PARAQUAT INDUCED CHANGES IN RESERVE CARBOHYDRATES, FATTY ACIDS AND OLEORESIN CONTENT OF YOUNG SLASH PINES

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Abstract. --Paraquat was fed into the terminal leaders of five-year-old slash pine trees and collected at weekly intervals for 4 weeks. Cytological observationsshowed a decrease in starch levels and a corresponding increase in content of oleoresin. Quantitative analysis indicated a decrease in starch accompanying increases in fatty acids, monoterpenes, and resin acids.

Additional keywords: Resin soaking, terpene biosynthesis, starch content, monoterpenes, resin acids.

INTRODUCTION

The induction of a 15 to 20 fold increase in oleoresin content of resin soaked xylem in paraquat treated pine stems raises many intriguing questions of a physiological nature. Our knowledge of stem anatomy and physiology of oleoresin formation in pines appears to exclude the possibility that resin soaking results from long range transport of oleoresin via vertical resin ducts to resin soaked areas. First, the length of vertical resin ducts is relatively short averaging less than six inches long with occasional ducts extending up to three feet in slash pine (Pinus elliottii Englem.) (unpublished, Brown and Helseth, 1966). Secondly, there appears to be no significant reduction of oleoresin content in xylem well above or below resin soaked areas, although there is a decreasing gradient of oleoresin away from these areas until normal levels are again reached along the stem. In addition, cytological observations on paraquat treated xylem indicates that membrane permeability of the secretory cells lining the resin ducts and associated vertical parenchyma cells is drastically altered. This allows outward leakage of oleoresin from these cells into adjacent tracheids by the path of least resistance, i.e., through the bordered pits. In untreated stems oleoresin secretion by the epithelial cells occurs preferentially into the duct, thereby maintaining pressures as high as 12 to 15 atmospheres within the normal undisturbed duct system. Hence, leaky ducts brought about by the effect of paraquat on membrane integrity and selectivity of parenchyma cells surrounding the ducts are not conducive to long range vertical movement or transport of oleoresin.

Cytological observations in the present study indicate that all living parenchyma cells within the xylem, especially the ray cells, in one-year-old shoots are induced by paraquat (in proper concentrations) to synthesize rather copious amounts of oleoresin. Certainly, under theinfluenceof paraquat normal cellular metabolism is altered resulting in oleoresin biogenesis by individual cells.

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From these general observations it is logical to deduce that the process of resin soaking in the xylem of paraquat treated trees occurs essentially *in situ* from (1) mobilized stored foods (carbohydrates, lipids, and protein) into translocatable and useable forms such as sugars, fatty acids, amino acids, and (2) from transport of current photosynthate (mostly sugars) from the crown by the phloem to local metabolic sinks in the xylem parenchyma via ray cells. The question of how much carbon comes from either source becomes of primary importance in understanding the physiology and biochemistry of paraquat induced oleoresin biogenesis, and seasonal aspects of the entire process will have to be thoroughly researched to provide a clear insight into the overall problem.

The present study had two primary objectives (1) to observe the cytological effects of a known concentration and quantity of paraquat on starch reserves and occurrence of oleoresin in treated stems and (2) to relate these observations to quantitative determinations of free carbohydrates, starch, fatty acids, monoterpenes, and resin acids within the same shoots.

MATERIALS AND METHODS

Twenty-four, five-year-old slash pine trees growing in a plantation at 8' x 10' spacing were selected for treatment during late July. Trees of comparable size and vigor, possessing healthy terminal leaders approximately 24 inches in length subtended by several strong lateral branches were randomly assigned to paraquat and control treatments.

Treated trees were fed 10 ml of 0.02 per cent (200 ppm) paraquat solution through a severed lateral branch at the base of the terminal leader through latex tubing fitted to a graduated 10 ml pipette. The pipette was attached temporarily to the terminal leader vertically above the severed branch to permit gravity flow of solution for uptake into the transpiration stream. The lateral branches were severed early in the morning to allow exudation of oleoresin, and recut at the time of fitting the latex tubes to prevent plugging of the cut xylem surface with exuded resin. After all trees were prepared, the pipettes attached to 12 trees were filled with 10 mls of paraquat, the remaining served as controls. Normally all of the solution would be taken up into the transpiration stream of the leader within 6-8 hrs. Following treatment, the terminal leaders of 3 treated and control trees were harvested at 7, 14, 21, and 28 days for cytological observations and for quantification of extractives.

The terminal leaders collected at weekly intervals were cut into three equal lengths and the upper 2-3 cm portion of each collected for cytological observations. The basal 10–15 cm portion of each shoot was quick frozen, and stored at –20° C. All of the extractions were made at the end of the fourth collection period.

Cytological Procedures

Fresh, free-hand, transverse and median longitudinal sections were made from each collected stem segment. The sections were stained with I_2KI for starch, and with 7% aqueous.cupric acetate for oleoresin. The latter stain is not specific for oleoresin, it also stains various fats and lipids;

however, the copious increase in oleoresin resulting in visible resin soaking of paraquat treated stems made it reasonably' safe to assume that most of the stained material in the lumen and cell walls of tracheids and in the parenchyma cells undergoing senescence was oleoresin and not storage fats or lipids. The latter were most frequently observed in fresh sections from control trees as small globules in the cytoplasm of parenchyma cells stained with cupric acetate. The thin sections (ca. 25-30 microns) were stained in small vials with I_2KI for 3-5 minutes, and with cupric acetate for 30 minutes, mounted in glycerol, and examined microscopically.

All sections were examined under the light microscope and visibly scored for content of starch and oleoresin in various tissues, namely; cortical parenchyma, vertical xylem and phloem parenchyma, xylem and phloem rays, and pith. Xylem tracheids were also scored for oleoresin content within the lumen of cells viewed in median longitudinal sections.

Extraction and Analysis of Resin & Fatty Acids:

A frozen five centimeter section of stem was defrosted, debarked, the wood reduced to mm square cubes and continuously extracted with anhydrous ether in a soxhlet extractor for eight hours. The extract was reduced, eluted, dried and methylated with .5N methanolic HCl. The methylated solution was exhaustively partitioned against n-hexane and the resulting hexane residue quantitatively eluted with benzene. After separating the benzene solution into equal parts and drying them, one part, which was further methylated with "Methyl 8" (Pierce Chemical Co.), was used for resin acid analysis and the other for fatty acid analysis.

Quantitative analysis for both fatty acids and resin acids was accomplished by gas chromatographic techniques. Fatty acids were analyzed using a SP-222-PS column (Supelco, Inc.) with methyl heptadecanoic as the internal standard. Resin acids were analyzed by ASTM method D 3008-72 using methyl benhenic acid as the internal standard. Results were expressed as mg/gm oven-dry weight.

Extraction and Analysis of Free Carbohydrate and Starch:

After weighing, the ether extracted wood was ground to pass through a 40 mesh screen, redried and 100 mg sample removed. Four 5 ml volumes of hot 70% ethanol were used to extract the free carbohydrates. Subsequently, the starch was extracted with 50% perchloric acid using techniques described by Hassid and Neufeld (1965). The starch precipitates were hydrolyzed (Pucher, Leavenworth and Vickery, 1948) and brought to a known volume.

The amount of free carbohydrate and starch present was determined by measuring absorbance at 620 mm following an anthrone reaction. The results were expressed as mg/gm oven-dry weight.

Extraction and Analysis of Monoterpenes:

A frozen 5 cm section of stem was debarked, reduced to mm square cubes and extracted with a 4 ml volume of anhydrous ether with vigorous shaking for 30 minutes in a closed container. This process was repeated anadditional

4 times and all ether volumes combined. Since a 5th repetition failed to remove a significant amount of monoterpene from the wood, 5 repetitions were considered to result in complete extraction. The combined extracts were brought to 25 ml by the addition of ether.

The ether extracts were analyzed by flame ionization on a Perkin-Elmer 3920 gas chromatograph coupled to a Hewlett-Packard 3380A recording integrator. Monoterpenes were separated on a Carbowax 20M column and identified by co-chromatography. Quantification was by external standard for each monoterpene. Results were expressed as mg/gm oven dry, extractive-free weight.

DISCUSSION OF RESULTS

Cytological Observations

In paraquat treated stems there is a distinct vertical gradient in disappearance of starch from the xylem ray cells (and to a slightly lesser degree from the vertical xylem parenchyma) corresponding to the concentration gradient of paraquat along the stem. The concentration of paraquat in the xylem is highest at the basal portion of the stem where it entered the transpiration stream and it diminishes upward because of paraquat's strong ionic affinity for cellulose cell walls (Brown and Nix, 1976). As the concentration of paraquat increases with time up the stem through continued release of bound molecules into the transpiration stream, the quantity of starch continues to decrease acropetally. It appears, therefore, that paraquat molecules entering the xylem ray cells and vertical xylem parenchyma has a rapid, almost immediate effect on the enzymatic conversion of starch into sugars and subsequently into other soluble metabolites for use in increased oleoresin synthesis. Concomitant with the observed decrease in starch there is a visible increase in oleoresin content, first at the base of the stem and then slowly extending upward as the concentration of paraquat increases acropetally (Tables 1 and 2).

Similar patterns of starch disappearance accompanied by increased synthesis of oleoresin were also observed in the parenchyma cells of all living tissues in which paraquat entered in sub-lethal concentrations. For example, in all of the paraquat treated stems the cortical cells, vertical phloem and ray parenchyma, and even pith cells showed similar patterns of starch disappearance accompanied by increased oleoresin formation for short distances above the site of paraquat entry. The increased oleoresin gradient in the phloem did not extend very far upward because the main pathway of paraquat movement is via the transpiration stream. Hence, at the concentration and quantity of paraquat used (0.02% - 10 ml) little of it moved radially across the cambium into the secondary phloem and cortex. This was substantiated in other trials where the concentration of applied paraquat was increased to 0.1, 0.5, and 1.0%. These increased concentrations were sufficient enough to obtain some movement of paraquat radially across the cambium into the adjacent phloem and cortex causing a distinct increased gradient in necrosis of these outer tissues corresponding to increases in paraquat concentration. At the 1.0% concentration (10 ml) all living cells throughout the terminal leader were killed outright within 10-14 days. Even the paraquat concentration used in this study (0.02%) killed all living cells at and immediately adjacent to the site of entry. These cells underwent rapid plasmolysis and death and contained distorted starch grains and other cellular components.

Table 1.--Cytological observations of starch content in different tissues of paraquat treated and untreated terminal leaders of slash pine during August, 1975.[a/]

Treatment	Days	Tissue[b/]					
		Xylem		Phloem		Cortex	Pith
		rays	vertical parenchyma[c/]	rays	parenchyma		
Paraquat	7	1	1	2	3	3	4
Control	7	2	2	2	3	3	4
Paraquat	14	<1	<1	<2	<2	2	<3
Control	14	<3	2	<3	3	3	<5
Paraquat	21	<1	<1	2	2	<2	<2
Control	21	<3	2	<3	<3	<3	<4
Paraquat	28	0	0	<2	2	<2	1
Control	28	3	2	3	3	3	4

[a/] Mean of three terminal leaders sampled at 12-15 cm above site of paraquat entry into shoot.

[b/] Numbers indicate average content of starch stained with I_2KI in individual cells of each tissue as follows: 0-nil; 1-sparce; 2-light; 3-moderate; 4-heavy; 5-excessive.

[c/] Includes parenchyma adjacent to epithelial cells surrounding ducts.

Table 2.--Cytological observations of oleoresin content in different tissues of paraquat treated and untreated terminal leaders of slash pine during August, 1975.[a/]

Treatment	Days	Tissue[b/]				
		Xylem		Phloem	Cortex	Pith
		rays	tracheids	rays		
Paroquat	7	<2	0	1	1	1
Control	7	1	0	1	1	1
Paraquat	14	<3	1	<2	1	1
Control	14	1	0	1	1	1
Paraquat	21	3	<2	2	2	3
Control	21	1	0	1	1	1
Paraquat	28	4	<4	<3	3	4
Control	28	1	0	1	1	1

a/ Mean of three terminal leaders sampled at 12-15 cm above site of paraquat entry into shoot.

b/ Numbers represent average content of oleoresin in tissues stained with cupric acetate; 0-absent; l-frequent small droplets; 2-larger, more diffuse droplets; 3-over half of individual cells filled with oleoresin; 4-cell lumens commonly filled.

From these observations it becomes increasingly clearer that the concentration of paraquat entering individual parénchyma cells, especially those of the xylem ray and vertical parenchyma, has a profound effect on the sequence of events leading to enhanced synthesis of oleoresin within these cells. If the concentration is optimal, stored food reserves appear to be mobilized into soluble forms followed by a gradual lysis of the cytoplasm with its enclosed cellular organelles including the nucleus and other residual Components of the protoplast. The carbon from these various sources ultimately is shunted into the terpenoid pathway before death of the cell (Brown and Clason, 1975). During the process of cellular lysis one can observe increases in oleoresin accumulation in individual ray cells followed by its movement through pits into the adjacent tracheids until ultimately all elements of the xylem in localized treated areas become resin soaked. The biochemical pathway by which these various sources of cellular carbon are converted into oleoresin formatio constitutes a large gap in our present knowledge of paraquat induced resin saaking in the xylem and other tissues.

Quantitative Observations

This preliminary study using three trees at each of the four weekly sampling times resulted in considerable quantitative variation among individual trees as indicated in the data presented in Table 3 and Figures 1, 2, and 3. Likewise, the variation could undoubtedly be substantially reduced by using more judicious sampling procedures prior to extraction of individual xylem samples. For example, in each treated *stem* the paraquat solution supplied through the severed lateral branch moved axially up the xylem for several centimeters before it began to spread out laterally into adjacent xylem cells. Had the xylem-at the basal portion of the stem been separated in half prior to extraction, extracting only the portion on the paraquat treated side, the amount of variation likely could have been reduced. As it was, the entire xylem of the lower portion of the stem was extracted and quantifie for free carbohydrates, starch, fatty acids, monoterpenes and resin acids.

These results, although quite variable, substantiate the cytological observations made on adjacent portions of the stem with respect to decreases in starch and increases in oleoresin content of the xylem (cf Tables 1, 2, and 3 and Figs. 2 and 3).

It is of interest that no significant differences occurred in free carbohydrates between treated and control trees. This could indicate that as rapidly as soluble sugars are released through hydrolysis of starch they are shunted into an alternate pathway in treated stems contributing to increased oleoresin formation.

The rather rapid increase of fatty acids with time (Fig. 1) suggests that storage forms of lipids are hydrolyzed which may also be diverted into the terpenoid pathway, Subsequent observations (unpublished, Clason) indicate that the initial increase in the pool of fatty acids observed in short-*term* experiments rapidly decrease during subsequent formation of oleoresin in paraquat treated stems.

Finally, the rather rapid increase in monoterpenes and resin acids with time (Figs. 3 and 4) although highly variable among individual trees, conform to the pattern of paraquat induced oleoresin formation in pines.

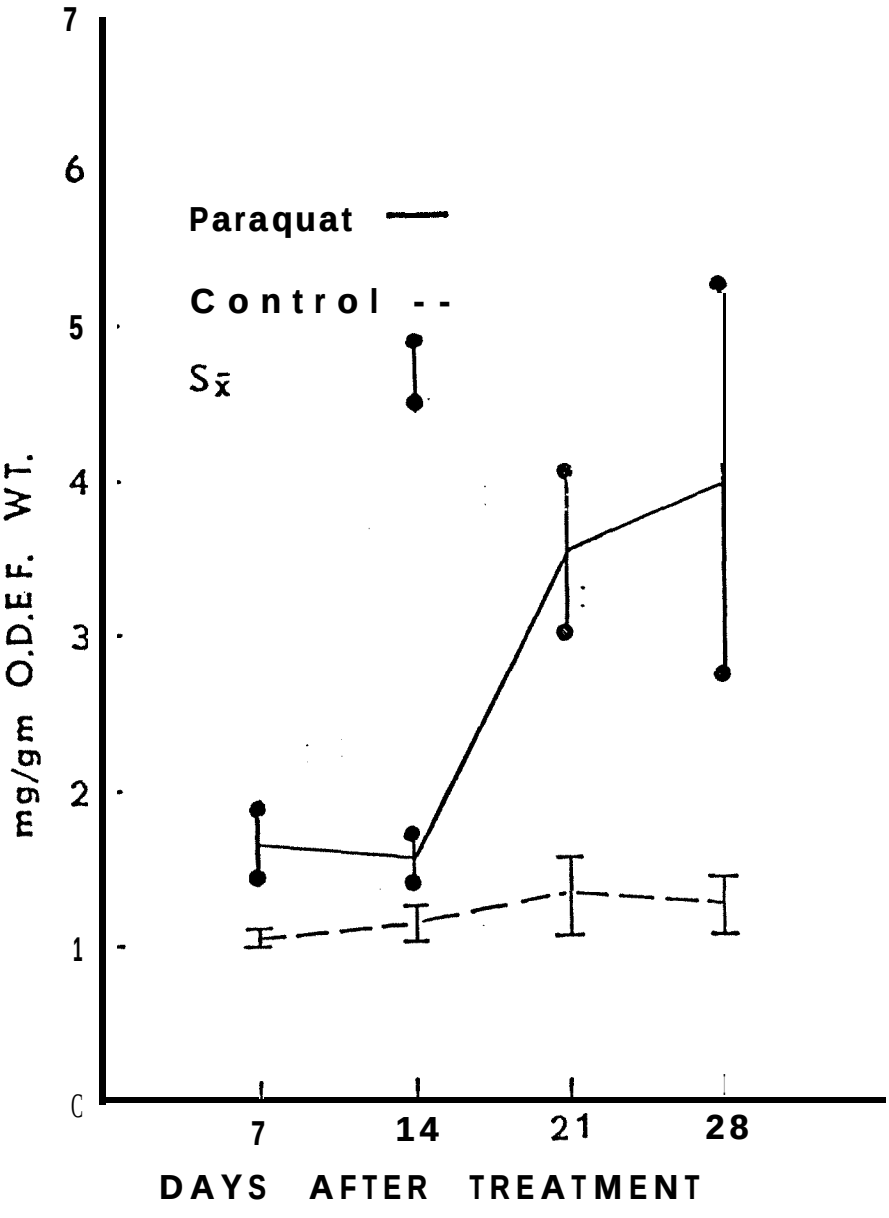

Figure 1. Free fatty acids from xylem of paraquat treated and untreated terminal leaders of slash pine during August, 1975.

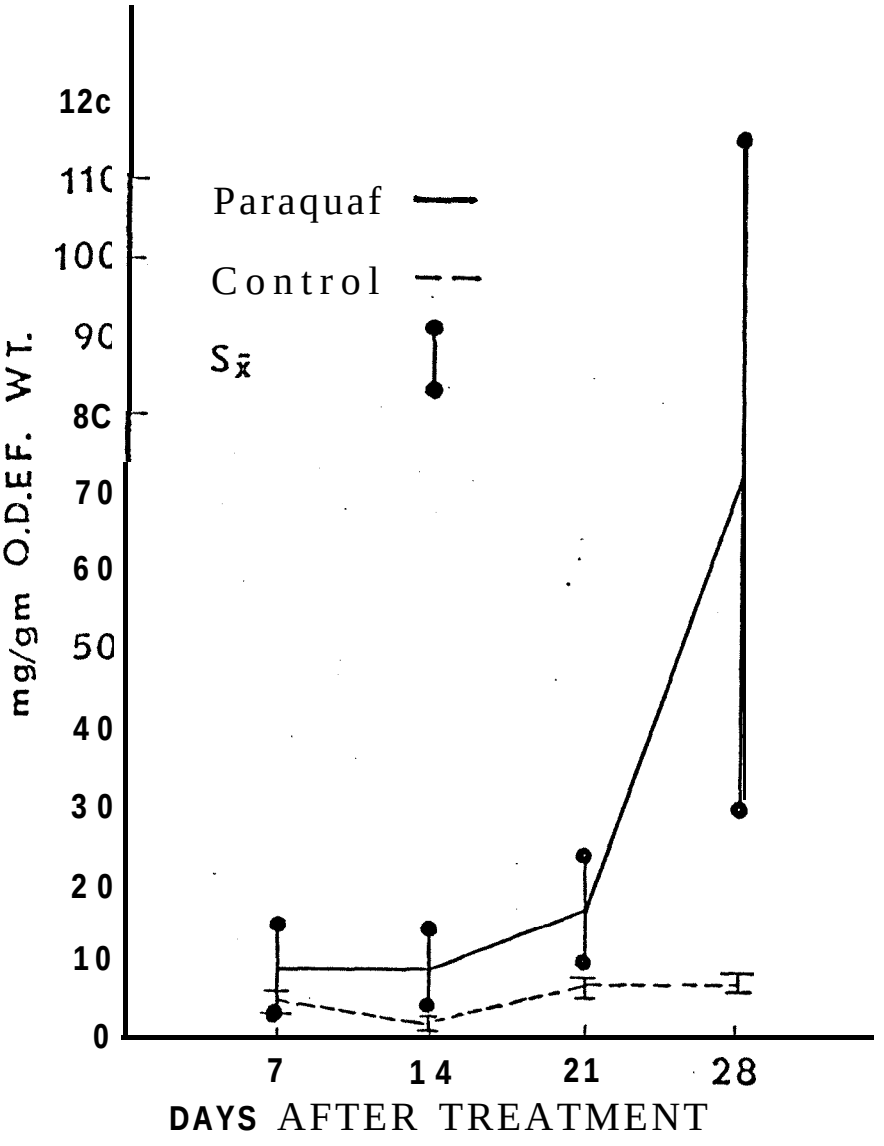

Figure 2. Monoterpenes from xylem of paraquat treated and untreated terminal leaders of slash pine during August, 1975.

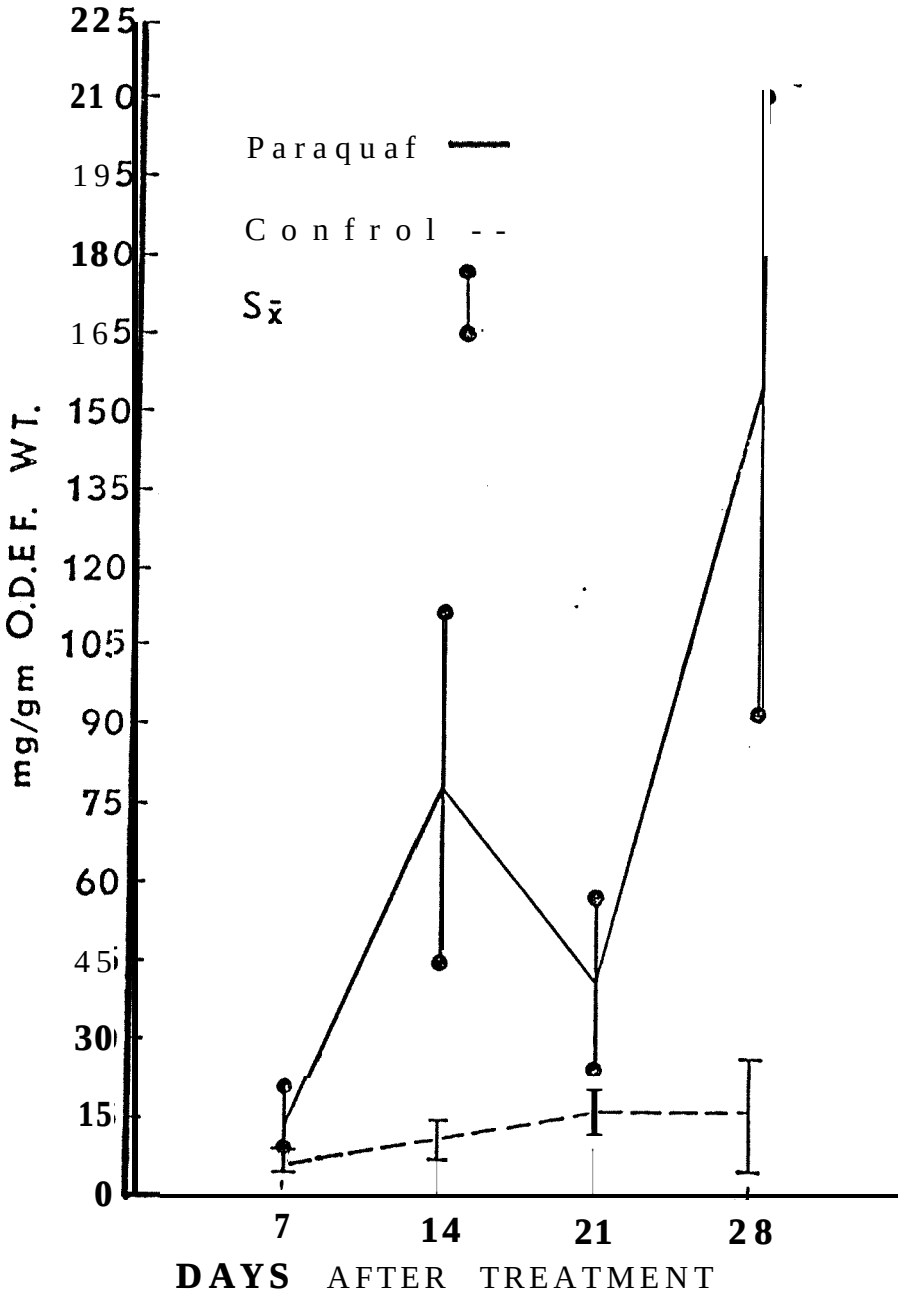

Figure 3. Resin acids from xylem of paraquat treated and untreated terminal leaders of slash pine during August, 1975.

Table 3. --Free carbohydrates and starch from xyiem of terminal shoots of five-year-old slash pine. [a/]

Treatment	Days after Treatment	mg/gm O.D.E.F. Wt. [b/] Carbohydrates	Starch
Paraquat	7	4.09 ± .45	1.56 ± .19
Control	7	3.58 ± .41	3.61 ± .17
Paraquat	14	4.33 ± .31	3.29 ± • 37
Control	14	2.66 ± .31	2.76 ± .78
Paraquat	21	5.36 ± .91	2.50 ± .80
Control	21	5.62 ± .43	4.20 ± .78
Paraquat	28	5.74 ± 1.35	2.40 ± 0
Control	28	5.77 ± .52	3.50 ± .25

[a/] From July-August treatment period

[b/] O.D.E.F. wt. = oven dry extractive free weight. Values given are means for 3 trees.

CONCLUSIONS

Although these observations confirm and partially explain the in situ process of oleoresin soaking in localized portions of paraquat treated slash pine stems, they do not provide quantitative data enabling one to ascertain what portion or ratio of the total carbon comprising paraquat induced oleoresin in the xylem comes from stored food reserves within the stem or what portion is directly supplied by current photosynthate. In attempting to solve this problem one must necessarily be concerned with the effects of paraquat on inducing mobilization of stored foods not only in localized cells directly influenced by the presence of paraquat as in the present study, but also in long-range mobilization of foods above and below the sites of paraquat application in tall, forest-grown trees. Additional studies relating to these aspects are currently in progress.

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