Bioassay of long fallow disorder by the *Azotobacter* plaque method

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Abstract

The *Azotobacter* plaque method was tested as a bioassay of long fallow disorder, which is primarily expressed as more severe deficiencies of zinc and phosphorus in crops grown after fallow. In a series of five experiments, a black earth soil that had been either fallowed or cropped to panicum (*Setaria italica* L.) was enriched with various rates of glucose, P, Zn, S, Mn and Fe, and moulded into plaques. Colony size of *Azotobacter chroococcum* growing on the plaques in response to the nutrient additions was rated after incubation.

Optimum glucose concentration for conducting the tests was 1% (w/w). When supplied with glucose, *Azotobacter* responded synergistically to P and S. Zinc was only slightly stimulatory, intensifying the melanin pigment of the colonies. There was no response to Mn and Fe. Maximum growth of *Azotobacter* in the two soils was obtained at the same rate of P (200 μg P / g soil) and of glucose (1% w/w) even though maximum growth in long fallow soil was less than in cropped soil. Higher nitrate concentration in the long fallow soil allowed greater competitive reduction of *Azotobacter* growth by non-nitrogen fixing fungi than in cropped soil. *Azotobacter* was responsive to P but less responsive to Zn than was linseed (*Linum usitatissimum* L.), the most sensitive crop species to long fallow disorder. No evidence was obtained by this method that long fallow disorder results from lower 'available' P and Zn in soil.

INTRODUCTION

A nutritional disorder related to previous cultural history can affect crops growing in black earth soils of the Darling Downs (Hewitt 1962). The severity of this disorder increases with the period of fallow preceding the crop (Leslie and Whitehouse 1966) and for this reason it is known as 'long fallow disorder'. Conversely double-cropping with little or no fallow between two crops can alleviate the problem in the second crop (Leslie and Whitehouse 1966). Linseed (*Linum usitatissimum* L.) is the most sensitive crop species (Duncan 1967c) but many other crop species are affected to varying extents by the disorder (Leslie and Whitehouse 1965; Duncan 1967a, b). Also a wide range of crop species can be used to break the fallow and reduce the disorder in a following crop of the same or more commonly a different species. In previous experiments on the phenomenon, panicum (*Setaria italica* L.) has been the preferred first crop in a double crop sequence to break long fallow disorder, and linseed the preferred second or test crop (J. K. Leslie, pers. comm. 1967).

With linseed, long fallow disorder is expressed primarily as zinc deficiency symptoms and plants respond to zinc fertilisers, but responses to phosphorus, potassium, and manganese also have been obtained in some field trials (Leslie and Whitehouse 1966). In glasshouse experiments, plants in long fallow soil have consistently responded to both zinc and phosphorus fertilisers, typically with a positive interaction between the two nutrients (Leslie and Whitehouse 1968). This type of response, by which the increase in growth due to the combination of two nutrients exceeds the sum of the responses to the nutrients applied individually, is termed 'synergistic' in this paper. Prior cropping of long fallow soil with panicum in pots alleviated the disorder in subsequently sown linseed,

provided the soil was left undisturbed between the two crops. However, disturbing the soil after the panicum crop by drying, grinding and re-potting resulted in disordered linseed plants (Leslie and Whitehouse, 1966).

Although plant responses to fertilisers indicate that fallowing increases the severity of zinc and phosphorus deficiencies, no significant differences in soil concentrations of these nutrients have been obtained with the usual chemical extractants for available P and Zn (Whitehouse 1967). This study was conducted to determine whether bioassay by the *Azotobacter* plaque method might indicate differences in available P and Zn in long fallow and cropped soils. The plaque method was devised by Winogradsky (1928) and Winogradsky and Ziemiecka (1928) to obtain macroscopic growth of *Azotobacter* colonies on soil itself rather than on artificial media, and to observe *Azotobacter* growth in response to inorganic nutrients and pH. The method was once widely used to bioassay phosphorus and other elements in soil (Ziemecka 1932; Young 1933; Halversen and Hoge 1942). Because black earth soils usually have high populations of *Azotobacter* (McKnight 1949; Thompson 1974) the plaque method can be used without inoculation. Also the chemical and physical properties of black earths are such that amelioration, with lime (Wieringa 1939) or kaolinite (Ziemecka 1932) which could alter nutrient availability, is unnecessary.

MATERIALS AND METHODS

Soils

The soil was a black earth of the Waco series (Beckmann and Thompson 1960) from a long fallow site (18 months fallow) near Mt. Maria (27°30′S; 151°28′E). The phosphorus status of the soil reported by Whitehouse (1967) was 74 µg bicarbonate-extractable P/g soil by Colwell's (1963) method and 404 µg acid-extractable P/g soil by the method of Kerr and von Stieglitz (1938). Soil pH was 8.6 (1:2.5 soil/water suspension). Cropped soil was produced from fallow soil by growing panicum (*Setaria italica*) in pots in the glasshouse. Both fallow and cropped soils were air-dried to 14% moisture content and ground with a glass mortar and pestle to pass a 2 mm mesh stainless steel sieve.

Preparation of plaques

The carbon source, glucose (1% w/w OD soil equivalent) was added to 50 g soil and mixed by shaking in a polythene bag. The required inorganic nutrients in solution were added as evenly as possible followed by enough distilled water to bring the soil to the desired moisture content (60 % w/w) for moulding plaques. The moist soil was kneaded within the polythene bag, removed and pressed into 90 mm diameter Petri dishes. The plaque surface was smoothed to a slightly convex, 'iced' finish with a moistened stainless steel spatula. Care was taken at all stages of preparation to prevent cross-contamination between different nutrient treatments and soils. Plaques of all treatments were prepared in duplicate. The plaques were incubated aerobically at 28°C over 1N H_2SO_4 to absorb any atmospheric ammonia, which might decrease the selectivity of the method for nitrogen-fixing *Azotobacter*.

Rating of *Azotobacter* growth

After incubation, obvious differences in growth of *Azotobacter* on plaques of different nutrient treatments were apparent. Plaques differed in appearance because of differences in colony size rather than colony number which was high, about 1000 per plaque, in all treatments where growth occurred. Therefore *Azotobacter* growth on the plaques was rated as follows:

        0   = no growth;

        0.5 = punctiform colonies only;

1   = mean colony diameter < 0.2 mm;

2   = mean colony diameter approximately 0.4 mm;

3   = mean colony diameter approximately 0.7 mm;

4   = mean colony diameter approximately 1.0 mm.

Once the rating system was established plaques were rapidly rated by visual comparison without need for individual measurements. Plaques were rated daily from 2 to 7 days after incubation.

Experiment 1: Response of *Azotobacter* in previously dried, cropped soil to phosphorus and zinc

This experiment was designed to test if *Azotobacter* responded synergistically to Zn and P fertilisers like linseed had in previous glasshouse experiments. In previous experiments, linseed in both long fallow and disturbed (dried and ground) cropped soil had grown poorly without fertiliser and had responded synergistically to Zn and P, but in undisturbed cropped soil it had grown well with only a small response to Zn and P fertilisers (J. K. Leslie, pers. comm. 1967). Air-dried, cropped soil (14 % moisture content) expected to suffer from long fallow disorder was used for this experiment. Experimental treatments were six levels of phosphorus (0, 50, 100, 200, 400 and 800 µg P/g soil applied as NaH_2PO_4) factorially combined with three levels of zinc (0, 23 and 90 µg Zn/g soil applied as $ZnSO_4$).

Experiment 2: Response of *Azotobacter* in dried, cropped soil to Zn, S and two sources of P

This experiment was designed to determine whether the response of *Azotobacter* growth to $ZnSO_4$ in Experiment 1 was to Zn or to S. In addition, this experiment tested whether acidic NaH_2PO_4 and alkaline Na_2HPO_4 as P sources might interact differentially in *Azotobacter* response to nutrients. Experimental treatments applied to air-dried, cropped soil were 'nil' phosphorus and 400 µg P/g soil as either NaH_2PO_4 or Na_2HPO_4, combined factorially with four sources of zinc and sulphur. That is, nil, $ZnSO_4$ (providing 23 µg Zn/g soil and 12 µg S/g soil), $ZnCl_2$ (23 µg Zn/g soil) and Na_2SO_4 (12 µg S/g soil).

Experiment 3: Response of *Azotobacter* in dried, long fallow soil to P, S, Zn, Fe and Mn

After the completion of Experiments 1 and 2 the results of the glasshouse experiment being run concurrently showed the soil that had been cropped to panicum produced healthy linseed plants irrespective of soil drying treatment (J. K. Leslie, pers. comm. 1967). Thus the results obtained in Experiment 2 were not valid for ascertaining if *Azotobacter* would respond to Zn additions in a soil that would produce zinc deficient linseed plants.

For this reason, air-dried long fallow soil that produced zinc deficient linseed plants in the concurrent glasshouse experiment was tested in Experiment 3 with factorial treatments of P, S, and Zn. Three additional treatments of Fe, Mn, and Fe+Mn were also tested in soil supplied with P, S and Zn. The treatments were as follows:

1. No inorganic nutrient;

2. P (400 µg P/g soil as NaH_2PO_4);

3. S (80 µg S/g soil as Na_2SO_4);

4. Zn (40 µg Zn/g soil as $ZnCl_2$);

5. P+S;

6. P+Zn;

7. S+Zn;

8. P+S+Zn;

9. P+S+Zn+Fe (65 µg Fe/g soil as FeCl$_3$);

10. P+S+Zn+Mn (35 µg Mn/g soil as MnSO$_4$);

11. P+S+Zn+Fe+Mn.

Experiment 4: Comparison of *Azotobacter* response to rate of phosphorus in long fallow and cropped soil

This experiment was designed to compare the P status of long fallow and cropped soil. Treatments were six levels of P (0, 50, 100, 200, 400 and 800 µg P/g soil) applied as NaH$_2$PO$_4$. All plaques received glucose (1% w/w) and a basal application of 80 µg S/g soil and 40 µg Zn/g soil as a mixture of ZnSO$_4$ and Na$_2$SO$_4$.

Experiment 5: Comparison of *Azotobacter* response to rate of glucose addition in long fallow and cropped soil

In all previous experiments, glucose had been added at a rate of 1.0% w/w. There was no evidence that this was optimal. Since the long fallow and cropped soils apparently differed in available nitrogen status, they might well require different levels of available carbon for maximum growth of *Azotobacter*. Six levels of glucose addition (0, 0.5, 1.0, 1.5, 2.5 and 5.0% w/w) were therefore tested on the two soils. A basal dressing of 800 µg P/g soil as NaH$_2$PO$_4$, with 80 µg S/g soil and 40 µg Zn/g soil as a mixture of ZnSO$_4$ and Na$_2$SO$_4$, was applied.

RESULTS

Experiment 1: Response of *Azotobacter* in previously dried, cropped soil to phosphorus and zinc sulphate

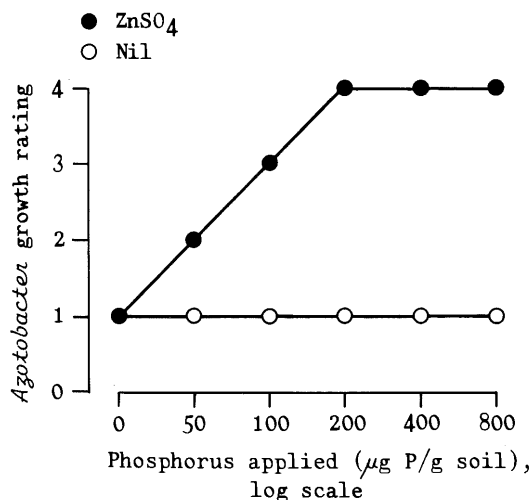Figure 1. Response of *Azotobacter* in plaques of previously dried, cropped soil to NaH$_2$PO$_4$ and ZnSO$_4$ after 3 days' incubation.

The response in growth of *Azotobacter* after 3 days' incubation to the nutrient treatments is given in Figure 1. There was a marked, synergistic response to P and $ZnSO_4$ similar to the type of response in growth of linseed. Without $ZnSO_4$ there was no response to P, but in the presence of $ZnSO_4$, *Azotobacter* responded to increasing rate of phosphorus, attaining maximum growth with 200 µg P/g soil. There was no difference between the two rates of zinc sulphate, apart from a somewhat darker melanin pigment in the *Azotobacter* colonies at the higher rate. On further incubation, colony size on plaques with $ZnSO_4$ and low rates of P increased until at 5 days all plaques with $ZnSO_4$ and P rated 4, and those with $ZnSO_4$ but no P rated 3. *Azotobacter* colonies on plaques with P but no $ZnSO_4$ did not grow beyond a rating of 1.

Thus *Azotobacter* responded to nutrients in a synergistic manner similar to linseed, and at this stage the plaque method appeared very promising as a bioassy for long fallow disorder. The primary limiting nutrient was Zn or S rather than P, despite the relatively high requirements for P by *Azotobacter chroococcum* (Becking 1961). However, it was necessary to check whether the marked response to $ZnSO_4$ was to Zn or to S.

Experiment 2: Response of *Azotobacter* in dried, cropped soil to Zn, S and two sources of P

The response in growth of *Azotobacter* after 3 days' incubation to the nutrient treatments is given in Figure 2. There was a marked synergistic response to S and P irrespective of whether the S was supplied as $ZnSO_4$ or Na_2SO_4 or the P was supplied as Na_2HPO_4 or NaH_2PO_4. There was no response to Zn either in the presence or absence of S. The two sources of P, one acidic and one alkaline, had no differential effect on possible response of *Azotobacter* to Zn. After 4 days' incubation, *Azotobacter* colonies on plaques with S had darker melanin pigment. On further incubation, *Azotobacter* colonies on plaques with S but no P continued to grow until they rated 4 at 7 days. Colonies with P but no S remained on a rating of 1.
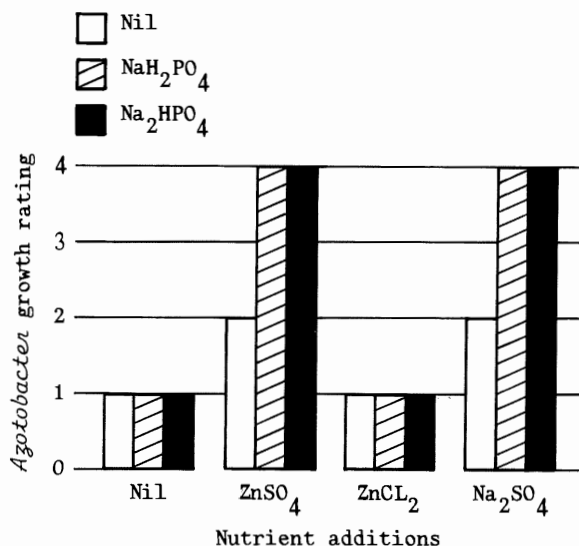

Figure 2. Response of *Azotobacter* in plaques of previously dried, cropped soil to Zn and S after 3 days' incubation.

Thus the primary limitation of *Azotobacter* growth in this air-dried, cropped soil was S, not Zn. Once the S deficiency was corrected, *Azotobacter* responded to P resulting in more rapid colony growth.

Experiment 3: Response of *Azotobacter* in dried, long fallow soil to P, S, Zn, Fe and Mn

Azotobacter colonies developed somewhat more slowly in this experiment than in the previous two, the best treatments rating only 2 after 3 days' incubation. Growth responses of *Azotobacter* to the nutrient treatments after 4 days' incubation are given in Figure 3. *Azotobacter* responded markedly to the combination of P and S but hardly at all to either element alone. With P alone there were many punctiform *Azotobacter* colonies (rated 0.5) while with S alone there was no growth of *Azotobacter*. The addition of Zn to treatments with both P and S slightly increased the proportion of *Azotobacter* colonies with darker melanin pigment. On further incubation for 7 days, maximum ratings did not increase above the 3 attained at 4 days. At 6 days a thin growth of filamentous fungi was observed on the surface of most plaques.

Figure 3. Response of *Azotobacter* in plaques of long fallow soil to P, S, Zn, Fe and Mn after 4 days' incubation.

The following comparisons can be made between results with long fallow soil in Experiment 3 and cropped soil in Experiments 1 and 2. With no mineral nutrient additions, *Azotobacter* grew to a rating of 1 in cropped soil but not in long fallow soil. On extended incubation, maximum growth was attained with S alone in cropped soil, but maximum growth required both P and S in fallow soil. *Azotobacter* appeared to grow more slowly and to attain a lower maximum growth rating in fallow soil than in cropped soil. Possibly maximum growth in fallow soil required greater additions than 400 µg P/g soil.

Experiment 4: Comparison of *Azotobacter* response to rate of phosphorus in long fallow and cropped soil

Growth responses of *Azotobacter* after 4 days' incubation to rate of P in long fallow and cropped soil are given in Figure 4. At any given P level, *Azotobacter* growth in fallow soil was less than in previously cropped soil. With subsequent incubation all treatments improved somewhat, but no growth occurred in fallow soil without added P, and at corresponding P levels there was always greater growth in the cropped than in the fallow soil. These results would indicate that the cropped soil was better supplied with available P than the fallow soil, except that maximum response in both soils was achieved with

the same rate, 200 µg P/g soil. Also, even with higher rates than 200 µg P/g soil, growth in the fallow soil did not reach that in the cropped soil. Thus it seemed that some factor other than available P was limiting growth in the fallow soil.
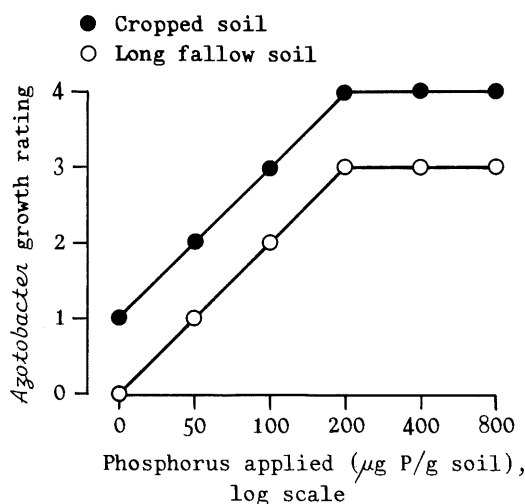

Figure 4. Response of *Azotobacter* in plaques of fallow and cropped soils to rate of phosphorus addition after 3 days' incubation .

In Experiment 4 differences in the surface appearance of the plaques were noticed. Whereas plaques of the cropped soil appeared dark-brown to black and shiny, the fallow plaques were greyish and matt in appearance. When examined under a plate microscope, all plaques of fallow soil had a thin, almost continuous weft of fungal mycelium, while cropped soil had hardly any. This fungal growth suggested that high available nitrogen in the long fallow soil plaques was reducing the selectivity of the added carbohydrate for the nitrogen-fixing *Azotobacter*.

Experiment 5: Comparison of *Azotobacter* response to rate of glucose addition in long fallow and cropped soil

Growth responses of *Azotobacter* after 3 days' incubation to rate of glucose added in long fallow and cropped soil are given in Figure 5. A similar, relative degree of growth was maintained with continued incubation. At the highest rate of glucose, particularly in the fallow soil, colonies of nitrogen-fixing *Clostridium* spp. grew. These commenced below the soil surface, raising and finally breaking it by gas production. There was much greater growth of fungi on all fallow soil plaques than on any cropped soil plaque.

It thus seemed that fungal growth in the fallow soil was preventing maximum development of *Azotobacter* colonies and that this could not be overcome by varying the suppply of available carbon through rate of glucose addition. The rate of glucose addition (1% w/w) used in all previous experiments was optimal for both soils. In view of these results both soils were analysed for mineral nitrogen. The fallow and cropped soils contained 130 and 10 µg NO_3–N/g soil respectively. Urea had been added to fallow soil from the field to grow the panicum in the glasshouse thus producing cropped soil. Fallow soil had been treated identically and kept in unplanted pots in the glasshouse as a control. Without crop extraction, the nitrified urea remained in the fallow soil in sufficient quantity for non-nitrogen fixing microorganisms to provide moderate competition for *Azotobacter* in the plaques.
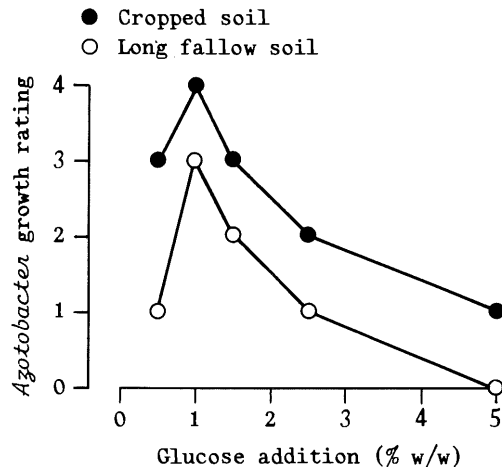
Figure 5. Response of *Azotobacter* in plaques of fallow and cropped soils to rate of glucose addition after 3 days' incubation.

DISCUSSION

In a parallel glasshouse experiment, linseed grew poorly in the long fallow but well in the cropped soil, despite air-drying the soil between crops. The addition of P and Zn fertilisers resulted in a marked synergistic response in linseed growth in the long fallow soil, doubling dry matter production to equal that in cropped soil, which did not respond to P and Zn fertilisers (J. K. Leslie, pers. comm. 1967). Thus the particular batches of long fallow and cropped soil used in this study were suitable to test whether the *Azotobacter* plaque method could indicate differences between long fallow disordered and healthy soils in available P and Zn. The plaque method has the advantage that black earth soils naturally contain high populations of *Azotobacter chroococcum* and have a neutral or alkaline pH so there is no need to add $CaCO_3$, which could alter available nutrient levels. A further advantage is that *Azotobacter chroococcum* does not produce strong acid reactions from glucose (Thompson and Skerman 1979), which also could alter the availability of certain nutrients. This has been a criticism of the *Aspergillus niger* bioassay (Donald, Passey and Swaby 1952).

Azotobacter colonies in both long fallow and cropped soils exhibited a marked synergistic response to P and S, with S being more limiting than P in the cropped soil and P being slightly more limiting than S in the long fallow soil. *Azotobacter* is known to require S in the form of sulphate for growth (Greaves and Anderson 1936), but that S was as limiting as P was unexpected as most emphasis in the literature has been placed on the *Azotobacter* plaque method to bioassay soil P (Sackett and Stewart 1931; Ziemecka 1932; Greene 1933; Young 1933; Halversen and Hoge 1942; Wieringa 1939). Initial impressions were that the long fallow soil was more deficient than the cropped soil in P, but the rate of P giving maximal growth was the same in both soils. The real difference between the long fallow and cropped soils was greater nitrate nitrogen in the fallow soil, which allowed non-nitrogen fixing fungi to compete with the nitrogen-fixing *Azotobacter*. This limited the maximum growth of *Azotobacter* in long fallow soil in response to increasing rate not only of P but also of glucose. Ziemecka (1932) also noted that high nitrate allowed non-nitrogen fixing bacteria to grow on soil plaques and to compete with *Azotobacter*. Although Whitehouse (1967) found no difference between the long fallow and cropped soils in available zinc and phosphorus measured by usual extraction methods,

he did find that the cropped soil contained 0.25 µg water-extractable P/g soil more than the fallow soil. It is unlikely this difference in water-soluble P would be sufficient to substantially affect *Azotobacter* growth, as Ziemecka (1932) found >4.4µg water-soluble P/g soil was required for *Azotobacter* growth in British soils. The concentration of zinc in water extracts of both soils was below the detection limit by the dithizone method (M. J. Whitehouse, pers. comm. 1986).

These experiments have shown that the growth of naturally occurring *Azotobacter* in Waco soil is responsive to phosphate and sulphate additions, but relatively insensitive to zinc. The greatest response by *Azotobacter* to zinc was intensified melanin pigmentation with little or no effect on colony size. Singh and Pathak (1962) showed that zinc stimulated growth and nitrogen fixation by *Azotobacter chroococcum* in both culture medium and soil. It seems that both long fallow and cropped soils can supply sufficient zinc for maximal *Azotobacter* growth in plaques. Thus, *Azotobacter* appears less sensitive than linseed to zinc deficiency. The nutrient elements Fe and Mn are required for growth of *Azotobacter* in culture media and can stimulate *Azotobacter* growth in some soils (Becking 1961). The availability of both elements usually decreases with increasing soil pH and both could be limiting *Azotobacter* growth in this soil of pH 8.6. However, no responses were obtained to additions of these elements.

Thus the *Azotobacter* plaque method was satisfactory for the bioassay of P but relatively insensitive for Zn. No difference in the apparent availabilty of either nutrient element between long fallow and cropped soil was established. This may be partly due to limitations of the method, for example sensitivity to differences in available nitrogen, but it may also be because no important difference in available P and Zn existed between long fallow and cropped soil. Recent research has shown poorer vesicular-arbuscular mycorrhizal colonisation of various crops including linseed after long fallow, resulting in poorer uptake of both P (Thompson 1987) and Zn (Thompson 1986) from the soil. This poorer uptake of nutrients results in deficiency symptoms and poor growth of the tops. Mycorrhizal hyphae extend centimetres away from root surfaces effectively tapping a larger volume of soil for non-mobile nutrients like P and Zn than do non-mycorrhizal roots. This mechanism can explain the phenomenon of long fallow disorder without requiring any difference between long fallow and cropped soils in concentrations of chemically available P and Zn. The effective physical availability of nutrients for uptake by a mycorrhizal root system is, of course, much greater than that for a non-mycorrhizal root system.

Although no difference was detected in available P and Zn between the long fallow and cropped soils by *Azotobacter* plaques, the method could well be of value as a bioassay of P and or S in black earths or vertisols in general. A possible *Azotobacter* bioassay would need to be calibrated against plant response to the particular nutrient in a set of soils of varying nutrient status.

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