METABOLIC CHANGES IN OXYGEN-DEFICIENT TOMATO ROOTS

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ABSTRACT

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by Morris G. Huck

Tomato plants were grown in chambers filled with a mist of nutrient solution suspended in air or other gas mixture which could be rapidly changed at will. When nitrogen gas was substituted for air in the root environment, the tomato leaves would wilt. The phenomenon was particularly apparent in the case of plants in early blossom stages when the "flooding" was done shortly after sunrise under conditions of high temperature and high light intensity.

Measurements of total water passing into the transpiration stream of these flooded plants (by disappearance from the chamber) showed that less water was taken up during flooding, and that the rate of water uptake decreased almost immediately after oxygen was removed from the root environment, while wilting symptoms did not appear until 30-90 minutes after the onset of "flooding." Use of deuterium-labelled water as a tracer to study water movement through the plant showed a tendency for the preferential

absorption of the heavier isotope. Water in the transpiration stream was often richer in deuterium than the water being supplied to the root system.

Radiophosphate studies confirmed earlier findings that phosphorylcholine is a major component of the exudate from freshly-decapitated tomato plants and that the label is present within 10-20 minutes after introduction of ^{32}P into the nutrient solution. Flooding did not appreciably impair synthesis of phosphorylcholine in extracts of root tissue, but it almost completely inhibited excretion of phosphorylcholine in the xylem exudate. Flooding of the root system stopped all phloem transport of newly-fixed ^{14}C-containing compounds from the leaves into the roots within one hour after onset of anaerobiosis.

Tissue injury resulting from ethanol toxicity occurs at a much later time after removal of O_2 from the root system than impairment of other physiological functions described above. Therefore its accumulation is believed to be more a symptom of flooding damage than an initial cause of the damage. Its accumulation is, however, directly related to blockage of the electron-transport system(s); it generally does not occur as a result of other environmental hazards such as mineral deficiency, low light intensity, etc.; it is a widely-occurring compound in many species of organisms; and it is relatively easy to sample and assay without the necessity for a preliminary solvent extraction of tissue.

Other compounds which might serve as indicators of the extent of flooding damage were investigated. Gamma-aminobutyric acid was found to accumulate in flooded tomato roots (but not in barley roots). It also accumulated in tomato roots subjected to a variety of other stressful conditions and was more difficult to sample and measure than ethanol. Much the same argument applies to any other intermediate compound related to biosynthesis, storage, utilization, or degradation in some phase of normal aerobic metabolism.

Coincidental nitrate reduction, in which nitrate ion from the soil solution serves as an alternate electron sink in the absence of molecular oxygen, was found not to occur in the roots of flooded tomato plants.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

It is widely known, among those who have had occasion to encounter the phenomenon, that flooding of the soil in which certain species of plants are growing can cause extensive injury to the plants. Farmers, especially those in regions of high rainfall, spend a sizeable portion of their efforts in tillage and drainage operations to insure that the supply of oxygen-containing air in the root-zone of the soil will be adequate for the needs of their growing plants.

The fundamental problems underlying this investigation have been: (a) an attempt to find or verify specific ways in which loss of molecular oxygen from the root-zone of tomato plants results in injury to the plant as a whole and (b) an exploration of the identity, toxicity, and manner of accumulation of compounds found in flooded plants which might serve as an indicator of flooding damage.

An understanding of the mechanisms of flooding injury should lead to the formation of intelligent speculations on practical ways to minimize susceptibility of the plants themselves to flooding injury and to reduce the damage to plants once flooding has occurred. In addition, it should

provide a means for increasing the accuracy of appraisals of the extent of damage. Use of a biologic property of the crop itself as a measure of flooding injury should be more closely correlated with expected yield reductions than a physical measurement made upon the soil system which is independent of biological variables.

II. LITERATURE REVIEW

One of the most remarkable phenomena which flooded tomato plants exhibit is a tendency to wilt within 30 to 90 minutes after onset of flooding--even though their roots are submerged in water. A review of early papers describing this phenomenon of wilting, epinasty, and recovery can be found in an article by Kramer (20). In more recent publications by the same author (21,22,23), the specific symptoms of flooding-induced wilting are described. The permeability of the root membranes to the passage of water is thought to diminish to such an extent that internal water stress in the xylem vessels is greater than the wilting-point for the leaves. When the guard-cells lose turgor sufficiently for the stoma to close, the shoots generally regain their normal appearance for the duration. If flooding is continued beyond a day or so, the angle between petiole and stem tends to widen and the leaves droop, the lower leaves turn yellow and then die; the upper leaves soon do the same. Only those plants bearing adventitious roots are able to survive. These plants suffer reduced growth but are less susceptible to later flooding treatment.

Work published within the past several years (24, 31) has shown that certain organic compounds such as

alkenylsuccinic acids will increase the water-permeability of root membranes to such an extent that the flooding-induced wilting can no longer be seen.

While Kuiper (24) believes the mechanism of this increase in permeability is a separation of lipid molecules in the root cell membranes of the sort proposed by Currier (9) or by Van Overbeek and Blondeau (45), Newman and Kramer (31) propose that the mode of action of the compounds is inhibition of certain metabolic reactions, such as those proposed by Zelitch (46) in the somewhat analagous case of stomatal opening. Kramer (31) notes that even dead roots have less resistance to water flow than oxygen-starved live roots. Just how it is that oxygen deficiency is capable of causing the membrane lipids to coalesce and impede water flow is not clear, but Rufelt (40) has shown that the addition of sodium diethyldithiocarbamate, a respiratory inhibitor, to roots growing in nutrient solution will cause a reduction in root pressure and transpiration in just the same way that a deficiency of molecular oxygen does. It would therefore seem that some sort of oxygen-requiring metabolic process is necessary for the maintenance of the integrity of the root membranes. If this process is blocked by anoxia or chemical inhibitors of respiration, the water permeability is reduced (at least temporarily until necrotic changes become evident); if certain stabilizing chemicals such as those discussed above are added, the permeability will

apparently remain unchanged even in the absence of oxygen in the root environment.

Since oxygen is required for production of metabolic energy to drive active ion accumulation processes, it would seem reasonable to suppose that uptake of mineral nutrients might be altered when the root system is deprived of oxygen. Further, if oxygen is required for translocation, even those mineral atoms entering the roots by diffusion or other passive processes might not be effectively passed on to the shoot. Tolbert et al. (28,44) have provided evidence which suggests that phosphorylcholine is an important constituent of the xylem sap of the tomato plant and that it is rapidly labelled with ^{32}P in short-time labelling experiments, which suggests that it may be actively involved in carrying newly-acquired phosphate to the shoot. Pate (36) has recently questioned whether similar experiments with tracers actually demonstrate that the organic compound isolated is the actively transported compound in vivo and whether the xylem is the vehicle of transport if it is actively transported. Tolbert, on the other hand, has noted that the phosphoryl choline is in fact in the xylem and not the phloem (unpublished observations) in experiments with squash plants which allow physical separation of the xylem and phloem tissue. In any event, compounds such as phosphorylcholine which can be converted from a non-polar zwitterion configuration to a highly polar configuration by minor structural changes in

the molecule must undoubtedly play a major role in any transport system in which the compound transported must be soluble in both polar and non-polar milieus.

Impairment of translocation functions in the flooded tomato root can apply to other organic molecules in addition to those involved in the assimilation of mineral nutrients. Many papers such as those of Curtis (8), Mason and Phillis (29) and others cited in the review by Kursanov (25) suggest that access to oxygen is almost an absolute requirement for phloem transport. If the supply of food and other essential organic intermediates produced in the leaves is cut off from the root system for any appreciable period of time, severe damage is likely to result.

Another class of injury mechanisms may be the failure of the flooded roots to synthesize some compound(s) required by the shoot. The recent work of Kende (18,19) and other papers cited there suggest that an auxin or kinetin-like substance synthesized in the root is essential for proper growth and development of the shoot. This position is substantiated by Kramer's observations (21) that only those tomato plants which rapidly develop adventitious roots are able to survive prolonged flooding. It would also follow as a logical consequence of Kursanov's (26) proposals regarding synthesis of amino acids and other biochemical intermediates in the root system from whence these pre-formed metabolites would be exported to the shoot. If essential

metabolites are in fact synthesized in the root system for export to the shoot and if these synthetic reactions require molecular oxygen, then lack of oxygen in the root environment would upset the plant's whole internal material-conversion apparatus.

Toxicity of such compounds as ethanol, which accumulates from use of alternate electron-sinks in the absence of molecular oxygen, is a possible explanation for the cause of injury from flooding. Speculation on what the plant root might use as an alternate electron-sink if it were deprived of molecular oxygen led Fulton (12) and other investigators cited there to the observation that ethanol accumulated in the root systems of plants deprived of oxygen for a few hours. The present investigation examined the possibility that an analagous process, similar to the "coincidental nitrate reduction" (4) observed in soil micro-organisms deprived of adequate molecular oxygen might be occurring in the roots of tomato plants. This could explain the increased levels of gamma-aminobutyric acid (GAB) which Fulton et al. (13) observed under flooding conditions. In any case, whether these compounds are a primary cause of injury or not, their measurement should constitute a tangible indication of the extent of utilization of alternate electron-transport pathways and hence of the probable extent of flooding injury.

In all likelihood, Kramer (21) has made an astute observation when he notes that "It is believed that injury to the shoots of flooded plants is complex in origin and has several causes. . . ."

III. MATERIALS AND METHODS

Plant Material

Tomato seeds (Lycopersicon esculentum Mill., var. Fireball) were germinated in flats and allowed to grow to approximately 10 cm. in height before transplanting to six-inch clay pots or to polyethylene freezer-containers containing aerated Hoagland's solution (16).

Plants grown in potting-soil were given water and mineral nutrients as required for vigorous growth until blossoms began to appear, at which time they were used for flooding treatment experiments. Flooding of potted plants consisted simply of setting the pot in a container of water on the greenhouse bench so that the water level was above the soil surface; in this manner gaseous diffusion of oxygen through the soil pore spaces to the roots was effectively blocked as shown by Fulton (12) and references cited there.

Plants grown in liquid nutrient-solution culture were allowed to grow approximately 3-4 weeks beyond the transplanting stage with daily additions of distilled water to replace transpiration losses and weekly replacement of the nutrient solution with freshly-prepared solution. Flooding experiments performed on solution-grown plants were generally done somewhat before the first blooms began

to appear, since the containers were so small that the plants did not grow well in them beyond the first few weeks after transplanting. "Flooding" of liquid-culture plants consisted of substituting nitrogen gas for the compressed air bubbled through the solution-containers.

When plants in full bloom with the roots exposed and readily accessible to experimental treatment were desired, nutrient-solution grown plants of some 30 cm. height were transferred to mist-chambers consisting of a lucite box with a sloping floor for return drainage and filled with a fine mist of Hoagland's solution generated by a commercial room-humidifier (See Fig. 1, below).

Fig. 1: Large mist-chamber with electrically-driven pump.

A sheet of black polyethylene was draped over the external surfaces of the chamber to exclude light and thus prevent algal growth, and several holes in the box provided for gaseous exchange with the greenhouse air. When flooding treatment was desired, compressed nitrogen gas from a tank was passed into the chamber through these holes. Fig. 2 (see next page) gives a detail view of the construction of the top of the box which facilitated transfer of the large plants into and out of the chamber without mechanical injury to the roots. This arrangement facilitated transplanting without injury to the plant and made it possible to get frequent weights on the entire plant including the root system. By the use of these large mist-chambers, vigorous growth was obtained through blossoming and well into fruit-set. Plants transplanted directly into the mist-chamber from the germination flat wilted badly and tended to have a very poor survival rate; growth for a few weeks in conventional nutrient-solution culture appeared to be necessary for successful adaptation of the plant to the mist-chamber environment. Probably root breakage incurred in removing the seedlings from the germination flats caused this transplanting problem. Maximum size of the plants was chiefly limited by physical space and self-shading of the tops. Occasional pruning of the roots was necessary to prevent them from growing into the humidifier unit and clogging the rotor.

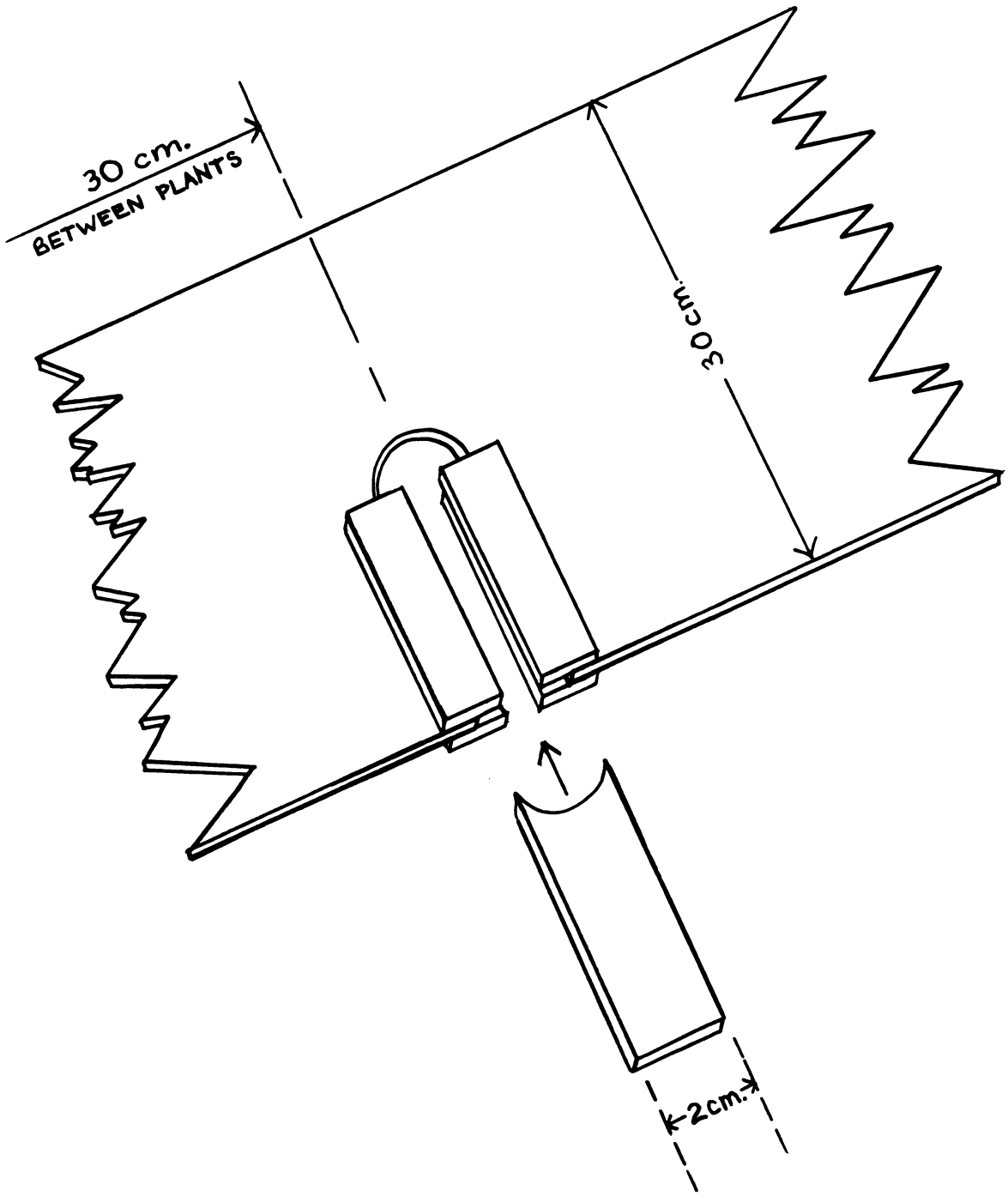

Fig. 2. Details of construction of top of mist-chamber

Apparently the presence of a gas-filled root environment in the vicinity of the surfaces of the mist-chamber grown roots greatly enhanced development of root hairs. Earlier workers (11,14) have reported great differences in root morphology as a result of variation in the amount of oxygen dissolved in a liquid-phase nutrient solution, but none of the roots reported by these workers morphologically resemble the mist-chamber grown roots (see Fig. 3 and 4). It would appear that having the moisture on the root surface in the form of a thin film surrounded by air produces a much more vigorous growth of root-hairs than having the roots in a completely liquid-phase culture system where oxygen must diffuse through bulk water in order to reach the roots.

Figure 3 shows typical root-tips in conventional nutrient-solution culture which has been continuously aerated since transplanting from the germination flat. The root-tips shown in Fig. 4 are identical in age and treatment, except that they were transplanted to a mist chamber 24 hours prior to photographing. The growth of root-hairs along that portion of the root which has been developed during the 24 hours of growth in the mist-chamber is quite striking.

For experiments involving water-uptake where a very small volume of liquid in contact with the root system was necessary for accurate measurements (to induce a large percentage change in the volume of water disappearing from the

Fig. 3: Root-tips from tomato plants grown in conventional liquid Hoagland's solution with vigorous aeration (with air).

Fig. 4: Root-tips from identically-treated tomato plants 24 hours after transplanting to a mist-chamber.

system), the minimum-volume mist chamber shown in Fig. 5 was designed.

A commercial nasal-mist aspirator circulated nutrient solution by spraying a mist of droplets onto the roots. The condensing droplets then dripped back into the reservoir at the bottom of the chamber and were recycled. Gaseous composition of the root atmosphere, of course, was identical with that used to power the atomizer; hence to initiate flooding, it was merely necessary to substitute a tank of nitrogen gas for the air-compressor supplying the aspirator. In an early version of this chamber, water level in the reservoir was held constant by replacement of losses to the plants by fresh nutrient solution from a volumetric flask, the amount of water needed to refill the flask to the calibration line being exactly equal to water usage during the time interval since the flask was last refilled. Water level in the chamber could be regulated by varying the height of the flask; only when the water level dropped so that a bubble of air could enter the flask would more water flow from the flask to the system. In later experiments requiring even greater precision of measurement, water level was held constant in a 1.5 mm. diameter tube below the reservoir and replacement was made manually from a burette calibrated to ± .05 ml. Readings made as often as once every 15 seconds gave extremely precise measurements of the rate of uptake of water by the plants in the system. As

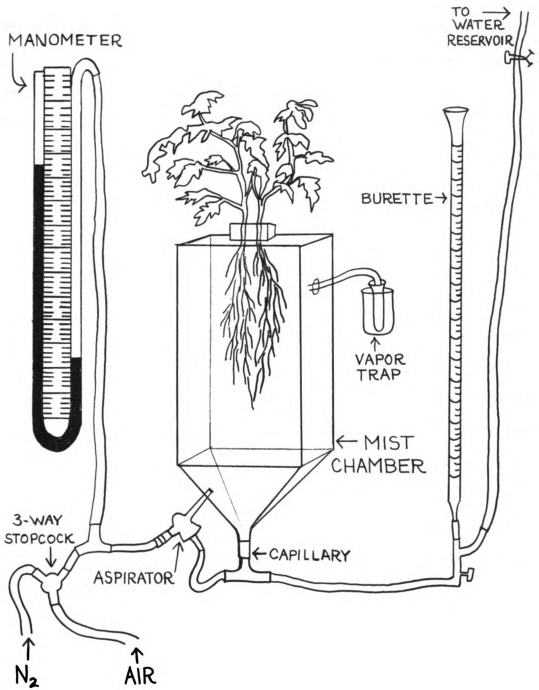

Fig. 5. Minimum-volume mist chamber.

explained in the Results section, the irregularity with which individual drops condensed on the roots and fell back into the level-tube formed the ultimate limit to the precision with which uptake measurements could be made with this system.

Labelling and Analytical Procedures

Water-uptake measurements using deuterium-labelled water were made with a Beckman IR-7 spectrophotometer by following the O-D stretch band at 2510 $cm.^{-1}$ in an Irtran-window cell with a path-length of 0.015 mm. (38). Water containing approximately 10% atom-fraction D_2O (v/v) was added to the small mist-chambers while using either compressed air or nitrogen gas as the input gas for the atomizers. Xylem exudate from decapitated plants was collected at frequent intervals and after 1-1/2 or 2-1/2 hours, intact plants were cut and the xylem sap extruded by the pressure-bomb technique of Scholander, et al. (42) modified to utilize a standard soil moisture pressure-chamber. Chewing gum formed an effective caulk to seal the stem into the exit-hole. In certain cases, water samples from the stem tissue were collected by squeezing the stem with pliers and collecting the resulting juice. After centrifugation of particulate debris, a specimen of the stirred supernate was scanned in the spectrophotometer. Preliminary experiments and consultation of charts showing absorption bands of common organic functional groups suggested that the assay would be a feasible one and

relatively free of interference from organic contaminants in the sap.

Radiophosphate labelling experiments were performed by adding approximately 200-500 μC. of ^{32}P labelled H_3PO_4 to the nutrient solution either in the polyethylene freezer containers used for liquid solution culture or to the small mist-chambers. In either event, the plants were grown in P-deficient Hoagland's solution for 3-5 days preceding the labelling itself; pH of the phosphorus-deficient Hoagland's solution was buffered near 6.0 by the citrate with which the iron was supplied. Usually the root atmosphere was adjusted to the desired oxygen concentration one hour prior to adding the radiophosphate to the solution. In this way, the roots would have had opportunity to more nearly equilibrate their enzyme systems with an anaerobic atmosphere before introduction of ^{32}P into the system. Thus, incorporation of label into pathways which require molecular oxygen for their functioning would be reduced. At times varying from 15 minutes to 3 hours after introduction of label into the system, samples were taken. Xylem exudate was collected by blotting it directly upon the origin of a two-dimensional paper chromatogram; leaf, stem, and root contents were assayed by boiling for 15-20 minutes in 70% aqueous ethanol followed by evaporation to dryness at room temperature in a stream of compressed air, resuspension in the same solvent, and chromatography utilizing the system

described by Benson et al. (5). Spots were located by exposure of the developed chromatograms to X-ray film and by use of a phosphomolybdate-blue spray test. Quantitation was done by counting the spots on the chromatograms with an end-window gas-flow proportional counter and by dry-ashing plant tissue samples at 500° C. for four hours or more followed by counting of the planchets with a Geiger-Mueller tube and scaler. Recovery studies showed essentially no loss of ^{32}P by this ashing technique.

Radiocarbon labelling experiments were done in several ways depending upon the compound containing the label which was to be introduced into the plant. For $^{14}CO_2$ fixation experiments, approximately 100 μC. per plant of the gas was generated inside a transparent polyethylene bag surrounding the shoot by reacting 5 N acid with pre-weighed solid barium carbonate. Fixation was generally allowed to continue for 15 minutes. For sucrose or glutamate labelling of phloem tissue, approximately 3 μC. per plant of the commercially-prepared (Calbiochem) substance in aqueous solution at 1μC./μl. was placed in a small flap prepared by peeling away the "bark" from the stem. A drop of the labelled compound was inserted in the flap and the exterior layers of the stem tissue replaced. The tissue was usually damp and sticky enough to stay in place with only a gentle touch to return it to its original position. Assay of radiocarbon-labelled compounds was performed as it was for the

radiophosphate compounds: extraction in boiling alcohol, two-dimensional paper chromatography, and autoradiography of the completed chromatograms. Due to the low energy of β-radiation emitted by the ^{14}C isotope, longer exposure times of the films were necessary and liquid scintillation counting (rather than end-window Geiger-Mueller tube counting) was necessary to achieve accuracy in quantitation.

Ethanol was measured with the Beckman GC-2a gas chromatograph equipped with the hydrogen-flame detector using the techniques detailed by Fulton (12).

Amino acid content of roots was estimated by boiling freshly-washed and blotted roots in distilled water for 5-10 minutes. After discarding the solid mass of roots, the hot water extract was adjusted to approximately pH 11 by addition of solid NaOH. Then Dowex 1-Cl resin was shaken with the alkaline solution for 24 hours at room temperature. The resin with attached anions was filtered off and washed with 10^{-2} M NaOH and then stirred in a small volume of water adjusted to pH 2 with HCl for several hours. After filtering off the resin, the eluted amino acids were concentrated further by evaporation to dryness in a stream of compressed air at approximately room temperature. The resulting solid matter was taken up in 100 µl. of distilled water (with heating in a water bath to dissolve); aliquots were spotted onto paper chromatograms which were then run in the two-dimensional system described previously. Following development,

the amino acids were visualized by ninhydrin spray and the intensity of the spot(s) compared to standards either visually or by elution and spectrophotometric assay of the eluate adjusted to constant volume with distilled water. In most instances the experimental treatments produced differences of several orders of magnitude in the amount of Gamma aminobutyric acid (GAB) present, so that a visual comparison of spot intensity was sufficient to provide an answer to the question: Which of the two treatments produced the greater amount of GAB? Because of the questions the experiments were designed to answer, numerical quantitation was often unnecessary.

IV. EXPERIMENTAL

Water Movement Through Flooded Plants

If, as Kramer's work (22) suggests, it is the increased resistance to water flow through the root system which causes internal water stress to increase beyond the wilting point before the stomata can close, it would seem reasonable to expect to find a reduction in the amount of water taken up by the plants during anaerobiosis. This reduced rate of water uptake should occur rapidly after oxygen is withdrawn from the root system and be clearly observable prior to the onset of wilting symptoms.

Table 1 presents the results of the experiment shown in Fig. 6, one of an early series of greenhouse experiments in which cumulative water uptake over periods of several hours was measured by observing disappearance of water from the volumetric supply flasks. This type of experiment was done with pairs of plants in the minimum-volume mist-chambers in the open greenhouse. An attempt was made to select periods of comparable weather and comparable times of day for comparison of flooded and control water usage values. The particular experiment reported in the table was chosen because of all the long-time uptake studies done in the open greenhouse, this was the only one in which

Fig. 6: Effects of aeration of tomato roots with 100% nitrogen gas. Photograph taken one hour after introduction of N_2 into Chamber A and Chamber C, hidden behind A on the left. Chamber B on the right is a control aerated with compressed air.

Table 1: Water usage on consecutive bright days by large tomato plants

Day and Time	Water Usage		
	Chamber A ml./hr.	Chamber B ml./hr.	Chamber C ml./hr.
Thursday AM	50.9	30.2	56.0
Thursday PM	41.5	19.6	77.6
Friday 9 AM--3 PM	50.5	leak	56.8
Saturday AM	*36.1	leak	*48.1
Saturday PM	44.3	leak	63.7
Sunday 11 AM--5 PM	49.0	46.2	63.8

*Nitrogen gas substituted for compressed air during this interval in Chambers A and C.

the weather was consistently good (bright and sunny) on the four consecutive days reported. Later experiments were conducted in a growth-chamber where variation due to weather changes was essentially eliminated.

Because of the large differences between plants, an average value would give little useful information. A more effective way of evaluating the data in Table 1 is to compare water usage of a given plant on the day of flooding with its own water usage rate on preceding and following days. This will tend to compensate for differences due to such factors as size of plant, etc. The water uptake rate of the flooded plants did not approach 0, but in this experiment as in all other experiments done within two hours of sunrise on a bright day using plants in the early blossom stage, there was a substantial reduction in the rate of water uptake in the flooded plants.

Since internal water stress within the plant would seem to be more important in causing or preventing wilting than total volume of water moving through the transpiration stream, it was felt that a kinetic experiment was necessary in order to establish the sequence of events. Clearly, if root resistance reduces flow of water into the plant while light intensity remains constant thereby holding the stomata at a constant width, the plant will soon begin to build up an internal water-deficit until wilting forces the stomata to close. If this is true, then the reduced rate of water uptake should be observable <u>before</u> the plants show signs of wilting.

By replacing the water-supply flasks shown in Fig. 6 with an automatic-refill burette as described on page 16 and in Fig. 5, it was possible to measure water uptake over consecutive intervals of as little as 15 seconds. Use of a three-way stopcock and a mercury manometer permitted interchange of compressed air and compressed nitrogen gas in the supply line to the atomizer at exactly the same pressure within 5 seconds or less. At a pressure of 30 cm. Hg, the atomizer used in these experiments delivered approximately 3.85 (±.10) liters of gas to the chamber per minute; with a mist-chamber volume of 18 liters, this means that the root environment could be completely displaced in approximately 4-1/2 to 5 minutes, neglecting the effects of mixing. Trapping of the gas displaced from the chamber in a dry-ice bath to freeze out moisture showed that water loss from this source was within an order of magnitude of the amount of water contained in an equivalent volume of saturated air at the chamber temperature (usually about 23° C.). No water droplets were escaping in the displaced air, and the rate of water loss to the chamber vents was small in comparison to the rate of water loss through the plant transpiration stream. Furthermore, there was no observable difference in rate of water loss to the vents when the chamber was supplied with nitrogen gas as compared to when it was supplied with compressed air, so that the rate of moisture loss to the vents would be constant in both flooded and non-flooded treatments.

Again, there was much individual variation between plants, so that the most meaningful comparisons are those made on the same plant before and after the beginning of "flooding" treatment.

Since the average amount of water used in a 15-second interval was generally on the order of 0.1 to 0.3 ml., consecutive 15-second water usage values tended to be somewhat erratic. Drops would accumulate on the roots and sidewalls of the chamber and then suddenly cascade back into the reservoir. During the next few seconds after the return of a drop to the reservoir, the level would rise above the mark in the capillary and it would be necessary to close the valve on the burette giving an apparent rate of water usage for that interval of 0. Then while awaiting the falling of another drop, the valve might have to be opened beyond the average position to maintain the level in the indicator capillary. Therefore it is necessary to report results over longer time intervals in order to average out the irregularities of the solution dripping from the roots and chamber walls back into the reservoir. Since approximately five minutes were required to displace the volume of the gas in the mist-chamber, this has been the interval used in calculation of the water usage rates reported in Table 2.

In this table, water uptake values for pairs of plants in minimum-volume mist-chambers are shown. Each experiment represents the first exposure after germination of

Table 2: Water uptake, averaged over consecutive 5-minute intervals. See text for details of experiment.

Series A: Early bloom, with two or more blossom clusters open. (Values are ml. of water use per chamber during a 5-minute interval.)

Experiment 1	Experiment 2	Experiment 3
--	--	3.25
4.20	--	2.75
4.90	3.75	2.80
4.80	2.50	2.20
4.50	2.85	3.20
4.20	3.40	2.40
Mean = 4.52, *S = 0.33	Mean = 3.12, *S = 0.56	Mean = 2.77, *S = 0.44
	Change to Nitrogen Gas	
--	†(2.45)	†(2.75)
4.30	2.10	2.65
4.00	2.45	2.50
4.00	2.60	2.42
3.85	2.45	2.50
3.80	--	2.65
Mean = 3.99, *S = 0.20	Mean = 2.41, *S = 0.18	Mean = 2.58, *S = 0.12

*S = Standard Deviation

†The first five minutes after change over are required to purge oxygen from chamber.

a healthy, vigorously growing root system to an anaerobic atmosphere. These experiments were performed in a growth chamber at a light intensity of approximately 1.4 gm.-cal. per cm^2 per minute and at a temperature of 23° C. Day length was 12 hours, and flooding was initiated in all cases at 2 hours after the beginning of the light period.

The data presented in Table 2 show that by the second or certainly by the third 5-minute interval following switchover to aeration with 100% N_2, the water uptake is generally less than the rate observed during aeration with compressed air. This reduction in water uptake rate apparently continues as long as one continues to make measurements or until the plant is severely wilted, at which time drastic changes in turgor cause rearrangements in many components of the transpiration system.

Table 3 demonstrates the importance of selecting appropriate plant material if the inhibition of water uptake phenomenon is to be demonstrated. Note that all values reported in Tables 2 and 3 are expressed as ml. disappearing from the chamber during a 5-minute interval. Therefore the young plants were transpiring nearly ten times as much water as the fruit-bearing plants, even though the amount of tissue was much less with the smaller plants. Erickson (10) has shown that yield reduction in tomato is most severe when flooding occurs during the early blossom stage; a high degree of correlation between inhibition of water movement and yield

Table 3: Water uptake, averaged over consecutive 5-minute intervals. See text for details of experiment.

Series B: Effect of variation in age of plants. All other experimental conditions as in Table 2.

Mature Plants, Many Fruits 350 g. tissue, 85 g. roots	Young Plants: First Blooms in Bud Stage. 48 g. tissue (including shoot + root)
0.80	9.90
0.95	8.20
0.90	9.90
Mean = 0.88, *S = 0.076	Mean = 9.33, *S = 0.97
Change to Nitrogen Gas	
†(1.30)	†(8.00)
1.15	8.40
0.90	8.50
1.50	7.30
--	8.50
Mean = 1.21, *S = 0.25	Mean = 8.14, *S = 0.47

*S - Standard Deviation

†The first five minutes after change over are required to purge oxygen from chamber.

reduction phenomena may exist, since plants at this same stage of development are apparently the most sensitive to impairment of water movement in an anaerobic root environment.

It is possible that much of the fluctuation between consecutive 5-minute interval water uptake values may be due to variation in the plant itself rather than variation in the random fall of drops to the bottom of the chamber. Raschke (39) has observed similar rapid fluctuations in stomata subjected to a variety of stress conditions.

Kramer (21) has reported that CO_2-flooded tobacco plants are more likely to wilt near noon while plants flooded with N_2 seem more susceptible to wilting in the morning. This observation was repeatedly confirmed with Fireball tomatoes during the course of the present investigations. If plants of the proper age were chosen, wilting could generally be induced in plants flooded within two hours of sunrise (or beginning of the light period in the case of chamber grown plants); plants flooded with nitrogen gas in the afternoon did not show signs of wilting at any time. The reason for this observation is completely obscure. Perhaps inhibition of respiratory activities upsets a different set of reactions from that upset by excessive carboxylation in the case of CO_2-flooded plants. Alternatively, the plant may be able to derive more oxidizing potential from photosynthetic activities after several hours of illumination and thus become less dependent upon molecular oxygen in the root environment.

The phenomenon of wilting due to morning anaerobiosis of the root system is clearly present. It was demonstrated repeatedly during the course of the present investigations. Wilting symptoms were more severe (provided the other conditions described above were met) under conditions of high temperature and high light intensity when the increased demand for water accelerated the development of an internal water stress. The data of Table 2 suggest that the resistance to flow of water through the root system is increased in the intact plant almost as quickly as the air can be purged from the root atmosphere; development of wilting symptoms requires up to an hour or more, depending upon temperature and light intensity.

Measurement of Water Uptake by Isotopic Tracer

Experiments by Ordin and Bonner (33) and by Ordin and Kramer (34) utilizing deuterium-labelled water to estimate the exact rate of uptake of water in excised *Avena* coleoptiles or *Vicia* root-tips, respectively, suggested that this technique would be a useful one for following uptake of water into intact plants as well. These papers report half-times for equilibration with external water across a distance of perhaps 0.1 mm. on the order of one minute or less. Since the primary objective in the present experiments was measurement of movement of water through the xylem to the shoot in order to explain the observed wilting phenomenon,

the experiments were done with intact plants or with intact root systems, the exudate from the freshly cut stump being considered a representative sample of what the shoot was receiving at a given time.

On the first attempt at measuring water movement in the xylem by the isotope technique, the results shown in Table 4 were obtained. In this instance, the shoot xylem sap was obtained from the N_2-flood plant by squeezing with pliers. (Since the shoot was wilted, it was not rigid enough to withstand the air pressure in the pressure-chamber and it kept sliding out the hole at about 10 or 12 atmospheres pressure.) The aerated shoot sap and all root sap specimens were collected by the modified Scholander (42) technique described in the Methods section. Sufficient exudate for analysis flowed freely from the cut ends of the root stumps.

Evaluation of the data presented in Table 4 suggested that the technique was a feasible one, but that there were certain discrepancies. The change in composition of the solution remaining behind in the mist-chamber suggested that the plants were selectively absorbing the deuterium in preference to the hydrogen. This was confirmed by the observation that the sap from the aerated, intact plant was enriched in deuterium over the solution used to label the roots (instead of being diluted by the water already in the plant). The 14% value for the shoot-sap squeezed out of

Table 4: D_2O uptake by tomato roots. March 2, 1967. (Data expressed as % D_2O, v/v)

	N_2 Gas % D_2O	Air % D_2O
Initial solution added to chamber	10.0%	10.0%
Solution in chamber after 150 min.	9.0	6.9
Exudate, 30-60 minutes	0	1.0
Exudate, 90-120 minutes	0	2.9
Decapitated root sap, 150 minutes	4.3	7.9
Intact root sap, 150 minutes	8.7	10.6
Shoot xylem sap, 150 minutes	>14.0 (off scale)	10.7

the anaerobic plant was a complete mystery; experimental error was the tentative working hypothesis.

Squeezing a section of plant tissue with pliers results in crushing of the tissue so that vacuolar contents are included in the sample as well as the xylem sap (which presumably composes the bulk of those samples collected by the differential air pressure technique). Pliers-squeeze samples contained a great deal of cellular debris, which was removed by centrifugation and decantation of the supernate. An identical pliers-squeeze sample from an identical tomato-shoot which had not been exposed to deuterium was run in parallel; the 2510 $cm.^{-1}$ absorbance of this control sample was essentially identical with a distilled water (H_2O) control, suggesting that the apparent high concentration of deuterium in this specimen was not due to organic contaminants in the tomato sap.

With this in mind, the experiment was repeated on an identical group of tomato plants the following week, with results summarized in Table 5. This experiment pointed out the same difficulties as the previous experiment summarized in Table 4 and emphasized the potential for erroneous interpretations of data.

Again, the aerated plants reduced the deuterium content of the solution in the mist chamber more than the anaerobic plants, but they also removed a larger volume of water from the chamber. The exact volume loss was not measured due to the experimental difficulty of measuring the cloud of droplets adhering to the sides of the chamber and condensing on the roots as described in the previous section.

Even though total volume of water taken up by the anaerobic plant was less, there was a higher percentage of deuterium in the shoot samples from the anaerobic plant. The pliers-squeeze sample, which presumably includes vacuolar contents, is enriched over the xylem sap sample from the same shoot in both the aerobic and the anaerobic plant, the differences being greater in the anaerobic plant where the root resistance to water flow was greater. The very first xylem exudate samples are higher in deuterium content than later exudate samples (the first few drops of exudate in the previous experiment had been discarded, so comparison was not possible). The decapitated root samples which were

Table 5: Uptake of D_2O by tomato roots. March 8, 1967.

	N_2 Gas	Air
	% D_2O, v/v	% D_2O, v/v
Initial solution in chamber	12.0%	12.0%
Soln. in chamber after 90 minutes	10.2	9.4
Exudate, 0-30 minutes (cumulative volume)	2.2 (0.15 ml)	5.3 (0.30 ml)
Exudate, 30-60 minutes (cumulative volume)	1.0 (0.55 ml)	0.7 (1.10 ml)
Exudate, 60-90 minutes (cumulative volume)	1.4 (0.75 ml)	1.4 (1.50 ml)
Exudate in cold-room, while waiting on shoot samples to be expressed	2.3	2.0
Decapitated roots, 90 minutes in D_2O in mist chamber followed by two hours in cold room before expression of xylem sap by air pressure	8.1	6.0
Intact roots, shoot cut at end of 90 minutes in mist-chamber with D_2O then two hours in cold room before expressing sap as above	9.8	8.3
Pressure-expressed sap from shoots, cut after 90 minutes exposure to D_2O and sap expressed immediately	5.7	5.4
Pliers-squeeze samples from same shoots collected immediately after above sample	8.2	6.1

set aside in the cold room continued to exude sap from the cut surface of the stump, and at the lower temperature the exudate contained a higher percentage of deuterium than the exudate collected from the root systems while in the mist chamber at greenhouse temperature (approximately 23° C.). It is clear that movement of water through the root of these tomato plants is a more complex phenomenon than the flow of water through a pipe.

One possibility for explaining these apparently bizarre results might be preferential adsorption of the deuterium onto some active surface in the plant in a manner similar to that reported by Jones and Hutcheson (17) for charcoal. Alternatively, it requires no great stretch of the imagination to suppose that the $\overset{H}{\underset{D}{>}}O$ molecule ionizes asymmetrically and that the D^+ ion moves through membranes and hydration shells more rapidly than the OH^- ion. Molecular and ionic-level interactions must be occurring which require the introduction of more sophisticated models to explain the observed data. It appears that at every step at which a membrane must be crossed, there is an enrichment of the deuterium fraction. The vacuolar contents seem to be enriched over the xylem sap. The xylem stream must be enriched over the water in the chamber, else the composition of the chamber water would remain constant. The first few molecules of water to pass through a given segment of a system appear to be enriched more than later increments of

water, in agreement with the findings of Carlbom, et al. (6) who proposed the use of barley seed absorption of water as a commercial means of preparation of deuterium. When the flow of water through the system stops, as in the root samples stored in the cold room, there would appear to be a desorption of deuterium from the sites to which it was initially bound until it is in equilibrium with the xylem sap.

In short, we can conclude that an intact plant is a more complex biological system than an excised root-tip or coleoptile. There are many compartments, each of which is in dynamic equilibrium with the other members of the system. The barriers between the "compartments" are selective, and the selectivity may not be constant over varying environmental conditions. When only the first and last components of such a complex system are analyzed, and when the simplifying assumptions of a steady-state system are not directly applicable (at least not without further proof), it is difficult to obtain useful information from the experiment. Further experiments must either be performed on a simpler system where greater control is possible, or if the interactions of the intact system are to be measured, much more data concerning intermediate reaction steps must be obtained.

Phosphorous Uptake Experiments

Uptake and metabolism of phosphate was observed in flooded and non-flooded plants using the techniques described in the experimental methods section. Several trials

were necessary to determine the amount of radiophosphate in the nutrient solution and the amount of xylem exudate to be collected on the paper chromatogram for optimum clarity of results.

Table 6 presents results from two of the clearest sets of xylem exudate chromatograms.

Exudate was blotted onto the origin of the chromatogram as it appeared on the cut stump for a period of approximately 15 minutes beginning with the time shown. A total of four complete sets of experiments of this type involving collection of exudates were performed which contained sufficient radioactivity for the chromatograms to be intelligible. In no instance was any compound other than inorganic orthophosphate or phosphorylcholine seen on the exudate chromatogram. Often the volume of exudate from the flooded plants was so small that it was necessary to mechanically squeeze the end of the cut stump to obtain a sample for chromatography. Little difference in chemical composition was noted between samples forcibly squeezed out of the plant and samples of exudate which flowed freely from the cut stump. In the case of aerated plants, there was always an abundant free flow of exudate. There was no appreciable amount of phosphorylcholine on the chromatograms of sap or exudate from any flooded root chormatogram, regardless of whether the top was freshly cut after three hours of flooding or whether decapitation had been performed at the time

Table 6: Radioactivity of spots on tomato root exudate chromatograms, expressed as counts per second on December 20.

	Flooded Root System			Aerobic Root System		
	P_i C/S	P-Choline C/S	% Org. C/S	P_i C/S	P-Choline C/S	% Org. C/S
November 21						
30-min. sample	4.4	0	--			
3-hr. sample	17.8	0.8	4	140	20	14
Freshly cut plant (3-hrs.)	1.6	trace	--	6.8	2.7	28
December 1						
15-min. sample	0	0	--	39	8.3	18
3-hr. sample	6.3	trace	--	122	31	20

P_i represents the radioactivity in the inorganic orthophosphate spot; P-Choline is the activity of the phosphorylcholine spot. The % organic column expresses the percentage of total radiophosphate which is present as phosphorylcholine. Due to the short half-life of the ^{32}P isotope, all counts are corrected to the same day so that relative levels of radioactivity can be compared.

of adding label to the nutrient solution. No appreciable difference was noted in the phosphorylcholine/orthophosphate distribution patterns between plants flooded in the mist chamber and plants flooded in conventional liquid nutrient-solution. The label was added in the morning on November 21 and in the afternoon on December 1; no difference in exudate composition was seen between experiments performed in the morning and those performed in the afternoon.

In four different sets of experiments, boiling 70% ethanol extracts were made from root, stem, and leaf tissue after 3-4 hours of exposure to radiophosphate in the presence or absence of oxygen in the root environment. Extracts of aerated root tissue generally contained spots having the R_f of glycerol phosphate, ethanol phosphate, inorganic phosphate, and phosphorylcholine as well as numerous other smaller and less intense unknown spots. Extracts from flooded root tissue contained all of the above, but here the homologous unknown spots (some 6-12 in most cases) generally had more activity than the tentatively identified spots of the aerated samples. Preliminary inspection of the flooded root chromatograms suggested that phosphoglycerylcholine might be present instead of phosphorylcholine on the flooded root chromatograms (the two compounds have very similar R_f values), but co-chromatography with the authentic compounds showed that phosphorylcholine was forming to nearly as great

an extent on the flooded chromatograms as on the aerated samples. The obvious conclusion is that while anaerobiosis prevents phosphorylcholine from being exuded onto the cut surface of a decapitated root system, it does not prevent formation of the compound within the root system. The fact that export of the phosphorylcholine is blocked, however, apparently induces other intermediate products of phosphate metabolism in the root to accumulate. Except for verification of the identity of phosphorylcholine on the anaerobic root samples (and the aerobic samples as well), no attempt at identification of the unknown spots was made.

Extracts from stem tissue are quite variable in composition; sometimes inorganic phosphate was almost the only spot present while at other times phosphorylcholine was a major spot in addition to the inorganic phosphate. In several instances a spot having an R_f near ethanolamine phosphate was quite prominent. In most cases, many of the same background spots seen on the root chromatograms were present, suggesting that the stem parenchymal cells were metabolically active. Ethanol phosphate, which is ubiquitously seen on phosphate chromatograms prepared from hot alcohol extractions is believed to result from esterification of the solvent during the extraction process. The overall impression of stem extract chromatograms is that they lie intermediate in composition between the root extracts and the xylem exudates.

Extracts of leaf tissue generally contained insufficient radioactivity to present a useful picture. Inorganic phosphate was the only compound which could be identified with certainty. On an occasional specimen performed at very high levels of radioactivity, there was a collection of unknown background spots very much like that seen in the stem extracts.

Autoradiograms prepared by placing freshly labelled leaf tissue against X-ray film (such as that shown in Fig. 7) showed that the bulk of the radioactivity reaching the leaves in these short-time experiments was in the region of the vascular bundles. No difference in the distribution of radiophosphate was seen in comparing autoradiograms from shoots of flooded and aerobic plants, although total amount of activity often differed markedly.

With the tomato plant, where the xylem and phloem are in such close proximity, it is impossible to distinguish which of the two classes of conducting tissue is involved in the transport of the ^{32}P.

Tolbert (unpublished results) has observed no phosphorylcholine formation in the peduncle exudate of squash plants while the stem exudate contained much ^{32}P-labelled phosphorylcholine. This difference is attributed to the

Fig. 7: Autoradiogram of ^{32}P-labelled tomato leaf. Aerated plant.

probability that the phloem exudate (peduncle exudate) contains no phosphorylcholine while xylem exudate (stem exudate) contains much of this compound.

Rapid scans of the shoots of flooded and non-flooded plants with the survey-meter suggested large differences in the amount of radioactivity moving up into the shoots in the presence or absence of root oxygen on certain days. Data in Table 7 are taken from planchet counts of ashed tissue on days when the survey meter indicated there was likely to be a difference.

Table 7: Planchet counts of ashed tomato tissue after ^{32}P-uptake from nutrient solution.

	cpm/mg. Tissue
December 15	
Air--leaf	4.4
N_2 --leaf	0.1
Air--root	122.6
N_2 --root	90.7
January 16	
Air--leaf	29.2
N_2 --leaf	11.4
Air--root	460.5
N_2 --root	114.6

The data presented are averages of duplicate counts on duplicate samples from pooled tissue specimens of each of

the several plants in a given treatment. Initial radioactivity levels in the nutrient solution of the N_2 and aerated treatment on a given day were identical, but each day's experiment may have had a different level of initial radioactivity from the previous day's experiment. On days when the survey meter showed that there was likely to be little difference between aerated and non-aerated plants, ashing and counting was omitted (since it seemed likely that differences would be insignificant on these days). It is interesting to note that on the days when there was a noticeable difference in shoot radioactivity, the experiment was done in the morning on a bright day--precisely the conditions required to induce wilting by blockage of water movement as discussed in a preceding section on page 33. On the two days reported in Table 7, the plants wilted within an hour or so after replacement of the aeration air with nitrogen--in fact, since the ^{32}P was not added until one hour after the beginning of flooding treatment (see Methods section), these plants were well on the way to wilting before the tracer was added to the chamber. On the other hand, the experiments in which the survey meter showed no noticeable difference in radioactivity of flooded and non-flooded plants were performed either in the afternoon or on cloudy days. It is as though there were no difference in shoot radioactivity unless the experiment had been done in such a way as to induce wilting.

All of the above observations could be explained if it were assumed that inorganic phosphate, which is water

soluble, is passively carried to the shoot in the xylem stream while phosphorylcholine is an organic metabolite which requires the presence of molecular oxygen in the root environment for its entry into the translocation apparatus. In those plants flooded under conditions which did not induce wilting, the soluble inorganic phosphate movement in the xylem could continue without interruption while the lack of oxygen could be preventing translocation of phosphorylcholine from the roots. Thus, the exudate samples would be principally composed of inorganic phosphate from the xylem stream while intermediate metabolites are backlogged in the root system. The total difference in radioactivity of the shoot samples would be small, since the xylem stream would be flowing freely in both flooded and non-flooded plants on these days. But in those plants in which flooding did induce wilting, the increased resistance to water movement caused by anoxia of the root media which induces wilting of the leaves would also interfere with the flow of the transpiration stream through the xylem and hence there would be a sizeable difference in the amount of radioactivity reaching the shoot during the labelling period when flooded plants were compared with non-flooded controls.

Downward Translocation of ^{14}C-Labelled Organic Compounds

The ^{14}C-labelling of flooded plants reported by Fulton, et al. (13) utilized plants which were grown in

potting soil and which required several hours for the O_2 in the root environment to be depleted. With the availability of the mist-chambers, it seemed desirable to repeat these experiments using plants which were flooded rapidly and note the intermediates which accumulated in shorter time intervals. The mist-chambers, with large plants, were filled with 100% N_2 for one hour prior to a 15-minute labelling period in which the leaves were exposed to $^{14}CO_2$ as described in the Methods section. When the plants were killed in alcohol after two hours of additional metabolism with the root system in a nitrogen environment, the roots of flooded plants were found to contain essentially no radioactivity while comparable roots of non-flooded control plants were found to have as much as 15-30% of the total radioactivity fixed during the labelling period present in the alcohol-soluble components of their root system. Thus, there was no downward translocation under anaerobiosis.

Chromatographic analysis of the aerated root extract showed that the bulk of the radioactivity remaining on the paper after three weeks exposure to X-ray film was at the origin--as though it were insoluble in the solvent systems used (5). Probably it was in the form of peptides, starch, pectins, or other insoluble storage compound(s). Total radioactivity fixed was approximately equal in aerated or flooded plants; the difference was chiefly in the shoot/root distribution of the newly fixed carbon. This observation was true in an experiment done in the morning and in another

experiment in which the labelling was done on a cloudy, overcast afternoon, so apparently the phenomenon is not so dependent upon water movement as is the upward translocation of radiophosphate. In view of published reports (8, 25,29) which note that oxygen is unequivocally required for phloem translocation, the observation would appear to be plausible.

Ethanol Toxicity

Since several reactions have been shown to occur in flooded plants within an hour or less after onset of flooding while ethanol does not accumulate in significant quantities in the xylem until several hours after the onset of flooding (10,12), it seemed reasonable to ask whether ethanol toxicity constitutes a mechanism of flooding injury or whether ethanol accumulation is merely a sympton of injury occurring from other reactions which simultaneously occur in flooded roots. The obvious means of answering this question is to place well aerated, healthy tomato root systems into an environment containing comparable levels of artificially supplied ethanol.

Since the minimum-volume mist-chambers are constantly re-supplied with fresh solution from the reservoir flask, they appeared to be a means of supplying a continuous, known amount of alcohol in the nutrient solution to the roots. But in practice, it was found to be experimentally impossible to maintain any appreciable level of ethanol in the small

mist-chambers. The solution is broken into a fine mist and thoroughly mixed with air several times per minute in these chambers, and apparently microbial oxidation of the ethanol proceeded much more rapidly than the tomato roots were able to take the ethanol-water mixture into their xylem. Gas-chromatographic analysis of xylem exudates from plants in this system consistently failed to show more than traces of ethanol regardless of the ethanol content of the nutrient-solution reservoir.

When ethanol was supplied to the tomato roots by mixing with the nutrient-solution in the large mist-chambers shown in Fig. 1, the xylem exudate was found to contain approximately 60-80% as high an ethanol concentration (in ppm) as the nutrient solution within 3-4 hours after the addition of ethanol to the nutrient solution. If the chambers were very clean, the initial addition of ethanol would generally remain present for 10 to 24 hours before completely disappearing. The rate of disappearance was apparently dependent upon temperature of the chamber and upon the amount of ethanol added. With each subsequent replenishment of ethanol, the gas-chromatograph assay showed a more rapid return to 0 ethanol concentration in solution, and the turbidity and foam in the nutrient solution (which presumably resulted from microbial growth) became apparent in a shorter time interval so that it was more and more difficult to hold the ethanol concentration at a given level in a well-aerated

mist-chamber the longer the time of the experiment (and hence, the heavier the microbial inoculum). During experiments in which only a single addition of 500 ppm. ethanol was made, no significant reduction in growth of the treated plants was noted. In the experiment reported in Table 8, the nutrient solution was maintained between 1,500 and 200 ppm. EtOH for a period of 96 hours, during which time the xylem exudate from decapitated plants in the same chamber was observed to be well over 500 ppm. on several occasions.

Table 8. Increase in weight of ethanol-treated, aerated tomato plants in the mist-chamber

	Plant #	Initial Weight gms	Final Weight gms	Increase gms	Increase %
Controls	a	65	88	23	35 %
	b	79	117	38	48 %
	c	83	132	49	59 %
	d	78	116	38	49 %
Ethanol	e	84	98	14	17 %
	f	107	135	28	26 %
	g	96	138	42	44 %
	h	73	86	13	18 %

Initial and final weights are reported in grams, and were obtained by quickly removing the entire plant from the mist-chamber and weighing it before adding the first increment of ethanol to the nutrient solution and again after 96 hours

of growth under ideal growing conditions in a growth chamber with the 12-hour day cycle described on page 29.

In a later experiment, ethanol was added to the nutrient solution at a concentration of 0.5% ethanol (5,000 ppm). Even though they were well aerated, the plants given this much ethanol soon lost turgor. Within 24 hours, the plants were dead.

From these observations, it can be concluded that ethanol itself can be toxic to plants even when they are well-aerated. Although it is experimentally difficult to maintain a given ethanol concentration in a well-aerated environment when microbial contamination is present, if the ethanol concentration is maintained sufficiently high for a sufficient period of time, the plant will suffer some damage.

As shown in the work described in the previous sections, water permeability, organic phosphate movement, and phloem transport are radically altered by anaerobiosis of the roots in times which are so short that the ethanol could scarcely have begun to accumulate in more than normal quantities. Because these changes occur first and ethanol accumulation occurs later, it would appear that the ethanol accumulation is a symptom rather than a cause of the initial injury from flooding. On the other hand, there can be little doubt that ethanol can exert toxic effects upon a living organism if it is present in sufficient concentration and duration; its quantitative estimation will certainly give

useful information regarding the extent of flooding injury. The point here is differentiation between cause and effect.

GAB Accumulation

A reasonable correlation between ethanol accumulation and yield reduction in flooded plants has been established (10, and references cited there), but the possibility remains open that there may be some other compound in the plant whose measurement would form an even better indication of the extent of injury to the plant. One possibility for such an indicator compound is gamma-aminobutyric acid (GAB), which Fulton, et al. (13) found in large quantities in the roots of flooded tomato plants. This observation was repeatedly confirmed during the course of the present investigations. In every experiment in which nitrate-fed, healthy tomato plants were subjected to an oxygen-deficiency of the root system for periods of approximately 30 hours or more, much more GAB was present in the hot-water extractable amino acids in the root systems of the flooded (oxygen-deficient) plants than in the controls.

When plants were grown under adverse conditions such as low light intensity (winter greenhouse without artificial illumination), phosphorus and/or magnesium deficiency, etc. the level of all free amino acids in the root system was much higher than in the roots of normal plants from other experiments in which growing conditions were more favorable.

In plants with these high levels of free amino acids, the amount of GAB in the controls was so high that there was little or no difference between the controls and the flooded plants.

In an attempt to produce plants having a "0" level of GAB, young tomato seedlings were grown to blossom stage on a sand-table held at a constant 30 cm. of water tension at all times. The level of GAB in these plants was approximately the same as in normally-grown greenhouse plants under favorable conditions of light, temperature, nutrient status, etc. It would appear that a certain finite but small amount of GAB is present at all times. In both the normally-watered control plants and the plants grown on the tension-table (12), the GAB level was quite low and would probably escape detection if one or more preliminary ion-exchange resin amino acid concentration steps were not used before chromatography.

Similar long-time flooding experiments with healthy barley plants showed that GAB did not accumulate in the roots of this species when it was flooded for comparable periods of time. Instead, another ninhydrin-positive compound having anionic properties at pH 11.5 but not at pH 2 and with an R_f very close to that of GAB appeared to accumulate in the flooded barley roots. Except for co-chromatography with authentic radioactive GAB to prove the difference in identity, no attempt at identification of this compound was made. GAB is therefore not a universal indicator of

flooding in all plant species. It accumulates in flooded tomatoes, but not in flooded barley roots. Possibly the compound which accumulates here is some other diamino acid similar to GAB, such as β-aminopropionic acid from aspartic acid.

The possibility that GAB might serve as a historical record of previous flooding of a plant was also considered. GAB is a stable compound and assays of dried roots stored on the laboratory shelf in air at room temperature for four months contained approximately the same level of GAB as freshly-harvested root tissue. The problem, however, is that in plants such as tomatoes where the fibrous root-system is being continually regenerated as the plant explores the soil volume, the tissue which was a part of the root a few weeks ago will have been sloughed off by harvest time. Also, as Kramer (21) has pointed out, tomato roots which are flooded for any appreciable time soon die; only those plants which are able to produce adventitious roots survive, and the adventitious roots soon take over as the principal root system. Hence, the historical record of root-flooding is soon lost to the decay organisms of the soil.

Fulton's ^{14}C work (12) also showed significant differences in the lipid components of the roots of flooded plants. It is likely that many of the unknown spots on the ^{32}P-labelled short-time flood chromatograms also involved lipids and lipid synthesis reactions. But here, too, the

problems of using a specific lipid or lipid precursor or degradation compound as an indicator of flooding injury would seem likely to meet the same difficulties as use of an amino acid as an indicator: (a) there are other environmental variables (besides flooding) which can influence lipid or amino acid metabolism, and (b) widely different species will probably have different pool-sizes of lipids, amino acids, and other metabolites. On the other hand, ethanol accumulation seems to be a more general phenomenon throughout all classes of organisms including members of the Angiospermata. Further, collection and analysis of samples is easier with ethanol than with GAB, phosphorylcholine or most other compounds which require solvent extraction of the tissue followed by concentration and condensed-phase chromatographic steps before the assay can begin. Ethanol measurements, however, are subject to the limitation that losses due to microbial activities can readily occur.

Speculations on the Origin of GAB

One hypothesis which could explain the accumulation of GAB in the roots of anaerobic tomato plants is that NO_3^- from the nutrient solution may be serving as an alternative electron-sink to substitute for molecular oxygen. If this were the case, the excess ammonium produced by nitrate reduction might be detoxified by reductive transamination (of α-Ketoglutarate) to glutamate and this in turn pulled out

of the active pools by decarboxylation to form GAB so that the nitrate-reduction and transamination reactions would continue in a forward direction. As a simple test of this hypothesis, plants were fed ammonium or nitrate salts while flooded and the relative levels of accumulated GAB were compared. In both tomato and barley experiments, there appeared to be a somewhat higher level of free amino acids with ammonia as the nitrogen source than when nitrate was fed. Tomatoes showed the typical picture of much more GAB in the nitrate-fed, long-time flooded plants than in the aerated control; the ammonium-fed plants had almost as much GAB in both aerated and flooded plants as was present in the flooded, nitrate-fed treatment. Much the same picture was seen in barley, except that the ninhydrin-positive compound accumulating in the flooded, nitrate-fed treatment did not co-chromatograph with labelled, authentic GAB as described above. If nitrate were serving as an alternate electron-acceptor in accordance with the hypothesis just described, then substitution of NH_4^+ for NO_3^- salts should have stopped the accumulation of this compound. If the NO_3^- had been serving as an alternate electron-sink, it would no longer be able to fulfill this function when it was absent from the nutrient solution. In the absence of the principal reaction of the sequence, there would be no reason for the plant to continue to transaminate and decarboxylate. The fact that pH was very difficult to control

in this experiment leaves it open to some question--the low pH (3.5-3.8) resulting from the exclusive uptake of cations in the ammonium-fed plants could have had the same effect as low light intensity or mineral nutrition deficiency as outlined earlier. But when these results are viewed in combination with others outlined above and below, the combined weight of evidence would seem to suggest that the source of GAB is not detoxification from use of nitrate as an alternate electron-sink, but rather a generalized breakdown of proteins resulting from cessation of the normal metabolic activities of the plant. This would also be consistent with the observation of Hewitt (15) that nitrate reductase activity disappears under anaerobic conditions.

In an attempt to follow the metabolic pathway proposed above, uniformly ^{14}C-labelled sucrose or glutamate was introduced into the phloem of the stem or freshly-cut petiole by the techniques outlined in the Methods section. The amount of translocation to the flooded root system was always very small, although fairly high percentages of added label sometimes reached the root system when aerated root systems were employed. Since the amount of label reaching the anaerobic roots was too small for paper chromatography of the compounds formed from the labelled sucrose or glutamate in the flooded root, an ion-exchange resin extraction of the alcohol-soluble components of the root system was made. These extracts and concentrates were then

counted by liquid scintillation techniques which enabled work at much lower levels of radioactivity. If the proposed NO_3^- reduction and transamination scheme had been operating in the flooded roots, then the percentage of radioactivity which did reach them should have been much higher in the amino acid fraction (resin-bound) than in the fraction of compounds which did not bind to the anion-exchange resin at pH 11. The results of this type of experiment are shown in Table 9:

Table 9: Radioactivity distribution in tomato root systems.

Compound Added	Aerated % of root radioactivity	Flooded in resin fraction
June 14		
Sucrose	49	30
Glutamate	52	67
July 16		
Sucrose	8.2	5.2
Glutamate	23.6	20.7

The labelled compound indicated was added via the phloem as described on page 20. The reason for the large differences in percent of radioactivity which reached the root that was subsequently recovered from the resin-bound fraction between the two experiments is not clear. The most likely reason is a difference in the age of the plants. The June 14 plants had fruit which was probably taxing the plant

system; the July 16 plants were flooded in early bloom and were in a stage of vigorous vegetative growth. In both cases, there is certainly no strong tendency for the carbon newly reaching the roots to be shunted off into free amino acids to detoxify large amounts of newly-reduced ammonium ion. Yet in each case, ninhydrin-sprayed chromatograms of the hot-water extracts of roots from the same plants showed much larger quantities of water-soluble GAB in the flooded roots than in the non-flooded plants. Clearly the GAB was not being formed from carbon which has recently arrived in the root system.

Biuret assay for amide linkages in extracts of flooded and non-flooded roots tended to suggest protein breakdown as the source of the free GAB, but experimental differences between replicate plants were as large or larger than the differences between flooded and control group averages (5%). To obtain meaningful results in answering the question as to whether GAB arises from the breakdown of pre-formed proteins, a fractionation procedure which favors measurement of the more labile forms of protein would facilitate observation of experimental differences. The published results of Fulton, et al. (13) provide an excellent example of an experiment of this nature. Since no significant amounts of carbon are likely to be translocated downward during flooding, the labelled GAB must have come from breakdown of pre-formed compounds which were in the root system at

the time he began his flooding treatment. Since the label was only in the roots some 12-24 hours before the onset of flooding, the presence of large amounts of activity in the GAB suggests that it must have arisen from relatively labile compounds or substances which had been assimilated only a few hours before flooding. Proteins, especially those with enzymatic activity, are generally considered to be in just such a state of constant turnover and resynthesis. If some circumstance (such as flooding) should prevent their continued resynthesis, the inevitable breakdown of existing enzymatic protein would liberate large amounts of free amino acids into the metabolic pools of the root systems. Conversion to GAB might be a defense mechanism whereby free amino acids are converted into a form which does not trigger the control mechanisms and shut off the synthesis or interconversions of other necessary metabolites, or it may occur because the control mechanisms fail to function normally in the absence of oxygen.

V. DISCUSSION

Experiments designed to elucidate the mechanism of flooding injury to tomato plants and to investigate the feasibility of ethanol assay as compared to other biological indicators of flooding damage have been performed. None of the possible mechanisms of flooding injury discussed in the Literature Review have been clearly ruled out; it would appear that the damage to plants flooded over long periods of time such as 24 hours or more is a composite of damages due to a variety of causes. When plants flooded for short times such as 1 to 2 hours are considered, it is suggested that the most rapidly observed changes are those having to do with membrane permeability and phloem transport. The resistance to the flow of water through the root system is often seen within minutes. One hour of flooding the root system is sufficient to effectively stop phloem transport and entry of organic phosphate into the translocation system.

In addition to the toxicity resulting from the solvent effects of ethanol accumulation, other forms of injury are also seen in plants flooded for longer times. Protein resynthesis in the root system is effectively blocked and large quantities of gamma-aminobutyric acid accumulate.

It is likely that many other chemical changes occur in long-time flooded roots. The cause of the changes could be a failure of the intra-cellular mechanism of material transport, a breakdown of membranes between cells, a shift in the equilibrium position of enzyme systems regulated by electron-transport substances, or other unknown causes, or any combination of these. In short, none of the proposed mechanisms for injury from flooding have been ruled out, and it appears likely that all of the mechanisms tested contribute to the observed injurious effects.

While there are probably many metabolic changes which occur in flooded plants, it is desirable to choose a process which is common to many species and which is affected much more strongly by flooding than by other environmental manifestations if one wishes a useful biological indicator of flooding damage. Ethanol is such a compound; other compounds considered, such as GAB, do not meet these criteria. In addition, ethanol can be sampled without the necessity for solvent extraction of the tissue and can be assayed very quickly and easily by gas chromatography. Compounds which are involved in biosynthesis, storage, or other aspects of the plant's metabolism are much more likely to be dependent upon extraneous environmental variables than a compound such as ethanol which is clearly and exclusively the product of anaerobic fermentation reactions.

VI. SUMMARY

It has been observed that tomato roots deprived of oxygen in the root environment undergo certain changes:

1. Translocation of newly-fixed carbon-containing products of photosynthesis to the roots is sharply reduced within one hour after the onset of flooding.

2. Translocation of phosphorylcholine from the roots into the xylem exudate is sharply reduced, although synthesis of the compound can continue even in the absence of root aeration.

3. The rate of water movement into the root system from the surrounding environment is reduced within 10 minutes of the onset of flooding, provided the flooding is done at the early blossom stage on a bright, warm day during the early morning.

4. Both aerated and non-aerated root systems apparently absorb deuterium hydroxide from their environment at a more rapid rate than they absorb water if both isotopes are present.

5. Gamma-aminobutyric acid accumulates in large quantities in root systems which have been flooded for periods of 24 hours or more. It is proposed that the source of this compound is degradation of pre-formed amino-acid

containing compounds, rather than use of nitrate as an alternate electron-sink in the absence of molecular oxygen.

It is likely that other changes in addition to those noted above occur in flooded tomato roots; the ones listed are merely those observed during the present investigations. The cause of the injury to the plant from flooding damage is believed to reside in a combination of the many changes observed and not in a single obstacle to the metabolism of the root system.

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