INFLUENCE OF NUTRITION ON THE ACTIVITIES OF SOME ENZYMES OF GLYCOLYSIS AND THE PENTOSE PHOSPHATE PATHWAY IN EXPANDING LUCERNE LEAVES

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Abstract

The yield response of lucerne to nitrogen and phosphorus was examined in relation to enzyme activities in young expanding leaves.

The response to nitrogen was associated with increased activity of phosphogluconate dehydrogenase, a key enzyme for the pentose phosphate metabolic route. Several glycolytic enzymes, particularly phosphofructokinase, showed corresponding decreases in activity. Yield response to phosphorus was associated with increased activities of glucose-6-phosphate and phosphogluconate dehydrogenases only at the highest level of applied nitrogen.

I. INTRODUCTION

The effects of nutrients on net assimilation in crops appear to be secondary to their effects on leaf area growth (Watson 1947, 1952; Watson, Thorne, and French 1963). That is, the efficiency of the assimilating system is less affected than its size. Assessment of the influence of nutrients on metabolic processes involved in leaf expansion is therefore basic to an understanding of this important nutritional control of crop productivity. Actively growing tissues require a steady supply of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for reductive biosynthetic processes. In leaves photosynthesizing chloroplasts produce 2 moles of NADPH per mole of oxygen evolved, but consume this same amount for each mole of CO_2 reduced (Kok 1965).

The pentose phosphate pathway is generally considered to be a major system of NADPH generation in addition to being a possible source of pentoses and other intermediates required for nucleic acid synthesis and growth. Goodwin (1960) has discussed the influence of NADP on the balance between the glycolytic and pentose phosphate sequences using the data of Potter and Niemeyer (1959) obtained with cell-free preparations from rat brain. He suggested that biosynthetic systems which consumed NADPH and regenerate NADP will stimulate the channelling of glucose 6-phosphate into the pentose phosphate pathway. A recent experiment has given an opportunity to examine some aspects of this suggestion in relation to the phosphorus and nitrogen nutrition of lucerne.

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II. Methods

The soil used was a 0–10-cm sample of a deep calcareous sand (N, 0·17%; P, 0·041%; sodium bicarbonate-soluble phosphorus, 31 p.p.m.; Northcote 1971, key Uc 1·11) from Marion Bay, Yorke Peninsula, South Australia. Polythene-lined enamel pots containing 2·5 kg soil were given a basal fertilizer dressing equivalent to the following rates (kg/ha): KCl, 251; $CuSO_4 . 5H_2O$, 12·5; $ZnSO_4 . 6H_2O$, 12·5; $MnSO_4 . 7H_2O$, 6; $(NH_4)_6Mo_7O_{24} . 4H_2O$, 1·5. Nitrogen and phosphorus treatments were calculated to supply ammonium sulphate at the equivalents of nil, 126, and 377 kg/ha, and superphosphate (8·6%P) at the equivalents of nil, 251, and 628 kg/ha. These treatments (replicated three times) were designated 0, 1, 3N and 0, 2, 5P respectively. The fertilizers were thoroughly mixed throughout the soil prior to potting. The soil was watered to 60% of field capacity and the pots were subsequently maintained at constant weight in a glasshouse. Lucerne seeds (*Medicago sativa* cv. Hunter River) were sown and thinned to four plants per pot at 10 days after germination.

The plants were harvested after 42 days growth. For enzyme assay a composite sample of fresh, young expanding leaflets was taken over three replicate pots. The remaining plant tops were oven-dried and this result taken as the dry matter yield, since a negligible amount was removed from individual pots for enzyme assay.

For enzyme assay, 100–400 mg fresh weight samples of the youngest expanding leaves were ground in 3 ml ice-cold buffer (250 mM sucrose, 100 mM Tris, 1 mM Mg^{2+}, 0·1 mM dithiothreitol, pH 7·6) in a chilled mortar. Homogenates were centrifuged at 30,000 *g* for 20 min and aliquots of the supernatant solution used for enzyme assays.

Glucosephosphate isomerase (GPI; E.C. 5.3.1.9), phosphofructokinase (PFK; E.C. 2.7.1.11), fructose-1, 6-diphosphate aldolase (E.C. 4.1.2.7), glucose-6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49), and phosphogluconate dehydrogenase (PGDH; E.C. 1.1.1.43) were assayed according to Scott, Craigie, and Smillie (1964). All assays were carried out at 25°C in a final reaction volume of 3 ml. Optical density changes at 340 nm were monitored in a Unicam SP800B recording spectrophotometer fitted with the SP850 scale-expansion accessory. Substrate and enzyme reagents were purchased from Sigma.

III. Results and Discussion

The yield response to phosphorus was small at the lowest level of nitrogen supply but became very highly significant at the 3N level (Table 1). Such data emphasize the need for superphosphate rates to be matched by an appropriate supply of nitrogen (or other potentially limiting nutrient) for optimal yields and the most efficient use of fertilizer.

Table 1

MEAN DRY MATTER YIELDS OF LUCERNE TOPS (MG/PLANT)

Phosphorus treatments	Nitrogen treatments			L.S.D. (log_{10} data)
	0N	1N	3N	
0P	210 (2·313)*	570 (2·751)	1110 (3·033)	$P > 0·05$, 0·117
2P	260 (2·419)	670 (2·831)	1430 (3·165)	$P > 0·01$, 0·168
5P	320 (2·498)	850 (2·929)	1660 (3·218)	$P > 0·001$, 0·343

* Mean log_{10} data.

The lucerne plants were not inoculated with *Rhizobium* and the roots of even the 0N treatment showed little evidence of nodulation at the end of the experiment. Only two to three nodules per plant were found on plants of the 3N treatment. The

significant nitrogen responses can therefore be taken to be due to the applied nitrogen treatments.

The response to nitrogen was associated with an increase in leaf size, as is commonly observed. Under these conditions it was expected that biosynthetic processes would be very active in the young expanding leaves, and a large demand for NADPH would exist.

Enzyme activities (Table 2) were calculated on a dry weight basis relative to leaf samples from each treatment on which percentage dry matter was determined. The data in Table 2 are for single assays, but checks made on several triplicate preparations showed good agreement (standard deviation $\pm 8\%$). Assays made on a concurrent pot trial in a similar soil from the same area were in general agreement with the values reported here. For these reasons I consider the data in Table 2 to be a valid estimate of the relative activities of the enzymes.

TABLE 2

ENZYME ACTIVITIES IN EXPANDING LUCERNE LEAVES

Treatments		Activities [μmoles min^{-1} (g dry wt.)$^{-1}$] of:				
P	N	GPI	PFK	Aldolase	G6PDH	PGDH
0P	0N	31·1	6·1	11·2	5·6	1·0
	1N	30·0	4·0	7·9	5·9	3·2
	3N	23·0	2·7	5·7	4·2	3·4
2P	0N	25·0	6·5	10·8	7·8	1·1
	1N	24·1	5·2	8·2	5·4	4·6
	3N	22·9	3·6	5·7	7·9	5·5
5P	0N	24·9	7·2	8·6	5·0	0·7
	1N	23·3	4·8	6·1	5·1	3·3
	3N	22·0	3·1	5·0	10·2	6·0

The key substrate for the system under consideration is glucose 6-phosphate, as shown in Figure 1. The enzymes PFK and PGDH have been considered to be important rate controllers for their respective metabolic routes (Atkinson 1966, 1969; Scrutton and Otter 1968) and the relative activities of these enzymes are important in determining how glucose 6-phosphate is utilized. It can be seen from Table 2 that PFK and PGDH consistently showed contrasting types of response to the increasing nitrogen treatments at all levels of phosphate. If these activities can be taken to be indicative of the relative potential flux through the metabolic routes, then these data do show increased activity of the pentose route under conditions which promote higher growth rates.

The response to nitrogen at all levels of phosphate supply was associated with an increase in the ratio of PGDH and PFK activities. This ratio was increased between phosphate treatments only at the highest level of nitrogen supply. These results are consistent with the hypothesis that growth-promoting conditions will stimulate the demands for NADPH (and also for pentoses required for nucleic acid

synthesis). It was only in the 3N series of phosphate response that there appeared to be a good correlation between the activities of both the G6PDH and PGDH enzymes in the pentose phosphate route. This might be anticipated since additional nitrogen is clearly required to take advantage of the available phosphate status of the soil used here.

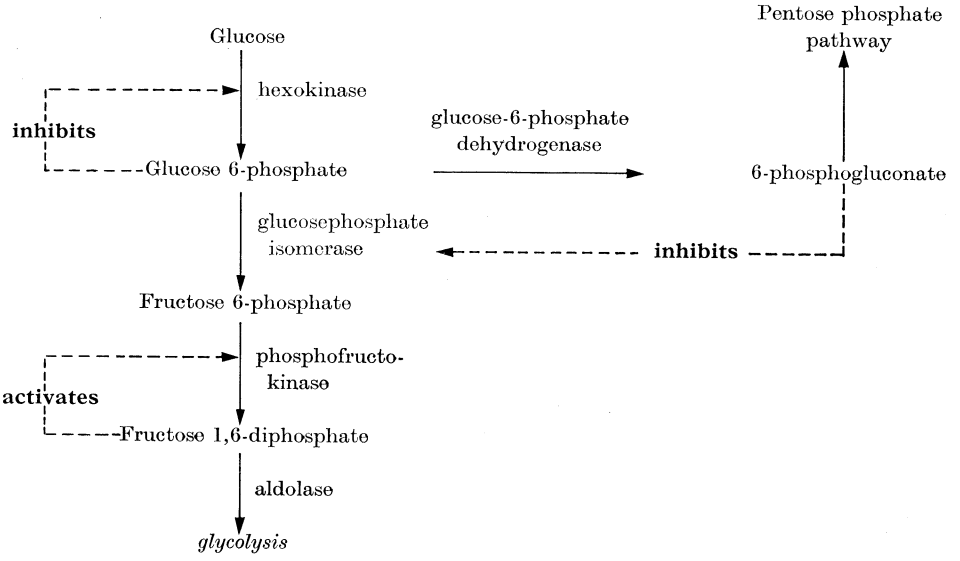

Fig. 1.—Reactions of glucose 6-phosphate.

The interrelationships of the enzymes and substrates under consideration are very complex. A full interpretation of the actual flux responses of the glycolytic and pentose phosphate sequences to the nutritional treatments is not possible without additional data on enzyme activities and metabolite concentrations together with *in vivo* measurement of metabolic flux through these routes (e.g. from C_6/C_1 ratio determinations using specifically labelled glucose, etc.) Published data (Long 1961) on the Michaelis constants of the enzymes considered here show that in both plant and animal tissues the pentose shunt enzymes G6PDH and PGDH have far greater substrate affinities ($K_m \simeq 10^{-5}$M) than the GPI and PFK enzymes ($K_m \simeq 10^{-3}$M). If these constants also apply to the lucerne enzymes, the system is potentially poised to run the pentose phosphate route at near saturation in young leaves. In order to compensate for this bias, and to counter the inhibition by 6-phosphogluconate, it would appear that the tissue maintains a high activity of the GPI enzyme (Table 2). Other systems of control which apply to the glycolytic enzymes have been discussed in detail by Atkinson (1966, 1969) and in Chance, Estabrook, and Williamson (1965).

Quantitative estimation of phosphate ester concentrations in lucerne leaves particularly esters present in low concentration such as 6-phosphogluconate) has proved very difficult because of interference from large amounts of yellow, anionic flavonoid pigments which separate along with the esters. It has now been found that these pigments present no such problem in wheat and ryegrass (*Lolium*) leaves.

In ryegrass the main water-soluble (red) pigments are cationic and easily separated from the esters. Such leaves will therefore be suitable for more detailed studies.

Several studies have demonstrated that the pentose phosphate respiratory pathway increases in importance as tissues differentiate. Its contribution to carbohydrate catabolism has been suggested to range from negligible values in juvenile tissue to approximately 25% in mature tissue (Bauer, Halliwell, and Langston 1968). Yamamoto (1963) found very high ratios of reduced to oxidized pyridine nucleotides in small expanding tobacco leaves, but low ratios occurred in old leaves. Expanding castor bean leaves similarly showed the greatest activity of the pentose route when compared with old tissue by the technique of C_6/C_1 recovery from specifically labelled glucose. Reductive biosynthetic metabolism in leaves may be expected to be maximal during the expansion phase. The enzyme assay results reported here are therefore relevant to the influence of nutrients on the processes of tissue development which affect crop growth.

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