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The Metabolism of Tubers of Cyperus Rotundus L. As Influenced by Oxygen Levels and 3-Amino-1,2,4-Triazole.

Rupert Dewitt Palmer

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THE METABOLISM OF TUBERS OF CYPERUS ROTUNDUS L.
AS INFLUENCED BY OXYGEN LEVELS AND 3-AMINO-1, 2, 4-TRIAZOLE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Botany, Bacteriology
and Plant Pathology

by
Rupert Dewitt Palmer
B. S., Mississippi State College, 1952
M. S., Mississippi State College, 1954
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ABSTRACT

Studies were conducted in the laboratory to determine certain metabolic pathways in nut grass, *Cyperus rotundus* L., as influenced by germination, oxygen gradients, and 3-amino-1, 2, 4-triazole (amitrol).

Either single tubers or three and five connected by rhizomes were exposed to 1, 10 and 20 per cent oxygen in combination with 1, 5, 10 and 15 per cent carbon dioxide. Germination of single tubers was greatest under 20 per cent oxygen and 15 per cent carbon dioxide. Tubers exposed to 1 and 10 per cent oxygen in mixture with 1 per cent carbon dioxide germinated 7.9 and 55 per cent less, respectively, than those under 20 per cent oxygen. Germination was depressed in 1 and 10 per cent oxygen with increased carbon dioxide levels.

A larger number of tubers germinated in the chain under the high oxygen gradients. Dominance was broken in the chain with oxygen concentration above 1 per cent. Different patterns of germination occurred in the chains under all oxygen levels. The respiration of dormant and germinated tubers was increased as oxygen concentration increased from 1 to 100 per cent. All respiratory studies were conducted using conventional manometric techniques.

Studies to determine the metabolism during germination revealed that the dormant tubers operated primarily on organic acids. Activities of catecholase, and isocitric dehydrogenase and certain Krebs cycle oxidases on "particles" were probably coupled with the organic acid metabolism. The germinated tuber possessed ascorbic acid oxidase. About equal activities of succinic and isocitric dehydrogenase were found on "particles" from the germinated tubers. Probably these
enzymes were coupled with carbohydrate metabolism. Ascorbic acid content decreased during germination which indicated an increase in ascorbic acid oxidation. The major change in metabolism appeared to take place at about five and one-half days of germination.

Polyphenoloxidase was established as the main terminal enzyme in the dormant tuber. Results obtained using the substrates, tyrosine, 3,4-dihydroxyphenylalanine (dopa), and p-cresol revealed that these compounds were oxidized by tissue preparations. Ascorbic acid, p-nitrophenol, benzoic acid, thiourea, diethyldithiocarbamate, and oxalic acid inhibited catecholase.

The activities of catecholase, ascorbic acid oxidase, dopa oxidase, peroxidase and catalase were determined in extracts from tubers which had received amitrol treatment in sufficient concentration to inhibit germination. The treatment period was 8 days.

Amitrol depressed the activity of ascorbic acid oxidase in tubers. Catecholase and dopa oxidase activities were depressed by amitrol treatment.

The highest level of amitrol held the activity of peroxidase in treated tubers at the same level found in the dormant tuber.

Catalase activity was 18.5 times higher in the control than in treated tubers.
INTRODUCTION

Although considerable applied research designed to provide control of nut grass has been conducted, very few fundamental studies have been carried out on this problem. This approach will lead to the exhaustion of the reservoir of basic information in developing applied approaches to problems.

At the present there is a poor understanding of the mechanisms of herbicidal action. The need is acute for a knowledge of the physiological processes involved in the response of nut grass to herbicides or environmental factors. One need is an understanding of factors which affect sprouting of dormant tubers in the field. A more exact knowledge is needed of the mechanisms involved in the control of dormancy in the nut grass system. An answer to these problems would provide a sound basis for the development of practical and reliable procedures for the use of herbicides to control nut grass.

Workers in the field of weed control agree that control methods should be based on physiological principles. The response of a particular plant to a specific herbicide usually varies with the stage of growth, and environmental conditions under which the plant is growing.

The purpose of this investigation was to obtain information on the metabolism of nut grass tubers at different stages of growth which might aid in determination of their sensitivity to herbicides. The response of nut grass to oxygen gradients was studied to determine the effects of oxygen on germination habits.

It was also the purpose of this study to obtain information on the possible mechanism of bud inhibition induced in nut grass by
3-aminol,2,4-triazole. The activities of certain enzymes extracted from tubers which received amitrol treatment were examined.
I. Effects of Oxygen Concentration on Plant Respiration and Plant Growth.

A. Plant respiration.

Stich (1891) was one of the earliest to examine the effects of reduced oxygen concentration upon the respiratory quotient (R. Q.). He found at 9 per cent oxygen that the R. Q. changed very little from the value obtained in air for wheat, maize and pea seedlings, but that a considerable rise in the R. Q. occurred at 3 per cent oxygen. The R. Q. for narcissus bulbs was close to 1.0 in air or 10.2 per cent oxygen, but was 2.4 in 7.5 per cent oxygen. Hackney (1943) found that the R. Q. of Granny Smith apples remained relatively constant in 1 to 3 per cent oxygen. The R. Q. of tomatoes did not change appreciably until the oxygen concentration was lowered to 2 per cent, and above 2 per cent the R. Q.'s were unity. Below 2 per cent they were higher than unity (Gustafson, 1936). Turner (1940) observed that carrot disks showed such rises at somewhat higher oxygen concentrations. With carrot slices in aerated water containing 1.5 per cent glucose the R. Q.'s rose a little, and the rise was much greater when the oxygen pressure was lowered. Carrot disks readily form alcohol, and according to James (1953a) may even do so to some extent in air, causing a rise of the R. Q. to 1.1.

James (1953a) postulated that anaerobic respiration was not limited in low oxygen levels. This type of respiration was gradually extinguished as the oxygen concentration rose. Its complete suppression would appear to need at least 3 per cent oxygen and considerably more in
some tissues. James' interpretation was confirmed by the use of terminal oxidase inhibitors. The inhibition rendered the enzyme inactive and reduced oxygen consumption. Carbon dioxide production was not cut down to a corresponding degree and the R. Q. rose.

According to James (1953a), Watson presented the most exact data on the relation of oxygen consumption and concentration. He worked with Bramley seedling apples maintained in an air flow at 22°C. Following the determination of steady rates of respiration in air different gas mixtures were introduced, and steady rates of respiration determined for each mixture, usually after 40 hours. It was found that oxygen uptake was greater at 25 and 30 per cent than at 20 per cent oxygen and below. Curves for rice seedlings, barley seedlings and onion roots showed the same general features.

Forward (1951) showed with young barley seedlings that oxygen uptake increased above one (an arbitrary value) for air when oxygen levels were up to 40 per cent. James (1953a) postulated that these results were due to the reaction of oxygen with a respiratory enzyme. Oxygen access for the enzyme would influence oxygen uptake at different concentrations; however, tissues with intracellular spaces would respond in increased oxygen uptake under higher levels of oxygen if the tissue carried an enzyme with a high affinity for oxygen. James and Beevers (1950) found that oxygen uptake of spadix slices from Arum was about 20 times faster than the rate for stalk slices. The stalk tissue of Arum reached oxygen saturation at about 5 per cent; whereas, the spadix slices were unsaturated at 100 per cent. It was found that the respiratory oxidases were different in the two tissues. The stalk possessed a metallo-enzyme of polyphenoloxidase type, while the spadix
contained no metallo-enzyme at all, and its respiration depended on an auto-oxidizable flavoprotein.

B. Plant growth.

Pfeffer (1899) stated that most aerobic plants failed to grow in oxygen concentrations below about 3 per cent, and that concentrations of oxygen above that in air often retarded growth. Growth was very commonly accelerated in oxygen concentrations between 3 and 21 per cent.

Leonard and Pinckard (1946) reported that cotton root growth was greater in air than in lower oxygen concentrations, and that below some minimum concentration, in the range of 0.5 to 2 per cent, growth ceased. The optimum concentration of carbon dioxide appeared to range between zero to 15 per cent when the oxygen concentration was maintained at 21 per cent. The absence of carbon dioxide did not appear to affect root elongation. Concentrations of carbon dioxide of 60 per cent and above prevented all root growth. Root growth was reduced also at 30 and 45 per cent carbon dioxide. Ninety or 100 per cent oxygen reduced root elongation. Shoot growth appeared to be uniform in both height and fresh weight from zero to 30 per cent carbon dioxide, reduced at 45 per cent carbon dioxide, and markedly reduced at 60 per cent carbon dioxide and above.

Valmis and Davis (1943) have found that growth of young rice roots attained a maximum in 3 per cent oxygen, and that even in 0.2 per cent oxygen growth was distinctly greater than in air. The growth of barley shoots was greater in air than in lower oxygen concentrations. Taylor (1942) and Lin (1946) reported that growth of young shoots and roots of wheat and rice was progressively reduced in oxygen concentration below 21 per cent, though to a less extent for the shoots than the roots.
Ranson and Parija (1955) used 7 day experiments at 20° C, and observed that a decrease in oxygen concentration from 21 to 3.5 per cent resulted in increased growth of shoot organs of wheat, barley, rice, bean, marrow, and buckwheat. This increase in growth was evident until the point of anaerobic respiration was reached at some oxygen concentration below 3.5 per cent. Below the extinction point growth was retarded progressively with an increase in the anaerobic component of the total metabolism. Root growth decreased with each decrease in oxygen concentration below 21 per cent. In 1.5 per cent oxygen development of root hairs and laterals was markedly retarded, and injury was often apparent. Growth rates of coleoptiles altered in response to change in oxygen concentration within 15 minutes. Experiments with the auxinometer showed that within one hour of the transfer from air the rate of growth in 3.5 and 8.5 per cent oxygen was often double that in air before transfer. Ranson and Parija suggested that the changes in growth rates resulted from changes in the auxin available for growth at the time of transfer from air to lower oxygen levels. They believed that reduction in oxygen concentration lead to a reduction in the oxidative destruction of auxin which might increase the effective auxin concentration for shoot growth and decreased root growth. They used detached coleoptiles in the presence of abundant indole acetic acid, and coleoptile growth was not markedly affected by oxygen concentration. This supported their hypothesis somewhat; however, two auxin assays provided no evidence of real value of the presence of more growth-promoting stimulant in extracts from coleoptiles growing in 3.5 and 8.5 per cent oxygen than in those growing in air.
Ferguson and Bond (1955) made a comparison of the effect of oxygen supply on the growth of nodulated *Trifolium pratense* plants dependent on nitrogen fixed in the nodules, and on nonnodulated plants provided with ammonium-nitrogen. The plants were grown in water culture with the root system only exposed to adjusted oxygen supply. The four levels of oxygen employed were 21, 12, 5 and 1 per cent. It was found that each reduction of oxygen supply resulted in a significant curtailment in growth of nodulated plants. In comparison the responses of ammonium-plants were less marked, indicating that the nodules required a higher level of external oxygen for effective functioning than do roots. The number of nodules was greater at 5 per cent oxygen. This suggested that the optimum oxygen level for early nodule development was well below that for the subsequent functioning of the nodules.

Allsopp (1949) observed no anatomical differences in the structure of growing shoot apices of *Dryopteris aristata*, and of plantings developing from detached meristems of rhizomes of *Onoclea sensibilis* and *Matteuccia struthiopteris*, when maintained in gas mixtures containing 6, 11, 21, and 45 per cent oxygen in nitrogen. In undiluted oxygen the rhizome lengths of *Matteuccia* and *Onoclea* developed abnormal periderm-covered, cushion-like growths from the detached meristems. When these structures were transferred to air, normal plantings arose endogenously from meristematic tissues which persisted within the periderm.

Denisen (1951) reported for artificial ripening of tomato fruits that the most rapid development of red color occurred at 20° C in an atmosphere containing 60 per cent oxygen in nitrogen. Oxygen content had a greater influence than temperature in the development of xanthophyll.
II. The Polyphenoloxidase System and Plant Respiration.

Boswell and Whiting (1933) reported that the addition of catechol to respiring potato slices caused a marked increase in oxygen uptake. This was the first direct demonstration that polyphenoloxidase was involved in the respiration of a higher plant tissue. They concluded that polyphenoloxidase was involved in approximately two thirds of the total respiration of potato tuber slices.

Baker and Nelson (1943) used protocatechuic acid and observed very little discoloration of the potato slices. They noted an increased rate of oxygen uptake which persisted for hours. A similar increase in the rate of carbon dioxide evolution was observed, and the R. Q. remained close to unity. Carbon dioxide could have hardly been evolved unless tyrosinase played the role of a respiratory enzyme.

Sreerangachar (1943) obtained evidence that polyphenoloxidase was involved in the respiration of tea leaves and stems. Bonner and Wildman (1946) found in spinach leaves that 90-100 per cent of the respiration was inhibited by p-nitrophenol, but this chemical had no effect on the cytochromes.

Bonner (1950) stated that polyphenoloxidase may be the terminal oxidase in higher plant tissues; however, since the enzyme was not found in all species, it cannot be the universal oxidase.

Robinson and Nelson (1944) used tyrosinase activity as a means of following the isolation of an active crystalline material from potato extracts. The crystalline material was found to be L-tyrosine by Schmalfus and Bumbacher (Dawson and Tarpley, 1951). Robinson and Nelson (1944) found in vitro by using ascorbic acid as a reductant in
L-tyrosine-tyrosinase systems that the role of L-tyrosine as a natural substrate and the participation of the two activities of tyrosinase in plant respiration may be explained in the following way: L-tyrosine in the presence of oxygen and tyrosinase is oxidized to L-3, 4-dihydroxyphenylalanine (dopa). Dopa is oxidized to dopa quinone by dopa oxidase (tyrosinase), and oxygen acts as the hydrogen acceptor. In the case of catechol, it is oxidized by catecholase to o-benzoquinone. The o-quinones formed can be rapidly reduced by ascorbic acid. Baldwin (1957) stated that glucose-6-phosphate dehydrogenase together with its substrate and triphosphopyridine nucleotide (TPN) could reduce the o-quinones. A small amount of diphenol, catechol, or dopa, or the corresponding quinones can be alternately reduced and oxidized many times, and thus can act as an intermediate carrier of hydrogen between an unlimited amount of reducing substrate on the one hand and molecular oxygen on the other. This system catalyzes a continuous uptake of oxygen and a simultaneous oxidation of reducing substrate in equivalent amounts.

Lerner and Fitzpatrick (1950) reported that in the absence of a reducing substrate, dopa quinone undergoes a spontaneous and rapid intermolecular change in which the nitrogen of the side chain attaches itself to the 6-position of the benzene nucleus with the formation of 5,6-dihydroxydihydroindole-2-carboxylic acid. This compound is readily oxidized by a reversible reaction to the corresponding quinone, hallochrome. Hallochrome is a red substance, and it is the first visible product formed in the reactions. Under physiologically alkaline conditions hallochrome decarboxylates and undergoes a rearrangement to form 5,6-dihydroindole. The indole compound is rapidly oxidized to the
corresponding quinone which has a purple color. The quinone then poly-
merizes to melanin. If the intermolecular rearrangement undergone by
hallochrome is quickened under acid condition, no decarboxylation
occurs and 5,6-dihydroxyindole-2-carboxylic acid is formed, and readily
converted to a melanin substance.

Hattori and Shiroga (1954) reported that *Stizolobuim hassjoo* con-
tains a specific oxidase, dopa oxidase, which did not oxidize tyrosine.
The oxidase was inhibited or retarded by adding the reduced form of
ascorbic acid, but these workers failed to determine whether the inhi-
bition consisted in reduction of dopa quinone or in preventing further
oxidation of this quinone. Young, pale green leaves contained less
ascorbic acid than adult green ones, and the blackening reaction was
more rapid and stronger in the former than in the latter. This could
probably be ascribed to the difference in the content of reduced
ascorbic acid.

Cantino and Horenstein (1954) demonstrated that catechol, tyrosine
and dihydroxyphenylalanine were oxidized by cell free preparations of
resistant sporangial plants of *Blastocladiella emersonii*. Catechol oxi-
dation by the enzyme could be coupled to reduction of either oxygen or
TPN, but not diphosphopyridine nucleotide. The enzymatic oxidation of
catechol required alpha-ketoglutarate as a co-factor. Apparently this
oxidation occurred by virtue of a coupled reaction between the reduction
of alpha-ketoglutarate to isocitrate by a TPN-specific isocitric dehy-
drogenase, and the oxidation of o-quinone, or trihydroxy benzene, to
hydroxy-o-quinone, by a TPN-specific quinone oxidase.

Middleton (1955) measured respiration and potassium uptake of beet
and potato disks in the presence of protocatechuic acid. He found that
this compound in a concentration from $10^{-3}$ to $3 \times 10^{-3}$ M at pH-6.0, stimulated both oxygen uptake and carbon dioxide output. This stimulation of gas exchange was accompanied by a reduction in potassium uptake. Polyphenoloxidase mediated the extra respiration in the presence of protocatechuic acid, but under 5 per cent oxygen this enzyme did not appear to play any part in the normal respiration. Potassium uptake by potato disks was light-reversibly inhibited by carbon monoxide, indicating the participation of cytochrome oxidase in salt uptake in the potato tissue.

III. The Effects of 3-Amino-1,2,4-Triazole (Amitrol) Upon Plant Growth.

Amitrol-$5\text{C}^{14}$ has been a very useful tool to determine the possible fate of amitrol in plants.

Anderson (1958) was the first worker to report on the translocation of amitrol-$5\text{C}^{14}$ in nut grass. When this compound was applied to the mother tuber in a chain of three, translocation and accumulation of $\text{C}^{14}$ occurred in all of the active meristematic tissues within 2 days after treatment. When a single plant was treated with amitrol-$5\text{C}^{14}$, $\text{C}^{14}$ translocated to and accumulated in meristems, such as active buds, sprouts, root tips, etc. $\text{C}^{14}$ was scarce or lacking in dormant buds, storage parenchyma and mature tissue in general. $\text{C}^{14}$ was found to move in the nut grass plant together with the photosynthetic food stream. This finding agreed with former work on translocation of plant growth regulators.

Rogers (1957a) applied amitrol-$5\text{C}^{14}$ to the foliage of soybean, Canada thistle and Johnsongrass. Translocation occurred throughout each of the plants during a 70-hour period. Accumulation was more
prominent in the younger leaves of Canada thistle. Johnsongrass was more resistant to amitrol than Canada thistle. The position of the meristematic regions in grasses impedes accumulation of large quantities of amitrol. Racusen (1958) found that amitrol-5C\textsuperscript{14} was rapidly transformed and C\textsuperscript{14} translocated from leaves of pinto bean seedlings. After 5 days, roughly one-half of the radioactivity had migrated elsewhere. Of the total radioactivity only 7 per cent was in the form of amitrol.

Aldrich and McLane (1957) described a method for detecting amitrol in plant tissue. By their method "free" and "bound" amitrol were found in plant preparations. The two fractions showed different Rf values, and both formed a yellow azo dye by a coupling reaction. Both fractions exhibited an absorption maximum of 4600 Å, as did a test tube preparation of the amitrol azo dye. This indicated to these workers that their test was specific for amitrol.

Racusen (1958) used a modification of the method of Aldrich and McLane (1957) in determining the fate of amitrol in plants. This test was more specific for amitrol. Analyses from pinto bean seedlings exposed to amitrol-5C\textsuperscript{14} showed that this compound was transformed into new products, X and Y. Compound X was the major product of amitrol metabolism. Both products were characterized by the same ring system and free amino group as the parent amitrol. Compound X and Y formed azo dyes by a coupling reaction.

The formation of compound X in the plant was a function of time, and the formation of both X and Y required healthy turgid leaf tissue.

Aldrich and McLane (1957) claimed their "bound" substance was a protein-amitrol complex. Racusen (1958) found, however, that both X
and Y was stable to hot HCL. This indicates that X and Y were not proteinaceous. Compound X, while having some toxicity when applied to plants was not as toxic as the parent amitrol. Compound Y did not appear toxic. Racusen believed the transformation of amitrol into X and Y was probably carried out by some enzyme system in the leaf. Attempts to isolate the active system in homogenates were unsuccessful. He surmised that the lower toxicity of compounds X and Y as compared with amitrol made it unlikely that either was an actual toxic principle. Perhaps these materials were simply detoxification by products and had no direct relation to the action of amitrol. On the other hand, they might be formed as a direct consequence by some toxic reaction of amitrol.

Rogers (1957b) observed that rates of amitrol sufficient to cause chlorosis in new growth do not cause a systemic chlorosis in older tissue. This suggested that the systemic reaction was not one of chlorophyll destruction. Therefore, presumably the action was an inhibition of chlorophyll synthesis. He also believed that amitrol might inhibit chlorophyll synthesis by the formation of complexes with the metals, iron and magnesium. The results of Hall et al. (1954) indicated that complexing of metals by amitrol was not important in chlorophyll synthesis.

Rogers (1957a) employed paper chromatography to separate a water extract from amitrol-treated plants. He determined radioactivity of fractions from the chromatogram with an amitrol-5C14 control. He found that little of the radioactivity in the soybean plant was present as amitrol, but was actually present in some other compound. Canada thistle and Johnsongrass were found to contain significant amounts of amitrol,
and a compound similar to the unknown in the soybeans, and one or two additional unknowns which were radioactive.

In a later study, Rogers (1957b) reported that the unknown radioactive compound common to all the plants and which contained most of the radioactivity in the treated soybean plants was a glucose adduct of amitrol.

Miller and Hall (1957b) reported that when amitrol-$^{14}C$ was applied to cotton, the $^{14}C$ translocated mainly to the younger tissue of the shoot of cotton. Translocation of $^{14}C$ occurred by the end of the first day and increased in the young growth up to seven days under greenhouse conditions. Acetone extracts containing all of the plastid pigments from treated plants accounted for about one-tenth of the radioactivity applied to the leaves. The extract was made one day after treatment. The major part of the activity remained in the acetone and water washings after transfer of the pigments to petroleum ether. This indicated that the activity was extracted with water or was associated primarily with constituents of the leaves other than pigments.

Rogers (1957b) investigated the possibility that amitrol, with structural similarity to porphobilinogen, may substitute for this compound when the four porphobilinogen units were fitted into a porphyrin ring. He used a biochemical system in which radioactive glycine was the substrate. Radioactive activity of the synthesized porphyrin would give a measure of the total amount of porphyrin synthesized. It was found that at a concentration considerably higher than that found in treated plants, amitrol did not inhibit synthesis of porphyrin rings. This evidence does not support the hypothesis that amitrol directly inhibits chlorophyll synthesis.
Tschudy and Collins (1957) observed that the ability of amitrol to effect two different porphyrin-containing compounds, chlorophyll and catalase, suggested a possible interference with porphyrin synthesis or inhibition of the activity of these porphyrin-containing compounds. These workers injected amitrol intraperitoneally in mice. Hepatic delta-aminolevulinic acid dehydrase activity was determined. They found that amitrol reduced the level of activity of this enzyme within 3 to 4 hours. The conversion of delta-aminolevulinic acid to porphobilinogen is catalyzed by the enzyme, delta-aminolevulinic acid dehydrase, an early step in the synthesis of porphyrin.

Heim et al. (1956) found that when amitrol was injected into rats, catalase activity dropped. Catalase from leaves was also lowered by amitrol application prior to enzyme isolation. The fact that amitrol was a very effective inhibitor in vitro implied that the action was in some way related to the metabolism of the organism.

Pyfrom et al. (1957) observed that a low concentration of amitrol, in a nutrient solution absorbed by potato plants or applied in suspension from barley leaves, markedly depressed catalase activity. Low concentrations of amitrol had no effect on the chlorophyll content of mature leaves from potato. Chlorophyll content was reduced when amitrol was present at the time of leaf differentiation. Catalase activity returned to normal following a disappearance of amitrol.

Rogers (1957b) presented cytological data from the corn leaf which indicated that chlorosis was due to a lack of chloroplasts other than some effect on chlorophyll per se. He suggested that amitrol inhibits the development of plastids from proplastids. Shive and Hansen (1958) found in the treated red pine needle that a very few
mesophyll cells had the infolded wall, and there was no chlorophyll patch which was evident in the control. The chlorophyll patch was absent in treated white spruce needles. The cells contained nuclei and non-pigmentary granular structures. The cells seemed to be arrested in the meristematic stage.

Miller and Hall (1957a) compared the performance of amitrol with eight of its salts or derivatives which were found to be the most successful in defoliating cotton and controlling regrowth. The phosphate salt gave the highest defoliation, and regrowth was inhibited most by the sodium salt in greenhouse tests. However, under field conditions there was no significant difference between the salts of amitrol and this compound upon defoliation. Amitrol and salts of amitrol generally caused an initial stimulation of respiration of Avena sections and cotton leaf discs, but after 26 hours oxygen uptake of treated leaf discs was approximately the same as controls. Miller and Hall (1957b) found that amitrol and the acetate, and phosphate salts of amitrol applied at 1500 ppm to the cotton plant reduced the relative amounts of both chlorophyllides a and b in regrowth. Amitrol treated plants showed twice as much anthocyanin in the regrowth leaves as in normal leaves; however, the inflections of the curves indicated little alteration in the properties of the anthocyanins due to amitrol treatment. The anthocyanin fractions from treated and untreated plants showed identical Rf values. Herbert and Linck (1957) found that amitrol increased respiration of leaf and rhizome sections of Canada thistle initially, but respiration decreased with time depending upon the concentration of amitrol, and on the time interval following application. The alteration of leaf tissue respiration was more than in the rhizome
tissue. Phosphate levels in the Canada thistle tissue were found to influence the changes in cell metabolism induced by amitrol.

McWhorter (1958) found that amitrol treatment of detached corn leaves by either surface application or vacuum infiltration had essentially no effect on either respiration or terminal oxidation. An increase in respiration was the case when amitrol was applied as a soil treatment to actively-growing corn. Chlorotic tissue obtained from soil treatment exhibited a much higher ascorbic acid oxidase than did control tissue. Polyphenoloxidase was slightly reduced in chlorotic tissue with cytochrome oxidase unaffected. Temperature and age of plants were factors which controlled somewhat the terminal enzyme in chlorotic corn tissue. Greater respiratory inhibition was obtained in chlorotic tissue with iodoacetic acid, malonic acid, and 2,4-dinitrophenol than in control tissue. Control and chlorotic tissues responded equally to sodium fluoride and sodium azide. Total carbohydrates, iron, manganese, nitrogen, phosphorous, and water were higher in chlorotic tissue. The respiratory quotient of chlorotic tissue grown at 70° F was indicative of organic acid metabolism, and total organic acids were lower in this tissue than in controls. Total activity of aldolase was greater in control material than in chlorotic plants.
METHODS AND MATERIALS

I. Source of Plant Material.

Tubers were taken from field soil and transplanted into pots in the greenhouse. Within a few months, an abundance of tubers had developed in the pots, and these tubers were used in the studies.

A. Preparation of whole tissue.

Several pots were emptied, and the soil was removed with water. Single tubers were separated from the tuber system for use in certain studies. Three and five tubers connected by rhizomes were also separated from the system. These materials were dipped into a 1 per cent solution of Vancide 51 and blotted with paper towels.

II. Germination of Single Tubers, and Three and Five Tubers Connected by Rhizomes in Gas Mixtures.

The gas mixtures used were 1, 10, and 20 per cent oxygen in combination with 1, 5, 10, and 15 per cent carbon dioxide, and nitrogen served as the inert gas. Oxygen, carbon dioxide, and nitrogen at 100 per cent were also used. The gas mixtures were prepared in tanks, and a separate tank was used for each mixture. For each treatment, four 125 ml Erlenmeyer flasks connected by tubing were used. Two flasks contained three single tubers each, and the other two flasks contained one chain of three tubers. Each treatment was replicated three times, and the complete test was triplicated. In a different experiment each of the four flasks contained one chain of five tubers. Each treatment was duplicated. The tubers were maintained on moist filter paper. The system of four flasks was evacuated for three minutes with an ordinary
laboratory water pump. A particular gas, or gas mixture, was then flushed into the system to refill it once a day for 17 days. The system was sealed with a clamp on the Tygon tubing and kept in the laboratory.

At the end of the 17th day, germination counts were made on single tubers. Patterns of germination were recorded on tubers in the chains. The data reported are averages of the experiments. The flasks containing the single tubers were opened and left in air for 11 days. The tubers were maintained on moist filter paper during this period. The flasks were opened in order to test the viability of tubers which had failed to germinate under low oxygen levels. Germination counts were made on the 11th day.

After the patterns of germination had been recorded for chains containing five tubers the dormant tubers were separated from the chains and returned to the flasks. These tubers remained under air for four days, and germination of these tubers was recorded.

III. Amitrol Treatment.

A. Shoot and root growth test.

Five grams of dormant tubers were placed in 125 ml Erlenmeyer flasks. Four flasks were used for each treatment. To each flask, amitrol was applied at $10^5$, $10^4$, $10^3$, $10^2$, $10^1$, and $10^0$ micrograms in five ml of complete Hoagland's nutrient solution at pH 5.7. The check contained no amitrol. The four flasks were connected in series with Tygon tubing. The system was flushed for two minutes with 100 per cent oxygen. Oxygen treatment was applied in order that germination could proceed faster. The tubing was sealed with a screw clamp, and the experiment was kept in the laboratory. The system was flushed once
every two days with 100 per cent oxygen. On the 8th day following
treatment, shoot and root lengths were taken on 20 tubers from each
treatment.

B. **Treatment of tubers with amitrol for enzyme assay.**

From the results of the shoot and root growth study, it was
decided to use amitrol at 8 and 16 mg in the same manner as described
under **Shoot and root growth test** (III A). Oxygen treatment was the
same. There were 8 flasks per treatment. This study was conducted at
24 ± 2° C and 310 foot candles of constant incandescent light. At the
end of the 8th day the tubers were utilized for the assays of ascorbic
acid oxidase, catecholase, dopa oxidase and peroxidase.

A different test was conducted by using the lower rate of amitrol
in Hoagland's solution at pH 5.7. The dormant tubers were placed in
Petri plates containing the amitrol. The test was kept under variable
room temperature, and constant fluorescent light at 53 foot candles in
the laboratory. At the end of the 8th day the tubers were prepared for
catalase assay. All tubers were washed in three liters of water before
they were homogenized. This was done to remove the nutrient solution
and amitrol.

IV. Preparation of Tissue Components.

A. **Tissue slices.**

Single tubers were selected at random and sectioned on the sliding
microtome. The slices were prepared at 30 microns. The slices were
immediately placed in distilled water, blotted with paper towels and
utilized in experiments. In certain studies, slices, 2-3 mm thick,
were prepared with a surgeon's scalpel.

B. Homogenates.

Five grams of the tubers to be homogenized were sliced 3-4 mm thick. These slices were placed in a semi-micro Waring blender containing 50 ml of a 0.05 M potassium phosphate buffer of pH 6.0. Unless otherwise stated, this buffer and pH were the same for all homogenate preparations. The tissue was homogenized for two minutes. A cardboard jacket contained adequate crushed dry ice around the base of the Waring blender. The temperature of the suspension inside the blender was maintained below 10° C. After blending the crude homogenate was used in preliminary studies, but these homogenates showed considerable endogenous oxidation. By filtering the homogenate through two layers of cheese cloth the crude fraction and endogenous oxidation were eliminated. The filtrate was centrifuged at 1000 x g for 10 minutes at 2° C. and used in the studies. A portion of the homogenate was frozen quickly in dry ice. In repeating an experiment this frozen homogenate was quickly thawed.

C. Preparation of "particles."

Forty to 50 grams of tubers were sliced 3-4 mm thick. The slices were placed in a pre-chilled chamber of the Omni mixer containing 250 ml of cold 0.5 M sucrose and 0.1 M potassium phosphate buffer of pH 6.0. The chamber was submerged in a constant temperature bath at 2° C. Homogenization was conducted for five minutes at 80 volts. Following homogenization the suspension was separated by a differential centrifugation procedure. The International refrigerated centrifuge and the Spinco model L ultracentrifuge were employed in the procedure (Figure 1).
The suspending buffer was 0.4 M sucrose plus 0.02 M potassium phosphate at pH 6.0. Residue 4 (Figure 1) was designated as "particles." When "particles" is used, it will refer to residue 4 which was obtained as described in the procedure. When "particles" were used in studies, a quantity was quickly thawed and diluted with buffer or used without dilution.

V. Determination of Oxygen Uptake and Carbon Dioxide Production.

Oxygen uptake and carbon dioxide evolution of whole tissue and slices, and oxygen uptake of "particles" and homogenates were determined by using a Warburg respirometer and conventional methods described by Umbreit et al. (1949). Except those experiments which state the specific temperature, all of the others were conducted at 25°C. Gas exchange is expressed as microliters per gram of fresh weight, microliters per gram of dry weight, and microliters of oxygen uptake per milligram of tissue nitrogen per hour ($Q_{O_2}(N)$), and microliters per milligram of nitrogen.

A. Under gas mixtures.

In experiments involving respiration under different oxygen concentrations and nitrogen a particular gas mixture was flushed through the Warburg flasks. Approximately one liter of gas was flushed through each flask after equilibration. The stopcocks of the manometers and flasks were closed, and gas exchange was determined.

1. Preparation of gas mixtures.

In order to maintain accurately the per cent oxygen in a mixture of oxygen and nitrogen, a quantity of oxygen higher than that desired
Suspension strained through one layer of cheese cloth

Residue 1  Supernatant 1
Discarded  Centrifuged at 1,000 x g for 10 minutes

Residue 2
Discarded  Supernatant 2
Centrifuged at 20,000 x g (minimum) for 15 minutes

Residue 3 "particles"
Discarded  Resuspended in same buffer centrifuged at 20,000 x g (minimum) for 30 minutes

Supernatant 4  Residue 4, "particles"
Discarded  Suspended in 10 ml of buffer and frozen

Figure 1. The differential centrifugation procedure followed to obtain "particles" from nut grass tubers.
was delivered into a tank. Nitrogen was delivered into the same tank. By reducing the pressure and delivering additional nitrogen the percentage of oxygen was decreased until the desired percentage of oxygen and nitrogen were obtained.

B. Whole tubers during germination and whole tubers under oxygen levels and nitrogen.

Four Warburg flasks each containing four tubers were employed for oxygen uptake and carbon dioxide evolution. Each flask received 3 ml of a buffer solution at pH 6.0 which was 0.05 M and 0.02 M with regard to potassium phosphate and sucrose, respectively. The determinations were carried out for 2.5 hours. Two flasks were employed for the anaerobic study.

1. Whole tubers during germination.

A determination of gas exchange was carried out on dormant tubers, and each day for six days, and again on the 10th day. The tubers were maintained on moist filter paper in Petri plates between determinations. Weights of the tubers were taken prior to each determination in order to determine growth from the time of dormancy. The data presented are average values of two experiments.

2. Whole tubers under oxygen levels and nitrogen.

Gas exchange was determined from dormant tubers in air, and in an atmosphere of 1 and 10 per cent oxygen in combination with nitrogen. Respiration was determined in 100 per cent oxygen and nitrogen. The flushing procedure was described Under gas mixtures (VA). Immediately following each determination the tubers for a particular treatment were placed on moist filter paper in four 125 ml Erlenmeyer flasks. The
flasks were connected with Tygon tubing. Once a day for six days the system was evacuated with an ordinary laboratory water pump for three minutes. A particular gas mixture, that is, the mixture or 100 per cent oxygen in which respiration was determined, was then flushed into the system to refill it, and the system was sealed with clamps. Nitrogen at 100 per cent was not used here. On the 6th day, gas exchange was determined in the same manner as when the tubers were dormant.

C. Slices 2-3 mm thick.

Cross sectional slices 2-3 mm thick were employed from dormant tubers. These sections, 35-40, were placed in the Warburg flasks containing 3 ml of 0.05 M potassium phosphate plus 0.02 M sucrose at pH 6.0. Duplicate flasks were employed and the test was duplicated. Gas exchange was determined after equilibration.

VI. Estimation of Activity and Inhibition of Catecholase.

A. General procedure.

A 2 ml aliquot of a homogenate was used in the determinations. The homogenate was prepared from tubers for a particular study. A 0.5 ml aliquot of an inhibitor, or catechol, was used in all studies. The final concentration of catechol and inhibitors is reported. Duplicate flasks were employed for each treatment, and a particular test was duplicated. The catechol and inhibitors were prepared in the buffer and adjusted to the proper pH. For studies in which the pH was different from 6.0, the homogenate was adjusted with NaOH or H2SO4. After equilibration the catechol in a single side arm, or catechol, and inhibitor in separate side arms of the vessel were tipped into the homogenate.
Manometric readings were recorded at 10 minute intervals for one hour, unless otherwise reported. For studies in which the pH was above 6.0, endogenous oxidation and autooxidation were determined and subtracted from total oxidation. The data reported for these studies are exogenous substrate oxidation.

1. Activity under oxygen levels.

Tissue slices at 30 microns were employed from dormant tubers. Thirty-five to 40 slices were floated in 2.5 ml of 0.05 M potassium phosphate plus 0.02 M sucrose of pH 6.0 in the main compartment of the Warburg flasks. The flasks were flushed with 1 and 10 per cent oxygen in mixture with nitrogen, and 100 per cent oxygen as described under gas mixtures (VA). The stopcocks were closed immediately, and catechol was tipped into the main compartment. The temperature was 30° C.

2. During germination.

In order to follow catecholase activity during germination, a large quantity of dormant tubers was placed in a moist chamber to germinate in water. Dormant tubers and germinated tubers with the most growth were selected every two days for 3 days for the enzyme source. Catecholase activity was determined in homogenates prepared from these tubers.

3. Inhibitors for catecholase.

Difficulty was encountered in the use of sodium diethyldithiocarbamate (dieca) as an inhibitor of catecholase. Initially this compound was used at pH 6.0 and 25° C, but under these conditions a positive pressure developed in the flask. James and Garton (1952) found that when dieca was used under slightly acid conditions, and at temperatures above 20° C a reaction product, carbon oxysulphide, was evolved which
yielded a positive pressure in the flask. The workers showed a very minimum positive pressure at pH 7.0 and 20° C; consequently, this inhibitor was used satisfactorily under these conditions.

Benzoic acid, thiourea and p-nitrophenol were used at pH 6.5. Catechol was used at the same pH value as the inhibitors.

VII. Estimation of Tyrosinase, Dopa oxidase and p-Cresolase.

A 2 ml aliquot of a homogenate from dormant tubers was used. A 0.5 ml aliquot of DL-tyrosine, L and DL-3,4-dihydroxyphenylalanine (dopa) and p-cresol were each placed in the side arm of duplicate flasks. These compounds were tipped into the homogenate following equilibrium. The final concentration of the compounds under this section is reported. These substrates were prepared in the same buffer as the homogenate. L and DL-dopa and p-cresol were adjusted to pH 6.0. DL-tyrosine was brought into solution with NaOH. A 0.5 ml aliquot of the alkaline tyrosine was tipped into the homogenate. The final pH was 6.5.

A. Tyrosinase of washed tissue slices.

Slices at 30 microns from dormant tubers were washed for 24 hours with running tap water. The slices were blotted with paper towel and 35-40 were floated in 2.5 ml of 0.05 M potassium phosphate buffer plus 0.02 M sucrose at pH 6.0 in the Warburg flasks. A 0.5 ml aliquot of alkaline aqueous L-tyrosine was tipped from the side arm 10 minutes after the manometers were closed. Water at the same pH as the L-tyrosine was tipped for the check treatment. The final pH was 6.3.
B. Estimation of dopa oxidase activity.


The method of Bailey (1958) was employed. Seven ml of 0.2 M potassium phosphate buffer at pH 6.0, and 2 ml of 0.5 per cent (W/V) gelatin were added to a test tube and placed in a 25° C constant temperature bath. Two ml of the same buffer containing 3.5 mg of L-dopa per ml were added followed by 1 ml of the homogenate. Absorbance was determined with the Beckman spectrophotometer with a number 470 filter and compared with a control containing enzyme, buffer and gelatin. The instrument was equipped with a thermostorer which maintained the temperature at 25° C.

Specific activity was computed by subtracting endogenous oxidation of the enzyme from total oxidation of the treatment, and dividing by the quantity of nitrogen in the 1 ml of homogenate, and multiplying by 100.

\[
\text{Specific Activity, S. A.} = \frac{(C_{At} - C_{Ae}) \times 100}{N}
\]

\( C_{At} \) = Absorbance change during reaction period in the presence of enzyme and L-dopa.

\( C_{Ae} \) = Absorbance change during reaction period of enzyme without added substrate.

\( N \) = Total nitrogen in the 1 ml of homogenate.

2. Manometric and colorimetric tests.

In order to test the influence of ascorbic acid upon oxygen uptake and hallochrome development or inhibition, these tests were employed.

A 2 ml aliquot of homogenate from dormant tubers was placed in the vessels. A 0.5 ml aliquot each of L-dopa and ascorbate in
buffer was placed in the side arms after being adjusted to pH 6.0. These materials were tipped simultaneously into the homogenate, and oxygen uptake was recorded. At the end of one half hour, samples of the reaction mixture in flasks, other than those used to measure oxygen uptake, were taken to determine absorbance with a number 470 filter. At the end of one hour a sample from all flasks was used for absorbance determinations with the same filter. Specific activity was determined from this data as described under Colorimetric test (VII B1).

VIII. Ascorbic acid Oxidase.

A 2 ml aliquot of a homogenate from dormant and germinating tubers was placed in the main compartment of the Warburg flask. The ascorbate was prepared in buffer and adjusted to pH 6.0 with NaOH. A 0.5 ml quantity was tipped from the side arm following equilibration. The final concentration is reported.

IX. Peroxidase.

Peroxidase was determined by a modified method of Stutz (1957). The buffer of the homogenate was adjusted from 0.05 M and pH 6.0 to 0.2 M and pH of 6.5 with a potassium phosphate buffer.

A 2 ml aliquot of this homogenate was added to a test tube, followed by 2.5 ml of freshly prepared pyrogallol. At zero time, 0.5 ml of hydrogen peroxide was pipetted into the tube. Optical density change was recorded at one minute intervals for five minutes in the Bausch and Lomb Colorimeter. A number 420 filter was used. The temperature was 25° C during the reaction period. The final concentration for pyrogallol and hydrogen peroxide was 0.25 M and 0.006 M,
respectively. These compounds were prepared in the buffer. The hydrogen peroxide was adjusted to pH 6.5 before adding to the buffer.

A similar procedure was followed to measure autooxidation of pyrogallol and endogenous oxidation of the enzyme. For these determinations the enzyme and pyrogallol were replaced by buffer.

Specific activity was computed by subtracting the sum of the autooxidation of substrate and endogenous oxidation of enzyme from total oxidation, and dividing by the total nitrogen in the 2 ml of homogenate, and multiplying by 100.

\[
\text{Specific activity} = \frac{(C_{At} - (C_{Ae} + C_{Aa})) \times 100}{N}
\]

\(C_{At}\) = Change in absorbance as a measure of total oxidation.

\(C_{Ae}\) = Change in absorbance as a measure of endogenous oxidation.

\(C_{Aa}\) = Change in absorbance as a measure of autooxidation.

\(N\) = Total nitrogen in the 2 ml of homogenate.

X. Catalase.

A modified procedure of Appleman (1951) was used for catalase determination.

The potassium phosphate buffer for the preparation of the homogenates was 0.2 M at pH 7.0. The hydrogen peroxide was prepared in the buffer after being adjusted to pH 7.0 with NaOH. A 2 ml aliquot of the hydrogen peroxide was placed in the main compartment of the Warburg flask, and 1 ml of homogenate was tipped from the side arm.

The final concentration of hydrogen peroxide was 1 M. Manometric readings were recorded at one minute intervals for four minutes. The temperature during the reaction was 20° C.
XI. Krebs Cycle Studies.

A. Dehydrogenase determinations.

Dehydrogenase determinations were made by using "particle" suspensions. The absolute dilution factor depended upon the turbidity of the solution and its effect upon colorimeter readings. Measurements of dehydrogenase activity were made under vacuum in Thunberg tubes by determining the rate of anaerobic reduction of Sodium-2, 6-dichlorophenolindophenol (DIP) as described by Umbreit et al. (1957b) and Price (1954). A Bausch and Lomb colorimeter was used with a number 630 filter. The temperature was 25° C during the determinations. Each component was prepared in glass distilled water. The acids were adjusted to pH 7.0. Specific activity was determined by obtaining the endogenous reduction of DIP in the presence of "particles" and buffer. Total reduction was determined in the presence of a 0.5 ml aliquot of "particle" suspension, substrate and co-factors. Endogenous reduction was subtracted from total reduction and divided by the total nitrogen in the 0.5 ml of "particle" suspension. This value was multiplied by 100. The formula is shown under Colorimetric test (VII B1).

The concentration of components for isocitric and succinic dehydrogenase activities are listed in the following protocol:

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>2 x 10^{-2} M</td>
</tr>
<tr>
<td>Potassium phosphate and sucrose</td>
<td>2 x 10^{-2} M, 0.4 M, respectively</td>
</tr>
<tr>
<td>pH 6.8</td>
<td></td>
</tr>
<tr>
<td>MgSO_4</td>
<td>2 x 10^{-3} M</td>
</tr>
<tr>
<td>DIP</td>
<td>5 x 10^{-5} M</td>
</tr>
</tbody>
</table>
Component Final Concentration
Isocitrate \(-\text{-}-\text{-}-\text{-}-\text{-}-\text{-}-\) \(2 \times 10^{-2} \text{ M}\)
DIP \(-\text{-}-\text{-}-\text{-}-\text{-}-\text{-}-\) \(5 \times 10^{-5} \text{ M}\)
Triphosphopyridine nucleotide (TPN) \(-\text{-}-\) \(1 \times 10^{-3} \text{ M}\)
\(\text{MgSO}_4\) \(-\text{-}-\text{-}-\text{-}-\text{-}-\text{-}-\) \(2 \times 10^{-3} \text{ M}\)
Potassium phosphate and sucrose \(\text{pH 6.8}\) \(-\text{-}-\text{-}-\text{-}-\text{-}-\text{-}-\) \(2 \times 10^{-2} \text{ M}, 0.4 \text{ M, respectively}\)

Water was added to bring the volume up to 4 ml.

B. Oxidase determinations.

The various co-factors and substrates were placed in the main compartment. The components were prepared in glass distilled water. The acids were adjusted to pH 7.0 before using. A 0.5 ml aliquot of the "particle" suspension was placed in the side arm of duplicate flasks and tipped into the main compartment after equilibrium.

The final concentrations of components for the three oxidases are listed in the following protocol:

Component Final Concentration
Isocitrate \(-\text{-}-\text{-}-\text{-}-\text{-}-\text{-}-\) \(1 \times 10^{-2} \text{ M}\)
Succinate \(-\text{-}-\text{-}-\text{-}-\text{-}-\text{-}-\) \(1 \times 10^{-2} \text{ M}\)
a-Ketoglutarate \(-\text{-}-\text{-}-\text{-}-\text{-}-\text{-}-\) \(2 \times 10^{-2} \text{ M}\)
a-Ketoglutarate plus Malonate \(-\text{-}-\text{-}-\text{-}-\text{-}-\text{-}-\) \(2 \times 10^{-2} \text{ M}, 1 \times 10^{-1} \text{ M, respectively}\)

For each oxidase the complete system contained in addition to one of the above substrates the following components:
Component Final Concentration

\( \text{MgSO}_4 \) \( 2 \times 10^{-3} \text{ M} \)

Potassium phosphate and Sucrose \( 2 \times 10^{-2} \text{ M} \), \( 0.4 \text{ M} \), respectively

pH 6.7

Adenosine diphosphate (ADP) \( 2 \times 10^{-3} \text{ M} \)

Adenosine triphosphate (ATP) \( 1 \times 10^{-3} \text{ M} \)

Diphosphopyridine nucleotide (DPN) \( 5 \times 10^{-4} \text{ M} \)

Triphosphopyridine nucleotide (TPN) \( 5 \times 10^{-4} \text{ M} \)

Cocarboxylase \( 1.23 \times 10^{-4} \text{ M} \)

Cytochrome C \( 6.83 \times 10^{-5} \text{ M} \)

Water was added to bring the volume up to 1.7 ml.

XII. Preparation of Dry Tissue.

Fresh whole tubers were dried in a convection oven set at 80\(^\circ\) C for 24 hours. Dry weights were taken, and the dried tubers were prepared in the Wiley mill equipped with a 60 mesh screen. The material was kept in dry flask containing stoppers and utilized for certain analyses.

Tissue slices were taken from the Warburg flasks and washed to remove the buffer. The tissue was dried as described above, and dry weight was taken.

XIII. Chemical Methods.

A. Total nitrogen.

Nitrogen in homogenates, dried tissue slices, and dry ground material from whole tubers was determined by digesting these materials with a \( \text{Na}_2\text{SO}_4 \), \( \text{H}_2\text{SO}_4 \) and \( \text{CuSeO}_3 \) mixture. The \( \text{NH}_3 \) was trapped in
H₂B₄O₇ and titrated with H₂SO₄ (Hiller, et al., 1948). A modified Pregl-Parnas-Wagner micro apparatus was employed for the liberation of the NH₃ from the digested sample (Jacobs, 1951a).

B. Fats and soluble carbohydrates.

Extractable fats were estimated according to the procedure of Jacobs (1951b). Soluble carbohydrates were extracted by the method of Jacobs (1951b) and estimated by the anthrone method described by Umbreit et al. (1957b).

C. Organic acids.

Estimations of total organic acids, malic acid and citric acid were carried out according to the procedure of Pucher et al. (1941). Oxalic acid was estimated by the method of Palmer (1955).

D. Ascorbic acid and dehydroascorbic acid.

Ascorbic acid was estimated by the procedure of Morell (1941). Dehydroascorbic acid was estimated according to the procedure of Roe et al. (1948).
EXPERIMENTAL RESULTS AND DISCUSSION

I. The Influence of 1, 10, and 20 Per Cent Oxygen in Mixture with 1, 5, 10, and 15 Per Cent Carbon Dioxide upon the Germination of Nut Grass Tubers.

A. Germination of single tubers.

Oxygen concentration below the air value decreased germination (Figure 2). About 80 per cent germination occurred under 20 per cent oxygen, and the highest concentration of carbon dioxide increased germination by 5 per cent. Tubers germinated 7.9 per cent less in 10 per cent oxygen, and the highest carbon dioxide concentration slightly reduced germination. The effects of carbon dioxide concentration in combination with 1 per cent oxygen are indicated by the decline in per cent germination for these treatments (Figure 2).

Tubers exposed to 100 per cent carbon dioxide or nitrogen failed to germinate.

Oxygen at 100 per cent induced 95 per cent germination. This is 15 per cent greater than those exposed to 20 per cent oxygen. At the end of the 6th day, 78 per cent of the tubers which were under 100 per cent oxygen had germinated, whereas 44 per cent had germinated under air. This possibly is indicative of an oxidase enzyme responding markedly to oxygen concentration. Tubers exposed to air for 11 days, after being under treatment for 17 days, showed an average germination of 90 per cent for all treatments. This would be expected if the tubers under low oxygen levels were removed from these treatments into air, and the tubers under air were exposed longer. This also illustrated that the tubers remained viable under 1 and 10 per cent oxygen,
Figure 2. The influence of 1, 10 and 20 per cent oxygen in mixture with 1, 5, 10 and 15 per cent carbon dioxide upon the germination of single tubers.
even though the per cent germination was low under these treatments.

B. Response of three and five tubers connected by rhizomes.

1. Three tubers in a chain.

In order to determine if different oxygen levels might influence dominancy in the chain, tubers which germinated in the chain were determined. It was found that several patterns of germination occurred under the oxygen concentrations (Figure 3A-F). Since these chains were broken at random from a system of nut grass, the stage of development of the tubers was not known in any of the chains. Under low oxygen concentration a larger number of the chains showed all tubers dormant, and this pattern was increased markedly as the carbon dioxide concentration increased (Figure 3A).

When one terminal tuber germinated, a larger number of this chain pattern was under 1 and 10 per cent oxygen. At the 1 per cent carbon dioxide concentration, there is an indication that oxygen level definitely controls this pattern of germination (Figure 3B). The central tuber germinated in the chain to a greater degree under 20 per cent oxygen than tubers under 1 or 10 per cent oxygen (Figure 3C).

The two terminal tubers germinated to a greater extent in 20 per cent oxygen. This pattern was higher in 10 per cent oxygen than in 1 per cent oxygen (Figure 3D). Carbon dioxide concentration did not influence the pattern appreciably. The same type of response to oxygen was the case where the 1st and 2nd tuber germinated (Figure 3E). The largest number of chains with 100 per cent germination occurred under 20 per cent oxygen and a carbon dioxide level of 15 per cent (Figure 3F).
Figure 3. The influence of 1, 10 and 20 per cent oxygen in mixture with 1, 5, 10 and 15 per cent carbon dioxide upon the number and position of germination of three tubers in a chain. The open circle opposite the figure letter represents the dormant tuber, and the solid circle represents the germinated tuber.
2. **Five tubers in a chain.**

At the lowest oxygen concentration the largest number of chains showed complete dormancy (Table 1). This pattern was followed by one in which one tuber germinated, and in some cases three tubers germinated. However, in 10 per cent oxygen there was a slight shift to patterns which showed mostly two and three, and in some cases four and five tubers germinated. Carbon dioxide concentrations appeared to have no predominating effect upon the patterns. The shift from a pattern which showed one tuber germinated to patterns which showed two, three, four, and five was striking in 20 per cent oxygen (Table 1).

The viability of the dormant tubers in a chain in which one to four tubers had germinated showed that in practically every case all of the tubers germinated; when these tubers were separated from the chain and were allowed to germinate for four days under air (Table 2). This demonstrates that the dominant tuber or tubers present in the chain apparently controlled the germination of the dormant tubers, because the dormant tubers germinated when they were released from the possible controlling mechanism of the germinated ones.

Dominancy in the chain was controlled by oxygen gradient. It was less under high oxygen levels and higher as the oxygen concentration was lowered. This response was probably due to changes in auxin available to the germinated tuber under high oxygen concentration. It is believed that the oxidative destruction of auxin was higher under the high oxygen concentrations which decreased the effective auxin concentration needed by the germinated tubers in order to exert dominancy. The reverse would have occurred under low oxygen concentration. This concept is supported by Ranson and Parija (1955) who have shown that
Table 1. The influence of various oxygen and carbon dioxide concentrations upon the number and position of germination of five tubers in a chain.

<table>
<thead>
<tr>
<th>Chain patterns</th>
<th>Treatment number 1/1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8 9 10 11 12</td>
</tr>
<tr>
<td>0-0-0-0-0</td>
<td>3 1 1 2 2/2</td>
</tr>
<tr>
<td>X-0-0-0-0</td>
<td>2 1 2 2 1 1</td>
</tr>
<tr>
<td>O-X-0-0-0</td>
<td>2 1 1 2 1 1</td>
</tr>
<tr>
<td>O-O-X-0-0</td>
<td>1 2</td>
</tr>
<tr>
<td>X-X-0-0-0</td>
<td>1 1 1 2 1 1 1</td>
</tr>
<tr>
<td>O-O-X-X-0</td>
<td>1 2 2 1 1</td>
</tr>
<tr>
<td>0-O-X-O-X</td>
<td>2 2</td>
</tr>
<tr>
<td>X-O-O-X-X</td>
<td>1 2</td>
</tr>
<tr>
<td>O-O-X-X-X</td>
<td>1 2</td>
</tr>
<tr>
<td>O-X-O-X-0</td>
<td>1 1 1 1 1</td>
</tr>
<tr>
<td>O-X-O-X-0</td>
<td>2 2</td>
</tr>
<tr>
<td>X-0-0-X-X</td>
<td>2 2</td>
</tr>
<tr>
<td>X-0-0-X-X</td>
<td>1 2</td>
</tr>
<tr>
<td>O-O-X-X-O</td>
<td>1 2</td>
</tr>
<tr>
<td>X-X-O-X-X</td>
<td>1 2</td>
</tr>
<tr>
<td>O-X-X-0-O</td>
<td>1 2</td>
</tr>
<tr>
<td>X-X-O-X-X</td>
<td>1 2</td>
</tr>
<tr>
<td>O-X-X-O-X</td>
<td>1 2</td>
</tr>
<tr>
<td>X-X-O-X-X</td>
<td>2 2</td>
</tr>
<tr>
<td>X-X-O-X-X</td>
<td>1 2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>L1 Treat. No.</th>
<th>Gas Mixture per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O2</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
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<tr>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
</tr>
</tbody>
</table>

1/2 The number of chains in a total of 8 chains per treatment which showed this pattern of germination.

1/3 The 0 represents the dormant tubers and the X represents the germinated tubers.
Table 2. The germination of dormant tubers separated from a chain of five tubers.

<table>
<thead>
<tr>
<th>Gas mixtures</th>
<th>Number tubers dormant in the chain</th>
<th>Number tubers germinated under air for four days</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂</td>
<td>CO₂</td>
<td>N₂</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>98</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>94</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>89</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>84</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>89</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>79</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>75</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>60</td>
</tr>
</tbody>
</table>
when coleoptiles of certain cereals were transferred from air the rate of growth in 3.5 and 8.5 per cent oxygen was often doubled that in air before transfer. Root growth decreased with each decrease in oxygen concentration below 21 per cent.

II. Respiration of Dormant and Germinated Tubers under 1, 10, 20 and 100 Per Cent Oxygen.

This study was conducted to obtain respiratory data on single dormant tubers, and germinated tubers kept under varying oxygen concentration.

There is the possibility that more oxidative destruction of auxin occurred under the higher oxygen concentrations. Germinated tubers showed an increase in oxygen uptake when oxygen concentration was increased. Carbon dioxide evolution was higher than oxygen uptake in 1 and 10 per cent oxygen, but the gas exchange was unity at 20 and 100 per cent oxygen (Figure 4B, C). Total gas exchange was higher at 20 and 100 per cent oxygen (Figure 4B).

Oxygen uptake for dormant tubers was low in 1 per cent oxygen, but it increased in 10 and 20 per cent oxygen, and it was considerably higher at 100 per cent oxygen (Figure 4A). Carbon dioxide evolution was high in 1 per cent oxygen and decreased when the oxygen concentration increased. The R. Q. 's for dormant tubers under 1, 10, 20 and 100 per cent oxygen were 4.5, 3.5, 2.8, and 1.9, respectively (Figure 4C). Total gas exchange increased with oxygen concentration (Figure 4A).

The fact that dormant single tubers, for the same period, germinated at a greater percentage under 100 per cent oxygen as compared to less germination under 20 per cent oxygen would indicate that oxygen
Figure 4. A and B-The influence of oxygen concentration upon the respiration of dormant and germinated tubers, respectively. C-The R. Q. s for the dormant and germinated tubers.
concentration was one factor that limited germination. Aerobic respiration was less in 1 and 10 per cent oxygen than in 20 per cent oxygen, and total germination was also less in 1 and 10 per cent oxygen. Thus, there appears to be a correlation between germination and aerobic respiration. Where respiration was high, per cent germination was high in high oxygen concentration. The combination of the inhibitory action of anaerobic carbon dioxide plus high carbon dioxide concentration possibly would account for the low percentage of germination of tubers under these conditions.

III. The Metabolism of Nut Grass During Germination.

A. Respiration and growth during germination.

In order to obtain more information about the respiration of nut grass, gas exchange was followed during germination. It was found that initially carbon dioxide evolution was considerably greater than oxygen consumption (Figure 5A). Dormant tubers had an R. Q. of 2.11 (Figure 5B). However, the rate of oxygen consumption increased with time and an R. Q. of unity was obtained by five and one-half days (Figure 5A, B). At this point the tubers were in the stage of maximum growth (Figure 6). The total gas exchange also increased with time over the experimental period (Figure 5A). Growth in fresh weight increased over the experimental period also (Figure 6). The high R. Q. values obtained in early stages of germination suggested that an organic acid metabolism was in operation, and possibly anaerobic respiration contributed to the evolution of some of the carbon dioxide. As germination proceeded, there appeared to be a rapid shift to a carbohydrate type metabolism.
Figure 5. A—Changes in oxygen consumption and carbon dioxide evolution by tubers during germination in water. B—The R. Q.'s derived from the gas exchanged during this period.
Figure 6. The increase in fresh weight of germinating tubers involved in the respiratory study.
Since the R. Q. values during the early stages of growth were indicative of organic acid metabolism, and in the later stages indicated a carbohydrate metabolism, analyses were conducted in order to determine possible changes in the quantity of certain storage materials during the germination of tubers.

Total organic acids were high in the dormant tuber and increased during the first two days, but a very striking decline occurred from two to four days. This was followed by a rapid increase from 6 to 10 days (Figure 7A). Citric acid, malic acid and oxalic acid were constant over the experimental period, but citric acid was higher in quantity than malic acid and the latter was higher than oxalic acid, (Figure 7A).

Fats followed a gradual decline in quantity until the 6th day, and increased to the 8th day (Figure 7B). Soluble carbohydrates showed a gradual decline after two days (Figure 7B). Total nitrogen showed a decline from the dormant tuber to four days, and increased slightly to a higher level (Figure 7C).

It is possible that during the early stages of germination the nut grass tuber does metabolize predominantly organic acids. It is apparent that the primary organic acids utilized in greater quantity would be those other than malic, citric and oxalic. Nitrogen and fats appeared to be used during this period also. The changes in organic acids and carbohydrates fit the changes in the R. Q. s. This is suggestive that organic acid metabolism was the case during the early stages of germination. Furthermore, it appears that carbohydrates were utilized in the later stages of growth. Total organic acids, fats and nitrogen were synthesized during germination, probably as a result
Figure 7. The influence of germination of tubers upon the changes in certain storage materials. C-The quantity of total nitrogen during germination. The tubers germinated in water.
of new growth or from storage carbohydrates.

In order to test the fact that oxygen gradients within the tuber might have been partly responsible for the high R. Q., tubers were sliced 2-3 mm thick, and respiration was determined on these slices. Total gas exchange was much greater by slices than by dormant tubers (Figures 8A and 5A). However, the R. Q. was 1.5 for the tissue slices (Figure 8B). These data show that when the tuber is sliced carbon dioxide evolution is not as great as in the dormant tuber, but the R. Q. would indicate perhaps an organic acid metabolism in the slices. Since the whole dormant tuber does have a high anaerobic respiration (Figure 8C), it is believed that some anaerobic respiration does take place in the dormant tuber. This anaerobic carbon dioxide would possibly account for that evolved above the quantity necessary to give an R. Q. of 1.5.

In attempts to study the enzymes responsible for the decarboxylation of organic acids in the Krebs cycle, "particles" from dormant and germinated tubers were employed. Isocitric and succinic dehydrogenase activities were estimated in the dormant and germinated tubers. The results of the activities are presented in Figure 9.

It may be seen that isocitric dehydrogenase was very actively associated with the "particles" from dormant tubers, but the activity was less on "particles" from germinated tubers. A point of significance is that isocitric dehydrogenase activity decreased with germination, whereas succinic dehydrogenase increased in activity (Figure 9). The total activities of isocitric and succinic dehydrogenase on "particles" from dormant tubers were approximately the same as the activities on "particles" from germinated tubers. It is
Figure 8. A-Oxygen uptake and carbon dioxide evolution from tissue slices at 2-3 mm thick. B-The R. Q.'s from the data in A. C-Anaerobic carbon dioxide measured from whole dormant tubers.
Figure 9. Isocitric and succinic dehydrogenase activities on "particles" from dormant and germinated tubers.
concluded from this information that the predominance of isocitric dehydrogenase in dormant tubers is perhaps responsible for the organic acid metabolism as indicated by the R. Q. s and organic acid analyses. The carbon dioxide evolved is probably a result of the decarboxylation of the oxalosuccinate produced by the active isocitric dehydrogenase. Furthermore, the a-ketoglutarate produced by the previous reaction is probably oxidatively decarboxylated. Since the "particles" demonstrated an active succinic dehydrogenase the Krebs cycle was probably in operation.

It is interesting that an R. Q. of unity was obtained in the germinated tubers at 6 days and that isocitric dehydrogenase and succinic dehydrogenase were about equally active (Figure 9). Thus, there appears to be a correlation between the shift to a carbohydrate metabolism and the shift to approximately equal activities of isocitric dehydrogenase and succinic dehydrogenase.

To test this system in more detail succinic, isocitric, and a-ketoglutaric oxidase activities were measured with "particles" from dormant tubers.

All of the intermediates used were oxidized by "particles" from dormant tubers (Table 3). The intermediates were oxidized in the presence of the various co-factors of the Krebs cycle. Succinate was oxidized two fold greater than isocitrate, and a-ketoglutarate was oxidized slightly less than succinate. Malonate inhibited the oxidation of a-ketoglutarate by 62 per cent (Table 3). It is concluded that the oxidation of isocitrate and a-ketoglutarate proceeded through the common intermediate succinate because malonate is an effective inhibitor of succinate. This demonstrates that this portion of the
Krebs cycle was in operation and probably is in some way responsible for the organic acid and carbohydrate metabolism of the nutgrass tuber during germination.

Table 3. The oxidation of Krebs cycle intermediates by "particles" isolated from dormant tubers.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Final concentration (M)</th>
<th>Microliters of $O_2$ uptake/ mg N/50 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isocitrate</td>
<td>0.01</td>
<td>154</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.01</td>
<td>338</td>
</tr>
<tr>
<td>$a$-Ketoglutarate</td>
<td>0.02</td>
<td>292</td>
</tr>
<tr>
<td>$a$-Ketoglutarate</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Plus malonate</td>
<td>0.01</td>
<td>112</td>
</tr>
</tbody>
</table>

B. The ascorbic acid inhibition of catecholase and dopa oxidase during germination.

On the basis of the response of single tubers and tubers in a chain to high oxygen concentrations, and the change in the R. Q.'s during germination, it was considered worthwhile to study some terminal oxidases. The homogenates were prepared from the dormant tuber and germinating tubers. This was done to determine if the change in R. Q.'s and dehydrogenase activities during germination could be coupled with a possible change in the terminal enzyme.

When the oxidation of catechol and ascorbate were examined in the dormant tuber, catechol oxidation was 6 times higher than ascorbate oxidation (Figure 10). As germination proceeded ascorbate oxidation increased with time, and at about five and one-half days the oxidation
Figure 10. The oxidation of catechol and ascorbate in homogenates prepared from tubers during germination in water.
of catechol and ascorbate oxidation were equal. The sum for the oxidation of the two substrates was approximately the same over the experimental period until five and one-half days (Figure 10). On the 8th day, ascorbate oxidation was 3.9 times greater than catechol oxidation.

Because catechol oxidation decreased during germination and ascorbate oxidation increased, it was thought that these changes in oxidation were due to the utilization of some reducing substance in the tissue, perhaps reduced ascorbate.

Ascorbate analyses were conducted on tubers during germination. Ascorbate was found at the highest level in the dormant tuber, whereas dehydroascorbate was low (Figure 11A). The ratio was 3.5 for ascorbate to dehydroascorbate in the dormant tuber (Figure 11B). As germination proceeded ascorbate decreased and dehydroascorbate increased, and on the 8th day of germination the ratio of ascorbate to dehydroascorbate was 1.5 (Figure 11A, B).

It was believed that the o-quinone formed from the oxidation of catechol was reduced in the presence of ascorbate; consequently, catecholase would absorb oxygen in this system and ascorbate would be oxidized. The quantity of oxygen uptake would be higher in a homogenate from the dormant tuber containing a high amount of ascorbate. This was confirmed by determining the rate of oxygen uptake in a homogenate from a dormant tuber. Catechol was used at a constant rate and ascorbate was used at variable rates. Typical results are shown in Figure 12. Oxygen uptake was roughly proportional to the ascorbate level. It was noticed that no color developed as a result of catechol oxidation as long as there was ascorbic acid in the flask. Since catechol is not
Figure 11. A-The quantity of ascorbate and dehydroascorbate determined from tubers during germination in water. B-The ratio of ascorbate to dehydroascorbate during germination.
Figure 12. The effect of adding different levels of ascorbate in the presence of catechol upon oxygen uptake in homogenates from dormant tubers.
likely to be the natural substrate for polyphenoloxidase in the tuber, dopa was used in the same manner as the study just described. It is thus evident that the higher the ascorbic acid concentration, the less was the oxidation due to dopa oxidase (Figure 13B). At the same time, however, the uptake of oxygen took place, and the quantity of oxygen absorbed is directly proportional to the quantity of ascorbic acid added, a remarkable fact, which might show the probable reduction of dopa quinone which is formed by the oxidation of dopa (Figure 13A). This oxidation and reduction might continue as long as ascorbic acid is available; hence the more ascorbic acid is added, the greater is the oxygen uptake and the less is the color strength of hallochrome (Figure 13B). The homogenate containing no ascorbic acid had a specific activity of 60, whereas ascorbate at the lowest concentration inhibited hallochrome formation by 33 per cent. The two higher levels of ascorbate inhibited hallochrome formation by 100 per cent (Figure 13B). An explanation of this is that dopa was dehydrogenated at its initial stage of oxidation by its oxidase giving rise to an ortho-quinone, and this in turn was reduced by the reduced form of ascorbic acid which inhibited hallochrome formation. It appears that the quantity of the reduced form of ascorbate is the determining factor for the oxidation of dopa in the tubers of nut grass. It is concluded from these results that the presence of ascorbate probably prevents the formation of melanin in these tubers; however, these tubers have a characteristic brown pigment in the epidermis. Perhaps catechol oxidation decreased during germination as a result of the reduction of ascorbic acid by other ascorbic acid oxidases. This is possible because dehydroascorbate level increased with an increase in ascorbate oxidation.
Figure 13. A-The influence of adding different levels of ascorbate in the presence of dopa upon oxygen uptake. B-The influence of ascorbate upon dopa oxidase activity in homogenates from dormant tubers.
IV. Polyphenoloxidase in the Dormant Tubers.

A. The oxidation of L-tyrosine by washed slices.

The results obtained thus far have shown that dormant tubers contain an active catecholase and dopa oxidase. It was the problem to determine more fundamental aspects of polyphenoloxidase in dormant tubers.

To examine the possibility that the amino acid, L-tyrosine, would be oxidized by washed tissue slices at 30 microns, L-tyrosine was tipped into flasks containing these slices. As may be seen in Figure 14, the $QO_2(M)$ was 3.2 times greater for the oxidation of L-tyrosine than the control. This indicates that tyrosinase was present in the tissue slices, and that perhaps a large portion of the soluble amino acid was washed from the slices. This could account for the marked oxygen consumption by the slices to added L-tyrosine.

B. The effect of pH on catechol oxidation.

Catechol oxidation at pH 2.5 was practically nil, but at pH 4 the $QO_2(N)$ was 20 (Figure 15). At the pH of 6.5 the $QO_2(N)$ was 2.6 times higher than at pH 6.0, and at pH 7.0 the activity was about 20 $QO_2(N)$, and no exogenous oxidation occurred at pH 8.0. These data point out that maximum oxidation of catechol in homogenates from dormant tubers took place at pH 6.5. Lerner and Fitzpatrick (1950) and Dawson and Tarpley (1951) reported that maximum activity of polyphenoloxidase from plants was at pH 6.5-6.7. Most of the studies with this enzyme from nut grass were conducted at pH 6.0 in order to eliminate endogenous oxidation and autocloration.
Figure 14. The oxidation of L-tyrosine by washed tissue slices at 30 microns from dormant tubers.
Figure 15. The effect of pH upon the oxidation of 0.024 M catechol in homogenates from dormant tubers.
C. Catecholase activity under oxygen gradient, and tyrosinase, p-cresolase, and dopa oxidase activities.

Dawson and Tarpley (1951) conveniently classify phenol oxidases from mushroom into monophenolase (cresolase), and o-dihydric phenolase (catecholase) and used p-cresol and pyrocatechol, respectively, to determine these activities. Their classification has been employed during these studies. Polyphenoloxidase is a general term applied to the mono- and o-dihydric phenolases.

The most active substrate for the enzyme from nut grass was catechol. Oxidation of catechol by tissue slices at 30 microns was markedly influenced by oxygen gradient (Figure 16A). Catecholase was most active under 100 per cent oxygen.

It is believed that because germination was more rapid under 100 per cent oxygen and that catechol oxidation was greater under 100 per cent oxygen tyrosinase in the tuber was the enzyme responding to high oxygen concentration. Furthermore, the fact that ascorbate was oxidized as the result of dopa oxidase activity this system might have acted as the terminal enzyme in the dormant tuber. Probably the isocitric dehydrogenase is coupled with tyrosinase which would make the aerobic system complete.

The monohydric phenol, p-cresol, and the phenolic amines, DL-tyrosine and DL-dopa, were less readily oxidized than the o-dihydric phenol, catechol (Figure 16B). DL-tyrosine was oxidized slightly more than DL-dopa, or p-cresol, but DL-tyrosine was used at pH 6.5, whereas the dopa and p-cresol were used at pH 6.0.

Bonner (1950), Baldwin (1957), Dawson and Tarpley (1951), and Lerner and Fitzpatrick (1950) agree that catechol is oxidized faster
Figure 16. A-The oxidation of catechol by tissue slices at 30 microns under different oxygen concentration. B-The oxidation of DL-Dopa, DL-tyrosine and p-cresol by homogenates from dormant tubers.
by the polyphenoloxidase system than the phenolic amines and monohydric phenols.

D. Inhibition of catecholase.

Most inhibition studies carried out with phenol-oxidizing enzymes have involved substances which form weakly dissociable complexes with copper. These include sodium cyanide, hydrogen sulfide, carbon monoxide, diethyldithiocarbomate, sodium azide, p-aminobenzoic acid, and various sulfhydryl compounds. Other types of inhibitors are (a) competitive inhibitors, including N-acetyl tyrosine and other substituted tyrosine derivatives, and (b) reducing substances such as ascorbic acid and sulfites (Lerner and Fitzpatrick, 1950). The per cent inhibition is expressed in the interpretation of the results on nut grass as the inhibition obtained at the end of the measurements.

Carboxy acids were shown by Krueger (1955) to inhibit phenolase activity at low pH values. He attributed this to complexation of some active center of the enzyme by the carboxylic acid anion. Oxalic acid at 0.02 M inhibited catecholase from nut grass tubers by 72 per cent at pH 4, and by 33 per cent at pH 6.0 (Figure 17A). For the same period, benzoic acid at 0.02 M and pH 6.5 gave only 19 per cent inhibition of catecholase (Figure 17C).

Bonner (1950) attributed the inhibition of polyphenoloxidase by p-nitrocatechol and p-nitrophenol to their resemblance to the natural substrates. At the end of one hour, an inhibition of 47 per cent was obtained when p-nitrophenol at 0.01 M was used to inhibit catecholase in nut grass, whereas thiourea at 0.02 M inhibited catecholase by 41 per cent (Figure 17C). It appears that p-nitrophenol was the most
Figure 17. Effect of various inhibitors upon the oxidation of catechol by homogenates from dormant tubers.
effective inhibitor used at pH 6.5. Greater inhibition was obtained with p-nitrophenol, and the concentration of this compound was one-half that of thiourea.

Dieca at 0.001 M gave 100 per cent inhibition of catecholase (Figure 17B). James (1953b) reported that dieca appeared to be the most effective chelating agent for distinguishing copper enzymes from cytochrome oxidase. He reported that dieca at 0.2mM had practically no effect on cytochrome oxidase. James and Garton (1952) obtained 100 per cent inhibition of ascorbic acid oxidase and polyphenoloxidase and 43 per cent inhibition of cytochrome oxidase with 0.001 M dieca at pH 7.0 and 20° C.

V. The Response of Nut Grass to Amitrol.

A. Root and shoot growth.

Hollingsworth and Ennis (1956) reported that when amitrol was applied to the shoots of nut grass this compound translocated to a majority of the dormant tubers in the system. Six months after application these tubers appeared to have healthy tissue, but they failed to produce shoots. From their information, amitrol appears to have inhibited germination of the tubers.

It was desired to examine the activity of some enzymes extracted from tubers which had received amitrol treatment in sufficient concentration to inhibit germination. To establish the rate of amitrol for the purpose in mind, this compound was applied at logarithmic rates as shown in Figure 18. It was found that amitrol at 10^4 micrograms inhibited both shoot and root growth. Consequently, for future studies this compound was used at 8 and 16 mg per 5 g of tissue.
Figure 18. The effect of amitrol concentrations upon the growth of the shoots and roots of nut grass tubers.
B. Enzyme activity determinations.

The activity used in the discussion is the total activity at the end of the measurements.

1. Ascorbic acid oxidase.

The activity level of ascorbic acid oxidase in treated tubers was roughly proportional to the amitrol concentration (Figure 19A).

Homogenates from the germinated control oxidized ascorbate 1.95 and 4.4 times greater, respectively, than homogenates obtained from tubers exposed to the low and high rates of amitrol.

2. Catecholase.

A point of interest is that catecholase was not predominant in the treated tubers, even though these tubers were not germinated (Figure 19B). Ascorbic acid oxidase was predominant (Figure 19A), and this would mean that similar changes occurred in the treated tubers as in a germinating control. In earlier findings dormant untreated tubers showed a higher catecholase level than ascorbic acid oxidase, and as germination proceeded ascorbate oxidation was greater than catecholase on the 6th day of germination (Figure 10).

It may be seen that the ratio of ascorbic acid oxidase to catecholase was 3.9 in the germinated tuber cultured in water during 8 days of germination (Figure 10), and 3.8 in the germinated tuber cultured in Hoagland's nutrient solution at pH 5.7 for the same period (Figure 19A, B). However, ascorbic acid oxidase was 6.9 times higher in tubers cultured in nutrient solution (Figure 19A), as compared to the oxidase from germinated tubers cultured in water (Figure 10). The oxidation of catechol was of the same magnitude in tubers cultured under the same conditions (Figures 19B and 10). It is interesting that the comparisons
Figure 19. A-The oxidation of ascorbate, and B-Catechol, by homogenates prepared from tubers which were exposed to amitrol treatments for 8 days.
were quantitatively the same. The reason that activities of these enzymes were higher in tubers cultured in nutrient solution probably was due to light intensity and quality, temperature, nutrition and the rate of growth, higher oxygen gradient or stage of growth at the end of the experimental period. These tubers were germinated in the presence of 95 to 100 per cent oxygen. The temperature was \(24 \pm 2°\) C and the light was 310 foot candles of constant incandescent for the tubers from which the data in Figure 19A, B were obtained, but 53 foot candles of constant fluorescent was the light for tubers from which the data of Figure 10 were obtained. The temperature was variable under laboratory conditions. Rate of growth was probably the most contributing factor, though no data are available on dry weights for comparison on tubers grown under these two conditions. Figure 10 and 6 support this concept because as germination proceeded and total fresh weight increased, ascorbic acid oxidase activity increased. In initial experiments, tubers which had germinated for 7 days in water showed an ascorbic acid oxidase activity of 248 microliters of oxygen per hour per gram fresh weight, whereas tubers which had germinated for 17 days showed 2200 microliters of oxygen on the same basis.

Catechol added to homogenates from control tissue was oxidized 1.4 and 1.9 times greater than the oxidation by homogenates from tubers treated with the low and high rates of amitrol, respectively (Figure 19B).

3. Dopa oxidase.

Dopa oxidase activity was 1.5 times greater in the dormant tuber than in the germinated tuber (Figure 20). This is consistent with the results obtained in previous studies. The level of activity in the
Figure 20. Dopa oxidase activity in homogenates from a dormant control, a germinated control, and in homogenates from tubers which were exposed to amitrol treatments for 8 days.
dormant tuber was 1.8 and 3.8 times higher than the activity for tubers treated with the low and high rates, respectively. On the basis of earlier results, it appears that amitrol has probably stimulated the oxidation of a reducing substrate or inactivated the enzyme responsible for dopa oxidation.

4. Peroxidase.

Peroxidase level was 2.5 times higher in the germinated tuber than in the dormant tuber (Figure 21). It is apparent from this data that the highest rate of amitrol controlled the activity of peroxidase at the same level of activity demonstrated by the dormant tuber, but the lowest rate of amitrol was slightly less effective. Consequently, by controlling germination, amitrol treatment controlled peroxidase activity.

5. Catalase.

The germinated tubers showed catalase activity to be 18.5 times higher than tubers treated with 8 mg of amitrol per 5 g of tubers (Figure 22). Without further detailed studies, conclusions would be inadequately drawn now from the effects of amitrol upon the activities of the various enzymes. Perhaps the present information will serve as a guide for further studies relating to the effects of amitrol upon the nut grass tuber.
Figure 21. Peroxidase activity in homogenates prepared from a dormant control, a germinated control and in homogenates prepared from tubers exposed to amitrol treatments for 8 days.
Figure 22. Catalase activity in homogenates prepared from germinated tubers, and prepared from tubers exposed to amitrol for 8 days.
SUMMARY

Attempts have been made to determine certain phases of the metabolism of nut grass. Metabolic processes have been investigated as influenced by germination of the tubers, oxygen gradients and amitrol treatment.

Germination of single tubers was markedly influenced by oxygen concentration. After 17 days of exposure the per cent germination was 34, 70 and 77, respectively, for 1, 10 and 20 per cent oxygen in mixture with 1 per cent carbon dioxide; however, as the carbon dioxide concentration increased, germination percentage decreased under 1 and 10 per cent oxygen and increased under 20 per cent oxygen. At the 15 per cent carbon dioxide level in mixture with 1, 10, and 20 per cent oxygen, the per cent germination was 17, 61, and 83, respectively.

When three and five tubers per chain were exposed to the same gas mixtures as the single tubers the total number of tubers which germinated in a chain was more under the higher oxygen levels. In many cases, 100 per cent of the tubers germinated, but at the low oxygen levels a greater number of the tubers remained dormant in the chain. Usually one or two tubers germinated at the low oxygen concentration. When the dormant tubers were separated from a chain which contained germinated ones, the dormant tuber germinated. This demonstrates that high oxygen concentrations broke dominance in the chain.

When a tuber germinated, growth of the terminal bud inhibited growth of the other buds on the tuber regardless of oxygen concentration. This was the case whether the germinated tuber was separate or in a chain. This showed that a different type of dominance exists in the
chain than in a single tuber, because if the single tuber responded similarly to those in a chain, oxygen concentration should have induced more buds to germinate in the single tuber.

Respiration of dormant tubers increased as oxygen gradient increased from 1 to 100 per cent. The same was true for the germinated tuber; however, total gas exchange was greater from the germinated tuber. The R. Q. s for the dormant tuber were 2.9 and 1.5, respectively, at the 20 and 100 per cent oxygen levels, but they were unity for the germinated tuber at these oxygen levels. If the dormant tuber was sliced, the R. Q. for these slices was 1.5 under air. Dormant tubers showed considerable anaerobic respiration.

The metabolism of nut grass during germination showed the following:

1. Dormant tubers showed an R. Q. of 2.11; however, oxygen consumption increased during germination and an R. Q. of unity was obtained in five and one-half days. Total gas exchange increased as germination proceeded.

2. Total organic acids, fats and total nitrogen decreased in quantity from the time the tubers were dormant until about four days after germination. These materials increased in quantity from the 4th day of germination to the 8th day.

3. Citric, malic, and oxalic acid quantities remained unchanged during germination, but the quantity of citric was higher than malic and malic was higher than oxalic.

4. Soluble carbohydrates increased from dormancy to the 2nd day and then decreased until the 8th day of germination.
5. Isocitric dehydrogenase associated with "particles" was more active in dormant tubers than in germinated tubers.

6. Succinic dehydrogenase associated with "particles" was more active in germinated tubers than in dormant tubers.

7. "Particles" from dormant tubers oxidized isocitrate, a-ketoglutarate and succinate. Malonate inhibited the oxidation of a-ketoglutarate by 62 per cent.

8. The relationship between catecholase and ascorbic acid oxidase showed that catechol oxidation was 6 times higher than ascorbate oxidation by the dormant tuber. As germination proceeded, ascorbate oxidation increased with time, and at about five and one-half days the oxidation of catechol and ascorbate was equal. On the 8th day, ascorbate oxidation was 3.9 times greater than catechol oxidation.

9. The ratio of ascorbate was 3.5 to dehydroascorbate in the dormant tuber. As germination proceeded, ascorbate decreased and dehydroascorbate increased, and on the 8th day the ratio of ascorbate to dehydroascorbate was 1.5.

It was of interest that the change in R. Q., organic acids, fats, nitrogen, catecholase, and ascorbic acid oxidase occurred at practically the same period during germination. Soluble carbohydrates, total organic acids, and ascorbic acid were utilized during germination.

These changes showed that dormant tubers metabolized predominantly organic acids. The catecholase, isocitric dehydrogenase, and Krebs cycle oxidase activities were coupled with organic acid metabolism. Ascorbic acid oxidase, succinic and isocitric dehydrogenase activities were coupled with carbohydrate metabolism.
Studies in establishing a polyphenoloxidase in dormant tubers showed the following:

1. Catechol was very actively oxidized by homogenates from dormant tubers. Catecholase activity on tissue slices was regulated by oxygen gradient.

2. L-tyrosine was actively oxidized by washed tissue slices.

3. Ascorbic acid inhibited the oxidation of catechol and dopa.

4. The maximum oxidation of catechol was at pH 6.5.

5. DL-tyrosine, DL-dopa and p-cresol were oxidized by homogenates from dormant tubers.

6. Dieca, thiourca, benzoic acid, p-nitrophenol, and oxalic acid inhibited catechol oxidation.

Preliminary studies concerning the mechanism of amitrol upon bud inhibition of nut grass revealed the following:

1. Ascorbic acid oxidase and catecholase were markedly depressed by amitrol treatment. The depression in activity was roughly proportional to amitrol concentration based on a germinated control and a treated tuber. Catecholase was greater than ascorbic acid oxidase in the dormant untreated tuber, but ascorbic acid oxidase was higher than catecholase in a treated ungerminated tuber or a germinated control. Thus, similar changes of these enzymes occurred in the treated tuber and in the germinated untreated tuber, although treated tubers did not germinate.

2. Amitrol treatment markedly depressed the activity of dopa oxidase. The activity of this enzyme was high in the dormant tuber, and decreased during germination, but amitrol
treatment depressed the activity even greater than germination. The treated tubers were not germinated but did not show as active dopa oxidase as the dormant untreated tuber.

3. Peroxidase activity was approximately the same for the dormant tubers and tubers exposed to the highest rate of amitrol. The activity of this enzyme was slightly higher from tubers exposed to the low rate of amitrol.

4. The germinated tuber exhibited a catalase activity of 18.5 times higher than tubers which were treated with 8 mg of amitrol.
BIBLIOGRAPHY


Roe, J. H., Mills, M. B., Olsterling, M. J. and Dawson, C. H. The determination of 2,3-diketo-1-gulonic acid, dehydroascorbic acid and ascorbic acid in the same tissue extract by the 2,4-dinitrophenylhydrazine method. J. Biol. Chem. 174:201-208. 1948.


Rogers, B. J. ATA—the action of 3-amino-1,2,4-triazole in plants. The Hormology, American Chemical Paint Company, ed. Pp. 10-12. 1957b.


BIOGRAPHY

Rupert Dewitt Palmer was born on January 28, 1929, in Winston County, Mississippi. He attended Calhoun High School, Winston County, Mississippi, and graduated in May, 1948.

He entered East Central Junior College, Decatur, Mississippi, in June, 1948, and graduated from that institution in January, 1950. He entered Mississippi State College, State College, Mississippi, in February, 1950, and received the Bachelor of Science degree from that institution in January, 1952. He continued his training at Mississippi State College and received the Master of Science degree in January, 1954.

He was employed by the Department of Plant Pathology and Physiology, Mississippi Agricultural Experiment Station at State College, Mississippi, in February, 1954, and remained in this employment until November, 1954.

He was married to Miss Reida White from Ethel, Mississippi, on August 22, 1954. He has one son, Robert Thomas Palmer, who was born on May 18, 1956.

He served in the U. S. Army, Medical Corps, from November, 1954, to September, 1956. He entered Louisiana State University in September, 1956, as a Graduate Research Assistant in the Department of Botany, Bacteriology and Plant Pathology. He is a candidate for the degree of Doctor of Philosophy in January, 1959.
EXAMINATION AND THESIS REPORT

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Major Field: Botany

Title of Thesis: The metabolism of tubers of Cyperus rotundus L. as influenced by oxygen levels and 3-amino 1, 2, 4-triazole.

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