EFFECTS OF ATRAZINE ON THE ASSIMILATION OF INORGANIC NITROGEN COMPOUNDS IN PLANTS AND MICROORGANISMS

by

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PREFACE

Some of the results described in this thesis have been published as follows:-

1. "Effects of atrazine on the assimilation of inorganic nitrogen in cereals"
   S. Mohanadas, W. Wallace and D.J.D. Nicholas
   Phytochemistry 17, 1021-1028 (1978)

2. "Effects of atrazine on the assimilation of inorganic nitrogen compounds in maize"
   S. Mohanadas, W. Wallace and D.J.D. Nicholas
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Finally, I wish to record my appreciation of the patience and support of my wife and two children.

DECLARATION

I hereby declare that the work presented in this thesis has been performed by myself and has not been submitted to any University for the award of any degree.

SUBRAMANIAM MOHANADAS

NOMENCLATURE AND ABBREVIATIONS

The major enzymes mentioned in this thesis are listed below with their numbers and systematic names as recommended by the Enzyme Commission (Florkin & Stortz, 1973).

Trivial Name	*E.C. Name and Number*
Adenosine triphosphatase	ATP phosphohydrolase E.C. 3.6.1.3
α-Amylase	1,4-α-D-Glucan glucanohydrylase E.C. 3.2.1.1
Glutamate synthase	L-Glutamine : 2-oxoglutarate amino-transferase (NADPH-oxidizing) E.C. 2.6.1.53
Glutamic-pyruvic transaminase	L-Alanine : 2-oxoglutarate amino-transferase E.C. 2.6.1.2
Glutamine synthetase	L-Glutamate : ammonia ligase (ADP-forming) E.C. 6.3.1.2
Nitrate reductase (NADH)	NADH : nitrate oxidoreductase E.C. 1.6.6.1
Nitrate reductase (NADPH)	NADPH : nitrate oxidoreductase E.C. 1.6.6.3
Nitrite reductase (NADPH)	NADPH : nitrite oxidoreductase E.C. 1.6.6.4
Peroxidase	Donor : hydrogen-peroxide oxido-reductase E.C. 1.11.1.7
Ribonuclease	Ribonucleate 3'-pyrimidino-oligonucleotidohydrolase E.C. 3.1.4.23
Starch phosphorylase	1,4-α-D-Glucan : orthophosphate α-glucosyltransferase E.C. 2.4.1.1

The plants mentioned in this thesis are listed below with their full binomial Latin names as Instructions to Authors for the Biochemical Journal [Biochem. J. (1973) 131, 1-20].

Trivial Name	*Binomial Latin Name*
apple	*Pyrus malus* L.
asparagus	*Asparagus officinalis* L.
barley	*Hordeum vulgare* L.
bush bean	*Phaseolus vulgaris* L. cv. 'Top Crop'
corn	*Zea mays* L.
cotton	*Gossypium hirsutum* L.
grape-vine	*Vitis vinifera* L.
kidney bean	*Phaseolus vulgaris* L. cv. 'Charlevoix'
maize	*Zea mays* L.
oat	*Avena sativa* L.
pea	*Pisum sativum* L.
peach	*Prunus persica* L.
rice	*Oryza sativa* L.
rye	*Secale cereale* L.
sorghum	*Sorghum vulgare* Pers. var.
soybean	*Glycine max* L.
spinach	*Spinacia oleracea* L.
sugar-cane	*Saccharum officinarum* L.
sunflower	*Helianthus annuus* L.
wheat	*Triticum aestivum* L.
wheatgrass	*Agropyron scabrum* L.

The standard abbreviations for chemicals and symbols in general follow either the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature [Biochem. J. (1966) 101, 1-7] or the Instructions to Authors for the Biochemical Journal [Biochem. J. (1973) 131, 1-20].

Chemicals

ADH	alcohol dehydrogenase
ATP	adenosine 5'-triphosphate
Butyl-PBD	2-(4'-tert-Butylphenyl)-5-(4"-Biphenyl)-1,3,4-Oxidazole
2,4-D	2,4-dichlorophenoxy acetic acid
DCMU	1,1-dimethyl-3-(3,4-dichlorophenyl)-urea
DD-H_2O	double glass-distilled water
EDTA	ethylenediamine tetraacetic acid (Na salt)
FAD	flavin-adenine dinucleotide
FMN	flavin mononucleotide
IAA	indoleacetic acid
MVH	methyl viologen, (reduced)
NADH	nicotinamide adenine dinucleotide, (reduced)
NADPH	nicotinamide adenine phosphodinucleotide, (reduced)
$Na_2S_2O_4$	sodium dithionite
POPOP	1,4-bis[2-(4-methyl-5'-phenyloxazolyl)]-benzene
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
TCA	trichloroacetic acid
Tricine	N-tris[Hydroxymethyl]-methyl Glycine; N-[2-Hydroxy-1,1-bis(hydroxymethyl)-ethyl]-Glycine

Symbols and Units

°C	degrees Celsius
Ci	curie
µCi	microcurie
d.p.m.	disintegrations per minute
µE	microeinstein
fr	fresh
ft-c	foot-candle
g	gram
mg	milligram
µg	microgram
g	unit of gravitation
h	hour(s)
LSD	least significant difference
ℓ	litre
ml	millilitre
µl	microlitre
m	metre
cm	centimetre
mm	millimetre
M	molarity
mM	millimolar
µM	micromolar
mmol	millimole
µmol	micromole
nmol	nanomole
min	minute
N	normality
%	per cent

lb	pound
in	inch
p.s.i.	pounds per square inch
r.p.m.	revolutions per minute
sec	second
v/v	volume per volume
v/w	volume per weight
w/w	weight per weight
wt	weight

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SUMMARY

SUMMARY

1 The application of sub-lethal amounts of atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-S-triazine] increased the growth of maize and barley. The elongation of roots of 7-day-old maize seedlings was increased within 4 h of adding 0.1μM atrazine either with or without nitrate. However, the fresh weights of roots and shoots of 7-day-old maize seedlings were enhanced by the herbicide over a 7 - 40 day period only in plants supplied with nitrate. When 100μM atrazine was supplied daily to maize, then the fresh weights of both roots and shoots were markedly depressed. Barley plants were more sensitive to atrazine than maize.

2 The uptake of nitrate was increased within 2 h of applying 0.1μM atrazine to maize seedlings. Of the other ions investigated (K^+, Ca^{2+}, Mg^{2+}, Cl^- and PO_4^{3-}), only K^+ content was enhanced by the herbicide. The uptake of Cl^- and PO_4^{3-} by the seedlings however was not affected by atrazine.

3 The rate of exudation as well as the nitrate and amino nitrogen contents of the xylem sap of maize plants grown with nitrate were increased by sub-lethal amounts of atrazine. Glutamine was a major compound of the xylem sap in maize plants grown with either nitrate or ammonium irrespective of atrazine treatment.

4 The activities of nitrate and nitrite reductases, glutamine synthetase and glutamate synthase were enhanced in both roots and shoots of nitrate grown maize and barley treated with sub-lethal amounts of atrazine. The herbicide did not directly affect the synthesis or the stability of nitrate reductase. The activities of glutamine synthetase and glutamate synthase were not affected by atrazine when the plants

were grown with ammonium.

5 The uptake of $^{15}NO_3^-$ and its incorporation into TCA-precipitable protein as well as into total nitrogen of roots and shoots of maize and barley respectively were increased when micromolar quantities of atrazine were included in the nutrient solutions. However, the incorporation of [^{14}C]-leucine into TCA-precipitable protein of detached leaves from 7-day-old barley seedlings was stimulated, only when combined nitrogen was omitted from either the culture solution or the *in vitro* incubation mixture containing the labelled amino acid.

6 Growth of *Escherichia coli*, *Anabaena cylindrica*, *Chlorella pyrenoidosa* and *Neurospora crassa* was depressed by the inclusion of atrazine (over the range 0.001 to 600μM) in the culture media. *E. coli* was more resistant to the herbicide than the other microorganisms since its growth was only reduced by 30% by 600μM atrazine. The growth of *N. crassa* and the two algae was completely inhibited by 330 and 10μM of the herbicide respectively.

7 Atrazine depressed the nitrate uptake in *A. cylindrica* as well as the respiration (to both nitrate and oxygen as terminal acceptors) in *E. coli*. The activities of nitrate reductase in *N. crassa* and *C. pyrenoidosa* as well as that of nitrogenase in *A. cylindrica* were also inhibited by the herbicide.

1. INTRODUCTION

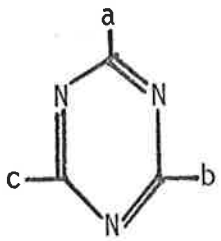
1. INTRODUCTION

1.1. Review of literature

1.1.1. Triazines

1.1.1.1. Structure

Atrazine is a triazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*S*-triazine] herbicide. The molecular structure given below is compared with two other commonly used triazine herbicides.

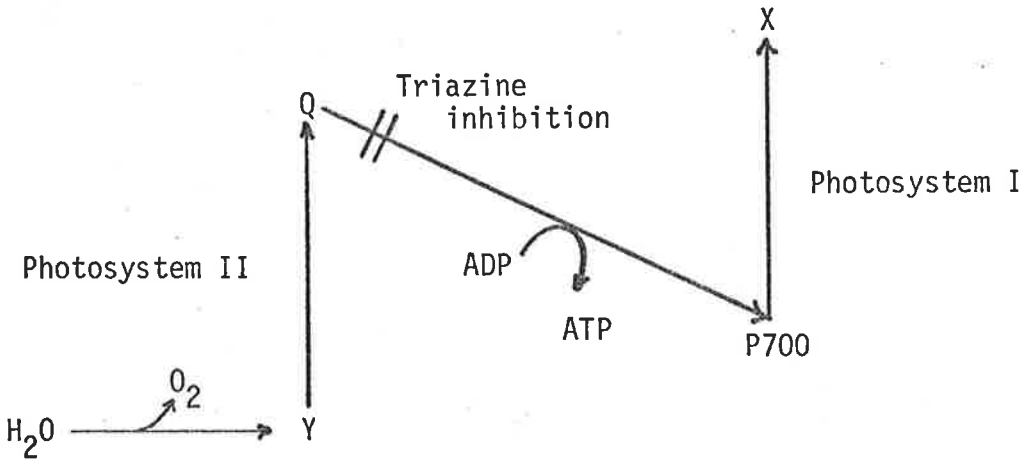


	a	b	c
Atrazine	Cl	NHC_2H_5	NHC_3H_7
Simazine	Cl	NHC_2H_5	NHC_2H_5
Prometone	OCH_3	NHC_2H_5	NHC_3H_7

They are used in general weed control but in addition, atrazine and simazine are selective weedkillers (Gast & Grob, 1960).

1.1.1.2. Mode of action

Atrazine is absorbed by roots (Gast & Grob, 1960; Graham & Buchholtz, 1968; Minshall, 1969; Rieder *et al*, 1970) but unlike simazine it also penetrates into leaves and stems because it is more water soluble (Gast & Grob, 1960). The herbicide is known to inhibit photosynthesis (Moreland *et al*, 1959; Ashton *et al*, 1960; Good, 1961; Moreland & Hill, 1962; Zweig *et al*, 1963) by its action on electron transport. The site of action of the triazines is near the photoreductant Q present in chloroplasts, as shown in Figure 1 (Zweig *et al*, 1963; Izawa & Good, 1972).



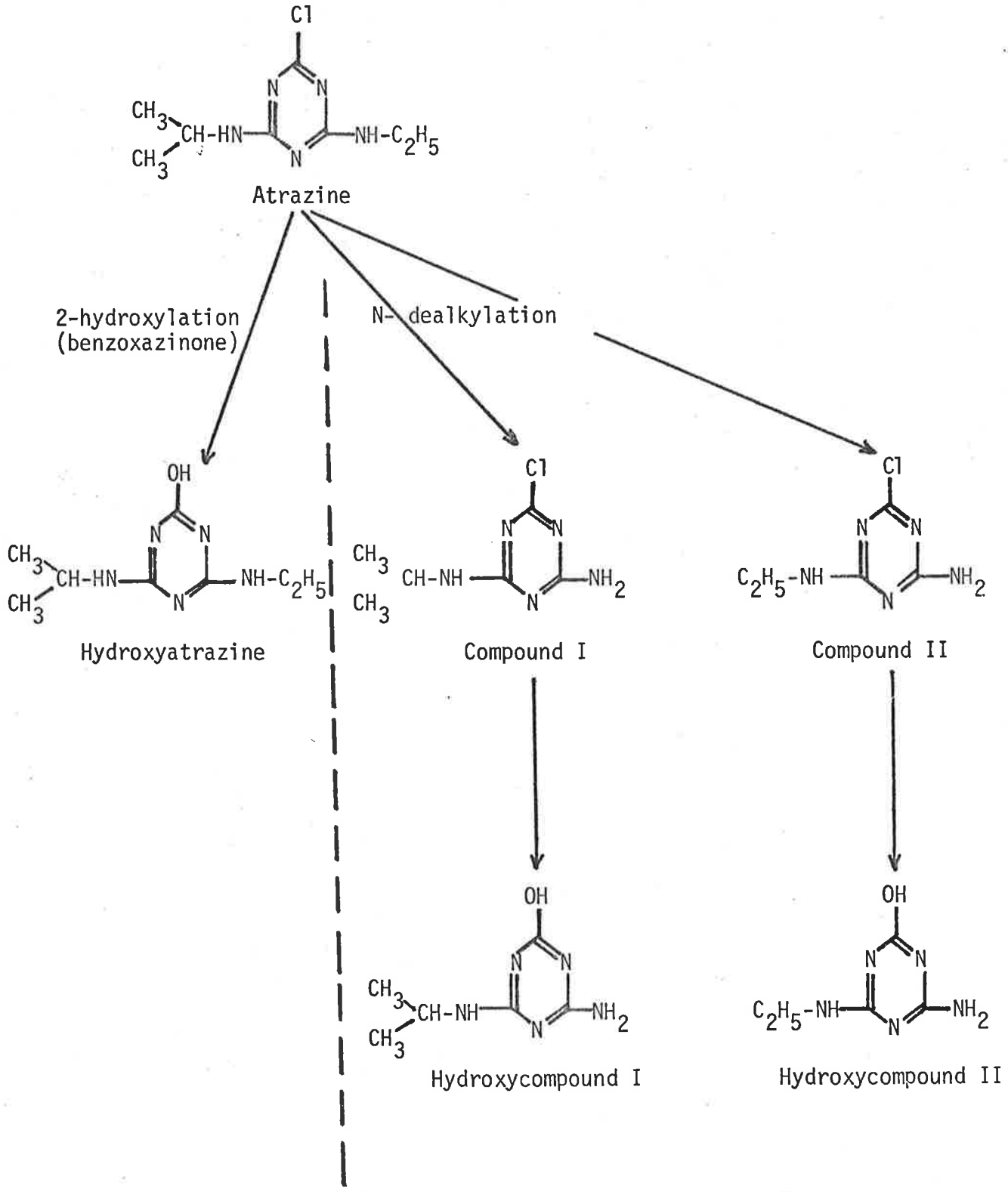
(Adapted from Izawa & Good, 1972)

Figure 1. *Effect of triazines on electron transport in chloroplasts*

Triazine herbicides inhibit the evolution of oxygen in the Hill reaction (Moreland *et al*, 1959; Moreland & Hill, 1962; Zweig *et al*, 1963) as well as ATP formation (Good, 1961) in photochemical reactions of chloroplasts in a variety of plants. Atrazine inhibited CO_2 fixation in excised leaves of kidney bean (Ashton *et al*, 1960; Zweig & Ashton, 1962). This herbicide was also shown to have several effects on the structure of the plant cells: including accelerated vacuolation of developing cells, modification of chloroplasts, reduced air spaces in mature primary leaves, cessation of cambial activity and reduced thickness of cell walls of sieve cells and tracheal elements of the stem (Ashton *et al*, 1963a & 1963b).

1.1.1.3. Detoxification

Atrazine is used as a selective weedkiller for various crops such as maize, grape-vine, fruit-trees, asparagus and sugar-cane (Gast & Grob, 1960). These tolerant plants are able to convert the absorbed atrazine into innocuous compounds. Detoxification of atrazine occurs in two ways in plants via (a) 2-hydroxylation and (b) N-dealkylation as shown in Scheme 1.



SCHEME I

Detoxification of atrazine in higher plants

The hydroxylation pathway results in a direct conversion of the highly phytotoxic atrazine to a completely non-phytotoxic hydroxyatrazine. The dealkylation pathway leads to less phytotoxic stable intermediates (compounds I and II). Subsequent metabolism of compound I and II can produce the completely non-phytotoxic hydroxycompounds I and II. The dealkylation pathway operates to some degree in most higher plants while the hydroxylation pathway for detoxification is found in species containing benzoxazinone - a cyclic hydroxamate. In species where both pathways exist, their relative activities may differ considerably. Thus in corn, a highly resistant plant to atrazine, the hydroxylation pathway is extremely active whereas the rate of dealkylation may be relatively low. In wheat the activities of both pathways are usually very low and the plant is highly susceptible to atrazine. In pea which is of intermediate susceptibility the dealkylation of atrazine occurs quite rapidly but the less phytotoxic products (compounds I and II) accumulated in relatively high concentrations. Therefore the pathways and rates of metabolism of atrazine are important in determining the tolerance of plants to the herbicide. The rate of conversion of the herbicide must be rapid enough to prevent the accumulation of lethal concentrations of atrazine in the plant and its metabolism must produce non-phytotoxic compounds (Shimabukuro, 1967).

1.1.2. Effects of sub-lethal amounts of triazines on higher plants

1.1.2.1. Growth

In a range of plants which are resistant to triazine herbicides a stimulation of growth and yield resulting from the addition of sub-lethal amounts of atrazine and simazine has been observed (Luckwill, 1967). These effects were not simply due to the elimination of weed competition (Ries *et al*, 1963; Gast & Grob, 1964) nor to the additional nitrogen content of the herbicide (Ries & Gast, 1965). Small amounts of triazines increased the yield of fruit trees and grapes (Gast & Grob, 1964), wheat (Ries *et al*, 1970), oat (Schweizer & Ries, 1969), sorghum (Tweedy *et al*, 1971) and wheatgrass (Kay, 1971).

Foliar application of simazine increased the fresh weight of shoots of pea and sweet corn (Wu *et al*, 1972) and leaves of bush bean (Singh *et al*, 1972a), five days after treatment. Atrazine increased the fresh weight of 28-day-old corn (Hiranpradit *et al*, 1972). In contrast to these increased fresh weight of plants associated with triazine treatment a corresponding increase in dry weight has not always been obtained. Thus simazine depressed the dry weight of roots and shoots of wheat, sorghum (Rottman *et al*, 1974), rice (Vergara *et al*, 1970), rye (Ries *et al*, 1967) and of leaves of soybean (Graham & Buchholtz, 1968) but that of corn remained unchanged (Hiranpradit *et al*, 1972). In some experiments this herbicide increased the dry weights of shoots of pea and sweet corn (Wu *et al*, 1972) and in roots and shoots of corn plants (Freney, 1965; Tweedy & Ries, 1967). Triazine herbicides increased the height of bean plants (Singh *et al*, 1972a) and the terminal lengths of apple trees (Ries *et al*, 1963).

The application of simazine delayed leaf senescence of rice plants resulting in an increase in the number of green leaves present 30 days after flowering (Vergara *et al*, 1970). Leaves of a variety of plants treated with herbicides appeared larger (Freney, 1965) and greener (Freney, 1965; Vergara *et al*, 1970; Hiranpradit *et al*, 1972) than those of the control plants.

1.1.2.2. Transpiration and respiration

Atrazine reduced transpiration of several species of plants (Smith & Buchholtz, 1962 & 1964; Graham & Buchholtz, 1968). In a range of plants treated with 5 - 100μM atrazine, a reduction in transpiration rate was observed within 1 - 3 h and after about 6 h, the rate of transpiration was reduced by 50% or more (Smith & Buchholtz, 1962 & 1964). The reduction of transpiration by atrazine treatment was associated with stomatal closure and an initial increase in water content of cotton, corn and soybeans (Wills *et al*, 1963). Stomatal closure in turn was linked to an increased concentration of intracellular CO_2 caused by the inhibition of photosynthesis by the herbicide

(Van Oorschot, 1965; Pallas, 1965). Simazine increased the rate of respiration in rye plants without affecting the respiratory quotient (Ries *et al*, 1967).

1.1.2.3. Uptake of nitrate and other ions

The triazine herbicides affected the uptake and accumulation of nitrate and other ions in plants. The uptake of nitrate was enhanced by the application of simazine to rye (Ries & Wert, 1972) and barley (Ries & Wert, 1972; Pulver & Ries, 1973); an early effect was recorded after 2 days of treatment of barley seedlings (Pulver & Ries, 1973). Simazine increased the nitrate nitrogen content of both roots and shoots of sorghum and wheat seedlings after 3 and 6 days respectively (Rottman *et al*, 1974). Corn grown in soil containing low levels of atrazine accumulated more N,P,K^{+} and Ca^{2+} per plant compared with untreated controls 28 days after the treatment (Hiranpradit *et al*, 1972). Simazine in solution culture enhanced the uptake of P,K^{+} and Mg^{2+} by corn plants after 35 days (Freney, 1965) whereas the herbicide did not affect either sulphate or phosphate uptake in barley (Wray *et al*, 1970 as cited by Pulver & Ries, 1973).

1.1.2.4. Amino acids and total nitrogen

The stimulation of growth by the triazine treatment of plants was generally accompanied by an increase in their amino acid, protein and total nitrogen content. The application of simazine increased all amino acids present in brown rice except for lysine which was depressed by 17% (Vergara *et al*, 1970). The other triazine herbicide, atrazine increased the total content of amino compounds in leaves of spinach and bush bean (Singh *et al*, 1972a & 1972b) and in all parts of bush bean plants (Singh *et al*, 1972b & 1972c; Janauer & Kinzel, 1976).

Substantial increases in protein content of corn, oat and wheat have been obtained from non-toxic applications of triazines in field trials (Freney, 1965; Ries *et al*, 1968). In addition, the triazine herbicides

enhanced the protein content of leaves of pea, sweet corn (Wu *et al*, 1972), spinach (Singh *et al*, 1972b), bean (Singh *et al*, 1972a) and both roots and shoots of barley (Ries & Wert, 1972; Pulver & Ries, 1973), rye (Ries *et al*, 1967; Ries & Wert, 1972) and corn (Freney, 1965) as well as in seeds of sweet corn, pea and bush bean (Singh *et al*, 1972b). As with increased accumulation of protein content, the total nitrogen was also enhanced by the application of triazines to oat, rye, barley (Ries & Wert, 1972) corn (Freney, 1965; Ries & Gast, 1965; Tweedy & Ries, 1967; Hiranpradit *et al*, 1972), grape plants (Gast & Grob, 1964) apple trees (Ries *et al*, 1963) and fruit trees (Gast & Grob, 1964).

Ries and associates (Ries *et al*, 1967; Tweedy & Ries, 1967; Pulver & Ries, 1973) studied the effects of nutritional and environmental factors on the increase in protein content of plants resulting from treatment with triazine herbicides. Simazine enhanced the protein content of corn (Tweedy & Ries, 1967) and rye (Ries *et al*, 1967) grown with nitrate but not when ammonium was the sole source of nitrogen. An increase in protein nitrogen was recorded in corn (Tweedy & Ries, 1967) and barley (Pulver & Ries, 1973) only when sub-optimal temperature and a low level of nitrate was supplied. There was a 20% increase in organic nitrogen content of simazine treated corn plants grown at low concentrations of nitrate (3.75mM) and at a low temperature (16 h day at 22.5°C and 8 h night at 17°C). The organic nitrogen content of corn was not affected by simazine treatment when supplied with a higher nitrate (7.5mM) or at a higher temperature - 16 h day at 28°C and 8 h night at 22.5°C (Tweedy & Ries, 1967). It has also been shown that light is an important parameter affecting the extent of triazine stimulation of nitrogen content of plants. Simazine increased the protein content of rye plants when illuminated with either 1200 or 2000 ft-c but there was no effect at a lower light intensity, 150 ft-c (Tweedy & Ries, 1967).

It has been proposed that triazines stimulate protein synthesis.

Simazine (10μM) enhanced the incorporation of [^{14}C]-leucine into protein by 33% in detached leaf segments of barley after a 12 h treatment (Pulver & Ries, 1973). Atrazine (0.022μM) significantly increased the [^{14}C]-leucine incorporation into protein of red kidney bean embryos within 1 - 2 h after treatment whereas an increase in the incorporation of [^{14}C]-ATP into RNA in the same tissues was only detected after 3 h (Bush & Ries, 1974). Ries and associates (Ries *et al*, 1967; Pulver & Ries, 1973; Bush & Ries, 1974) found that the application of triazines to plants stimulated protein synthesis as well as the uptake of nitrate and nitrate reductase activity. They suggested that the primary effect of the triazines was a stimulation of protein synthesis which then resulted in an increased uptake and assimilation of nitrate.

1.1.2.5. Carbohydrates

The application of sub-lethal amounts of simazine substantially reduced the total soluble sugar and starch content of rice plants. Total carbohydrates were depressed 4 days after the herbicide treatment by more than 50% (Vergara *et al*, 1970). Foliar application of atrazine (10μM) resulted in a decrease in starch as well as total and reducing sugars in leaves of bush bean (Singh *et al*, 1972a), sweet corn and pea seedlings (Wu *et al*, 1972). Ries *et al* (1967) found that there was an increased respiration associated with a lower rate of carbohydrate accumulation in rye plants and suggested that there was a greater energy requirement in simazine treated plants. Pulver & Ries (1973) later established that an increase in protein content by the herbicide treatment was accompanied by a reduction of soluble carbohydrates. Singh & Salunkhe (1970) suggest that triazine produced a metabolic condition whereby carbohydrates were utilized for protein synthesis.

1.1.2.6. Enzymes

The accumulation of total nitrogen in plants resulting from the application of sub-lethal amounts of triazines enhanced nitrate reductase activity (Ries *et al*, 1967; Tweedy & Ries, 1967). The enzyme activity in

leaf extracts of corn (Tweedy & Ries, 1967) and rye (Ries *et al*, 1967) grown at low level of nitrate and at low temperatures increased almost 10-fold after a 7 day exposure to simazine. Singh & Salunkhe (1970) and Wu *et al* (1972) established that a foliar application of triazines not only enhanced the activity of nitrate reductase but also the activities of glutamic-pyruvic transaminase, adenosine triphosphatase, α-amylase and starch phosphorylase. The activity of each of the five enzymes was significantly increased in leaves of bush bean plants, 5 days after treatment with either atrazine or simazine (Singh & Salunkhe, 1970). Triazines also enhanced the activities of nitrate reductase and glutamic-pyruvic transaminase but not ribonuclease in leaves of pea and sweet corn (Wu *et al*, 1972). In contrast, the application of high levels of atrazine (5 - 100μM) increased the extractable nitrate reductase activity but decreased the *in vivo* reduction of nitrate in detached leaves of barley (Aslam & Huffaker, 1973). In a similar study higher amounts of atrazine (50μM) inhibited nitrite reduction and depressed the *in vivo* activities of nitrate and nitrite reductases (Finke *et al*, 1977).

1.1.2.7. Auxin-type metabolism

The atrazine effects on the stimulation of growth, greening of leaves and delayed senescence suggested that this herbicide may influence auxin metabolism in plants as suggested by Ebert & Van Assche (1969). They established that atrazine (10^{-6} to 10^{-8}M) increased the IAA-peroxidase activity in oat coleoptile whereas very low amounts of herbicide (0.5×10^{-10} to 0.5×10^{-21}M) depressed the enzyme. They suggested that tne inhibition of enzyme activity raised the level of endogenous IAA resulting in a stimulation of growth. Atrazine (10^{-8}M) stimulated the elongation of the developing embryos of red kidney bean (Bush & Ries, 1974). This herbicide appears to act in a similar way to growth regulators such as 2,4-D or naphthenic acid. These growth regulators increased growth and protein synthesis in plants (Huffaker *et al*, 1967; Wort *et al*, 1973). Chromatin

isolated from 2,4-D treated soybeans has been found to support increased RNA synthesis (O'Brien *et al*, 1968). Similarly the application of atrazine to 4-day-old etiolated soybean seedlings 6 h before the isolation of chromatin enhanced the chromatin directed RNA synthesis by 65% (Penner & Earley, 1972).

1.1.3. Effects of atrazine on microorganisms

Triazine herbicide applied at recommended field rates to soil (4 lbs/ha) to eliminate weeds did not affect the nitrifying bacteria (Caseley & Luckwill, 1965). However, higher concentrations of atrazine and simazine (100μM) delayed the nitrite production in *Nitrosomonas europaea*. *Nitrobactor agilis* was more sensitive than *Nitrosomonas* to these herbicides and the nitrite utilization in the former was completely inhibited by 100μM atrazine. Nitrogen fixation in *Azotobactor vinelandii* was not affected by the addition of high amounts of atrazine e.g. 100μM (Caseley & Luckwill, 1965). As with nitrifying bacteria triazines applied at normal field rates did not affect the growth of *Bacillus subtilis* (Breazeale & Camper, 1972; Thomas *et al*, 1973).

The triazine herbicides had greater inhibitory effects on green algae than soil bacteria. Atrazine at 5μM level completely inhibited the growth of *Chlorella* sp, *Chlamydomonas* sp (Arvik *et al*, 1973; Valentine & Bingham, 1976), *Anabaena*, *Schizothrix calcicola*, *Chlorococcum* sp (Arvik *et al*, 1973), *Scenedesmus quadricauda* and *Euglena gracilis* (Valentine & Bingham, 1976). Atrazine as low as 0.05μM had no effect on these microorganisms but at 0.25μM, growth was inhibited by about 20%. Growth of *Chlorella* sp has been used as an index of herbicide toxicity (Addison & Bardsley, 1968; Kratky & Warren, 1971) and 330μM atrazine completely depressed growth within 24 h (Ashton *et al*, 1966). At this concentration cell division, chlorophyll formation and starch production were inhibited in chloroplasts of *Chlorella vulgaris* (Ashton *et al*, 1966).

The site and mode of action of triazines as a photosynthetic inhibitor in algae are similar to their effects on higher plants. Atrazine as low as 0.05μM restricted $^{14}CO_2$ fixation by 30% in *Chlorella pyrenoidosa*, *Scenesdesmus quadricauda*, *Chlamydomonas reinhardtii* and *Euglena gracilis* but at 5μM the herbicide completely inhibited photosynthesis (Valentine & Bingham, 1976).

1.2. Aim of the study

The aim of the present study was to examine the mechanism whereby atrazine at sub-toxic levels affects growth and nitrogen assimilation in maize and barley.

The main parameters investigated included the effects of the herbicide on

(a) growth,

(b) uptake of nitrate and other ions,

(c) synthesis of amino acids, amides and protein,

(d) enzymes involved in the assimilation of nitrate.

In addition, the effects of atrazine on growth and nitrate reductase and nitrogenase activities in some microorganisms were also determined.

2. MATERIALS AND METHODS

2.1. Biological Materials

2.1.1. Higher plants

The following plants were used in this study:-(a) maize (*Zea mays* L, hybrid variety DSC1) supplied by the De Kalb Shand Seed Co., Tamworth, N.S.W., Australia and (b) barley (*Hordeum vulgare* L) kindly supplied by the Department of Agronomy of this Institute.

2.1.2. Microorganisms

The following microorganisms were used in this work:-(a) *Escherichia coli* (Strain B), (b) *Anabaena cylindrica* (Strain 1403/2A) obtained from the Culture Centre of Algae & Protozoa, Cambridge, U.K., (c) *Chlorella pyrenoidosa* (Strain CU211/8b, ATCC, 11469) and (d) *Neurospora crassa*, macroconidial Wild type 5297a.

2.1.3. Enzymes

Alcohol dehydrogenase was purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.).

2.2. Reagents

2.2.1. Chemicals

NADH, NADPH, FAD, FMN, MV, cycloheximide, 6-methyl purine, cysteine, alanine, B.S.A., Tricine, Butyl-PBD were obtained from the Sigma Chemical Co. (St. Louis, Missouri, U.S.A.).

2,5-Diphenyloxazole (PPO) and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (POPOP) were obtained from Packard Co. (Chicago, Illinois, U.S.A.).

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-S-triazine],

(GESAPRIM, 80% ATRAZINE) was kindly provided by CIBA-GEIGY Australia Ltd.

All other chemicals were of the highest purity available.

2.2.2. Isotopes

L-[U-^{14}C]Leucine (330mCi/mmol) purchased from The Radiochemical Centre, Amersham, Buckinghamshire, England. $K^{15}NO_3$ (31.25% enrichment) obtained from Office Industrial De L'Azote, France.

2.3 Culture Solutions and Buffers

The full strength Hoagland's solution without combined nitrogen contained in 1ℓ (ml/l):- M KCl, 5.0; M $CaCl_2$, 5.0; M $MgSO_4$, 2.0; M KH_2PO_4, 1.0; Fe (CIBA-GEIGY 138 Fe, 100 mg/l), 1.0 and trace element stock, 1.0 ml. The trace element stock contained the following (g/l):- H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.25; $CuSO_4.5H_2O$, 0.08 and $Na_2MoO_4.H_2O$, 0.025.

When 4.5mM NO_3^- was used as a source of nitrogen, 1.5 ml of M KNO_3 + 1.5 ml of M $Ca(NO_3)_2$ were added to the half strength Hoagland's solution but only 1.0 ml of each M KCl and M $CaCl_2$ respectively were included. When lower levels of KNO_3 + $Ca(NO_3)_2$ were used e.g. 0.5 - 1.0mM, then only 1/10th strength Hoagland's solution was employed. Ammonium chloride was used as a source of ammonium nitrogen viz. either 4.5mM NH_4Cl in 0.5 strength Hoagland's solution or 0.5mM NH_4Cl in 0.1 strength Hoagland's solution as indicated in the text. In hydroponic experiments, the culture solutions were supplemented with $FeSO_4$ (1 mg/l of the appropriate strength Hoagland's solution) (Christ, 1974).

All buffers and aqueous solutions were prepared in double glass-distilled water. NADH and NADPH (in 0.025M phosphate buffer, pH 7.5) and both casein (3% w/v) and cysteine in 50mM phosphate buffer, pH 7.5 were prepared just prior to use. Alcohol dehydrogenase was prepared in 0.1M phosphate buffer, pH 7.0.

The stock solutions of 0.1M phosphate buffer was prepared by the method of Gomari (1955). The pH of the buffers was determined using a Beckman H5 pH meter.

2.4. Growth of Plants

2.4.1. Liquid cultures

Seeds of *Zea mays* L were surface sterilized in 50% (v/v) ethanol followed by 0.05% (w/v) mercuric chloride solution and then rinsed thoroughly with sterile distilled water. The washed seeds were germinated on aseptic 1% (w/v) agar plates at 28°C in the dark and 50% relative humidity. At the 4-day-old stage, the seedlings were transferred into 0.1 strength Hoagland's solution without combined nitrogen in black plastic trays (30 x 20 x 6 cm). Thirty seedlings were supported with pieces of sponge in holes in the lid of the container. The solutions were aerated and the plants grown in a phytotron with 16 h light periods at an intensity of 350$\mu E/m^2$/sec at 22°C followed by 8 h dark periods at 17°C.

Seven-day-old seedlings grown in the pretreatment culture solutions as described above were then supplied with nitrate or ammonium in dilute Hoagland's solution as indicated for each experiment. When lower amounts of combined nitrogen were used (0.5 - 1mM of either nitrate or ammonium), they were supplied in 0.1 strength Hoagland's solution and this solution was replenished daily. However, when higher concentrations of nitrogen were used viz. 4.5mM, a 0.5 strength Hoagland's solution was employed which was changed every 3 days. The pH of the nitrate culture solution was adjusted daily to 4 with N H_2SO_4 and to 6 with N KOH when ammonium was the nitrogen source. During one day, the pH did not alter by more than 1.0 unit.

2.4.2. Sand cultures

Surface sterilized seeds of maize planted in black plastic pots

(16 x 16 cm) containing washed sand were watered daily for 14 days with 0.5 strength Hoagland's solution without combined nitrogen. These plants were thinned to 6 per pot and 0.5 strength Hoagland's solution containing 4.5mM of either nitrate or ammonium and in addition atrazine (as indicated for each experiment) was supplied daily. The plants were grown in a phytotron as described previously (Section 2.4.1.).

In experiments with barley (*Hordeum vulgare* L), the seeds were soaked in 0.2% (w/v) $HgCl_2$ solution for 3 min followed by several rinses with sterile distilled water. They were then planted in sand either in black plastic trays (21 x 15 x 5 cm) for short term experiments or in black plastic pots (16 x 16 cm) for long term experiments. Hoagland's solution (0.1 strength) without combined nitrogen was added daily for the first 7 days. This was then followed with a nitrogen supply and atrazine as indicated for each experiment. When barley was grown for a longer period, 7-day-old seedlings were thinned to 6 per pot and 0.5 strength Hoagland's solution containing 4.5mM NO_3^- was supplied. The plants were grown in a phytotron as described in Section 2.4.1.

2.5. Culture of Microorganisms

2.5.1. *Escherichia coli* was grown in a culture solution described in Table 1 containing graded amounts of atrazine (0, 0.001, 0.01, 0.1, 1, 10, 100, 300 and 600µM respectively). At the highest level (600µM), two applications of atrazine each at 300µM were added to the culture solutions at 0 time and after 2 h incubation respectively.

Aerobic cultures: When grown aerobically, 100 ml of culture medium was dispensed into a 250 ml Erlenmeyer flasks, sterilized in an autoclave (Smith Industries, South Australia) at 17 lbs pressure per sq in for 30 min, cooled and then inoculated with 5 ml of cell-suspension from a starter culture at the end of its growth phase (absorbance at 660nm was approximately 1).

TABLE 1

Composition of a culture solution for Escherichia coli

Solutions of glucose and Na-citrate were sterilized together in an autoclave separately from the rest of the medium.

Chemicals	wt/l
	(g)
"Bacto-Tryptone" (Difco)	5
Yeast extract (Difco)	5
Glucose	10
KNO_3	1.7
$(NH_4)_2SO_4$	0.7
Na-citrate	3
K_2HPO_4	3
Trace elements	(mg)
$MgSO_4.7H_2O$	50.0
$CaCl_2.2H_2O$	0.5
$MnCl_2.4H_2O$	5.0
$FeSO_4.7H_2O$	2.0

They were then clamped to a wrist-action shaker adjusted to 150 strokes/min. The bacteria were grown at 30°C. In growth studies with atrazine, aliquots of the cultures (3 ml) were withdrawn every 3 h up to 24 h after inoculation and turbidity was measured at 660nm in a Shimadzu (QV-50) spectrophotometer (Section 2.12.1.).

Anaerobic cultures: When *E. coli* was grown anaerobically, 250 ml of culture medium was dispensed into a 250 ml Erlenmeyer flask, sterilized, cooled and then inoculated with 5% (v/v) inoculum from a starter culture as described previously. The bacteria were grown at 30°C and aliquots of cultures (3 ml) were withdrawn aseptically at intervals and the turbidity was measured as described for aerobic cultures.

When cells were required for enzyme assays, they were grown anaerobically with and without atrazine (300μM) in 10ℓ pyrex jars containing 8ℓ of culture solutions at 30°C. They were sparged with N_2 gas (0.5 ml per min per ℓ medium). After a 12 h incubation period the cells were harvested in a Sorvall RC2-B refrigerated centrifuge fitted with a continuous-flow rotor (SS-34). They were washed twice with 20mM phosphate buffer (pH 7.5). All operations were carried out at 2°C.

2.5.2. *Anabaena cylindrica* was grown in a medium described in Table 2. A culture of *A. cylindrica* was maintained on 1.5% (w/v) agar slopes containing the culture medium. A loopful of the alga was transferred to each of 100 ml of sterilized liquid cultures in 250 ml Erlenmeyer flasks. They were grown for 7 days at 30°C and at a light intensity of 200μE/m^2/sec provided by a bank of cool white fluorescent lights. Filaments of alga from this starter culture were dispersed in fresh media and a thick suspension was gently mixed in a glass homogenizer. One ml of this suspension contained about 0.2 g fresh weight.

Aliquots (5 ml) of this cell-suspension were added to 100 ml aliquots of

TABLE 2

Composition of a culture solution for Anabaena cylindrica

Salts	wt/l
	(g)
$MgSO_4.7H_2O$	0.123
$CaCl_2.2H_2O$	0.037
NaCl	0.117
KCl	0.373
KH_2PO_4	0.087
K_2HPO_4	0.585
KNO_3	10.1
Trace elements	(mg)
Fe(EDTA complex)	2.5
$MnSO_4.4H_2O$	0.25
$(NH_4)_6MoO_{24}.7H_2O$	0.05
$ZnSO_4.4H_2O$	0.025
$CuSO_4.5H_2O$	0.01
H_3BO_3	0.025
$Co(NO_3)_2$	0.005

The pH was adjusted to 7.5 and sterilized

media in 250 ml Erlenmeyer flasks containing graded amounts of atrazine (0, 0.001, 0.01, 0.1, 1, 10 and 100μM respectively). After 5 days growth at 30°C the alga was harvested.

2.5.3. *Chlorella pyrenoidosa* was grown in a medium described in Table 3. A culture of C. *pyrenoidosa* was maintained on agar slopes and a starter culture was grown for 7 days by a similar procedure as that described for A. *cylindrica* (Section 2.5.2.).

Aliquots (10 ml) of this starter culture were added to 100 ml aliquots of sterilized culture solutions in 250 ml Erlenmeyer flasks containing atrazine and grown for 5 days as described for A. *cylindrica* (Section 2.5.2.).

2.5.4. *Neurospora crassa* was grown in a modified Westgaard-Mitchell culture solution described in Table 4. Spores of *N. crassa* from an agar slope were dispersed in a 50 ml of sterile culture medium. Aliquots (3 ml) of a thick spore suspension were transferred into 100 ml sterilized liquid cultures in 250 ml Erlenmeyer flasks containing graded amounts of atrazine (0, 0.001, 0.01, 0.1, 1, 10, 100 and 330μM respectively). They were clamped to a wrist action shaker adjusted to 100 strokes/min. The felts were harvested after 3 days growth at 30°C.

2.6. Growth Measurements

2.6.1. Length

The elongationsof root tips (either cut roots or intact roots) of 7-day-old maize seedlings were measured under a dissecting microscope. Seedlings with primary root tips intact were transferred into Petri dishes (2 x 14 cm - one plant per Petri dish) in 75 ml of culture solution as described in the legend of each experiment. The primary root was fixed in position by a small rubber bung and the Petri dish with the root tip was viewed under the dissecting microscope. The field of the microscope was

TABLE 3

Composition of a culture solution for Chlorella pyrenoidosa

Salts	wt/l
	(g)
$MgSO_4.7H_2O$	0.123
$CaCl_2.2H_2O$	0.037
NaCl	0.117
KCl	0.373
KH_2PO_4	0.47
K_2HPO_4	0.27
Na-citrate	0.5
$(NH_4)_2SO_4$	0.2
KNO_3	0.8
Trace elements	(mg)
Fe(EDTA complex)	2.5
$MnSO_4.4H_2O$	0.25
$(NH_4)_6MoO_{24}.7H_2O$	0.05
$ZnSO_4.4H_2O$	0.025
$CuSO_4.5H_2O$	0.01
H_3BO_3	0.025
$Co(NO_3)_2$	0.005

The pH adjusted to 6.9 with 2% (w/v) K_2CO_3 and sterilized

TABLE 4

Composition of a culture solution for Neurospora crassa

Chemicals	wt/l
	(g)
Glucose	20
KNO_3	1
KH_2PO_4	1
$MgSO_4.7H_2O$	0.5
NaCl	0.1
$CaCl_2.2H_2O$	0.1
Trace elements	(mg)
$ZnSO_4.7H_2O$	5
Na-citrate	5
$FeSO_4$	1
$CuSO_4.5H_2O$	0.25
$MnSO_4.4H_2O$	0.05
H_3BO_3	0.05
$Na_2MoO_4.2H_2O$	0.05
Biotin	0.01

The pH adjusted to 6.5 with 2% (w/v) K_2CO_3 and sterilized

calibrated to 1 cm with an eye-piece (100 divisions = 1 cm). The tip of the primary root was focussed at 0 reading at 0 h and the elongation of the root tip was observed hourly over 8 h. The experiment was conducted in an illuminated room (200$\mu E/m^2/sec$) at 20°C. The growth of root tip was measured in mm. The growth of cut root tips were also measured by the same technique.

2.6.2. Fresh weight

2.6.2.1. Maize and barley

In liquid culture experiments, the roots and shoots of plants were collected and the roots were washed thoroughly with distilled water and surface dried on paper. Fresh weight of roots and shoots of each plant was recorded separately. In sand culture studies, 6 plants were grown in each pot and their roots were collected after washing the sand with water. Since the roots of the plants were not easily separated, they were weighed together.

2.6.2.2. *Neurospora crassa*

Felts were washed with distilled water in a sintered Buchner funnel, blotted with filter paper and fresh weights were recorded for each treatment.

2.6.3. Dry weight

The materials used for fresh weight measurements in maize, barley and *Neurospora* respectively were dried in an oven at 80°C and cooled in a dessicator to a constant weight.

2.6.4. Turbidity

The turbidity of culture solutions containing either *E. coli* or *C. pyrenoidosa* was measured at 660nm in a spectrophotometer (Section 2.12.1.).

2.6.5. Chlorophylls

Chlorophyll content of C. *pyrenoidosa* was determined according to Strain *et al* (1971) using an acetone extract (80% v/v) and measuring the absorbance (A) at 665 and 649nm respectively in a spectrophotometer (Section 2.12.1.). The formula given by them ($6.45 \times A_{665} + 17.72 \times A_{649}$) was used to calculate the µg chlorophyll content of the samples.

2.6.6. Carotenoids

Carotenoid pigments extracted in 80% (v/v) acetone for 3 min were measured at 435nm in a spectrophotometer (Section 2.12.1.).

2.7. Uptake of Ions

2.7.1. Nitrate

2.7.1.1. Higher plants

A batch of 40 maize seedlings (7-day-old) was transferred into fresh Hoagland's solution (0.1 strength) containing 1mM NO_3^- (Section 2.4.1.). Aliquots (0.5 ml) of nutrient solution were withdrawn hourly and the depletion of nitrate from this solution was determined by estimating their content of nitrate by the *E. coli* nitrate reductase method as described in Section 2.12.3.

2.7.1.2. Microorganisms

A piece of *N. crassa* felt was transferred into a fresh culture solution containing 1mM NO_3^- (Section 2.5.4.). The depletion of nitrate from the nutrient solution was estimated hourly as described previously (Section 2.7.1.1.).

Nitrate uptake by *E. coli* (dissimilation) was measured by estimating the amounts of nitrite produced in the medium (Section 2.12.4.). The uptake

of nitrate by A. *cylindrica* was monitored continuously using nitrate electrode as described in Section 2.12.2.

2.7.1.3. Metabolic pool

Root tips (0-20 mm; 0.5 g) of maize seedlings were washed well with distilled water and placed in Warburg's flasks (10 ml) containing 3 ml of 0.1M phosphate buffer, pH 7.5. The flasks were then evacuated and the contents were flushed with N_2 gas for 2 min and incubated at 30°C in the dark. Aliquots of samples (about 0.1 ml) were withdrawn from time to time using a gas-tight Hamilton micro-syringe and the nitrite content determined (Section 2.12.4.).

2.7.2. K^+, Ca^{2+}, Mg^{2+}, Cl^- and PO_4^{3-}

Roots and shoots of maize were extracted with hot distilled water in a mortar and pestle (Section 2.9.1.1.) and the clear supernatant fractions were assayed for K^+, Ca^{2+} and Mg^{2+} using an Atomic Absorption Spectrometer, Varion Techtron,Model AA4 according to the methods in the laboratory manual provided with the instrument.

Chloride in extracts of roots and shoots of maize was determined by titrating with standard $AgNO_3$ (Chapman & Pratt, 1961). In this procedure, 1 g fresh weight of plant material (either root or shoot) was ground with 0.25 g CaO (fresh) and 2 ml distilled water. They were digested at 550°C for 2 h in a muffle furnace. After cooling, 10 ml of hot water was added and the sample kept on a hot plate. Each sample was then mixed well with a glass rod and filtered through a Buchner funnel. Further 10 ml aliquots of hot water were added and the final volume made to 50 ml. Aliquots (10 ml) were titrated against 0.05N $AgNO_3$ using 1% (w/v) K_2CrO_4 solution as an indicator.

Phosphate in the hot water extracts of roots and shoots of maize was measured according to the method of Murphy & Riley (1962). Aliquots (50 µl) of extracts of roots and shoots (1 g fresh weight extracted with 5 ml of hot

water) were added to 4 ml of a mixed reagent and the final volume was made to 25 ml with distilled water. The mixed reagent contained the following in 50 ml:- (1) 25 ml of 5N H_2SO_4, (2) 7.5 ml of ammonium molybdate (2 g in 50 ml distilled water), (3) 15 ml of 0.1M ascorbic acid and (4) 2.5 ml of potassium antimonyl tartarate (27.5 mg in 10 ml distilled water). The absorbance of the resulting molybdenum blue complex was measured at 882nm in a spectrophotometer (Section 2.12.1.). The readings were compared with a standard calibration graph over the range 1-4 µg phosphate.

2.8. Xylem Sap

2.8.1. Collection

Plants were cut at the base of the shoots and the bleeding sap was collected into test tubes with Pasteur pipettes. The first drop of the xylem sap was discarded and the sap was collected over the first hour. The test tubes were weighed before and after the collection of the xylem sap to determine the volume of the xylem exudation.

2.8.2. Analysis of amino acids

The individual amino acids were determined in a Beckman Amino Acid Analyser, Model 119 (Beckman Instrument, Inc., California) using the lithium buffer procedure as published in the Instructions Manual supplied with the instrument.

2.9. Enzyme Techniques

2.9.1. Preparation of cell-free extracts

2.9.1.1. Higher plants

Roots and shoots of maize and barley and scutella of maize were homogenized at 2°C in a chilled mortar and pestle with pre-cooled extracting buffer, pH 7.5 (5 ml: 1 g fr wt sample). Some acid-washed sand was added to

facilitate the grinding. In some experiments a mechanical homogenizer (Ultra-turrax TP 18/2) was used. The crude extracts obtained by both the methods were squeezed through miracloth (Miltown, New Jersey, U.S.A.) and then centrifuged at 27,000 x *g* for 15 min in a Sorvall RC2-B refrigerated centrifuge (2°C) using SS-34 rotor. The supernatant fraction was retained for enzyme assays.

The basic medium for the preparation of cell-free extracts for assaying nitrate and nitrite reductases, glutamine synthetase and glutamate synthase was 50mM phosphate buffer, pH 7.5. When extracting for nitrate reductase, the phosphate buffer contained in addition 0.5mM Na-EDTA as well as casein and cysteine as shown in Table 5. The extraction medium for glutamate synthase contained either (1) 0.5mM Na-EDTA and 1mM cysteine in 50mM phosphate buffer (pH 7.5) or (2) 5mM Na-EDTA and 12.5mM mercaptoethanol in 0.1M Tricine (pH 7.5) as indicated in Section 2.9.2.4.

2.9.1.2. Microorganisms

The medium used for extracting nitrate reductase was 50mM phosphate buffer (pH 7.5) containing 0.5mM Na-EDTA.

The felts of *N. crassa* were homogenized in a cold mortar and pestle with the extracting medium (2 ml: 1 g fr wt) and a clear supernatant fraction was collected as described in Section 2.9.1.1. The cells of *C. pyrenoidosa* were suspended in the extracting medium (2 ml: 1 g fr wt) and disrupted by passing them through a chilled Aminco French pressure cell (American Instrument Co., Maryland, U.S.A.) twice at 20,000 p.s.i. (Hughes *et al*, 1971). The homogenate was centrifuged at 27,000 x *g* and a clear supernatant fraction was prepared as described in Section 2.9.1.1.

TABLE 5

*Amounts of casein and cysteine in the phosphate buffer used to extract nitrate reductase from roots and shoots of maize and barley respectively**

Tissue	Casein (%,w/v)	Cysteine (mM)
Maize		
Roots	3	5
Shoots	3	20
Barley		
Roots	3	1
Shoots	3	1

* Neither casein nor cysteine was included in the extraction medium for the scutella of maize

2.9.2. Enzyme assays

2.9.2.1. Nitrate reductase

Nitrate reductase activity was determined by measuring the rate of nitrite production. Assay mixture for the enzyme determination contained the following in a total volume of 1.5 ml:- (1.1 - x) ml of 0.025M phosphate buffer, pH 7.5; 0.2 ml of 0.1M KNO_3; 0.2 ml of NADH (4 mg/ml of phosphate buffer, pH 7.5) and x ml of enzyme preparation (usually 0.1 - 0.3 ml; Section 2.9.1.1.). The reaction mixture was incubated for 30 min at 25°C, it was stopped by adding 0.1 ml of alcohol dehydrogenase (0.3 mg protein/ml of 0.1M phosphate buffer, pH 7.0) followed by 0.1 ml of N acetaldehyde to oxidize the excess NADH. The amount of nitrite produced was determined as described in Section 2.12.4.

The *in vivo* enzyme activities of scutella and root tips (0-20 mm) of maize seedlings was determined in Thunberg tubes (1.0 x 15 cm) containing 2.5 ml of phosphate buffer, pH 7.5 (0.2M), 1.0 ml of nitrate (0.5M), 1.25 ml of distilled water and 0.25 ml of n-propanol. Freshly harvested samples of roots or scutella (0.5 g) were added and infiltrated with the reaction mixture using either a bench water pump (12-15 mm of Hg) or a rotary high vacuum oil pump (1-5 x 10^{-3} mm of Hg; Edwards "Speedivac",Model 2S50) for 2 min. Care was taken to keep the tissues submerged in the reaction mixtures. The Thunberg tubes were then shaken in a reciprocating water bath at 25°C and aliquots (0.5 ml) of incubation medium were removed every 30 min. The Thunberg tubes were re-evacuated after sampling. The amount of nitrite in the aliquots was determined as described in Section 2.12.4.

The nitrate reductase was assayed for *C. pyrenoidosa* as described for higher plants. In *N. crassa*, NADPH was used as an electron donor and 1.5 ml of assay mixture contained the following:- 0.9 - x ml of 0.1M phosphate buffer, pH 7.5; 0.3 ml of 0.1M KNO_3; 0.15 ml of FAD (0.8 mg/10 ml); 0.15 ml of NADPH (2 mg/ml buffer) and x ml of enzyme preparation (Section 2.9.1.2.). The assay

was carried out as described for higher plants.

2.9.2.2. Nitrite reductase

Nitrite reductase activity was determined by following the rate of nitrite utilization by the procedure of Sawhney & Nicholas (1975). The assays were conducted anaerobically in test tubes (1 x 7.5 cm) stoppered with rubber Suba-seal caps (size 17, Wm. Freeman, Barnsley, Yorks, U.K.). The reaction mixture in a final volume of 2 ml contained the following:- 1.2 ml of 50mM phosphate buffer, pH 7.5; 0.1 ml of MV (7.56 mg/10 ml); 0.1 ml of 2mM $NaNO_2$ and 0.5 ml of enzyme preparation (Section 2.9.1.1.). The test tubes were evacuated and flushed with high purity N_2 gas. Then the reaction was started by adding 0.1 ml of freshly prepared $Na_2S_2O_4$ (11.17 mg $Na_2S_2O_4$ in 1 ml of 1% (w/v) $NaHCO_3$) using a gas-tight micro-syringe. After 30 min incubation at 25°C, the reaction was terminated by removing the Suba-seal caps and the tubes were shaken vigorously until MVH was completely oxidized. Nitrite was determined in an appropriate aliquot of the reaction mixture as described in Section 2.12.4.

2.9.2.3. Glutamine synthetase

Glutamine synthetase activity was determined by the rate of formation of γ-glutamyl hydroxamate by the method of Shapiro and Stadtman (1970). The enzyme assay was carried out in a test tube (1 x 7.5 cm) and the reaction mixture in a final volume of 2 ml contained the following:- 0.2 ml of enzyme preparation (Section 2.9.1.1.); 0.3 ml of distilled water and 0.5 ml of a 2-fold concentrated assay mixture. This concentrated assay mixture (freshly prepared each day) in a final volume of 25 ml (stock) contained the following (ml):- Imidazole-HCl buffer (1M, pH 7.0), 2.0; freshly prepared glutamine (0.1M), 1.5; $MnCl_2$ (0.1M), 1.5; freshly prepared Na-ADP, 2.0; K-arsenate (1M, pH adjusted to 7.0 with M KOH), 1.0; freshly prepared hydroxylamine-HCl (2M), 1.5 (neutralized before adding with 1.5 2N NaOH). Incubation was carried out at 37°C for 15 min. The reaction

was terminated and a colour was developed by adding 2.0 ml of the following mixture:- 12 ml contained 4.0 ml of 10% (w/v) $FeCl_3$; 1.0 ml of 24% (w/v) TCA; 0.5 ml of 6N HCl and 6.5 ml of distilled water. The tubes were centrifuged at 2,500 x *g* for 15 min in a bench centrifuge (M.S.E.) and the absorbance of the colour was measured at 540nm in a Turner spectrophotometer (Section 2.12.1.).

2.9.2.4. Glutamate synthase

The rate of synthesis of glutamate was followed in this assay as described by Miflin and Lea (1975). The reaction was carried out anaerobically in test tubes (1 x 7.5 cm) stoppered with rubber Suba-seals (size 17, Wm. Freeman, Barnsley, Yorks, U.K.). The reaction mixture in a final volume of 1 ml contained 0.1 ml of 50mM glutamine, 0.1 ml of 50mM α-ketoglutarate, 0.1 ml of MV (1 mg/ml), 0.2 ml of enzyme preparation (Section 2.9.1.1.), 0.25 ml of distilled water and 0.2 ml of buffer (25mM either Tricine or phosphate, corresponding to the buffer used to extract the enzyme). The test tubes were evacuated using a bench water pump and the contents were flushed with high purity N_2 gas. The reaction was started by adding 0.05 ml of $Na_2S_2O_4$ in 1% (w/v) $NaHCO_3$ (8 mg/ml) and incubated at 30°C for 20 min. Then the reaction was terminated by removing the Suba-seal caps and shaking until the MVH was fully oxidized and then 0.1 ml of absolute ethanol was added. Aliquots (0.05 ml) were spotted onto a Whatman 3MM paper (45 x 55 cm) and the amino acids were separated by running the chromatogram overnight (12 - 15 h) in 75% (w/w) phenol in the presence of ammonia vapour (Miflin & Lea, 1975). When cysteine was used in the extraction medium, the chromatogram was run for a longer period (about 24 h) to separate it from glutamate. The chromatogram was dried overnight at constant room temperature in a fume cupboard and then dipped in a cadmium acetate - ninhydrin solution (Atfield & Morris, 1961). This solution was freshly prepared by the following procedure. Cadmium acetate (A.R. grade; 0.05 g) was dissolved in a mixture of distilled water (5.0 ml)

and glacial acetic acid (1.0 ml). Acetone (50 ml) was added and the mixture shaken until any precipitate formed had dissolved. Ninhydrin (A.R. grade; 0.5 g) was dissolved in the mixture.

The chromatogram was then dried again at room temperature and finally at 80°C for 4 min. Glutamate was located by reference to a standard sample of glutamate (10 µg) dissolved in 1% (w/v) $NaHCO_3$ (10 mg/ml). Paper strips containing the glutamate spots were cut out and placed into vials containing 3 ml of methanol. The vials were sealed and shaken for 3 h. The colour extracted was compared with the absorbance reading of the standard at 500nm in a Shimadzu (QV-50) spectrophotometer (Section 2.12.1.).

2.9.2.5. Nitrogenase

The nitrogenase activity in A. *cylindrica* was determined by the reduction of C_2H_2 by the cells of this alga by a modified method of Nicholas & Deering (1976). Aliquots (1 ml) of thick suspension of *Anabaena* cell as prepared in Section 2.5.2. were transferred into 10 ml conical flasks each containing 1 ml of fresh culture solution without combined nitrogen (Section 2.5.2.). The flasks were fitted with Quickfit necks (C14) with single sidearms with an entry port each fitted with a Suba-seal cap (Freeman, Yorks, England). Quickfit taps connected the flasks to a six-place manifold which was in turn connected to an oil pump for evacuation as well as to a source of C_2H_2 in He through taps as described by Elleway *et al* (1971). The flasks were evacuated and then flushed with a gas mixture containing 5% (v/v) C_2H_2, 10% (v/v) O_2 and 85% (v/v) He. The flasks were kept at 30°C in a reciprocater over a bank of cool white fluorescent lights (200µE/m^2/sec). Samples of the gas phase in the flasks (0.5 ml each) were withdrawn from time to time through the Suba-seals using a gas-tight syringe. The C_2H_4 formed was determined in a Philips 4000 series gas chromatograph fitted with Poropak R columns (2 m by 2 mm) at 75°C with a flow rate of 24 ml carrier N_2/min.

2.9.3. Induction experiments with nitrate reductase

Leaves of 7-day-old barley seedlings were cut and kept base down in a 150 ml vial containing 40 ml of treatment solutions as described in the text for the particular experiment. The leaves were infiltrated with the treatment solutions by placing the vials and the contents in a dessicator (18 x 30 cm) and evacuating with an oil pump (Section 2.9.2.1.). The vials and the contents were then kept in a phytotron (Section 2.4.1.) and the leaf samples removed at various time intervals were analysed for nitrate reductase activity (*in vitro*) as described in Section 2.9.2.1.

2.9.4. *In vivo* and *in vitro* decay of nitrate reductase

For the *in vivo* experiments, maize seedlings were given a 3 h pretreatment with nitrate (4.5mM) and atrazine (0.1μM) and the loss of enzyme activity was followed. This was done by transferring the seedlings to a nitrate-free Hoagland's solution (0.1 strength) and batches of seedlings were removed for (*in vitro*) enzyme assay (Section 2.9.2.1.) at the time periods indicated. For the *in vitro* enzyme studies, leaves of non-atrazine treated seedlings grown with nitrate were homogenized with extraction solutions (Section 2.9.1.1.) containing atrazine (0, 25 and 100μM) and analysed for nitrate reductase activity (Section 2.9.2.1.).

2.10. Studies with $^{15}NO_3^-$

Maize and barley seedlings were supplied with 3mM $K^{15}NO_3$ (Section 2.2.2.) in 0.5 strength Hoagland's solution as described in the text for each experiment. The roots and shoots were harvested separately as described in Section 2.6.2.1. and the incorporation of $^{15}NO_3^-$ into total nitrogen (Kjeldahl method) and into TCA-precipitable protein was determined using the AEI MS2 mass spectrometer (Stanley & Nicholas, 1969).

Kjeldahl nitrogen was determined as follows:- fresh samples of roots and shoots (0.5 - 1 g) ground in a mortar and pestle with 5 vol of 50mM phosphate

buffer, pH 7.5 were transferred into 100 ml Kjeldahl flasks containing 2 g HgO-K_2SO_4 catalyst. Then 2 ml of 36N H_2SO_4 was added and the contents digested on electric heaters in a fume cupboard. When the contents were clarified, they were heated for a further 15 min, removed, cooled for about 10 min and 10 ml of distilled water added. The total nitrogen in the digest was determined by distillation and titration as follows:- Distillation was carried out in a Markham apparatus. Aliquots (5 ml) of digest were added to distillation flask through the funnel and about 10 ml of 40% (w/v) NaOH and 0.5 g Devarda's alloy (about 45% Al, 50% Cu and 5% Zn) added. The funnel was then washed with a little distilled water. The distillate (about 10 ml) was collected into a 50 ml Erlenmeyer flask containing 10 ml of 2% (w/v) boric acid. The distillate containing NH_3 was titrated against a standard H_2SO_4 (N/70) using 0.1 ml of indicator (0.033% w/v bromocresol green and 0.066% w/v methyl red in ethanol). The total nitrogen content was calculated from the amounts of standard acid used for titration. Then the content of the flask was concentrated to about 2 ml over a hot plate after adding 1 ml of 36N H_2SO_4. The ^{15}N enrichment was determined in the mass spectrometer.

The incorporation of $^{15}NO_3^-$ into TCA-precipitable protein was determined as follows:- the soluble protein was extracted by grinding the tissue in a mortar and pestle with 50mM phosphate buffer (pH 7.5) and centrifuging at 27,000 x *g* for 30 min (Section 2.9.1.1.). The proteins in the supernatant fraction were precipitated with 10% w/v TCA (final concentration) and collected by centrifuging as described previously. The precipitate was dissolved in 5 ml of distilled water and the total nitrogen present was determined by the Kjeldahl method described previously. The ^{15}N enrichment in the final reaction mixture was determined by means of a mass spectrometer.

2.11. Incorporation of [^{14}C]-leucine into protein

Detached leaves of barley seedlings were placed base down in 150 ml vials containing 20 ml of treatment solution (including ^{14}C-labelled leucine) as described in the text for each experiment. The leaves were then infiltrated

with reaction mixtures as described in Section 2.9.3. and the vials and their contents were placed in a phytotron (Section 2.4.1.). The leaves were collected at the time periods as indicated and the soluble proteins were extracted as described in Section 2.10. The precipitated protein was collected on a 0.22μm millipore filter, washed with 5% (w/v) TCA and then 95% (v/v) ethanol and then air dried. The dried filters were then placed horizontally at the bottom of glass scintillation vials (Packard Instrument Co., Chicago, Illinois, U.S.A.). The 5 ml of scintillation fluor consisting of PPO (0.3% w/v) and POPOP (0.03% w/v) in toluene was pipetted into the vials and these were assayed in a Packard Tri-Carb liquid scintillation spectrometer, Model 3375. Channels ratios were used to determine the counting efficiency (usually about 85%) by comparison with standard ^{14}C-toluene samples provided by the Packard Instrument Co., Chicago, U.S.A.

2.12. General Techniques and Determinations

2.12.1. Spectrophotometer

Absorbance measurements were made either in a Shimadzu (QV-50) spectrophotometer using glass cuvettes, 1 cm light path-length, or in a Turner spectrophotometer, Model 330 using a vacuum emptying glass cuvette, 1 cm light path-length.

2.12.2. Nitrate and oxygen electrodes

The uptake of nitrate was measured with a nitrate electrode (Orion, Model 93-07) and a reference calomel electrode (Radiometer K 401). The uptake of oxygen was measured with an oxygen electrode. These electrodes were inserted through ports in a lid of a vessel (8 ml) constructed of perspex. The reaction mixture was maintained at a constant temperature of 30°C by circulating water through the outer jacket. The nitrate and oxygen electrodes were connected to a Beckman expanded-scale pH meter. Oxygen uptake and the response (potential) of the nitrate electrode were recorded

simultaneously by using a two-channel Rikadenki B381 potentiometric recorder. Microorganisms (*E. coli* or A. *cylindrica*) were added to the incubation mixture in the vessel through a small centre port in the lid. The contents were continuously stirred magnetically. The recordings were compared with the calibration graph of the standard substrates.

2.12.3. Nitrate

An enzymic method using a dissimilatory nitrate reductase from *E. coli*, Strain B was used (McNamara *et al*, 1971) and the nitrite produced from nitrate was determined (Section 2.12.4.). The reaction mixtures (2 ml) dispensed in test tubes (1.5 x 10 cm) contained 0.5 ml of 0.1M phosphate buffer (pH 7.25), 1 ml of 1M Na-formate, 0.4 ml of *E. coli* enzyme preparation as described by McNamara *et al*, 1971. Aliquots from the appropriate nutrient solutions were then added to the reaction mixtures and incubated at 45°C for 2 h. The nitrite formed was then determined. The enzyme was calibrated against standard solutions of nitrate (0 - 200nmol).

2.12.4. Nitrite

Nitrite was determined by the procedure of Hewitt & Nicholas (1964). To the test solution containing nitrite, 1 ml of 1% (w/v) sulphanilamide in N HCl followed by 1 ml of 0.01% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride were added. The absorbance of the red azo dye formed was measured after 15 min at 540nm in a Turner spectrophotometer (Section 2.12.1.).

2.12.5. Protein

Protein was measured by a modified method of Lowry *et al* (1951) using bovine serum albumin as a standard. Water soluble protein was extracted from roots and shoots of maize and barley respectively as described in Section 2.10. They were treated with cold TCA (final concentration 10% w/v) and left in ice for 10 min. The precipitated protein was collected by centrifugation as described in Section 2.10. They were then treated with

0.8 ml of 2% (w/v) Na_2CO_3 in 0.1N NaOH followed by 4 ml of a mixed reagent. This reagent contained the following in a total volume of 52 ml:- 50 ml of 2% (w/v) Na_2CO_3 in 0.1N NaOH; 1 ml of 0.5% (w/v) $CuSO_4.5H_2O$ and 1 ml of 1% (w/v) sodium potassium tartrate. The protein in this mixed reagent was incubated at 37°C for 15 min and 0.5 ml of 1:1 Folin-Ciocalteu phenol reagent (Fisher Scientific Company, New York) was added. After 30 min at room temperature, the absorbance of the blue colour formed was read at 730nm in a Shimadzu (QV-50) spectrophotometer (Section 2.12.1.).

3. RESULTS

3. RESULTS

3.1. Maize and Barley

3.1.1. Growth of maize and barley

3.1.1.1. Fresh weights

Fourteen-day-old maize seedlings grown with 0.5mM NO_3^- in 0.1 strength Hoagland's solution had a greater fresh weight than those grown with an equivalent amount of ammonium chloride or without combined nitrogen (Table 6). The inclusion of 0.1μM atrazine in the culture solutions enhanced the growth of plants grown with nitrate by 26% (roots) and 31% (shoots). Atrazine had no effect on seedlings grown without combined nitrogen and the yields of roots and shoots of seedlings grown with ammonium chloride were slightly depressed by the herbicide. Atrazine (1μM) in the nutrient solution also stimulated the growth of maize plants grown for 40 days in sand culture supplied with 4.5mM NO_3^- in 0.5 strength Hoagland's solution (Table 7). Increases in fresh weight of roots and shoots resulting from the herbicide treatment were 34 and 22% respectively. Again atrazine did not increase the fresh weight of roots and shoots of plants grown with either 4.5 or 2.0mM NH_4Cl.

The effect of atrazine in stimulating growth of maize over a 7 day period is shown in Figure 2. In this experiment, 7-day-old seedlings in liquid culture were given 4.5mM NO_3^- and 0.1μM atrazine and the herbicide increased the fresh weight of roots and shoots within a day of applying the herbicide. In all the studies described above where the fresh weight of plants grown with nitrate was increased by atrazine treatment, the dry weight was unchanged.

In barley plants grown in sand culture with 4.5mM NO_3^-, increased fresh weight of roots and shoots was observed following one application of

TABLE 6

Effect of atrazine on the fresh weight of maize seedlings grown with nitrate and ammonium in liquid culture

Seven-day-old maize seedlings were grown in liquid culture containing 0.1 strength Hoagland's solution without a source of nitrogen as described in Section 2.4.1. From the 8th day onwards 10 seedlings in each tray of liquid culture were supplied with either nitrate [0.15mM $Ca(NO_3)_2$ + 0.2mM KNO_3] or ammonium chloride in 0.1 strength Hoagland's solution and 0.1μM atrazine as indicated. The seedlings were harvested after a subsequent 7 days growth.

Nitrogen source	Fresh weight (g)					
	Omit atrazine			Atrazine		
	Roots	Shoots	Total	Roots	Shoots	Total
None	0.71	0.67	1.38	0.68	0.65	1.33
Nitrate (0.5mM)	0.84	0.85	1.69	1.06	1.11	2.17
Ammonium (0.5mM)	0.64	0.78	1.42	0.58	0.71	1.29

Least significant difference $LSD_{95\%}$ for roots = 0.093 and $LSD_{95\%}$ for shoots = 0.068

TABLE 7

Effect of atrazine on the fresh weight of maize plants grown with nitrate and ammonium in sand culture

Maize plants were grown in sand culture for 40 days, supplied daily with 0.5 strength Hoagland's solution as described in Section 2.4.2. For the first 14 days, they were given no combined nitrogen and it was followed with a daily supply of nitrogen as indicated. Atrazine (0.1μM) was included in the culture solution. Plants were harvested after 40 days growth.

Nitrogen source	Fresh weight (g)					
	Omit atrazine			Atrazine		
	Roots	Shoots	Total	Roots	Shoots	Total
Nitrate (4.5mM)	17.17	15.73	32.90	23.02	19.15	42.17
Ammonium (4.5mM)	14.78	15.61	30.39	12.41	13.48	25.89
Ammonium (2.0mM)	8.81	7.76	16.57	9.77	8.22	17.99

LSD for roots = 2.96 and LSD for shoots = 0.86

FIGURE 2

Effect of atrazine on the fresh weight of maize seedlings over a period of 7 days

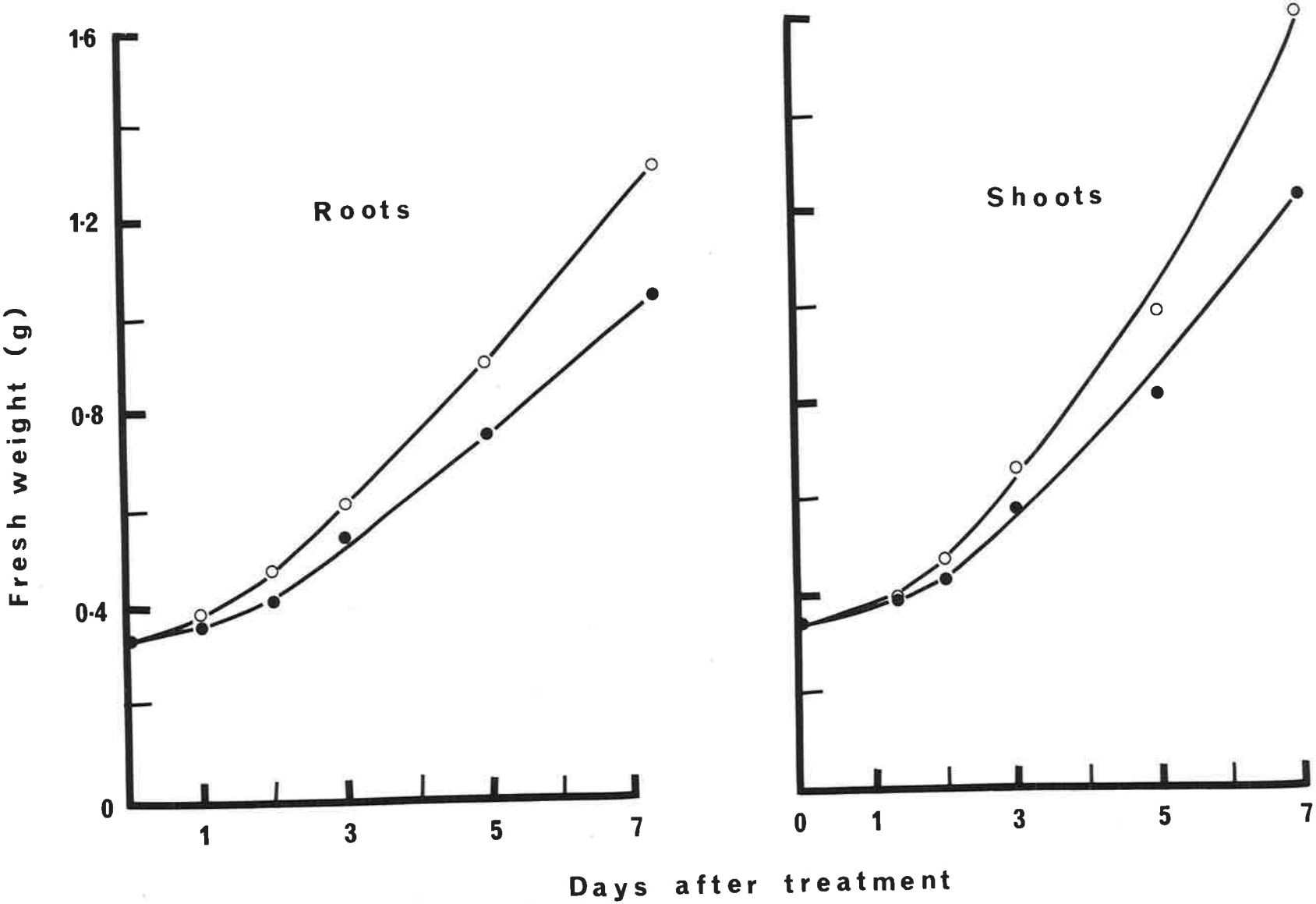
Seven-day-old maize seedlings were grown in liquid culture containing 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.1. From the 8th day onwards, 40 seedlings in each tray were supplied with 1.5mM KNO_3 + 1.5mM $Ca(NO_3)_2$ in 0.1 strength Hoagland's solution and one batch supplied with 0.1μM atrazine. Batch of 6 seedlings were harvested for analysis as indicated. Further experimental details are given in Section 2.6.2.1.

$LSD_{95\%}$ for roots = 0.035 and for shoots = 0.032

●——● Omit atrazine

o——o Atrazine

Fig 2


Roots
Shoots
Fresh weight (g)
1·6
1·2
0·8
0·4
0
1
3
5
7
0
1
3
5
7
Days after treatment

1μM atrazine (Table 8). The fresh weight of roots and shoots of barley plants harvested 2 days after the herbicide treatment were increased by 18 and 8% respectively whereas after 21 days more substantial increases were recorded viz. 53% for roots and 58% for shoots. One application of 2μM atrazine suppressed growth of roots and shoots by 12 and 39% respectively. In a separate experiment, the application of 1μM atrazine in the nutrient solution of barley plants daily for 21 days resulted in a complete dessication of the leaves. However, maize plants in sand culture tolerated daily applications of 10μM of the herbicide in the nutrient solution for 25 days while 100μM suppressed growth (Table 9). Senescence was delayed in older leaves of plants supplied with 1, 10 or 100μM atrazine.

3.1.1.2. Root length of maize

Elongation of primary root tips of 7-day-old maize seedlings was observed hourly over an 8 h period. The application of 0.1μM atrazine in the nutrient solution had significantly increased the elongation of intact primary root tips at 4 h after treatment (Figure 3). However this elongation of root tips resulting from the herbicide treatment was similar in seedlings grown with or without 1mM NO_3^-. The rate of elongation of root tips in control plants (4 - 8 h) was 6.25 mm/h whereas that of the atrazine treated plants was 7.5 mm/h as shown in Figure 3. In experiments with excised roots of 7-day-old maize seedlings, the application of 0.1μM atrazine again increased the elongation of root tips (Figure 4). The rate of growth of excised root tips was 2 mm/h for control and 3.4 mm/h for atrazine treatment. The herbicide increased the elongation of the root tips significantly starting from the 5th h after treatment as shown in Figure 4. The effect of atrazine on the total length of the primary root is illustrated in Figure 5. Seven-day-old seedlings were supplied with 4.5mM NO_3^- and 0.1μM atrazine in the liquid culture solution. Atrazine increased the length of primary root significantly from the 1st day onwards after treatment. The rate of growth of the primary root during the first two days after treatment was 20 mm/day for control and 30 mm/day for

TABLE 8

Effect of atrazine on the fresh weight of barley

Barley seedlings were grown for the first 7 days in sand culture supplied with 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.2. The seedlings were then given one application of nutrient solution containing 4.5mM NO_3^- and atrazine as indicated, followed by a daily treatment of the same nutrient medium without atrazine. Plants were harvested as indicated.

Days after treatment	Roots or Shoots	Fresh Weight			
		Atrazine (μM)			
		0	0.5	1	2
2	Roots (mg)	103	-	122	-
	Shoots (mg)	63	-	68	-
	Total (mg)	166	-	190	-
21	Roots (g)	1.04	1.44	1.59	0.91
	Shoots (g)	1.23	1.76	1.94	0.75
	Total (g)	2.27	3.20	3.53	1.66

TABLE 9

Influence of atrazine level on the fresh weight of maize

Maize plants were grown in sand culture for the first 14 days in 0.5 strength Hoagland's solution without combined nitrogen as described in Section 2.4.2. From the 15th day onwards, the plants were supplied daily with 0.5 strength Hoagland's solution containing 1.5mM $Ca(NO_3)_2$ + 1.5mM KNO_3 and atrazine at 0, 1, 10 and 100µM respectively. The plants were harvested after 40 days growth.

	Fresh weight (g)			
	Atrazine (µM)			
	0	1	10	100
Roots	15.0	21.8	15.9	6.7
Shoots	12.2	13.8	14.4	9.7
Total	27.2	35.6	30.3	16.4

FIGURE 3

Effect of atrazine on the elongation of the primary root of maize seedlings

Seven-day-old maize seedlings were grown in liquid culture containing 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.1. The seedlings were then transferred into 250 ml capacity Petri dishes (one plant per Petri dish) containing 1mM NO_3^- and 0.1μM atrazine as indicated, in 75 ml of 0.1 strength Hoagland's solution. Root elongation was measured as described in Section 2.6.1.

$LSD_{95\%}$ for nitrate treatment = 1.67

$LSD_{95\%}$ for omit nitrate treatment = 1.73

▲——▲ Omit nitrate, omit atrazine

△——△ " " , + "

●——● + " , omit "

o——o + " , + "

Fig 3

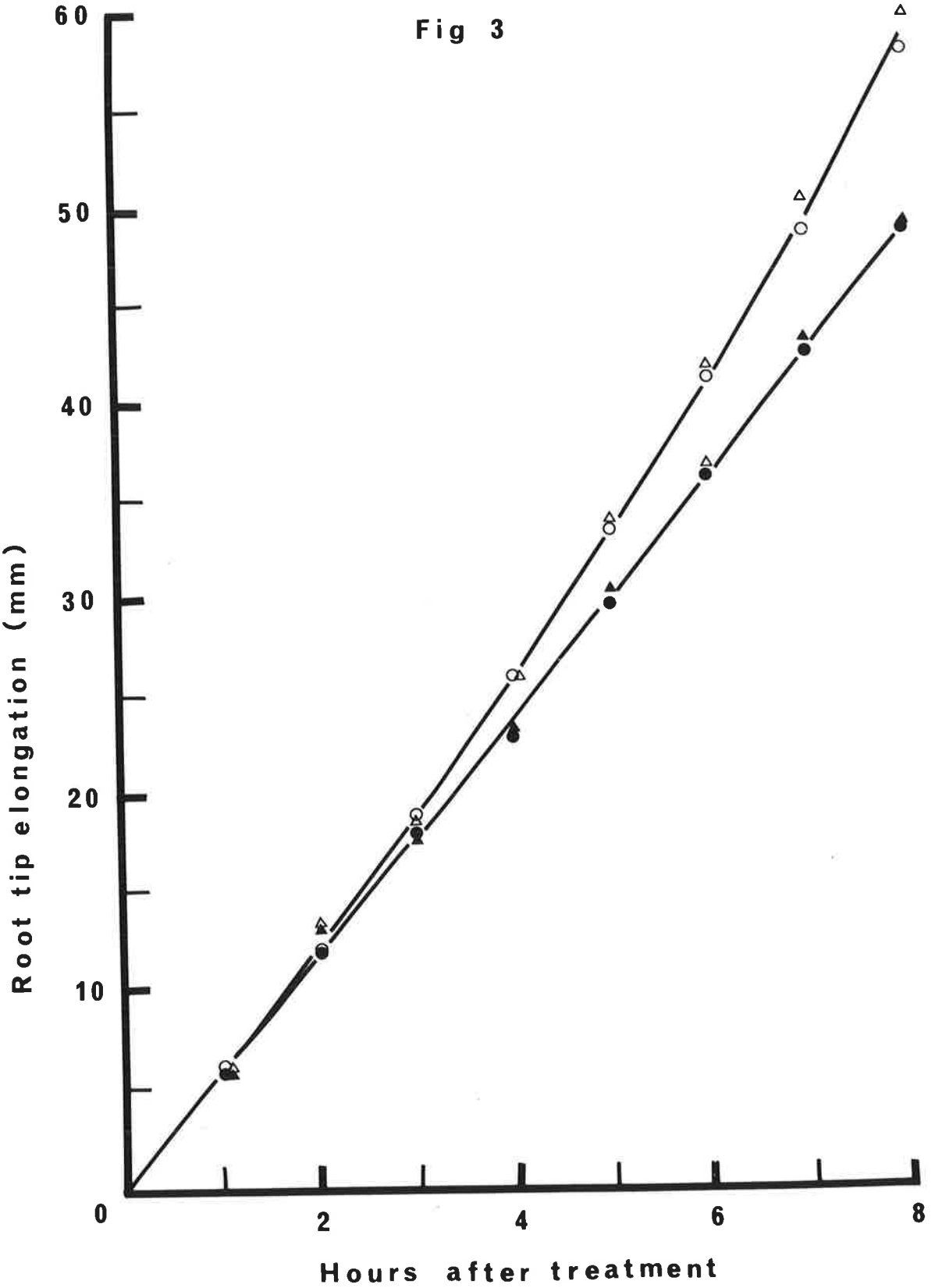

60
50
40
30
20
10
0
Root tip elongation (mm)
2
4
6
8
Hours after treatment

FIGURE 4

Effect of atrazine on the elongation of cut root tips of maize seedlings

Seven-day-old maize seedlings were grown in liquid culture containing 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.1. Primary root tips were then isolated and transferred into 60 ml capacity Petri dishes (one root tip per Petri dish) containing streptomycin sulphate (120 µg), sucrose (2% w/v) and atrazine (0.1µM) in 18 ml of 0.1 strength Hoagland's solution. Root tips elongation was measured as described in Section 2.6.1.

$LSD_{95\%} = 0.75$

●——● Omit atrazine

o——o Atrazine

Fig 4

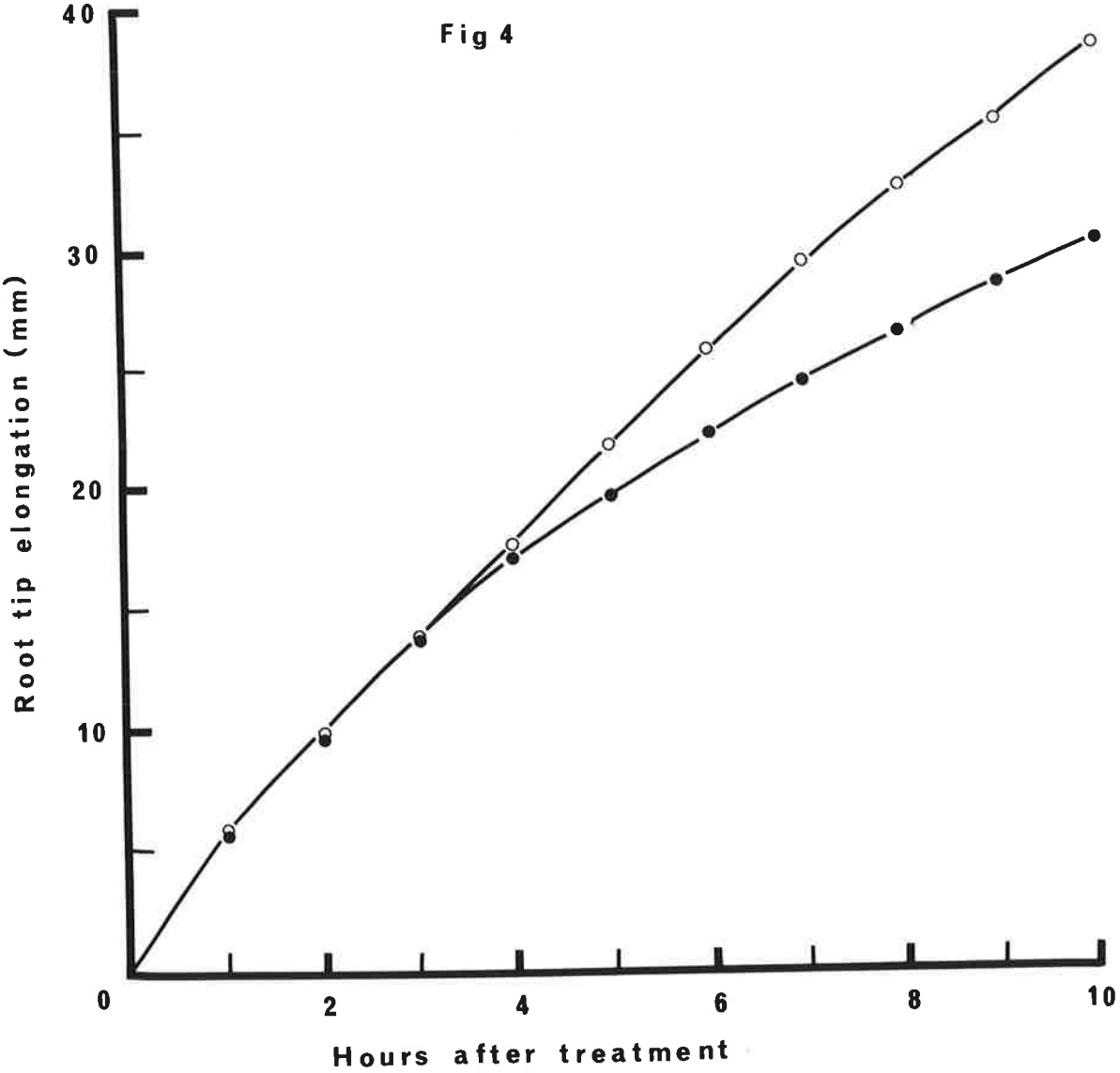

40
30
20
10
0
2
4
6
8
10
Root tip elongation (mm)
Hours after treatment

FIGURE 5

Effect of atrazine on the length of primary root of maize seedlings over a period of 7 days

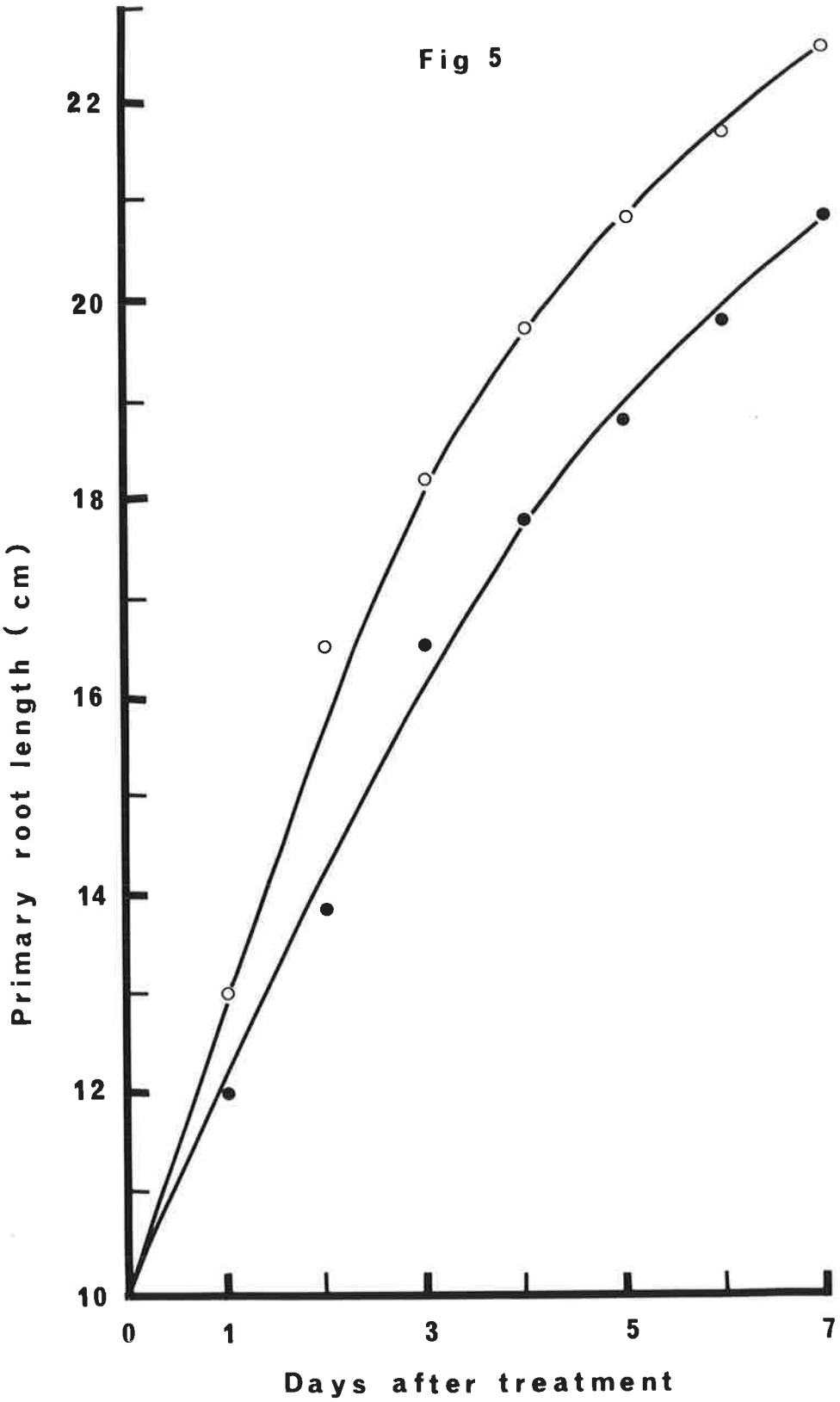
Seven-day-old maize seedlings were grown in liquid culture containing 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.1. All seedlings used in the experiment had a root length of 100 mm ± 5 mm on the 8th day. Ten such seedlings in each tray were supplied with 1.5mM KNO_3 + 1.5mM $Ca(NO_3)_2$ in 0.1 strength Hoagland's solution and 0.1μM atrazine. The length of the primary root was measured in mm each day over the next 7 days. The experiment was conducted in a phytotron at the usual growth condition (Section 2.4.1.).

$LSD_{95\%}$ = 9.4

●——● Omit atrazine

o——o Atrazine

Fig 5


22
20
18
16
14
12
10
Primary root length (cm)
0
1
3
5
7
Days after treatment

atrazine treated seedlings. However, the growth rate of primary root declined after the 3rd day of treatment, when the adventitious roots started to appear (4 roots per plant). The growth of adventitious roots was linear over the remaining period of the experiment (2 - 7 days) and atrazine treatment significantly increased their growth also (Figure 6).

3.1.2. Nutrient uptake in maize

3.1.2.1. Nitrate

3.1.2.1.1. Uptake by seedlings

Seven-day-old maize seedlings grown without combined nitrogen in liquid culture as described in Section 2.4.1. were transferred into 1mM NO_3^- in 0.1 strength Hoagland's solution (pH 4) with and without atrazine (0.1µM). The depletion of nitrate from this nutrient medium was determined as described in Section 2.12.3. The uptake of nitrate from the culture solution showed an initial lag phase and after 4 h a steady rate of nitrate uptake was observed as shown in Figure 7. The inclusion of atrazine in the nutrient solution increased the uptake rate of nitrate by the plants at 2 h after treatment. The steady state uptake rates for control and atrazine treatments were 1.31 and 1.72 µmol NO_3^-/g fr wt/h respectively.

3.1.2.1.2. Nitrate content of roots and shoots

A summary of several studies on the nitrate content of extracts of roots and shoots of maize plants is shown in Table 10. In experiment A, 7-day-old seedlings supplied with 0.5mM NO_3^- and 0.1µM atrazine in the culture solution were harvested after one week. Atrazine increased the nitrate content of roots by 105% and shoots by 92%. Similar results were obtained with extracts of roots and shoots of 40-day-old plants (experiment C). Here the plants grown in sand cultures were provided with 4.5mM NO_3^- and 1µM atrazine daily in the nutrient solution from the 15th day onwards. In experiment B, the uptake of nitrate in roots and shoots was examined in

FIGURE 6

Effect of atrazine on the length of adventitious roots of maize seedlings over a period of 7 days

Experimental details as in Figure 5. The adventitious roots appeared only 2nd day after treatments.

$LSD_{95\%}$ = 5.1

●——● Omit atrazine

o——o Atrazine

Fig 6

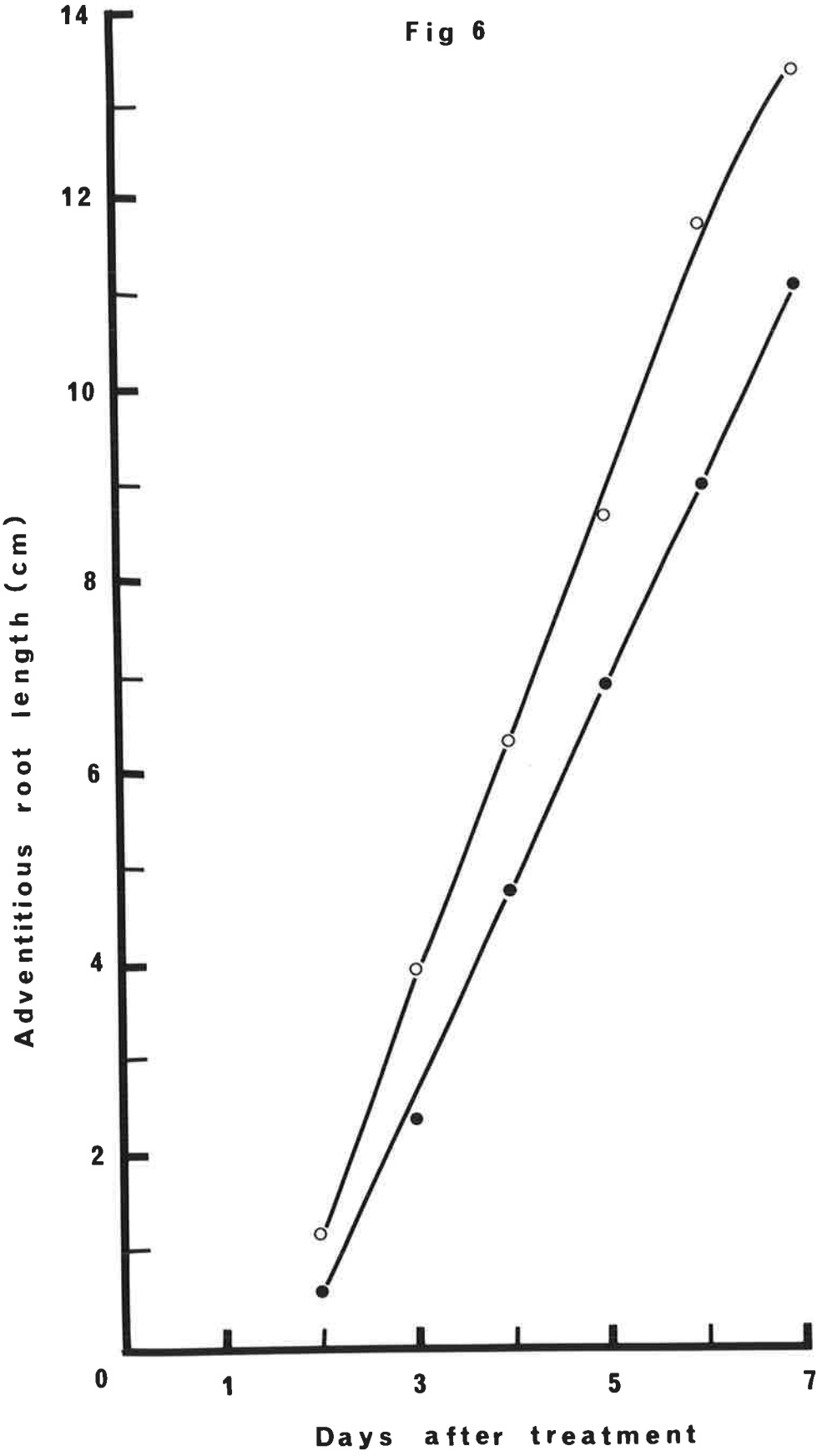

14
12
10
8
6
4
2
0
1
3
5
7
Adventitious root length (cm)
Days after treatment

FIGURE 7

Effect of atrazine on the rate of uptake of nitrate by maize seedlings

Seven-day-old maize seedlings were grown in liquid culture supplied with 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.1. One batch of 40 seedlings was then transferred into a fresh Hoagland's solution (0.1 strength) containing 1mM NO_3^- and 0.1μM atrazine while another batch of 40 seedlings was treated with the same nutrient solution but without atrazine. The uptake of nitrate from the nutrient solution by the seedlings was determined using *E. coli* nitrate reductase enzyme as described in Section 2.7.1.1. The rate of uptake of nitrate (μmol NO_3^-/g fr wt/h) is plotted against time (h).

●——● Omit atrazine

o——o Atrazine

Fig 7

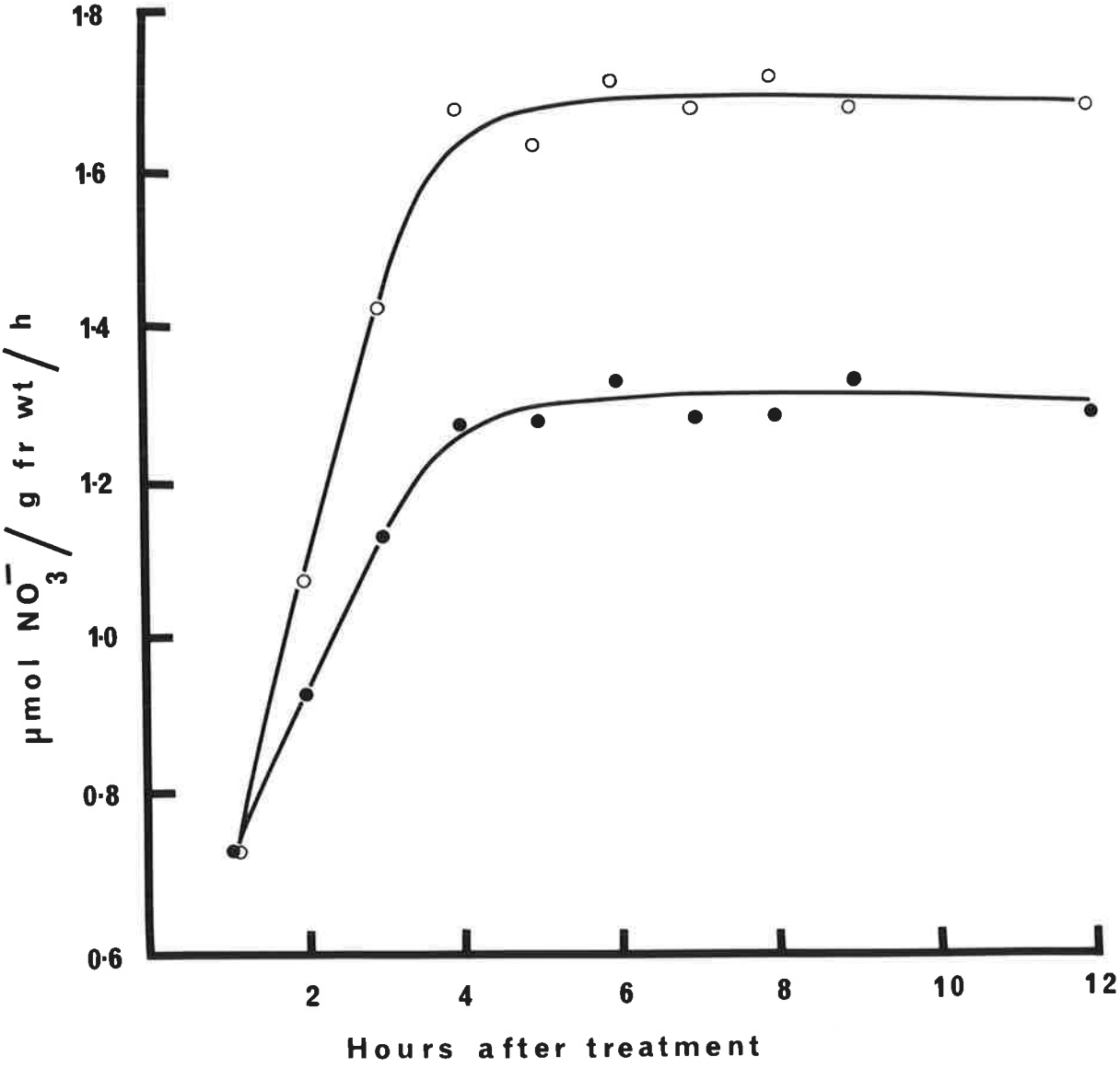

1·8
1·6
1·4
1·2
1·0
0·8
0·6
µmol NO_3^- / g fr wt / h
2
4
6
8
10
12
Hours after treatment

TABLE 10

Effect of atrazine on nitrate content of roots and shoots of maize

In experiments A and B, 7-day-old seedlings were grown in liquid culture supplied with 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.1. From the 8th day onwards, they were supplied with equimolar $Ca(NO_3)_2$ and KNO_3 (0.5mM NO_3^- for experiment A and 4.5mM NO_3^- for experiment B) and 0.1μM atrazine in 0.1 strength Hoagland's solution. In experiment C, the maize plants were grown in sand culture provided with 4.5mM NO_3^- in 0.5 strength Hoagland's solution as described in Table 7.

Experiment	NO_3^- level (mM)	Age at harvesting (days)	No. of days after treatment for harvesting	NO_3^- content (mg/g fr wt)			
				Roots		Shoots	
				Omit atrazine	Atrazine	Omit atrazine	Atrazine
A	0.5	14	7	0.97	2.0	0.48	0.92
B	4.5	8	1	1.4	2.0	0.37	0.55
		9	2	2.8	3.4	0.73	1.1
		10	3	3.3	5.8	1.1	1.7
		12	5	3.8	5.5	1.3	1.7
		14	7	3.3	5.0	1.15	1.8
C	4.5	40	25	0.4	0.95	0.2	0.34

7-day-old seedlings over a further period of 7 days. The plants were supplied with 4.5mM NO_3^- and 0.1μM atrazine in liquid culture and the plants were harvested as indicated (Table 10 and Figure 8). The nitrate content per g fr wt of roots and shoots increased up to the 3rd day and then remained relatively constant. The nitrate content of the roots was always higher than that of the shoots.

3.1.2.1.3. Metabolic and storage pools of nitrate

According to Ferrari *et al* (1973) the measurement of anaerobic nitrite production in the absence of exogenous nitrate indicates the size of the metabolic or active pool of nitrate. Measurements of this active pool in the root tips of 14-day-old plants grown with 0.5mM NO_3^- with and without atrazine are shown in Figure 9. The herbicide increased the rate of nitrite production as well as the final amount of nitrite accumulated, which is a measure of the active pool of nitrate: 61 and 79 nmol/g fr wt for control and atrazine treated plants respectively. To test whether the atrazine effect resulted in a leakage of nitrate from the storage pool to the metabolic pool, the following experiment was undertaken (Table 11). Root tips (0 - 4 cm) from 14-day-old maize seedlings grown with a higher nitrate supply viz. 4.5mM were sampled. Atrazine was only supplied in the reaction mixture. Nitrite production was measured after 3.5 h incubation under anaerobic conditions. Addition of 0.1μM atrazine at the beginning of the anaerobic incubation or after a 2 h incubation did not increase the anaerobic nitrite production. Therefore the herbicide did not result in a leakage or redistribution of nitrate from the storage to the metabolic pool. However, in the study of the active pool of nitrate as outlined in Figure 9, maize seedlings had been supplied with atrazine (0.1μM) for a week.
A comparison of the active pool of nitrate with total nitrate content is shown in Table 12. The size of the storage pool in the root tips was about 275 times larger than the metabolic pool for plants grown with or without atrazine . The herbicide increased the metabolic pool of nitrate as well as

FIGURE 8

Effect of atrazine on nitrate content in roots and shoots of maize

Histogram for data in Table 10, experiment B.

Open histogram:- omit atrazine

Shaded histogram:- atrazine

Fig 8

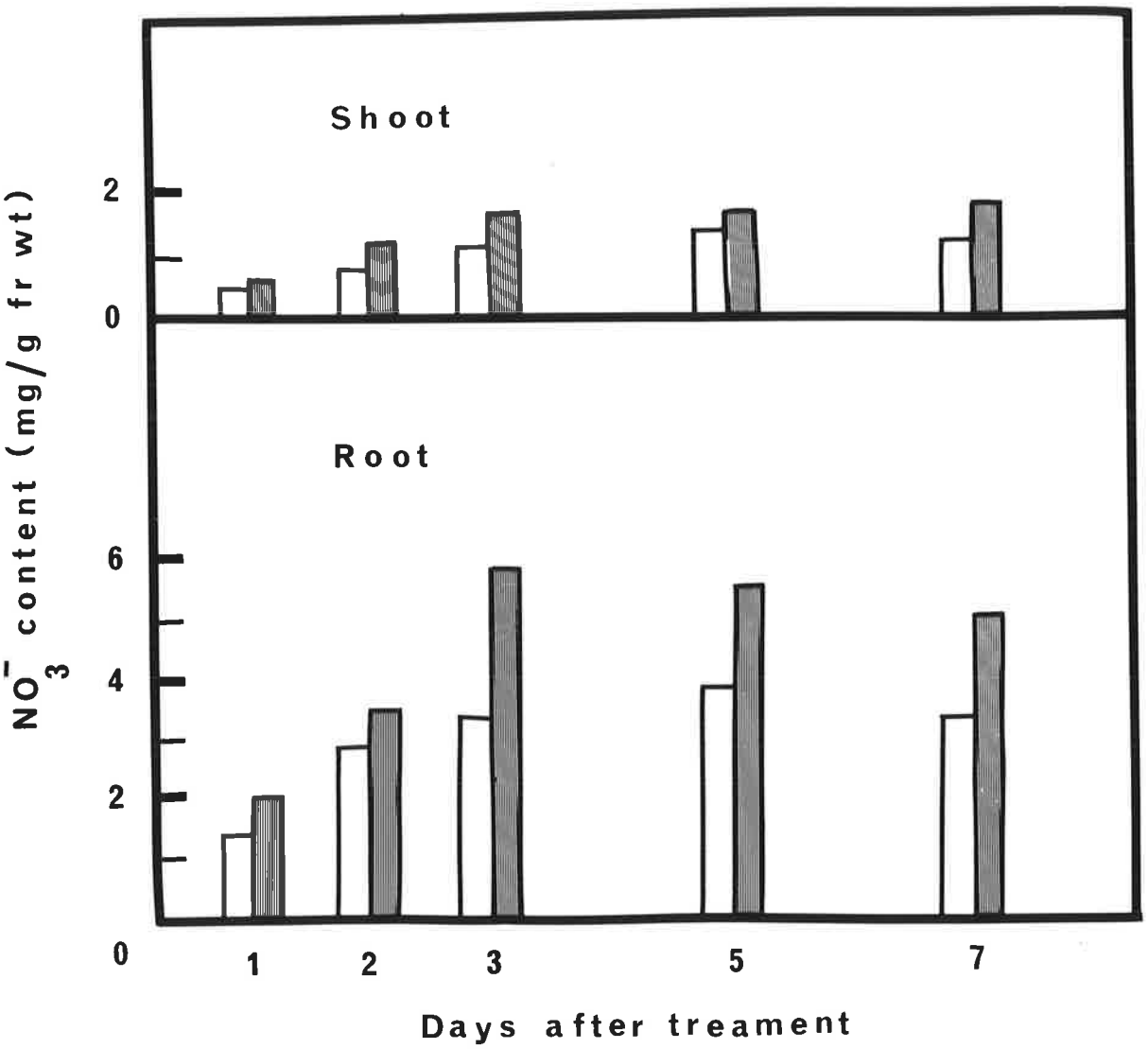

Shoot
Root
2
0
6
4
2
0
NO_3^- content (mg/g fr wt)
1
2
3
5
7
Days after treament

FIGURE 9

Effect of atrazine on the metabolic pool of nitrate in root tips of maize

Seven-day-old seedlings were grown in 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.1. They were then transferred into a fresh 0.1 strength Hoagland's solution containing 0.5mM NO_3^- and 0.1μM atrazine for the next 7 days. The roots were washed with distilled water and root tips (0 - 20 mm) were placed in Warburg flasks containing 0.1M phosphate buffer (pH 7.5) as described in Section 2.7.1.3. The flasks were then evacuated and flushed with N_2 gas and the production of nitrite during the anaerobic incubation was measured as described in Section 2.12.4.

●——● Omit atrazine

o——o Atrazine

Fig 9

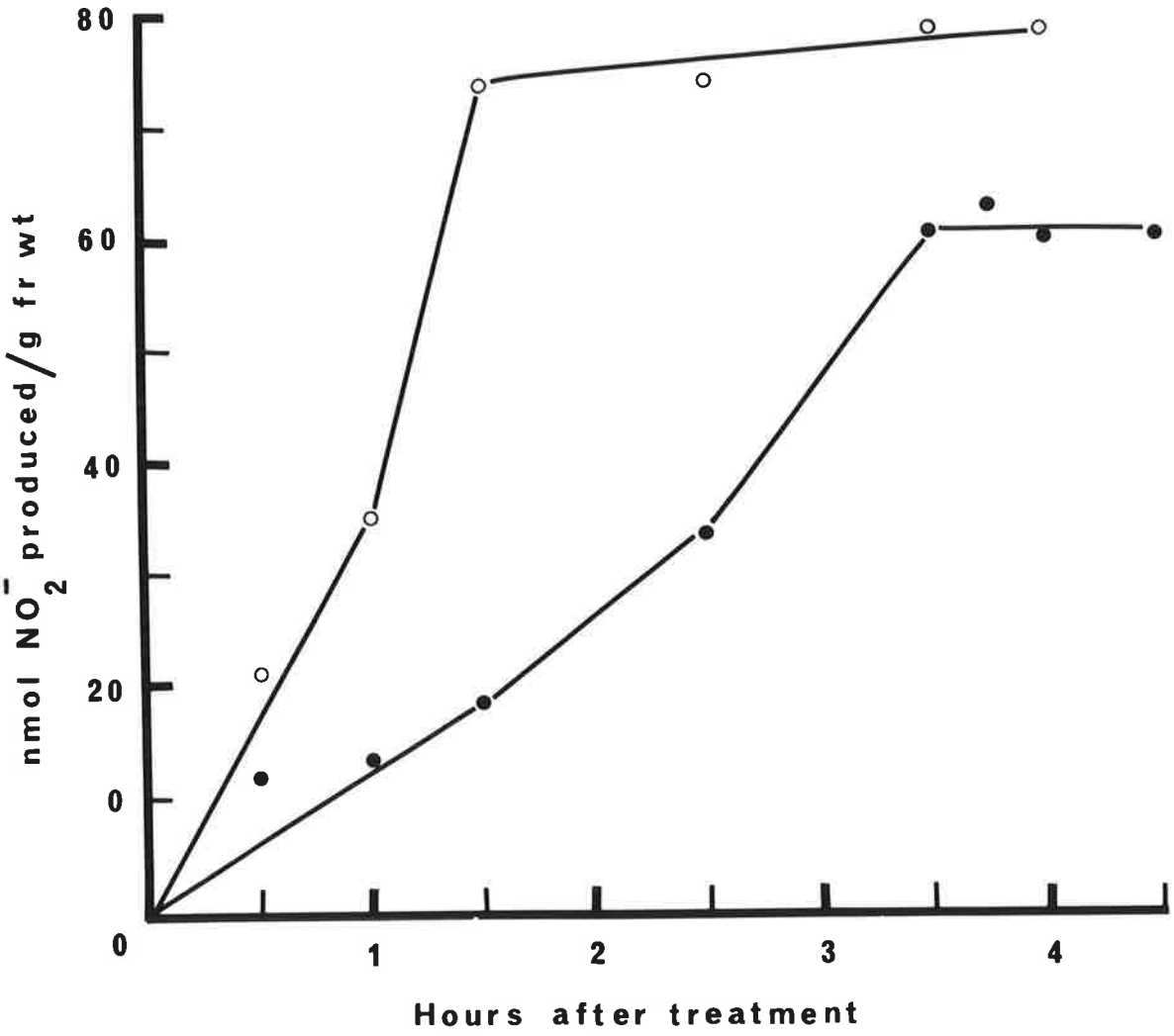

80
60
40
20
0
0
1
2
3
4
nmol NO_2^- produced/g fr wt
Hours after treatment

TABLE 11

Effect of atrazine on the production of nitrite in root tips of maize under anaerobic conditions

Fourteen-day-old maize seedlings were grown in liquid cultures containing 4.5mM NO_3^- as described in Section 2.4.1. Nitrite production in the root tips under anaerobic conditions was measured as described in Section 2.7.1.3. after a 3.5 h incubation period.

Expt	Reaction procedure	Production of nitrite (nmol NO_2^-/g fr wt)
(A)	Standard procedure (for anaerobic nitrite production as described in Section 2.7.1.3.)	700
(B)	As in (A) but 0.1μM atrazine added in the reaction mixture	670
(C)	As in (A) but 0.1μM atrazine added after 2 h incubation	660

TABLE 12

Effect of atrazine on the metabolic pool and total nitrate content of root tips of maize

	Nitrate content (nmol/g fr wt)	
	Omit atrazine	Atrazine
Metabolic nitrate	61	79
Total nitrate	16,100	22,300

Data for root tips examined in Figure 9.

the total nitrate content in root tips of maize seedlings.

3.1.2.2. Other cations and anions

The root and shoot samples that were analysed for nitrate content in experiment B, Table 10 were also analysed for K^+, Ca^{2+}, Mg^{2+}, Cl^- and PO_4^{3-} (Table 13). The accumulation of K^+ was always enhanced by the atrazine treatment and maximum increases were recorded at 3 days. This pattern of accumulation was similar to that observed for nitrate but the effect of the herbicide was less for K^+ than for nitrate, only 30% increase in roots and 13% in shoots on the 3rd day after the initial application of nitrate (4.5mM) and atrazine (0.1μM). The herbicide treatment increased the uptake of Ca^{2+} and Mg^{2+} in roots only on the 3rd day after treatment, 39 and 23% respectively, but was without effect on these divalent cations in shoots. The herbicide had no effect on the uptake of phosphate or chloride in roots and shoots.

3.1.3. Xylem sap of maize

3.1.3.1. Rate of exudation

When maize shoots were excised, the exudates from the cut roots could be collected. Fourteen-day-old maize seedlings grown in liquid culture with 0.5mM NO_3^- secreted more xylem sap (60 μl/plant/h) than did those grown with 0.5mM NH_4Cl (42 μl/plant/h) as shown in Table 14. Atrazine increased the rate of sap secretion of seedlings grown with nitrate by 70% while it had no effect on those supplied with ammonium chloride. Maize plants (40-day-old) grown in sand cultures, secreted more xylem sap than did the 14-day-old seedlings (Table 14). Here again plants supplied with nitrate exuded more xylem sap than those given ammonium chloride. The response of the 40-day-old plants to atrazine treatment was similar to that described for 14-day-old seedlings i.e. atrazine increased the rate of exudation of xylem sap only in plants grown with nitrate.

TABLE 13

Effect of atrazine on ion accumulation in root and shoot of maize

Ion	No. of days after atrazine treatment	Age of maize (days)	mg ion/g fr wt			
			Roots		Shoots	
			Omit atrazine	Atrazine	Omit atrazine	Atrazine
K^+	1	8	1.08	1.25	2.00	2.17
	2	9	1.46	1.58	2.67	3.00
	3	10	1.92	2.50	3.67	4.17
	5	12	2.25	2.67	3.67	3.83
	7	14	2.17	2.42	3.83	4.16
Ca^{2+}	1	8	0.09	0.09	0.21	0.21
	2	9	0.11	0.14	0.34	0.34
	3	10	0.18	0.25	0.48	0.52
	5	12	0.21	0.23	0.52	0.52
	7	14	0.22	0.21	0.55	0.64
Mg^{2+}	1	8	0.08	0.08	0.16	0.16
	2	9	0.11	0.11	0.18	0.19
	3	10	0.13	0.16	0.19	0.19
	5	12	0.13	0.14	0.17	0.16
	7	14	0.12	0.12	0.16	0.16
Cl^-	1	8	0.97	0.97	2.13	2.39
	2	9	1.24	1.33	2.30	2.30
	3	10	1.33	1.50	2.48	2.57
PO_4^{3-}	1	8	0.33	0.39	0.39	0.40
	2	9	0.48	0.36	0.45	0.42
	3	10	0.48	0.42	0.42	0.39
	5	12	0.45	0.48	0.39	0.42
	7	14	0.42	0.45	0.36	0.36

The root and shoot samples analysed were those described in experiment B of Table 10

TABLE 14

Effect of atrazine on the rate of exudation of xylem sap

The 14-day-old maize seedlings were grown in 0.1 strength Hoagland's solution containing 0.5mM NO_3^- or 0.5mM NH_4Cl and 0.1μM atrazine as described in Section 2.4.1.

The 40-day-old maize plants were grown in sand provided with 0.5 strength Hoagland's solution containing 4.5mM either NO_3^- or NH_4Cl and 1μM atrazine as described in Section 2.4.2.

Xylem sap was collected as described in Section 2.8.1.

Expt	Age of plants (days)	Exudation of xylem sap (μl/plant/h)			
		Nitrate grown plants		Ammonium grown plants	
		Omit atrazine	Atrazine	Omit atrazine	Atrazine
(A)	14				
(1)		60	110	40	35
(2)		60	100	42	37
(3)		55	100	40	35
(B)	40				
(1)		200	390	150	160
(2)		220	420	170	200
(3)		200	370	150	140

3.1.3.2. Amino acid and amide content

The amino compounds of the xylem sap of plants grown with nitrate and ammonium respectively are compared in Table 15a. In the 40-day-old plants grown in sand, there were higher amounts of amino compounds (27.8 μmol/ml) in plants given ammonium compared with those supplied with nitrate (10.7 μmol/ml). The main amino compound in the sap was glutamine, which was 60 and 72% of the total amino compounds in plants grown with 4.5mM nitrate or ammonium respectively (Table 15a). The composition of other amino compounds in plants grown with this amount of nitrate and ammonium respectively were as follows (% of total amino compounds):- serine (12 and 8%), asparagine (4.5 and 5%) and alanine (5 and 3%). Other amino compounds in Table 15a constitute less than 5% of the total amino compounds. In plants grown with 4.5mM ammonium (Table 15a) glutamine was 213% higher than in exudates of those supplied with nitrate. Other amino compounds which were significantly increased in plants grown with ammonium compared with those given nitrate were asparagine (184%), glutamic acid (150%), tyrosine (148%), phenylalanine (367%) and lysine (113%). The amounts of aspartic acid, proline and half cystine were similar in the sap of plants grown with either ammonium or nitrate nitrogen.

Atrazine treatment increased the total amino compounds of the exudates of plants grown with nitrate by 82% but the herbicide slightly decreased (10%) the amino compounds of plants grown with ammonium. Atrazine treatment increased asparagine (149%), glutamine (88%), alanine (102%), arginine (112%), lysine (91%), histidine (92%), phenylalanine (87%), leucine (75%) and aspartic acid (51%) in the xylem sap of nitrate grown plants.

Maize plants grown for 40 days with 2mM ammonium (Table 15b) had a lower total amount of amino compounds in the sap (5.1 μmol/ml) compared with those grown with 4.5mM ammonium (Table 15a) but atrazine again had no effect on the total amino content.

The effects of atrazine on the xylem sap of 14-day-old maize seedlings

TABLE 15a

Effect of atrazine on the amino acid and amide contents of xylem sap collected from 40-day-old maize plants supplied with 4.5mM nitrate and ammonium nitrogen respectively

Maize plants were grown in sand as described in Section 2.4.2. From the 15th day onwards the plants were supplied daily with 0.5 strength Hoagland's solution containing either 4.5mM nitrate [1.5mM $Ca(NO_3)_2$ + 1.5mM KNO_3] or 4.5mM ammonium chloride, supplemented with 1μM atrazine as indicated. After 40 days growth xylem sap was collected and analysed for amino compounds as described in Section 2.8.2.

Amino compounds	nmol amino compound per ml of xylem sap			
	4.5mM nitrate grown plants		4.5mM ammonium grown plants	
	Omit atrazine	Atrazine	Omit atrazine	Atrazine
Aspartic acid	232	350	240	344
Threonine	259	410	442	398
Serine	1,308	2,054	2,146	1,792
Asparagine	475	1,181	1,350	1,328
Glutamine	6,392	11,994	20,015	17,786
Proline	40	59	37	40
Glutamic acid	202	307	505	476
Glycine	103	192	148	116
Alanine	504	1,016	723	606
Valine	303	439	541	511
Half Cystine	77	72	78	70
Methionine	9	14	16	15
Isoleucine	71	103	108	121
Leucine	63	110	116	119
Tyrosine	31	43	77	75
Phenylalanine	15	28	70	58
γ-Aminobutyric acid	24	29	55	79
Ornithine	49	53	150	98
Lysine	235	450	501	430
Histidine	85	155	134	109
Tryptophan	25	21	39	34
Arginine	175	371	315	273
Total	10,677	19,451	27,806	24,878

TABLE 15b

Effect of atrazine on the amino acid and amide contents of xylem sap collected from 40-day-old maize plants supplied with 2.0mM ammonium

Maize plants were grown as described in Table 15a but with 2.0mM ammonium. Xylem sap was collected and analysed for amino compounds as described in Section 2.8.

Amino compounds	nmol amino compound per ml of xylem sap	
	Omit atrazine	Atrazine
Aspartic acid	154	128
Threonine	71	77
Serine	300	297
Asparagine	144	220
Glutamine	3,582	3,826
Proline	-	-
Glutamic acid	139	106
Glycine	18	5
Alanine	135	98
Valine	196	207
Half Cystine	-	-
Methionine	7	10
Isoleucine	26	25
Leucine	26	24
Tyrosine	27	22
Phenylalanine	8	8
γ-Aminobutyric acid	37	29
Ornithine	29	29
Lysine	66	82
Histidine	-	5
Tryptophan	25	36
Arginine	37	24
Total	5,127	5,258

grown with 4.5mM nitrate are shown in Table 16. The sap of these younger plants contained almost the same amount of total amino compounds as the 40-day-old plants previously described (Table 15a). Glutamine was again the major component and was present at the same level as in the older plants i.e. 60% of the total. The sap from the younger plants contained higher amounts of asparagine, alanine and lysine but less serine than those of older plants. Atrazine treatment increased the level of all the amino compounds but the following were very markedly increased:- glutamine, alanine and all the basic amino compounds. The application of 0.1μM atrazine increased the total amino compounds by 146% viz. 20.7 and 8.4 μmol/ml for atrazine and non-atrazine treatment respectively.

3.1.3.3. Transport of nitrogen fractions from roots to shoots

A summary of the effect of atrazine on the organic and inorganic nitrogen fractions of the xylem sap from 40-day-old maize plants is presented in Table 17. Organic nitrogen only was transported in the sap of plants grown with ammonium whereas in nitrate grown plants, both organic nitrogen (91.6% of the total nitrogen) and inorganic nitrogen (8.4%) were present. In nitrate grown plants, atrazine increased the organic nitrogen by 86% and inorganic (nitrate) nitrogen by 63%. Nitrate was not detected in the xylem sap of maize plants grown with ammonium and atrazine decreased the organic nitrogen contents of these exudates by 10%. Neither nitrite nor protein was detected in the xylem sap of plants grown with either nitrate or ammonium.

The results in Table 18 show that the rate of transport of total organic nitrogen of xylem sap in the absence of the herbicide was more in ammonium grown plants than those given nitrate. However,atrazine increased the rate of exudation of xylem sap as well as the total amount of amino compounds in plants grown with nitrate but not in those given ammonium (Section 3.1.3.1.). It follows that in the presence of atrazine, more total organic nitrogen per h (190 μg N/plant/h) was transported to the shoots of nitrate grown plants than in those supplied with ammonium with or without

TABLE 16

Effect of atrazine on the amino acid and amide contents of xylem sap collected from 14-day-old maize seedlings supplied with 4.5mM nitrate

Fourteen-day-old seedlings were grown in 0.1 strength Hoagland's solution containing 4.5mM NO_3^- and atrazine (as indicated) for 7 days as described in Section 2.4.1. Xylem sap collected and analysed for amino compounds as described in Section 2.8

Amino compounds	nmol amino compound per ml of xylem sap		
	Atrazine (μM)		
	0	0.05	0.1
Aspartic acid	162	270	185
Threonine	223	388	415
Serine	641	1,415	1,236
Asparagine	1,028	1,595	1,919
Glutamine	4,910	9,048	12,984
Proline	160	321	271
Glutamic acid	252	470	542
Glycine	40	75	62
Alanine	553	2,368	2,112
Valine	40	88	84
Half Cystine	127	137	127
Methionine	4	20	19
Isoleucine	37	84	80
Leucine	32	118	106
Tyrosine	12	51	47
Phenylalanine	8	38	29
γ-Aminobutyric acid	-	-	4
Ornithine	18	124	66
Lysine	87	202	233
Histidine	23	70	122
Tryptophan	35	68	47
Arginine	49	189	180
Total	8,441	17,139	20,770

TABLE 17

Effect of atrazine on various nitrogen fractions of the xylem sap

The xylem sap was obtained from 40-day-old maize plants described in Table 15a and the amounts of organic nitrogen calculated from the data in the table.

Types of nitrogen	µg N per ml of xylem sap			
	4.5mM nitrate grown plants		4.5mM ammonium grown plants	
	Omit Atrazine	Atrazine	Omit Atrazine	Atrazine
Organic nitrogen				
(a) ninhydrin positive amino nitrogen	150	272	390	348
(b) amide nitrogen of asparagine and glutamine	96	185	307	274
(c) other nitrogen of amino acid	15	29	22	23
(d) total	261	486	719	645
Inorganic nitrogen				
Nitrate	24	39	0	0
Total nitrogen	285	525	719	645

TABLE 18

Effect of atrazine on the rate of transport of nitrogen compounds in the xylem sap

	Nitrate grown plants		Ammonium grown plants	
	Omit Atrazine	Atrazine	Omit Atrazine	Atrazine
Rate of exudation of xylem sap (µl/plant/h)	200	390	150	160
Organic nitrogen in the xylem sap (µg N/µl xylem sap)	0.261	0.486	0.719	0.645
Rate of transport of organic nitrogen through xylem sap (µg N/plant/h)	52	190	108	103
Nitrate nitrogen in the xylem sap (µg N/µl xylem sap)	0.024	0.039	0	0
Rate of transport of nitrate nitrogen through xylem sap (µg N/plant/h)	5	15	-	-

Data shown was compiled from Tables 14, 15a and 17

atrazine (approximately 105 µg N/plant/h). In addition to organic nitrogen, nitrate grown plants also transported nitrate, which was also increased by the herbicide treatment (viz. 15 and 5 µg N/plant/h with and without atrazine respectively).

3.1.4. Enzymes assimilating nitrate in maize and barley

3.1.4.1. Nitrate reductase

3.1.4.1.1. Cell-free extracts of roots, shoots (maize and barley) and scutella (maize)

Extracts of 3-day-old maize root tips (0 - 2 cm) and scutella respectively were prepared either in a mortar and pestle or with a mechanical homogenizer (2.9.1.1.) for assay of nitrate reductase. With both roots and scutella higher enzyme activity was recorded in extracts prepared in a mortar and pestle (Table 19). The *in vitro* nitrate reductase activities of root tips were 620 and 528nmol NO_2^- produced/g fr wt/h in the extracts prepared in the mortar and pestle and by mechanical homogenizer respectively. Extraction for longer periods in mechanical homogenizer e.g. 60 sec resulted in a decline in enzyme activity as shown in Table 19. Similar results were obtained for the scutella. The enzyme activity in the root tips was more than twice than those of root tips. Almost 90% of the total nitrate reductase activity recovered was obtained in the initial supernatant fraction of roots and scutella respectively when a mortar and pestle was used for enzyme extraction (Table 20).

Nitrate reductase activity was detected in extracts of 10-day-old barley roots only when casein was included in the extraction medium (Table 21). The enzyme activity was also increased by 50% in extracts of shoots prepared with 2% (w/v) casein and was more stable. In a separate study, only 6% of the initial enzyme was lost during storage at 0°C for 24 h in extracts of barley shoots when a higher concentration of casein (3% w/v) was included in

TABLE 19

A comparison of mortar and pestle and mechanical homogenizer for the extraction of nitrate reductase from root tips and scutella of 3-day-old maize seedlings

Maize seedlings were grown in Petri dishes containing 1% (w/v) agar and 0.1 strength Hoagland's solution with 5mM NO_3^- for the first 3 days from germination as described in Section 2.4.1. The root tips (0 - 2 cm) and scutella were harvested and cell-free extracts were made with either a mortar and pestle or a mechanical homogenizer (Section 2.9.1.1.) at the time periods shown. Nitrate reductase activity was assayed as described in Section 2.9.2.1.

Samples analysed	Nitrate reductase activity (nmol NO_2^- produced/g fr wt/h)				
	M & P	MH.5	MH.15	MH.30	MH.60
Root tips (0 - 2 cm)	620	528	514	510	480
Scutella	1,790	1,533	1,573	1,168	1,013

M & P: Mortar and Pestle
MH.5 : Mechanical Homogenizer for 5 sec
MH.15: " " " 15 "
MH.30: " " " 30 "
MH.60: " " " 60 "

TABLE 20

Nitrate reductase activity in various supernatant fractions in cell-free extracts prepared in a mortar and pestle of 3-day-old maize root tips and scutella respectively

Maize seedlings were grown for 3 days and cell-free extracts of root tips and scutella respectively prepared in a mortar and pestle as described in Table 19. The extracts were centrifuged at 27,000*g* for 15 min at 0°C and the 1st supernatant was collected. The pellet obtained in this centrifugation was resuspended in fresh extraction medium and ground again in a mortar and pestle. This extract was again centrifuged and the 2nd supernatant fraction was obtained. The pellet thus obtained was extracted and centrifuged again to obtain the 3rd supernatant fraction. All three supernatant fractions were assayed for nitrate reductase activity as described in Section 2.9.2.1.

Samples analysed	Nitrate reductase activity (nmol NO_2^- produced/g fr wt/h)		
	1st Supernatant	2nd Supernatant	3rd Supernatant
Root tips (0 - 2 cm)	637	69	0
Scutella	1,467	219	0

TABLE 21

Effect of casein on nitrate reductase activity in extracts of roots and shoots of barley seedlings

Seven-day-old barley seedlings were grown in sand without combined nitrogen as described in Section 2.4.2. They were then supplied with 4.5mM NO_3^- in 0.5 strength Hoagland's solution. Roots and shoots were harvested when the seedlings were 10-day-old and extracts made with 0.5mM Na-EDTA, 1mM cysteine in 0.5M phosphate buffer (pH 7.5) containing the casein level indicated. These were assayed for nitrate reductase activity (Section 2.9.2.1.) just after preparation and after storage for 24 h in ice.

Tissue	Casein level in the extraction medium (% w/v)	Nitrate reductase activity (nmol NO_2^- produced/g fr wt/h)	
		0 h	After 24 h
Roots	0	0	0
	2	960	400
Shoots	0	2,790	520
	2	4,300	1,890

the extraction medium. In a further study using barley leaves, it was shown that the inclusion of either 1mM cysteine or 3% (w/v) casein in the extraction medium increased the enzyme activity in extracts by 123 and 82% respectively (Table 22). The maximum increase was produced when both cysteine and casein were included in the extraction medium. In maize, maximum activity and stability of nitrate reductase for root and shoot samples was achieved with 5 and 20mM cysteine respectively in the extraction medium (Table 23). The inclusion of cysteine (2mM) in the extraction solution did not enhance the enzyme activity of the maize scutella. When 5mM cysteine was added in the extraction medium, enzyme activity was reduced in maize scutella.

3.1.4.1.2. *In vivo* and *in vitro* assays of the enzyme in maize roots

When a root tip sample was placed in a Thunberg tube containing nitrate and 5% (v/v) n-propanol in 0.1M phosphate (pH 7.5), nitrite accumulation was detected (Figure 10). Evacuation of the tube enhanced the rate of nitrite accumulation; the higher vacuum created by an oil pump produced higher enzyme activity than the water pump evacuation as shown in Figure 20. Similar findings were obtained with the maize scutella samples.

Rhodes & Stewart (1974) used an *in vivo* assay method for nitrate reductase in which the tissue was first subjected to a freeze-thaw procedure. In Table 24 it is shown that the *in vivo* nitrate reductase activity of maize root tips was reduced by the freeze-thaw step. As shown earlier by Rhodes & Stewart (1974), the addition of NADH into the assay medium enhanced the production of nitrite by the freeze-thawed sample whereas the inclusion of the FMN and $Na_2S_2O_4$ decreased the nitrite production in both standard and freeze-thaw *in vivo* assay procedure (Table 24). It is also shown in the Table that the freeze-thaw step released the enzyme from the tissue and it was recovered in the supernatant fraction after centrifugation.

Results of *in vitro* nitrate reductase activity (using mortar and pestle) were compared with those of the *in vivo* activity (oil pump evacuation) for

TABLE 22

Effect of casein and cysteine on nitrate reductase activity in extracts of leaves of barley seedlings

Seven-day-old barley seedlings were grown in sand without combined nitrogen as described in Section 2.4.2. Leaves were detached and kept in vials containing 5mM NO_3^- in 0.1 strength Hoagland's solution and they were vacuum infiltrated for 2 min with this medium. The vials and their contents were then incubated in a phytotron (Section 2.4.1.) for 12 h. The enzyme was prepared with the various extraction media shown and assayed for nitrate reductase activity (Section 2.9.2.1.).

Experiments	Extraction medium	Nitrate reductase activity (nmol NO_2^- produced/g fr wt/h)
(1)	0.5mM Na-EDTA in 0.05M phosphate buffer (pH 7.5)	1,892
(2)	As (1) with 3% (w/v) casein	3,440
(3)	As (1) with 1mM cysteine	4,214
(4)	As (1) with 3% (w/v) casein and 1mM cysteine	6,106

TABLE 23

Effect of cysteine on nitrate reductase activity in extracts of roots, shoots and scutella of maize seedlings

Scutella were harvested from 3-day-old maize seedlings grown in 1% (w/v) agar containing 5mM NO_3^- in 0.1 strength Hoagland's solution as described in Section 2.4.1.

Roots and shoots were harvested from 14-day-old maize seedlings which were grown for the first 7 days without combined nitrogen followed by 4.5mM NO_3^- in 0.1 strength Hoagland's solution as described in Section 2.4.1.

Extracts were made with the media indicated and nitrate reductase activity was assayed as described in Section 2.9.2.1.

Tissue	Cysteine (1mM) in the extraction medium *	Nitrate reductase activity (nmol NO_2^- produced/g fr wt/h)	
		0 h	After 24 h
Roots	0	1,378	284
	5	2,033	840
	20	1,909	762
Shoots	0	455	227
	5	2,722	2,194
	20	2,608	2,346
Scutella	0	1,962	1,093
	2	1,948	888
	5	1,279	528

* Extraction medium contained 0.5mM Na-EDTA, 3% (w/v) casein in 50mM phosphate buffer (pH 7.5)

TABLE 24

A study of in vivo assay procedures for nitrate reductase

Maize root tips (0 - 2 cm) were harvested from 3-day-old seedlings grown in nutrient agar (1% w/v) containing 5mM NO_3^-. In the freeze-thaw technique 0.5 g root tips were placed in a thin-walled test tube (1.5 x 10 cm) which was then immersed in liquid nitrogen for 1 min and then thawed for 1.5 min in a water bath at 30°C. This procedure of freeze-thawing was repeated 6 times and then substrate solutions (5 ml of assay mixture) were added. 5 ml of standard assay mixture (techniques 1 and 2) contained 0.5µmoles of potassium nitrate and 1% (v/v) n-propanol in phosphate buffer (pH 7.5). In techniques 3, 4 and 5, 5 ml of this assay mixture also contained either (FMN and $Na_2S_2O_4$) or NADH as indicated. The freeze-thaw treatment was followed by vacuum infiltration of the assay mixtures with an oil pump. They were then incubated at 25°C for 1 h and the nitrite produced measured (Section 2.12.4.).

No.	Techniques	Nitrate reductase activity (nmol NO_2^- produced/g fr wt/h)
(1)	Standard *in vivo* assay method (using oil pump evacuation) as described in Figure 10.	216
(2)	Technique (1) but root tips were immersed in the substrate medium after freeze-thawing (6 cycles) as described in the legend.	75
(3)	Technique (2) but the substrate medium contained 1.2µmoles of NADH in addition.	151
(4)	Technique (1) but the substrate medium contained in addition 1.5µmoles of FMN and 5 mg $Na_2S_2O_4$.	4.7
(5)	Technique (2) but the substrate medium contained in addition 1.5µmoles of FMN and 5 mg $Na_2S_2O_4$ (Rhodes and Stewart, 1974).	28
(6)	Root tips placed in 0.1M phosphate buffer (pH 7.5), freeze-thawed for 6 cycles and centrifuged at 30,000*g* for 15 min. Nitrate reductase activity was assayed in the supernatant fraction as described in Section 2.9.2.1.	201

FIGURE 10

Effect of evacuation procedures on an in vivo assay of nitrate reductase

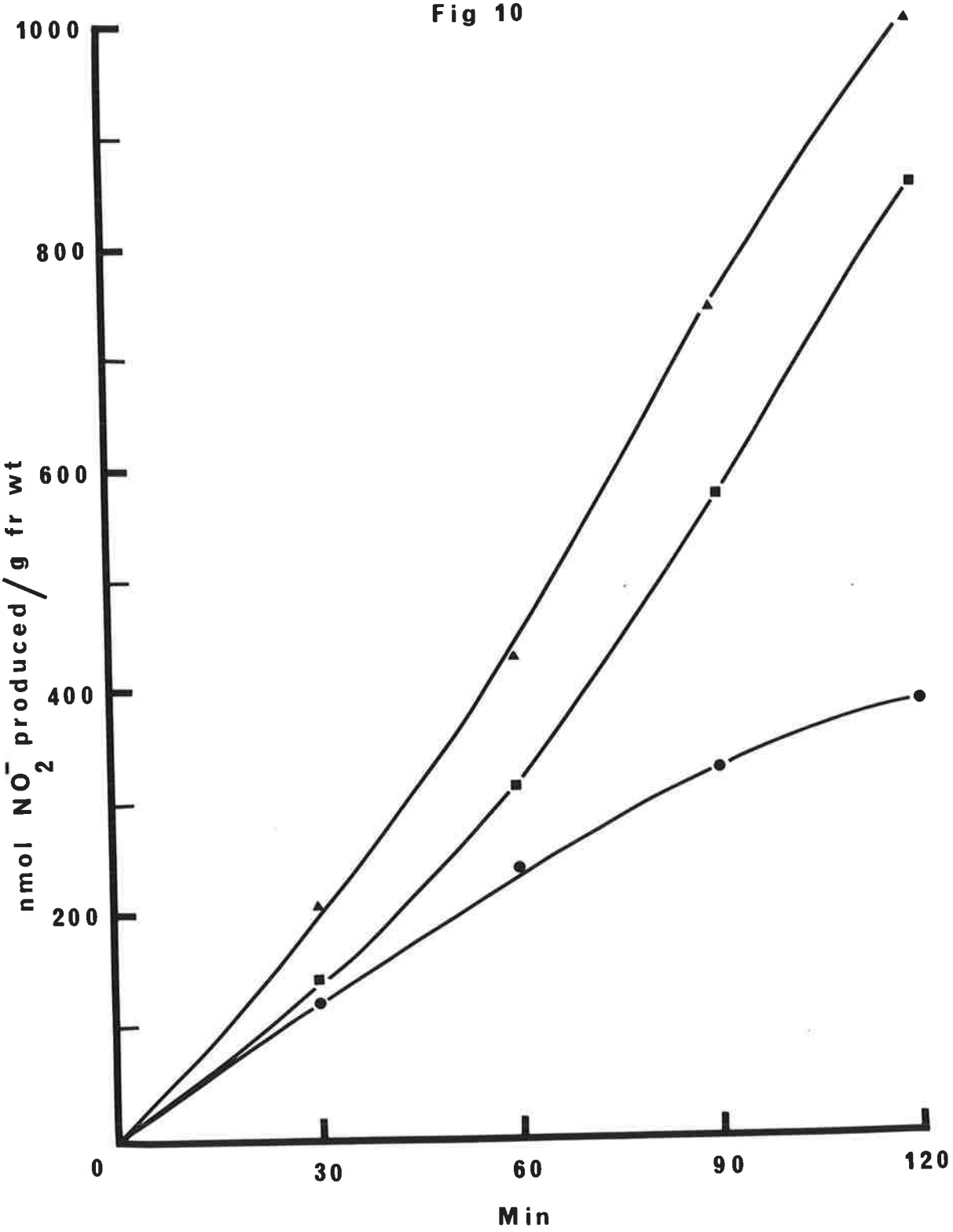
Root tips (0 - 2 cm) were obtained from 3-day-old maize seedlings grown in Petri dishes containing 1% (w/v) agar and 0.1 strength Hoagland's solution with 5mM NO_3^-. Root tips (0.5 g) were then placed in Thunberg tubes together with 2.5 ml of 0.2M phosphate buffer, 1.0 ml of 0.5M KNO_3, 1.25 ml of distilled water and 0.25 ml of n-propanol, pH 7.5. The tubes were then evacuated using either a water pump (12 - 15 mm of Hg) or an oil pump ($1 - 5x10^{-3}$ mm of Hg) for 2 min as described in Section 2.9.2.1. Evacuated tubes were then kept in a shaking water bath at 25°C and aliquots (0.5 ml) of incubation medium were removed every 30 min and nitrite production measured as described in Section 2.12.4. Thunberg tubes were evacuated every time after samples removed.

●——● No evacuation

■——■ Water pump evacuation

▲——▲ Oil pump evacuation

Fig 10



root tips (0 - 2 cm) of 3-day-old maize seedlings (Table 25). The seedlings were grown in Petri dishes containing 1% (w/v) agar and 5, 20 and 50mM KNO_3 in 0.1 strength Hoagland's solution respectively. Higher enzyme activity in the root tips reflected the increased supply of nitrate in the nutrient solutions. The *in vitro* and *in vivo* activities were similar and a constant ratio (approximately 1) was maintained at all nitrate levels as shown in Table 25.

3.1.4.1.3. Enzyme induction in leaves of barley seedlings

The production of nitrate reductase in detached leaves of maize as a function of time is shown in Figure 11. The cut ends of detached leaves were immersed in 0.1 strength Hoagland's solution containing various concentrations of nitrate (0, 1, 3, 5 and 10mM) and incubated in a phytotron (Section 2.4.1.). The nitrate reductase activity of leaf extracts increased following the nitrate treatment. Inclusion of atrazine in the incubation solution enhanced the induction of enzyme in detached leaves of maize and barley as shown in Figures 12 and 13 respectively. The cut leaves were placed in vials containing incubation solution (0.1 strength Hoagland's solution with 5mM NO_3^-) and graded amounts of atrazine (0, 5, 10, 25, 50 and 100µM respectively) and kept in a phytotron for 12 h (Section 2.4.1.). Atrazine at 10µM produced the maximum effect on nitrate reductase activity (26% increase over non-atrazine treatment) of detached leaves of maize (Figure 12) whereas in barley leaves, the maximum production of enzyme (32% increase over non-atrazine treatment) was obtained with 25µM atrazine (Figure 13).

The nitrate reductase activity was significantly increased within 16 h in intact leaves of barley seedlings (7-day-old) supplied with 1µM atrazine in the nutrient solution as shown in Figure 14a. There was a lag period up to about 4 h followed by an increase in enzyme activity in both control and atrazine treatments. At the 16th h, atrazine treatment increased

TABLE 25

A comparison of in vitro and in vivo assays for nitrate reductase in root tips of maize seedlings

Maize root tips (0 - 20 mm) were harvested from 3-day-old seedlings grown on 1% (w/v) agar in 0.1 strength Hoagland's solution. The nitrate concentrations in the agar medium were 5, 20 and 50mM respectively. Nitrate reductase activity was assayed in root tip by both *in vitro* and *in vivo* methods as described in Section 2.9.2.1.

Nitrate concentrations in agar (mM)	Nitrate reductase activity (nmol NO_2^- produced/g fr wt/h)		
	In vitro assay	*In vivo* assay	Ratio *In vitro*/*In vivo*
5	557	546	1.02
20	960	843	1.14
50	1,174	1,311	0.9

FIGURE 11

Time course for the production of nitrate reductase in detached leaves of maize with various concentrations of nitrate

Maize seedlings (14-day-old) were grown in 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.1. Young leaves (3rd from emergent leaf) were cut and their bases immersed in a set of 4 vials (150 ml capacity) each containing 40 ml of 0.1 strength Hoagland's solution with 1, 3, 5 or 10mM NO_3^- respectively. The leaves were then vacuum infiltrated with the incubation medium for 2 min and kept in phytotron (Section 2.4.1.). Nitrate reductase activity was assayed in cell-free extracts of leaves at time periods indicated as described in Section 2.9.2.1.

●——● 1mM NO_3^-

o——o 3 " "

▲——▲ 5 " "

■——■ 10 " "

Fig 11

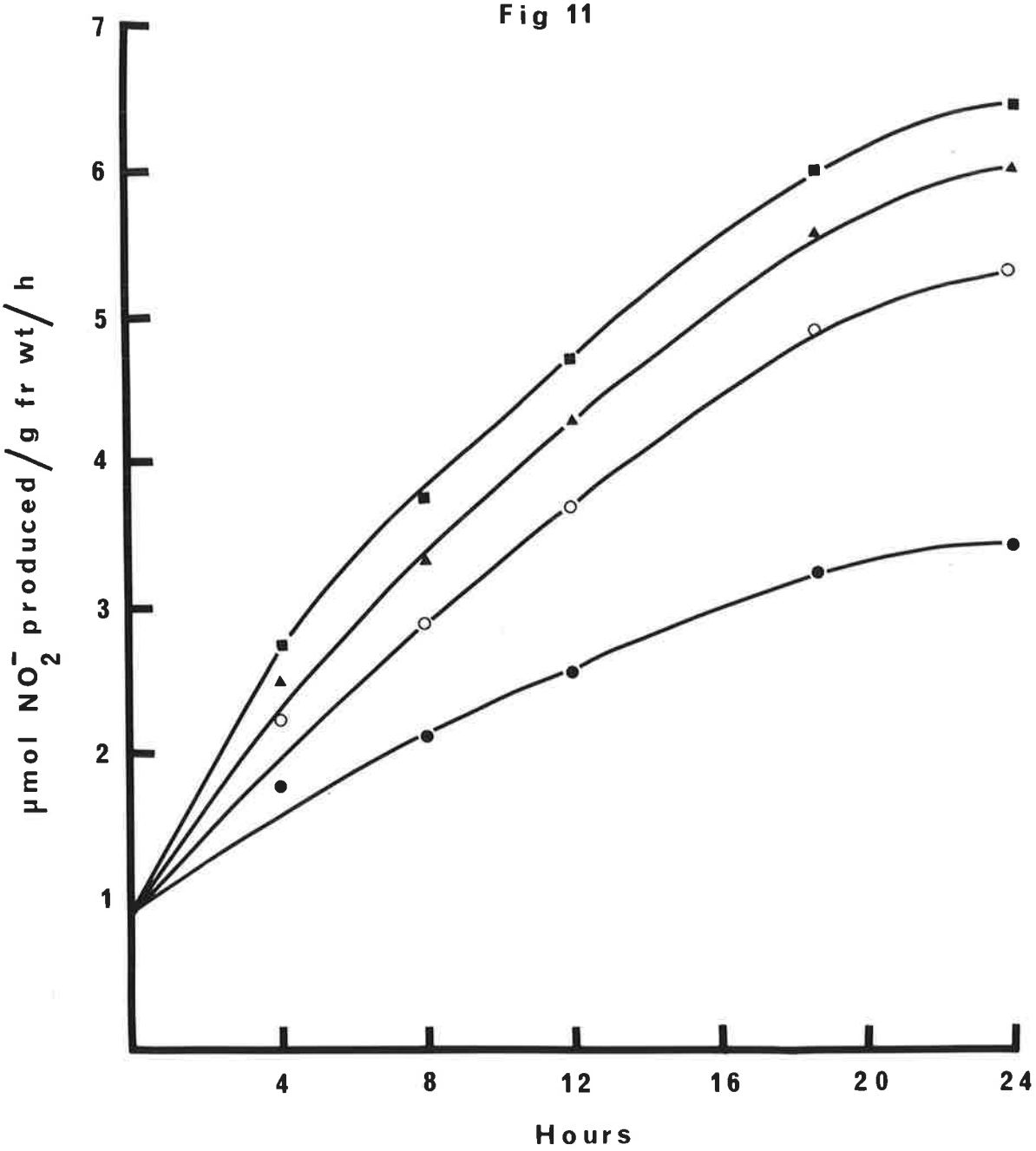


FIGURE 12

Effect of atrazine on the production of nitrate reductase in detached maize leaves

Maize seedlings (14-day-old) were grown in 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.1. Young leaves (3rd from emergent leaf) were cut and their bases immersed in a set of 6 vials (150 ml capacity) each containing 40 ml of 4.5mM NO_3^- in 0.1 strength Hoagland's solution. Graded amounts of atrazine were included in the vials as indicated. The leaves were then vacuum infiltrated with the incubation medium for 2 min and then kept in a phytotron (Section 2.4.1.) for 12 h. Nitrate reductase activity was assayed in cell-free extracts of leaves as described in Section 2.9.2.1.

Fig 12

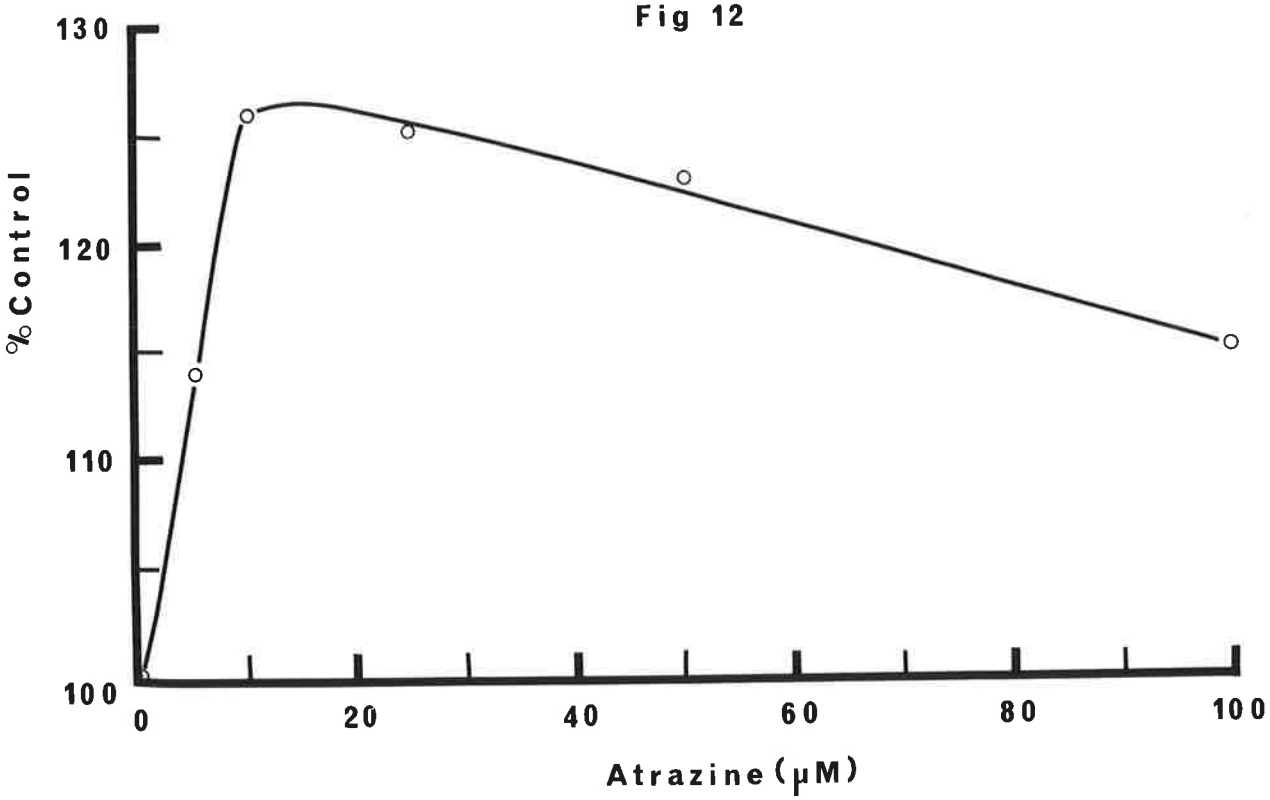


FIGURE 13

Effect of atrazine on the production of nitrate reductase in detached barley leaves

Barley seedlings (7-day-old) were grown in sand supplied with 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.2. Leaves were detached and treated as described in Figure 12. The nitrate reductase activity was determined as described in the same Figure.

Fig 13

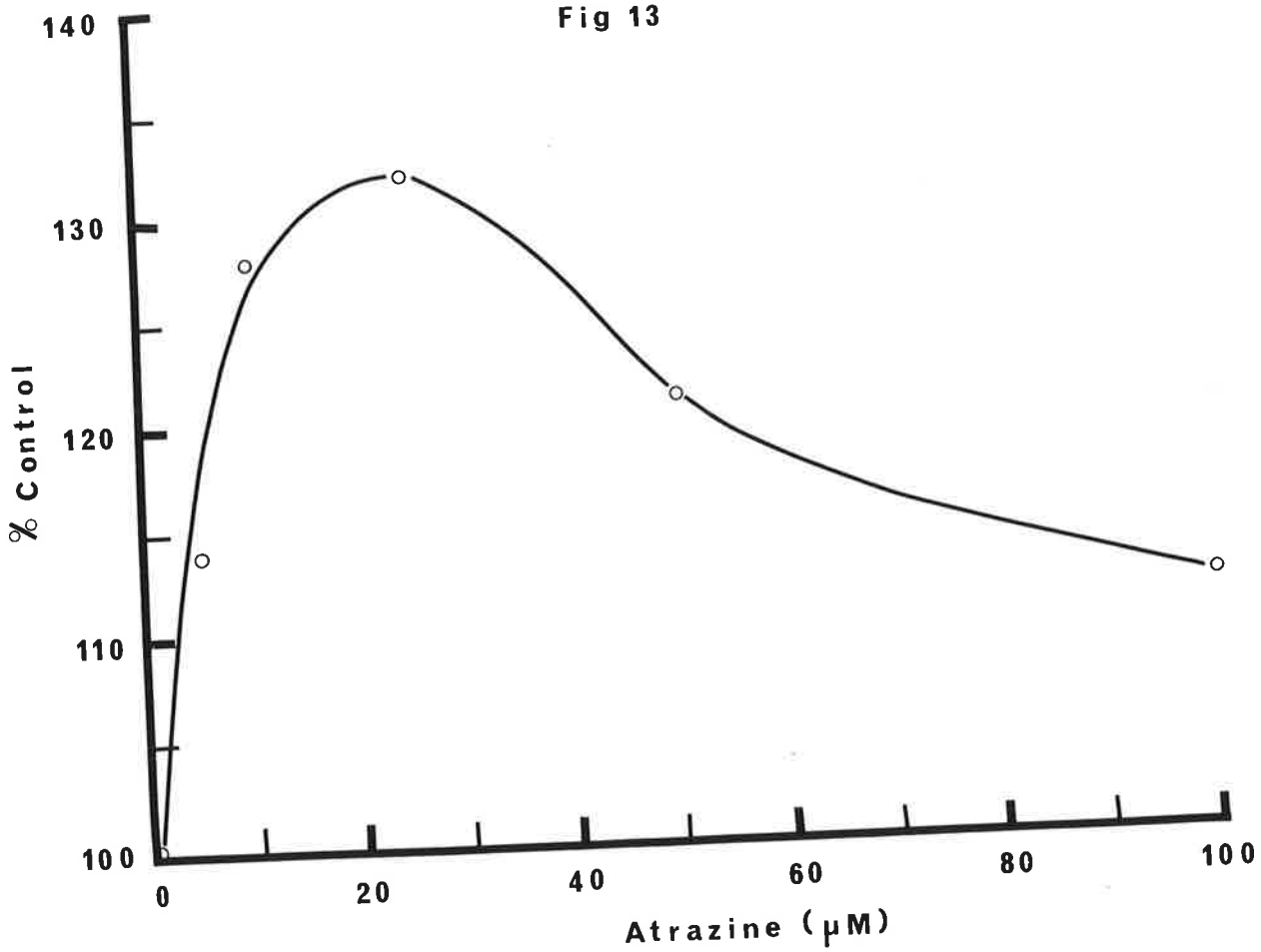


FIGURE 14a

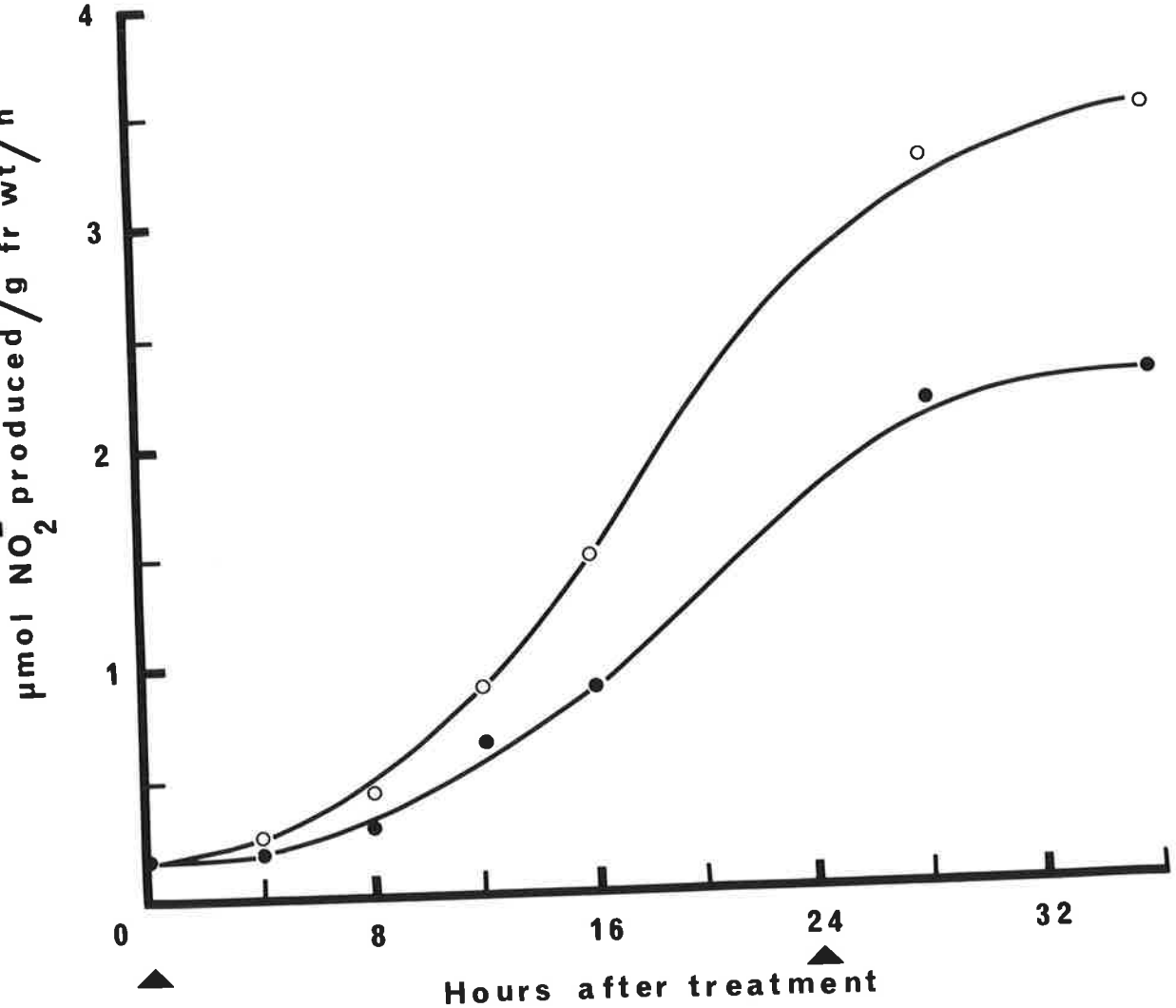
Effect of atrazine on the induction of nitrate reductase in intact leaves of barley seedlings, (a) over a period of 0 - 36 h

Seedlings (7-day-old) grown in sand culture provided with 0.1 strength Hoagland's solution without combined nitrogen were subsequently supplied with 4.5mM NO_3^- and 1μM atrazine in their nutrient solutions at 2 days as indicated by arrows (0 and 24 h). The nitrate reductase activity was determined in extracts of leaves at the times indicated as described in Section 2.9.2.1. Control plants were grown without atrazine.

●——● Omit atrazine

o——o Atrazine

Fig 14a


4
3
2
1
0
µmol NO_2^- produced/g fr wt/h
8
16
24
32
Hours after treatment

the induction of nitrate reductase activity in leaves of barley seedlings by 70% over those without atrazine application. This stimulation of enzyme production by the herbicide was observed over a longer period (14 days) when 1μM atrazine was supplied to 7-day-old seedlings on 3 successive days as shown in Figure 14b. In this investigation the enzyme activity increased steadily over the first 6 days after treatment and then the rate of increase declined gradually between 6 and 14 days in both atrazine and non-atrazine treatments. Atrazine increased the enzyme activity in leaves of barley seedlings by 50% after 14 days of treatment compared with the control leaves. However, when 1μM atrazine was supplied to the seedlings each day up to the 14 days, the enzyme activity increased up to the 6th day and then decreased (Figures 15 & 16). The application of 0.5μM atrazine increased the enzyme activity to the same extent as did 1μM of the herbicide but over a longer period (9 days). Higher levels (>1μM) of the herbicide resulted in a smaller stimulation of the enzyme and these effects were apparent for short periods, e.g. 2 days for 5μM atrazine (Figure 16). No enzyme activity was detected in plants supplied with atrazine in the absence of nitrate.

3.1.4.1.4. Enzyme synthesis in detached leaves of barley

To determine whether atrazine increased the activity of preformed nitrate reductase rather than the rate of its synthesis, isolated leaves from 7-day-old seedlings were treated with cycloheximide (Figure 17) and 6-methyl purine (Figure 18) for 6 h followed by the addition of atrazine in a medium containing 4.5mM NO_3^- in 0.1 strength Hoagland's solution. Both cycloheximide (at two concentrations 6 and 12 μg/ml) and 6-methyl purine (67 μg/ml) prevented the synthesis of nitrate reductase and under these conditions atrazine did not increase the activity of the enzyme. The nitrate reductase activity was increased for atrazine treatments when inhibitors were absent (Figures 17 & 18) which suggests that a continued synthesis of protein is

FIGURE 14b

Effect of atrazine on the induction of nitrate reductase in intact leaves of barley seedlings, (b) over a period of 0 - 14 days

The seedlings (7-day-old) were grown as described in Figure 14a but atrazine (1μM) was added for 3 days as indicated by arrows (0, 1 and 2 days). The nitrate reductase activity was determined in extracts of leaves at the times indicated as described in Section 2.9.2.1. Control plants were grown without atrazine.

●——● Omit atrazine

o——o Atrazine

Fig 14b

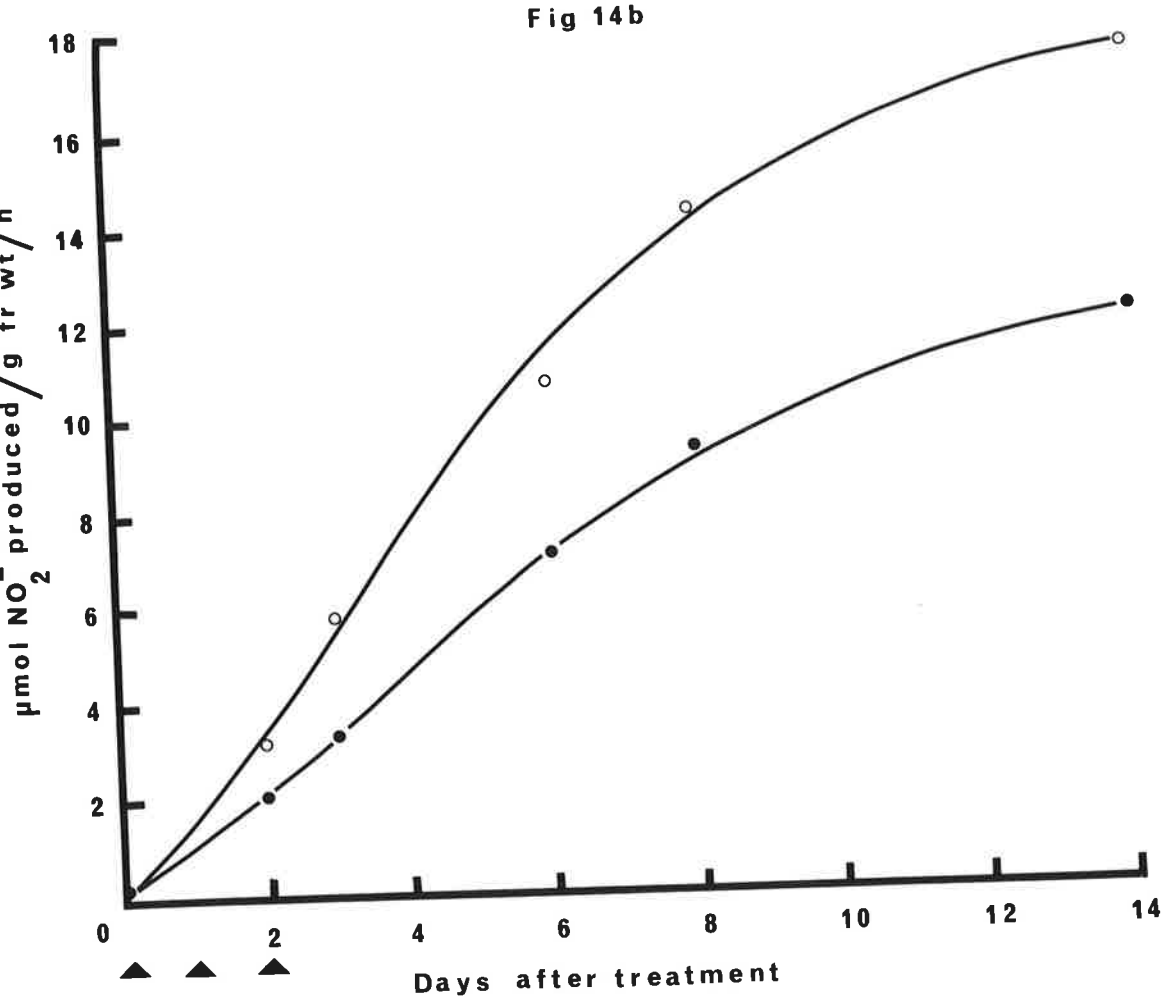

18
16
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
µmol NO_2^- produced/g fr wt/h
Days after treatment

FIGURE 15

Effect of daily application of 1μM atrazine on the production of nitrate reductase activity in leaves of barley seedlings

Seedlings (7-day-old) grown in sand cultures supplied with 0.1 strength Hoagland's solution without combined nitrogen were then supplied daily with 4.5mM NO_3^- and 1μM atrazine in their nutrient solutions. The nitrate reductase activity was determined in extracts of leaves at the times indicated as described in Section 2.9.2.1. Control plants were grown without atrazine.

●——● Omit atrazine

o——o Atrazine

Fig 15

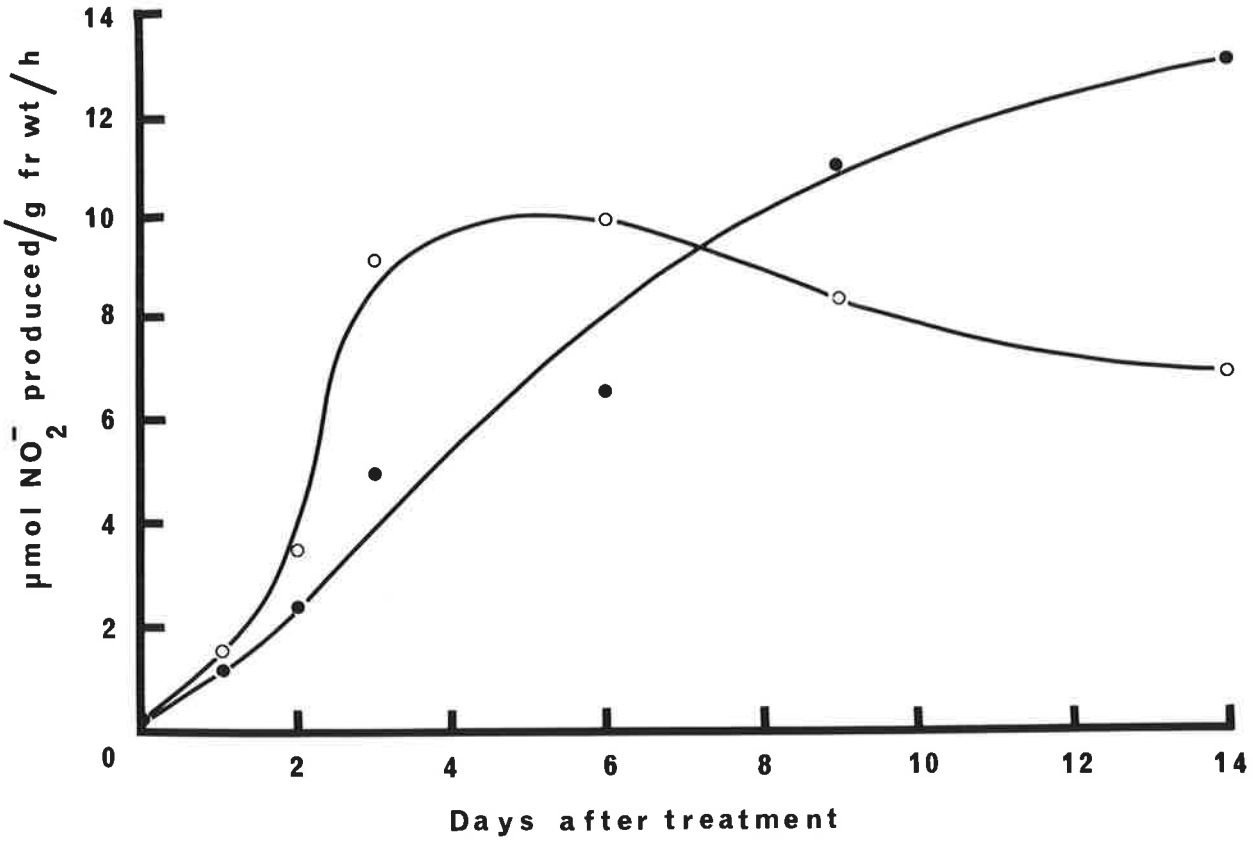

µmol NO_2^- produced/g fr wt/h
0
2
4
6
8
10
12
14
Days after treatment

FIGURE 16

Effect of graded amounts of atrazine on nitrate reductase activity in leaves of barley seedlings

Batches of 5 trays of seedlings (7-day-old) were grown in sand cultures supplied with 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.2. They were then treated daily with 0.5 strength Hoagland's solution containing 4.5mM NO_3^- and 0, 0.5, 1, 3 and 5μM atrazine respectively. Extracts of leaves were assayed for nitrate reductase activity at the time periods indicated as described in Section 2.9.2.1. The concentrations of atrazine in μM is given in the histogram viz. 0, 0.5, 1, 3 and 5.

Fig 16

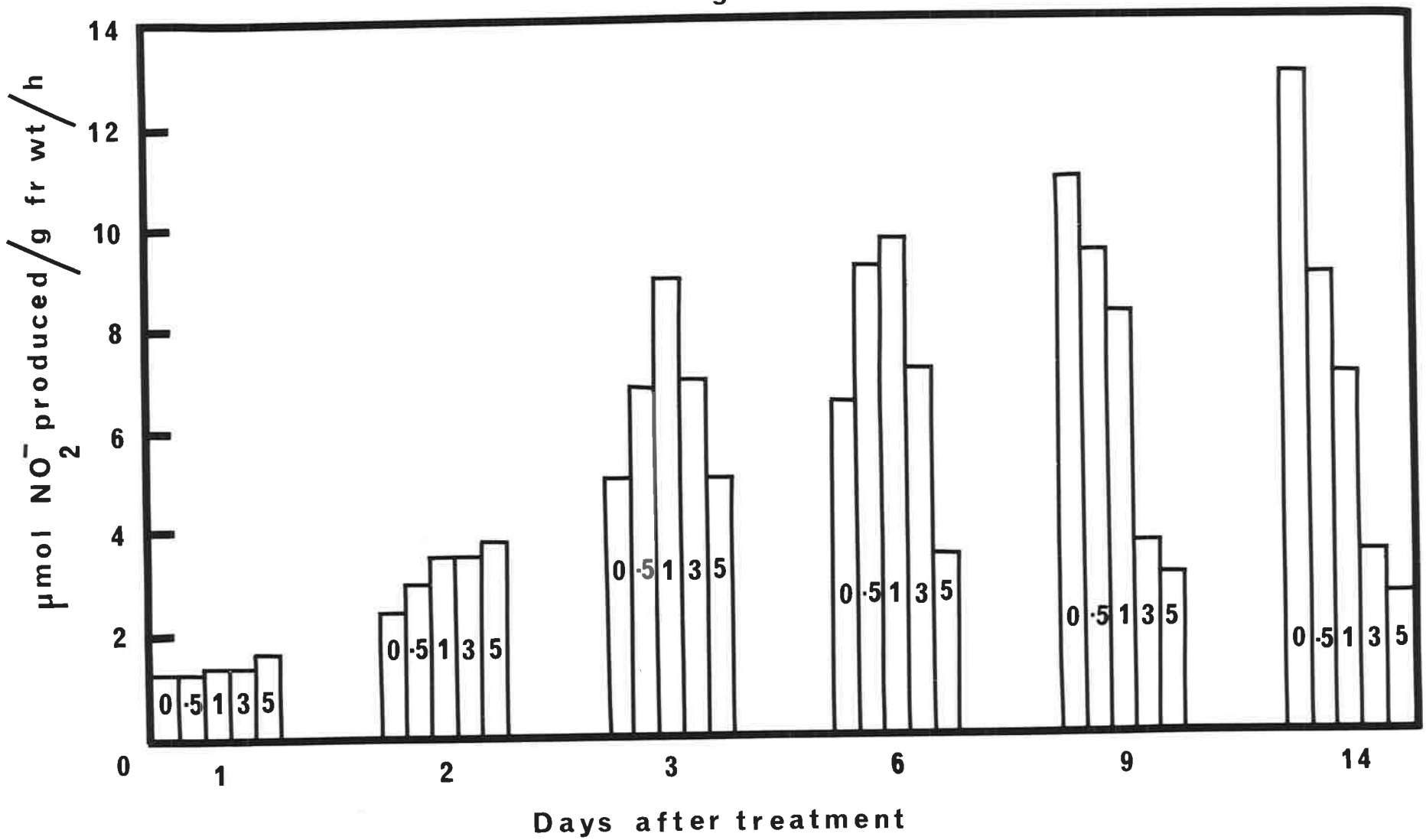

µmol NO2- produced/g fr wt/h
14
12
10
8
6
4
2
0
0 ·5 1 3 5
1
2
3
6
9
14
Days after treatment

FIGURE 17

Effect of atrazine on the production of nitrate reductase in detached barley leaves supplied with nitrate and cycloheximide

Detached leaves from 7-day-old seedlings grown without combined nitrogen (Section 2.4.2.) were kept in a vial containing 4.5mM NO_3^- in 0.1 strength Hoagland's solution for 6 h as described in Section 2.9.3. The leaves were then transferred to 4.5mM NO_3^- Hoagland's solution (0.1 strength) containing 6 µg or 12 µg cycloheximide/ml and left for 6 h (first arrow). Finally they were placed in fresh solutions containing NO_3^- (4.5mM), cycloheximide (6 µg or 12 µg/ml) and 1µM atrazine in Hoagland's solution (0.1 strength) for a further 21 h (second arrow). Cell-free extracts of leaves were analysed for nitrate reductase activity at the times indicated as described in Section 2.9.2.1. For the control treatment, leaves were kept in nutrient solution without inhibitor.

●——● 4.5mM NO_3^-

o——o 4.5mM NO_3^- and 25µM atrazine

△——△ 4.5mM NO_3^-, 25µM atrazine and 6 µg/ml cycloheximide

▲——▲ 4.5mM NO_3^- and 6 µg/ml cycloheximide

□——□ 4.5mM NO_3^-, 25µM atrazine and 12 µg/ml cycloheximide

■——■ 4.5mM NO_3^- and 12 µg/ml cycloheximide

Fig 17

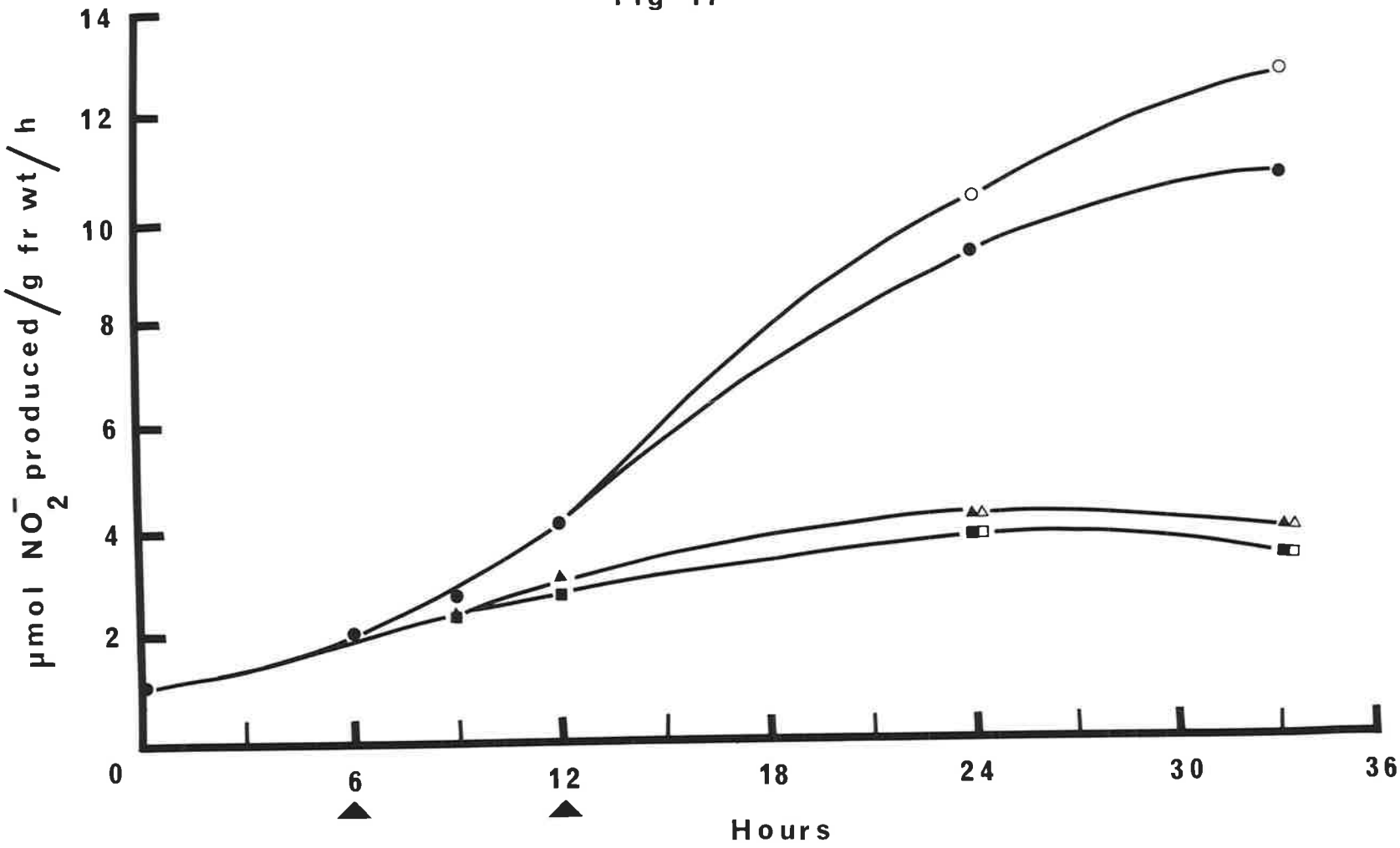


FIGURE 18

Effect of atrazine on the production of nitrate reductase in detached barley leaves supplied with nitrate and 6-methyl purine

Detached leaves from 7-day-old seedlings grown without combined nitrogen (Section 2.4.2.) were kept in a vial containing 4.5mM NO_3^- in 0.1 strength Hoagland's solution for 6 h as described in Section 2.9.3. The leaves were then transferred to Hoagland's solution (0.1 strength) containing NO_3^- (4.5mM) and 6-methyl purine (67 µg/ml) and left for 6 h (first arrow). Finally they were placed in fresh solutions containing NO_3^- (4.5mM), 6-methyl purine (67 µg/ml) and 1µM atrazine in Hoagland's solution (0.1 strength) for a further 22 h (second arrow). Cell-free extracts of leaves were analysed for nitrate reductase at the times indicated as described in Section 2.9.2.1. For the control treatments, leaves were kept in nutrient solutions without inhibitor.

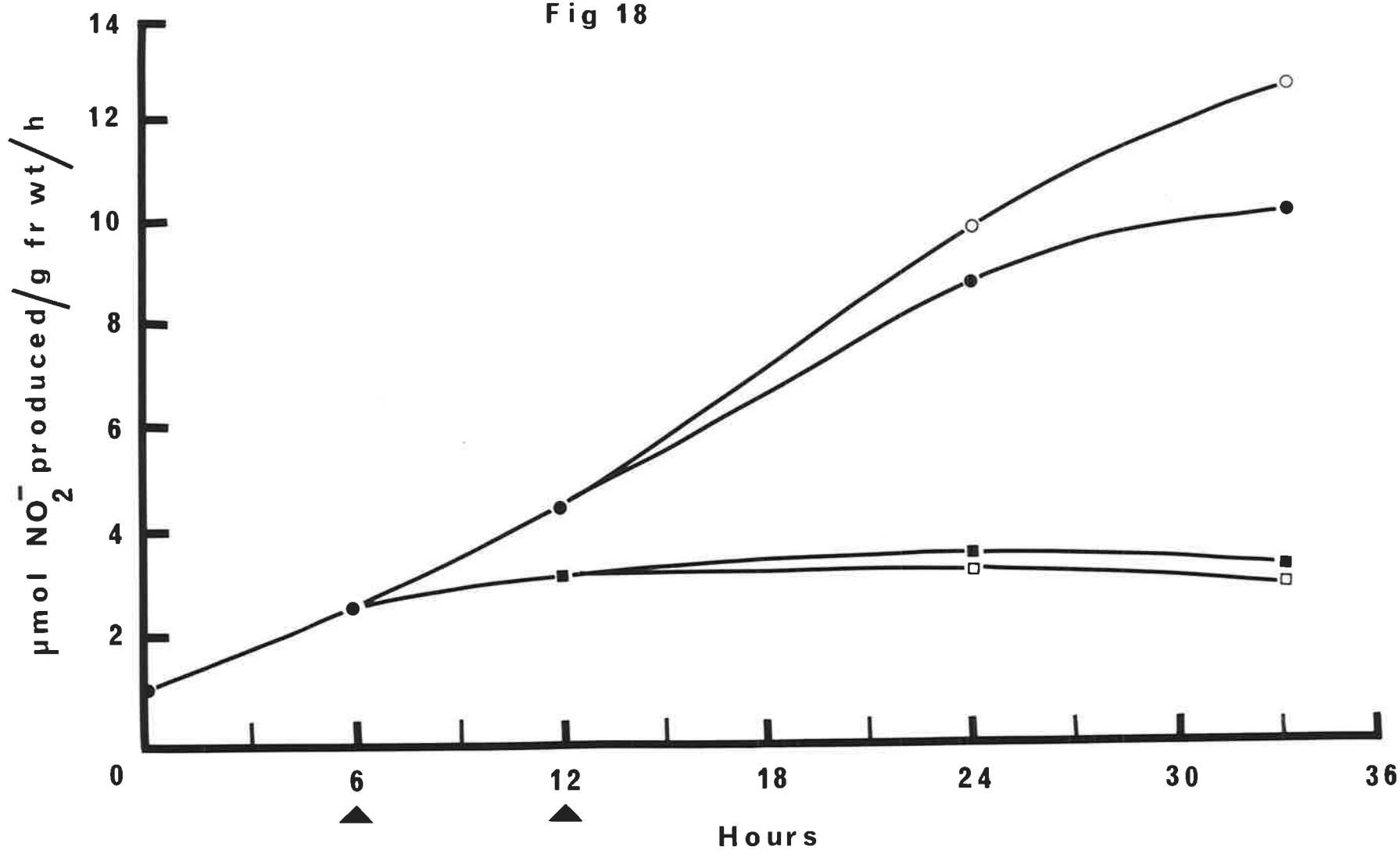
●——● 4.5mM NO_3^-

o——o 4.5mM NO_3^- and 25µM atrazine

■——■ 4.5mM NO_3^- and 67 µg/ml 6-methyl purine

□——□ 4.5mM NO_3^-, 25µM atrazine and 67 µg/ml 6-methyl purine

Fig 18



necessary for the stimulation of the enzyme by the herbicide.

3.1.4.1.5. *In vivo* and *in vitro* decay of enzyme activity in maize

In experiments carried out to investigate the effect of atrazine on the *in vivo* decay of nitrate reductase activity in 7-day-old maize seedlings, they were given an initial treatment of nitrate (4.5mM) and atrazine (0.1μM) for 3 h. The seedlings were then transferred into fresh culture solutions lacking nitrate and during this time the enzyme activity in roots was assayed over a period of 8 h as shown in Figure 19. In some experiments, tungstate (100μM) was included in the liquid cultures after the treatments with nitrate. Atrazine did not prevent the loss of *in vivo* nitrate reductase activity in plants supplied with or without tungstate. The loss of enzyme activity was faster in seedlings treated with tungstate (halflife 3.6 h) than in others (halflife 6 h) irrespective of atrazine treatments.

To determine the effect of atrazine on the *in vitro* decay of nitrate reductase, a time course for the loss of enzyme activity in cell-free extracts of leaves was examined at 25°C (Figure 20). Seedlings (7-day-old) were supplied with 4.5mM NO_3^- for 2 days and on the 3rd day cell-free extracts of leaves were prepared with atrazine in the extracting buffer solutions as described in Section 2.9.1.1. The rates of decay of enzyme activity were the same for control and the atrazine treatments implying that the herbicide did not affect the inactivation of nitrate reductase *in vitro*.

3.1.4.2. Nitrate and nitrite reductases, glutamine synthetase and glutamate synthase

The application of atrazine increased the activities of nitrate and nitrite reductases, glutamine synthetase and glutamate synthase in roots and shoots of 14-day-old maize seedlings grown with nitrate as a source of nitrogen (Table 26). The activity of nitrate reductase was increased most

FIGURE 19

Effect of atrazine on the in vivo decay of nitrate reductase activity of maize seedlings

Maize seedlings (7-day-old) grown without combined nitrogen as described in Section 2.4.1. were given 4.5mM NO_3^- and 0.1μM atrazine in 0.1 strength Hoagland's solution for 3 h. They were then transferred into fresh nutrient solutions without nitrate. Two sets of seedlings were given tungstate (100μM) after the induction with nitrate (Section 2.9.4.). Atrazine treatment was continued throughout the experiment. Nitrate reductase activity was assayed (Section 2.9.2.1.) in root extracts over a period of 8 h as indicated.

●——● Omit atrazine

o——o Atrazine

Δ----Δ Omit atrazine, plus tungstate

Δ----Δ Atrazine, plus tungstate

Fig 19

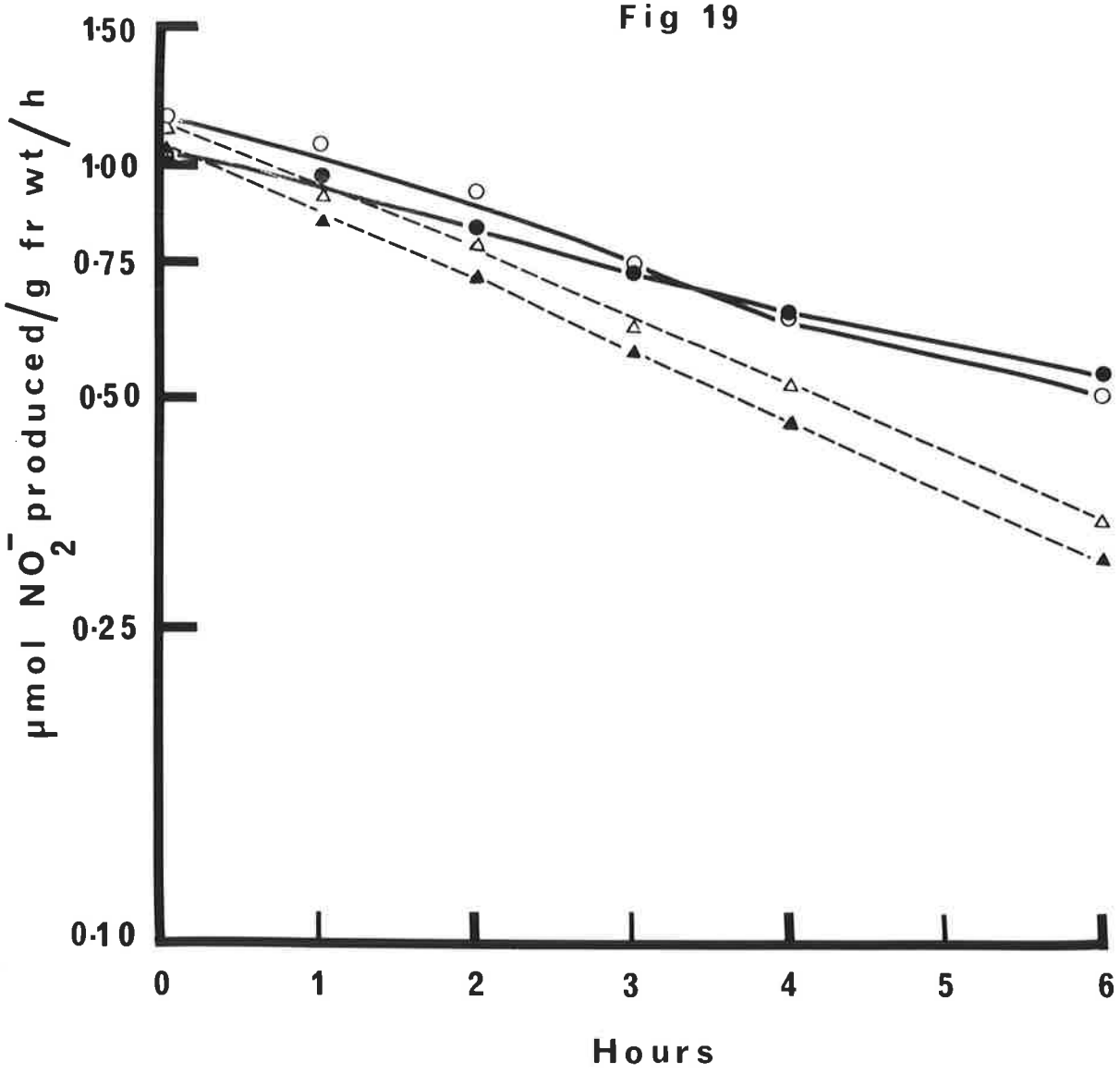

1·50
1·00
0·75
0·50
0·25
0·10
µmol NO2- produced/g fr wt/h
0
1
2
3
4
5
6
Hours

FIGURE 20

Effect of atrazine on the decay of nitrate reductase activity in cell-free extracts of maize leaves at 25°C

Maize seedlings (7-day-old) grown in sand cultures without combined nitrogen (Section 2.4.1.) were subsequently given 4.5mM NO_3^- in 0.5 strength Hoagland's solution daily for 2 days. Cell-free extracts of leaves were prepared on the 3rd day after the application of atrazine using three extraction solutions viz. 0.5mM Na-EDTA, 1mM cysteine, 3% (w/v) casein and 0, 25 and 100µM atrazine respectively in 50mM phosphate buffer (pH 7.5). The reaction mixtures were kept in a water bath at 25°C (Section 2.9.4.) and assayed for nitrate reductase activity at the time periods indicated as described in Section 2.9.2.1.

●——● Omit atrazine

o----o 25µM atrazine

Δ····Δ 100µM atrazine

Fig 20

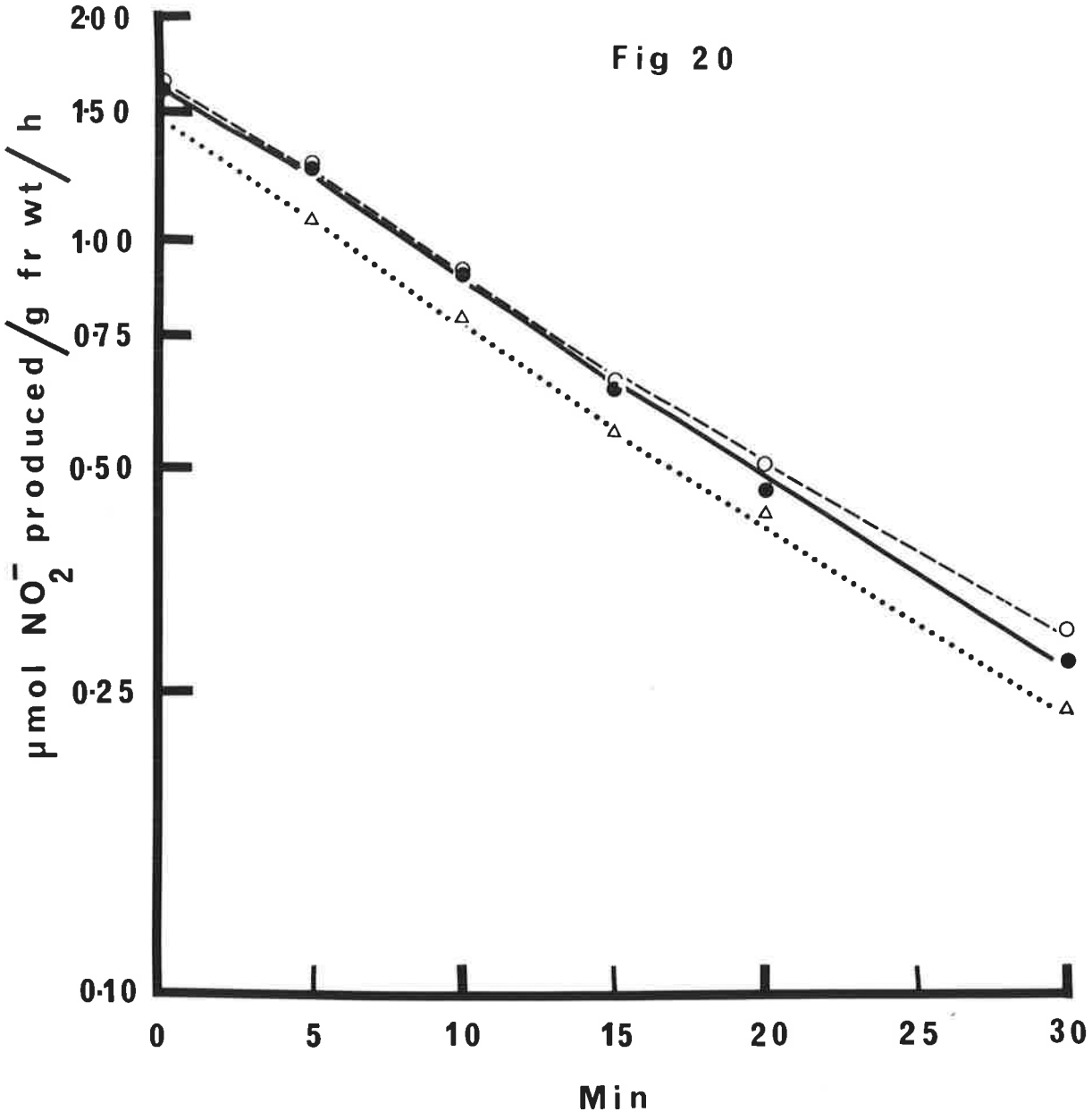

2·00
1·50
1·00
0·75
0·50
0·25
0·10
µmol NO_2^- produced/g fr wt/h
0
5
10
15
20
25
30
Min

TABLE 26

Effect of atrazine on the activities of nitrate and nitrite reductases, glutamine synthetase and glutamate synthase in maize seedlings grown with nitrate

Maize seedlings (7-day-old) were grown in liquid cultures containing 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.1. They were then given 4.5mM NO_3^- and 0.1μM atrazine in the nutrient solutions and harvested after a further 7-day growth. Enzymes were determined in cell-free extracts as described in Section 2.9.2.

Enzymes	Enzyme activities			
	Roots		Shoots	
	Omit Atrazine	Atrazine	Omit Atrazine	Atrazine
Nitrate reductase (μmol NO_2^- produced/ g fresh weight/h)	1.1	2.3	1.8	2.9
Nitrite reductase (μmol NO_2^- utilized/ g fresh weight/h)	17.9	25.5	19.9	29.6
Glutamine synthetase (μmol glutamyl hydroxamate produced/ g fresh weight/h)	80.0	112.0	96.0	144.0
Glutamate synthase (μmol glutamate produced/g fresh weight/h)	53.2	67.8	147.0	172.2

markedly by atrazine treatment in roots (109%) and to a lesser extent in shoots (61%). The activities of other enzymes were increased by the herbicide by about 40 - 50% in both roots and shoots of maize seedlings except for glutamate synthase where the increases were less, viz. 27 and 17% for roots and shoots respectively (Table 26). Activities of these enzymes were then compared in leaves of 7-day-old barley seedlings supplied with either nitrate or ammonium as a source of nitrogen together with atrazine as described in Section 2.9.2. (Table 27). Atrazine treatment increased the activities of these enzymes in leaves of nitrate grown barley plants and the results were similar to those for maize seedlings described earlier. When ammonium was the sole source of nitrogen, the activities of nitrate and nitrite reductases were very low as expected and atrazine did not stimulate them nor the activities of glutamine synthetase and glutamate synthase.

3.1.5. Incorporation of $^{15}NO_3^-$ into cell nitrogen in maize and barley

Seven-day-old barley seedlings grown in sand were supplied with 3mM $K^{15}NO_3$ and 1μM atrazine in 0.1 strength Hoagland's solution. About 80% of the total ^{15}N recovered in the plants on the 3rd and 5th days after the application of the tracer was found in the shoot (Table 28). On the 3rd day 35% of the total ^{15}N in roots and shoots was incorporated into TCA-precipitable protein whereas the corresponding value for the 5th day was increased to 58%. The inclusion of atrazine in the nutrient solutions enhanced the uptake of labelled nitrate into both roots and shoots by 20% after 3 days and by 85% after 5 days. On the 5th day the incorporation of $^{15}NO_3^-$ into TCA-precipitable protein and into total nitrogen of roots was increased by 47 and 54% respectively by the herbicide treatment. In shoots the corresponding values were 114 and 93% respectively.

A stimulatory effect of atrazine on the incorporation of $^{15}NO_3^-$ into TCA-precipitable protein and into total nitrogen was observed in leaves of barley at 2 days after its application to the roots medium with

TABLE 27

Effect of atrazine on nitrate and nitrite reductases, glutamine synthetase and glutamate synthase in leaves of barley seedlings grown with either nitrate or ammonium chloride as a source of nitrogen

Seven-day-old barley seedlings grown in sand cultures (without the supply of nitrogen compound as described in Section 2.4.2.) were treated daily with either [1.5mM $Ca(NO_3)_2$ + 1.5mK KNO_3] and 1μM atrazine or 3mM NH_4Cl and 1μM atrazine both in 0.5 strength Hoagland's solution. Leaves were collected at 72 h after the application of nitrogen and atrazine. Enzymes were assayed in cell-free extracts as given in Section 2.9.2.

Enzymes	Enzyme activities			
	Nitrate treated plants		Ammonium treated plants	
	Omit Atrazine	Atrazine	Omit Atrazine	Atrazine
Nitrate reductase (μmol NO_2^- produced/ g fresh weight/h)	4.47	8.1	0.34	0.26
Nitrite reductase (μmol NO_2^- utilized/ g fresh weight/h)	11.0	17.2	5.84	4.12
Glutamine synthetase (μmol glutamyl hydroxamate produced/ g fresh weight/h)	136.0	192.0	120.0	116.0
Glutamate synthase (μmol glutamate produced/g fresh weight/h)	73.4	99.9	65.3	61.2

TABLE 28

Effect of atrazine on $^{15}NO_3^-$ uptake and incorporation into TCA-precipitable protein and into total nitrogen in barley

Seven-day-old barley seedlings were grown in sand without combined nitrogen in the nutrient solutions as described in Section 2.4.2. These were treated with 3mM $K^{15}NO_3$ and 1μM atrazine for the next 3 days and for a further 2 days with $K^{15}NO_3$ only. Roots and shoots were harvested separately on the days indicated and analysed for $^{15}NO_3^-$ incorporation into the TCA-precipitable protein and into total nitrogen as described in Section 2.10.

Plant materials	No. of days of $^{15}NO_3$ treatment	μg $^{15}NO_3^-$ N/g fresh weight			
		TCA-precipitable protein		Total nitrogen	
		Omit Atrazine	Atrazine	Omit Atrazine	Atrazine
Roots	3	33	39	62	72
	5	57	84	103	159
Shoots	3	69	81	220	265
	5	214	457	400	771
Roots and shoots	3	102	120	282	337
	5	271	541	503	930

$K^{15}NO_3$ (Figure 21). An almost 3-fold increase in uptake was observed throughout the 10 days period of the investigation. At all stages most (about 80%) of the ^{15}N was recovered in the TCA-precipitable protein fraction which was not influenced by the herbicide.

The data from a study on the uptake of $^{15}NO_3^-$ by maize seedlings similar to that described above for barley is shown in Table 29. In contrast to barley, a larger amount of the total labelled nitrate incorporated by maize plants was found in the roots especially on the first day (approximately 68%). Here again, the inclusion of 0.1μM atrazine in the cultures increased the incorporation of labelled nitrate into both TCA-precipitable protein and total nitrogen in roots and shoots. On the 3rd day, atrazine treatment increased the incorporation of $^{15}NO_3^-$ into roots by 28% and into shoots by 18%.

3.1.6. Incorporation of [^{14}C]-leucine into detached barley leaves

Detached leaves were used to study the effects of atrazine on the incorporation of [^{14}C]-leucine into TCA-precipitable protein. The detached leaves from 7-day-old seedlings grown without combined nitrogen were inserted into vials containing [^{14}C]-leucine and atrazine in phosphate buffer (pH 7.5) for 12 h as described in Section 2.11. Atrazine markedly increased the incorporation of ^{14}C-labelled leucine into protein and the effect already apparent within 2 h of adding the herbicide was sustained for a further 12 h (Figure 22). At 12 h the increased incorporation of the tracer into protein resulting from atrazine treatment was about 45 - 57% (Figure 22 and Tables 30 & 31). However, the results in the Tables (30 & 31) show that this enhanced incorporation of [^{14}C]-leucine into protein of detached leaves was only obtained in the absence of a supply of inorganic nitrogen to the seedlings and to the detached leaves. When the seedlings were grown with nitrate or ammonium, a higher level of [^{14}C]-leucine incorporation was found in detached barley leaves and this

FIGURE 21

Effect of atrazine on the incorporation of $^{15}NO_3^-$ into TCA-precipitable protein and into total nitrogen in barley seedlings

Seven-day-old barley seedlings grown in sand cultures (without the supply of nitrogen compounds as described in Section 2.4.2.) were treated for the next three days with 3mM $K^{15}NO_3$ in half-strength Hoagland's solution containing 1µM atrazine (as indicated by the arrows). They were then maintained on unlabelled nitrate [1.5mM $Ca(NO_3)_2$ + 1.5mM KNO_3] in half-strength Hoagland's solution without atrazine. Leaves were analysed at the 2nd, 3rd, 6th and 10th day respectively after the first application of the tracer and atrazine. The incorporation of $^{15}NO_3$ into protein and total nitrogen was determined as described in Section 2.10.

A $^{15}NO_3^-$ incorporated into total nitrogen

●——● Omit atrazine

o——o Atrazine

B $^{15}NO_3^-$ incorporated into TCA-precipitable protein

▲----▲ Omit atrazine

Δ----Δ Atrazine

Fig 21

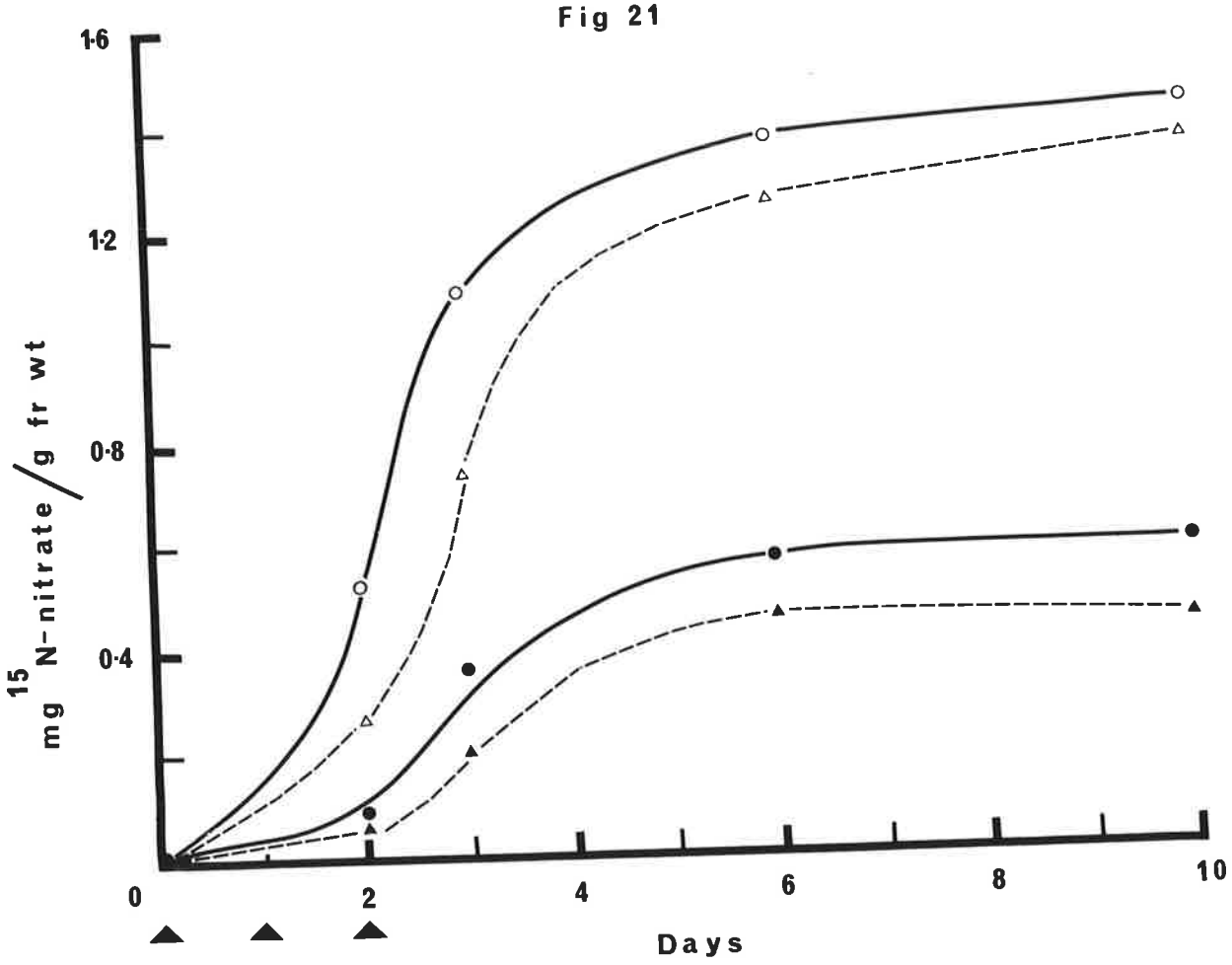


TABLE 29

Effect of atrazine on $^{15}NO_3^-$ uptake and incorporation into TCA-precipitable protein and into total nitrogen in maize

Eleven-day-old maize seedlings were grown in liquid cultures without combined nitrogen as described in Section 2.4.1. From the 12th day onwards, 3mM $K^{15}NO_3$ and 0.1μM atrazine were added to the nutrient solutions. Roots and shoots were harvested on the days indicated and analysed for $^{15}NO_3$ incorporation into the TCA-precipitable protein and into total nitrogen as described in Section 2.10.

Plant materials	No. of days after $^{15}NO_3^-$ treatment	μg $^{15}NO_3^-$ N/g fresh weight			
		TCA-precipitable protein		Total nitrogen	
		Omit Atrazine	Atrazine	Omit Atrazine	Atrazine
Roots	1	123	135	265	293
	3	341	435	581	747
Shoots	1	71	84	129	140
	3	375	443	586	685
Roots and shoots	1	194	219	394	433
	3	716	878	1,167	1,432

FIGURE 22

Effect of atrazine on the incorporation of [^{14}C]-leucine in detached leaves of barley seedlings grown without combined nitrogen

Barley seedlings were grown for the first 7 days in sand cultures supplied with 0.1 strength Hoagland's solution without combined nitrogen (Section 2.4.2.). Leaves were detached on the 8th day and the cut ends immersed in incubation solutions containing 2μCi [^{14}C]-leucine and 25μM atrazine in 0.05M phosphate buffer (pH 7.5) and placed in a phytotron at 22°C and 350μE/m^2/sec (Section 2.11). The leaves were collected at time periods indicated and the incorporation of [^{14}C]-leucine into water-soluble proteins was determined in leaves as described in Section 2.11.

●——● Omit atrazine

o——o Atrazine

Fig 22

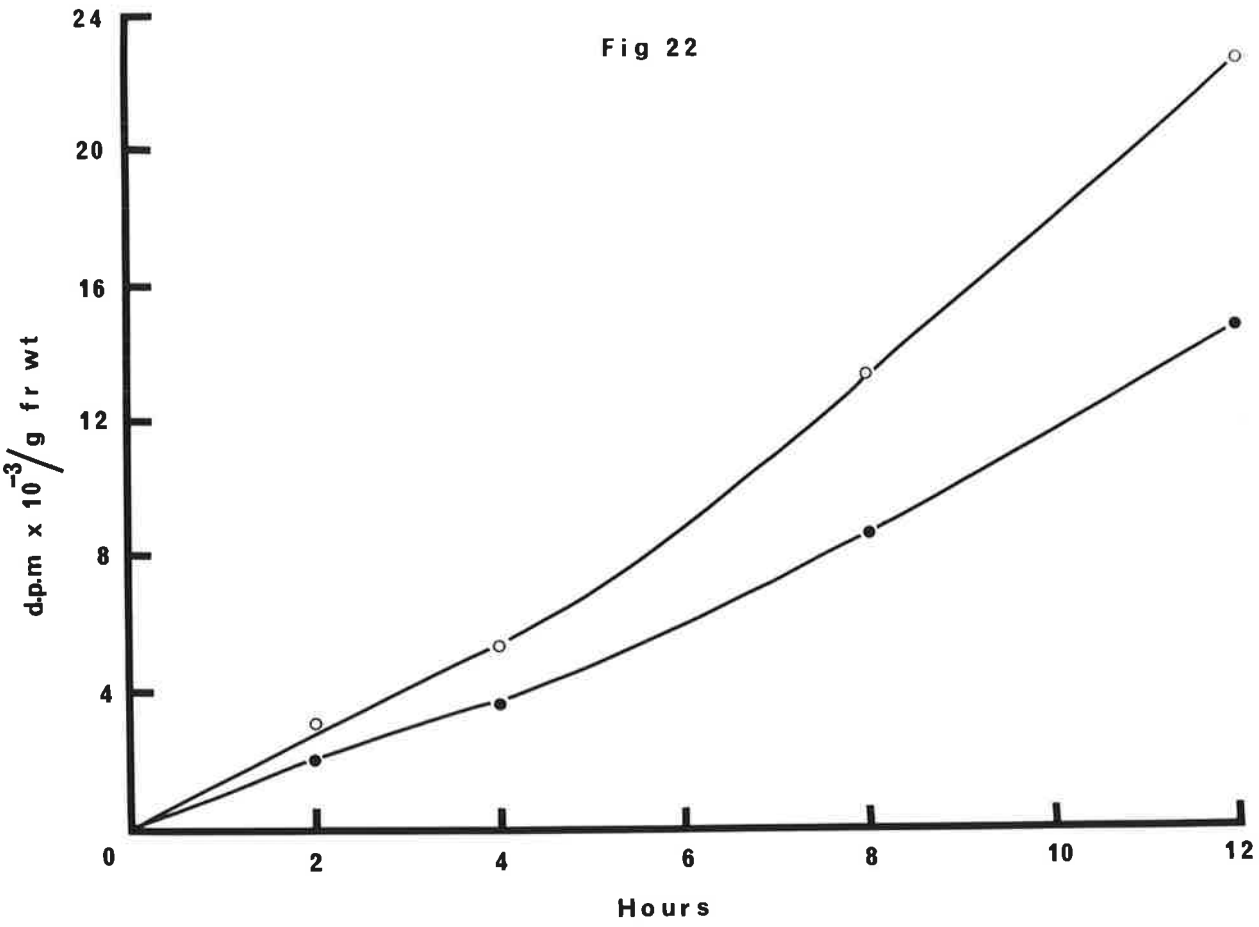


TABLE 30

Effect of atrazine on the incorporation of [^{14}C]-leucine into detached leaves of barley seedlings grown with either nitrate or ammonium as a source of nitrogen

Barley seedlings were grown for 5 days in 4 trays supplied daily with 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.2. On the 6th and 7th day, the seedlings were supplied with either equimolar potassium and calcium nitrate or ammonium chloride as indicated. Leaves were detached on the 8th day and the cut ends immersed in incubation solutions containing 2μCi [^{14}C]-leucine and 25μM atrazine in 0.05M phosphate buffer (pH 7.5). The vials containing the cut leaves were kept in a phytotron at 22°C and 350μE/m^2/sec. The incorporation of [^{14}C]-leucine into protein was determined in leaves after a 12 h incubation (Section 2.11.).

Source of nitrogen in the nutrient solutions	[^{14}C]-Leucine incorporation into protein dpm/mg protein		
	Atrazine μM		% Increase
	0	25	
Omit N	5,073	7,590	49
4.5mM NO_3^-	8,055	8,446	5
15mM NO_3^-	7,205	7,881	9
4.5mM NH_4^+	7,239	7,302	1

TABLE 31

Effect of atrazine on the incorporation of [^{14}C]-leucine into detached leaves of barley seedlings grown without combined nitrogen but supplied with either nitrate or ammonium in the [^{14}C]-leucine incubation solutions

Barley seedlings were grown for 7 days in sand cultures supplied with 0.1 strength Hoagland's solution without combined nitrogen as described in Section 2.4.2. Leaves were detached on the 8th day and the cut ends immersed in incubation solutions containing 2μCi [^{14}C]-leucine, 25μM atrazine in 0.05M phosphate buffer (pH 7.5) and a nitrogen supply as indicated. The vials containing the cut leaves were kept in a phytotron at 22°C and 350μE/m^2/sec. The incorporation of [^{14}C]-leucine into protein was determined in leaves after a 12 h incubation period (Section 2.11.).

Source of nitrogen in the incubation solutions	[^{14}C]-Leucine incorporation into protein dpm/mg protein		
	Atrazine μM		% Increase or Decrease
	0	25	
Omit N	6,163	8,936	+45
4.5mM NO_3^-	10,723	8,961	-16
4.5mM NH_4^+	8,241	7,021	-15

was not affected by the atrazine treatment (Table 30). Similarly in detached leaves of seedlings grown without combined nitrogen, but supplied with either nitrate or ammonium in the incubation solutions, a high rate of [^{14}C]-leucine incorporation was again obtained (Table 31) and atrazine marginally reduced (15%) the rate of incorporation of ^{14}C-labelled leucine.

3.2. Microorganisms

3.2.1. Growth of *Escherichia coli*, *Neurospora crassa*, *Anabaena cylindrica* and *Chlorella pyrenoidosa*

The results in Table 32 show that substantial amounts of the herbicide were required to depress the growth of *E. coli* whether grown in air or anaerobically when it utilized nitrate instead of O_2 as a terminal electron acceptor. Thus 300μM atrazine inhibited growth by only about 25% after a 6 h incubation in both experiments. The growth reverted to a normal rate however, when incubated further as shown in Figure 23.
In *N. crassa*, 330μM atrazine almost completely restricted growth and the fungus was therefore more readily affected by the herbicide than was *E. coli* (Table 32 and Figure 24). The data in Figure 24 shows that lower amounts of herbicide only marginally depressed the growth of *N. crassa* e.g. 9% inhibition by 10μM atrazine. The algae however were markedly affected by atrazine; 10μM completely inhibited growth (Table 32). *A. cylindrica* grown with nitrate was more sensitive to atrazine than those fixing dinitrogen, viz. 0.1μM atrazine inhibited 45% of growth of nitrate grown *A. cylindrica* whereas the corresponding value for the N_2 fixing cultures was 23% (Table 32 and Figure 25). The growth of *C. pyrenoidosa* was most affected by the herbicide than the other microorganisms described (Table 32). Turbidity measurement in Figure 26 show that 1μM atrazine inhibited the growth of *C. pyrenoidosa* by about 80% whereas 10μM completely restricted the growth after 5 days of treatment. The data in Figure 26 show that the

TABLE 32

Effect of atrazine on the growth of E. coli, N. crassa, A. cylindrica and C. pyrenoidosa

These microorganisms were grown in culture media described in Section 2.5. supplied with graded amounts of atrazine as indicated. The growth of these organisms were determined as follows:- *E. coli* (turbidity), *N. crassa* (fresh weight), *A. cylindrica* (carotenoid content) and *C. pyrenoidosa* (turbidity). Additional experimental results were obtained from Figures 23, 24, 25 and 26 respectively.

Microorganisms and nitrogen source	Growth Period	Atrazine (μM)	Growth (% of control)
E. coli			
(1) NH_4^+ (aerobic)	6 h	0.001	100
		0.1	98
		300	77
		600	67
(2) NO_3^- (anaerobic)	6 h	0.001	100
		0.1	98
		300	81
		600	70
N. crassa			
(1) NO_3^-	3 days	0.01	100
		0.1	98
		10	91
		100	70
		300	4
A. cylindrica			
(1) NO_3^-	5 days	0.001	85
		0.01	70
		0.1	55
		1	40
		10	1
(2) N_2	5 days	0.001	94
		0.01	89
		0.1	77
		1	65
		10	4
C. pyrenoidosa			
(1) NO_3^-	5 days	0.001	83
		0.01	47
		0.1	41
		1	21
		10	0

FIGURE 23

Effect of atrazine on the growth of Escherichia coli

E. coli strain B was grown in culture solutions aerobically (100 ml in 250 ml Erlenmeyer flask) as well as anaerobically (250 ml in 250 ml Erlenmeyer flask) and the culture solutions contained atrazine (0 and 300μM respectively) as described in Section 2.5.1. When grown aerobically, the flasks and their contents were clamped to a wrist-action shaker adjusted so that the cells were well aerated (150 strokes/min). The cultures were incubated at 30°C and aliquots of samples were withdrawn at the times indicated for turbidity measurements as described in Section 2.6.4.

□——□ Aerobic cultures

Δ----Δ Anaerobic cultures

Fig 23

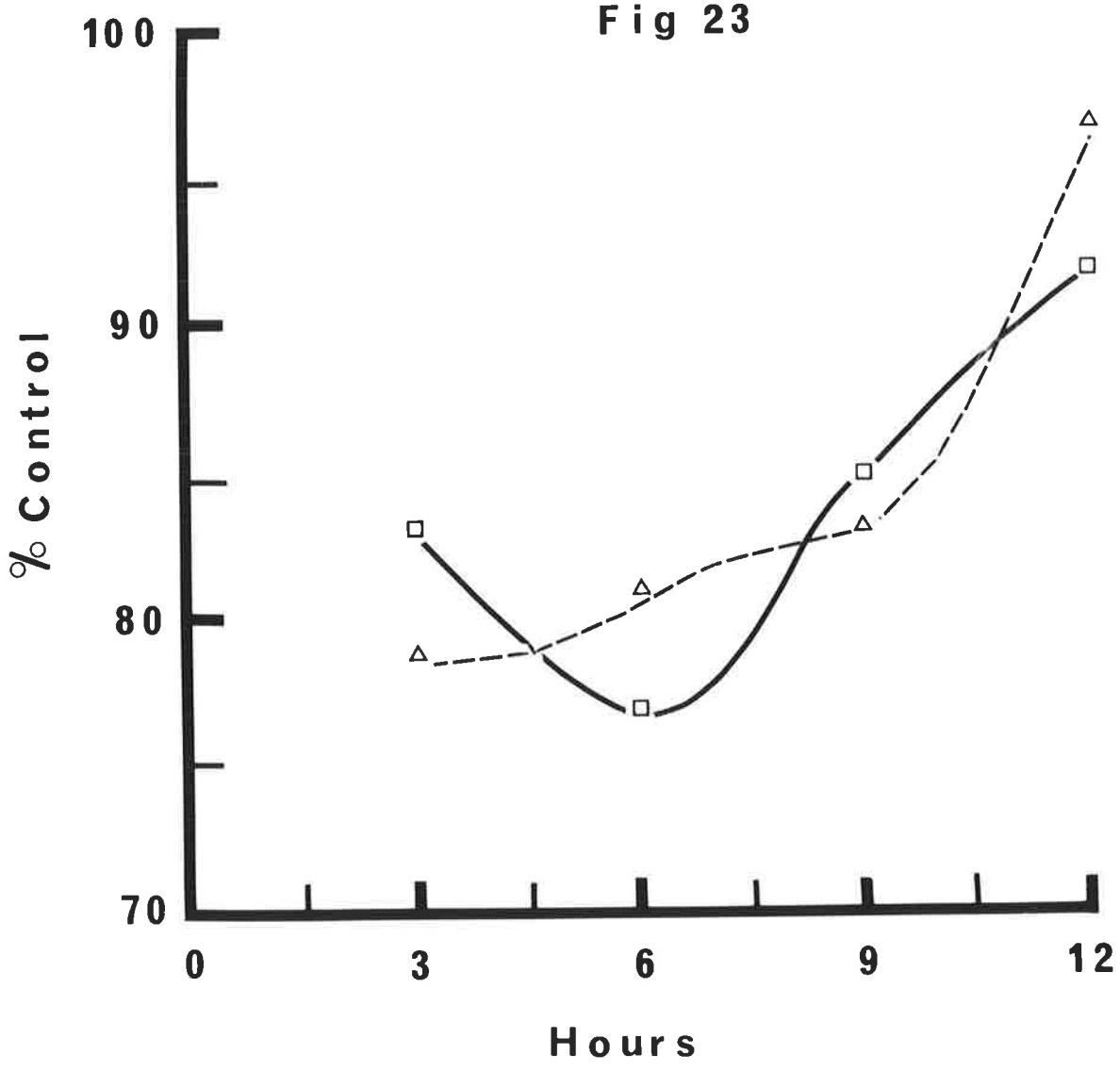


FIGURE 24

Effect of atrazine on the growth of Neurospora crassa

The fungus was grown in a modified Westgaard-Mitchel medium (100 ml in 250 ml Erlenmeyer flask) containing graded amounts of atrazine (0.01, 0.1, 1, 10, 100, 300 and 330μM respectively) as described in Section 2.5.4. They were clamped to a wrist-action shaker adjusted to aerate the culture solutions (100 strokes/min). After 3 days growth, the felts were harvested and fresh and dry weights determined (Section 2.6.2.2. & 2.6.3. respectively).

Fig 24

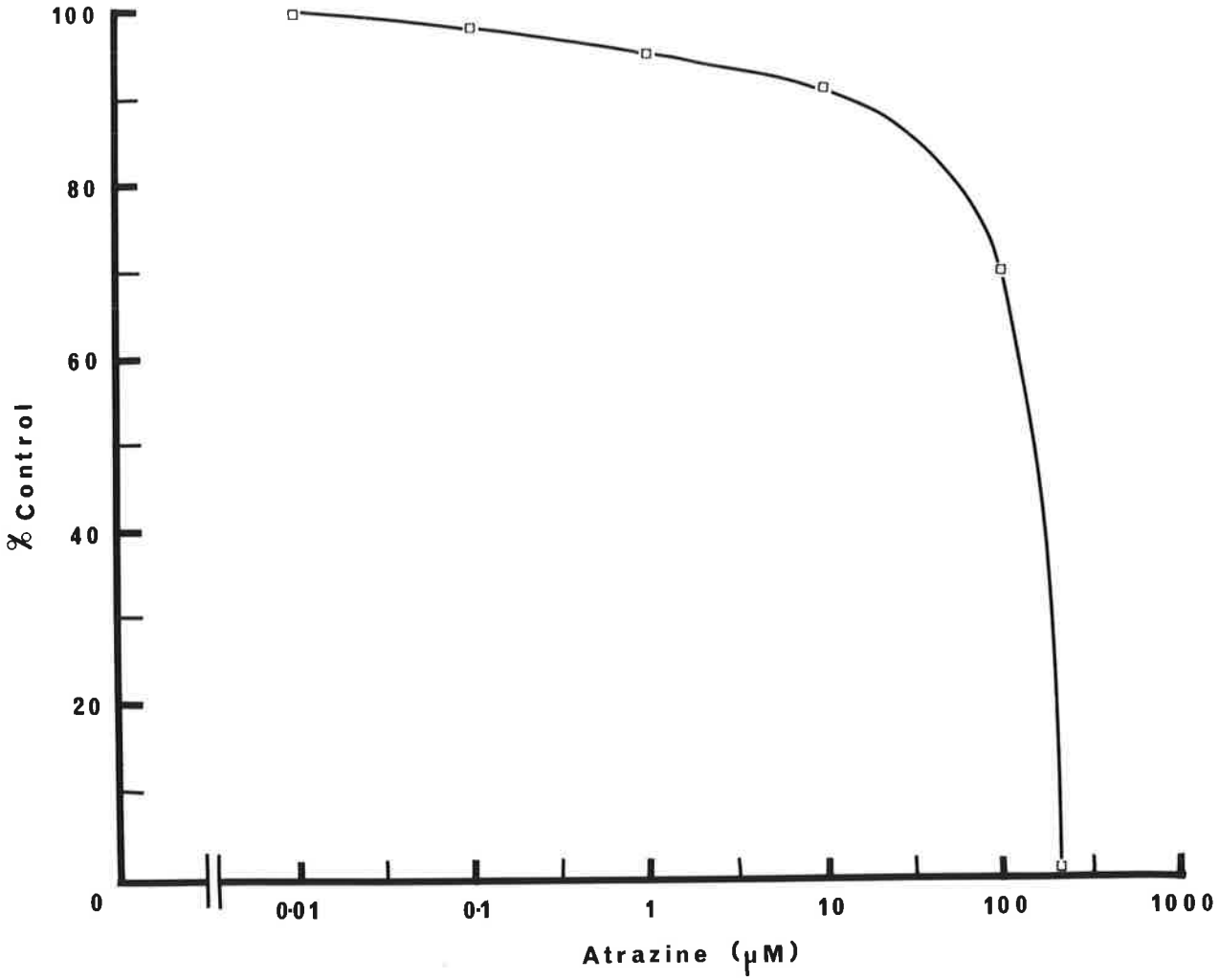


FIGURE 25

Effect of atrazine on the growth of Anabaena cylindrica

The alga was grown either without combined nitrogen or with NO_3^- in the culture solutions (100 ml in 250 ml Erlenmeyer flasks). Graded amounts of atrazine (0.001, 0.01, 0.1, 1 and 10μM respectively) were included in the culture solutions as described in Section 2.5.2. The cultures were exposed to a bank of cool white fluorescent lights (200μE/m^2/sec). They were aerated with a stream of sterile air at 30°C. After 5 days growth, carotenoids were determined as described in Section 2.6.6.

□——□ N_2 fixing cultures

Δ----Δ NO_3^- growing "

Fig 25

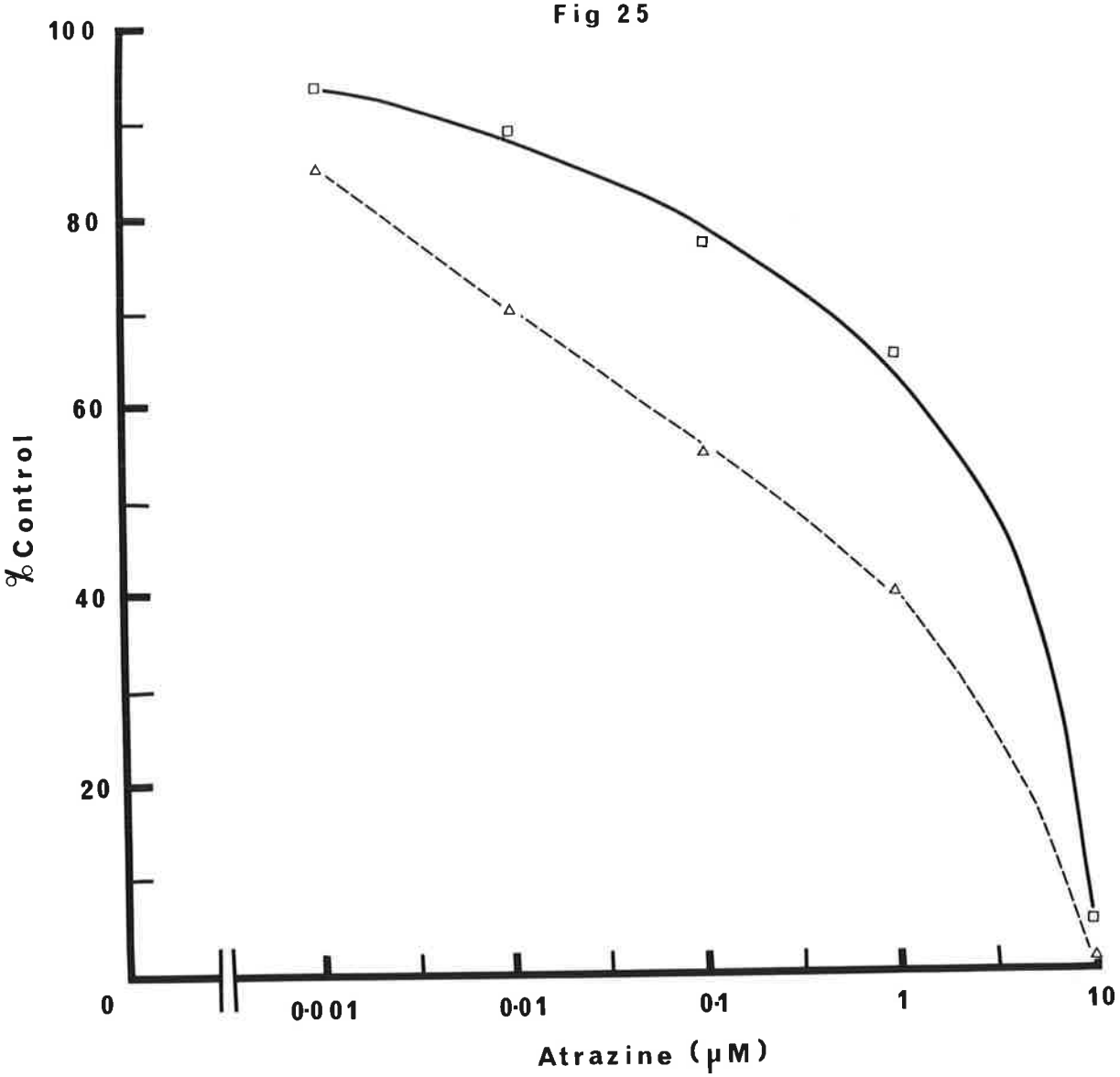


FIGURE 26

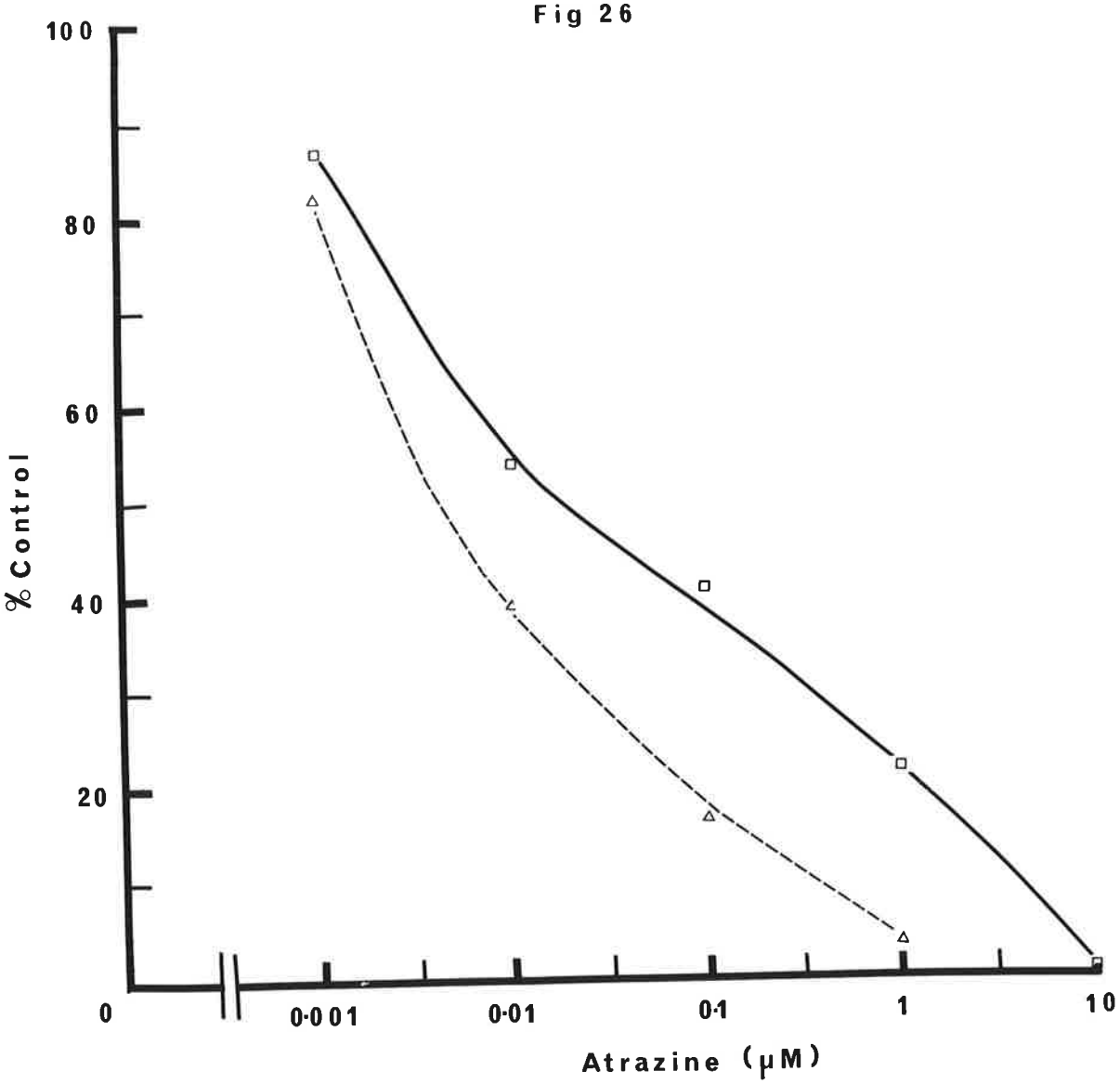
Effect of atrazine on the growth of *Chlorella pyrenoidosa*

The alga was grown in culture solutions containing nitrate (100 ml in 250 ml Erlenmeyer flask) and graded amounts of atrazine (0.001, 0.01, 0.1, 1 and 100μM respectively) were supplied as described in Section 2.5.3. The cultures were exposed to a bank of cool white fluorescent lights (200μE/m^2/sec). They were aerated with a stream of sterile air at 30°C. After 5 days growth, samples were removed for turbidimetric measurement (Section 2.6.4.) and for chlorophyll determination (Section 2.6.5.).

□——□ Turbidimetric measurement

Δ----Δ Chlorophyll content

Fig 26



growth of *C. pyrenoidosa* measured by chlorophyll content was less than that determined by turbidity measurement, indicating that a primary effect of the herbicide was on chlorophyll production.

3.2.2. Nitrate uptake in *E. coli*, *N. crassa* and *A. cylindrica*

E. coli utilizes nitrate as a terminal electron acceptor for respiration when grown in the absence of air. Under aerobic conditions however the bacterium utilizes O_2 for respiration. Nitrate and O_2 uptake by *E. coli* were monitored simultaneously in a special reaction vessel fitted with NO_3^- and O_2 electrodes as described in Section 2.12.2. The incubation solutions in the vessel contained Na-formate and NO_3^- in phosphate buffer (pH 7.5) as described in the text for Figure 27. The washed cells of *E. coli* grown anaerobically with and without atrazine were injected in turn into the reaction mixture and NO_3^- and O_2 concentrations in the solutions monitored continuously. Atrazine (300μM) reduced the rate of O_2 uptake by 67% as shown in Figure 27. Cells grown without atrazine utilized 56 μg atom oxygen/g fr wt/h whereas the corresponding value for atrazine grown cells was 18. The uptake of NO_3^- was very low in the presence of O_2 but when it was depleted from the reaction mixture, NO_3^- was readily taken up. Here again the herbicide decreased the rate of uptake of NO_3^- by 48% viz. 58 and 30 μmol NO_3^-/g fr wt/h for control and atrazine treated cells respectively.

Unlike *E. coli* (B), *A. cylindrica* and *N. crassa* assimilate NO_3^- into cell nitrogen compounds. The uptake of NO_3^- was determined in *N. crassa* supplied with amounts of atrazine (0.1μM) which did not inhibit the growth. The fungus grown for 3 days as described in Section 2.5.4. was transferred into 250 ml Erlenmeyer flasks containing 100 ml of fresh culture media (1mM NO_3^-) with and without atrazine. Aliquots of culture solutions were withdrawn hourly over a 12 h period and the NO_3^- therein determined as described in Section 2.12.3. Atrazine (0.1μM) did not affect the uptake

FIGURE 27

Effect of atrazine on the utilization of NO_3^- and O_2 by cells of Escherichia coli

The bacteria was grown anaerobically with and without atrazine (300μM) in 10 litre pyrex jars containing 8 litres of culture solutions through which N_2 gas was bubbled as described in Section 2.5.1. The cells harvested after an 8 h incubation were washed with 20mM phosphate buffer (pH 7.5) to remove nitrite as described in Section 2.5.1. The cells (0.1 g fr wt) were injected into a special reaction vessel fitted with NO_3^- and O_2 electrodes (Section 2.12.2.) and containing KNO_3 (20 μmoles) in 8 ml of 50mM phosphate buffer (pH 7.5). The vessel was maintained at 30°C by circulating water (30°C) through the outer jacket and the contents were stirred magnetically. The NO_3^- and O_2 levels in the incubation solutions were monitored continuously as described in Section 2.12.2.

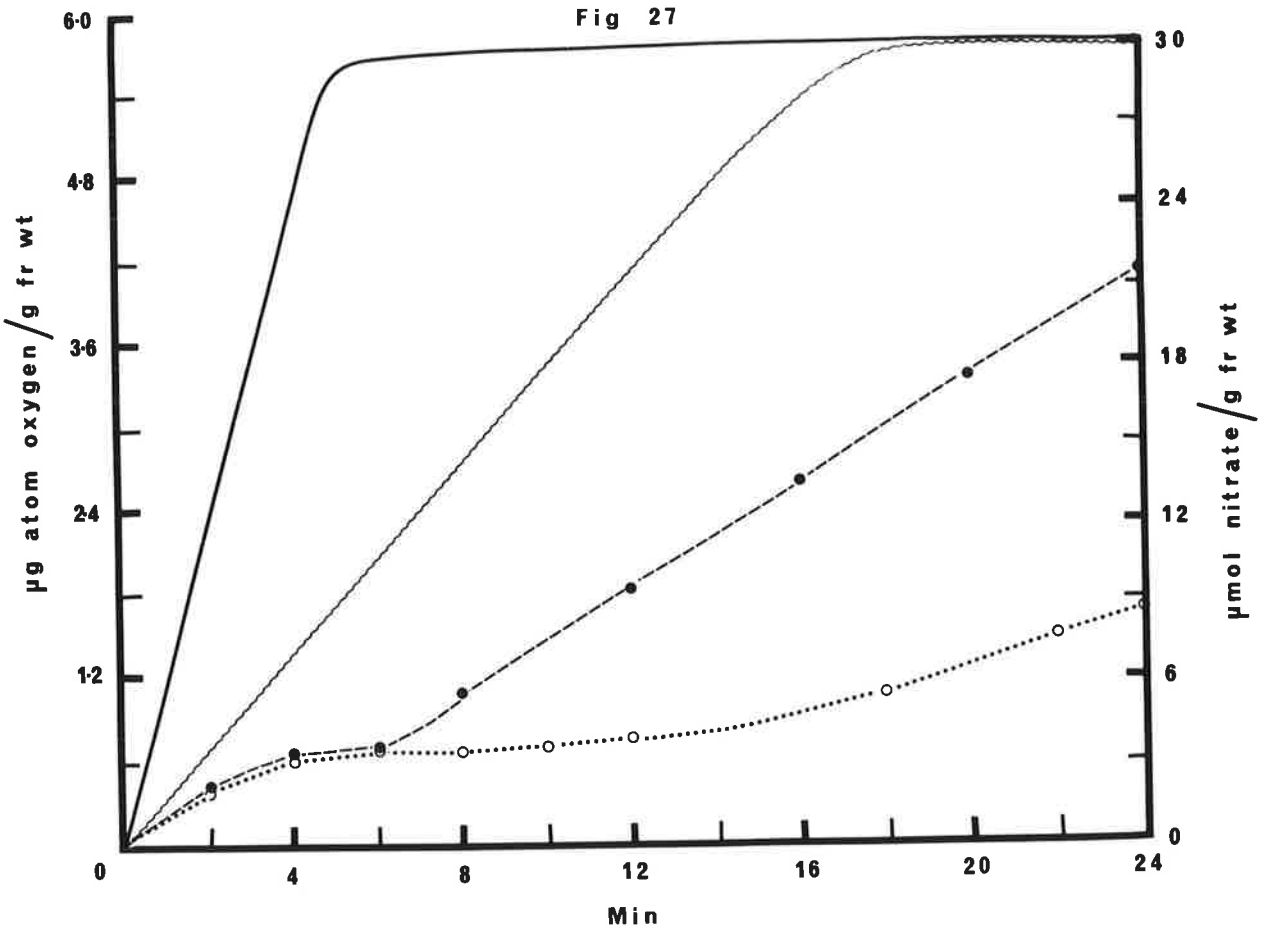
——— O_2 ; omit atrazine

~~~~ O_2 ; atrazine

o----o NO_3^- ; omit atrazine

o····o NO_3^- ; atrazine
~~~~

Fig 27


6·0
4·8
3·6
2·4
1·2
0
µg atom oxygen /g fr wt
30
24
18
12
6
0
µmol nitrate /g fr wt
0
4
8
12
16
20
24
Min

of NO_3^-. A steady rate of NO_3^- uptake was reached at 6th h of incubation (0.8 µmol NO_3^-/g fr wt/h) which was similar irrespective of atrazine treatment.

In A. *cylindrica*, the uptake of NO_3^- was studied in the special reaction vessel fitted with NO_3^- and O_2 electrodes as described for *E. coli*. The incubation solutions in the vessel contained nitrate and phosphate buffer (pH 7.5) as described in Section 2.7.1.2. A thick suspension of alga prepared as described in Section 2.5.2. was transferred into the vessel containing incubation solution and kept under a source of light (200µE/m^2/sec). The NO_3^- and O_2 concentrations in the incubation solutions were monitored continuously. The rate of NO_3^- uptake in A. *cylindrica* was 0.66 µmol NO_3^-/g fr wt/h (Figure 28). Atrazine was added to the culture solutions in the vessel at the time periods indicated in the Figure 28. Addition of 0.01µM atrazine reduced the rate of NO_3^- uptake by 31% (0.44 µmol NO_3^-/g fr wt/h) whereas 10µM completely restricted the uptake of NO_3^-. The O_2 concentration in the reaction mixture also changed during the course of the experiment. Initially before atrazine was introduced into the reaction mixture, the alga produced O_2 (34 µg atom oxygen/g fr wt/h) as in the Figure 28. The addition of 0.01µM atrazine (10 min interval) reduced the evolution of O_2 to 4 µg atom oxygen/g fr wt/h. However, when 10µM atrazine was added O_2 was utilized at the following rate:- 21 µg atom oxygen/g fr wt/h.

3.2.3. Enzymes

3.2.3.1. Nitrate reductase in *C. pyrenoidosa* and *N. crassa*

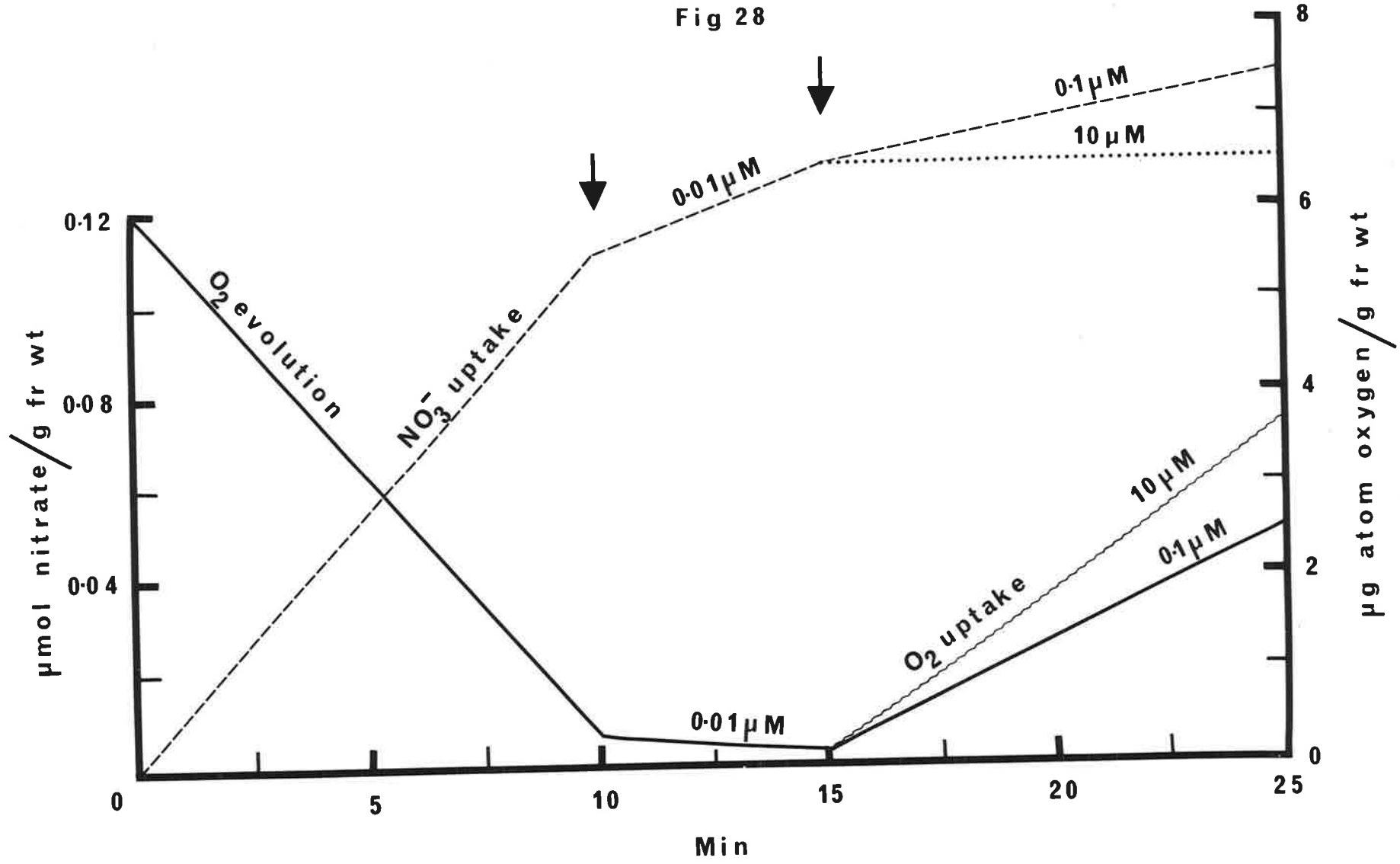
C. pyrenoidosa and *N. crassa* were grown for 2 and 4 days respectively in culture solutions containing nitrate and graded amounts of atrazine as described in Section 2.5.3. and 2.5.4. respectively. In *N. crassa*, the application of 100µM atrazine reduced enzyme activity by 30% whereas in *C. pyrenoidosa*, the herbicide at only 1µM level depressed the enzyme by

FIGURE 28

Effect of atrazine on the uptake of NO_3^- and O_2 evolution and uptake in Anabaena cylindrica

A. *cylindrica* was grown for 4 days and a thick suspension of the alga was prepared as described in Section 2.5.2. An aliquot (1 ml) of this suspension (0.2 g fr wt) was transferred from a Hamilton gas tight syringe into a special reaction vessel fitted with NO_3^- and O_2 electrodes (Section 2.12.2.) and containing 7 ml of fresh culture solutions with 1mM NO_3^- (Section 2.5.2.) They were exposed to light (200μE/m^2/sec) at 30°C and the contents were continuously stirred magnetically. The levels of NO_3^- and O_2 in the reaction mixtures were monitored continuously as described in Section 2.12.2. Atrazine was introduced into the reaction mixtures at 0.001μM at 10 min (first arrow) and 0.1 and 10μM at 15 min (second arrow) respectively after the start of the experiment.

Fig 28


0·12
0·08
0·04
0
µmol nitrate/g fr wt
O2 evolution
NO3- uptake
0·01µM
0·1µM
10µM
0·01µM
O2 uptake
10µM
0·1µM
0
5
10
15
20
25
Min
8
6
4
2
0
µg atom oxygen/g fr wt

44% (Table 33).

3.2.3.2. Nitrogenase in A. *cylindrica*

The nitrogenase activity in A. *cylindrica* was determined by the C_2H_2 reduction technique (Nicholas & Deering, 1976). The alga was grown for 4 days without combined nitrogen and a thick suspension of filaments was prepared as described in Section 2.5.2. Aliquots (1 ml) of this suspension were transferred into Warburg flasks fitted with Suba-seals and containing graded amounts of atrazine in fresh culture solutions without combined nitrogen. The flasks were then evacuated, flushed with gas mixture containing 5% (v/v) C_2H_2, 10% (v/v) O_2 and 85% (v/v) He and placed in an illuminated reciprocator. Samples of the gas phase in the flasks withdrawn at the time periods indicated in Figure 29 were assayed for C_2H_4 (Section 2.9.2.5.). The results show that there was a steady rate of C_2H_4 production up to the 10th h of incubation and subsequently it declined in both control and atrazine treatments. Atrazine at all concentrations (between 0.005 to 150μM) depressed the production of C_2H_4 by the alga; the 0.005μM level reduced the C_2H_4 production by 10% after 10 h whereas the corresponding value for 150μM atrazine was 41% (Figure 29).

TABLE 33

Effect of atrazine on nitrate reductase activity in N. crassa and C. pyrenoidosa

N. crassa was grown for 2 days in a Westgaard-Mitchell medium supplemented with atrazine as described in Section 2.5.4. *C. pyrenoidosa* was grown for 5 days in culture solutions (Section 2.5.3.) containing graded amounts of atrazine as indicated. Cell-free extracts of these microorganisms were assayed for nitrate activity as described in Section 2.9.2.1.

Organism	Nitrate reductase activity (nmol NO_2^- produced/g fr wt/h)				
	Atrazine (μM)				
	0	0.001	0.1	1	100
N. crassa	895	-	757	-	620
C. pyrenoidosa	1,566	1,392	1,305	870	-

FIGURE 29

Effect of atrazine on acetylene reduction by Anabaena cylindrica

The alga was grown for 4 days without combined nitrogen and a thick suspension of the filaments was prepared as described in Section 2.5.2. An aliquot (1 ml) of this suspension was transferred into 10 ml Warburg flasks with single sidearms fitted with Suba-seals (Section 2.9.2.5.), each flask containing 1 ml of fresh culture solution (without combined nitrogen) with graded amounts of atrazine (0.005, 0.01, 10 and 150μM respectively). The flasks were then evacuated and flushed with a gas mixture containing 5% (v/v) C_2H_2, 10% (v/v) O_2 and 85% (v/v) He as described in Section 2.9.2.5. The flasks were kept at 30°C on a reciprocator over a bank of cool white fluorescent lights (200μE/m^2/sec). Samples of the gas phase in the flasks (0.5 ml each) were withdrawn at the time intervals indicated and C_2H_4 production determined in a Philips 5000 series gas chromatograph as described in Section 2.9.2.5.

●——● Omit atrazine

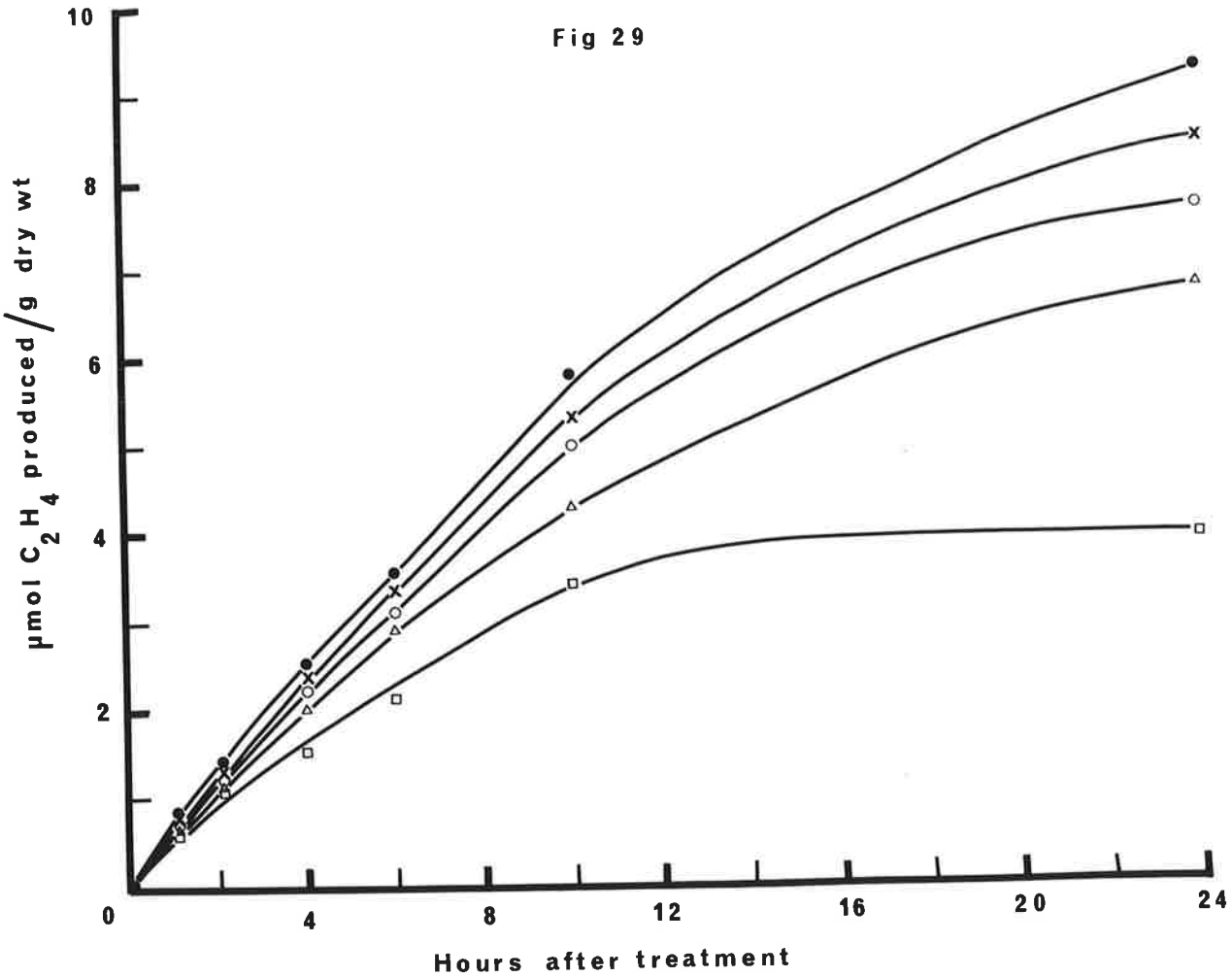
x——x 0.005μM atrazine

o——o 0.01 " "

Δ——Δ 10 " "

□——□ 150 " "

Fig 29


µmol C_2H_4 produced/g dry wt
10
8
6
4
2
0
4
8
12
16
20
24
Hours after treatment

4. DISCUSSION

4. DISCUSSION

4.1. Atrazine effectson maize and barley

Atrazine is well known as a phytotoxic weedicide but at sub-lethal levels, as shown in this thesis for maize and barley, it can promote growth and nitrate assimilation in plants.

One of the earliest effects of atrazine was a stimulation of the elongation of root tips of 7-day-old maize seedlings observed about 4 h after herbicide treatment. This effect may be similar to the increased elongation of the developing kidney bean embryos resulting from atrazine treatment reported by Bush & Ries (1974). Although it was found in maize that the stimulation of root elongation occurred either in the presence or absence of nitrate, a subsequent increase in fresh weight of seedlings due to atrazine was obtained only in nitrate grown plants. No such effect was obtained with ammonium grown plants. Similarly Ries *et al* (1967) showed that simazine increased total protein content in rye plants grown with nitrate but not when ammonium was the sole nitrogen source.

It is of interest that although the atrazine treatment increased the fresh weight of maize and barley seedlings, it did not increase the dry weight. This is in agreement with the results of Hiranpradit *et al* (1972) with corn and could be due to either an increase in respiration rate without affecting the respiratory quotient as found in rye plants treated with simazine (Ries *et al*, 1967) or increased synthesis of protein at the expense of carbohydrates (Wu *et al*, 1972; Pulver & Ries, 1973). Alternatively the increase in fresh weight but not dry weight could be caused by a reduced water loss by transpiration reported in several species of plants treated with triazines (Smith & Buchholtz, 1964). However, it is noteworthy that in the present study, the increase in fresh weight of the atrazine treated plants was accompanied by increases in protein content as well as the uptake

and assimilation of nitrate, which makes it unlikely that it resulted simply from an increase in water content of the plants.

It was observed in the current study that atrazine treated maize plants were taller and sturdier than the controls and in addition the leaves were broader, greener and senescence was delayed. A similar observation was made by Hiranpradit *et al* (1972) and they suggested that atrazine affects directly or indirectly either the number of chloroplasts or the amount of chlorophyll in the plants. They also reported that atrazine increased the synthesis of protein in chloroplasts as well as the amount of chlorophyll nitrogen per plant and suggested that the higher protein content of treated plants could be accounted for, by the increased protein synthesis in the chloroplasts. Freney (1965) and Vergara *et al* (1970) also found that the senescence of leaves was delayed by triazine treatments. This effect on senescence may be linked to the influence of atrazine on auxin metabolism of plants as suggested by Ebert & Van Assche (1969).

Although maize is considered to be a resistant plant (Shimabukuro, 1968), daily application of 100μM atrazine resulted in reduced growth. Barley is more susceptible to atrazine than maize and daily application of 2μM of the herbicide resulted in complete dessication of leaves and eventually death of the plant.

The increase in fresh weight observed in nitrate grown maize plants but not in those supplied with ammonium suggests that the main effect of atrazine may be on either the uptake and transport of nitrate or nitrate assimilation or both. In contrast to nitrate, ammonium is absorbed faster than nitrate (Yoneyama *et al*, 1975) and is reduced in the roots before transport of the reduced nitrogen compounds to the shoots (Muhammad & Kumazawa, 1974). Higinbotham (1973) claims that cations like ammonium are passively absorbed by plants whilst nitrate and other anions are accumulated against an electrochemical potential gradient so that they are actively

transported into the roots.

The results reported in this thesis suggest that atrazine enhanced the rate of nitrate uptake by maize seedlings within 2 h of treatment. Pulver & Ries (1973) also showed that simazine increased the uptake of nitrate in barley seedlings but the earliest effect recorded by them was 2 days after treatment. The atrazine stimulation of nitrate uptake was accompanied by a higher accumulation of nitrate in both roots and shoots of maize seedlings. This effect was further confirmed by studies with $^{15}NO_3^-$ where atrazine enhanced the uptake and incorporation of this compound into protein as well as into total nitrogen in both maize and barley seedlings.

Nitrate taken up by the roots of plants treated with atrazine accumulated in both the vacuolar storage pool and the cytoplasmic active pool as defined by Ferrari *et al* (1973). In the present study there was no evidence that the herbicide altered the distribution of nitrate between the storage pool and active pool. Such changes in the distribution of nitrate were recorded when corn tissues were exposed to either *Helminthosporium carbonum* toxin (Yodder & Scheffer, 1973) or glucose (Aslam & Oaks, 1975).

In addition to nitrate, it has been reported that the uptake of K^+, Ca^{2+}, Mg^{2+} and PO_4^{3-} was stimulated by triazines (Freney, 1965; Hiranpradit *et al*, 1972). However, in the present study on maize, only the uptake of K^+ was increased but to a lesser extent than for nitrate. The herbicide did not affect the uptake of either Cl^- or PO_4^{3-}. Wray *et al* (1970) also found that simazine stimulated the uptake of nitrate by barley plants but the SO_4^{3-} and PO_4^{3-} were not affected. The stimulation of nitrate uptake by atrazine reported here thus appears to be a specific effect on this ion and the effect on K^+ was probably secondary. An increased uptake of nitrate could result in a charge difference across the root surface and therefore cations like K^+ could move into roots down this

electrochemical potential gradient (Higinbotham, 1973). Blevins *et al* (1974) found that the pH of the cell sap increased when nitrate accumulated and this was accompanied by an increase in the synthesis of malic acid. They suggested that malic acid would then provide counterions for the increased K^+ uptake.

Atrazine significantly increased the content of amino acids transported through the xylem sap of nitrate grown maize plants, almost to the levels recorded in the ammonium grown plants. It is well known that ammonium plants synthesize higher amounts of amino acids in the roots than plants supplied with nitrate (Bekmukkamedova, 1961; Ingversen & Ivanko, 1971) but it is of interest that with atrazine treatment, the synthesis of amino acids in nitrate grown plants was increased to that of ammonium plants. Indeed when the increased rate of exudation of xylem sap of nitrate grown plants due to atrazine treatment is also considered, the total output of amino compounds from the roots of nitrate plants is higher than that of ammonium plants. In the present study, almost 90% of the nitrogen compounds transported via the xylem sap of nitrate grown maize plants were amino compounds irrespective of atrazine treatment. In a comparative study on a range of plant species Pate (1972) found that maize transported relatively high amounts of inorganic nitrogen compounds (65%) through the xylem sap. The high ratio of amino nitrogen to nitrate nitrogen transported through xylem sap of maize in the present investigation may be due to the sub-optimal growth conditions employed for growing maize (4.5mM NO_3^- and temperature 17°C night and 22°C day).

In the present investigation the amino compounds in the xylem exudate of maize were found to be primarily the amides, glutamine and asparagine with the former predominating. The main amino acids were serine, alanine, valine, lysine and threonine. It is surprising that serine which is considered to be synthesized in the leaves (Beevers, 1976) was found in

substantial amounts in the root exudate. Possibly it has been recycled through the roots as discussed by Pate (1977). Serine has not been previously reported as a significant constituent of the xylem sap: indeed, Ivanko & Ingversen (1971) did not list it in their detailed analysis of xylem sap from maize seedlings. Before the development of the improved buffer systems used in this thesis which allow for the separation of the amides from threonine and serine, the amount of the latter may have been underestimated.

The increase in nitrate and amino acid content of plants was accompanied by an enhanced protein synthesis as shown in the $^{15}NO_3^-$ studies. Since the ratio of $^{15}NO_3^-$ incorporation into protein to $^{15}NO_3^-$ incorporation into total nitrogen was similar in control and atrazine treated plants, it appears that the herbicide did not specifically increase protein synthesis. In maize seedlings a day after the initial treatment with the tracer, roots accumulated a higher level of $^{15}NO_3^-$ than did shoots but the ratio of $^{15}NO_3^-$ incorporation into protein to $^{15}NO_3^-$ incorporation into total nitrogen was higher in shoots than in roots. This is in agreement with the data of Ashley *et al* (1975) for wheat seedlings treated with $^{15}NO_3^-$ for 9 h. After longer periods e.g. 3 or more days, the total $^{15}NO_3^-$ incorporation in shoots was more than in roots in both maize and barley.

A study of the effects of triazines on the rate of protein synthesis was undertaken by Ries & associates (Pulver & Ries, 1973; Bush & Ries, 1974). They followed the incorporation of [^{14}C]-leucine into protein as an indicator of protein synthesis. Pulver & Ries (1973) found that simazine increased the incorporation of [^{14}C]-leucine into protein in detached leaf segments of barley seedlings grown in dilute Hoagland's solution. Simazine did not alter the uptake of leucine and only 20% of that taken up was incorporated into protein. In contrast it is shown in this thesis that atrazine did not increase the [^{14}C]-leucine incorporation into protein in detached leaves of barley seedlings grown with 4.5mM of either NO_3^- or NH_4^+

as a source of nitrogen. However, the herbicide enhanced the incorporation of ^{14}C-labelled leucine into protein in detached leaves of barley seedlings grown without combined nitrogen. Since Pulver & Ries (1973) did not state the amount of nitrate supplied to the barley seedlings, their results cannot be compared with the results presented in this thesis. It appears therefore that the primary effect of atrazine is not on the rate of protein synthesis. The increased rate of incorporation of [^{14}C]-leucine into protein in the nitrogen deficient leaves given atrazine may be linked to changes in the availability of endogenous amino acids by the herbicide.

There are several reports in the literature that plants treated with triazine herbicides have an enhanced level of nitrate reductase (Tweedy & Ries, 1967; Ries *et al*, 1967; Singh & Salunkhe, 1970). This has been confirmed in the present studies on maize and barley and it has also been shown that atrazine (a) did not increase the activity of preformed enzyme (*in vivo*), (b) did not stimulate the production of enzyme (*in vivo*) in the absence of nitrate, (c) was without effect on nitrate reductase when added to the assay mixture *in vitro* and (d) did not arrest the decay (*in vitro* or *in vivo*) of the nitrate reductase. The latter is not in accord with the finding of Hewitt as cited by Luckwill (1967) that the herbicide simazine increased the stability of nitrate reductase. The effect of atrazine on nitrate reductase would thus appear to be on the rate of its synthesis and it is suggested in this thesis that this results from the enhanced uptake of nitrate.

Aslam & Huffaker (1973) reported that while the activity of extractable nitrate reductase in detached barley leaves was increased by atrazine, its *in vivo* activity was decreased. They suggested that the decrease in *in vivo* activity resulted in a higher accumulation of nitrate in the tissue which in turn induced the synthesis of higher levels of nitrate reductase. They used a relatively high amount of herbicide (0.1mM) and obtained a similar

result with DCMU, a known inhibitor of photosynthesis. In a more recent study, atrazine at approximately 0.05mM inhibited nitrite reduction in a number of plants (Finke *et al*, 1977). At these relatively high levels of herbicide, photosynthesis was severely inhibited and therefore the supply of reducing equivalents (ferredoxin) for the nitrite reductase was impaired. In the present investigation with sub-lethal amounts of atrazine, the enhanced activity of nitrate reductase has been correlated with an increase in both uptake and incorporation of ^{15}N labelled nitrate into protein and into total nitrogen.

The activities of nitrite reductase,glutamine synthetase and glutamate synthase were also increased in maize and barley. This confirms that there was a higher rate of nitrate reduction and amino acid synthesis in the treated plants. Singh & Salunkhe (1970) and Singh *et al* (1972a) found that triazines increased the activities of adenosine triphosphatase, glutamic-pyruvic transaminase, α-amylase and starch phosphorylase in addition to nitrate reductase in leaves of bush bean plants. This was associated with the decrease in starch, total and reducing sugars and an increase in protein and amino acid content of the treated plants. They suggested that triazines stimulated the utilization of carbohydrates for the synthesis of amino acids and proteins. They did not however determine what were the early effects of triazines on plant metabolism. In the present investigation, atrazine enhanced the uptake of nitrate within 2 h of treatment. It is likely that atrazine stimulation of the uptake of nitrate would result in an increased utilization of carbohydrates for the production of energy and carbon skeletons to facilitate the uptake and assimilation of nitrate into amino acids and protein.

Ebert & Van Assche (1969) report that low amounts of atrazine depressed the IAA-peroxidase activity in oat coleoptiles and they suggested that inhibition of this enzyme could raise the endogenous IAA, resulting in a

stimulation of growth. It is possible that some auxin like effect on growth could explain the early effect of atrazine on root elongation which was independent of a supply of combined nitrogen. However, the main action of the herbicide on the growth of maize and barley plants was obtained only when nitrate was given as a source of nitrogen. This would link the involvement of atrazine to some aspect of nitrate assimilation.

4.2 Effects of atrazine on microorganisms

One of the main difficulties with the higher plant studies was to measure accurately the early effects of atrazine on the uptake of nitrate as well as the enhancement of growth and to distinguish which process was affected first. Some studies were therefore undertaken with microorganisms where nitrate uptake and growth could be monitored more accurately. The microorganisms selected were two procaryotes (*Escherichia coli* and *Anabaena cylindrica*) and two eucaryotes (*Chlorella pyrenoidosa* and *Neurospora crassa*). However, in no case was there a stimulation of either growth or enhancement of nitrate uptake by atrazine.

Growth of *E. coli*, *A. cylindrica*, *C. pyrenoidosa* and *N. crassa* was not affected by very low amounts of atrazine (10^{-9}M). Higher concentrations were very toxic to both algae but *E. coli* was more tolerant which is in agreement with results of other investigators (Breazeale & Camper, 1972; Arvik *et al*, 1973; Valentine & Bingham, 1976).

Atrazine at sub-lethal levels did not affect the uptake of nitrate in *N. crassa* whereas the herbicide increased the nitrate uptake in maize and barley seedlings. The application of higher amounts of atrazine (0.1 - 10μM) depressed the uptake of nitrate by *A. cylindrica*. Respiration to either O_2 or NO_3^- was also restricted in *E. coli* treated with atrazine. The activity of nitrate reductase which was increased in higher plants by sub-lethal amounts of atrazine was however reduced in *C. pyrenoidosa* and

N. crassa grown with the herbicide (0.001 - 100μM). Although *E. coli* and *N. crassa* were relatively resistant to atrazine, there was again no stimulation of either growth or nitrate uptake resulting from the application of the herbicide. Microorganisms therefore differ markedly from higher plants where atrazine selectively increased the uptake of nitrate which in turn was assimilated at a higher rate into amino acids and protein.

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EFFECTS OF ATRAZINE ON THE ASSIMILATION OF INORGANIC NITROGEN IN CEREALS

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Key Word Index—*Zea mays*; *Hordeum vulgare*; Gramineae; maize; barley; atrazine; nitrate uptake; amino compounds in xylem sap; nitrate assimilating enzymes; nitrate and amino acid incorporation into proteins.

Abstract—The inclusion of sub-lethal amounts of the herbicide atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine] in the nutrient solution supplied to maize and barley increased the growth of the root and shoot and the uptake of nitrate. The activities of nitrate and nitrite reductases, glutamine synthetase and glutamate synthase were enhanced and the amino acid and nitrate contents of the xylem sap increased. All these effects of atrazine were found only in plants grown with nitrate as the nitrogen source. The uptake of $^{15}NO_3^-$ and its incorporation into protein in the root and shoot of maize and barley seedlings was significantly greater in the atrazine treated plants. However, a stimulation in the incorporation of leucine-[^{14}C] into TCA-precipitable protein of detached leaves from 7-day-old barley seedlings was obtained only in the absence of a supply of combined nitrogen either in the culture medium or in the *in vitro* incubation mixture containing the labelled amino acid.

INTRODUCTION

There are several reports that *s*-triazine herbicides applied in sub-lethal amounts to a variety of plants increase growth and nitrogen contents [1–11]. These effects were obtained in plants grown with nitrate but not with ammonium salts as a source of nitrogen [3]. A stimulation of RNA synthesis in isolated chromatin [12], an enhanced protein synthesis [11], a decrease in sugar content [5] and a delayed senescence [6] have also been associated with atrazine treatments of plants. Thus the action of these herbicides on nitrate assimilation may be indirect and their initial effect may be on protein synthesis or on carbohydrate metabolism. Studies with maize and barley reported in this paper are concerned with the action of the *s*-triazines on nitrate assimilation.

RESULTS

Growth

Maize seedlings grown with 0.5 mM NO_3^- had significantly greater fresh weight yield than those grown with an equivalent amount of ammonium chloride (Table 1). Atrazine (0.1 μM) supplied in the culture solutions enhanced the fr. wt of nitrate grown plants by 26% in roots and 31% in shoots. It had no effect on seedlings grown without nitrogen and the yields of roots and shoots of ammonium grown seedlings were slightly depressed. Atrazine also stimulated growth in maize plants after 40 days growth in sand cultures supplied with 4.5 mM NO_3^- in 0.5 strength Hoagland's solution supplemented with 1 μM of the herbicide, the increase in fr. wt of roots and shoots being 32 and 21% respectively. Although the fr. wt of the nitrate grown plants was increased by atrazine treatment, the dry wts were unchanged. In barley plants grown in sand culture with 4.5 mM nitrate and given one application of atrazine (1 μM) when the plants were 1-week-old, increased growth was again observed. The application of 1 μM atrazine daily in the nutrient solution of barley plants proved toxic but maize plants in sand culture tolerated 10 μM of the herbicide and 100 μM suppressed growth. There was delayed senescence in the older leaves of plants supplied with either concentration.

Table 1. Effects of atrazine on the fr. wt of maize seedlings grown with nitrate and ammonium

Nitrogen source	Fresh weight (g)					
	Omit atrazine			Atrazine		
	Roots	Shoots	Total	Roots	Shoots	Total
None	0.71	0.67	1.38	0.68	0.65	1.33
Nitrate (0.5 mM)	0.84	0.85	1.69	1.06	1.11	2.17
Ammonium (0.5 mM)	0.64	0.78	1.42	0.58	0.71	1.29

7-day-old maize seedlings were grown in liquid culture containing 0.1 strength Hoagland's soln without a source of nitrogen as described in Experimental. From the 8th day onwards 10 seedlings in each tray of liquid culture were supplied with either nitrate [0.15 mM $Ca(NO_3)_2$ + 0.2 mM KNO_3] or NH_4Cl in 0.1 strength Hoagland's soln and 0.1 μM atrazine as indicated. The seedlings were harvested after a subsequent 7 days growth. Least significant difference $LSD_{95\%}$ for roots = 0.093 and $LSD_{95\%}$ for shoots = 0.068.

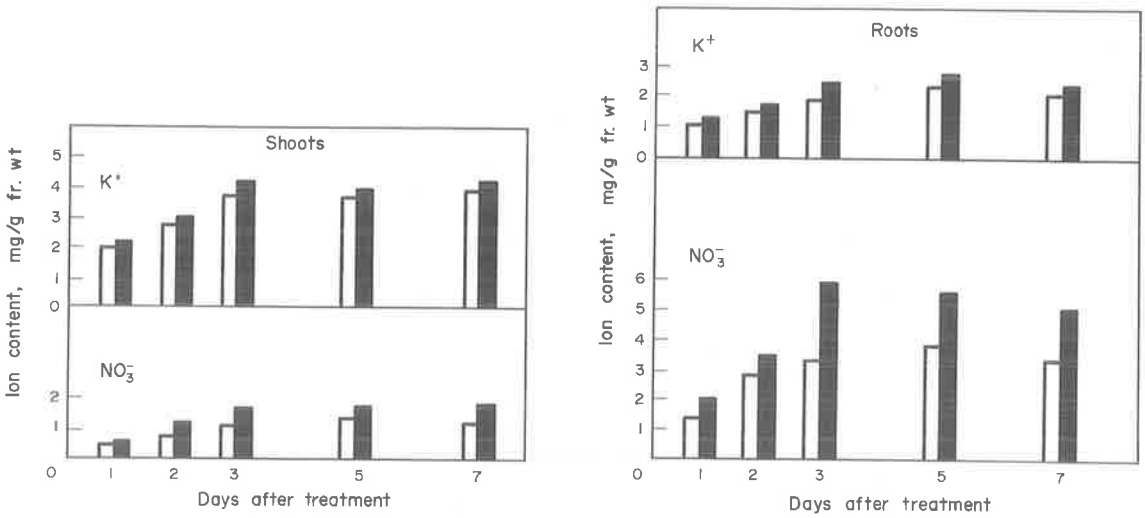

Fig. 1(a and b). Effects of atrazine on the uptake of NO_3^- and K^+ in maize seedlings. The 7-day-old seedlings were grown without nitrogen as described in Experimental. From the 8th day onwards, they were supplied with 4.5 mM NO_3^- [1.5 mM $Ca(NO_3)_2$ + 1.5 mM KNO_3] and 0.1 μM atrazine in 0.5 strength Hoagland's soln. The seedlings were harvested at various times after the application of nitrate and atrazine on the 8th day, as indicated. Open histogram, without atrazine; solid histogram, with atrazine.

Nutrient uptake

When 7-day-old maize seedlings were transferred into 0.5 strength Hoagland's solution containing 1.5 mM $Ca(NO_3)_2$ and 1.5 mM KNO_3, the nitrate content per g fr. wt in the roots and shoots respectively increased up to the 3rd day and then remained relatively constant for the next 4 days (Fig. 1a and b). A similar pattern was observed for the uptake of K^+, Ca^{2+} and Mg^{2+} whereas PO_4^{3-} accumulation in roots and shoots remained relatively constant at *ca* 0.4 mg PO_4^{3-} per g fr. wt. The

Table 2. Effects of atrazine on the amino acid and amide contents of xylem sap collected from maize plants supplied with nitrate and ammonium nitrogen

Amino compounds	nmol amino compound per ml of xylem sap			
	Nitrate grown plants		Ammonium grown plants	
	Omit atrazine	Atrazine	Omit atrazine	Atrazine
Aspartic acid	232	350	240	344
Threonine	259	410	442	398
Serine	1310	2050	2150	1790
Asparagine	475	1180	1350	1330
Glutamine	6400	12000	20000	17800
Proline	40	59	37	40
Glutamic acid	202	307	505	476
Glycine	103	192	148	116
Alanine	504	1020	723	606
Valine	303	439	541	511
Half cystine	77	72	78	70
Methionine	9	14	16	15
Isoleucine	71	103	108	121
Leucine	63	110	116	119
Tyrosine	31	43	77	75
Phenylalanine	15	28	70	58
γ-Aminobutyric acid	24	29	55	79
Ornithine	49	53	150	98
Lysine	235	450	501	430
Histidine	85	155	134	109
Tryptophan	25	21	39	34
Arginine	175	371	315	273
Total	10700	19500	27800	24900

Maize plants were grown in sand cultures as described in Experimental. From the 15th day onwards the plants were supplied daily with 0.5 strength Hoagland's soln containing either 4.5 mM nitrate [1.5 mM $Ca(NO_3)_2$ + 1.5 mM KNO_3] or 4.5 mM NH_4Cl, supplemented with 1 μM atrazine as indicated. After 40 days' growth xylem sap was collected and analysed for amino compounds as described in Experimental.

uptake of nitrate was always enhanced by atrazine and maximum increases in both roots and shoots (76 and 62% respectively) were recorded at 3 days (Fig. 1 a and b). A similar pattern was observed for K^+ accumulation but the effect of atrazine was less than for nitrate, i.e. 30% for roots and 13% for shoots on the 3rd day (Fig. 1a and b). Increases in Ca^{2+} and Mg^{2+} were observed in roots only on the 3rd day but at a later stage the herbicide was without effect. The uptake of Ca^{2+} and Mg^{2+} into shoots and PO_4^{3-} into roots and shoots was not affected by the atrazine treatment. During the period of this experiment, the growth rate increased in atrazine treated plants by 38% in roots and 43% in shoots.

Nitrogen content of xylem sap

Fourteen-day-old maize seedlings grown in liquid cultures with 0.5 mM nitrate as a source of nitrogen secreted more xylem sap (60 µl/plant/hr) than did those grown with 0.5 mM ammonium chloride (42 µl/plant/hr). A relatively small volume of exudate was recorded in seedlings grown without added nitrogen (6 µl/plant/hr). Atrazine increased the rate of xylem sap secretion in nitrate grown seedlings by 70% while it had no effect on ammonium grown plants. Similar results were obtained with older plants grown in sand cultures after 40 days growth.

There was a larger amount of amino compounds (27.8 µmol/ml) in the sap of 40-day-old ammonium grown plants compared with those grown with nitrate (10.7 µmol/ml) Table 2. The dominant compound was glutamine (60 and 72% of the total amino compounds in plants grown with nitrate and ammonium chloride respectively). The composition of other amino compounds in plants grown with nitrate and ammonium chloride respectively are as follows (% of total amino compounds), serine (12 and 8%), asparagine (4.5 and 5%) and alanine (5 and 3%). In ammonium grown plants glutamine makes up the greater proportion (72%) of the total amino compounds of the xylem sap and is 213% higher than in exudates of plants grown with nitrate only. Other amino compounds showing significant increases in the ammonium plants compared with nitrate grown plants were asparagine (184%), glutamic acid (150%), tyrosine (148%), phenylalanine (367%), γ-aminobutyric acid (129%), ornithine (206%) and lysine (113%). Similar amounts of aspartic acid, proline and half cystine were recorded in the sap of plants grown with either ammonium or nitrate nitrogen. Atrazine treatment resulted in an 82% increase in the total amino compounds of the exudates of plants grown with nitrate but in a corresponding treatment of plants grown with ammonium salts, there was a 10% decrease. Atrazine treatment increased asparagine by about 149%. In addition to the other amide, glutamine (88%) the following amino acids were also increased as a result of atrazine treatment, alanine (102%), arginine (112%), lysine (91%), histidine (82%), phenylalanine (87%), leucine (75%) and aspartic acid (51%). Even in ammonium grown plants where atrazine treatment decreased most of the amino acids the level of aspartic acid was increased by 43%. Maize plants grown for 40 days with 2 mM ammonium chloride had a lower amino nitrogen content in the xylem sap (5.1 µmol/ml) than those grown with 4.5 mM ammonium chloride but atrazine again had no effect.

The effects of atrazine on the organic and inorganic nitrogen fractions of xylem sap are presented in Table 3. Organic nitrogen was the main component transported in the xylem sap of plants grown with ammonium chloride whereas in nitrate grown plants, both organic nitrogen (91.6% of the total nitrogen) and inorganic nitrogen (8.4%) were detected. In nitrate grown plants atrazine increased the organic nitrogen and nitrate nitrogen transported through xylem sap by 86 and 63% respectively. Nitrate was not detected in the xylem sap of maize plants grown with ammonium salts and atrazine decreased the organic nitrogen content of these plants by 10%. Neither nitrite nor protein was detected in the xylem sap.

The total content of soluble amino nitrogen in the 80% ethanol extracts of roots and shoots was not affected by atrazine treatment (*ca* 80 µmol/g fr. wt in roots and shoots respectively irrespective of atrazine treatment).

Enzyme activities

The application of atrazine increased the activities of nitrate and nitrite reductases, glutamine and glutamate

Table 3. Effects of atrazine on various nitrogen fractions in the xylem sap

Types of nitrogen	µg N per ml of xylem sap			
	Nitrate grown plants		Ammonium grown plants	
	Omit atrazine	Atrazine	Omit atrazine	Atrazine
Organic nitrogen				
(a) ninhydrin positive amino nitrogen	150	272	390	348
(b) amide nitrogen of asparagine and glutamine	96	185	307	274
(c) other nitrogen of amino acid	15	29	22	23
(d) total	261	486	719	645
Inorganic nitrogen				
Nitrate	24	39	0	0
Total nitrogen	285	525	719	645

The xylem sap was obtained from 40-day-old maize plants described in Table 2. The various organic nitrogen fractions were calculated from the data in Table 2.

Table 4. Effects of atrazine on the activities of nitrate and nitrite reductases, glutamine synthetase and glutamate synthase in maize seedlings grown with nitrate

Enzymes	Enzyme activities			
	Roots		Shoots	
	Omit atrazine	Atrazine	Omit atrazine	Atrazine
Nitrate reductase (μmol NO_2^- produced/g fr. wt/hr)	1.1	2.3	1.8	2.9
Nitrite reductase (μmol NO_2^- utilized/g fr. wt/hr)	17.9	25.5	19.9	29.6
Glutamine synthetase (μmol glutamyl hydroxamate produced/g fr. wt/hr)	80.0	112.0	96.0	144.0
Glutamate synthase (μmol glutamate produced/g fr. wt/hr)	53.2	67.8	147.0	172.2
Average fr. wt six plants (g)	1.17	1.60	1.14	1.73

Maize seedlings grown with nitrate and atrazine in the nutrient solutions as described in Fig. 1 were harvested after a 7-day treatment. Enzymes were determined in cell-free extracts as described in Experimental.

synthetases in roots and shoots of 14-day-old maize seedlings grown with nitrate (Table 4). The activity of nitrate reductase was increased most markedly by atrazine in roots (109%) and to a lesser extent in shoots (61%). The activities of other enzymes were increased by the herbicide by about 40–50% in both roots and shoots except for glutamate synthase where the increases were less, viz. 27 and 17% for roots and shoots respectively. Seven-day-old barley seedlings grown without added nitrogen as described in the Experimental were supplied with nitrate or ammonium chloride as a source of nitrogen together with atrazine for the next 3 days (Table 5). The activities of these enzymes in nitrate grown barley leaves were enhanced by atrazine treatment and the results are similar to those of the nitrate grown maize seedlings described earlier. When ammonium was the nitrogen source, the activities of nitrate and nitrite reductases were very low as expected and atrazine did not stimulate them nor the activities of glutamine synthetase and glutamate synthase.

Assimilation of $^{15}NO_3^-$

Seven-day-old barley seedlings were supplied with 3 mM $K^{15}NO_3$ and 1 μM atrazine in the nutrient solu-

Table 5. Effects of atrazine on nitrate and nitrite reductases, glutamine synthetase and glutamate synthase in leaves of barley seedlings grown with either nitrate or ammonium chloride as a source of nitrogen

Enzymes	Enzyme activities			
	Nitrate treated plants		Ammonium treated plants	
	Omit atrazine	Atrazine	Omit atrazine	Atrazine
Nitrate reductase (μmol NO_2^- produced/g fr. wt/hr)	4.47	8.1	0.34	0.26
Nitrite reductase (μmol NO_2^- utilized/g fr. wt/hr)	11.0	17.2	5.84	4.12
Glutamine synthetase (μmol glutamyl hydroxamate produced/g fr. wt/hr)	136.0	192.0	120.0	116.0
Glutamate synthase (μmol glutamate produced/g fr. wt/hr)	73.4	99.9	65.3	61.2

7-day-old barley seedlings grown in sand cultures (without the supply of nitrogen compound as described in Experimental) were treated daily with either [1.5 mM $Ca(NO_3)_2$ + 1.5 mM KNO_3] and 1 μM atrazine or 3 mM NH_4Cl and 1 μM atrazine both in 0.5 strength Hoagland's soln. Leaves were collected at 72 hr after the application of nitrogen and atrazine. Enzymes were assayed in cell-free extracts as given in Experimental.

Table 6. $^{15}NO_3^-$ uptake and incorporation into TCA-precipitable protein and into total nitrogen in barley

Plant materials	No. of days of $^{15}NO_3^-$ treatment	µg $^{15}NO_3^-$ (N/g fr. wt) TCA-precipitable protein Omit atrazine	Atrazine	Total nitrogen Omit atrazine	Atrazine
Roots	3	33	39	62	72
	5	57	84	103	159
Shoots	3	69	81	220	265
	5	214	457	400	771
Total	3	102	120	282	337
	5	271	541	503	930

7-day-old barley seedlings were grown in sand cultures without added nitrogen in the nutrient solutions as described in Experimental. These were treated with 3 mM $K^{15}NO_3$ and 1 µM atrazine for the next 3 days and for a further 2 days with $K^{15}NO_3$ only. Roots and shoots were harvested separately on the days indicated and analysed for $^{15}NO_3^-$ incorporation into the TCA-precipitable protein and into total nitrogen as described in Experimental.

tions. Roots accumulated about one-fifth of the labelled nitrogen on the 3rd and 5th day after the application of $^{15}NO_3^-$ (Table 6). The inclusion of atrazine in the nutrient solutions enhanced the total uptake of $^{15}NO_3^-$ by 20% after 3 days and by 85% after 5 days. On the 5th day the incroporation of $^{15}NO_3^-$ into TCA-precipitable protein and into total nitrogen was increased by atrazine treatment in roots by 47 and 54% respectively as well as in shoots by 114 and 93% respectively. The increased incorporation of $^{15}NO_3^-$ into TCA-precipitable protein and into total nitrogen was detectable in leaves of barley within 2 days of adding the herbicide and $K^{15}NO_3$ (Fig. 2). Unlike barley, maize accumulated a higher proportion of the labelled nitrate nitrogen in the roots as shown in Table 7. On the 1st day, 67% of the nitrate nitrogen accumulated in roots and on the 3rd day 50%. Here again, the application of 0.1 µM atrazine increased the uptake of $^{15}NO_3^-$ as well as the incorporation of labelled nitrate into TCA-precipitable protein and into total nitrogen in both roots and shoots. On the 3rd day, the uptake of $^{15}NO_3^-$ was increased (23%) by atrazine as was the incorporation into protein in roots and shoots (28 and 18% respectively).

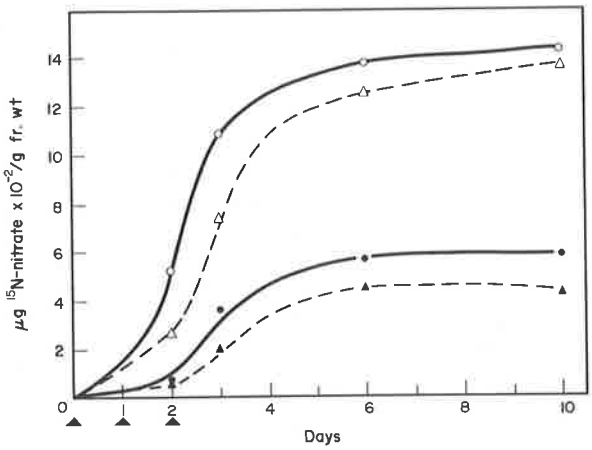

Fig. 2. Effect of atrazine on the incorporation of $^{15}NO_3^-$ into TCA-precipitable protein and into total nitrogen. 7-day-old barley seedlings grown in sand cultures (without the supply of nitrogen compounds as described in Experimental) were treated for the next three days with 3 mM $K^{15}NO_3$ in half-strength Hoagland's soln containing 1 µM atrazine (as indicated by the arrows). They were then maintained on unlabelled nitrate [1.5 mM $Ca(NO_3)_2$ + 1.5 mM KNO_3] in half-strength Hoagland's soln without atrazine. Leaves were analysed at the 2nd, 3rd, 6th and 10th day respectively after the first application of the tracer and atrazine; the incorporation of $^{15}NO_3^-$ into protein and total nitrogen was determined. $^{15}NO_3^-$ incorporated into total nitrogen, ●——● omit atrazine and ○——○ atrazine; $^{15}NO_3^-$ incorporated into TCA-precipitable protein, ▲-----▲ omit atrazine and △-----△ atrazine.

Incorporation of L-*leucine*-[U-^{14}C]

Detached leaves from 7-day-old barley seedlings grown without added nitrogen were inserted into vials containing leucine-[^{14}C] and atrazine in phosphate buffer (pH 7.5) for 12 hr, as described in Experimental. Atrazine increased the incorporation of leucine-[^{14}C] into TCA-precipitable protein of these leaves by *ca* 47% (Table

Table 7. $^{15}NO_3^-$ uptake and incorporation into TCA-precipitable protein and into total nitrogen in maize

Plant materials	No. of days after $^{15}NO_3^-$ treatment	µg $^{15}NO_3^-$ (N/g fr. wt) TCA-precipitable protein Omit atrazine	Atrazine	Total nitrogen Omit atrazine	Atrazine
Roots	1	123	135	265	293
	3	341	435	581	747
Shoots	1	71	84	129	140
	3	375	443	586	685
Total	1	194	219	394	433
	3	716	878	1170	1430

11-day-old maize seedlings were grown in liquid cultures without nitrogen as described in Experimental. From the 12th day onwards, 3 mM $K^{15}NO_3$ and 0.1 µM atrazine were added to the nutrient solutions. Roots and shoots were harvested on the days indicated and analysed for $^{15}NO_3^-$ incorporation into the TCA-preciptable protein and into total nitrogen.

Table 8. L-Leucine-[U-^{14}C] incorporation by detached leaves.
(a) Nitrogen supply in culture medium

Source of nitrogen	Leucine-[^{14}C] incorporation into protein (dpm/mg protein) Atrazine μM 0	25	% Increase
Omit N	5070	7590	49
4.5 mM NO_3^-	8060	8450	5
15 mM NO_3^-	7210	7880	9
4.5 mM NH_4^+	7240	7300	1

Barley seedlings were grown for 5 days in 4 trays supplied daily with 0.1 strength Hoagland's soln without nitrogen. On the 6th and 7th day, the seedlings were supplied with either equimolar KNO_3 and $Ca(NO_3)_2$ or NH_4Cl as indicated. Leaves were detached on the 8th day and the cut ends immersed in incubation solns containing 2 μCi leucine-[^{14}C] and 25 μM atrazine in 0.05 M Pi buffer (pH 7.5). The vials containing the cut leaves were kept in a phytotron at 22° and 350 μE/m^2/sec. The incorporation of leucine-[^{14}C] into protein was determined in leaves after a 12 hr incubation as described in Experimental.

(b) Nitrogen supply in leucine-[^{14}C] incubation medium

Source of nitrogen	Leucine-[^{14}C] incorporation into protein (dpm/mg protein) Atrazine μM 0	25	% Increase or decrease
Omit N	6160	8940	+45
4.5 mM NO_3^-	10700	8960	−16
4.5 mM NH_4^+	8240	7020	−15

Barley seedlings were grown for 7 days in sand cultures supplied with 0.1 strength Hoagland's soln without nitrogen. Leaves were detached on the 8th day and the cut ends immersed in incubation solns containing 2 μCi leucine-[^{14}C], 25 μM atrazine in 0.05 M Pi buffer (pH 7.5) and a nitrogen supply as indicated. Samples were incubated for 12 hr as described above.

8a and b). The leucine-[^{14}C] incorporation into leaves of plants grown without nitrogen and atrazine was about 5–6 × 10^3 dpm/mg protein. The uptake of labelled leucine into protein was higher in leaves of similar seedlings grown with nitrate or ammonium but without atrazine as in Table 8a (7.2–8 × 10^3 dpm/mg protein) and in detached leaves of plants grown without a nitrogen source but supplied with either nitrate or ammonium in the incubation solution as in Table 8b (8.2–10.7 × 10^3 dpm/mg protein). This increased incorporation of leucine-[^{14}C] was comparable to that in atrazine treated leaves of barley seedlings grown without added nitrogen (7.5–9 × 10^3 dpm/mg protein). Atrazine did not stimulate the incorporation of leucme-[^{14}C] into leaves of seedlings grown with a nitrogen source (Table 8a) or in detached leaves of seedlings grown without a nitrogen source but supplied with either nitrate or ammonium chloride in the incubation solution (Table 8b).

DISCUSSION

Atrazine stimulated the growth of maize and barley in agreement with previous reports on a variety of plants [1–5]. The growth response was observed in plants supplied with nitrate but not with those given ammonium salts or no combined nitrogen. Ries *et al.* [3] have shown that simazine increased protein in rye plants grown with nitrate but not when ammonium was the sole nitrogen source. In experiments described herein, increased growth resulting from atrazine treatment was correlated with an increased nitrate content and enhanced level of its assimilation in roots and shoots. This effect was confirmed by the uptake and incorporation of $^{15}NO_3^-$ into protein and total nitrogen of roots and shoots respectively.

In addition to the increased accumulation of nitrate, it has been reported that the uptake of K^+ [6, 8], Ca^{2+} [6], Mg^{2+} [8] and PO_4^{3-} [6, 8] are also stimulated by triazines. In the present study it is shown that the effect of atrazine on the uptake of K^+ by maize was less marked than for nitrate. Atrazine however had no effect on the accumulation of PO_4^{3-}. Wray *et al.* [13] also concluded that simazine had a specific effect in stimulating nitrate uptake in barley since it did not affect SO_4^{2-} and PO_4^{3-}. The stimulation of nitrate uptake by atrazine reported herein appears to be a specific effect so that the increased accumuation of other ions e.g. K^+ is probably secondary. It has also been shown that the uptake of nitrate in barley seedlings resulted in an augmented accumulation of K^+ [14].

The differential effect of atrazine on the growth of maize plants supplied with nitrate compared to those given ammonium may be explained in terms of the uptake and utilization of these compounds in plants. Nitrate and other major anions are probably actively accumulated, but cations like ammonium may be passively absorbed [15]. In recent studies with maize roots Yoneyama *et al.* showed that the uptake of ^{15}N labelled ammonium was more rapid than ^{15}N labelled nitrate [16] and the uptake of the anion was more temperature sensitive [17]. There is evidence in several plant cells for the induction by nitrate of a nitrate transport system which involves protein synthesis [18]. It is also known that the absorption of nitrate by barley roots was decreased by inhibitors of respiration and oxidative phosphorylation [19]. The enhancement of nitrate uptake by the triazines could be due to their effects on the nitrate carrier system and its associated metabolic activity.

Pulver and Ries [10] have reported an increased incorporation of leucine-[^{14}C] in detached barley leaves resulting from simazine treatment and suggest that the herbicide stimulated protein synthesis which then increased nitrate uptake. In the present study with detached barley leaves atrazine was found to enhance the incorporation of leucine-[^{14}C] into TCA-precipitable protein but only in the absence of a nitrogen supply. The inclusion of nitrate or ammonium in the plant culture solutions or in the leucine-[^{14}C] incubation mixtures *in vitro* however gave a higher rate of incorporation of the isotope which was not further increased by atrazine. It does not appear to us that the primary action of atrazine is on protein synthesis. The stimulation of the rate of leucine-[^{14}C] incorporation into the nitrogen deficient leaves may be due to some alteration in the availability of the endogenous amino acids.

The triazines are non-polar molecules and are rapidly taken up by plant roots [20] and transported to the shoot [21]. Because of their lipophilic nature, they could exert some effect on membrane permeability. In addition to the increase in total nitrate uptake, they could favour a higher proportion of nitrate in the active (cytoplasm) vs storage (vacuole) pool in the cell. Glucose and light have

been shown to mediate similar changes in the distribution of nitrate in barley leaves [22]. Ammonium unlike nitrate is not accumulated in the plant since it is rapidly metabolised. Thus its assimilation would not be expected to be altered by triazine affecting the localisation of ammonium ions in the cell.

A striking effect of atrazine on maize plants grown with nitrate, described in this paper, is the increase in the amino acid content of the xylem sap. It is stimulated almost to the level of that in the ammonium grown plants. This suggests that as for plants grown with ammonium, a relatively high rate of amino acid synthesis occurs in roots of plants grown with nitrate and atrazine and the higher rate of amino acid synthesis is correlated with the enhanced uptake of nitrate by the herbicide. Glutamine was the dominant amino compound in the xylem sap of nitrate and ammonium grown plants but asparagine was the compound most affected by atrazine treatment of maize plants grown with nitrate and atrazine (149% increase). The maize plants used in this work were grown at a sub-optimal temperature and with a low nitrate regime. This may be the reason for the relatively high ratio of amino nitrogen to nitrate nitrogen in the xylem sap, the reverse is usually the case in maize plants grown in more normal conditions [23].

It is well established in several plants [3–5] that the activity of nitrate reductase is increased in those treated with triazines. Aslam and Huffaker [7] however reported that while the activity of nitrate reductase in detached barley leaves was increased, its *in vivo* activity was decreased. In their experiments however they used a relatively high concentration of herbicide (0.1 mM) which would inhibit the growth of barley. In a more recent study [24] with an *in vivo* assay for nitrate and nitrite reductases, atrazine at *ca* 0.05 mM inhibited nitrite reduction. At these relatively high levels of herbicide photosynthesis was severely inhibited and therefore the supply of reducing equivalents (ferredoxin) for the nitrite reductase impaired. In the current work with sub-lethal amounts of atrazine, the enhanced activities of the nitrate assimilatory enzymes have been correlated with increased incorporation of ^{15}N labelled nitrate into protein and into total nitrogen.

One other important characteristic of the triazine effect is that the increase in fr. wt and protein content is not correlated with an increased dry wt. Wu *et al.* [5] have shown that triazine treated plants had decreased starch and soluble carbohydrate levels which would explain why the dry wts are not increased by the herbicide treatment. It is quite likely that even at sub-lethal concentrations of the herbicides, there is some inhibition of photosynthesis and the plants suffer some carbon stress. It is interesting that such conditions are associated with a higher rate of nitrate assimilation and growth in maize and barley plants treated with the herbicide.

EXPERIMENTAL

Liquid cultures of maize seedlings. Seeds of *Zea mays* L. (Hybrid variety DSC1) supplied by the De Kalb Shand Seed Co., Tamworth, N.S.W., Australia, were surface sterilised as described previously [25]. They were germinated on 1% (w/v) agar at 28° in the dark. At the 4-day-old stage, the seedlings were transferred into 0.1 strength Hoagland's soln without added nitrogen in 30 × 20 × 6 cm trays. 30 seedlings were supported in holes in the lid of the container with pieces of sponge. The solns were aerated and the plants grown with 16 hr light periods at an intensity of 350 μE/m²/sec at 22° followed by 8 hr dark periods at 17°. 7-day-old seedlings grown in pretreatment culture solns as described above were supplied with either NO_3^- or NH_4Cl in dil. Hoagland's soln. When nitrogen compounds viz. 0.5 mM of either NO_3^- [0.15 mM $Ca(NO_3)_2$ + 0.2 mM KNO_3] or NH_4Cl was supplied in 0.1 strength Hoagland's soln, the liquid cultures were replenished daily. When higher concns of nitrogen were used viz. 4.5 mM NO_3^- [1.5 mM $Ca(NO_3)_2$ + 1.5 mM KNO_3] or NH_4Cl, the liquid cultures were changed every 3 days. Hoagland's soln was supplemented with $NaMoO_4$ (0.01 μg Mo/ml) and $FeSO_4$ (1 μg Fe/ml). The pH of the NO_3^- culture solns was adjusted daily to 4 with N H_2SO_4 and with N KOH to 6 when NH_4Cl was the nitrogen source. During one day, the pH did not alter by more than 1 unit.

Sand culture expts. Surface sterilised seeds of maize were planted in black plastic pots (15 × 16 cm) containing washed sand and watered daily for 14 days with 0·5 strength Hoagland's soln without added nitrogen. These plants were thinned to 6 per pot and 0.5 strength Hoagland's soln containing 4.5 mM NO_3^- [1.5 mM $Ca(NO_3)_2$ + 1.5 mM KNO_3] or 4.5 mM NH_4Cl and 1 μM atrazine was supplied daily. The plants were grown as described previously. In expts with barley (*Hordeum vulgare* L.) the seeds were soaked in 0.2% (w/v) $HgCl_2$ for 3 min followed by 5 rinses with sterile H_2O. They were then planted in sand in plastic trays (21 × 15 × 5 cm). One-tenth Hoagland's soln (without nitrogen) ws added daily for the first 7 days. From the 8th day onwards the seedlings were supplied with a nitrogen source and atrazine as indicated in the tables. The atrazine sample (Gesaprim, 80% Atrazine) was provided by Ciba-Geigy Australia Ltd.

Xylem sap. Plants were cut at the base of the shoots and the bleeding sap was collected into test tubes using Pasteur pipettes. The first drop of the xylem sap was discarded and the sap was collected over the first hr. The amino nitrogen in the sap was determined by the ninhydrin reaction [26] and individual amino acids in a Beckman Amino acid Analyser, Model 119 with a lithium citrate buffer system to allow direct measurement of glutamine and asparagine.

Measurement of nitrate and other ions. Nitrate was determined by the procedure of ref. [27] using a nitrate reductase enzyme prepared from *E. coli*, strain B. Phosphate was determined by the molybdenum blue method of ref. [28]. An atomic absorption spectrophotometer, was used to determine K^+, Ca^{2+} and Mg^{2+}.

Enzyme assays. Cell-free extracts of roots and shoots respectively were prepared in an extraction medium containing 0.5 mM Na-EDTA, and 3% (w/v) casein in 50 mM Pi buffer (pH 7.5). In addition cysteine (either 5 mM for roots or 20 mM for shoots) was included in the extraction medium. Extracts were analysed for *in vitro* nitrate reductase activity by the method of ref. [25], nitrite reductase activity by the procedure of ref. [29], glutamine synthetase activity by the method of ref. [30] and the glutamate synthase activity as described in ref. [31].

Incorporation of $^{15}NO_3^-$. The seedlings were supplied with 3 mM $K^{15}NO_3$ (31.25% enrichment, Office Industrial De L'Azote, France) in 0.5 strength Hoagland's soln as described in each expt (see Tables). The roots and shoots were harvested separately and the incorporation of $^{15}NO_3^-$ into total nitrogen (Kjeldahl) and into TCA-precipitable protein was determined by MS.

Incorporation of leucine-[U-^{14}C] (330 mCi/mmol, obtained from the Radiochemical Centre, Amersham, U.K.), into TCA-precipitable protein was studied in detached leaves of 7-day-old barley seedlings. The supply of nitrogen compounds either to the barley seedlings or to detached leaves in vials is described in Table 8a and b. The detached leaves were immersed in incubation solns in the vials (25 μM atrazine was included because this amount produced maximum stimulation of nitrate reductase activity in detached barley leaves) and these were placed in a phytotron at 22° and at a light intensity of 350 μE/m²/sec. The leaves collected after a 12 hr incubation were rinsed ×3 with

H_2O. Soluble proteins were extracted by the method of ref. [10]. The pptd protein was collected on a 0.22 μm millipore filter, washed with 5% TCA and 95% EtOH and then air dried. The dried filters were then placed in vials containing 5 ml of scintillation fluor [0.8% (w/v) butyl-PBD in toluene] and ^{14}C incorporation was determined in a liquid scintillation spectrometer.

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