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Mediation of rapid electrical, metabolic, transpirational, and photosynthetic changes by factors released from wounds. I. Variation potentials and putative action potentials in intact plants. II. Mediation of the variation potential by Ricca's factor. III. Measurements of CO_2 and H_2O flux

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Mediation of rapid electrical, metabolic, transpirational, and photosynthetic changes by factors released from wounds. I. Variation potentials and putative action potentials in intact plants

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Damaging representative plants from five angiosperm families by heating or crushing a small portion of a single leaf results in an electrical change which may spread throughout the shoot. In *Mimosa*, similar changes have previously been identified as variation potentials.

Except in one of the five plants, a variation potential is often accompanied by brief fluctuations which may propagate either basipetally or acropetally and which have many of the properties of action potentials.

The spread of a variation potential as described in *Mimosa* is due to the concommitant spread of a chemical substance in the transpiration stream. In this paper, it is shown that the spread of the purported variation potential is compatible with movement of material in the transpiration stream. In the next paper causation by a substance or group of substances, at present called Ricca's factor, is demonstrated.

Introduction

As early as 1916, Ubaldo Ricca showed that crushing or burning a leaf of the motile plant *Mimosa pudica* releases a substance or substances which can spread through the xylem to adjacent leaves, causing them to droop suddenly. Such sudden collapse of the leaves evidently discourages insects from feeding on them (Daniel Janzen, personal communication). Houwink (1935) provided strong evidence that passage of Ricca's agent in the xylem of *Mimosa* produces in adjacent tissue an electrical response described as a variation potential. He showed that either the agent itself or the variation potential it initiates is capable of triggering an action potential which may propagate ahead of the variation potential.

The experiments of Ricca, Houwink, and several subsequent workers were reviewed in 1973 by Pickard, who suggested that Ricca had discovered a hormone or group of hormones of widespread distribution in higher plants, coordinating some aspects of plant response to insect depredation, to breakage or abrasion, and perhaps to water deficit. Pickard also reviewed several publications from Russian laboratories that presented sketchy data suggesting that differences in the gas exchange of an undamaged leaf might result when neighboring leaves are damaged. Meanwhile, work of Desbiez (1973) indicated that the variation potential or its correlates can play a role in the correlative inhibition of buds of *Bidens*.

The present set of papers shows for representative higher plants from five families that damage releases material that can travel rapidly through the plant, accompanied by electrical changes, and that arrival of the material in a leaf blade rapidly increases catabolism and rapidly decreases both transpiration and photosynthesis. The first paper describes extracellular electrical features of the variation potential and associated propagating spikes or putative action potentials.

Materials and Methods

(1) Plants

(A) Taxa

Most experiments were carried out on plants of tomato (*Lycopersicon esculentum* cv. Bonnie Best), but key experiments were repeated on morning glory (*Ipomoea hederacea* cv. Scarlett O'Hara), pumpkin (*Cucurbita pepo* cv. Jack-O-Lantern), cocklebur (*Xanthium strumarium* L.), and cotton (*Gossypium hirsutum* cv. Auburn M.).

(B) Terminology

Leaves of all plants were numbered starting with the first leaf above the cotyledons; internodes and nodes were similarly numbered. Nomenclature used for the compound leaf system of *Lycopersicon* is that of Correll (1962) and of d'Arcy (1973) and is partially presented in Fig. 4.

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(C) Growing Conditions

Soil was compounded from 36 parts by volume topsoil from the Missouri River bottomland, 18 parts peat moss, 18 parts sand or horticultural perlite, 9 parts dry cow manure, and 2 parts steamed bone meal. The soil was then sterilized and placed in sterilized clay pots, and presoaked seeds were planted in it.

Fertilizer containing 16 parts N, 9 parts P, and 5 parts K was applied: each pot received 0.5 g 2 weeks after planting and 1 g each week thereafter. *Gossypium* plants received a variable supplement of calcium and extra iron.

Plants were grown in chamber model MB-60 or model MB-60b (Percival Refrigeration and Mfg. Co. Inc., Boone, IA 50036) on a cycle of 10 h dark at 21 °C and 14 h light at 27 °C. Light intensity at soil level in the absence of plants was about 30 W m^{-2} and was provided by a combination of four 60-W incandescent lamps and 12 fluorescent tubes (either F48-T12-VHO Gro-WS or F48-T12-CW-1500 from Sylvania, Inc., Mountain View, CA 94040). Relative humidity ranged from 60 to 90%.

(II) Experimental Assembly

Each experimental plant was set with its pot in a Petri dish of water on a vibration-damped steel plate inside an open-fronted screen cage. The stem was held in place with a rubber-coated clamp fixed to a rigid vertical support. Above the cage were suspended two Gro-lux fluorescent lamps (F96T12-Gro-VHO-WS, Sylvania) and (or) two 250-W tungsten halogen lamps (e.g., 250 Q/CL, Westinghouse Electric Corp., Lamp Divn., Bloomfield, NH 07003); in later experiments, these were replaced with four Agro-lite tubes (F40/AGRO) from Westinghouse. An air cooler circulated the air and minimized buildup of heat. During experiments on darkened plants, a dim green light was used to monitor electrode contacts and recording equipment.

(III) Monitoring Equipment

(A) Electrodes and Salt Bridges

Nonpolarizable Ag–AgCl electrodes were routinely connected to the plant by means of a short agar-coated cotton thread protruding from a small glass pipette containing 0.1 *M* KCl gelled with 1% agar. The wick was set against the epidermis. With stems, it was usually placed as close as possible to one of the major vascular bundles entering the leaf at the node above. With petioles, it was generally placed on the adaxial surface. It was equilibrated for more than 10 min after positioning to achieve good electrical contact and hence a steady baseline signal. A tiny drop of 0.1 *M* KCl was added whenever necessary to keep the wick moist.

Some experiments were duplicated with a different kind of salt bridge: a pipette with a tip diameter of 4–10 μm filled with the gelled saline was driven into the stem or petiole to a depth of 0.5–2 mm. Resultant recordings were similar to those obtained with the wick-tipped salt bridges, although the amplitude of the signals was frequently slightly larger. The advantage of the larger signal was, however, outweighed by the greater difficulty in maintaining contact between a set of fragile, finely drawn-out pipette tips and the plant. Moreover, baselines recorded through such pipettes tended to be somewhat unsteady.

Reference electrodes connected to the plant were bridged the same way as recording electrodes, but those stationed in the wet soil made contact through an extra, larger salt bridge.

Electrical contact was routinely tested by waving an ungrounded hand within 10–15 mm of the contact points.

(B) Electronic Devices

The signal from a recording electrode was passed through an electrometer–amplifier of 10^{13} Ω input resistance (either a Picometric model 181, Instrumentation Lab, Inc., Watertown, MA 02172, or a 10 × amplifier developed especially for the purpose).

Amplifier output was usually passed through a simple RC low-pass filter constructed with five cascaded stages and having a rise time (10 to 90%) of 100 ms, a passband of 0 to 2.8 Hz, and insignificant voltage reduction.

Filtered signals were recorded on Mark 200 oscillographs from Brush Instrument Division of Gould, Inc., Cleveland, OH 44114, using chart speeds ranging from 5 mm min^{-1} to 1.0 mm s^{-1}. To facilitate alignment of simultaneous recordings on two or more oscillographs, the input jacks for the event marker pens of each oscillograph were shorted with a switch to mark synchronous index lines at appropriate intervals.

Results

(I) Response to Localized Thermal Damage

(A) Choice of Stimulus

Preliminary experiments compared the responses of *Lycopersicon* plants to two stimuli which might be expected to produce similar types of localized damage: (1) the tip of a terminal leaflet was wilted by dipping for 30 s into 70–90 °C water and (2) the tips of the terminal and subterminal leaflets were wilted by passing a flaming match back and forth 10–50 mm below the tips for 15 s. The effects observed spreading away from the locus of stimulation were similar for the two stimuli, but since the flaming match produced larger and more uniform responses, it was selected for extensive replication.

(B) General Pattern of Response

When one leaf of a tomato plant which had been electrically grounded through the soil was treated with a flaming match, a voltage fluctuation soon spread past electrodes recording from adjacent, undamaged leaves. Figure 1 is a photograph of a pair of recordings from a representative experiment in which electrode wicks were situated 35 mm apart on the petiole of leaf 4 while leaf 3 was stimulated at the arrow. In this particular experiment, the first sign of the electrical disturbance is a synchronous rise in the voltage level 12 s after the start of stimulation (a recording artifact to be explained in association with Fig. 3). Then, the electrode nearer the

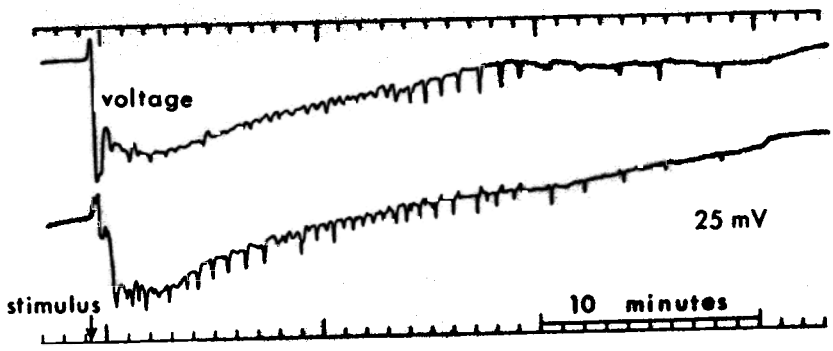

FIG. 1. Photographs of paired recordings of a variation potential, accompanied by numerous spikes, traveling up the petiole of the fourth leaf of a potted *Lycopersicon* plant with reference electrodes in the soil. The arrow indicates the application of a thermal stimulus to leaf 3. The first (more basal) and second (more apical) electrodes were separated by 35 mm. Time ticks represent 60-s intervals.

stressed leaf registers a rapid downward swing of the voltage 20 s after the start of stimulation, within 30 s reaching a value about 35 mV below the baseline and about 41 mV below the most positive excursion of the recording artifact. Also, at about 30 s after the start of stimulation, the electrode positioned 35 mm farther from the point of stimulation begins to register a similar downward deflection. Characteristically, the signals from a pair of electrodes connected to a given petiole are similar though by no means identical.

From the data of Fig. 1, it is difficult to measure how fast the fluctuation is spreading since there are spikes on the initial, falling phase of the fluctuation; however, in many cases (e.g., Figs. 2 and 3) the initial drop of the voltage was relatively smooth, and velocity could be approximated by comparing the times at which the two electrodes registered 25% of their maximum negative deflection (measuring from the peak of positive deflection). Typically, velocity up the petiole of the illuminated plant ranged from 3 to 10 mm s^{-1}; the average velocity was 6 mm s^{-1}. Velocity up the petiole of the darkened plant averaged 4 mm s^{-1}; aside from the difference in velocity, there was no reproducible difference between electrical events in the dark and in the light.

The 'peak' of the fluctuation tends to be a broad one, as is well illustrated in Fig. 1, and return of the fluctuation toward the baseline is slow and quite variable from experiment to experiment. A number of smaller, briefer, downward deflections can be seen during the return of the major deflection.

(1) The Variation Potential

We identify the major deflection with the variation potential which Houwink (1935) (see also review by Pickard (1973)) described in *Mimosa pudica*. Although experiments of Cheeseman and Pickard (1975) indicate that the extracellularly recorded event represents the collective depolarizations of a great many cells, we wish to use Houwink's term in describing the extracellularly measured, slowly changing envelope of electrical activity with which the present experiments on intact plants deal.

To establish whether the variation potential initiated by sudden heating could spread throughout the plant, two series of experiments were carried out using six electrodes to monitor voltage in various parts of the shoot and recording at relatively high chart speed to emphasize the pattern of propagation.

In the first series, one pair of electrodes was placed on the petiole of the leaf which was to be stimulated, a second pair was placed on the petiole of a neighboring leaf, and a third pair was placed on the stem with one near the apex and one near the soil. A typical experiment from this series is illustrated in Fig. 2. Clearly, the signal moves basipetally out of the stimulated leaf and spreads into the shoot above. A fluctuation is also seen in the shoot basal to the stimulated leaf. In the experiment of Fig. 2 as in all replicates, this downward-moving fluctuation is of apparently brief duration and looks as though it might consist mainly of a putative action potential. Indeed, it is not impossible that this is the case in this instance, but in similar experiments in which the leaf with electrodes C and D was

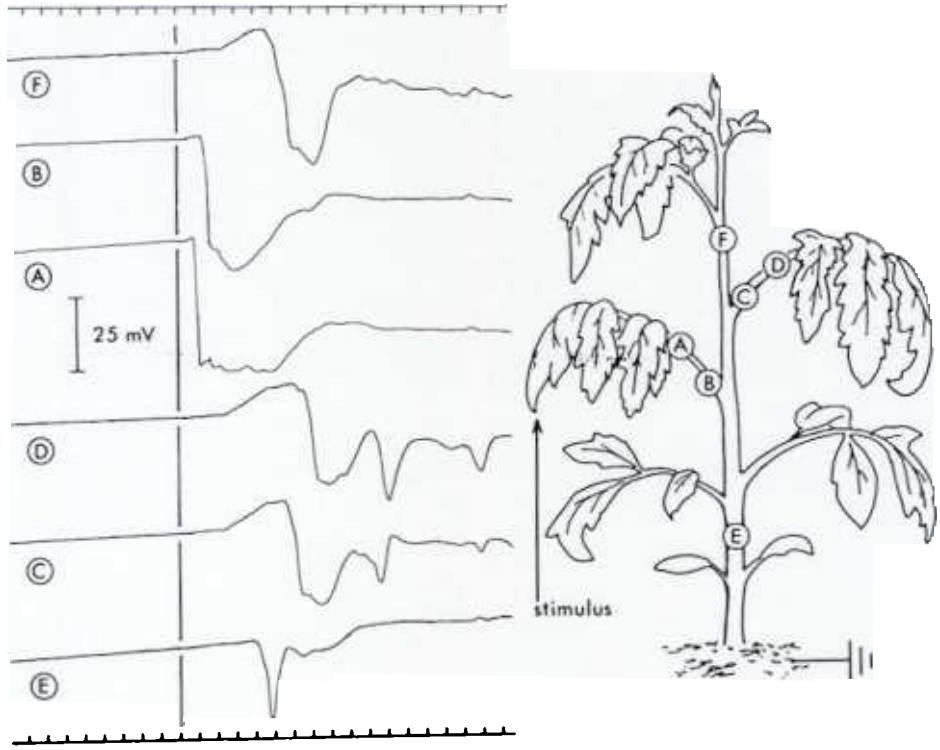

FIG. 2. A spreading variation potential detected by six electrodes placed as indicated in the sketch at the right. (A short burst of putative action potentials occurred after a considerable lag and is not included in the figure.) Separations of A and B, B and E, B and F, B and C, and C and D are 60, 56, 55, 42, and 50 mm, respectively (the drawing is not to scale). Time ticks represent 12-s intervals; the time of application of stimulus to the third leaf is marked by a vertical line.

stimulated rather than that with electrodes A and B, the variation potential consistently moved basipetally past D and C, and acropetally past B and A. Moreover, as will be demonstrated below, the differential recording which occurs when a fluctuation passes between the recording and ground electrodes results in a premature, artificial reversal of the signal. In sum, variation potentials can move basipetally in the stem when damage is by sudden heating but are probably rapidly attenuated when they thus move counter to the normal direction of the transpiration stream.

In the second series, all electrodes were placed on a single leaf: two were positioned on the petiole, one on the rachis, and the remaining three on the apical regions of the midveins of lateral and terminal leaflets. Then, either a neighboring leaf or one of its own leaflets was stimulated. This second type of experiment extends the conclusions from the first, indicating that under the specified conditions variation potentials can spread both in and out of the leaflets.

Although the extracellularly recorded envelope cannot be expected to give a precise indication of the time course of events in the cells which contribute to it, nevertheless it is worthwhile to assess artifacts by varied positioning of the reference electrode. The experiment of Fig. 1 illustrates that variation potentials measured on a petiole–rachis with respect to a reference electrode in the soil can have an apparent early, simultaneous positive-going component. If this upward component is due to the occurrence of the variation potential between the reference electrode and the measuring electrodes as it passes from the petiole of the stimulated leaf into and along the stem, it should be essentially absent in recordings for which the reference electrode is placed at the tip of the unstimulated leaf to which the measuring electrodes are connected. However, an upward component should

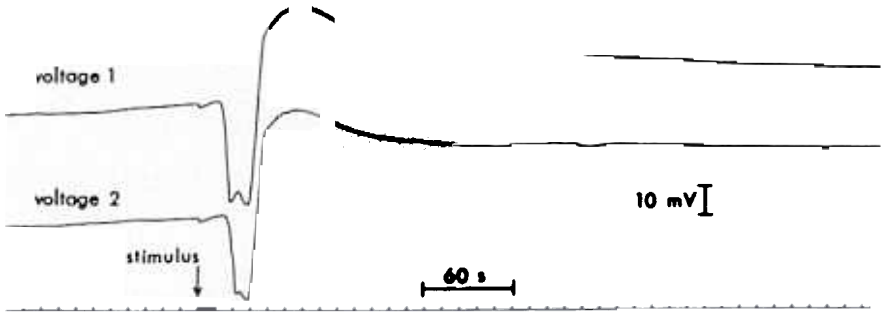

FIG. 3. A variation potential recorded from the petiole–rachis of a leaf with respect to a reference at its tip. The electrodes were on leaf 3, and the stimulus was applied to leaf 4; time ticks indicate 12-s intervals. Note the absence of a floating-up artifact between the times of stimulation and arrival of the variation potential under the electrodes, but note the artifactual (see text), essentially simultaneous ascent of the two signals immediately after the inverted peak, in this particular recording to a value well above the original baseline.

appear as the variation potential passes beyond the measuring electrodes toward the reference. The final voltage readings will be the differences between changes under the electrodes on the petiole–rachis and changes in the tissue between them and the reference placed on the terminal leaflet, which is itself active. This prediction is borne out in experiments typified by that of Fig. 3. It may be checked that the somewhat more complex set of recordings in Fig. 2 shows artifacts consistent with this interpretation. Many similar experiments with the several genera suggested that it is not possible to prevent the measured signal from 'floating' when electrical activity occurs between the measuring and reference electrodes, even if a large volume conductor is positioned between the plant and the reference electrode.

A further important observation on the variation potential is that if two heat stimuli are administered in rapid succession, the first tends to eliminate the ability of the plant to respond to the second. However, over a period of hours plants may recover enough from the influence of even a strong stimulus to respond well to a second stimulus.

(2) Putative Action Potentials

In *Mimosa*, according to the work of Houwink (1935) and Sibaoka (reviewed in 1969), only one action potential usually accompanies a variation potential, and it tends to propagate at a higher velocity than the variation potential so that it continually gains lead as the two fluctuations move along the petiole. In *Lycopersicon* grown under the conditions of the present study, the inverted crest of the variation potential often bears one or more extra peaks or shoulders, hinting that the front edge of the extracellular envelope may cover both action potentials and a variation potential (e.g., Figs. 1, 5). However, the similarity of velocities of these two kinds of fluctuations and the apparently irregular propagation of the presumed action potentials make extracellular separation difficult.

In contrast, the spikes which occur in trains during the returning phase of the variation potential are often readily identified as putative action potentials because they may rise cleanly from a rather smooth background voltage and may propagate for long distances. The trains of spikes often propagate out of the damaged leaf and into the neighboring undamaged leaves; for example, Fig. 4 shows one acropetally propagating putative action potential from such a train. Sometimes, however, the trains appear to originate in undamaged leaves. Rarely, it appears that a train of signals of a characteristic apparent height and shape may be propagating in one direction when a train of signals of different apparent height and shape begins to propagate in the same petiole but in the opposite direction. This suggests that there are multiple channels which can carry the putative action potentials.

As illustrated in Fig. 5, the shape of the spikes generally appeared simpler and the amplitude was greater when recording was accomplished through glass-tipped pipettes inserted into the tissue rather than through superficial wick-tipped pipettes. However, the inconvenience of this recording method with a large, rather floppy plant precluded its extensive use.

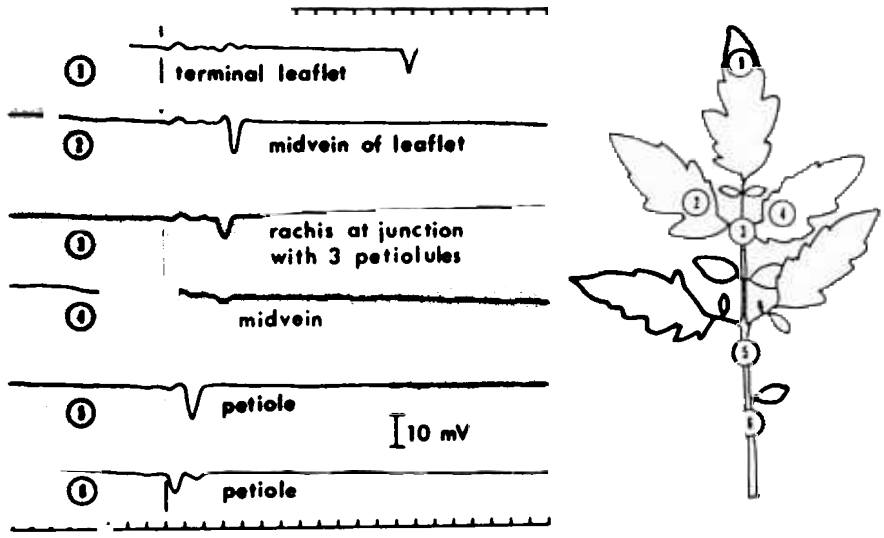

FIG. 4. A set of recordings of a putative action potential which arose late in the course of a variation potential and which propagated into an undamaged leaf on an intact plant grounded through the soil. Time ticks indicate 12-s intervals. Numbers on the right-hand tracing of the leaf used in this specific experiment correspond to the six electrode positions. Electrode separations were as follows: 6 to 5, 35 mm; 5 to 3, 80 mm; 3 to 4 and 3 to 2, 20 mm; 3 to 1, 110 mm (this was a large leaf from an older plant). The vertical line is a convenient reference against which to measure propagation. Note that velocity and amplitude decrease as the putative action potential passes farther from the stem. In addition to illustrating a discrete putative propagating action potential, the recordings show clear floating-up artifacts; the trace from electrode 4 appears to contain nothing but such artifacts. (This figure also illustrates parts of the pinnately compound *Lycopersicon* leaf. In this paper the term leaflet designates a major leaflet of which the tracing has 5; the petiolar, rachiolar, and petiolular interstitial leaflets were neglected because of their small size.)

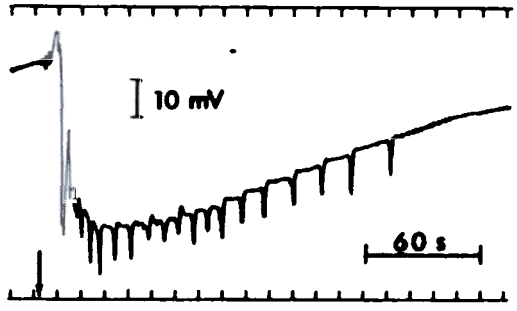

FIG. 5. Variation potential with associated putative action potentials recorded through a simple saline-filled pipette; the pipette was inserted in the petiole of leaf 3 and the stimulus was applied to leaf 4. The reference electrode was in the soil. Time ticks indicate 12-s intervals.

It must be emphasized that the spikes do not always appear to propagate over long distances. Sometimes, when three electrodes are positioned in a row along a stem or petiole, some spikes appear to propagate under all three electrodes, some appear to propagate only as far as the second, and some do not appear to pass beyond the first. In more than half of the recordings in which two electrodes were positioned on a petiole, the spikes were too numerous and closely spaced to permit any judgment about direction of propagation (e.g., Fig. 1). In many of these petiolar recordings, there is no evident correlation of spikes registered by the two electrodes even when reasonably long intervals separate the spikes. In still other recordings, especially those from mildly stimulated plants, spikes were very small if evident at all.

It cannot be overstressed that evaluation of the direction of propagation requires a subtle appreciation of the 'float-up artifact.' 'Floating' can actually cause spikes to appear to propagate in a direction opposite to that which is observed with proper location of the reference electrode. Therefore, to hope to assess direction in an extracellular recording, unless very sharp, well separated signals are observed propagating very slowly as shown in Fig. 4, it is necessary to record from a long and regular train with reference electrode alternating between the apical and basal regions of the tissue under study. Even then,

especially if there are poorly propagating signals mixed with propagating ones, it is not always possible to analyze the propagation of the spikes.

The time of occurrence of the spikes in relation to the variation potential is not predictable, either in the stimulated or unstimulated leaf. Sometimes (e.g., Figs. 1, 5) the spikes begin with the drop of the variation potential, but other times an interval as long as 20 min may occur between the drop of the variation potential and the appearance of the first spike of a train.

Putative action potentials as well as variation potentials can propagate into the leaflets at least as far as the dwindling of the midveins (Fig. 4; first, second, and fourth traces). The spikes are harder to record convincingly along the midveins than along the petiole or rachis, partly because the problem of 'floating' is more complex in the leaflets and partly, it appears, because the propagation tends to die out as the midveins become thinner.

(C) Other Plants

Localized thermal damage to leaves of illuminated or darkened *Cucurbita*, *Gossypium*, and *Xanthium* resulted in spreading variation potentials and accompanying spikes which were essentially the same as those observed in *Lycopersicon*. Experiments with *Ipomoea* evidenced standard variation potentials, but in only 1 out of 27 replicates were distinct spikes seen on the leading edge of the fluctuation. In several recordings, there were a few spikes on the return slope, but these were impossible to identify with the putative action potentials seen in other plants because they were of trivial amplitude and were not observed to propagate.

(II) Response to Localized Mechanical Damage

(A) Putative Action Potentials

If a leaf of *Lycopersicon* is excised by cutting through the petiole close to the stem, a putative action potential may propagate along the stem, away from the site of injury, without the company of a variation potential. If the cut elicits no putative action potential, slight squeezing of the stump with a pair of forceps may elicit one. Such putative action potentials are typically large under an electrode placed near the point of excision but are smaller under more distant electrodes. Figure 6A illustrates a typical putative action potential with an amplitude of −26 mV in a recording made 15 mm from the base of a crushed stump and −6 mV in the paired recording made 60 mm farther from the stump. The velocity of propagation was about 1.5 mm s^{-1}.

In addition similar putative action potentials may be elicited by localized application of ice-cold water or icy dilute Hoagland's solution either to an intact plant or, more simply, to the base of an excised illuminated leaf to which electrodes have been attached (cf. Van Sambeek *et al.* 1976). However, the effectiveness of sudden chilling is much more sporadic than that of mechanical injury. Figures 6C and 6D show two putative action potentials propagating away from the point of application of 0.1 × Hoagland's macronutrient solution chilled to 0 °C.

(B) Variation Potentials

If a petiole or its stump is crushed close to the stem with a pair of forceps, a small variation potential will frequently result in the stem. Variation potentials can be produced easily in excised leaves: a leaf is taped firmly to a support, with its base immersed in a beaker of water or dilute Hoagland's solution and its blade brightly illuminated, electrodes are then attached to the petiole–rachis, and the petiole is crushed lightly with tweezers just above the solution level. Very slight squeezing may only produce a putative action potential, but moderate crushing usually produces a variation potential such as shown in Fig. 6B. Whether the stimulus is heating or crushing, petioles and stems tend to initiate a weaker variation potential than do heated leaf blades. In general, regardless of the kind of stimulus which triggers them, small variation potentials diminish in amplitude as their front moves away from the point of origin.

Variation potentials do not spread well from bruised illuminated blades in contrast with their spread from blades wilted with a flaming match, but in the latter case the spread is facilitated by the disturbance of the transpiration stream which doubtless results from heating water in the leaf (cf. Results, section I). In this connection, it should be recalled that the variation potential initiated by heating can move both in and out of leaves and up and down the stem but that it appears to diminish rapidly when moving down the stem against the regular direction of water flow.

Although crushing of leaf blades does not usually produce a large, suddenly dropping variation potential in the rest of the shoot, it may conspicuously desensitize a plant to further

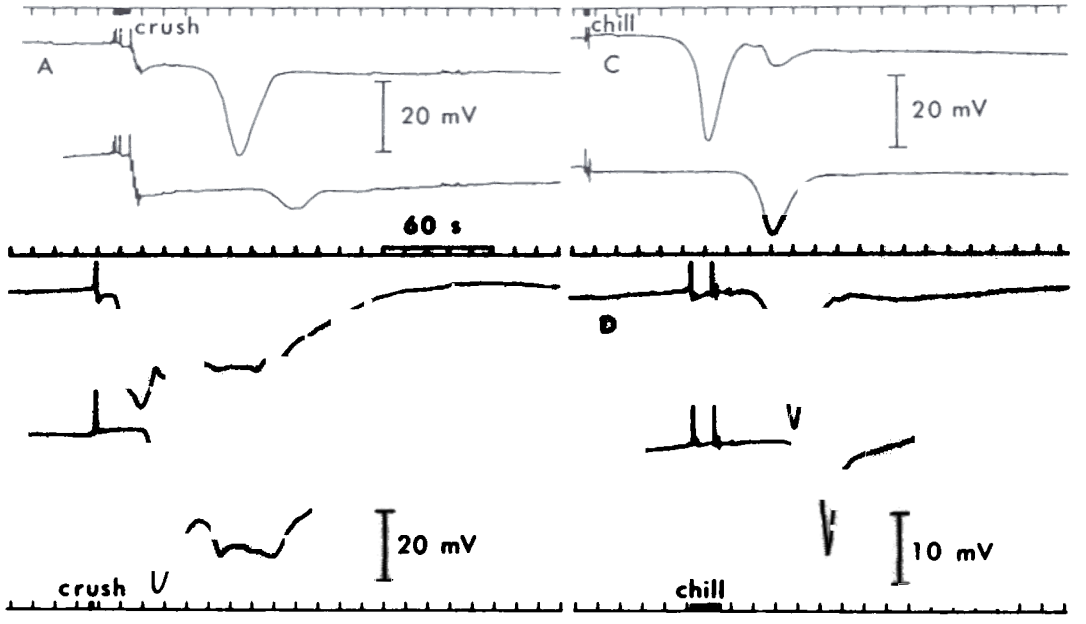

FIG. 6. (A) Action potential propagating up a stem of *Lycopersicon* after excision of a petiole about 20 mm distant from the stem and light crushing of the tip of its stump. First electrode, 15 mm distant from base of petiole, second 45 mm distant from first; propagation speed about 1.5 mm s^{-1}. Wick of reference electrode on apical leaf; attenuation of signal explains lack of late float-up. Truncated stimulus artifacts indicate time of cutting and crushing; baseline shifted during crushing resulting from jiggling of stem against pipette wicks. (B) Variation potential propagating up a petiole–rachis of an illuminated excised leaf of *Lycopersicon* after squeezing of the base with forceps. The basal cut surface of the petiole was immersed in 0.1 × Hoagland's macronutrient solution, and the wick of the reference electrode was placed at the tip of the apical leaflet. Electrode separation was 35 mm. (C and D) Two examples of action potentials propagating up petioles of excised *Lycopersicon* leaves after application of iced 0.1 × Hoagland's macronutrient solution to cut ends at times indicated by truncated stimulus artifacts. In both cases the electrode separation was 35 mm; the wick of the reference electrode was at the leaf apex, and float-up is presumed absent owing to signal attenuation during propagation. Propagation velocity about 1.5 mm s^{-1} in C and about 1 mm s^{-1} in D.

stimulation by either crushing or heating. Thus, it might be supposed that the effects of crushing blades may under some circumstances spread, but too slowly to permit reproducible extracellular detection. Rough handling of a plant also tends to desensitize it to standard stimuli; it was for this reason that equilibration was always allowed between the setting-up and running of an experiment. It is not known whether mild mechanical stimulation resulting from gentle handling triggers small or slow responses of the same sort which are seen so dramatically when a leaf is cut, bruised, or mashed.

(C) Other Plants

The above experiments have been carried out primarily with *Lycopersicon*. However, results with *Cucurbita* were somewhat similar. In this plant, propagating disturbances considered to be action potentials are readily elicited by sudden cooling of the hypocotyl (cf. Fig. 5, Pickard (1973)). Mechanical stimulation results in fluctuations similar to those of Figs. 6A and 6B.

Certain Russian authors (referenced in Pickard (1973)) have reported reproducible repetitive propagating spikes in *Cucurbita* shoots when the roots are immersed in salt water. We too have observed conspicuous repetitive spiking in hypocotyls when salt water was applied to their roots (though fluctuations were relatively few and weak when salt water was applied to the base of the hypocotyl itself). In no case did the spikes evoked by applying salt to the roots give evidence of propagation, nor did they occur in the roots themselves. It is possible that the reports of propagating repetitive spikes were based on failure to interpret artifacts resulting from misplaced reference electrodes. In any event, it

is not clearly established how closely these salt-elicited fluctuations can be compared with those elicited by localized chilling or damage.

Cutting, crushing, and chilling *Ipomoea* consistently failed to produce action potentials.

Discussion

The artificial means of eliciting spreading electrical fluctuations used in these experiments have important counterparts in nature. Wounding is a common occurrence in the world of plants: it occurs when insects feed, pathogens invade, herbivores graze, or strong winds blow. Appropriate responses to wounding have considerable survival value, and possible roles for the variation potential or its underlying causal agent in coordinating wound responses therefore deserve study.

The main experiments have been carried out with the expectation that relatively drastic stimulation would provide sudden, large, readily measurable electrical effects of a sort which might occur in a more subtle way after much milder but more prolonged stimulation. Moreover, they have been designed in such a way that if the response were mediated by chemical material, there would be ample opportunity for it to gain access to the transpiration stream. In the second paper of this series, it will be demonstrated that the variation potential can indeed be mediated by dispersal in the transpiration stream of a factor or set of factors of the kind discovered by Ricca in 1916.

Acknowledgments

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CHEESEMAN, J. M., and B. G. PICKARD. 1975. Depolarization of cell membranes in leaves of *Lycopersicon* by Ricca's factor. Plant Physiol. 56: 81 (Suppl.).

CORRELL, D. S. 1962. The potato and its wild relatives. Texas Research Foundation, Renner, Texas.

D'ARCY, W. G. 1973. Family 170. Solanaceae. *In* Flora of Panama. Part IX. *Edited by* R. E. Woodson, Jr., and R. W. Schery. Ann. Mo. Bot. Gard. 60: 573–780.

DESBIEZ, M.-O. 1973. Précisions sur le mécanisme des corrélations entre bourgeons cotylédonaires induites par des stimulations mécaniques du cotyledon chez *Bidens pilosus* L. Z. Pflanzenphysiol. 69: 174–180.

HOUWINK, A. L. 1935. The conduction of excitation in *Mimosa pudica*. Recl. Trav. Bot. Néerl. 32: 51–91.

PICKARD, B. G. 1973. Action potentials in higher plants. Bot. Rev. 39: 172–201.

RICCA, U. 1916. Solution d'un problème de physiologie: la propagation de stimulus dans la sensitive. Arch. Ital. Biol. (Pisa), 65: 219–232.

SIBAOKA, T. 1969. Physiology of rapid movements in higher plants. Annu. Rev. Plant Physiol. 20: 165–184.

VAN SAMBEEK, J. W., and B. G. PICKARD, with participation by C. E. ULBRIGHT. 1976. Mediation of rapid electrical, metabolic, transpirational, and photosynthetic changes by factors released from wounds. II. Mediation of the variation potential by Ricca's factor. Can. J. Bot. 54. This issue.

Mediation of rapid electrical, metabolic, transpirational, and photosynthetic changes by factors released from wounds. II. Mediation of the variation potential by Ricca's factor

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An extract of *Lycopersicon* leaf tissue applied to the base of an excised *Lycopersicon* leaf causes a variation potential to spread through the leaf. This spread of an electrical wave is closely dependent on the spread of the extract via the transpiration stream, and the amplitude of the variation potential depends on the concentration of extract applied. Extract from 5–10 mg fresh leaves diluted to 1 ml elicits threshold response, and saturation is accomplished with a concentration about 40 times greater. Evidently, the active factor or factors are effective at very low concentrations. A variety of substances cause electrical disturbances when applied to the excised leaf, but plant extract is the only tested material which under the given conditions desensitizes the leaf to an application of saturating extract 5 min after the initial application.

The active factor or set of factors has been extracted from plants in several families and appears to be closely related to the substance implicated by Ricca in 1916 in trauma-induced closure of *Mimosa* leaves. In recognition of this early discovery, the material is at present called Ricca's factor.

Introduction

In the first paper of this series, it was shown that localized damage to a leaf can cause an electrical fluctuation identified as a variation potential to spread throughout the plant. The present paper justifies the identification by describing the preparation and assay of a plant extract containing a factor or set of factors which, during its distribution via the transpiration stream, produces the electrical fluctuation.

Materials and Methods

(I) General Aspects

Most of the techniques and equipment as well as the methods of growing plants have been described in the preceding paper (Van Sambeek and Pickard 1976).

However, to obtain large quantities of *Lycopersicon* shoots for extraction of factor, plants were grown as described in Materials and Methods, section I(C), of the preceding paper until they reached a height of 0.2–0.3 m. They were then transferred to clay pots of about 0.2 m diameter and set on a shelf in front of a window with full southern exposure. They were used for extract whenever convenient but always before they attained a height of 1 m.

Mimosa pudica and *Pisum sativum* cv. Alaska were also grown under window light.

(II) Extraction

The procedure for preparing crude extract was essentially the same for roots, fruits, stems, and leaves. Tissue was excised rapidly, weighed immediately, combined with deionized water,[2] and ground in a Waring blender. The resultant puree was boiled for about a minute and then filtered through a sheet of Whatman No. 1 or No. 4 paper into an ice-cold flask. The filtrate was then frozen and thawed, centrifuged for about 15 min at about 10 000 *g*, and decanted. After the volume of the extract was measured, its concentration was indexed as milligrams of fresh tissue per millilitre final volume of extract (hereafter referred to as mg-equivalents/ml). The extract was lyophilized or diluted as desired and frozen for storage.

Large amounts of crude extract were prepared at one time so that many experiments could be carried out with the identical batch. The batch of *Lycopersicon* leaf extract used for most of the final experiments, including those of Figs. 2–5, contained 26 μeq Na^+ and 99 μeq K^+ per gram extracted.

In a special experiment, *Lycopersicon* leaf extract was divided during preparation into two lots, one of which was not boiled but which was centrifuged and then bioassayed immediately and after a lapse of 2 h. No

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[2]For *Mimosa*, boiling water was used, after Hesse *et al.* (1957).

difference was found in the activities of boiled and unboiled samples so it may be concluded that for *Lycopersicon* the function of boiling the extract is to facilitate precipitation of debris.

It was checked that the extract did not lose activity during standard autoclaving or long-term storage at subzero temperature.

(III) Bioassay

Plants were handled extremely gently during preparation for the bioassay. Using a sharp razor, the stems were cut under water to avoid drawing air into the xylem. The upper portion of the shoot was kept dry, for once wetted, the cuticle withdrew spreading films of water from the electrode wicks which were later placed on it, and short-circuiting could occur. Fully or mostly expanded leaves were cut close to the stem, and maintaining them at an angle close to that at which they had grown, their cut ends were submerged under tap water that had been aerated to permit escape of chlorine. For at least ½ h, they were maintained under low-level illumination. During this period they were secured with a soft thread or a thin strip of tape against 10-mm-wide wooden slats fixed at an angle of 30° from the vertical; their adaxial surfaces were forward and their ends were now submerged in 0.1 × Hoagland's macronutrient solution (0.5 m*M* $Ca(NO_3)_2$, 0.5 m*M* KNO_3, 0.2 m*M* $MgSO_4$, and 0.1 m*M* KH_2PO_4). Then the lights described in the preceding paper were turned on.

Two variations were made on this basic procedure. First, for leaves of *Ipomoea* the standard solution was reduced to half strength. Second, the motile leaves of *Mimosa* were excised in air at the base of the pulvinus and recut under water at the junction of the pulvinus and petiole. The petioles were slipped through holes of 5 mm diameter in a wooden stand and secured with a small amount of caulking compound, and the cut ends were submerged in the standard solution.

After 2–8 h of equilibration under the lights, two recording electrodes were applied to the petiole (or, in the case of *Lycopersicon*, to the rachis between two major pairs of leaflets). A reference electrode was applied to the tip of the leaf (or for *Lycopersicon* the terminal leaflet and for *Mimosa* the tip of a rachiolus). The method of electrical recording has been described in the preceding paper. After recording for at least 10 min, with a relatively steady baseline for at least 5 min, the dilute Hoagland's macronutrient solution was withdrawn and within 10–15 s a test solution was applied. If the test solution was abundant the cut ends were submerged, but if it was limited, a 10- to 50-μl drop was dispensed from a syringe onto the petiole just above the cut face and allowed to run over it. The drop was watched carefully, and if it dwindled to a thin film during the treatment period, solution was reapplied.

Responses in the bioassay were assessed by measuring the voltage drop from the most positive point on the record after application of solution to the most negative voltage that developed within 5 min, exclusive of spikes which at half amplitude had a duration of less than 30 s. It should be noted that because of the necessity of placing the reference electrode at the leaf tip, fall of the voltage may be prematurely terminated by the passage of the variation potential toward and under the reference electrode, as described in the preceding paper. Thus some measurements may represent minimal estimates, but this source of error is probably no greater than the other sources of variability associated with the bioassay.

For quantitative determinations, all bioassays for a given sample were repeated using at least five leaves with no more than three of the five leaves used on a single day. Since two voltage recordings were obtained from each leaf, each determination is thus an average of the results of at least 10 recordings.

(IV) Measurement of Radioactivity Introduced into the Transpiration Stream

To check the temporal relation between the distribution of [^{32}P]phosphate in the transpiration stream and the spread of the variation potential, special frames which held sheets of nylon milliner's net were built to support *Lycopersicon* leaves upright, accessible for the placement of electrodes on the adaxial side and for the placement of windows of Geiger-Müller tubes on the other. The windows were mounted behind a sheet of Plexiglas 6 mm thick; each was centered behind a slit 2.5 mm high and 30 mm wide. Slit centers were 35 mm apart. The distance of the lower slit from the base of the petiole was adjustable. Short electrode wicks were positioned in the groove that runs up the rachis, directly opposite the centers of the slits.

One Geiger-Müller counter was survey meter model 2652 from Nuclear Chicago (Des Plaines, IL 60018); its counting efficiency was about 10% and its background was low (21 counts/min). The second counter was an older machine with a slightly lower efficiency and a high background (98 counts/min). In half of the experiments, this counter was placed next to the distal electrode and in half it was placed next to the proximal electrode.

$KH_2{}^{32}PO_4$ in H_2O, specific activity 1 Ci/mmol, was obtained from New England Nuclear, Boston, MA 02118. To extract at a concentration of 200 mg-equivalents/ml was added enough ^{32}P to give about 4.4×10^7 disintegrations/s ml. A 5-ml aliquot of the radioactive solution in a beaker shielded inside a thick Plexiglas container was slipped under the petiole to replace the beaker containing 0.1 × Hoagland's solution. A Plexiglas plate mounted under the counting tubes shielded them from the beaker during its transfer and when it was in experimental position.

Each electrode signal together with the voltage output of its associated Geiger-Müller counter was monitored on a dual-channel Brush Mark 220 oscillograph at a chart speed chosen to facilitate measurement of the electrode signal (125 mm/min). The two Geiger-Müller counter outputs were simultaneously monitored by a third oscillograph running at a higher chart speed (5 mm s^{-1}) to increase resolution of the counts. The charts of the three recorders were coordinated at appropriate intervals by activating a relay to short the event marker inputs.

Results

(I) Experiments with Lycopersicon

(A) Response of Excised Leaves to Leaf Extract

(1) Typical Variation Potentials

Initially, it was of interest to check whether excised leaves respond to the standard heat

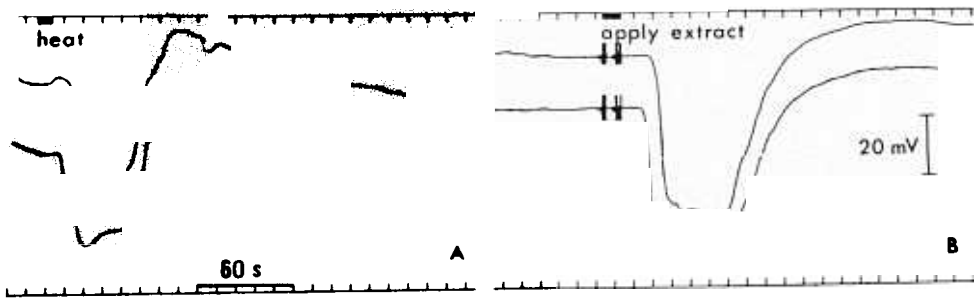

FIG. 1. (A) Control: a variation potential spreading up the rachis of an excised leaf prepared for bioassay but stimulated by heating a lower leaflet; electrode separation, 20 mm. (B) Variation potential resulting from application of extract in standard bioassay fashion. First burst of truncated electrical noise resulted from removal of equilibrating solution, second burst resulted from replacement with 200 mg-equivalents/ml extract. Electrode separation, 20 mm. Neither the variation potential of A nor that of B shows obvious putative action potentials, but these were frequently present, especially when high concentrations of extract were applied. Note the rapid apparent simultaneous return of the signals from the peak resulting from passage of the variation potential beyond the recording electrodes and under the reference electrode situated at the tip of the apical leaflet.

stimulus used in the first paper in this series in the same way as do intact plants. Therefore, in several experiments a lower leaflet of an excised leaf that had been set up for bioassay was heated with a flaming match while voltage was monitored. Figure 1A shows a photograph of a representative pair of recordings of a variation potential passing up the rachis immediately after stimulation; the electrical response is indeed sensibly the same in excised leaves and intact plants (cf. Fig. 3, Van Sambeek and Pickard 1976).

When a crude extract of *Lycopersicon* leaves is applied at an appropriate concentration to the base of a leaf set up for bioassay, a variation potential again results. Figure 1B is a photograph of a typical pair of recordings after application of extract at a concentration index of 200 mg-equivalents/ml.

As demonstrated in the preceding paper and noted again in Materials and Methods, Bioassay, voltage changes detected by the reference electrode at the leaf tip are subtracted from those detected under the recording electrodes. Because of the variability of the former, the late portion of the signal is a complicated composite. In general, therefore, the meaningful parameters of variation potentials in bioassay leaves are arrival times, rise times, and peak amplitudes.

As a further check that the bioassayed responses are not peculiar to excised leaves, another set of experiments was carried out on soil-rooted plants. A lower leaf was removed, and a test solution was applied to the cut surface of the remaining short petiolar stump while voltage was recorded from a higher internode or leaf. Application of 1/10-strength Hoagland's macronutrient solution produced no response, but application of extract at a concentration of 200 mg-equivalents/ml could cause a typical variation potential to spread up the shoot. Extract also produced large typical variation potentials when applied to the bases of shoots that had been detached from the roots at soil level.

(2) Dose-response Curves

The plot of Fig. 2 illustrates how the amplitude of the variation potential depends on the concentration of applied extract. Concentrations of 5 mg-equivalents/ml or less produce no noticeable effect, but between about 10 and 100 mg-equivalents/ml the plot of average amplitude of the variation potential rises sharply and then begins to level off. In the experiment shown in Fig. 2 (one of four closely comparable replicates based on four separate extracts), the amplitude of the variation potentials elicited by the saturating concentration ranged between -20 and -50 mV and averaged about -35 mV. In a parallel experiment in which one leaflet of the lowest pair of interstitial leaflets was thermally damaged, the average response seen in 30 recordings from 15 leaves was -34 mV.

Although the amplitude of the variation potential provides the only quantitatively reliable index of the concentration of applied extract, there is a second feature of the variation potential which also tends to increase with the concen-

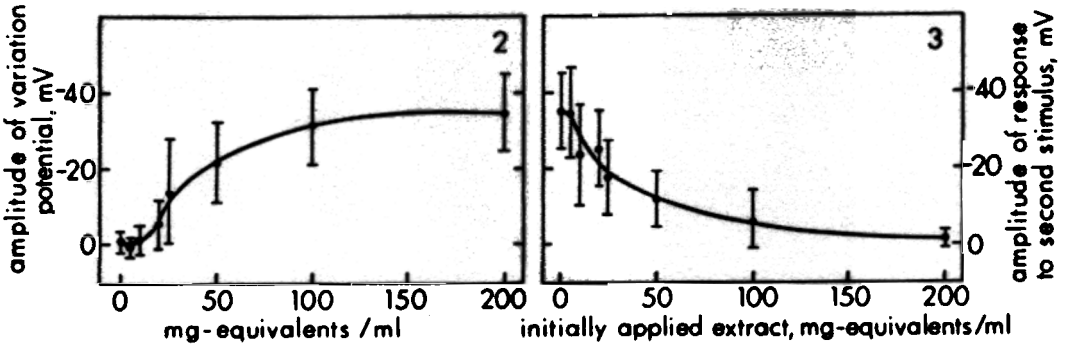

FIG. 2. Bioassay response as a function of the concentration of crude extract. The standard deviation of the sample population rather than the standard error (standard deviation of the mean) is presented to emphasize the large scatter of the experimental recordings; five leaves (10 recordings) per sample. FIG. 3. Desensitization of the bioassay leaf to further stimulation as a function of the concentration index of applied extract. The second, challenging application is of 200 mg-equivalents/ml extract and follows 5 min after the first. The standard deviation is shown for each sample population; 10 recordings per sample.

tration of applied extract: the number and size of accompanying putative action potentials. While it is rare to see any indications of putative action potentials when low concentrations are applied, spikes are common at near-saturating concentrations.

(3) Desensitization

If extract at a concentration of 200 mg-equivalents/ml is applied to a leaf 5 min after evocation of a variation potential by an initial application of extract, the amplitude of the variation potential evoked by the saturating concentration will be reduced by an amount about equal to the amplitude of the first response. Figure 3 illustrates this graphically. Thus, application of a standard 200-mg-equivalents/ml extract 5 min after application of an unknown solution provides a check of the primary assay response, and this procedure was therefore incorporated into the routine bioassay protocol.

The persistence of the desensitization demonstrated in Fig. 3 was not quantified, but preliminary experiments suggested that the return to initial sensitivity may require some hours.

(B) Correlation of the Movement of the Electrical Fluctuation and of the Extract

There are two possible explanations for the spread of the electrical fluctuation: (1) the extract, acting at the base of the petiole, triggers a self-propagating electrical wave and (2) the extract, spreading throughout the leaf, causes local electrical changes as it goes. Only the second explanation accords with previously established usage of the term variation potential. To distinguish between the two possibilities, a set of experiments was carried out to establish what correlation might exist between the movement of the extract and the movement of the electrical wave. The extract was labeled with $KH_2{}^{32}PO_4$ and applied to the cut base of a petiole while at each of two points on the rachis a Geiger-Müller counter monitored the arrival of the radioactivity and an electrode monitored the arrival of the voltage fluctuation.

Figure 4A shows a photograph of a portion of a typical set of 125-mm/min recordings. The uppermost and lowermost traces are the recordings of the radioactivity registered by the basal and apical Geiger-Müller counters, and the two center traces are the companion recordings of voltage. At the time marked by the first arrow, the control solution was removed from the base of the petiole (the stable voltage baseline is disrupted by this manipulation). At the second arrow, the beaker containing ^{32}P-labeled extract was slipped under the petiole (resulting in a rapid return of the voltage to the original baseline). Between the arrows, as the beaker of labeled extract was being moved into position, the Geiger-Müller counters recorded a slight rise in the background level of radioactivity.

In less than ½ min after application of labeled extract, it is conspicuously visible that both the radioactivity and the voltage are shifting in the basal recordings, and in less than 1 min there is a clear shifting in the apical recordings. Under these circumstances, rapid movement of ^{32}P-

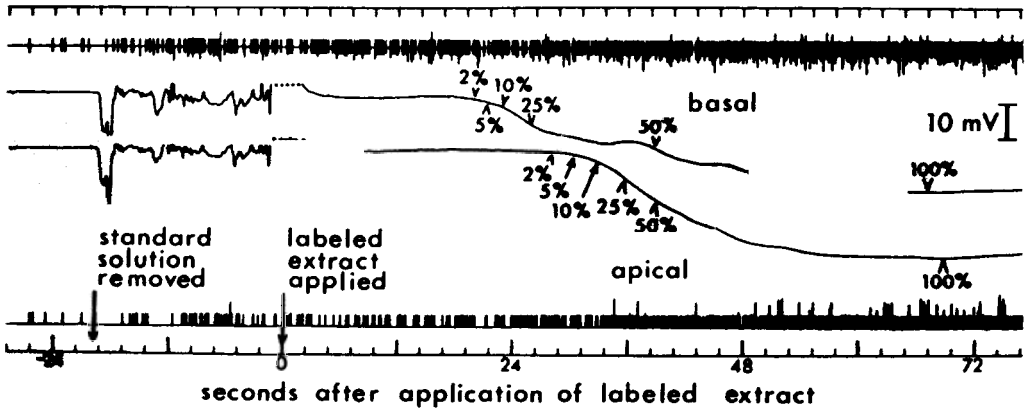

FIG. 4A. Recordings of the progress of radioactively labeled extract and of the variation potential up the petiole–rachis of an excised leaf. Outer traces, Geiger-Müller counter output; middle traces, voltage record. The count rate and the variation potential can be seen to increase together both in the paired basal recordings and in the paired apical recordings. The detectors were centered on the rachis 105 and 140 mm above the cut base of the petiole. The interwindow velocity of the variation potential is 4.0 mm s^{-1}.

labeled extract outside the transpiration stream is unlikely, and the velocity of the label is of the correct magnitude for the transpiration stream. Since the electrical wave and the wave of radioactivity reach the recording points at about the same time, their movements are evidently closely coupled. Even from these very qualitative observations, then, support is provided for the second of the two hypotheses under test: namely, the electrical fluctuation is a local response to the spread of the extract in the transpiration stream. However, to strengthen this support, replicate experiments were subjected to statistical analysis.

(1) Statistical Determination of the Arrival of Radioactivity

The counts in each 1-s interval of every 5-mm s^{-1} Geiger-Müller recording were plotted as a function of time from the application of extract. This is illustrated for the early portion of the same experiment shown in Fig. 4A by the upper pair of graphs in Fig. 4B. To a good approximation, every plot could be considered to consist of three parts: (1) a set of points falling along a straight, nearly horizontal path, (2) a set of points falling along a linearly rising path, and (3) a set of points which fell along a path of decreasing slope. The first part of the plot must represent the background activity detected during the movement of the front of radioactivity up the petiole–rachis to the window, the second part must represent the initial movement of the front past the window (and early leakage of tracer from the xylem to the surrounding tissue), and the third part of the curve must represent the period well after the front of radioactivity has passed the window when tracer is accumulating in the tissue because of leakage out of the xylem. The intersection of

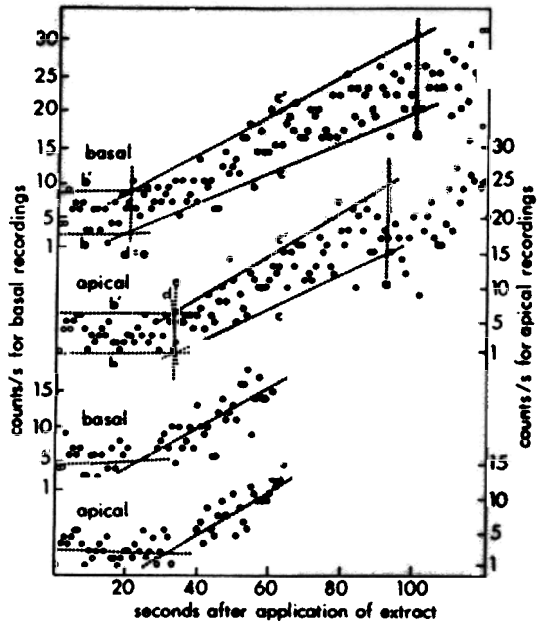

FIG. 4B. Upper plots are of the counts per second measured on the accelerated recordings corresponding to the outer recordings of Fig. 4A. Lower plots illustrate the fitting of regression lines to ascertain their intercepts as estimates of the times of arrival of the labeled extract at the two counter window slits. The interwindow velocity of the radioactivity is 4.4 mm s^{-1}, corresponding closely with the velocity of the variation potential.

the first two parts must represent the arrival of the radioactive tracer at the window.

This intersection was evaluated iteratively for each plot by making a preliminary graphical estimate and then using it as the basis for a statistical determination. The initial graphical step was to draw a vertical line (marked *a* in Fig. 4B) approximately indicating the end of the linearly rising part of the curve; all points beyond this were then ignored. Next, the points of the first part of the plot were delimited by two lines (marked *b* and *b'*) which were constrained to be horizontal, and the points of the second part of the plot were delimited by two lines (*c* and *c'*) which were not constrained to be parallel. (Points that lay well away from the main cluster were ignored.) A line segment, *d*, was drawn to connect the intersections of *b* and *c* and of *b'* and *c'*, and its midpoint, through which was drawn a vertical line, *e*, was considered the first, graphical iteration of the arrival time of the tracer.

For the second, statistical iteration, points of the first two parts of the curve were represented by linear regression lines and the intercept of the lines was determined. As illustrated in the lower two plots of Fig. 4B, all points between the time of application of the extract and the graphically estimated intercept were used for the fitting of the first regression line, excepting occasional points that fell outside the horizontal bounds. (It was then checked that the excluded points did fall outside the 95% confidence interval for the regression line.) Selection of points for the calculation of the second linear regression line was slightly more involved since (1) it was supposed that the front of radioactivity could not be perfectly abrupt but must represent a region of increasing slope and (2) the slope of the late portion of the curve might begin to decrease imperceptibly long before a change can be seen by drawing the envelope of the scattered points. Reduction of possible bias by these two factors was accomplished by basing the regression analysis on only those points falling in the interval from 0.1 to 0.5 of the way between lines *e* and *a* (again, see the lower plots of Fig. 4B). Points which fell well outside the enveloping lines *c* and *c'* were ignored. Finally, the intercept of the two straight lines was determined; in the case of Fig. 4, values are 24 s for the basal recording and 32 s for the apical recording.

If in a particular recording the initial background was relatively high, as was often the case with the older Geiger-Müller counter, and if the second phase of the curve had a relatively low slope, the recording was of low reliability. Therefore, recordings were rejected if the 68% confidence interval of the first regression line for the 1-s interval preceding the intercept was not less than 15 times the value of the slope of the second regression line.

(2) Determination of the Arrival of the Electrical Fluctuation

Two factors complicate the estimation of the arrival of the electrical fluctuation. First, as is readily apparent from the voltage recordings of Figs. 1 and 4A, the waveform is variable. Because of this problem, no recording was used unless its purported variation potential had an amplitude greater than −20 mV and a reasonably smooth fall unaccompanied by spikes. Second, the extracellular electrodes undoubtedly pick up a signal some small distance away from the wick as a result of electrotonic spread. For this reason, several indices of arrival were compared: the times required for a 2%, 5%, 10%, 25%, and 50% fall from the baseline (cf. Fig. 4A). Certainly, the 2% fall is difficult to determine with accuracy because the early rise of the voltage wave is slow, and it is apt to precede activity of cells directly under the electrode wick because of electrotonic run-up. The 5% and 10% falls would seem to be more reliable because they are easier to measure and because the relative contribution from distant electrical activity is likely to be smaller. On the other hand, shoulders and minor peaks of the curve are often prominent by the time 50% of the fall has been attained, and the second half of the fall, though indexed for completeness by the 75%, 90%, and 100% fall times (Fig. 4A), is highly variable. As noted in Results, section I(B), of the preceding paper, the smoothest, most regular, and most easily measured portion of the typical variation potential is the region in which the potential has attained about 25% of its maximum amplitude.

(3) Correlation of the Arrival of the Label and the Electrical Wave

One simple way of correlating the electrical signal with the tracer is to compare their velocities between the two measuring points. Averaging the 12 experiments in which all four recordings passed the selection criteria discussed in sections 1 and 2, the interwindow velocity of

the radiotracer was found to be 3.7 mm s^{-1}, with a standard error of ± 0.7 mm s^{-1}. The average interelectrode velocity of the variation potential, based on the 25% fall, was 3.5 $\pm$ 0.6 mm s^{-1}. The close comparability of these two numbers suggests that the movement of the electrical fluctuation is closely correlated with the movement of the extract in the transpiration stream.

A more searching test of the relation of voltage to radioactivity is presented in Fig. 5, in which the percentage attainment of full amplitude of the variation potential is plotted against time, with the calculated time of arrival of radioactivity set to zero. Only the 38 paired voltage and radioactivity recordings which met the criteria of sections 1 and 2 were used. The average calculated arrival of radioactive label coincides with the interpolated 4% rise of the variation potential. This is of course a satisfactory indication that the arrivals of the radioactivity and the variation potential are closely linked.

Further, one cannot imagine that the arrival of the electrical event triggers the arrival of the radioactivity, whereas it does seem reasonable that the arrival of the isotopically labeled extract triggers the occurrence of the electrical event. There are enough theoretical problems in the determination of both the arrival of electrical activity and the arrival of radioactivity to account for an even larger difference in indices of arrival times than is seen. Electrotonic run-up may precede the arrival of the electrical signal directly under the electrode wicks. A bias in the same direction will result if, as seems likely, the simplifying assumption of section 1 that the linear rise of radioactivity begins abruptly is erroneous. Countering these particular probable biases is the evident need for active material to leak from the dead conducting channels of the xylem to the surrounding live cells for membrane depolarization to occur. All in all, any attempt to make much of either a lead or a lag of a few seconds would be frivolous.

Thus, the electrical fluctuation is correctly identified as a variation potential.

(C) Specificity of the Bioassay

The variation potential is the externally measured manifestation of the depolarization of the membranes of a large population of cells (Cheeseman and Pickard 1975). Since many substances which might influence transmembrane potentials could potentially occur in crude leaf extract, it is of interest to consider the effects of several kinds of substances applied as test solutions in the bioassay.

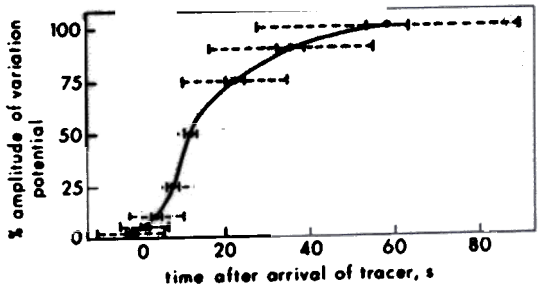

FIG. 5. The fall of the variation potential plotted against time after the calculated arrival of radioactivity. Both the standard deviation of the mean and of the sample population are indicated for each mean percentage of full amplitude.

(1) Solutions of High Osmolarity

Because a crude extract of 100 or 200 mg-equivalents/ml must contain a high concentration of osmotically active material, it was of particular interest to test the effects of common osmotic agents. Both KCl and mannitol were ineffective at low concentrations, but at high concentrations they produced responses which closely resembled extract-induced variation potentials and which could have associated spikes. For example, 1 *M* mannitol produced responses averaging -12 mV in amplitude, and 1 *M* KCl (2 equivalents/litre) produced an average response of -22 mV while 100 m*M* KCl produced an average response of -7 mV. However, in marked contrast with application of 200 mg-equivalents/ml extract, application of these solutions caused the bioassay leaves to wilt within a short while. KCl solutions of 50 m*M* strength caused no wilting and caused average responses of -3 and 0 mV, respectively; only extensive replication could permit judgment of whether the value of -3 mV is significantly different than the value of -1.3 mV recorded from water controls.

It is quite likely that high osmotica cause depolarization directly, but it is also possible that part of their effect is due to release of a specific active agent by stressed cells. Regardless of the interpretation of the effect, it is clear that any agent putatively active at low concentrations cannot be tested in the presence of a large amount of other solute.

(2) Known Depolarizing Agents

In some systems, sugars and amino acids can be cotransported with hydrogen ions and, when applied suddenly, can cause dramatic depolarizations (Slayman and Slayman 1974; Etherton and Nuovo 1974). Therefore, sugars and amino acids were tested in the bioassay.

D-Glucose, D-fructose, D-galactose, and also myoinositol were applied individually in the bioassay at a concentration of 10 m*M*, but they produced no response. When the four carbohydrates were mixed together and applied at a total concentration of 40 m*M* (with a contribution of 10 m*M* from each), an average response of −8 mV was measured. However, these concentrations were each far above those measured in 200 mg-equivalents/ml extract, using gas chromatography (Mary Ellen Pusateri, unpublished data). When the mix was diluted to a still high value of 4 m*M*, no response resulted. Sucrose (10 m*M*) was also tested, but it produced either no response or at best a few millivolts.

Amino acids, some of which were noted by Fitting (1930) to give positive results in a *Mimosa* leaf closure bioassay, were also tested in the *Lycopersicon* assay. Cysteine, at the relatively high concentration of 20 m*M*, gave responses as great as those of a saturating concentration of leaf extract. Serine (20 m*M*) and glycine (20 m*M*) each produced a response of about −20 mV. However, these results do not suggest that amino acids could contribute importantly to the effect of the leaf extract because the challenging application of 200 mg-equivalents/ml extract 5 min after application of these amino acids resulted in a full-sized variation potential. Moreover, the leaf tissue contained only about 2 μmol of free amino acids per gram (Corinne E. Ulbright, unpublished data).

Metabolic inhibitors may also depolarize cell membranes, and therefore KCN was tested at concentrations of 1, 20, and 50 m*M*. At 1 m*M* no effect was observed, but at the higher concentrations KCN could cause a drop equal to or greater than that expected from 200 mg-equivalents/ml extract. However, the drop of the voltage was extremely slow under both recording electrodes and the propagation of the effect was also slow. From the failure of the potentials to return toward zero within the 10-min period of observation, it may be supposed that the tissue under the reference electrode was not strongly affected during that time. During the slow depolarization, many large spikes or putative action potentials were observed. In several instances, a challenging solution of 200 mg-equivalents/ml extract was applied 5 min after the application of KCN, and little desensitization of the response to the extract was observed.

Salicyclic acid, which probably uncouples mitochondrial electron transport and which causes depolarization of cell membranes (Glass and Dunlop 1974), was also tested. No appreciable response was elicited by a 10-m*M* solution during the 5-min observation period.

(3) Reducing Agents

The reducing agents glutathione and dithiothreitol produced results indistinguishable from that of water when applied at concentrations of 1 m*M*. Ascorbic acid, found by Hesse *et al.* (1957) to be active in the *Mimosa* leaf closure bioassay at a concentration of 100 m*M*, could produce responses of about −30 mV at 100 m*M* but on the average only resulted in a drop of 7 mV when applied at 10 m*M*.

(4) Common Hormones

The plant hormones abscisic acid, gibberellic $acid_3$, and indoleacetic acid could not be discriminated from water when tested at a concentration of 100 μ*M*.

(D) Effective Concentration of Extract: a Suggestion that the Active Agent is Hormonal

About 93% of the fresh tissue from which the extract was prepared was water. If one were to assume for purposes of argument that all of the dry matter is a single compound active in the bioassay and having a molecular weight of 1000, the compound would elicit a measurable response at a concentration of less than 10^{-3} *M*. Of course, the concentration applied to the base of the petiole in the bioassay is likely to be diluted before it initially reaches the living cells near the recording electrodes, as is compatible with the finding of Cheeseman and Pickard (1975) that threshold electrical responses are elicited by a 10-fold lower concentration if application is made to sectioned tissue rather than to entire leaves. Thus, according to this argument, the postulated compound would be active at a concentration below 10^{-4} *M*. Moreover, allowing realistically for the great diversity of molecules in the plant as well as for an incomplete extraction of the dry matter of the leaf into the crude extract and an arbitrary assignment of molecular weight, it is probable that the active material

TABLE 1. Bioassay with several taxa

Plant	Concentration index, mg-equivalents/ml	Replicate recordings	Amplitude, −mV	Standard deviation
Ipomoea	200	32	7	5
Mimosa	100	28	17	16
Xanthium	100	10	31	12
Gossypium	100	10	17	12

must be present at considerably lower concentrations. By definition, if a chemical agent is released at one site in the plant and at low concentrations brings about physiological changes at distant sites, the agent is a hormone; the totality of the evidence of this and the preceding paper points to the existence of a factor or set of factors that meets the definition. However, until the material can be highly purified and chemically well characterized, it is premature to decide whether it is a hormone.

(E) Root, Fruit, Stem, and Petiole Extracts

Extracts of green *Lycopersicon* fruits, of ripened fruits, of petioles, of leaf blades, and of stems all showed activity and 'specificity' in the bioassay. On the other hand, there was considerably less activity per unit weight of root tissue extracted from hydroponically grown plants than found in the other tissues, making it difficult to separate the influence of a specific agent from that of contaminating salts, amino acids, and the like.

(II) Experiments with Other Plants

(A) Response of Leaves of Various Plants to Their Own Extracts

As documented in the preceding paper, several kinds of plants produce similar variation potentials when stimulated by localized heating. Thus, it is of interest to check for the ability of some different extracts to elicit variation potentials in the kind of leaves from which they were derived. Table 1 shows that extracts of *Ipomoea*, *Mimosa*, *Xanthium*, and *Gossypium* all elicit variation potentials in leaves of their respective taxa. The variation potentials of *Ipomoea* leaves stimulated with *Ipomoea* extract are fairly low, but not enough work has been carried out on these several kinds of leaves to know if the experimental conditions have been suitably optimized.

(B) Response of Leaves to Extracts from Other Species

Preliminary experiments showed that extracts from *Ipomoea*, *Mimosa*, *Xanthium*, *Gossypium*, *Cucurbita*, and *Pisum* are electrically active on leaves of *Lycopersicon*, though not in all cases as active as extract from *Lycopersicon* itself. The several extracts tended to desensitize *Lycopersicon* leaves to *Lycopersicon* extract. When *Lycopersicon* extract was tested on *Ipomoea*, *Mimosa*, and *Gossypium*, there were relatively small responses, whereas the response of *Xanthium* was comparable with that of *Lycopersicon* itself. These experiments reinforce the interpretation that there is a widespread class of compound which is responsible for variation potentials and associated effects. The data are compatible with the interpretation that there is more than one member of the class; it is, however, unwise to make ado over this compatibility because so many problems can confound experiments with crude extracts.

Discussion

(I) General Remarks

In the first paper of this series, it was demonstrated that damage to *Lycopersicon*, *Ipomoea*, *Gossypium*, *Xanthium*, and *Cucurbita* produces a traveling electrical response which was identified with the variation potential shown by Houwink (1935) to spread through a damaged *Mimosa* plant. Houwink produced reasonably compelling evidence that the electrical wave accompanies the distribution via the transpiration stream of an unidentified hormone implicated in the control of leaf closure by Ricca in 1916 (see review by Pickard (1973)). In the present paper, it has been checked that plant extract can cause a wave of voltage fluctuation in *Mimosa*, and it has been further shown that the five nonmotile plants under study respond to extract of their own leaves with similar voltage fluctuations. By using *Lycopersicon* as a model, it has been demonstrated that the voltage fluctuation is a rapid local response to the distribution of extract by way of the transpiration stream; therefore, the fluctua-

tion meets all the criteria for a variation potential established by Houwink with *Mimosa*.

We propose that at present the unidentified factor(s) responsible for the variation potential be called after Ricca. Because the material is transported through the plant and at low concentrations brings about physiological effects at sites distant from its point of release, Ricca's factor may indeed be a hormone or class of hormones. However, until purification and characterization of Ricca's factor are completed, it is impossible to know whether the physiological activity is due to a single factor, a class of closely related factors, or to several unrelated agents acting synergistically. It is unwise to assume that the simplest interpretation, namely, that Ricca's factor is a class of hormones, is correct. However, should further experimentation favor this simple interpretation, we propose that the hormone be named Riccanin in recognition of its original discoverer.

(II) Earlier Work on Ricca's Factor

The method used to prepare a crude extract of Ricca's factor was modified from the method of Hesse *et al.* (1957). These workers had in turn based their studies on an earlier publication, that of Soltys and Umrath (1936). The factor Hesse *et al.* describe was unstable in fresh extracts in *Mimosa*, though they believed it to be abundant and stable in dried leaves of tea (*Thea chinensis*). They prescribed boiling of the extract to inactivate a degrading enzyme and working in an atmosphere of nitrogen to prevent oxidation, but in our work with *Lycopersicon* extracts we have been able to let freshly pureed leaf material sit in air at room temperature for 2 h without detectable change of activity. Boiling has, however, proved to be a useful step in precipitating protein and other unwanted materials from the pureed leaves. In its stability, our extract corresponds to that of Umrath (1927) and Soltys and Umrath (1936), with whose conclusions Hesse *et al.* disagreed. The active agent does disappear during prolonged incubation unless kept sterile, so it is presumably subject to microbial degradation.

(III) Possible Roles for Ricca's Factor

It is widely known that wounding triggers a variety of important processes. Of particular interest are those which may participate in plant response to herbivorous insects and to pathogens. The catenas of events leading from an act of damage to the final responses are not in general thoroughly worked out. The possibility that Ricca's factor plays a mediational role in some of these responses therefore deserves attention.

However, at this stage of study it is desirable that Ricca's factor not be characterized simply as a wound factor. As our knowledge of regulatory agents expands, a variety of diverse roles often becomes apparent for compounds which were identified in a limited context.

Ricca's factor was discovered because of its almost instantaneous causation of the variation potential. Should it turn out to be a genuine hormone or class of hormone, this immediate response might serve as a useful system for investigating primary hormone action.

Acknowledgments

We thank Richard Burne for his participation in some of the early experiments of this study. We acknowledge much invaluable stimulating discussion with Joseph E. Varner. We are grateful to Mary Ellen Pusateri, Charles Jeff Mauvais, and Carol C. Van Sambeek for contributing superb technical assistance during later phases of the work. We are grateful to the Shell Development Co., Modesto, CA 95352, for a gift of abscisic acid.

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CHEESEMAN, J. M., and B. G. PICKARD. 1975. Depolarization of cell membranes in leaves of *Lycopersicon* by Ricca's factor. Plant Physiol. 56: 81 (Suppl.).

ETHERTON, B., and G. J. NUOVO. 1974. Rapid changes in potentials of oat coleoptile cells induced by amino acids and carbohydrates. Plant Physiol. 54: 49 (Suppl.).

FITTING, H. 1930. Untersuchungen über endogene Chemonastie bei *Mimosa pudica*. Jahrb. Wiss. Bot. 72: 700–775.

GLASS, A. D. M., and J. DUNLOP. 1974. Influence of phenolic acids on ion uptake. IV. Depolarization of membrane potentials. Plant Physiol. 54: 855–858.

HESSE, G., B. BANERJEE, and H. SCHILDKNECHT. 1957. Die Reizbewegungen der Mimosoideen und ihre Hormone. Experientia, 13: 13–19.

HOUWINK, A. L. 1935. The conduction of excitation in *Mimosa pudica*. Rec. Trav. Bot. Néerl. 32: 51–91.

PICKARD, B. G. 1973. Action potentials in higher plants. Bot. Rev. 39: 172–201.

RICCA, U. 1916. Solution d'un problème de physiologie: la propagation de stimulus dans la sensitive. Arch. Ital. Biol. (Pisa), 65: 219–232.

SLAYMAN, C. L., and C. W. SLAYMAN. 1974. Depolarization of the plasma membrane of *Neurospora* during active transport of glucose: evidence for a proton-dependent cotransport system. Proc. Natl. Acad. Sci. U.S. 71: 1935–1939.

SOLTYS, A., and K. UMRATH. 1936. Über die Erregungssubstanz der Mimosoideen. Biochem. Z. 284: 247–255.

UMRATH, K. 1927. Über die Erregungssubstanz der Mimosoideen. Planta (Berl.), 4: 812–817.

VAN SAMBEEK, J. W., and B. G. PICKARD. 1976. Mediation of rapid electrical, metabolic, transpirational, and photosynthetic changes by factors released from wounds. I. Variation potentials and putative action potentials in intact plants. Can. J. Bot. 54. This issue.

Mediation of rapid electrical, metabolic, transpirational, and photosynthetic changes by factors released from wounds. III. Measurements of CO_2 and H_2O flux

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By using intact plants from five angiosperm families, shifts in CO_2 and H_2O exchange have been demonstrated to follow the arrival of a variation potential in unharmed leaves shortly after adjacent leaves are damaged.

Whether in the light or dark, the first change is a brief burst of CO_2, which may well be due to a sudden breakdown of bicarbonate dissolved in the extracellular solution.

In the dark, a rise in metabolic CO_2 output becomes evident during the decay of the initial burst, and the output remains elevated for at least several hours.

In the light, a biphasic drop in both transpiration and net CO_2 uptake is underway before the initial CO_2 burst has subsided. Since the decrease in CO_2 uptake is larger than the increase in output in the dark, it must be due in part to decreased net photosynthetic CO_2 fixation. It is plausible but as yet unproven that the biphasic photosynthetic change is a consequence of the biphasic closure of the stomata. In any case, curtailment of both transpiration and net photosynthesis, like the enhancement of dark metabolism, persists for a number of hours.

Introduction

In the preceding papers of this series, it has been shown that localized injury of a leaf or stem results in the release of substances which can spread throughout the shoot. The spreading is indicated by an essentially instantaneous electrical response, the variation potential.

The purpose of this paper is to demonstrate that large shifts in gas exchange also rapidly follow the spread of the chemical and electrical wave.

Materials and Methods

(I) General Aspects

The methods of growing plants, the methods of taking electrical measurements, and the general experimental parameters have already been described (Van Sambeek and Pickard 1976; Van Sambeek *et al.* 1976).

(II) Air Flow System

After setting up a plant as described in Materials and Methods, section II, of Van Sambeek and Pickard (1976), one of its leaves was mounted in a flow-through system for measurement of water vapor release and CO_2 release or uptake (Fig. 1). The leaf (or in the case of *Lycopersicon* some portion of the pinnately compound leaf) was suspended in a 100- or 200-ml transparent plastic chamber by a screen of about 6 × 6 mm grid and sealed in with slightly warmed caulking agent (Mortite brand of the Mortell Co., Kankakee, IL 60901). The leaf chamber was fixed normal to the incident light.

Ambient air from an adjoining, rarely occupied room was passed by a diaphragm pump through a 50-litre surge tank and then cooled in a condensor to the 0–8 °C dew point, thus reducing the water content to 5–8 mg l^{-1}. The air stream was next warmed to room temperature and divided to pass part of it through the leaf chamber. The flow through the chamber was typically 8.4 ml s^{-1}, except in the anaerobic experiments, in which it ranged from 1 to 3 ml s^{-1}. Air coming out of the chamber passed first over a hygrosensor, then over a thermistor, through a drying tube containing either $CaCl_2$ or $CaSO_4$, through a flow meter, and finally into a differential infrared CO_2 analyzer (again, see Fig. 1).

All leaves were equilibrated in the chamber for at least an hour before gas-exchange measurements were begun. For the experiments on darkened plants, the light was turned off 20–30 min before stimulation. For experiments on darkened anaerobic plants, the air stream was replaced with O_2-free gas at the same time. The basal rate of net CO_2 and H_2O flux was recorded for at least 20 min before stimulation.

At about the time when gas measurements were begun, two electrodes were positioned on the petiole or petiole-rachis of the leaf enclosed in the chamber, and frequently an extra electrode was positioned on the leaf to be stimulated (Fig. 1). Recording techniques have been detailed by Van Sambeek and Pickard (1976).

(III) Determination of Transpiration Rate

The hygrosensor over which the air stream from the leaf chamber was passed was a probe containing both a LiCl sensor and a thermistor (sensor model 15-2011A, powered by a modified model 15-3050 hygrometer, Hygrodynamics subsidiary of American Instrument Co., Silver Spring, MD 20910). The probe was mounted in a sealed Plexiglas tube of 100 ml volume in such a way that the air stream was forced directly across the sensing

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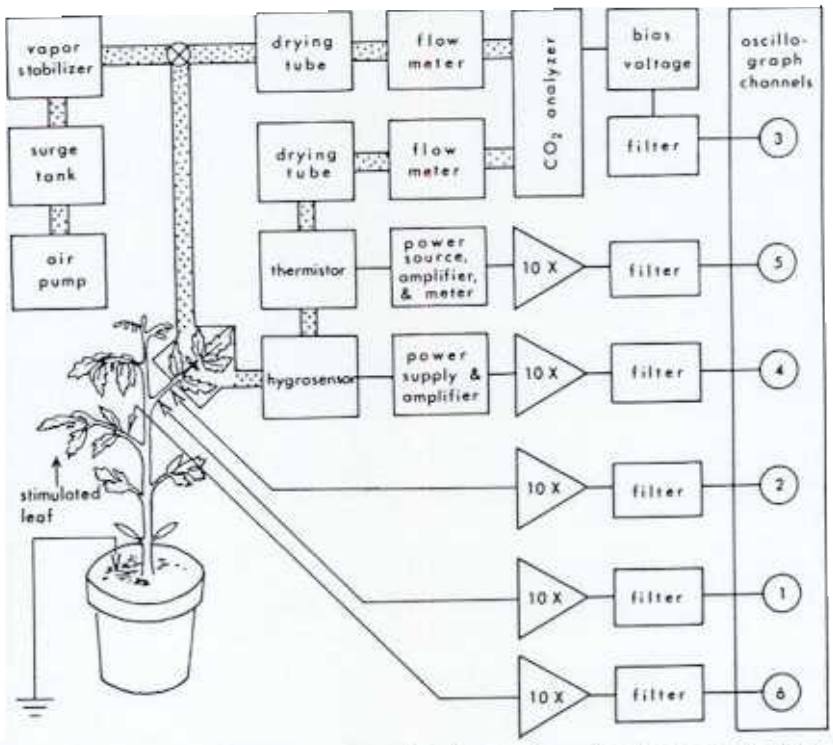

FIG. 1. Block diagram of instrumentation, with plant in place. For *Lycopersicon*, either leaf 3 is stimulated with leaf 4 enclosed in the transparent chamber for measurements (as shown) or 3 is measured while 4 is stimulated. Oscillograph channels 1, 2, 3, and 4 correspond to the four recordings in Figs. 2A and 2B.

elements. At a flow rate of 8.4 ml s^{-1} and a temperature of 25 °C the lag time to 2% deflection, considered as the sum of the time required for a change to flow from the leaf chamber to the mounted hygrosensor plus the time for the hygrometer to respond, was 3 s. Under these same conditions the response time (2 to 98%) of the hygrometer for a change of 7.0 μg water vapor per millilitre of air (a change causing the meter to deflect 50% of full range) was 200 s. Because the thermistor of the Hygrodynamics probe had a slow response time and because its signal could not be recorded concurrently with that of the LiCl sensor, an independent thermistor was often mounted in the air stream just beyond the Hygrodynamics probe (model 409 probe, with model 43-TD Tele-Thermometer, Yellow Springs Instrument Co., Yellow Springs, OH 45387).

The rate of transpiration for the enclosed leaf was calculated by converting the calibrated hygrometer output to water vapor concentration, subtracting the concentration in the air stream entering the leaf chamber from that leaving, and multiplying the remainder by the flow rate.

(IV) Determination of Rate of Net CO_2 Uptake or Release

Differential CO_2 measurements were made with an infrared carbon dioxide analyzer provided with a pair of 343-mm sample cells (models 215 B and 109510, Beckman Instruments, Inc., Fullerton, CA 92634). The stability of the zero point of the analyzer was checked frequently by means of an air line which bypassed the chamber containing the leaf (not indicated in Fig. 1). Periodically, the analyzer was calibrated to assure that with the most sensitive scale a full deflection occurred for a change of 50 nl CO_2 per millilitre of gas. The calibration mixtures of CO_2 in N_2 were obtained initially from Matheson Gas Products, a division of Will Ross, Inc., East Rutherford, NJ 07073, and later from Air Products and Chemicals, Inc., Tamaqua, PA 18252. At a flow rate of 8.4 ml s^{-1} the lag time to 2% deflection, considered as a sum of the time required for a change to flow from the leaf chamber to the cell of the analyzer (cf. Fig. 1) plus the time for the analyzer to respond, was 12 s. At this same flow rate, the response time (2 to 98%) of the analyzer for a change of 25 nl CO_2 per millilitre of gas was 18 s.

Values for CO_2 concentration in the air stream from the leaf chamber were converted to rates of uptake or release (usually expressed as negative uptake) by multiplying by the flow rate.

(V) Recordings

Instrument outputs were passed through low-pass filters, amplified 10 × if desired, and recorded on synchronized Brush Mark 220 oscillographs.

Before photographing CO_2 and H_2O recordings for Figs. 2, 5, and 6, the lag times to 2% instrument response were compensated by displacing the recordings with respect to the stimulus markers.

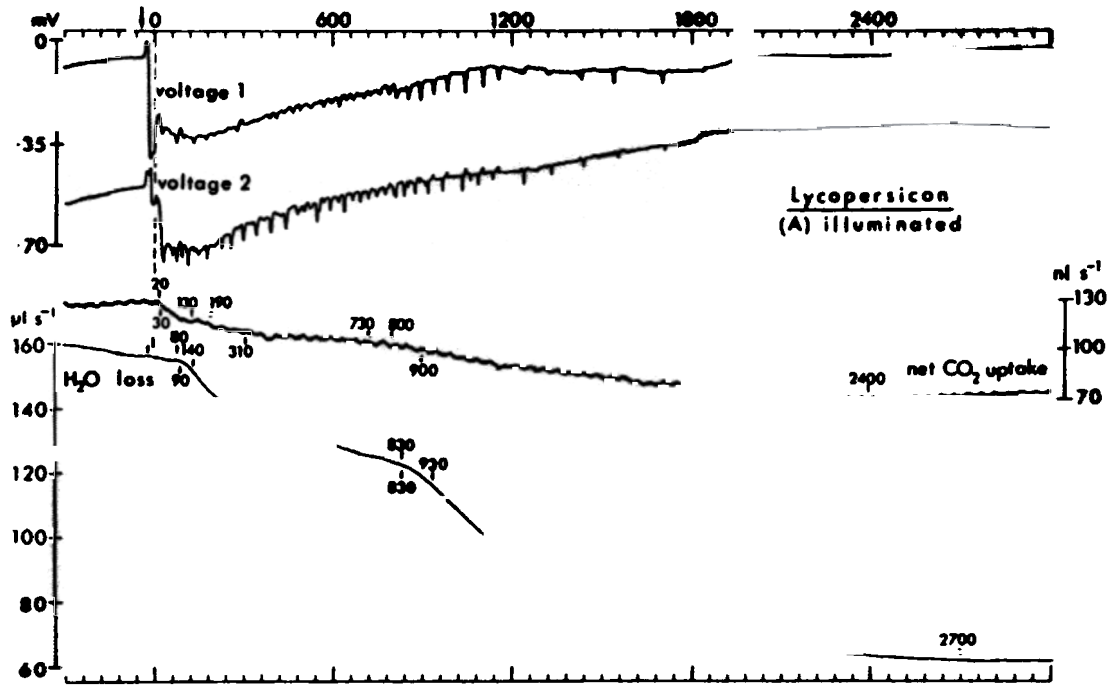

FIG. 2. Photographs of recordings of changes in net CO_2 uptake and transpiration after the spread of a variation potential from a damaged to an undamaged leaf of *Lycopersicon* in the light (A) and in the dark (B). Times of stimulation are indicated by arrows. Zero time was chosen as the time at which the variation potential is calculated to have attained 25% of its amplitude at the junction of the rachis and the three leaflets enclosed in the gas-exchange chamber. Arrival times calculated as explained in the text were 40 and 43 s after stimulation for A and B respectively. The more basal electrode (voltage record 1) and more apical electrode (voltage record 2) were separated 35 mm for A and 25 mm for B. Gas records are marked at times of initiation of a change, 10% change, and completion of change; the first mark on the transpiration record of A indicates a small peak associated with stimulation. For A, enclosed blade area was 33 cm^2, dry weight was 60 mg. Incoming water vapor concentration was 5.0 mg l^{-1} and ambient air temperature was 31 °C. For B, blade area was 48 cm^2, dry weight was 98 mg. Incoming water vapor concentration was 8.0 mg l^{-1} and temperature underwent a linear drift from 31 to 29 °C. The dotted line indicates the stable, temperature-corrected value of transpiration before stimulation. After stimulation, transpiration dropped rapidly so that the measured water vapor was in a low range within which the scale on the left is applicable. The irregularity of the transpiration curve of A between about 180 and 660 s was atypical and so was ignored in processing the data; a break occurs in the curve between about 1380 s and 1560 s because the recording pen went off scale, necessitating the location of the trace at 1560 s by comparison with hygrometer readings.

Results

(1) General Pattern of Response for Lycopersicon

Whether a *Lycopersicon* plant is in the light or in the dark, changes in net CO_2 uptake or release and in transpiration occur shortly after the arrival of a variation potential in a leaf. Figure 2A is a photograph of recordings from a typical experiment with *Lycopersicon* in the light. The first two traces show the variation potential propagating into the leaf after standard stimulation of its neighbor. To facilitate comparison, the experiment is the same one used to illustrate a typical variation potential in Fig. 1 of the first paper of the series (Van Sambeek and Pickard 1976). A zero time for the events in the leaf chamber has been established as the time at which the variation potential is calculated to pass into the petiolules of the three enclosed leaflets, indexing its arrival as the time at which 25% amplitude has been attained and assuming that its velocity along the petiole–rachis is constant at 6 mm s^{-1} (cf. first paper).

Shortly after the arrival of the variation potential in an illuminated leaf, the net CO_2 uptake

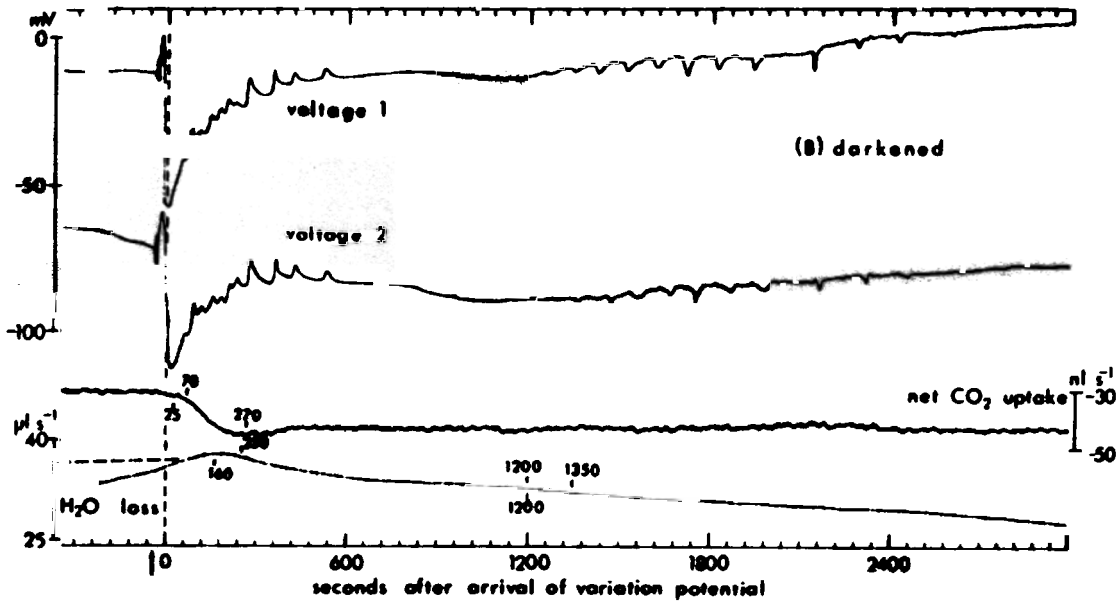

FIG. 2B.

begins a triphasic decline. The first drop is just noticeable at 20 s, the second drop becomes noticeable at 130 s, and the third, much delayed drop is noticeable at 800 s. The decline is completed by 2400 s.

Approximately coinciding with the second drop in net CO_2 uptake is a decrease in transpiration; it is just noticeable at 80 s. After an atypical irregularity in the curve, at 830 s another change of slope indicates the beginning of a second, very large drop which is finally complete at about 2700 s.

Figure 2B illustrates a similar experiment performed at about the same time of day on a plant in the dark. The zero time is calculated on the basis of an average velocity of the variation potential of 4 mm s^{-1} in the dark. In about 25 s, the recording of net CO_2 uptake (which is of course negative in the dark) begins to fall. A trough is reached at 270 s, and then the level returns to a steady value which is considerably lower than the baseline. Transpiration is low in the darkened plant; nevertheless, a first decline begins at about 160 s, and there is a hint that a second phase of decline may begin at about 1200 s.

To establish the kinetics of these changes more firmly, 25 replicate experiments were run in the light, and 19 were run in the dark.

Averaging within the two sets was carried out in two ways. First, gas flux was measured every 60 s starting from the time of stimulation, and time averages were taken for each set of points. Second, critical points were established (i.e. visually determined points of initiation and completion of each fall and points of 10%, 50%, and 90% decrease as well as points of maximum return, where applicable), and both time and magnitude were averaged for each critical point. It is important that both methods yielded closely comparable plots. The first method is more direct but is not as searching as the second, for it tends to blur reproducible patterns in which time as well as amplitude is variable, reducing the magnitude of changes. Yet, all changes consistently seen in individual records did appear in the curve connecting time-averaged points. The second method more realistically depicts the amplitude of the changes, and Figs. 3A and B present plots obtained with it.

(II) The Initial Burst of CO_2

Allowing for the different rates at which materials released into the transpiration stream can be distributed in the dark and light, indexed by the differences in variation potential velocity, the initial drops in net CO_2 uptake appear closely comparable in the two sets of experiments of Fig. 3. However, in the dark there is partial return of the curve to a steady value, while in the

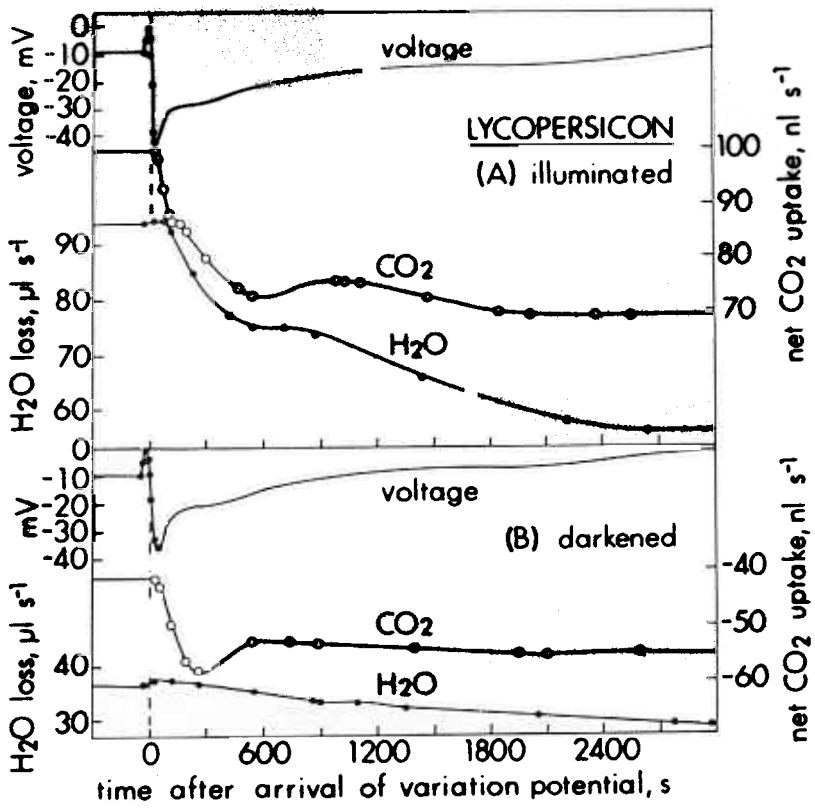

FIG. 3. Composite plots of (A) 25 experiments such as illustrated in Fig. 2A and (B) 19 experiments such as illustrated in Fig. 2B. Critical values used to approximate the passage of the variation potential through the junction of the rachis and the three enclosed major leaflets are the points of beginning, 50%, and full float-up, and the points of 10%, 25%, 50%, 90%, and full fall; beyond this, voltage records have only qualitative significance and were sketched in on the basis of amplitude averages made at 60-s intervals. The critical points for net CO_2 uptake and transpiration are described in the text. About half of the CO_2 and H_2O recordings of B lacked critical points beyond about 700 s; for later times, critical points of the other half of the recordings were found and averaged, and the amplitudes of the remaining recordings at these average critical times were then averaged in with the primary set of critical amplitudes. Average temperature was 28 and 26 °C; incoming water vapor was 10.0 and 11.1 mg l^{-1}; average area of enclosed blades was 28 and 32 cm^2, and average dry weight was 62 and 80 mg for A and B respectively.

light a further drop occurs before such a return would be permitted expression.

On the basis of the present evidence, it cannot be ruled out that the initial drop in the curves, which begins on the average at 30 s in the light and at 60 s in the dark, represents a metabolic burst. However, when it is considered that the measuring technique tends to smooth the shape and damp the amplitude of any sudden change which propagates through the leaf, it seems more plausible that this burst is the result of an abrupt decrease in the amount of CO_2 held as salts of carbonic acid in the extracellular solution in the leaf. Such a shift might of course result from the direct passage from the damaged leaf of a material (such as the hydrogen ion) affecting the equilibrium concentrations of carbonic acid and its ions, but it might also result from the local release of such a substance during the variation potential.

(III) Increased CO_2 Production in the Dark

The most notable feature of CO_2 output from the darkened plants of Fig. 3B is that after a tendency to recover from the initial burst it settles toward a steady value which after 9 min is about 29% higher than the baseline. This new level persists for many hours. The simplest in-

terpretation of this increase in CO_2 output is that it is due to a metabolic shift, for example, an increase in respiration. The shift probably begins a monotonic time course during the initial CO_2 burst. Extrapolating the presumed metabolic part of the curve backwards, it seems likely that the rise begins rather early after the arrival of the materials from the damaged cells and of the concomitant variation potential. This impression that the rise must begin early is in accord with the fact that the CO_2 efflux of the entire leaf is averaged by the collecting system, whereas the variation potential is sweeping slowly through the leaf.

It seems likely that metabolic CO_2 output is also enhanced in the light, but this cannot be determined on the basis of Fig. 3 since a postulated increase would be hidden under the dramatic light-dependent CO_2 changes.

(IV) Decrease of Net CO_2 Uptake in the Light

After the immediate drop of the curve which has been interpreted as passive CO_2 release, there is a biphasic drop of net CO_2 uptake in the light. Figure 3B shows the first decrease clearly underway by 3 min, on the average, but the origins of this decrease are obscured by the early CO_2 burst as well as by the likelihood that it overlies a change in dark metabolism. By the time that this first decline in the light has reached its greatest extent, the average total reduction in net CO_2 uptake under these experimental conditions is 26%. Then, after a slight recovery, the net uptake drops still further, settling within 20 min at a value about 31% below the baseline. This new level persists for many hours.

Even assuming that the same increase in metabolic CO_2 release observed in the dark occurs in the light, the final level of reduction in light-dependent net CO_2 uptake would be 13%. Doubtless, this value reflects a drop of at least this magnitude in the net amount of photosynthetic carbon fixation. It should be pointed out that the illumination used in these experiments was low in comparison with that from the sun in the middle of a clear summer day and that photosynthetic changes of much greater magnitude might be expected under increased illumination.

(V) Reduced Transpiration

Even in the dark (Fig. 3B), when basal transpiration is low, damaging the neighboring leaf causes some decline in transpiration. Because it is so slight in absolute magnitude, it does not justify extensive analysis. In the light, on the other hand, the biphasic drop in transpiration is conspicuous (Fig. 3A).

The beginning of the first transpirational drop and the beginning of the first presumably light-dependent drop in CO_2 uptake are closely linked. The just noticeable drop in transpiration and the 10% achievement of the first drop are on the average at 80 s and 110 s, respectively. The first 'light-dependent' CO_2 drop, evaluated without any attempt to correct for simultaneous changes in passive CO_2 release or metabolic CO_2 production, is noticeable on the average at 170 s and achieves 10% fall at 190 s. While it is possible that the initial transpirational change is a response to the early CO_2 burst which does not depend on light, it is perhaps more likely that the transpirational changes are primary and that the reduction of net CO_2 uptake may be a consequence of stomatal closure.

On the average, the second drop in transpiration begins at 730 s, achieves a 10% fall at 870 s, and has come to completion at 2600 s. The final drop in net CO_2 uptake in the light is first evident at 1050 s, after a partial recovery from the preceding drop. It achieves 10% fall at 1100 s and is completed at about 2000 s. There is a great deal of variability in the individual experiments. Moreover, although on the average the transpirational drop precedes the final fall in net CO_2 uptake, this was not always evident in individual experiments. Nevertheless, it is quite possible that the second 'light-dependent' change in net CO_2 uptake occurs because of further stomatal closure. It is clear that the superposition of several CO_2 effects necessitates great caution in interpreting both the time courses of component changes and their relation to stomatal changes.

The ultimate average reduction in transpiration rate in the light is 41%. Like the curtailment of net CO_2 uptake in the light, this reduction is not a short-lived transient but persists for at least several hours.

(VI) Correlation with Variation Potential

The shifts in rates of net gas exchange illustrated in Figs. 2 and 3 were never observed in the absence of a variation potential, which has been demonstrated (Van Sambeek *et al.* 1976) to be a correlate of the spread of material released from the damaged leaf. In a few four-electrode experiments in which large plants were given mild

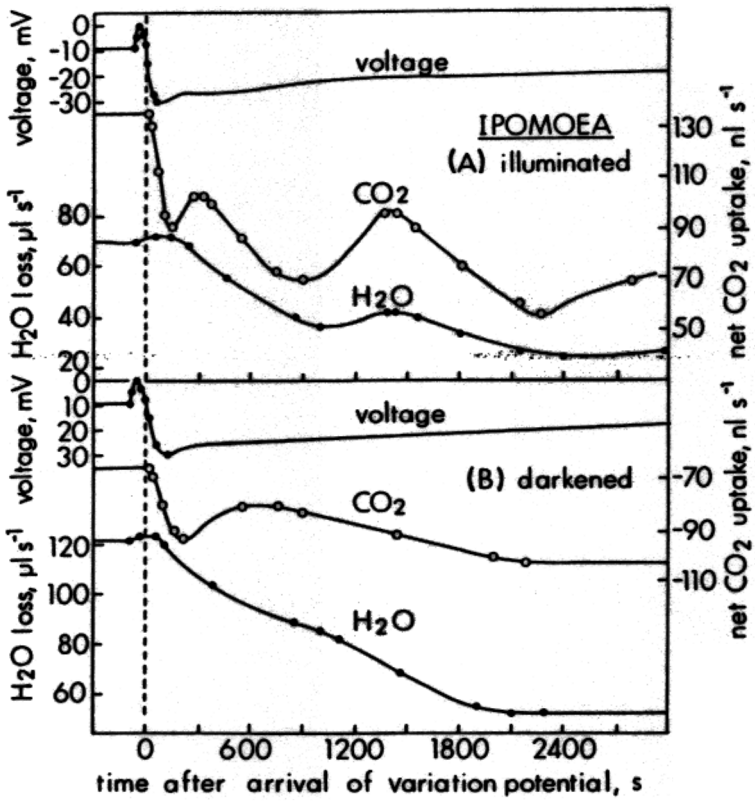

FIG. 4. Composite plots of 15 experiments with *Ipomoea* in the light and 12 experiments in the dark. Data processing analogous to that of Figs. 3A and 3B. (A) Leaves 1 to 3 on 3-week-old plants were used. Average temperature of air stream, 22 °C; average incoming water vapor concentration, 8.0 mg l^{-1}; average blade area, 36 cm^2; average dry weight, 70 mg. (B) Respective values are 27 °C, 5.0 mg l^{-1}, 44 cm^2, and 91 mg. Leaves 5 to 9 on 5-week-old plants were used. Between A and B there are enough differences in plant age and experimental parameters that baselines for repiration, photosynthesis, and transpiration cannot be compared.

stimulation, variation potentials were detected passing up the stem but not into the petiole of the leaf enclosed for measurement of gas exchange; in such cases, CO_2 and water vapor baselines remained stable.

Moreover, the shifts were not correlated with the occurrence of the putative action potentials: the shifts occurred at predictable times, whereas trains of putative action potentials might occur either soon after stimulation or after a long delay.

(VII) Other Plants

The experiments described for *Lycopersicon* were carried out on *Gossypium* with results indistinguishable except for minor differences in the timing and magnitudes of the various events.

Experiments were also run with *Ipomoea*, a plant which exhibits variation potentials which seem to have few if any putative action potentials in their company. The leaves of the older vines grown in window light responded to the standard stimulus in much the same manner as did leaves of *Lycopersicon*. The CO_2 flux and transpiration of leaves of younger plants grown in the environmental chamber tended to oscillate even without specific stimulation; nevertheless, as shown by the composite graphs of Fig. 4, the same general pattern of behavior could be discerned in spite of the occurrence in the illuminated experiments of rather large peaks between the first and second drops in the transpiration recordings and the corresponding drops in the CO_2 recordings and in spite of a tendency for a slight recovery by damped oscillation after the second of these two drops. Thus, it is shown

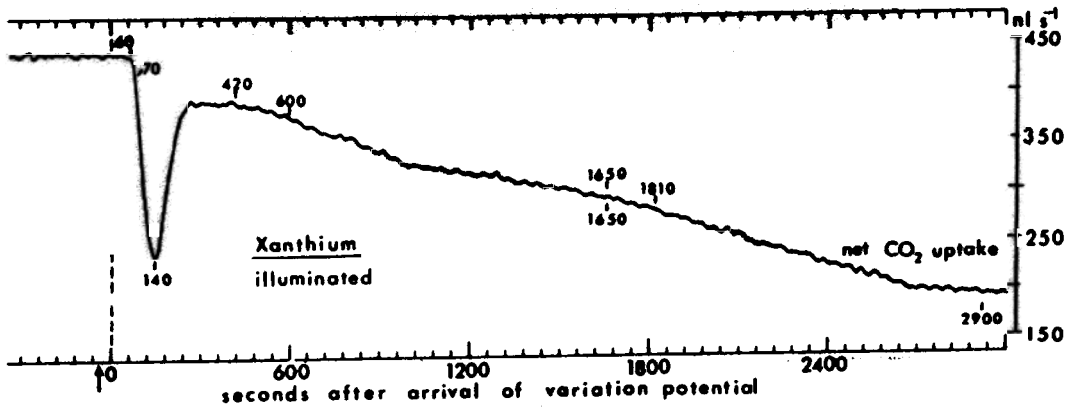

FIG. 5. Net CO_2 uptake by *Xanthium* leaf in the light. Temperature of air stream, 30 °C; incoming water vapor, 4.5 mg l^{-1}; blade area, 76 cm^2; dry weight, 190 mg. The large burst of CO_2 also appeared in experiments in the dark.

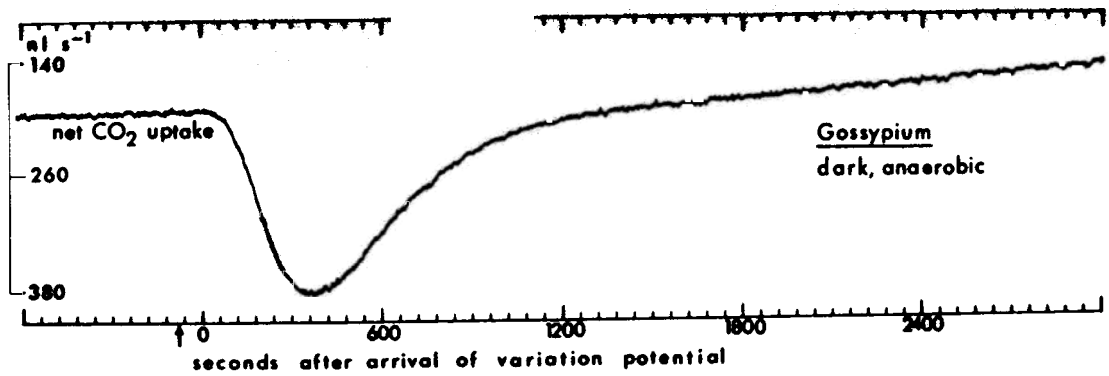

FIG. 6. Burst of anaerobic CO_2 release after arrival of a variation potential in a leaf of *Gossypium*. Arrow indicates time of stimulation, and zero of scale indicates 25% rise of variation potential under the electrode just outside the gas-exchange chamber. Flow rate of gas was slow: about 2.5 ml s^{-1}. Leaf dry weight, 564 mg; blade area, 78 cm^2.

even more clearly with *Ipomoea* than with *Lycopersicon* that the pattern of shifting gas exchange does not depend on the occurrence of putative action potentials.

Experiments carried out on *Xanthium* gave results generally comparable with those with *Lycopersicon*, although the events tended to be better separated in time and larger in magnitude. In particular, the initial burst of CO_2 was a much greater one and also much more variable. A typical burst is illustrated in Fig. 5. In the most extreme of the five experiments performed in the light, the CO_2 output expressed as net uptake peaked at -150 nl s^{-1}, having begun at a baseline of 610 nl s^{-1}. It is hard to imagine such a burst arising from any metabolic activities.

A further experiment on the large initial burst of CO_2 was carried out to see if the burst could occur in an oxygen-free atmosphere. Both *Gossypium* and *Xanthium* plants were placed in the dark, and through the leaf chamber was passed N_2 with 375 µl CO_2 per litre (guaranteed by the manufacturer to have <1 part in 10^6 contamination with O_2). As can be seen in Fig. 6 for a representative experiment with *Gossypium*, the enclosed leaf was capable of carrying on fermentation in the dark for some while, and when its neighbor was stimulated, the enclosed leaf released a substantial burst of CO_2. The baseline of a fermenting leaf tended to slope slowly toward zero both in prestimulated plants and in unstimulated controls, and when the burst subsided after a stimulus, it was always to a value and slope close to that extrapolated from the baseline. Thus, the initial burst can occur in the absence of respiration, whereas the increase pre-

sumed to be metabolic cannot be sustained when respiration is prevented. While the data do not completely eliminate the possibility that the burst might be due to glycolysis or to some form of fermentation, this experiment greatly strengthens the interpretation that the initial burst is due to a release of CO_2 from dissolved carbonate.

In overview, the reason that the most exhaustive experiments of this study were carried out on *L. esculentum* cv. Bonnie Best was because other work in this lab such as that of papers I and II, the intracellular work of Cheeseman and Pickard (1975), and ongoing purification of Ricca's factor has centered on this plant and because other work on wounding (Ryan and Green 1974) and mechanically induced hardening (Mitchell *et al.* 1975) has been carried out on it as well. However, comparisons with results from four other species have been of great importance. Although it is notable that multiple shifts in CO_2 and H_2O exchange occur very rapidly after arrival of a variation potential in *Lycopersicon*, shifts seen under the conditions of study in some of the other plants were somewhat larger and better separated. This better resolution of individual changes has permitted substantiation of the basic pattern described for *Lycopersicon* while demonstrating its generality.

Discussion

It is clear that a localized wound can have a dramatic effect on the gas exchange of an entire shoot if substances from the damaged cells gain entry to the transpiration stream.

The damaging stimulus used in the present experiments was a severe one chosen because it gave rise to large, sudden, spreading, readily measured responses. However, it seems likely that a slower but sustained release of material into the transpiration stream may have consequences which sum to similar magnitude. Indeed, even much smaller and more localized responses might be of great significance to the plant.

The wave of changes which can result from damage raises three major, specific questions.

First, what is the immediate cause of the rapidly enhanced respiration and what sequence of events mediates it? Rapid enhancement of respiration by wounding has been noted in the literature from time to time, but there has been less exploration of the mechanism of enhancement than is warranted by the importance of this subject. There are two noteworthy studies of special relevance. First, the work of Laties's lab on *Solanum* tubers (e.g. Laties *et al.* 1972; Hasson and Laties 1976*a*, 1976*b*) has demonstrated that in this tissue, cutting enhances the breakdown of phospholipids and galactolipids and the α-oxidation of fatty acids immediately. Moreover, Laties and Theologis find that the 'signal' resulting from cutting moves several millimetres inward from the wound surface within a few minutes (personal communication). Second, a rather different respiratory increase seems to occur in leaves of *Nicotiana*: Macnicol (1976) showed that when leaf discs are cut, starch depolymerization accelerates and pyruvate kinase is activated. Again, the respiratory enhancement spreads inward from the cut surface rapidly.

Thus, there seem to be at least two different respiratory pathways which may be enhanced by a stimulus spreading rapidly from damaged to undamaged tissue. It would appear useful on the one hand to check whether the findings of Laties or of Macnicol are applicable to the tissues used for the present study or whether enhancement is effected by means of yet another pathway. On the other hand, it would be interesting to try to apply the methodology of separating sites of stimulus release and action to the experimental systems of Laties and of Macnicol.

Whether the stimulus for increased catabolism is the depolarization of cell membranes which underlies the variation potential, whether it is due to the factor or set of factors which causes the variation potential, or whether it is due to yet another kind of material released from the damaged cells can probably be established by fractionating and assaying plant extract.

A second problem which is raised is the cause of the initial burst. Is it in fact due to release of CO_2 from bicarbonate? Perhaps working with either whole leaves or carefully cut and washed pieces of leaf equilibrated in buffers of different pH might confirm this interpretation. If so what brings about the release? Again, testing the effects of fractionated extract seems a promising route of study.

A third problem is the relation of the biphasic reduction of net CO_2 uptake in the light to the two phases of reduction in transpiration, that is, presumably, to biphasic stomatal closure. Which events are primary? It is interesting to speculate that Ricca's factor (Van Sambeek *et al.*

1976) and the depolarization of cells its causes (Cheeseman and Pickard 1975) may play a role in at least one of the phases of stomatal closure, but a test of the idea must await identification of this operationally defined agent. It is likely that abscisic acid is released into the transpiration stream: does it trigger one of the phases of closure? On the other hand, it is not excluded that one or both phases of closure are due to inhibition of photosynthesis. The third problem, then, like the first two, requires the purification of factors from damaged cells and an assessment of their direct and indirect effects.

In part, the importance of the processes described in this paper is that they are triggered with very little delay. However, excepting the initial burst of CO_2, they can also persist for many hours and could therefore have a long-lasting effect on plant performance.

In a more speculative vein, it may be suggested that some of the early catabolic, transpirational, and photosynthetic changes or the events that mediate them might lead in turn to the later and often more specialized responses that plants make to damage by pathogens, herbivores, and physical factors.

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CHEESEMAN, J. M., and B. G. PICKARD. 1975. Depolarization of cell membranes in leaves of *Lycopersicon* by Ricca's factor. Plant Physiol. 56: 81 (Suppl.).

HASSON, E. P., and G. G. LATIES. 1976*a*. Purification and characterization of an A type phospholipase from potato and its effect on potato mitochondria. Plant Physiol. 57: 148–152.

——— 1976*b*. Separation and characterization of potato lipid acylhydrolases. Plant Physiol. 57: 142–147.

LATIES, G. G., C. HOELLE, and B. S. JACOBSON. 1972. α-oxidation of endogenous fatty acids in fresh potato slices. Phytochemistry, 11: 3403–3411.

MACNICOL, P. K. 1976. Rapid metabolic changes in the wounding response of leaf discs following excision. Plant Physiol. 57: 80–84.

MITCHELL, C. A., C. J. SEVERSON, J. A. WOTT, and P. A. HAMMER. 1975. Seismomorphogenic regulation of plant growth. J. Am. Soc. Hortic. Sci. 100: 161–165.

RYAN, C. A., and T. R. GREEN. 1974. Proteinase inhibitors in natural plant protection. Recent Adv. Phytochem. 8: 123–139.

VAN SAMBEEK, J. W., and B. G. PICKARD. 1976. Mediation of rapid electrical, metabolic, transpirational, and photosynthetic changes by factors released from wounds. I. Variation potentials and putative action potentials in intact plants. Can. J. Bot. 54. This issue.

VAN SAMBEEK, J. W., and B. G. PICKARD, with participation by C. E. ULBRIGHT. 1976. Mediation of rapid electrical, metabolic, transpirational, and photosynthetic changes by factors released from wounds. II. Mediation of the variation potential by Ricca's factor. Can. J. Bot. 54. This issue.