ground fertilizer applications. Therefore, the amount of nitrogen applied in the KNO_3 spray should be considered when preparing the yearly nitrogen budget of the citrus grove. If this is not done, excessive nitrogen may adversely affect the fruit quality of oranges and grapefruit.

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Alachlor Effects on Plant Nitrogen Metabolism and Hill Reaction

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Intact wheat (*Triticum aestivum* L.) plants were utilized to study the effect of alachlor [2-chloro-2′,6′-diethyl-*N*-(methoxymethyl) acetanilide] on nitrate reductase, nitrate, amino acid, and water-soluble protein. Alachlor may stimulate or inhibit both nitrate ion uptake or nitrate reductase activity, depending on the rate used, order of exposure, or the total time of exposure. The nitrate reductase activity was affected more than the nitrate content. Alachlor addition after nitrate caused a greater reduction in nitrate content and nitrate reductase activity than if added before the nitrate was. Nitrate reductase activity was inversely proportional to alachlor exposure time. The amino acid content was decreased with an increase in total exposure time to alachlor. High concentrations of alachlor depressed the amino acid content and protein level, while the lower concentration did not. Alachlor did not inhibit the Hill reaction in isolated wheat chloroplasts.

Since a substituted urea has been shown to inhibit protein synthesis and some other herbicides affect nitrate reductase, the effect of alachlor [2-chloro-2′,6′-diethyl-*N*-(methoxymethyl) acetanilide] on nitrate reductase, the enzyme substrate, nitrate, and two products, the amino acids and water-soluble protein were determined. The action of alachlor on the Hill reaction of isolated wheat chloroplasts was also investigated.

Nitrate is the primary form of nitrogen available to higher plants for protein synthesis and the enzyme nitrate reductase initiates the reduction process. Nitrate reductase is the rate-limiting step in this reduction process (Beevers and Hageman, 1969). Nitrate reductase is substrate inducible and thus its level of activity should be an index of the reduced nitrogen available to the plant for protein synthesis.

α-Chloroacetimide herbicides have been shown to inhibit protein synthesis. Protein synthesis in root tips has been inhibited and growth of cucumber roots reduced by propachlor (Duke, 1967). ATP formation or respiration was not affected. The inhibition of nascent protein formation was believed to result from the failure to transfer the amino-acyl-S-RNA to the polypeptide chain. Alachlor has been shown to stimulate growth and ^{14}C-leucine incorporation into root protein in excised cucumber tissue (Edmondson, 1969). Polyribosome formation was not affected by alachlor, while the amount of RNA and DNA increased in root tissue of treated cucumbers. Alachlor reduced proteinase activity 69% in cucumber cotyledons with very little effect on ribonuclease activity in pregerminated embryos.

Other families of herbicides such as 2,4-D and simazine have been shown to affect the enzyme nitrate reductase. The nitrate reductase level was increased in corn and reduced in cucumber in cell-free extracts from corn and cucumber plants sprayed with varying levels of 2,4-D (Beevers *et al.*, 1963). Nontoxic levels of simazine added to the root zone of corn plants, grown under both suboptimal temperatures and low nitrate levels, increased the nitrogen content and dry weight of plants by 20–25% (Tweedy and Ries, 1967). These increases were associated with increased nitrate reductase and were attributed to increased nitrate absorption, nitrate assimilation, or both.

The Hill reaction is inhibited by some herbicides. In the Hill reaction, when illuminated, chloroplasts perform oxygen evolution using water as an electron donor (Bonner and Varner, 1965). Monuron, a substituted phenylurea herbicide,

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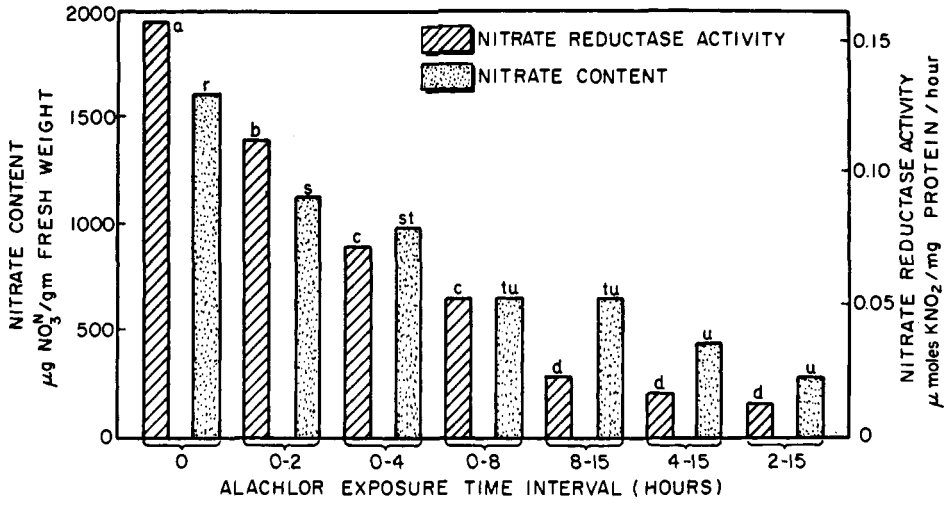

Figure 1. Nitrate content and nitrate reductase activity as affected by exposure time to alachlor (average of 0.05 and 0.0005 m*M* conc) during a 15-hr induction peroid. Alachlor removed after the exposure time

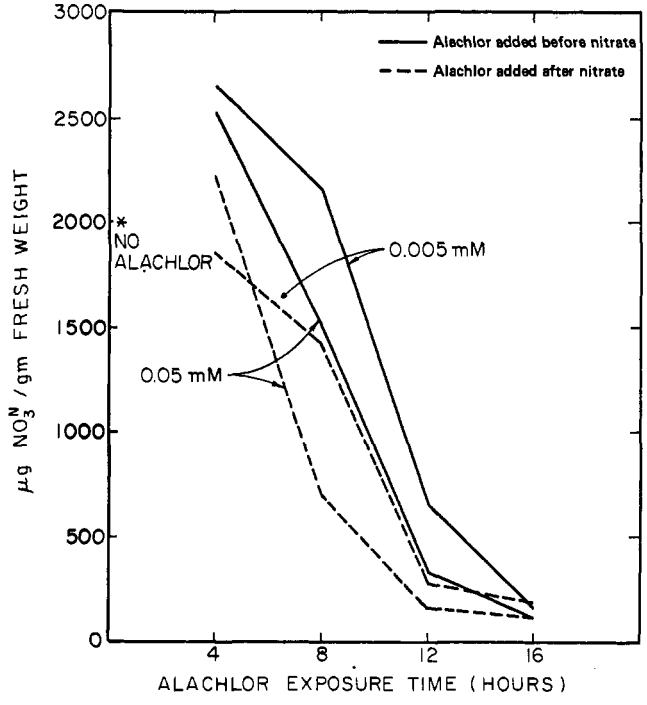

Figure 2. Influence of alachlor on nitrate content with the alachlor added either before or after nitrate, and then removed at the end of the exposure period

has been shown to inhibit the Hill reaction in isolated chloroplasts (Wessels and Van der Veen, 1956). More than 200 acylanilides, thiocylanilides, acylamides, ureas, and thioureas were investigated as potential Hill reaction inhibitors (Good, 1961). The only feature common to all the Hill reaction inhibitors was the presence of an imino hydrogen. The imino hydrogen must not only be present but also accessible if a substance is to have significant inhibitory action. The acylanilides, which have an imino hydrogen, are much stronger inhibitors of the Hill reaction than the phenylcarbamates, but are not quite as inhibitory as the phenylureas (Moreland and Hill, 1961).

Conceivably there could be a relationship between the inhibitory effects of herbicides on the Hill reaction and the supply of nitrogen reductants since light has been shown to be promotive of nitrate reduction. However, it is not clear whether the influence of light on nitrate metabolism results from direct effects on nitrate reductase, the availability of nitrate, the generation of a reductant for nitrate, or some combination of these effects (Beevers and Hageman, 1969).

PROCEDURES

Nitrate Reductase and Associated Constituents. Wheat plants for nitrate reductase and associated determinations were grown in a growth chamber, arranged as a factorial in a randomized block design with four replications. Technical alachlor was used in all of the studies at designated rates. A 30–24°C day–night temperature regime was used. A 14-hr day with a light intensity of 2000 ft-candles was used and the light source was a combination of incandescent and fluorescent lamps.

Nitrogen status of the wheat plants prior to induction and effect of alachlor exposure time intervals were studied. Three-day-old seedlings were pretreated for 7 days either with nitrogen supplied as $(NH_3)_2CO_3$ at 5 m*M* concentration or with no nitrogen in a Hoagland's nutrient solution. The solutions were replaced with a Hoagland's solution containing either alachlor, nitrate [5 m*M* $Ca(NO_3)_2$ and 5 m*M* KNO_3] or both for an induction period of 17 hr.

The influence of various alachlor concentrations (0, 0.005, and 0.05 m*M*) and exposure time intervals applied either before or after nitrate was studied. The object of these studies was to ascertain whether the alachlor might be affecting nitrate uptake or nitrate reductase synthesis *per se*.

Alachlor exposure time was considered, along with order of enzyme induction and alachlor rates (0, 0.005, and 0.05 m*M*). Wheat was germinated in perlite (Zonolite Division, W. R. Grace & Co., Chicago, Ill.) for 4 days and then transferred to jars containing a nitrogen-free Hoagland's solution for 6 days. Then either alachlor, nitrate, or both were introduced for a set period of time during a reaction period of 15 or 16 hr.

A cell-free preparation was obtained and nitrate reductase determinations were made using the method of Hageman and Flesher (1960), as modified (Croy and Hageman, 1970). Nitrate content (Wooley *et al.*, 1960), amino acid as α-amino nitrogen (Yemm and Cocking, 1955), and protein as a 5% TCA precipitation of the water-soluble protein (Lowry *et al.*, 1951) were determined.

Hill Reaction Analysis. Wheat chloroplasts 14-days-old were isolated by grinding 5 g of fresh leaves in 30 ml of a

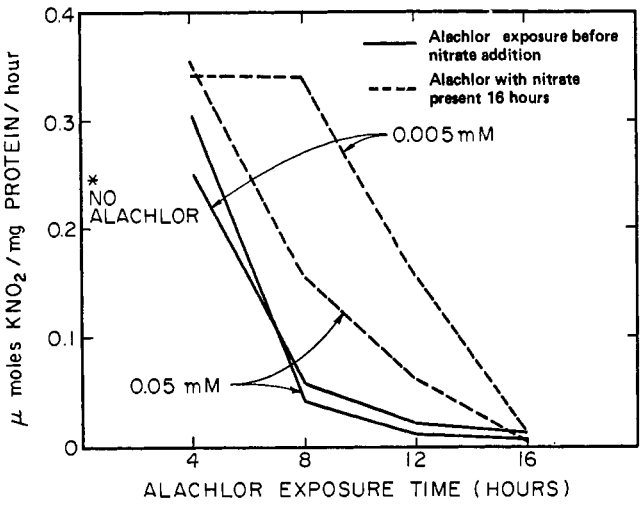

Figure 3. Influence of alachlor on nitrate reductase activity with the alachlor added either before or after nitrate, and then removed at the end of the exposure period

medium containing 10 m*M* of EDTA, 450 m*M* of sucrose, 50 m*M* of boric acid, and 30 m*M* of citric acid adjusted to pH 7.2 with NaOH. The extract was filtered through cheesecloth, centrifuged at 121 × *g* for 5 min, and the precipitate discarded. The chloroplasts were collected by centrifugation at 4000 × *g* for 25 min and resuspended in 10 ml of the original buffer solution. The reaction mixture contained 0.2 ml of the chloroplast suspension, 1.0 ml of 50 m*M* KCl, 0.2 ml of 2,6-dichlorophenolindophenol (145 mg/l.), and 4.6 ml of buffer with alachlor at the desired concentration replacing 1 ml of the buffer. The change in optical density at 590 mμ was measured after illumination for 30-sec intervals for a total of 3 min with a 150 W outdoor type spot lamp at 18 cm from the tube containing the chloroplast. The tube of chloroplast was placed in a filtering flask with tap water running through it to keep the chloroplast at a constant temperature of 24°C.

Table I. Influence of Alachlor Concentration and Exposure Time on Nitrate Content and Nitrate Reductase Activity of Wheat[a]

Alachlor conc, m*M*	Nitrate content[b] No nitrogen[d] pre-exposure	Nitrate content[b] Nitrogen[e] pre-exposure	Nitrate reductase[c] No nitrogen[d] pre-exposure	Nitrate reductase[c] Nitrogen[e] pre-exposure
Alachlor present for entire 17-hr period				
0	142 a[f]	118 ab	0.74 a	0.63 ab
0.005	77 cd	100 bc	0.59 bc	0.45 c
0.5	12 e	40 de	0.25 d	0.24 c
Alachlor present for last 16 hr of exposure period				
0	122 b	90 c	0.82 a	0.41 b
0.005	178 a	142 b	0.81 a	0.46 b
0.5	4 d	74 c	0.31 c	0.22 c
Alachlor present for first hour of exposure period				
0	120 b	143 b	0.74 a	0.51 b
0.005	130 b	200 a	0.72 a	0.48 b
0.5	22 d	73 c	0.31 c	0.26 c

[a] Nitrate was present in the Hoagland media for the complete 16-hr exposure period. [b] μg NO_3N/g fresh weight. [c] μmoles KNO_2/mg protein/hr. [d] No nitrogen present in 7-day preexposure period. [e] 5 m*M* NH_4 as N source in 7-day preexposure period. [f] Means having the same letters are not significantly different according to Duncan's Multiple Range Test at 5% level of probability.

Table II. Average Amino Acid and Water-Soluble Protein Level as Affected by Exposure Time to 0.005 and 0.5 m*M* Alachlor

Time interval of alachlor exposure, hr	Amino acids, μg of amino N/g fresh wt	Water-soluble protein, mg protein/g fresh wt
0	852 ab[a]	51.9 a
0–2	1249 a	52.0 a
0–4	931 ab	52.5 a
0–8	748 b	48.8 b
8–15	685 b	53.5 a
4–15	592 b	51.8 a
2–15	764 b	51.4 a

[a] Means having the same letters are not significantly different at 5% level according to Duncan's Multiple Range test.

RESULTS AND DISCUSSION

Nitrate Reductase and Associated Studies. The effects of alachlor varied depending on the alachlor concentration and treatments (Table I). The presence of alachlor for the entire period (17 hr) in general reduced nitrate content, with the greatest reduction occurring at the highest alachlor concentration (0.5 m*M*). Nitrate content for plants receiving the low alachlor concentration (0.005 m*M*) was higher than the control in two treatments—the nitrogen pretreatment with alachlor present for the first hour of the induction and no nitrogen pretreatment with alachlor present the last 16 hr of the induction. Nitrate reductase activity was not affected by the low concentration of alachlor when present either the first hour or the latter 16 hr of the induction period. The low concentration of alachlor depressed nitrate reductase activity when present for the entire induction period, and the higher concentration reduced nitrate reductase activity in all induction intervals.

In a number of measurements, plants receiving the nitrogen pretreatment had lower nitrate reductase activity and nitrate levels than the no nitrogen pretreatment. The plants also showed slight to moderate stunting. The ammonia ions may inhibit plant responses, particularly nitrate uptake (Weissman, 1951).

The effect of pulses of alachlor (0.005 or 0.5 m*M*) at the initiation and in the latter portion of 15-hr induction periods was measured. Alachlor was removed after the exposure time interval. An increase in total exposure time to alachlor or an increase in concentration caused a reduction in nitrate content and nitrate reductase activity, with the higher concentration being more inhibitory (Figure 1). Alachlor was less inhibitory if added during the first half of the induction period than in the second half. The reduction in nitrate reductase activity may be a reflection of the reduced nitrate content.

The amino acid levels were reduced by the presence of alachlor, although not significant at the 5% level of probability (Table II). The levels were reduced in plants exposed to alachlor for 8 hr or longer. Exposure to only 2 hr of alachlor actually increased the amino acid level. Protein content was lowered only when alachlor was present for the first 8 hr of the experiment.

Plants were exposed to alachlor for varying time periods before or after nitrate was added to the solution (Figure 2). Exposure of plants to alachlor at both concentrations for 4 hr before the plants were given nitrate caused a significant stimulation of nitrate uptake, while additions for longer than 8 hr reduced uptake. Nitrate uptake was greater when alachlor was present before the nitrate than when added after the nitrate.

A stimulation of nitrate reductase activity occurred with 4 hr of alachlor exposure after 12 hr of nitrate. An increased

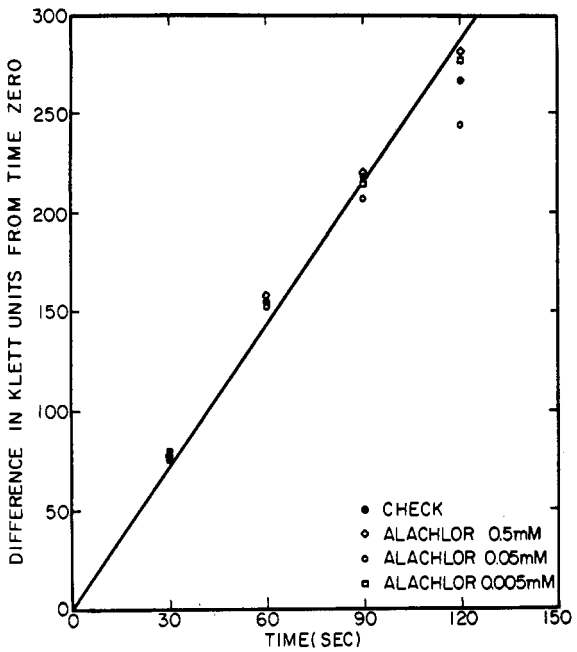

Figure 4. Effect of alachlor on the Hill reaction of isolated wheat chloroplasts

time exposure of alachlor after nitrate caused a significant reduction in nitrate reductase activity. Plants receiving an alachlor treatment before nitrate had much lower levels of nitrate reductase activity than those plants receiving alachlor after nitrate (Figure 3).

The nitrate ion concentration at 8 hr was higher in the 0.005 m*M* alachlor treatment than in no alachlor treatment, and yet nitrate reductase was much lower (Figures 2 and 3). The nitrate concentration was depressed somewhat by alachlor at 0.05 m*M*, while the nitrate reductase was depressed significantly. These data indicate that the induction of nitrate reductase was being inhibited by alachlor.

In a developing seedling the induction of nitrate reductase is set into motion with the absorption of nitrate and light. About the same time the roots begin absorbing nutrients from the soil, the coleoptile is approaching or emerging through the soil surface. The wheat coleoptile tissue is capable of absorbing alachlor; therefore, in the development of a seedling in soil where alachlor has been applied before seedling emergence, the uptake of nitrate and induction of nitrate reductase may occur before, after, or simultaneous to the absorption of alachlor.

The above data indicate that if uptake of nitrate and alachlor at a sufficient concentration occurred simultaneously, the nitrate content and nitrate reductase activity could be substantially reduced. If the uptake of nitrate precedes alachlor absorption, low alachlor concentrations could stimulate nitrate uptake, while higher rates could be inhibitory. The nitrate reductase activity could be reduced if alachlor concentrations were sufficiently high. If alachlor uptake occurred prior to enzyme induction, the nitrate content could be enhanced or reduced, depending on the concentration of alachlor and exposure period before nitrate uptake. The enzyme activity could be reduced drastically if the exposure to alachlor occurred in sufficient time prior to nitrate uptake.

Hill Reaction. Inhibition of the Hill reaction in isolated wheat chloroplasts did not occur with the addition of alachlor at concentrations of 0.5, 0.05, and 0.005 m*M* (Figure 4). The literature indicates that the presence of a free imino hydrogen atom is required for the inhibition of the Hill reaction. Since the imino hydrogen is replaced with a methoxymethyl group in alachlor, the lack of inhibition would be consistent with the hypothesis that an imino hydrogen atom is needed for inhibition.

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