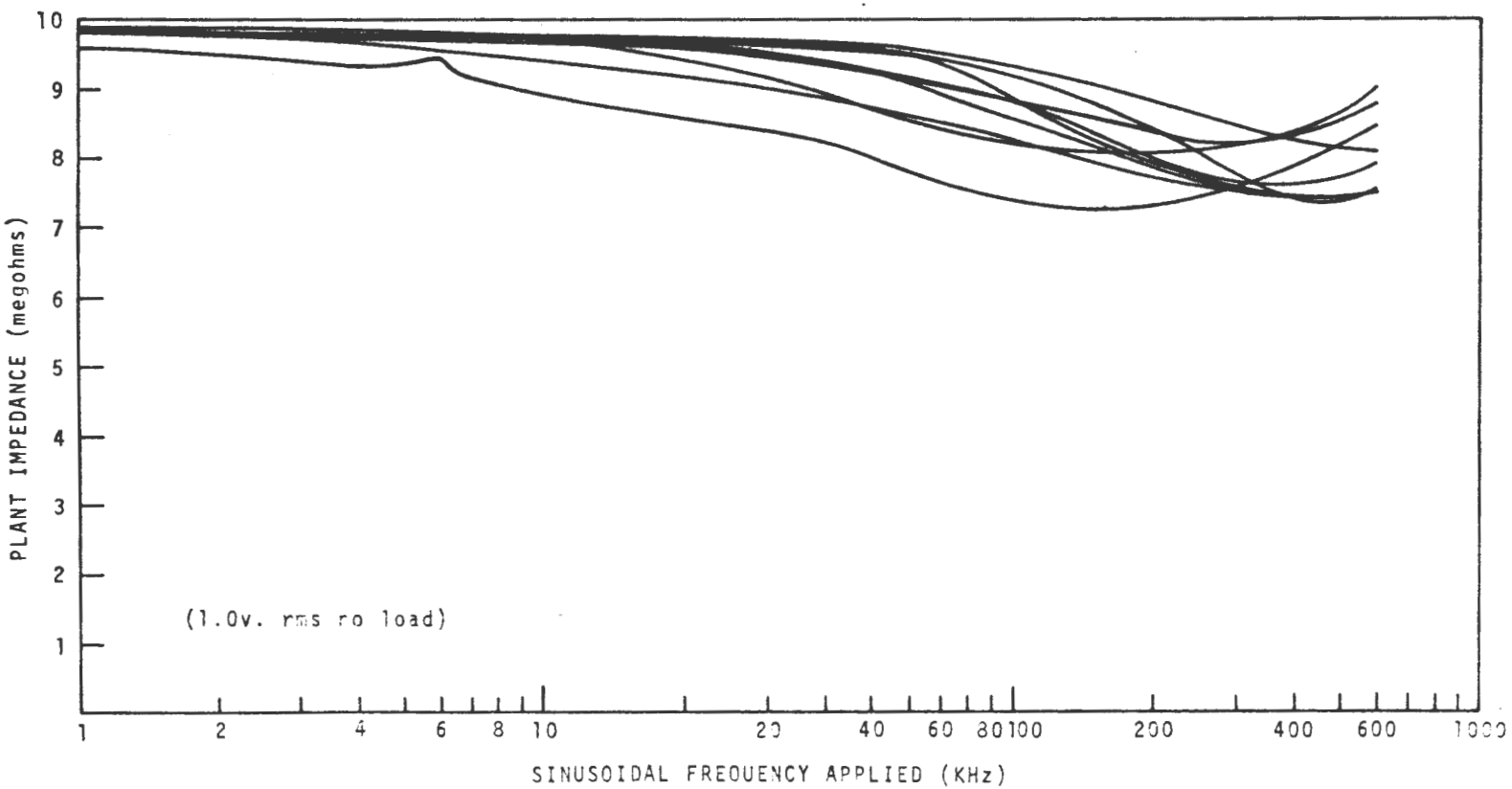

Figure 2. Values of impedance for 9 different tomato plants (L. esculentum 'Rutgers')

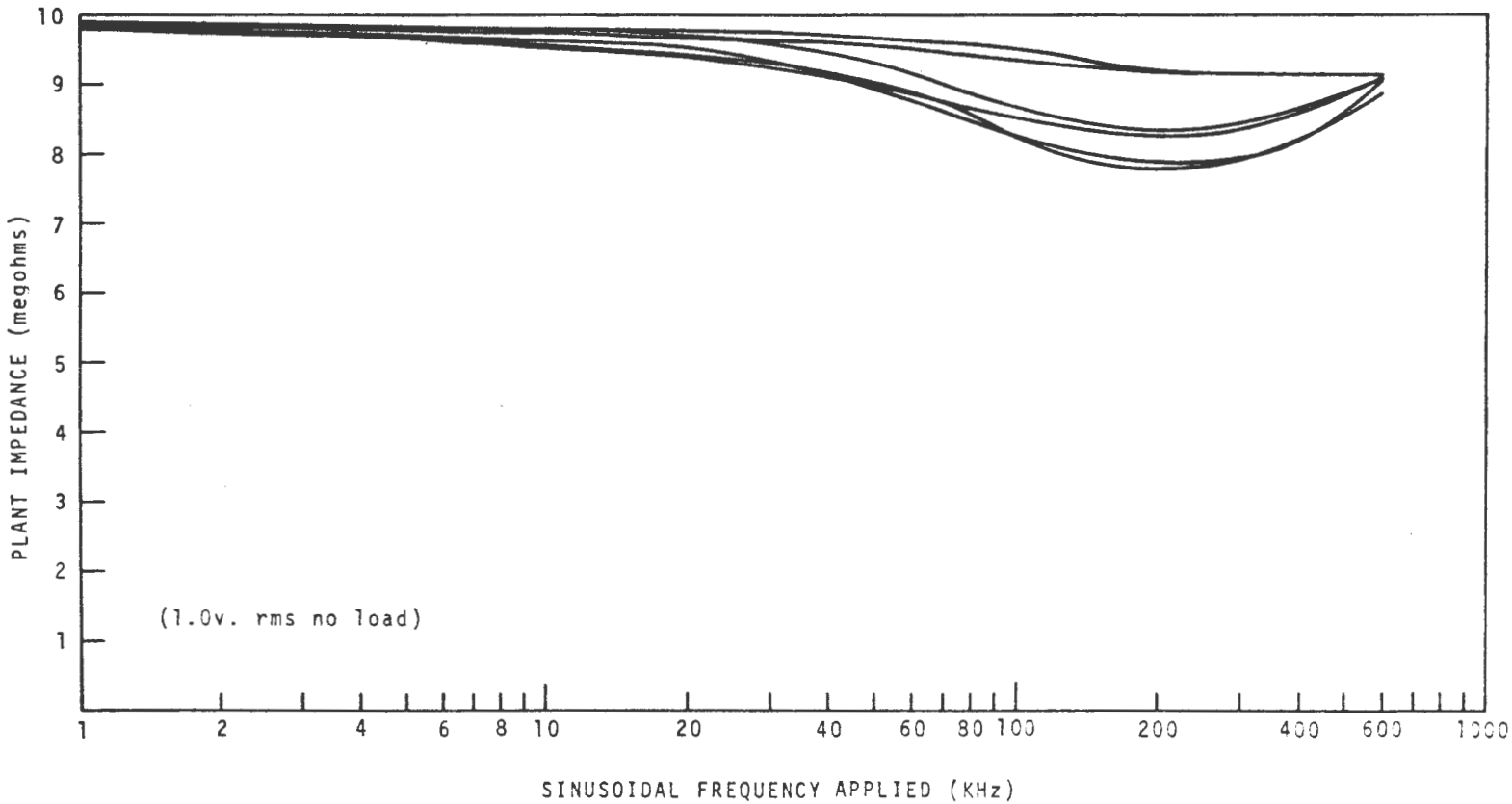

Figure 3. Change of impedance with frequency for 6 different zinnia plants (Z. elegans 'Canary Yellow')

B. Plant Biopotential Tests

1. Methods

Electrodes constitute a key component in successful electrical coupling to plants for effective monitoring of plant biopotentials. Alternatives to piercing pins were sought to avoid the possibility of injury to plant tissues that would alter the desired signals, or complications that could arise from measurements such as those between the upper and lower surfaces of a thin plant member such as a leaf.

Measurement of self-produced biopotentials involves considerably lower levels of signal voltage than the external voltages applied for impedance measurements in the preceding tests. Since the biopotentials are in the range of a few millivolts, noise signals and stray signal pickup become more of a problem especially if the electrode system is high impedance. Low electrode impedance will assist in obtaining the desired signals. A highly conductive interface between the electrode and the plant tissue is also required to get a low impedance system.

The larger the area of contact between electrode and plant tissue, the lower will be the impedance. This needs an electrode contact system that will accommodate indentations, undulations, pubescence or other irregularities of plant surfaces for best conduction. Such accommodation could be either liquid or soft electrodes, or a deformable interface

between solid electrodes and the plant surface.

a. Electrodes Among the possible electrode choices, the following were considered:

(1) Liquid junctions and "salt bridges." These definitely would conform to the upper surfaces of a horizontal leaf and can have low impedance when containing an electrolyte. But for nonpolarization with d-c signals the contact connection would need to be something more than a simple wire. The liquid should not be phytotoxic to plant tissues because any plant cells killed would alter the resistance. While a "physiological saline" solution of 0.85% sodium chloride (NaCl) is isotonic to animal tissues and has been used by some researchers, the sodium level would be undesirably high for plants. It was desired in this program to try to be as isotonic and nontoxic to the plant tissues as practical; therefore, the best choice appeared to be 0.1 N potassium chloride (KCl). A further problem with the use of a liquid arises from its evaporation; this was found to cause a perceptible drift in electrical potentials. In addition, a cup or other type of container would be necessary to retain the liquid on the desired area, especially the bottom side of a horizontal leaf.

(2) Dropping mercury electrode. This would resist polarization and conform to irregular surfaces, but

would not "wet" waxy leaf surfaces and is considered quite toxic. Since its operation depends on a downward dropping, contact to the underside of a horizontal leaf would be a problem.

(3) Flexible gels or pastes that dry out quite slowly. This would include silica gel with its water of hydration, polyvinyl alcohols, lithium chloride gels, dimethyl formamide with perchloride (which would resist polarization), ethylene glycol gel or propylene glycol gel; the latter would also be an effective solvent for wax on leaf surfaces. However, most of these would be phytotoxic.

A nontoxic gel was made from agar. When not in use its water loss was reduced by storing it in a closed container in a refrigerator. Its conductivity was safely improved by incorporation of 0.1 N KCl. It is subsequently described in detail.

(4) Polynuclear hydrocarbons, particularly those that would wet any leaf wax. Predominantly this would involve pyrene iodide in a suitable carrier gel or tetracyano-dimethane quinone (TCMQ). Both of these would resist polarization, but would be more phytotoxic than the 0.1 N KCl.

(5) Fibrous wicks, strings or fabrics impregnated with conductive materials. Graphite impregnation was found to result in rather erratic conductivity, as

evaluated by a Grass Instruments Co. TIM-1 tissue impedance meter. In this device an a-c signal source was used to circumvent polarization problems and a calibrated meter indicated total circuit impedance when below 15,000 ohms. The Dow Co. conductive resin ECR-34 provided acceptable impedance of 5,000 to 6,000 ohms for a cotton cylinder 1½ cm in diameter and 8 cm long. However, when it was allowed to air dry the resistance increased to between 200,000 ohms and 1 megohm. The ECR-34 also had to be diluted 1:100 to reduce visible phytotoxicity. Even at the diluted concentration, it could be a disturbance to the plant over a period of time.

(6) Nonpolarizing contacts. Most popular have been zinc sulfate ($ZnSO_4$), calomel, and "chloridized silver." The latter two were found, in previous work conducted by the author, to have a tendency to be photovoltaic; this would be important if light were used as a stimulus. No photovoltaic effects were observed for Ag/AgCl, as long as the AgCl coating over the silver wire was unbroken. The utility of these contacts lies primarily in their combination with some of the foregoing electrode choices. The first two of the nonpolarizing contacts usually include a liquid junction to the plant, with its attendant problems of confinement of the liquid, signal drift from

evaporation, and isolation of phytotoxic substances.

(7) Solid surface electrodes. Such electrodes are commonly used on animals, usually with conductive interfaces. Medical electrocardiography (ECG) often uses 1-3 cm diameter nickel-plated, stainless steel, silver, or chrome-plated flat disk electrodes with a conductive "ECG jelly", usually with crystals of NaCl to help abrade the outer layer of skin of animals when the electrodes are "rubbed in" for better contact.

Backster (1) used similar flat disks in making contact to the upper and lower surfaces of plant leaves; these disks were held in place with a pseudo "C-clamp." He used agar-impregnated gauze pads between the 2 x 3 cm stainless steel plates and the leaf surface. However, both these electrodes and similar disks of solder used earlier in this I.S.U. project had impedances of approximately 100,000 ohms, and were thus prone to an adverse signal/noise ratio. For plants, such electrodes also exclude free movement of air and moisture to and from the covered area.

An early practical arrangement used by the author was a composite electrode that made a soft contact to the surfaces of a plant leaf through a wick which conveyed conductive liquid from a reservoir to the leaf surfaces. The flow and quantity were controlled so that the liquid did not drip off and had a reduced amount of evaporation. The reservoir for the isotonic 0.1 N KCl was contained in a section of 0.635 cm ($\frac{1}{4}$-inch) O.D.

soft glass tubing that had been drawn to a narrow capillary section at one end. A wick was worked through this small end to provide a continually moist path to the leaf surface. Since some calomel electrodes employ asbestos fibers sealed in glass, asbestos was tried first in the form of asbestos sleeving but was found too inflexible and had poor wicking action.

Next, 1.588 mm (1/16-inch) diameter Refrasil fiberglass sleeving (type B-24A) was epoxyed in place and gave better results. With a hollow center the sleeving was over-generous in liquid flow for downward-slanting electrodes. Slitting the sleeve and wrapping it with nylon thread yielded good wicking action. Some cotton strings also gave good wicking action. In both cases, they were epoxyed into the capillary ends of sections of glass tubing.

For a nonpolarizing contact in this electrode, a silver-silver chloride wire was held in the center of the KCl reservoir by a cork in the larger end of the miniature pipette, as shown in Figure 4. The 7½ cm length of 20-gauge silver wire had been made the anode in a saturated sodium chloride solution and a thin layer of silver chloride electrodeposited. A short end of the silver wire protruded from the cork on the side away from the solution and the center conductor of RG-174 U coaxial cable was soldered to it for a signal lead.

The coax shield was "floated" at the electrode end and grounded at the amplifier input. Initially, both the soldered joint and the glass tube were covered with black electrical

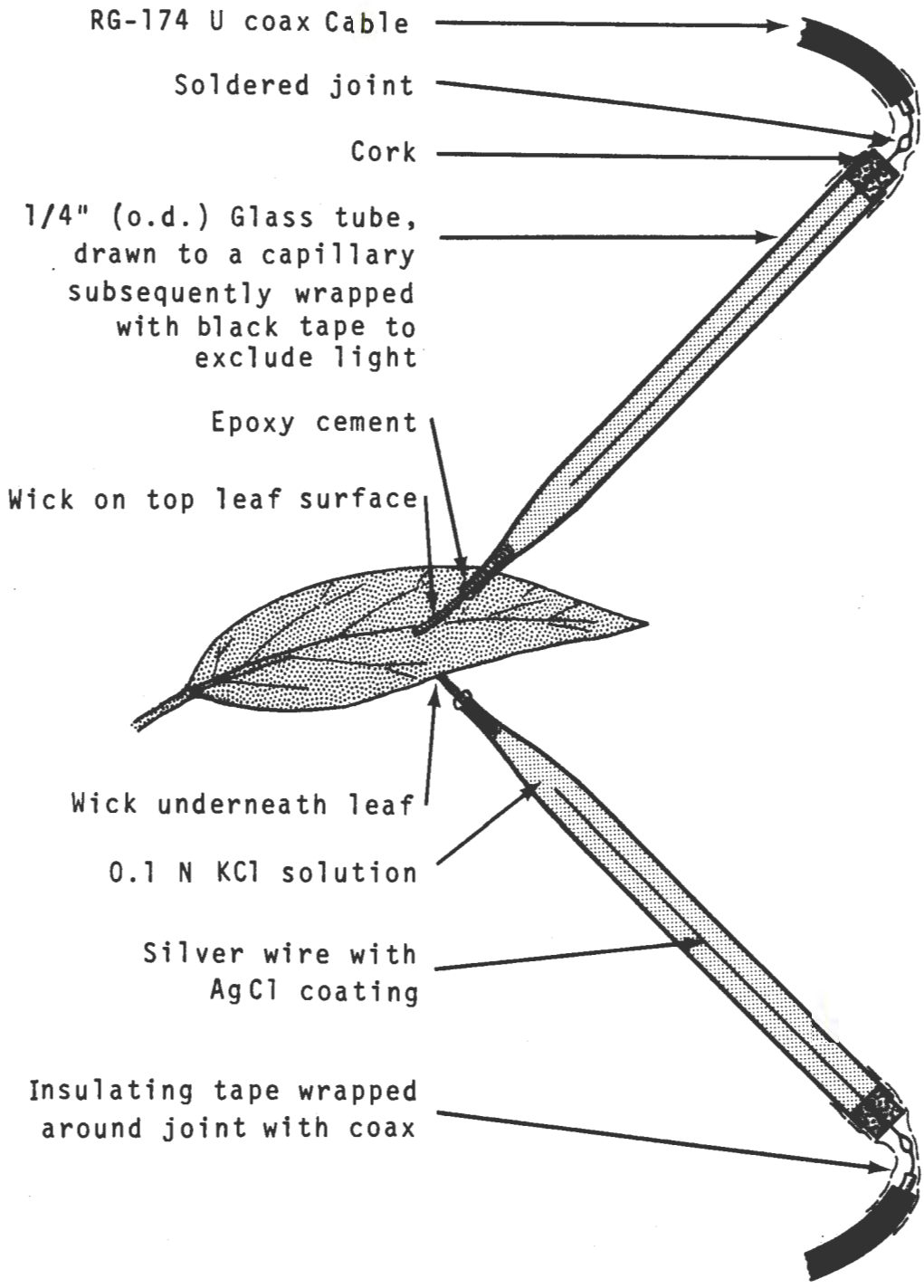

Figure 4. Plant surface electrode, pipette type (high impedance); for detecting biopotential signals between top and bottom of a plant leaf, in vivo

tape, both as insulation and to exclude light, but it was subsequently proven that the silver chloride coating was sufficiently homogenous to preclude photovoltaic effects.

Several species of plants were used for testing, including Aglaonema commutatum, Philodendron micans 'Bronze Beauty', and Zinnia elegans 'Canary Yellow', the last being shown in use in Figure 5. When light was used as a stimulus, initial tests were attempted by turning the overhead room lights on and off, or leaving the plants in a darkened room and illuminating them with a flashlight. As was later established, both light sources were well below the 600 ft-c illumination intensity which was found necessary for good response. The tests did show, however, that the electrodes were of sufficiently high impedance to be vulnerable to artifacts and stray pickup.

The TIM-1 tissue impedance meter indicated something greater than 15,000 ohms, the upper limit of the TIM-1, when a pair of pipette electrodes had their wicks touching in a pool of 0.1 N KCl. Such a procedure was also used when setting the zero signal level of the recording system. Testing one electrode between the end of the wick and the lead wire gave comparatively high resistance. In order to localize the source of the high impedance, a pair of Ag/AgCl center electrode wires were tested in a solution of 0.1 N KCl and showed negligible resistance. The high resistance was found to come primarily from the long narrow wick, which was not helped by the small area of contact on a leaf surface.

Figure 5. Pipette electrodes on leaf of *Zinnia elegans* 'Canary Yellow' *in vivo*

Negligible offset voltage was needed from the Grass preamplifier, and the electrodes were relatively stable with time, even with recording system sensitivities of 0.5 mv/cm or 0.1 mv/cm, providing there were no transient movements of personnel in the vicinity. However, if the electrode wicks became dry or excess liquid accumulated and evaporated or dripped off the leaf pronounced drift or discontinuities appeared in the tracings.

In spite of the foregoing operational deficiencies, it later was learned that Dr. B. Pickard (26) had independently developed small pipette electrodes that were quite similar to those just described. Her 4 cm-long glass pipettes were about 4 mm in diameter with a 0.5 mm opening at the small end through which protruded a cotton thread impregnated with 1% agar. The rest of the pipette was filled with 0.1 N KCl, and a Ag/AgCl wire was placed in the center as a nonpolarizing electrode. She accommodated the high impedance with extensive shielding, which included a double-screen cage, and a special preamplifier adjacent to the plant. She reduced evaporation drift by having a humidifier constantly blowing on the plant and electrode.

Subsequent efforts of the author proceeded toward development of low impedances electrodes which resulted in a better signal/noise ratio and fewer shielding problems. Moist agar gel, under gentle pressure, was found to conform reasonably well to most leaf surfaces. Its resistance was reduced from 1000 ohms between 1 cm^2 metal plates to no measurable

resistance on the TIM-1 by incorporating 0.1 N KCl. Tests were made to find the optimal amount of 0.1 N KCl to add to the agar gel.

One percent by weight of agar powder into a 0.1 N KCl aqueous solution was slow to gel and was still soft after two days; it also had a rough surface. Four or 5% agar resulted in a gel that was rough and grainy. Two or 3% agar resulted in better gels that became firm in ½ hour, but the 3% was bubbly. Therefore, the 2% was established for further electrode work.

A recessed area or cup was employed to contain agar gel to press against the plant surface. Electrical contact was accomplished by a flat silver wire spiral embedded in the agar. This spiral had a continuous layer of AgCl electrolytically deposited over its surface. Pressure of the agar electrodes against the plant surface for firm but nondamaging contact was accomplished by a threaded screw arrangement.

A series of improvements were made in developing agar electrodes. In general, the succeeding versions were smaller; other improvements included a swivel arrangement for the agar container, so that the agar would not be rubbed off as the pressure adjusting screw was turned. It was also found necessary to make the connections between the Ag/AgCl spiral and RG-174 U coax cable center conductor external to the agar compartment. This precluded electrolytic action and generation of local galvanic voltages.

The majority of the tests were run with electrodes designated Agar III and Agar IV, which differed only in physical size and were constructed as shown in Figure 6. Swivel action with firm positioning was accomplished by means of a banana plug/jack arrangement. This also permitted the electrodes to be easily removed from their support arms and stored in a sealed jar with moist atmosphere in a refrigerator. This greatly reduced the drying out of the agar and thus prolonged the useful life of the electrodes. Previously, it was necessary to go through the cumbersome procedure of putting the combined electrode/support arms/spacer assembly in a large plastic bag when refrigerating, then reassemble for use. The electrode assembly details are stretched out as an exploded view in Figure 6 for clarity.

For Agar III a block of lucite, 1.8 cm by 1.8 cm and 3 cm long, was drilled and tapped at one end to accommodate the banana jack. This was a mechanical connection and was insulated from the 1.3 cm diameter cavity for the agar drilled in the other end. The active cavity had a side hole just big enough to pass a 20-gauge silver wire. A silver wire spiral was again formed and treated as before to have a AgCl film. While the completed spiral was held in place, the cavity was poured full of 2% agar-0.1 N KCl gel, then the gel was mounded up to protrude beyond the edge of the plastic block enough to contact the plant surface without the holding block interfering.

The banana plug that mated with the banana jack in the

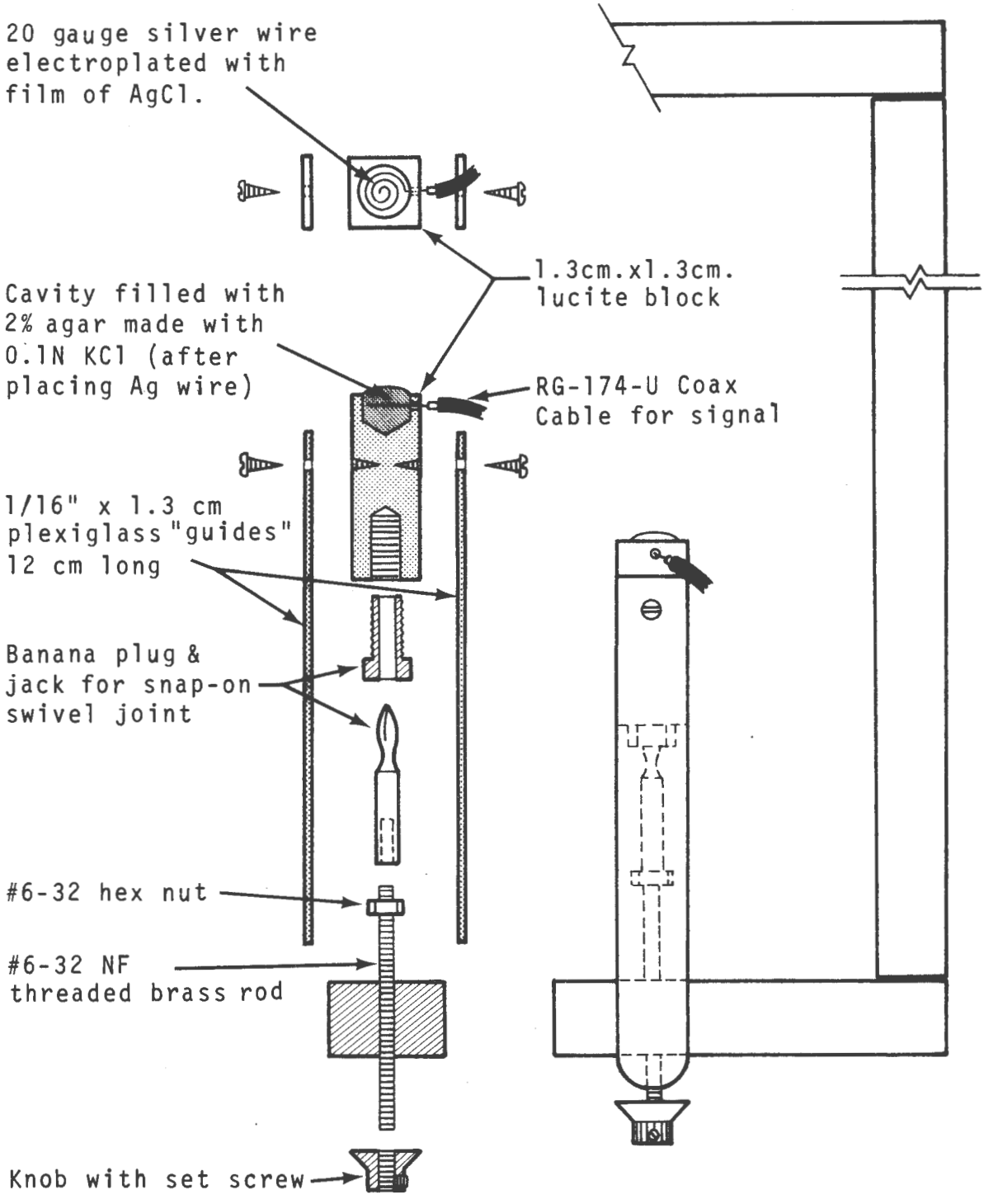

Figure 6. Plant surface electrodes, Agar III type (low impedance); for detecting biopotential signals between top and bottom of a plant leaf, *in vivo*

plastic block was threaded and locked with a nut onto a brass rod that passed through the top support arm, then a knob was securely attached to the protruding end of the rod to make it easy to rotate. This was used to advance or withdraw the electrode from the leaf surface. The plastic electrode block was prevented from rotating as the rod was turned by flat plastic guides of 1.588 mm (1/16-inch) thick lucite fastened to each side of the electrode and extending to straddle the support arm. An identical arrangement was used on the lower support arm for the electrode that went to the underside of the leaf.

All lucite components were left clear to reduce leaf shadowing. The RG-174 U coax cable signal lead had its center conductor soldered to the silver wire of the electrode spiral where it protruded out the side of the plastic block, thus keeping the connection out of the agar cavity. The shield was floated at the electrode end and grounded at the preamplifier input.

To provide better support on the opposite side of the leaf for offset electrodes, a flat piece of 3.175 mm (1/8-inch) thick lucite was cut and shaped so it could be held by a clamp from the ringstand and fitted under the leaf below the top electrode. The portion of this "plate" that was beneath the leaf was drilled as full of holes as structural integrity would permit to allow as much air and moisture as possible to reach the leaf undersurface.

For support over the top of the leaf against the bottom electrode, a plastic pill bottle of methyl methacrylate and 20 mm in diameter was utilized. A 12 mm hole was drilled in the center of the bottom, thus leaving a ring of plastic, and the sides were cut out except for three 5 mm "straps" 120° apart, leaving a sort of open cage. When heated in an oven the top ends of these vertical straps softened enough to be bent to a common center point. A hole was drilled through all three when overlapped, and a 6-32 threaded rod run through and retained by nuts for support. The result was a 40 mm high open "cage" of plastic suspended from an overhead arm extending from the ringstand. This arrangement permitted most of the light, air, and humidity to reach to top surface of the leaf.

The Agar III electrodes had the same desirable low impedance and soft contact to a plant leaf as their predecessors. Impedance values, as determined by the TIM-1, for electrodes on opposite sides of a plant leaf were in the range of 2,000 to 5,000 ohms. When the electrodes were offset, e.g. 2 to 3 cm center-to-center, the path resistance was somewhat higher, but the light/dark response was greater.

When pepper plants were tested it was found that the 60 cycle hum pickup was reduced when the amplifier system ground was extended to the soil in the pot with the plant. This was adopted as standard practice for all operations.

Agar IV electrodes were improved detachable agar electrodes with the smaller dimensions of Figure 6. The smaller

size of about one-half the area of contact of Agar III caused less shadowing of a leaf.

Additionally, the suggestion had been made that the silver wire used in the spiral of the electrode might have an undesirable high copper content so a precautionary procedure was added. With application of a low voltage, the silver wire was first used as a cathode in a weak HCl solution having a pH around 6.5, to electrolytically remove the worst of the copper from the surface of the wire. Thereafter, the previous procedure was followed, namely shaping the wire to a spiral then cleansing it with a mild acid and distilled water rinse after it had been handled. Next, it was made the anode in the saturated NaCl bath, at low voltage, to form the AgCl film.

Better support under a plant leaf for the top electrode was designed from a piece of fiberglas window screening stretched across a small wooden frame, 35 mm wide by 90 mm long, and 90 mm deep, with a small turnbuckle arrangement to maintain adequate tension in the screening. This screening had more "give" to accommodate the leaf and greater area of openings than the flat rigid plastic.

A further refinement in the testing procedure was initiated at the same time as the Agar IV version. The plant, electrodes, and supporting stands and lights were enclosed in a box 61^2 cm by 91 cm high. The sides were a wooden framework covered with black greenhouse shading cloth which excluded light, yet allowed sufficient room air passage to preclude

excessive heat build up and permitted equilibrium of air moisture. The removable top also contained a vent fan and a pair of 2-lamp fluorescent fixtures totaling 80 watts. The black cloth was detachable at its seam, providing easier access for set up and adjustments. This enclosure permitted light/dark cycling without the need to turn off room lights.

In operation it was found desirable to lower the fluorescent fixtures half way down in the box toward the plants. However, this provided only a nominal 400 ft-c at plant leaf level, so an auxiliary light was included in the enclosure. A microscope illuminator with its narrow light beam focused on the leaf-electrode junction served to raise the total light level at that point to 600 ft-c and aided the plant response, but was not enough light to overheat the leaf, avoiding the complications of filtering the light through water or other heat-absorbing measures. A thermometer was also inserted from the side of the enclosure at plant level to permit monitoring of the ambient air temperature. The enclosure proved quite satisfactory and permitted light/dark cycles independent of the laboratory regime.

The electrodes were the most reliable and satisfactory to use of those devised to date here or reported in the literature. It was determined that any shrinkage or abrasion of the agar surfaces could be amended by application of more drops of the hot 2% agar-0.1 N KCl solution, since extra gel could be remelted at 82°C and remained liquid for several degrees below

this temperature. If properly done the new and original agars bonded adequately. It has appeared best to let the protruding agar cool and stiffen for a half day or more before using.

Evaluations were made of the completed electrode response to changes in light level, changes in temperature, and time stability. A pad of gauze wetted with 0.1 N KCl was placed between a pair of operational electrodes connected to operating circuitry. The electrodes were subjected to alternating dark/light (600 ft-c) cycles of several minutes duration each. The only perceptible change was approximately 0.1 mv between contacts, with insignificant differences over a span of 1 hour. The amplitude changes were of an order of magnitude lower than the plant biopotentials in the ensuing tests, and the time period was more than adequate for plant growth manifestation. Therefore, the inherent electrode changes were not considered to affect the results significantly.

Studies were made of the effects of heat changes on the electrodes when operating in a complete system with a zinnia plant and 600 ft-c of illumination. This was done by means of heated air from a Master Appliance Corp. (model HG301-J) heat gun. With an overall temperature change from 25°C to 42°C in small gradual increases, the overall net electrical potential change was less than 1 mv. Since the air temperature within the test enclosure normally stayed within a few degrees, the results of the 17°C differential test implied that temperature changes in normal tests would not significantly affect

electrode response to biopotentials.

b. Electronic presentation Additional equipment was required to provide the necessary amplification for the relatively small plant biopotentials and to yield a permanent visual record. D-c amplification was preferred, so that even slow changes or voltage level shifts could be monitored, since a-c coupled amplifiers would pass only relatively rapid changes in plant signals. It was further desired not to impose a load on the plant under test.

These requirements were met with Grass[1] equipment. A model 7P1B preamplifier with chopper feedback for stability, was used at 10 meghom input impedance. Its bandwidth was d-c to 40 Hz for $\frac{1}{2}$ amplitude, with a maximum sensitivity of 10 microvolts/cm of recording pen deflection. Inherent noise and signal drift of the preamplifier were 3 microvolts and 3 microvolts/hour respectively. It was followed by a Grass model 7B polygraph with a driver-amplifier having a bandwidth of d-c to 75 Hz, a pen oscillograph using curvilinear paper, and a 12-speed pushbutton electric shift chart drive. The combined amplifier and pen system was linear within +5% or -10% from d-c to 45 Hz. Signal amplitude was down 50% at 70 Hz. Such equipment capabilities greatly reduced the likelihood of unintentional variations from equipment artifacts. This placed more dependence on techniques and signals from the test plants.

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[1]Grass Instrument Co., Quincy, Mass.

A 60 Hz notch filter on the driver amplifier was utilized mainly to reduce hum from the fluorescent lights. Calibration signals of ± 100 mv and 1-second time marker pips were available if desired on the paper chart record.

Adjustment of the offset voltage from the preamplifier facilitated centering of the signal on the chart for the best display. Fixed steps of gain change on the preamplifier permitted rapid change of signal scale factor to accommodate either wide signal swings, or finer examination of small phenomena. For most tests, 1 mv/cm sensitivity was found to be the best compromise. Normal runs were made at a chart speed of 10 mm/min, but were slowed to 2½ mm/min for night runs, and were speeded up to as much as 100 mm/sec to record transient phenomena.

Numerous zinnias were tested prior to exposure to a pollutant. Some plants served as controls and were never exposed, thereby providing reference for any changes with time or age. Others were subsequently exposed to 2,4-D in various established concentrations, or tested after 2,4-D exposure only when normal biopotential patterns were sufficiently established. All the biopotential measurements were made with the potted plant in the enclosed box which rested in a screened hood, next to the Grass polygraph within the air-conditioned laboratory. Control and test plants were taken to isolated treatment chambers for the pollutant exposures.

The procedure for making a biopotential test sequence of

either treated or untreated plants was essentially the same. While the Grass polygraph was warming up, the agar electrodes were removed from their humidified container in a refrigerator, fastened to their support arms, and connected to the input cable. It was found that stable operation was reached sooner if a pad of gauze moistened with a KCl solution was placed between the electrodes. Presence or absence of light was found to make negligible difference. The recorder was run at its lowest speed while the tracing reached a stable level with any necessary adjustments of the offset voltage to center the tracing; this usually required 10 to 15 minutes.

Next, a potted plant was put into the enclosure and one of its leaves selected and substituted for the gauze between the electrodes. Pressure on upper and lower leaf surfaces was just enough to keep the leaf from being movable since too much pressure would eventually result in damage to the leaf tissue.

A lead from the amplifier system ground was put into the soil of the pot, and the ringstand and metal frames of the lights were also grounded. During all connections and changes the Grass amplifier was switched to standby mode to prevent possible overloading from transients.

With the plant connected and polygraph on, the plant-electrode system was allowed to stabilize on the chart tracing, usually requiring 20-30 minutes. The enclosure cloth was closed and internal lights either on or off, depending on what the intended stimulus would be. Meanwhile, a record was

made of the plant number and its history (variety, treatment, maturity status, etc.), which amplifier connection (G_1 or G_2) went to the top or bottom electrode, amplifier system sensitivity (gain setting), any offset voltage (and its polarity), air temperature in the enclosure near the plant, and the chart speed that would be used when a test was started and stimulus applied.

When a test of a plant specimen was begun, the wall clock time was noted as the stimulus was initiated, i.e. lights either switched on or off, and an event marker "pip" applied to the chart paper at that point.

During a test, periodic notations were made at the respective points on the strip chart of the time and air temperature in the plant enclosure. If initial surges of a biopotential tracing ran the risk of going off-scale, it was deemed better to reduce the amplifier gain by a fixed and noted increment to preserve the waveshape rather than to change the offset voltage and thereby artificially shift the tracing.

For light/dark cycling over extended periods, an a-c line driven electric timer (Paragon JW-60-0) was used to turn the lights on and off. Its maximum period was one hour, which could be apportioned to any combination of lights on/off totaling one hour. The exhaust fan in the enclosure was arranged to go on and off with the lights, which aided in dissipating the heat from the lights for more even inside air temperature.

At the end of each test the paper chart drive would be stopped and the amplifier put on standby before the electrodes were backed off from the leaf. The plant was then removed. Both the plant leaf and the electrode surfaces were rinsed with distilled water at the conclusion of usage, as a precaution against drying of the electrode or damage to the leaf surface.

2. Results

The biopotential results that follow were obtained by the stimuli of illumination (ON stimulus) or termination of illumination (OFF stimulus) at the 600 ft-c level previously indicated.

a. Stability The possibility of light causing a photovoltaic response of the electrodes themselves was dispelled by the test procedures already covered. A similar temperature stability test of the electrodes that were previously described was run over a 17°C range as tabulated in Table 1.

As indicated before, the results of these checks showed that light and temperature change effects on the electrodes were of a lesser order of magnitude than the effects of intentional stimulation, so should not adversely affect the validity of the results.

After the stability of the electrodes to light and temperature changes was established, the complete biopotential operation with the plant included was examined. The time stability and repeatability of biopotential responses for a

Table 1. Effect of temperature on self-generated potentials of Agar III electrodes in direct contact with themselves

Ambient temperature °C	Electrode potential change millivolts
26-31	+0.3
32-35	+0.2
36-39	-0.2
40-43	+0.2

given plant under repeated stimuli were both apparent from prolonged cyclical runs. Using the electric time clock, five or more complete ON/OFF cycles were obtained for some of the plants; for a given plant, the ON/OFF patterns were essentially "carbon copies" of each other over periods as long as 10 hours. An example is shown in Figure 7 as typified by Zinnia No. 55. No fatigue of the plant or electrode was apparent.

b. Chart records Biopotential chart graphs obtained with the agar electrodes had characteristic patterns. All plants tested at adequate light levels had an immediate reaction when lights were turned on or off. The biopotential level, whether positive or negative, appeared to overshoot in the other direction. The amplitude tended to decrease with time, and the period of the resulting waveforms increased with time. The phenomenon was more pronounced in some plants than others, and some had the opposite polarity. However, the

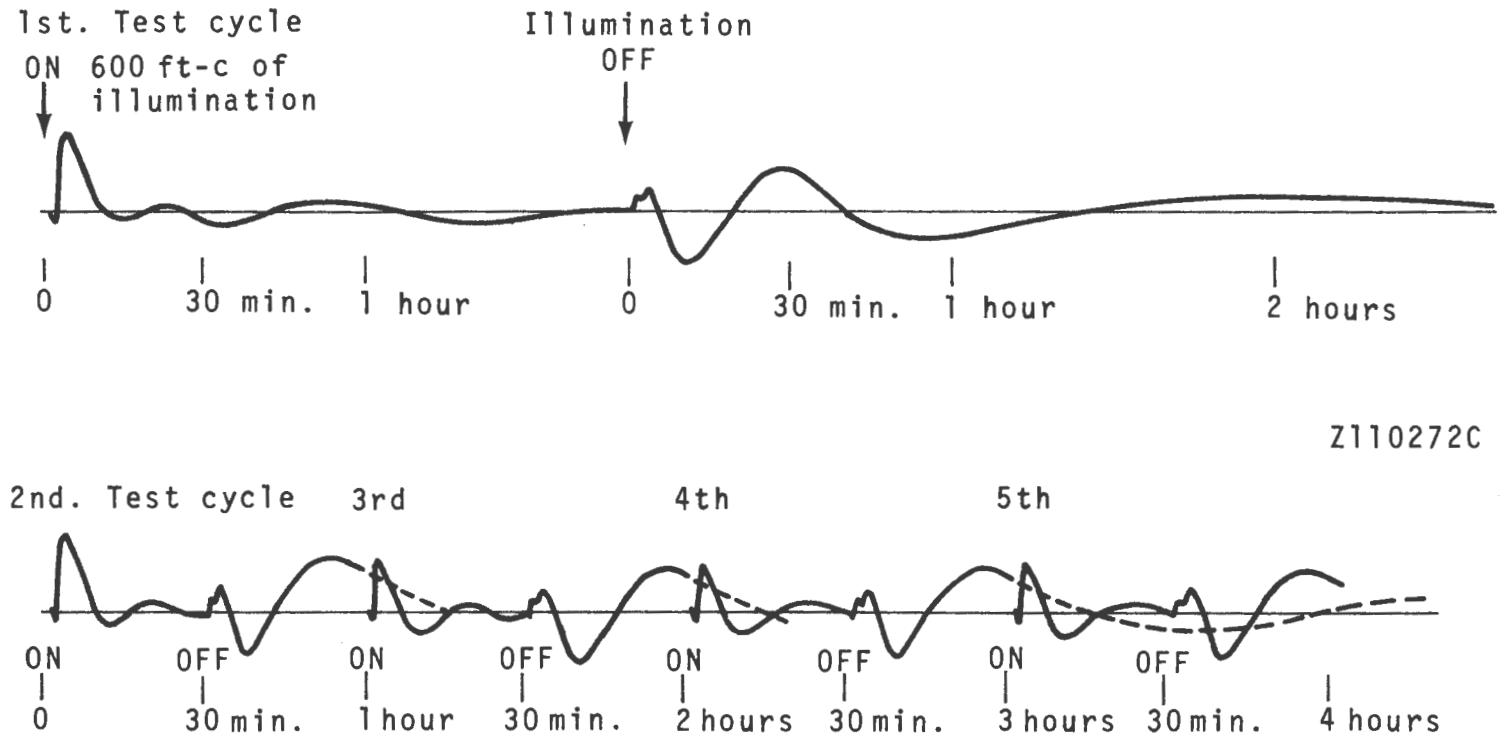


Figure 7. Condensed biopotential waves obtained from Zinnia elegans No. 55. An automatic time clock changed light stimulus between off and on every 30 minutes from the second cycle on, thus foreshortening the natural curve

general pattern was similar: the curves had the appearance of an overreaction in one direction to the change in illumination, followed by an opposite correction that overshot to a lesser extent.

After the "wave train" had damped out and reached something of a steady state value, the initiating stimulus was intentionally reversed. Thus, if initially the lights were turned on they were next turned off, or vice versa, and another wave train started usually of opposite polarity. A generalized representation of this phenomenon is presented in Figure 8.

Since faster chart speeds gave better definition of the biopotential curves at the time of stimulus application, the actual recordings were usually done at paper chart speeds of 10 mm per minute or 25 mm per minute. Some of the more pertinent chart sections have been manually condensed to the equivalent of approximately ½ mm per minute for purposes of presentation.

Figure 9 shows some condensed curves for Z. elegans 'Canary Yellow' No. 45. The waveforms when the lights were turned on were fairly typical of most zinnias and several other varieties of plants; more differentiation sometimes appeared in the waveform when the lights were turned off. Figure 9A shows typical curves for both the ON and OFF stimuli for the zinnia before any treatment.

Figure 9B depicts the results from the same plant under the same stimuli after being exposed to 2,4-D (50 ppm for 10 min) approximately 20 hours before testing. Besides the

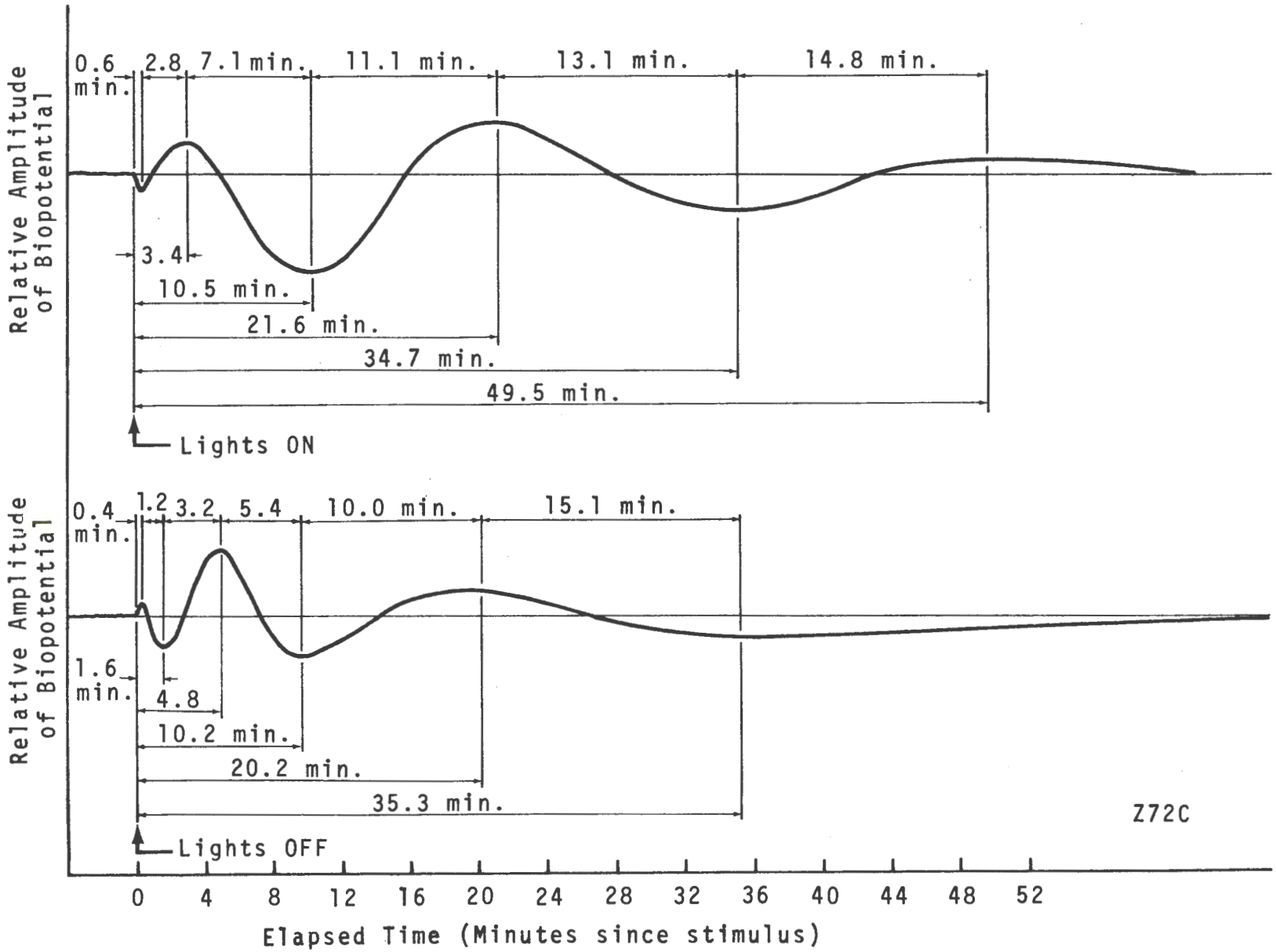
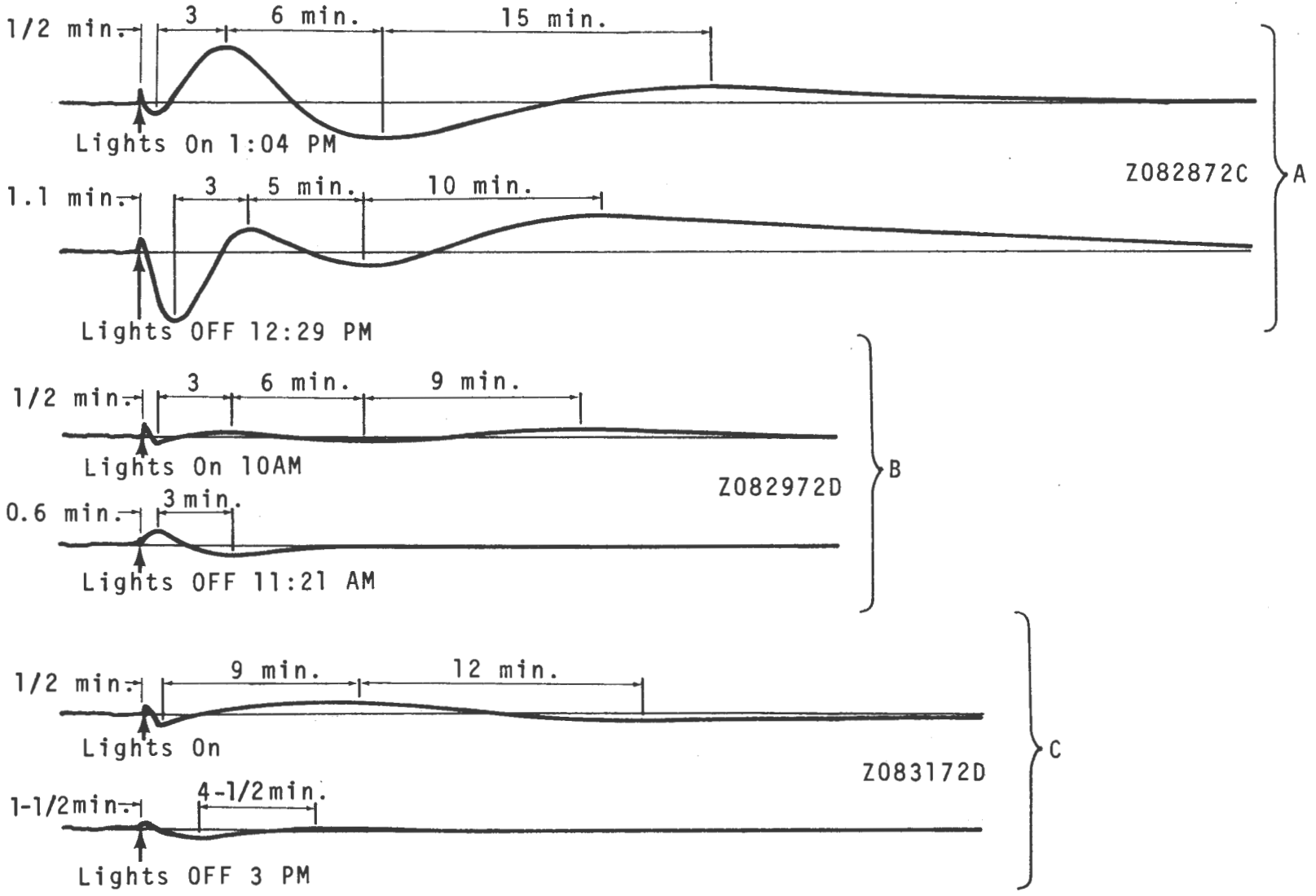

Relative Amplitude of Biopotential
0.6 min.
2.8
7.1 min.
11.1 min.
13.1 min.
14.8 min.
3.4
10.5 min.
21.6 min.
34.7 min.
49.5 min.
Lights ON
Relative Amplitude of Biopotential
0.4 min.
1.2
3.2
5.4
10.0 min.
15.1 min.
1.6 min.
4.8
10.2 min.
20.2 min.
35.3 min.
Lights OFF
Z72C
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Elapsed Time (Minutes since stimulus)

Figure 9. Biopotential responses from Zinnia No. 45 *Z. elegans* 'Canary Yellow' using Agar III electrodes and stimuli of 600 ft-c of illumination ON or OFF:

A. Before treatment

B. One day after exposure to 50 ppm of 2,4-D for 10 min.

C. Three days after exposure to 50 ppm of 2,4-D for 10 min.


1/2 min.
3
6 min.
15 min.
Lights On 1:04 PM
1.1 min.
3
5 min.
10 min.
Lights OFF 12:29 PM
Z082872C
A
1/2 min.
3
6 min.
9 min.
Lights On 10AM
0.6 min.
3 min.
Lights OFF 11:21 AM
Z082972D
B
1/2 min.
9 min.
12 min.
Lights On
1-1/2min.
4-1/2min.
Lights OFF 3 PM
Z083172D
C

obvious reduction of biopotential amplitude (sensitivity remained at 2 mv per cm), there were definite changes in the times between crests and troughs of the waves, both for a stimulus ON and stimulus OFF.

It may be noted for the OFF stimulus of Figure 9B that the predominant crest was upward rather than downward as in Figure 9A before treatment. Three days after treatment (Figure 9C) neither wave form displayed appreciable activity. For the ON wave train the upward 3-minute wave crest had essentially disappeared.

Zinnia elegans 'Canary Yellow' No. 46 waveforms are reproduced by two figures, the first showing the response before treatment, and the second after treatment. Figure 10 covers both ON and OFF stimuli prior to treatment. Both waveforms are comparable to those of Zinnia No. 45, with slight differences in the wave periods perhaps due to plant-to-plant variation. The lights OFF waveform for Zinnia No. 46 before exposure (Figure 10) does differ from the comparable curves for Zinnia No. 45 (Figure 9A) after the first trough. The next crest occurs after 3 more minutes for both. But for Zinnia No. 46 it is at a low level and goes into a mild dip in another 3 minutes compared to the more pseudo-sine wave like pattern of Zinnia No. 45 after 3 and 5 minute intervals. It could be postulated that a secondary wave train (a harmonic?) of lesser amplitude may have been asserting enough influence to partially combine and alter the primary wave train.

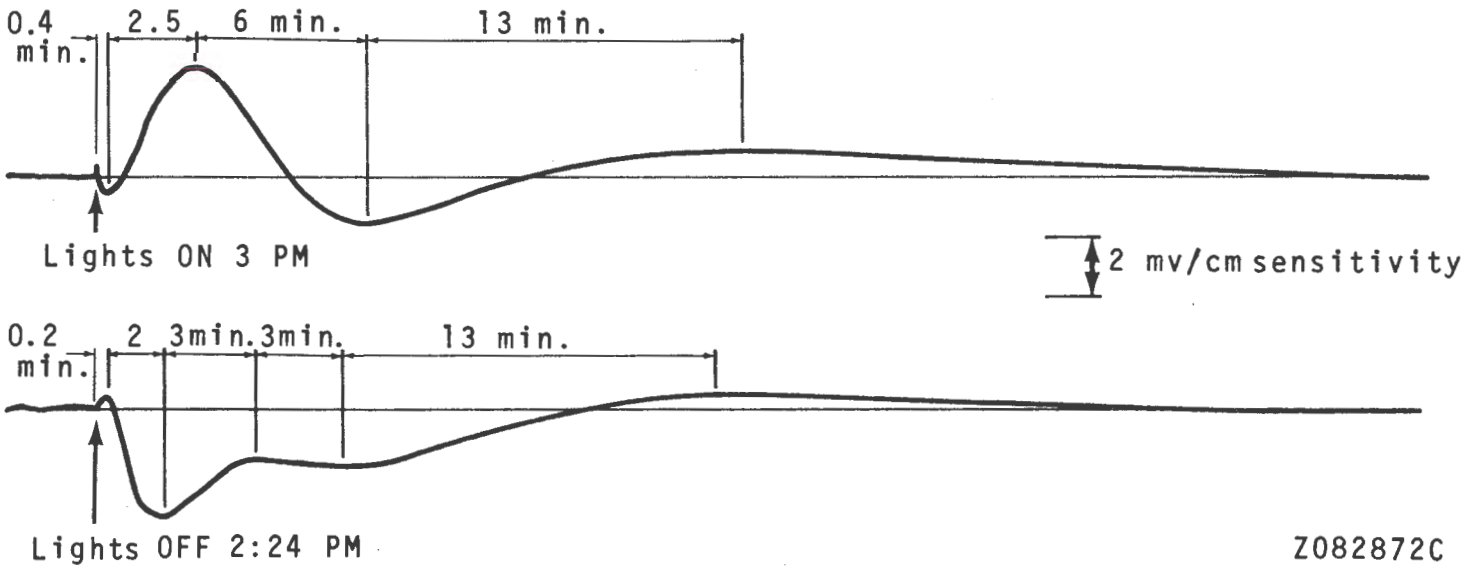


Figure 10. Biopotential responses from Zinnia No. 46 Z. elegans 'Canary Yellow' using Agar III electrodes and stimuli of 600 ft-c of illumination ON or OFF; before treatment

The most consistent change after 2,4-D exposure (Figure 11) was the change in wave periods for the lights OFF stimulus; the second crest and trough changed from a 3-minute interval each for the treated, to 5 minutes and 9 minutes respectively for one day after exposure. The first trough which occurred 2 minutes after the initial brief crest in both the untreated and one-day post exposure, dropped to 1 minute for the 3-day post exposure curves. All of the wave amplitudes were obviously lower on all 3-day runs, for both ON and OFF conditions.

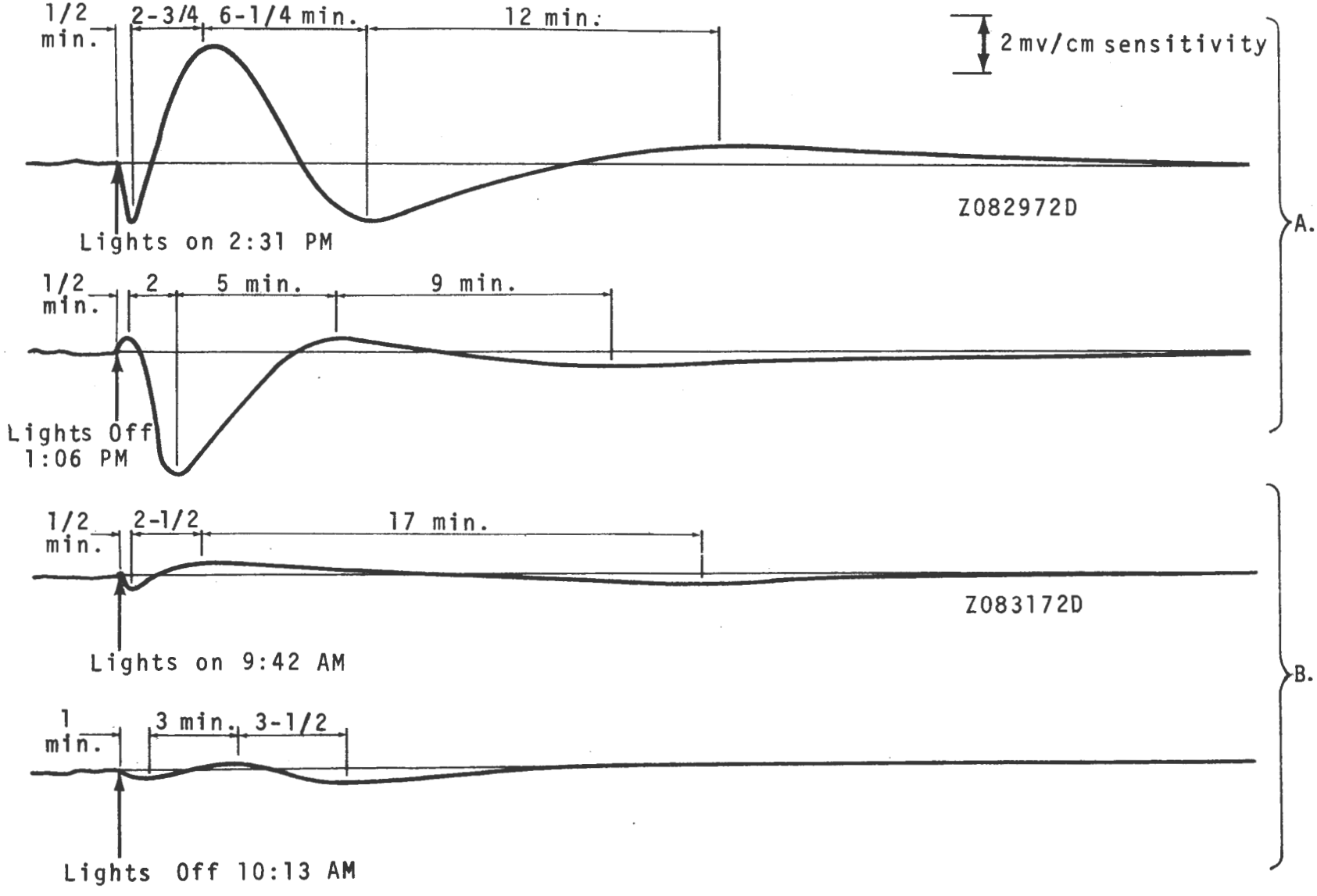
Exposure of Z. elegans 'Canary Yellow' to just ozone (O_3) treatment did not alter the biopotential waveshapes for lights ON stimulus, but did result in a unique waveform for the lights OFF response. Figure 12 shows this for Zinnia No. 43 one day after exposure to 0.5 ppm of ozone for 100 minutes. After an initial surge when the lights were turned off, the tracing drops, then starts to level off at the point marked Q, before continuing its downward movement. To date, only the ozone-exposed specimens have exhibited this characteristic.

Many of the tracings had sharp initial spikes when the stimulus lights were first turned on. It was determined that the split-second pen deflections corresponded to the momentary static in a nearby radio when the fluorescent starter first attempted to ignite the fluorescent bulbs. Therefore, these brief initial spikes should not be attributed to plant reactions, but rather to spark static pickup. While

Figure 11. Biopotential responses from Zinnia No. 46 *Z. elegans* 'Canary Yellow' using Agar III electrodes and stimuli of 600 ft-c of illumination ON or OFF:

A. One day after exposure to 50 ppm of 2,4-D for 10 min.

B. Three days after exposure to 50 ppm of 2,4-D for 10 min.


1/2 min.
2-3/4
6-1/4 min.
12 min.
2 mv/cm sensitivity
Z082972D
Lights on 2:31 PM
A.
1/2 min.
2
5 min.
9 min.
Lights Off 1:06 PM
1/2 min.
2-1/2
17 min.
Z083172D
Lights on 9:42 AM
B.
1 min.
3 min.
3-1/2
Lights Off 10:13 AM

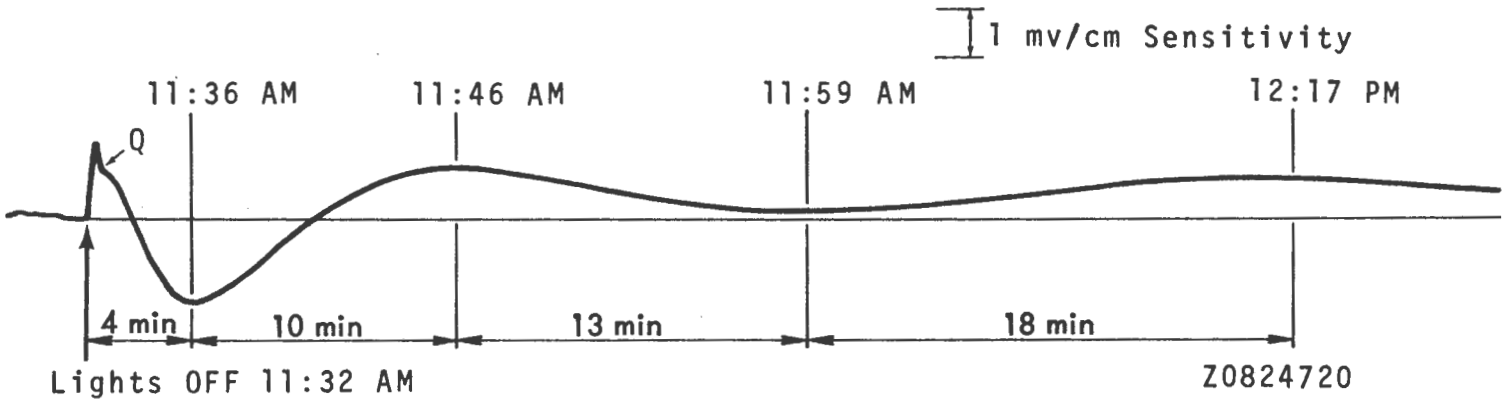


Figure 12. Biopotential responses to ozone with Zinnia No. 43 Z. elegans 'Canary Yellow', using Agar III electrodes, upon termination of illumination

indications of the air temperature in the vicinity of the plant leaf under test were noted on the strip charts, this did not appear to be a significant factor.

c. Additional tests Tests on an untreated zinnia (No. 48) involved the additional consideration of electrode placement. After 4 hours of normal recordings involving several ON/OFF cycles with both electrodes on one leaf with the usual electrode offset, the electrode on the top side of the leaf was moved to the top of the other leaf of the same axil pair. A complete ON/OFF cycle was then run. The ON stimulus produced wave trains comparable in wave phase and amplitude to those when both electrodes were on the same leaf. However, application of the OFF stimulus resulted in the introduction of a substantial peak at the initiation of the waveforms, instead of an initial trough. This alteration of wave persisted throughout the remainder of the wave train. A comparable test with electrodes on the plant stem resulted in similar wave shapes, but with considerably lower amplitudes. Apparently the oscillatory wave trains appear in other if not all parts of a plant in similar or slightly modified form.

d. Patterns of wave forms The first classification of of biopotential wave forms was based on whether they followed an ON stimulus or an OFF stimulus. Further distinction was made according to whether the initial wave started upward (on the chart paper) or downward from what was taken to be a "neutral" position just before the application of the stimulus.

For the OFF stimuli, 71% (40 of 56) of the instances analyzed had an initial upward movement. By comparison, application of the lights ON stimuli resulted in 83% (40 of 48) initial downward movements of the biopotential waveforms. The Grass polygraph was customarily operated with negative-going voltage being displayed as an upward deflection on the chart paper. Thus, when illumination was applied, the top surface of the leaf became negative with respect to the bottom surface, then reversed for the other half of the cyclic wave. Biopotentials were of the order of several millivolts for peak values.

Since time intervals of wave peaks or troughs were considered likely to have significance, both time intervals between a given peak (or trough) to adjacent trough (or peak) were examined, as well as total elapsed time from stimulus initiation to each successive peak or trough. There was no consistent distinction, for either basis of measurement, between time periods for initially upward wave trains as compared to initially downward wave trains.

Averages of the time intervals for the less complex wave trains were made for both control (untreated) and treated specimens and are tabulated in Table 2, with standard deviations and sample sizes. These wave trains were similar to those previously shown in Figure 8.

e. Comparison of waves By the use of Student's t-test of significant mean differences, comparisons were made between the biopotential wave periods for untreated plants, and plants

Table 2. Mean values ± standard deviations of time intervals of wave periods for biopotentials of *Zinnia elegans* as depicted in Figure 8.

Wave half-cycle:	1st	2nd	3rd	4th	5th	6th
Time period:	Minutes	Minutes	Minutes	Minutes	Minutes	Minutes
I. Untreated (control) plants						
A. Stimulus: Lights ON (600 ft-c)						
(1) Adjacent peak-to-trough	0.6±0.7	2.8±0.6	7.1±1.3	11.1±4.2	13.1±7.6	14.8±9.1
(2) Total elapsed time since stimulus (cumulative)	0.6	3.4	10.5	21.6	34.7	49.5
Sample size:	46 waves	45	46	34	22	10
B. Stimulus: Lights OFF (termination of 600 ft-c)						
(1) Adjacent peak-to-trough	0.4±0.5	1.2±1.1	3.2±2.8	5.4±3.4	10.0±4.4	15.1±4.0
(2) Total elapsed time since stimulus	0.6	1.6	4.8	10.2	20.2	35.3
Sample size:	24 waves	42	42	38	39	34

II. Treated plants

A. Stimulus: Lights ON (600 ft-c)

(1) Adjacent peak-to-trough

0.6±0.3	2.2±0.8	6.1±3.7	7.9±4.6	10.8±8.2

(2) Total elapsed time since stimulus (cumulative)

	0.6	2.8	8.9	16.8	37.6
Sample size:	88 waves	89	89	69	43

B. Stimulus: Lights OFF (termination of 600 ft-c)

(1) Adjacent peak-to-trough

0.5±0.6	1.8±2.2	4.5±4.1	6.2±4.4	7.9±3.9

(2) Total elapsed time since stimulus (cumulative)

	0.5	2.3	6.8	13.0	20.9
Sample size:	81 waves	99	99	84	51

that had received specific treatments. Significance was tested at the 5% level and involved comparisons of the time length of waves for the first half-cycle through the fifth half-cycle. The lights ON responses were calculated separately from the lights OFF responses. The respective half-cycles were taken as the time from the peak of a wave to the trough of the next wave. Since some wave trains did not last as long as others, the sample size decreased for the later wave half-cycles. The standard deviations and sample sizes used were the same as those shown in Table 2. Since sample sizes were usually different for control compared to treated specimens, the equations for unpaired observations and unequal variances were used. The results are compiled in Table 3. The "t" values are for Student's t-test based on the null hypothesis and for significance should be greater than the "t'" values calculated from table values for the 5% probability level and corrected for unequal sample size.

Table 3. Comparison of biopotential wave periods of Zinnia elegans for possible significance at the 5% level between control plants and treated plants

Wave ½-cycle	t	t'	Interpretation
		Lights ON	
1st	0.374	1.998	no significance at 5% level
2nd	2.743	2.005	significant difference between wave periods for treated vs. control plants
3rd	2.259	1.999	significant difference between wave periods for treated vs. control plants
4th	3.453	2.019	significant difference between wave periods for treated vs. control plants
5th	2.262	2.053	significant difference between wave periods for treated vs. control plants
		Lights OFF	
1st	0.966	2.041	no significance at 5% level
2nd	2.393	1.999	significant difference between wave periods for treated vs. control plants
3rd	2.717	2.004	significant difference between wave periods for treated vs. control plants
4th	1.139	2.010	no significance at 5% level
5th	2.395	2.019	significant difference between wave periods for treated vs. control plants

IV. DISCUSSION

In fulfilling the objectives of this project to examine plant variables some definite bioelectrical responses were obtained. Since two different bioelectrical measurements were used, correspondingly different response patterns resulted. Impedance measurements involved amplitude of the voltage drop across the test plant vs. frequency of the applied signal; biopotential measurements consisted of the plants' endogenous signal voltage vs. time. Furthermore, the external voltages used for impedance measurements were applied by means of piercing pins into the stems of the plants, whereas the self-generated biopotentials were obtained from the plant leaf surfaces by agar electrodes.

Amplitudes of the biopotential oscillatory pattern were not sustained but decreased after each stimulus. The rate of amplitude reduction was suggestive of damped electrical oscillations whose amplitude envelope decayed exponentially. If an analogy could be made to underdamped electrical circuits, this exponential decay would be $e^{-(t/2RC)}$ for parallel resonance, or $e^{-(Rt/2L)}$ for series resonance. It was not readily apparent what plant mechanisms, if any, might correspond to electrical inductance L or capacitance C. However, in a simple electrical analogy, the frequency or period between waves is taken as constant. In all oscillatory biopotential waveforms obtained to date, this <u>period</u> also increased,

possibly at some exponential rate, but apparently with a more complex relationship.

Attempts to find significance in the rate of period increase have so far been inconclusive. Neither was it deemed advisable to attempt comparisons of wave peak amplitudes between different plants or plant treatments. As previously mentioned, some variations in wave train amplitudes could come from equipment or operational variables such as contact resistance, drying of electrodes, or variation of electrode pressure on the leaf. However, wave periods are more a function of inherent plant responses and therefore, have more useful potential for interpretation.

A. Advantages and Disadvantages

The use of pins piercing the plant stem for impedance tests was simple, fast, and installation was not critical. Alternatively, relatively soft or deformable surface contacts avoided damage to plant cells from the piercing action but were more dependent on adjustment. An agar gel had an advantage of stability over a plain liquid, where drift could occur as a result of evaporation. The addition of 0.1 N KCl improves the conductivity of such an interface material, without subjecting the plant tissues to possible toxicity. However, for some plant species the surface resistance could be high enough to present noise problems. Examples might be waxy leaf surfaces or leaves with a cuticle or outer cell layers of sclerenchyma-

type cells intervening between the electrodes and the vascular system.

Another problem was the possible polarization of the electrodes. For stainless steel pins, this was avoided by the application of a-c as the signal source, although nonpolarizing hybrid contacts could be devised using Ag/AgCl, calomel, or similar electrodes. Use of a vacuum tube voltmeter to measure the signal drop across the plant being tested was an application of more recent technology that early experimenters lacked.

For biopotential measurements, low impedance electrodes were highly advantageous, since the signal voltages were considerably lower than for resistance tests. High impedance electrodes had been quite susceptible to stray pickup, resulting in artifacts and excessive noise. The agar electrodes provided sufficiently low impedance, as a result of 0.1 N KCl retained in the agar, and a favorable area of contact. Use of 0.1 N KCl also provided minimal toxicity and favorable tonicity toward the plant tissues. Polarization was avoided by the use of the Ag/AgCl center contact. Agar had the advantage of being sufficiently conformable to avoid significant injury to the plant surface.

Consideration was given to the use of smaller agar electrodes so that the composite biopotential signal would be picked up from a smaller group of plant cells. Thus, there might be less likelihood of diverse activities among them and hence a less complex signal. One of the limits for this is

the increase in electrode resistance as the area of contact is reduced. Such resistance is inversely proportional to the diameter of the area of contact.

Stability of the agar electrodes was good with respect to exposure to light, temperature variations, and time, provided that there were no breaks in the AgCl coating of the silver wire. Quite a few Ag/AgCl spirals had to be fabricated before a good one was obtained. Since no commercial sources of the described agar electrodes are known, the difficulty of fabrication appears to be a distinct drawback.

B. Significance of Results

The two chosen methods of plant monitoring tend to complement each other. Different aspects of plant processes were involved in the two approaches, so that results of one technique augment results of the other. Self-generated biopotentials involve more natural physiological activities of living plant tissue than do the comparative resistances of either living or dead plant tissues. Both methods were utilized for the same basic reason of examining the effects of stresses upon plants. From this study it appears possible to distinguish between certain living cultivars by their characteristic impedance curves. It is postulated that the amount of voltage drop across a portion of a living plant being subjected to a signal over an a-c frequency range is the result of differences in the kind and degree of organization of plant tissues. The size and

composition of vacuolated cells, fluidity and composition of protoplasmic strands within and between cells, and particularly the composition and condition of the cell membranes would be expected to be influenced by cultivar, stage of growth, and any specific treatment.

The impedance tests represent a biological phenomenon that was examined and preliminary results obtained. Future use could arise from accumulation of a sufficiently large data base for statistical analysis of uniformly collected results. Additional isolation of variables could permit more discernible differences that would then provide a basis for better significance and interpretation, using this technique.

The oscillatory type of response of biopotentials to the initiation or cessation of illumination has received negligible attention in this country. However, some physical occurrences in plants have been reported that could have a bearing on the oscillatory biopotentials obtained. Cowan (10) in 1972 described how physical oscillations (either sustained or damped) of the stomata have been detected by direct observations of the plant leaves or by inference from measurements of changes in related plant properties. Both Cowan and Barrs (2) also reported on allied phenomena that were studied, including transpiration rate, net photosynthetic rate, leaf diffusivity, leaf water status, osmotic pressure, and leaf temperature.

The studies of Cowan and Barrs regarding physical oscillations of plant mechanisms have indicated a possible damping of

wave amplitudes only, and did not include damping of the wave-form periods which was quite evident in the electrical signals obtained here. Barrs (2) reported consistent periods of stomatal mechanical oscillations within the range of 2½ to 50 minutes depending on the plant. This compares favorably with the periods encountered with the biopotentials, although the latter had the period lengthening that was not mentioned for the physical observations. In general, amplitude damping after a stimulus indicates some measure of stability, whereas sustained oscillations imply some degree of instability, positive feedback, or a forcing function. There did not appear to be sustained oscillations of the electrical biopotentials of the plant. The observed pattern may represent an overshoot or overcompensation of physiological mechanisms that "damp out" with time.

The wave periods tended to fall into a pattern with respect to time, regardless of initial polarity of a wave. From Table 2 and Figure 8, the first peak or trough was approximately one-half minute after the stimulus was applied; the next trough (or peak) usually came between 1½ and 3 minutes later. The third peak or trough occurred approximately 3 to 7 minutes after the second. Further periods were of longer duration (e.g. 10-20 minutes) and were less predictable.

Interpretation of effects of 2,4-D treatment was complicated by the timing of application. Some of the plants were, of necessity, exposed "en masse" or simultaneously on the same day.

However, each plant had to be tested individually on the Grass polygraph, usually requiring the better part of a day for each one. Hence, the time interval between exposure and recording kept lengthening between successive plants. The elapsed time after nonlethal 2,4-D treatments is probably a factor but it was not found to be sufficiently consistent in its effects to permit extrapolation or compensation for its variance.

Since other researchers have visually observed physical oscillations of stomata, the question arises why electrical biopotential oscillations have received so little attention in the literature. It is possible that the experimental techniques of many researchers may have unintentionally suppressed significant mechanisms, which might include stomatal activity, transpiration, or similar phenomena. Use of high-impedance electrodes with their greater susceptibility for pickup of artifacts could have resulted in the obscuring of some patterns. Factors that could have aided the current results include the use of low-impedance electrodes having soft contacts, which were offset from each other and with porous tissue supports. The latter should have reduced the suppression of stomatal activities or the hinderance of air or moisture circulation, compared to the solid plate or similar electrodes often used by other researchers. Subsequent to the testing reported here, excellent substantiation for these results has appeared in the literature. During final writing of this report, an article by Maslobrod was recently translated

from the Russian (22). His technique was similar to that used here, although the report in English was not as detailed as would be desired.

It may be postulated that several physiological processes in the plants are likely to influence the obtained responses simultaneously, to give composite waveforms. Specific tests to screen out particular plant processes would narrow down the individual cause-effect portions of the mixed responses observed. However, Table 3 indicated that for most of the biopotential cycles, significant statistical differences already existed at the 5% level of probability between the time periods of treated and untreated plant waveforms.

The reproducibility of the biopotential responses obtained thus far is encouraging to the further use of bioelectric techniques. The impedance tests suggested distinctive dispersion patterns for a given cultivar, and the biopotential oscillatory patterns for a given setup repeated themselves cycle after cycle for several hours.

The results of this project indicate that bioelectronics can provide relatively fast nondestructive measurement of plant responses, particularly in the case of biopotential response to illumination. Good opportunities exist for additional interpretation and insight into various plant physiological responses to given stimuli and/or stresses. Bioelectronics may prove as valuable for work with plants as it has already proven to be for people and other animals. This

is particularly true for tests *in vivo* or *in situ* where an analysis that requires destruction of plant tissues is impractical or undesirable. Bioelectronic outputs also lend themselves to remote or continuous monitoring or central data processing.

V. SUMMARY

Electronic monitoring of representative horticultural plants *in vivo* was investigated in a search for response from living plants to environmental stresses that would be faster than the production of visible growth symptoms. Tissue impedance was measured and self-generated biopotentials were recorded from active living plant tissues with biomedical equipment and techniques comparable to those used for medical ECG and electroencephlograms (EEG).

Several techniques were employed. These included a-c impedance measurements of plants from 1 KHz through 600 KHz and the recording of plant biopotentials in response to changes in illumination. Plant varieties tested included Canary Yellow zinnias and Rutgers tomatoes. Test treatments involved exposures to different levels of 2,4-D or ozone in sublethal to lethal dosages at known levels of temperature and illumination.

Nonpolarizing electrodes were developed to conform to plant surfaces, to be nondestructive, and nonphytotoxic. Low impedance biopotential sensors using 2% agar gel with 0.1 N KCl and Ag/AgCl resulted in much less noise and better signals than wick-type pipettes.

Distinctive biopotential patterns associated with onset and termination of illumination of plants were obtained. They resembled oscillatory waves that were damped in both wavelength

and amplitude. Evidently the plant metabolic processes represent complex control mechanisms having frequency damping. The periodicity of the waveforms was considered to be more significant than amplitude, since there were a number of experimental factors that could affect the amplitude of the damped waves. Presumed action potential spikes were observed on some occasions.

Future work with these and other bioelectric techniques hold promise of better insight into physiological changes within a plant, plus possibilities of usage for environmental monitoring.

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