MOLYBDENUM AS A PLANT NUTRIENT. X. SOME FACTORS AFFECTING THE ACTIVITY OF NITRATE REDUCTASE IN CAULIFLOWER PLANTS GROWN WITH DIFFERENT NITROGEN SOURCES AND MOLYBDENUM LEVELS IN SAND CULTURE[1]

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Nitrate reductase enzymes were obtained in partially purified states from *Neurospora crassa* and soybean by Nason and Evans (16) and Evans and Nason (8) who showed also that the enzyme is widely distributed with greatly differing activity in several higher plants. The presence and role of molybdenum as a specific component of these enzymes was shown by Nicholas and Nason (17, 18, 19).

Evidence has been presented elsewhere by Agarwala (1), Agarwala and Hewitt (3), Hewitt and McCready (13) and Nicholas, Nason and McElroy (19) that molybdenum is also required by plants and fungi when provided with nitrite, ammonium compounds, urea, or other sources of nitrogen and that the role of molybdenum in nitrate reductase may not entirely explain its function as an essential element. This conclusion, together with the evidence of Evans and Nason (8), who reported that they obtained no responses to different nitrogen sources or molybdenum supplies in regard to their effects on the activity of nitrate reductase, suggested that further investigation of some of the factors affecting the activity of the enzyme was desirable.

The investigations reported here were made on cauliflower, as this plant has been extensively used at Long Ashton in previous studies concerning molybdenum, with special reference to the origin of the disorder known as whiptail (2, 3, 10, 11).

MATERIALS AND METHODS

Cauliflower plants (*Brassica oleracea* L. var. *botrytis* cv. Majestic) were grown in sand culture using methods already fully described (3, 9). Nitrogen was given as nitrate or as nitrite or ammonium sulphate usually at 12 millimoles/liter. Molybdenum was given at 0.05 ppm, 10 to 20 ppm or was omitted. The residual level in the molybdenum omitted treatments was probably the equivalent of 0.00001 to 0.00002 ppm. Plants grown at this level developed typical severe symptoms of whiptail already described (2, 3). The symptoms were produced in all three nitrogen treatments but plants grown with nitrate showed also the characteristic mottling and necrosis that occur under these conditions (3, 13).

ENZYME EXTRACTION AND SAMPLING: Nitrate reductase was extracted by grinding fresh tissue for 15 minutes with neutral acid-washed silica sand and three times its weight of extractant at 0° C in a chilled mortar. The extracting reagent in 1954 was 0.1 M phosphate buffer pH 7.0 containing 10^{-4} M cysteine and ethylenediamine tetraacetate (EDTA) (18). In 1955 the buffer pH was 8.8. The ground tissues were filtered through muslin and the extract was centrifuged at 20,000 × g at 0° C and kept in ice or used immediately. Activity observed in these extracts was also compared with that obtained by grinding at 0° C in mechanically driven close-fitting glass macerators, by disintegration in a high speed blendor, or by macerating in a hand operated Ten-Broeck pattern glass macerator.

In 1954 the leaf lamina of entire plants was used except that the two oldest or other senescent leaves and those under about 2 cm in length were discarded. In 1955 plants were subdivided into pairs of leaves from adjacent nodes and into root, stem and petiole which were examined separately. It was not possible to sample at one time from all treatments. Simultaneous comparisons were made in regard to molybdenum status whilst the nitrogen treatments were sampled consecutively on three or more occasions in random order. In some tests the plant material was stored at −18° C before assay but the same storage treatment was used in all comparative tests. There did not appear to be any loss of nitrate reductase over 24 hours under these conditions.

NITRATE REDUCTASE ASSAY AND PREPARATION OF REAGENTS: Two tenths or 0.5 ml of extract were used almost immediately in the following reaction system: 1.0 ml 5 % sodium nitrate; 0.1 ml reduced diphosphopyridine nucleotide (DPNH 1 mg/ml); 0.05 ml boiled extract of pig heart acetone powder; 2.5 or 2.8 ml of 0.2 M phosphate buffer pH 7.3; distilled water to produce 5.15 ml. The assays were carried out at 27° C for 20 minutes in 1954 or for 10 minutes at 28° C in 1955. The respective rates of reaction were constant over these intervals. Nitrite was estimated as described by Evans and Nason (8) with the addition of sufficient hydrochloric acid to obtain maximum colour development. Blank estimations were carried out by omitting DPNH and all assays were made in duplicate. Activities were expressed as total activity: millimicromoles of nitrite produced in 10 or 20 minutes per 0.2 or 0.5 ml of extract and as specific activity: millimicromoles nitrite in one minute per mg

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Table I

Effect of Method of Grinding on Nitrate Reductase Activity in Cauliflower Leaf Extracts *

Expt	Method of grinding	Ratio of buffer to leaf wt		
		1/3	1/4	1/5
1	Mortar	154	...	200
	N.I.R.D. blendor	...	...	177
2	Mortar	133	...	...
	N.I.R.D. blendor	...	...	140
	Mechanical glass	91	47	...
3	Mortar	84	...	110
	Mechanical glass	26	37	20
4	Mortar '	96	97	102
	Ten-Broeck	59	...	80
5	Mortar	137	...	...
	Mechanical glass	73	...	...
6	Mortar	179	...	...
	Mechanical glass alone	133	...	136
	With ground glass	79	...	58

* Millimicromoles nitrite produced in 10 min per gm leaf wt.

protein. Protein was estimated colorimetrically by the biuret reaction (21) or turbidimetrically in 1954, and turbidimetrically after precipitation with 2 % trichloracetic acid in 0.06 M ammonium sulphate in 1955.

The presence of nitrite in solutions of analytical reagent grade sodium nitrate and other reagents was minimised by preparing daily a fresh solution of nitrate to which 0.5 mg ammonium chloride was added followed by boiling for three minutes, and also by selecting reagents for minimum nitrite content. The DPNH was prepared from 60 to 75 %. DPN obtained from yeast or commercially. It was reduced in 0.04 M pyrophosphate buffer containing 0.2 M ethyl alcohol by addition of crystalline alcohol dehydrogenase prepared from yeast as described by Racker (20). The pH was maintained between 9.8 and 10.0 with 2 N KOH during the reaction which was observed spectrophotometrically at 340 mμ. Approximately 100 mg actual DPN were used in 70 ml and about 70 to 80 % of the DPN was reduced. At the conclusion of the reaction the solution was heated for 5 minutes in a boiling water bath, cooled rapidly and stored at –18° C. The pig heart extract used to provide FAD (8) was prepared by boiling one part of an acetone powder of pig heart in three parts of water for 5 minutes, centrifuging and storing at - 18° C.

Acid Phosphatase Assay: Acid phosphatase activity was also determined in some extracts. The two enzymes showed markedly different trends in activity in regard to the regions from which they were extracted. This disposed of the possibility that large differences observed between the activity of nitrate reductase in samples from different regions might be due merely to a variable degree of disruption of the cells in different extractions. Acid phosphatase was assayed as the rate of hydrolysis of phenolphthalein phosphate at 27° C over a 15-minute period at pH 5.4 in 0.075 M acetate buffer. The reaction was stopped by adding 1 ml 15.5 % trichloracetic acid. The colour of liberated phenolphthalein was developed on addition of 1 ml N sodium hydroxide to bring the solution to pH 10 approximately and was measured with a Spekker absorptiometer within 10 minutes, using green filters and a calibration curve prepared from phenolphthalein.

Results

Effect of Grinding Procedure and pH: Results in table I show that grinding in a mortar with sand or in the high-speed blendor gave the highest ac-

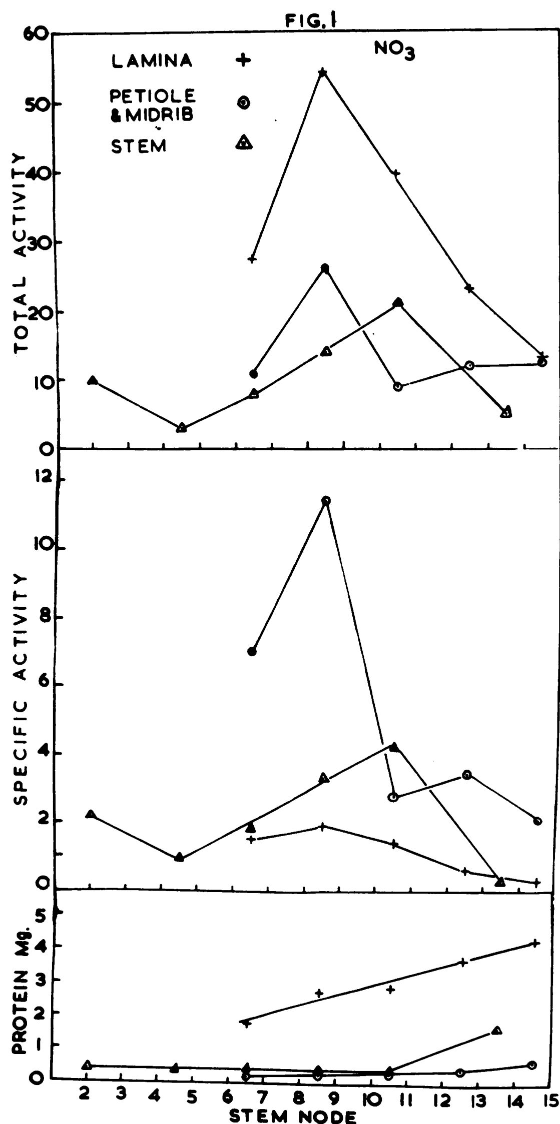
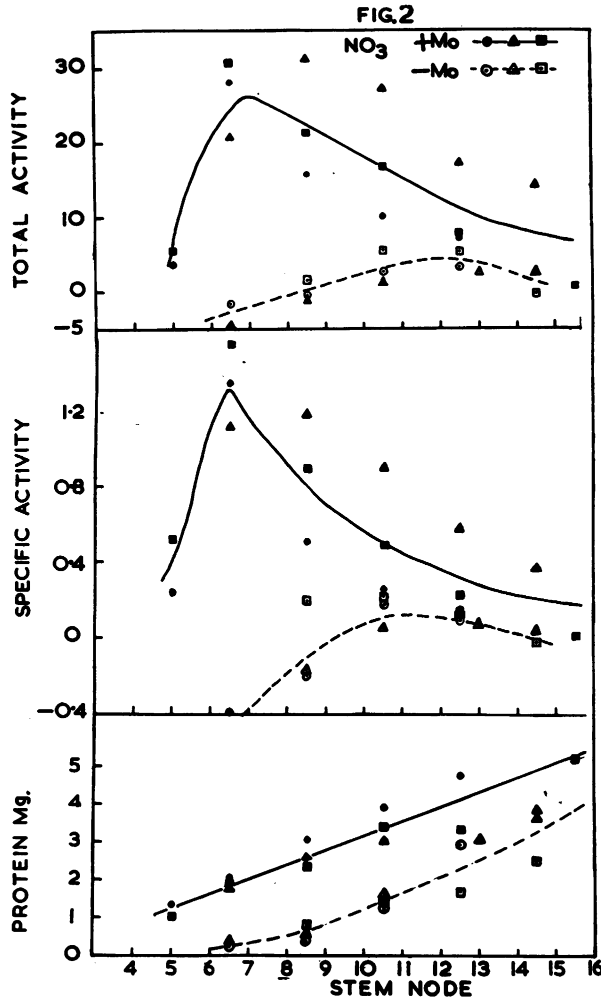


Fig. 1. Nitrate reductase in different regions of cauliflower plants grown with nitrate.



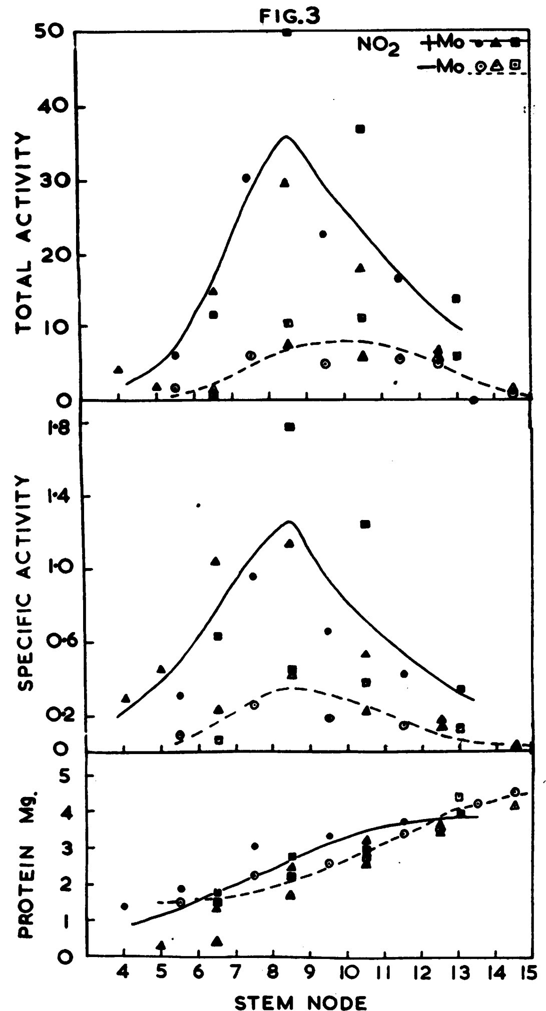


FIG. 2. Effects of leaf position and molybdenum supply on distribution of nitrate reductase activity and protein in cauliflower grown with nitrate. Mean curves for 3 separate experiments shown as squares, circles and triangles, respectively.

FIG. 3. Effects of leaf position and molybdenum supply on distribution of nitrate reductase activity and protein in cauliflower grown with nitrite. Mean curves for 3 separate experiments shown as squares, circles and triangles, respectively.

tivities. Extracts prepared with the glass macerators were, however, usually low in activity and the inclusion of ground glass appeared to be still more unfavourable. The ratio of extracting fluid to tissue weight over a range between 3/1 and 6/1 did not greatly affect the activity per unit leaf weight.

The activity of the centrifuged extract was greatest with a sharp optimum between pH values of 7 and 7.4 in phosphate buffer for cauliflower grown with nitrate in contrast with purified soya bean enzyme for which the optimum was about 6 (8).

DISTRIBUTION OF ACTIVITY AND PROTEIN: The activities observed were the net activities without correction for effects of nitrite reductase. Preliminary tests indicated that nitrite reductase activity was relatively high in roots and low in aerial parts under the conditions of assay. Inclusion of hydroxylamine hydrochloride from one half, up to double the amount used by Evans and Nason (8) to inhibit nitrite reductase did not appreciably affect the observed activity of nitrate reductase in leaf extracts. It was concluded that nitrite reductase activity of these extracts was not sufficient to interfere with the general conclusions reached in this work.

In plants given nitrate with molybdenum (figs 1, 2), the net activity was greatest in mature fully expanded, though not senescent leaves, adjacent to the oldest two or three leaves remaining on the plant at the time. Net activity decreased progressively in younger leaves. The distribution in leaves of plants grown with nitrite or ammonium sulphate and molybdenum (figs 3, 4) was similar to that in plants

given nitrate, but the decrease from the maximum was relatively less sharp in young leaves of plants given ammonium sulphate. In plants aged 16 to 20 weeks the maximum activity was usually found in leaves at nodes 6 and 7 or 8 and 9 (figs 1, 2, 3).

The distribution of net nitrate reductase activity in petioles (fig 1) resembled that in leaves with somewhat lower values. In stems the maximum occurred in younger sections than in leaves. No nitrite production could be detected using extracts of roots. Measurements on loss of nitrite in the presence and absence of added nitrate, suggested that nitrate reductase activity was low in roots.

The distribution of extractable protein differed from that of nitrate reductase and increased from oldest to youngest leaves (fig 1 to 4). The changes were marked in laminae and small in petioles. The protein extracted from stems was the same for lower and mid-stem sections and increased markedly in the youngest stem region. The distribution of specific activity resembled that of total activity, but the changes were relatively smaller between old regions and those with maximal activity and relatively greater between these and the young regions. Specific activity figures were especially high for petioles owing to the low protein values (fig 1).

Age of Plant: The net total activities in leaves of plants grown with 10 to 20 ppm molybdenum and the three sources of nitrogen in 1954 (table II) were considerably less for plants aged 20 weeks than those aged 12 weeks sampled at the same time. The specific activities, however, were similarly in both age groups for nitrite and ammonium treatments due to

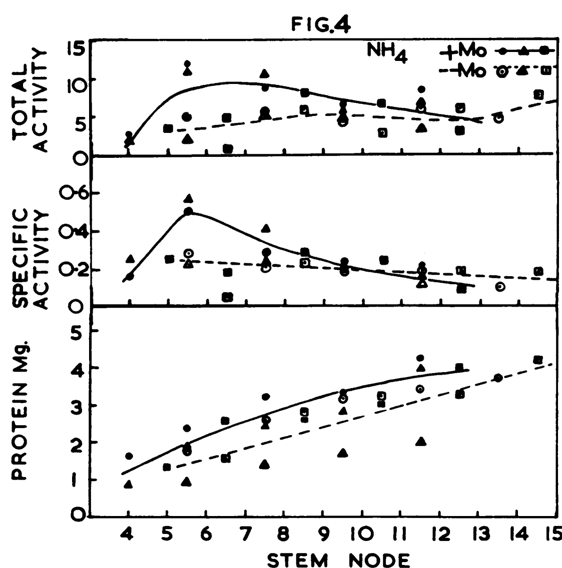


Fig. 4. Effects of leaf position and molybdenum supply on distribution of nitrate reductase activity and protein in cauliflower grown with ammonium sulphate. Mean curves for 3 separate experiments shown as squares, circles and triangles, respectively.

Table II

Effects of Nitrogen Source and Age of Plant on Nitrate Reductase Activity in Leaves of Cauliflower Grown in 1954

Nitrogen supply	Age of plants, wks	Total activity*	Protein, mg/0.5 ml	Specific activity**
Nitrate				
Normal level	12	21.6	3.2	1.68
	20	12.5	2.3	1.36
Double level	20	17.7	3.2	1.38
Nitrite	12	15.8	3.4	1.16
	20	13.5	2.8	1.20
Ammon. sulphate	12	10.4	3.0	0.87
	20	5.9	1.7	0.87

* Millimoles nitrite in 10 min per 0.2 ml extract.
** Millimoles nitrite in 1 min per mg protein.

the lower protein extracted from the older plants, but specific activity also was decreased in the older plants grown with nitrate.

Effects of Nitrogen Source and Molybdenum Level: The net total and specific activities (figs 1 to 4, tables II and IV) were decreased in the order nitrate > nitrite > ammonium sulphate often with significance at 0.1 % in plants aged 12 weeks given 10 ppm molybdenum in 1954. In tests with plants aged 20 weeks the total activities were similar with nitrate and nitrite and much less with ammonium sulphate. Plants aged 20 weeks given double the normal nitrate level had higher total and similar specific activities compared with those given the lower level. In 1955 the maximum values and distribution of net total activities were similar in plants given nitrate or nitrite in the presence of 0.05 ppm molybdenum. In plants given ammonium sulphate the maximum values were between one-third and one-sixth of those observed in the other two nitrogen treatments (figs 2, 3, 4). The changes in amounts of protein extracted from leaves at successive nodes were similar in the three nitrogen treatments in the presence of molybdenum and the effects of nitrogen source on relative specific activities resembled in general their effects on total activities.

In the absence of molybdenum (figs 2, 3, 4) the differences in net total activities between the different nitrogen sources were smaller. Values in leaves of plants given nitrite or ammonium sulphate, however, were perceptibly greater than those in plants given nitrate, possibly because the protein extracted from successive leaves was much less in the nitrate treatment than in the other two. For the same reason the specific activities were similar for the different nitrogen treatments in the molybdenum deficiency series.

Omission of molybdenum markedly decreased net total nitrate reductase activity in nitrate and nitrite treatments (figs 2, 3), but the effect was much smaller in plants given ammonium sulphate, where values even with molybdenum, were generally low (fig 4).

Total activities were distributed more uniformly in deficient than in normal plants, and maxima were less marked especially in plants given ammonium sulphate. In plants given nitrate without molybdenum (fig 2) activity usually increased from oldest to youngest leaves and net activities in old leaves were sometimes apparently negative. This was attributed to a slight excess of nitrite reductase activity over that of nitrate reductase in these plants. The relative effect of molybdenum status on nitrate reductase activity varied greatly and ranged from a ratio of over 15 to 1 to less than 1.5 to 1 in different regions of plants grown with or without molybdenum. The maximum values in molybdenum deficient plants tended to occur in leaves that were attached at higher nodes than those on normal plants.

Molybdenum deficiency markedly decreased the protein extracted from leaves of plants grown with nitrate (fig 2). Smaller differences were also observed in plants grown with nitrite or ammonium sulphate where values observed in leaves from any pair of nodes on molybdenum deficient plants often compared fairly closely with those in leaves from the pair of next older nodes on the normal plants (figs 3, 4). Specific activities were also generally decreased by molybdenum deficiency especially in plants grown with nitrite or ammonium sulphate, and did not show the marked maximum values at intermediate stem nodes observed in normal plants (figs 3, 4).

Effect of Leaf Infiltration by Molybdate on Nitrate Reductase and Acid Phosphatase: Leaves were removed from molybdenum deficient plants and divided into half laminae. One half was infiltrated several times whilst immersed in a 5×10^{-5} M solution of sodium molybdate by alternate evacuation and admission of air, until apparently watersoaked, and

Table III

Effects of Infiltration of Paired Half-Leaves of Molybdenum Deficient Cauliflower Plants with 10^{-5} M Molybdate on Nitrate Reductase Activity *

Source of leaf	Nitrogen treatment	Infiltration treatment			
		Water		Molybdate	
		Range	Mean	Range	Mean
Random leaves	Nitrate	1.0–10.8	3.85	– 1.4–21.4	6.60
Random leaves	Nitrite	1.2– 7.7	4.04	5.3–14.1	9.08
Leaves from successive nodes					
(i)	Nitrite	3.3– 7.2	5.14	2.6–12.8	7.28
(ii)	"	2.7–10.1	5.68	2.7– 8.9	5.90
(iii)	"	3.7– 9.1	6.40	5.6–10.8	8.67
Random leaves	Ammon. sulphate	2.0– 7.2	4.60	3.0–11.4	8.22

* Millimicromoles nitrite produced in 10 min by 0.5 ml extract.

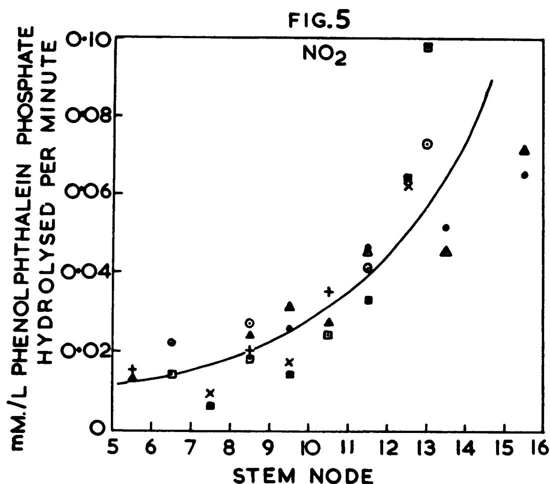


Fig. 5. Effect of leaf position on distribution of acid phosphatase activity in 8 sets of assays with cauliflower plants grown with nitrite. Paired half leaves: ▲ ■ ● ⊙ Infiltrated with molybdate. + × △ ⊡ Infiltrated with water.

the other half was similarly infiltrated with water as a control. The fluid absorbed was determined by the change in weight. Before extraction the leaves were kept in a saturated space for 18 to 24 hours during which they remained turgid and eliminated the surplus water. Several tests were made with single leaves chosen at random from lower nodes, and a few tests were made in which a series of leaves was sampled in relation to stem nodes. The results are summarised in table III. Infiltration by molybdenum increased the activity in most of the tests regardless of the nitrogen treatment but the magnitude of the response varied greatly in different leaves. No response to molybdenum was observed when it was added at the time of maceration. Marked increases in nitrate reductase activity have been observed in whole plants and detached leaves two to three hours after introducing molybdenum or nitrate in later experiments by E. J. Hewitt.

Acid phosphatase activity was measured in the infiltration experiments and results are shown in figure 5. The distribution was quite distinct from that of nitrate reductase and closely resembled that for protein shown in figures 2, 3, and 4. The acid phosphatase values increased in a regular manner from old to young leaves and suggested that the extraction procedure used was valid and reproducible. No effect of molybdate infiltration on acid phosphatase activity was observed.

Effect of Light and Darkness on Nitrate Reductase: These tests were prompted by two considerations, namely (i) the possibility that prolonged darkness might cause sufficient protein loss to decrease enzyme activity; and (ii) the conclusion by Burström (6) and others, that light has a specific function in nitrate reduction in some plants. Plants

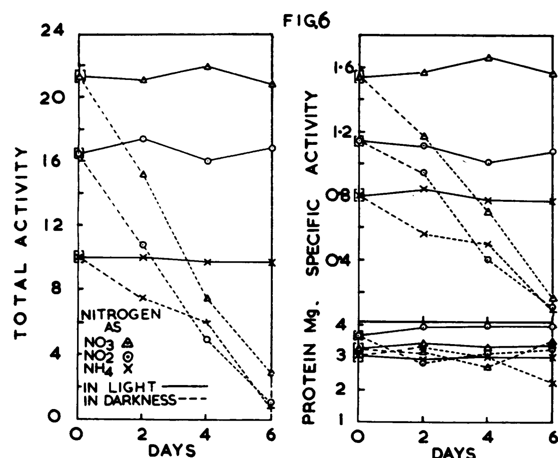


FIG. 6. Nitrate reductase activity in leaves of cauliflower grown with different sources of nitrogen in daylight or darkness.

aged 13 weeks which had been grown with nitrate, nitrite or ammonium sulphate at the high molybdenum levels in 1954 were placed in darkness in the greenhouse or were otherwise allowed to grow in the normal way. Total and specific nitrate reductase activities were determined in triplicate samples from normal and darkened plants at two-day intervals. The results are shown in figure 6. There were marked decreases in the total and specific activities significant at the 0.1 % level for plants of each nitrogen treatment whilst they were kept in darkness. Total extractable protein did not show changes of corresponding magnitude. Similar results were observed with plants aged 21 weeks in the second experiment (fig 7) which was not replicated because of shortage of material. It was found that when normal light was restored there were rapid increases in the total and specific activities in plants from each nitrogen treatment. The changes that were detectable during the first three hours of daylight continued over a period of 24 hours until the following day but the main increases occurred in the first 12 hours. There also appeared to be a net synthesis of protein during the first 6 hours of daylight, which produced slightly higher values than those observed initially.

EFFECTS OF EXCLUSION OF AIR: As the maintenance of organised metabolic activity sometimes depends on normal respiration, the effects of exclusion and reintroduction of atmospheric oxygen were tested. Plants grown with each of the nitrogen treatments were enclosed in transparent airtight plastic covers that were sealed around the outside of the pots. Nitrogen was passed rapidly through a tube into the interior to displace air and a slow stream of nitrogen was then maintained to exclude air as far as possible for 2 days. At the end of this period the plants were sampled and nitrate reductase activity was estimated. Results for plants aged 13 weeks in December, 1954 are given in table IV. In the second experiment in February 1955 with plants aged 21 weeks, the changes that occurred after the restoration of normal atmospheric conditions were also observed and are shown in figure 8. Total and specific net activity of nitrate reductase was greatly decreased by exclusion of air.

TABLE IV

EFFECTS OF 48 HOURS EXCLUSION OF AIR ON NITRATE REDUCTASE ACTIVITY IN LEAVES OF CAULIFLOWER PLANTS AGED 13 WEEKS (1954 EXPT)

NITROGEN SUPPLY	ATMOSPHERE	TOTAL ACTIVITY *	PROTEIN, MG/0.5 ML	SPECIFIC ACTIVITY **
Nitrate	Air	22.0	3.1	1.77
	N_2	11.1	3.6	0.77
Nitrite	Air	18.5	3.4	1.36
	N_2	8.0	2.8	0.71
Ammon. sulphate	Air	9.0	3.4	0.66
	N_2	8.0	3.9	0.51

* Millimoles nitrite produced in 10 min by 0.2 ml extract.

** Millimoles nitrite produced in 1 min per mg protein.

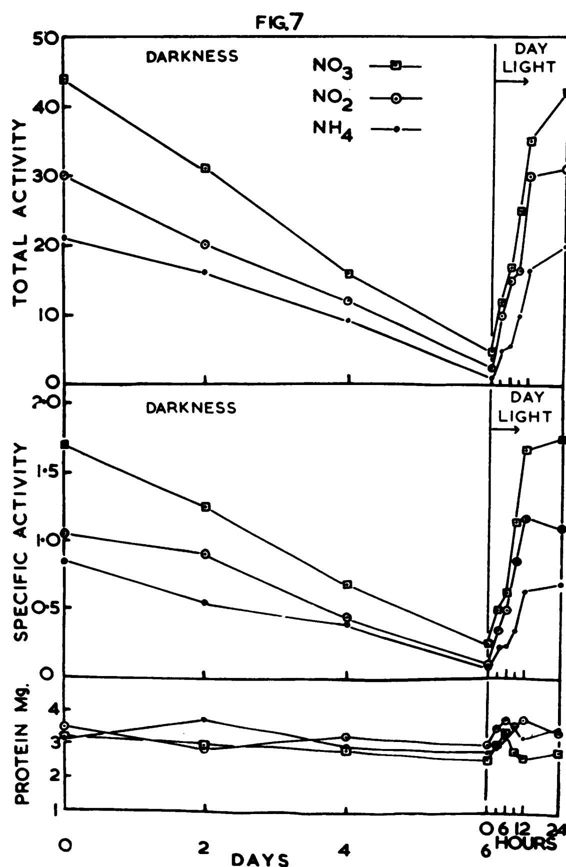


FIG. 7. Effect of darkness and restoration of light on nitrate reductase activity in leaves of cauliflower grown with different sources of nitrogen.

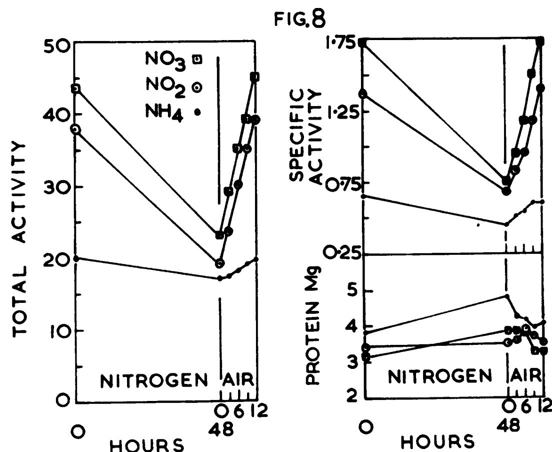


FIG. 8. Effect of exclusion and re-admission of air on nitrate reductase activity in leaves of cauliflower grown with different sources of nitrogen.

The activity was also rapidly restored on re-admitting air to the plants. No consistent trends were observed for the amounts of extractable protein.

DISCUSSION

The net nitrate reductase activity in cauliflower is determined by several factors. The pronounced peak in activity associated with mature but not senescent leaves of normal plants is noteworthy. This distribution was practically independent of the type of nitrogen nutrition and clearly distinct from that of another enzyme, acid phosphatase. The distribution of total extractable leaf protein and nitrate reductase are relatively independent at some stages, although in ageing plants total activity fell as leaf protein decreased. The total activities observed in cauliflower appeared to exceed considerably those reported by Evans and Nason (8) for soya bean and many other plants when the data are recalculated on a comparable basis. Maximal activity occurred in the primary and young trifoliate leaves of soya bean in contrast to the mature leaves of cauliflower.

The effect of molybdenum supply was consistent with existing knowledge regarding the role of molybdenum in the enzyme (17, 18). The failure of Evans and Nason (8) to observe an effect of molybdenum on the enzyme in young soya bean plants might have been due to the molybdenum reserves in large seeded legumes which may delay deficiency effects for a whole generation (12). It is also apparent that the effect of molybdenum may be small or negligible in regions possessing only minimal activity. It was found in cauliflower, as in fungi (19) that the enzyme as such is apparently not produced by tissues that have developed in the absence of sufficient molybdenum and cannot be immediately reactivated in disrupted cells. Work with fungi (19) has shown that 3 hours are required before the synthesis of new enzyme can be demonstrated after the addition of molybdenum to deficient mycelia. The activity in cauliflower was also increased when detached leaves were infiltrated with molybdate and increases have been observed since by Hewitt after 2 hours. In intact tomato plants the effect of adding molybdenum may be observed in two hours às nitrite production from the accumulated nitrate in the deficient tissues (22). A similar response to the addition of molybdenum to excised discs from molybdenum deficient cauliflower plants may also be inferred from experiments on respiration by Ducet and Hewitt (7). The effects of growing the plants with different sources of nitrogen also suggest adaptive features in the production of the enzyme. As with fungi (19), production of the enzyme was much greater in the presence of nitrate or nitrite than of ammonia, but was not completely suppressed by this last treatment as observed in the fungi (19).

Evans and Nason (8) suggested that the effect of light on nitrate reduction described by Burström (6) was consistent with the photochemical reduction of pyridine nucleotides by illuminated chloroplasts and their utilisation by nitrate reductase. The results obtained here show that exclusion of light from the plants may cause also a temporary loss of enzyme activity in extracts even when adequate reduced pyridine nucleotides are present. In this connection Stoy (23, 24) recently concluded that added riboflavin catalyses a photo-reduction of nitrate in irradiated wheat leaf extracts and provides a more effective electron donor for nitrate reductase in this system than reduced pyridine nucleotides. There are other examples also of the effects of light on enzyme production, as for example the observation of Appleman and Pyfrom (4) that red and blue light exert different effects on catalase production by etiolated barley plants.

Analogous changes to those observed in darkness and light were observed when air was excluded and re-admitted, but it cannot yet be concluded that they were in fact due to the same cause in both sets of treatments. Since this work was ended, Morton (15) observed independently that anaerobic conditions caused similar reversible changes in nitrate reduction capacity in fungi. He concluded that the enzymes responsible were not lost and resynthesised but were only reversibly masked by the anaerobic conditions. Removal of free carbohydrate from the medium or addition of ammonia produced similar reversible effects to those of anaerobic conditions. Lenhoff, Nicholas and Kaplan (14) have found that the relative proportions of flavin and haematin enzymes in *Pseudomonas fluorescens* also are determined by atmospheric conditions as well as by the available metals in the culture medium. Such changes contrast markedly with the tendency for enzymes such as invertase and peroxidase to persist even after extensive proteolysis in detached leaves kept in darkness as observed by Axelrod and Jagendorf (5). It may be concluded from this work that nitrate reductase is a highly labile enzyme in living cells as well as in vitro.

Work is now in progress, using this enzyme as a model, to investigate adaptive aspects of enzyme activity in higher plants as revealed by changes in nutritional or environmental conditions.

Summary

1. Cauliflower plants cv. Majestic were grown in sand culture with deficiency, normal or excess levels of molybdenum and with nitrogen supplied as nitrate, nitrite or ammonium sulphate.

2. Nitrate reductase was extracted by grinding leaves with sand in a mortar or by maceration in a high speed blendor with buffer solution at 0° C. These methods gave comparable activities but grinding in mechanically driven close fitting glass macerators produced low activities. The optimum pH was about 7.2 in phosphate buffer extracts.

3. The net nitrate reductase activity was maximal in mature leaves and was markedly lower in both senescent and rapidly expanding young leaves.

4. Extracts from leaves possessed greater net total activity than those from stems or petioles, and that of roots was extremely low. The specific activity per mg protein was much greater in petioles than in leaves or stems.

5. The nitrogen source also determined the level of nitrate reductase activity. This was high in plants given nitrate and usually high in those given nitrite. Plants given ammonium sulphate possessed markedly lower but not negligible activities.

6. In regions showing maximal activity, plants grown without molybdenum possessed much lower net total and specific nitrate reductase activities than the normal plants. In regions showing minimal activity these differences were small. Plants given excess molybdenum did not appear to possess more activity than normal plants. The effect of molybdenum supply mentioned above was greatest with plants given nitrate and least with those given ammonium sulphate.

7. Infiltration of molybdenum into leaves of deficient plants resulted in increases in nitrate reductase activity in 18 to 24 hours.

8. Nitrate reductase activity was greatly depressed by prolonged darkness during two to six days and recovered rapidly within a few hours of restoring the plants to light.

9. Exclusion of air for 48 hours also caused marked decreases in activity; and readmission of air led to rapid recovery in activity.

10. Changes in activity were not closely related to the total soluble protein content of the tissues. The distribution of the enzyme in plants grown with different molybdenum levels and nitrogen sources was markedly different from the distribution of extractable protein which was, however, similar to that of another enzyme—acid phosphatase.

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TRANSLOCATION OF ORGANIC SUBSTANCES IN TREES. I. THE NATURE OF THE SUGARS IN THE SIEVE TUBE EXUDATE OF TREES[1]

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Most investigators regard sucrose as the only form in which carbohydrates are translocated in plants. Some, however, suggest that hexoses are also translocated. It is beyond the purpose of this paper to list all these publications, a good bibliography will be found in the review of Crafts (1), and more recent publications are listed in the work of Ziegler (13).

One major difficulty in translocation work is the fact that possibilities of obtaining material that is actually transported are very limited. Chemical analyses based on tissue extractions yield substances which originate not only from sieve tubes but also from other types of cells in phloem and non-phloem tissues. There are only two ways of obtaining more or less pure sieve tube exudate. One method developed recently (7, 8) uses cut-off stylets of aphids which were feeding on the phloem. The other method, tapping the sieve tube system of trees by cutting into their bark, was first described by Th. Hartig (4) in 1860. It is this method that has been used in the present paper. There is plenty of evidence that the exudate obtained in this way is actually translocated material; Hartig found by microscopic observation that the exudate does come from the sieve tubes. This was later confirmed by Huber and Rouschal (5). Hartig observed that a second cut always yields sap if it is applied above the first one, below the first cut, however, a second cut usually failed to yield exudation (4). Sixty years later, Münch extended these studies and found that the sieve tube turgor is released up to several meters above a tapping cut (9). Ziegler supplied hexoses to the inner bark of trees (Robinia) and subsequently found them in the exudate below, but not above the point of application (13). The reason we referred to "more or less pure" sieve tube exudate is the possibility that exudate may elute traces of impurities from the cut surface during the tapping process.

Using chromatographic methods, Wanner found in 1953 that sucrose is the only sugar in the sieve tube exudate of *Robinia Pseudo-Acacia* L. and *Carpinus Betulus* L. (11). Testing exudate of 10 other European tree species, Ziegler also found only sucrose (13). However, in the course of a study on phloem translocation in white ash (*Fraxinus americana* L.), the results of which will be published elsewhere, it has been found that sucrose is by no means the only sugar in the exudate. It constitutes only 1/10 to 1/5 of the total sugar content. The other sugars identified are members of the raffinose family: raffinose, stachyose verbascose. In an attempt to learn whether white ash is an exceptional case, the sieve tube exudate of 16 tree species was collected and analyzed. The results of this survey are presented here.

EXPERIMENTAL

Sieve tube exudate was obtained by cutting into the inner bark of the tree with a sharp knife. In older trees it was necessary to remove the dead bark with an axe first. The tapping cut must penetrate the region of the functioning sieve tubes, which are located a fraction of a millimeter outside the cambium. Too deep a cut, however, severs the xylem, which, when it is under negative pressure, will immediately draw the exudate back with a hissing sound. The samples were either directly transferred onto the paper chromatograms or collected in a vial and immediately frozen in a Thermos bottle containing an ice-salt mixture. Exudate from white ash and red oak was collected from many individual trees during the summer and fall of 1955 and 1956. The samples from all other tree species were collected during the month of September 1956.

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