TITLE **RHIZOSPHERE SOIL ENZYMES AS INDICATORS OF PERTURBATIONS CAUSED BY ENZYME SUBSTRATE ADDITION AND INOCULATION OF A GENETICALLY MODIFIED STRAIN OF *PSEUDOMONAS FLUORESCENS* ON WHEAT SEED**

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Rhizosphere soil enzymes as indicators of perturbations caused by enzyme substrate addition and inoculation of a genetically modified strain of Pseudomonas fluorescens on wheat seed

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SUMMARY -Comparative assays for determining chitobiosidase, N-acetyl glucosaminidase, acid phosphatase, alkaline phosphatase, phosphodiesterase, aryl

sulfatase and urease activities from small samples of soil were developed. The enzyme assays and ATP biomass assessments were used to monitor perturbations caused by the presence of *Pseudomonas fluorescens* in the rhizosphere of wheat. Microbial biomass as well as the measured enzyme activities decreased with depth, except for acid phosphatase activity which was similar at all depths. A combined substrate mix addition of urea, colloidal chitin and glycerophosphate significantly increased N-acetyl glucosaminidase, chitobiosidase, aryl sulfatase and urease activities but did not cause a significant difference in acid and alkaline phosphatase and phosphodiesterase activities. Inoculation of seeds with *P. fluorescens* resulted in significant increases in rhizosphere chitobiosidase and urease activities at 5-20 cm depth and a significant decrease in alkaline phosphatase activity. Inoculation with the bacterium in the presence of substrate mix gave opposing effects to those treatments without substrate mix addition: chitobiosidase, aryl sulfatase and urease activities were significantly lower and alkaline phosphatase was significantly higher at the 5-20cm depth interval with inoculation of bacteria. Biomass values for the combined bacteria and substrate mix treatment were significantly higher than the substrate mix alone treatment.

INTRODUCTION

Genetically modified microorganisms (GMMs) might be deployed for use in biocontrol or in plant growth stimulation, but before they can safely be released, a better understanding of the effect that such releases will have on the soil and especially rhizosphere ecosystems must be assessed (Smit *et al.,* 1992).

Most attempts to monitor the effects of microbial introductions to the rhizosphere have centred on microbial enumeration of specific populations, often aided by molecular techniques (e.g. Tsushima *et al.,* 1995). These methods rely on large changes, as microbial numbers measured on a log scale require differences of between 2 and 3 fold (0.3 and 0.5 on a log scale) to be significant, and do not provide a wide picture of the overall effect of the introduced microbe on the whole ecosystem. De Leij *et al.,* (1993b) used colony development for the quantitative assessment of r and K strategists from different habitats, but these methods also rely on culturable micro-organisms. Molecular detection methods do no more than monitor or detect specific micro-organisms and do not give any indication of the actual effect of the inoculum on the ecosystem (Pickup, 1991; Van Elsas and Waalwijk, 1991; Tsushima *et al.*, 1995 and for a review see Morgan, 1991). Marker genes can be deployed, for example to track potential luminescence (Meikle *et al.,* 1994), however these only provide information about the activity and ecology of a specific microorganism in soil, and do not indicate if a gene is actually expressed in soil.

Previous work using the genetically modified *Pseudomonas fluorescens* SBW25 strain carrying the *lac*ZY and *xyl*E functions has concentrated on the ecology of the released organism (De Leij *et al.*, 1994a 1995), and has relied upon culturable methods to assess impacts on specific microbial groups (De Leij *et al.*, 1994b). This, however, does not give an overall view of the impact of the GMM upon the functioning of the ecosystem as a whole.

The measurement of perturbations with soil biochemical variables, such as enzyme activities, may be an alternative way of monitoring overall effects of the introduced GMM on the ecosystem, in a more sensitive and comprehensive way. Soil phosphatase activity has been shown to be an important indicator of the effects of soil management systems and of the organic matter content of the soil (Jordan *et al.*, 1995). Doyle and Stotzky (1993) found no difference in enzyme activities (aryl sulfatase, phosphatases and dehydrogenase) when an *Escherichia coli* strain was introduced into soil, this is perhaps not surprising as the work was not conducted under relevant conditions for the release of GMMs into soil. The metabolic activity of the *E. coli* strain was likely to be low since no substrate, be it rhizodeposition or soil amendments, was present that could be used as a substrate source. Furthermore, *E. coli* is not a soil organism, and is unlikely to establish a viable population after introduction into soil. In contrast to that work, Mawdsley and Burns (1994) successfully used soil enzyme measurements to detect perturbations caused by a *Flavobacterium* spp inoculated onto wheat seedlings, finding increased activity of α -galactosidase, β-galactosidase, α-glucosidase and β-glucosidase.

We have developed and used a number of simple enzyme assays to detect perturbations resulting from different soil treatments, including the introduction of a genetically modified *Pseudomonas fluorescens* SBW25 strain and substrate amendments. The aim of this experiment was to deduce whether these assays are sensitive enough to measure perturbations caused by microbial inoculation, and to uncover the extent of any perturbation. Specific attention was paid to the validation of soil biochemical techniques as a method of monitoring the effects of inoculation. Differences in rhizosphere soil biomass (measured by ATP content) and several key soil enzyme activities with microbial inoculation or in exaggerated conditions (addition of the enzyme substrates, chitin, urea and glycerophosphate, to soil) were measured.

MATERIALS AND METHODS

Soil description

The soil used was a silty loam of the Hamble series, taken from an agricultural site at Littlehampton (West Sussex, UK). It has been cultivated with wheat for the past 4 y. The pH of the soil was 6.0, its carbon content was 1.4% by weight, and its particle ratio was 15:68:17 for clay: silt: sand, respectively.

Experimental systems used

Intact soil cores held in PVC tubes (60 cm x 15 cm dia), were extracted from the ground. The cores were stored vertically in a trench in the field from which they were taken, until required. Subsequently the cores were left to settle in the glasshouse for 14 days prior to the start of the experiment. For experimental purposes, soil cores were placed in shallow trays filled with wet capillary matting. Each tray was supplied with deionised water via separate pumps which regularly added water from separate tanks, excess water freely drained back to the supply tanks. The capillary matting around the cores set on the trays was covered in polyethylene sheeting to reduce evaporation. Additional water was added from the top of the cores (150 ml per core every 48 h) to ensure the top soil did not dry out, and to disseminate bacteria and amendments through the soil profile. The soil surface of each core was covered with plastic balls (1.5 cm dia) to reduce evaporation. The

lighting regime in the glasshouse was set at a photoperiod of 16 h. The cores were shaded by a coat of Aluminium foil. Temperatures in the glasshouse were unregulated; the maximum recorded daytime temperature was 32^{o}C and the minimum night time temperature was 8^{o}C.

Experimental design.

Three replicates (1 per tray, spaced in a randomised block design) of each of six treatments were assembled as follows: (1) unamended soil cores, considered to be unamended non rhizosphere soil; (2) soil cores amended with a substrate mix, consisting of a solution containing 2% w/v colloidal chitin (prepared by the method of Shimatiara and Taiguchi (1988)), 2% w/v urea and 2 % w/v glycerophosphate. Every 10 days 100 ml was added instead of the water addition in the normal watering regime; (3) soil cores sown with wheat seed (var. Axona), 6 seeds were sown per core. The emerged seedlings were randomly reduced to 3 after 7 days growth; (4) seed and substrate mix addition; (5) seeds inoculated with *Pseudomonas fluorescens* SBW 25 EeZY which has the marker genes *lacZY*, kan^{r} and *xylE* (De Leij *et al.,* 1993a) (supplied by Dr M. J. Bailey, NERC Institute of Virology and Environmental Microbiology, Oxford) which was originally classified as *P. aureofaciens*. The bacterium was grown to late exponential phase in tryptone soya broth before being introduced to the wheat seed in a 0.75% gum guar solution resulting in a concentration of 10^{8} cfu per seed^{-1}; (6) seeds treated as in treatment 5, with substrate mix addition to the cores as in treatment 2.

Sampling

Cores were harvested after 60 days, cut open lengthways and for experimental purposes, divided into three arbitrary depth levels, 5-20, 20-35 and 35-50 cm (large depth intervals were required to provide sufficient rhizosphere soil for analysis). The soil was excavated to reveal the roots and approximately 5 g of rhizosphere soil, (defined as soil no more than 2 mm from the root) was scraped directly from the root surface, (using 2 mm dia wire) within the designated depth intervals. Non rhizosphere soil was scraped from within the analogous cores from similar depths. The soil samples were either immediately assayed or stored for up to 48 h at 4^{o}C prior to assay.

Enzyme extraction

Acidic-pH active extracellular enzymes were extracted by a method modified from Wirth and Wolf (1992). The buffer consisted of 0.5 M sodium acetate set at pH 5.5 using acetic acid, with NaN_3 (1 mg ml^{-1}) added to prevent microbial growth. Alkaline-pH active enzymes were extracted using 0.2 M sodium orthophosphate buffer (containing NaN_3, 1 mg ml^{-1}) set at pH 8 using NaOH. Five ml of buffer g^{-1} of moist soil was used for the extraction. The soil suspensions were mixed for 1 h on a carousel rotor before being centrifuged at 4000 rev min^{-1} for 15 minutes. The supernatant (enzyme extract) was decanted off into clean test tubes and kept at 4^{o}C until required on the same day.

Enzyme assays

N-acetyl glucosaminidase (NAGase) and chitobiosidase activities were measured by methods modified from Trosmo and Harman (1993). To 1 ml of the pH 5.5 enzyme extract in disposable 11 ml centrifuge tubes, 1 ml of p-nitrophenyl β-D-N,N'-diacetylchitobiose (pNDC, chitobiosidase substrate) (100μg ml^{-1}) or 1 ml of p-nitrophenyl N-acetyl-β-D-glucosaminide (pNAG, NAGase substrate) (200μg ml^{-1}), both made in the acetate buffer, was added. Subsequently the mixtures were incubated in a water bath at 37^{o}C for 24 h.

Acid phosphatase activity was assayed by a similar method to the NAGase and chitobiosidase methods, using 25 mM p-nitrophenyl phosphate as the substrate in the same buffer. The reaction took place in a shaking water bath set at 200 strokes min^{-1} for 1 h at 37 ^{o}C.

Alkaline phosphatase activity was assayed using the pH 8 enzyme extract and 25 mM p-nitrophenyl phosphate in the phosphate buffer (pH 8). The incubation time was 2 hours in a shaking water bath set at 200 strokes min^{-1} at 37 ^{o}C.

Aryl sulfatase and phosphodiesterase activities were measured by a method similar to the alkaline phosphatase assay, using 25 mM p-nitrophenyl sulphate as the substrate for the sulphatase assay and 5 mM bis(p-nitrophenyl) phosphate for phosphodiesterase with an incubation time of 24 hours in a water bath at 37 ^{o}C.

Activity in all the above assays was terminated by the addition of 1 ml of cold 0.4 M $NaHCO_3$, which also serves to enhance the yellow colour of the p-nitrophenol released by enzymatic action, and centrifuged for 15 min at 4000 rev min^{-1}. The amount of p-nitrophenol released was measured at 400 nm in a spectrophotometer against separately incubated enzyme extract and substrate, which were mixed at the end of incubation and terminated by the same method as the samples. Standard curves of 1mg ml^{-1} up to 100mg ml^{-1} p-nitrophenol in acetate or phosphate buffer with $NaHCO_3$ added were plotted to calculate the amount of p-nitrophenol.

Urease activity was measured by a modification of the method of Gianfreda *et al.*, (1994) using sodium orthophosphate buffer (pH 8) without NaN_3 and the enzyme extract described earlier. The reaction took place in a shaking water bath set at 200 strokes min^{-1} for 2 h at 37 ^{o}C. The phenol-hypochlorite method of Weatherburn (1967) was used for the KCl (2 M) terminated assay, to measure NH_3^+ production.

ATP biomass was measured by a modification of the method of Webster *et al.*, (1984) using ATP solutions as standards, and the biomass conversion factor of Tate and Jenkinson (1982) of biomass C = 171 x soil ATP content.

Statistical analysis

A multivariate analysis of variance (two way ANOVA) was used to compare: substrate mix addition vs. no addition against depth; microbial inoculation vs. non inoculation

against depth, both in the presence and absence of substrate mix. Students T-tests were also used to analyse the data from the 5-20 cm depth, where higher activities and differences were expected.

RESULTS

The *Pseudomonas fluorescens* inocula had established as an effective population of approximately 5.8 x 10^5 g^{-1} of root in all the inoculated treatments, the ecology and survival of the same strain on the same crop in the same soil type using the same microcosm has been described in depth previously (De Leij *et al* 1993a, 1994a and b) and has also been described upon field release De Leij *et al* (1995).

Urease activity was significantly ($p < 0.01$) higher at the 5-20 cm depth interval than the two deeper soil intervals (Fig 1). This effect was most pronounced in the substrate mix-amended treatments. In general, addition of substrate mix caused an increase in urease activity ($p < 0.01$), the largest being a 6 fold increase in the seed plus substrate mix treatment over the seed treatment. Microbial inoculation (without substrate mix addition) caused an approximately 20% increase ($p < 0.05$) in activity at 5-20 cm depth. Microbial inoculation in the presence of substrate mix resulted in a 50% reduction of the urease activity of the analogous treatment without inoculation, ($p < 0.01$) .

N-acetylglucosaminidase activity decreased significantly ($p < 0.01$) with depth in all the treatments and increased ($p < 0.05$) when the substrate mix was added to the soil (Fig. 2), however, an interaction between depth and substrate mix addition was not found. Chitobiosidase activity significantly ($p < 0.01$) decreased with depth (Fig. 3). This effect was very evident when substrate mix was added, with a resulting sharp increase in all

activities, (most prominently at 5-20 cm depth) which produced a more pronounced depth effect (interaction between depth and substrate mix, $p < 0.01$), which was contrary to the NAGase activity. The most striking difference was found between the unamended seed treatment and the seed + substrate mix treatment, which showed a 5-fold increase with substrate mix addition. The overall effect caused by the substrate mix addition was highly significant ($p < 0.01$). Microbial inoculation (without substrate mix addition) increased ($p < 0.01$) the chitobiosidase activity at 5-20 cm depth compared to the seed alone treatment. Inoculation in the presence of substrate mix resulted in a lower ($p < 0.05$) activity than the substrate mix treatment without the inoculum.

Aryl sulfatase activity decreased in general ($p < 0.01$) with depth (Fig. 4) although the seed and the non rhizosphere (control) treatments did not have any significant differences between the 5-20 cm and 20-35 cm depth intervals. Substrate mix amendment caused an overall increase in activity ($p < 0.05$). Activity was higher ($p < 0.05$) in the seed + substrate mix treatment than the seed + microbial inoculation + substrate mix treatments, in the 5-20 cm depth interval and an interaction was found between the depth effect and substrate mix addition effects ($p < 0.05$).

Acid phosphatase activity was not significantly affected by depth or any of the treatments (data not shown). Phosphodiesterase activity significantly decreased ($p < 0.01$) with depth in all treatments (Fig. 5). The phosphodiesterase data were variable (as seen by the large error bars) and thus no other significant effects could be distinguished. Alkaline

phosphatase activity decreased ($p < 0.01$) with depth (Fig. 6). In the rhizosphere soil alkaline phosphatase activity was higher ($p < 0.01$) without the added microbial inoculum than with the inoculum at 5-20 cm depth. However when substrate mix was added, alkaline phosphatase activity was enhanced ($p < 0.01$) in the presence of the inoculum.

Biomass decreased with increasing depth ($p < 0.01$) (Fig. 7). Substrate mix addition caused a reduction in the amount of ATP ($p < 0.05$) measured. Microbial inoculation in the presence of substrate mix resulted in a higher ATP content than the seed + substrate mix treatment ($p < 0.01$).

DISCUSSION

The enzyme assays we described allowed a range of enzymes and a large number of samples to be assayed over a relatively short period (2 days) from small quantities of soil, in a less labour intensive manner than the existing methodology. Other methods require much larger amounts of soil and are more labour intensive, for example the methods of Tabatabai (1982) often require relatively large amounts of soil, and rely on toluene additions to prevent microbial growth. The modifications of our method made it possible to measure the activity of seven or more soil enzymes in the same amount of time required for three enzymes by established methods. The requirement for only small quantities of soil is important where samples produced are small, as is often the case when working with rhizosphere soil. Two grams of soil is sufficient for up to 10 enzymes to be assayed (enzymes higher in activity can be assayed with less enzyme extract). The methods described require only one set of soil samples to be weighed for several enzymes, and the use of centrifugation instead of filtration requires much less effort. It should be possible to determine the activity of almost any soil enzyme, for which a p-nitrophenyl substrate analogue is available using the methods described here. Incubation times of enzyme assays can be increased for less active soils, including most sandy soils.

It must be noted, however, that the methodology described was designed purely for comparative assays, where samples and treatments taken from one soil type can be

directly compared. In general soil enzyme activity measurements are only a measure of the potential activity of a given enzyme in soil and not the *in situ* activity in the natural soil system, where activity is impaired by absorption and immobilisation of enzymes by soil particles and organic matter, which differ between soils (Gianfreda and Bollag 1994). The assays we have devised are not intended as a method for determining the actual or the maximum enzyme activities of the soil samples, as many of the enzyme assays are displaced from their optimal pH for activity and desorption from soil (Quiquampoix *et al.*, 1993).

Soil enzymes will not necessarily be at their optimal pH *in situ* given the variation in micro-environments that exist in soil (Gianfreda and Bollag, 1994 and Kanazawa and Filip, 1986). Also the natural soil system is not saturated with enzyme substrate as it is in assay incubations. It is not necessary to create optimal activity conditions for assays in comparative studies, as long as a standard methodology is used. However, it is possible to make some statistical comparisons of enzyme activities between soil types where several treatments have been used. This can be done by normalising the data into arbitrary units, for example, ranking the enzyme activities of the different treatments or considering each data point as a proportion of the data point with the highest activity.

The decrease in activities with increasing depth in all but the acid phosphatase activity corresponds with the decreasing biomass in the deeper soil layers. The lack of a depth effect on acid phosphatase activity is most likely the result of this enzyme predominantly

being secreted by plant roots (and associated mycorrhiza and other fungi) (Tarafdar and Marschner, 1994), thus in rhizosphere soil there is unlikely to be much difference in the amount of enzyme secreted by the plant roots. This also supports the theory that the amount of immobilised soil enzymes is a measure of cumulative activity during the growth of plant roots and soil biochemical processes, as immobilised enzymes are relatively stable until the soil is disturbed.

The addition of substrate mix, which included substrates for urease, chitobiosidase and NAGase caused a direct effect (increase) on the activities of these enzymes which were induced in the presence of their respective substrates. The influence on aryl sulfatase activity (increase) is more complex as specific substrates were not added for this enzyme, therefore the effect must have been indirect. It is possible that soil aryl sulfatase is directly linked to microbial activity as has been suggested by a number of authors working with several enzymes (e.g. Nannipieri *et al*., 1983). However the significant interaction between the depth and substrate mix addition found in the sulfatase activity indicates an alternative cause of the increased aryl sulfatase activity. A similar pattern and effect was found in the chitobiosidase activity. The fact that chitin was added as a colloid, unlike the other two substrates which were in solution, meant that the chitin was unable to percolate down to the deeper soil layers. This is supported by the fact that the upper regions of microcosms amended with the substrate mix were discoloured white (the same colour as colloidal chitin). The substrate-depth interaction in chitobiosidase activity can also be attributed to the presence of chitin predominantly in the upper soil regions,

whereas degradation products of chitin are more soluble and could reach deeper areas of the soil core, thus stimulating NAGase activity in deeper soil layers. The chitin substrate may have caused a direct induction of sulfatase production in the microbiota, which contain trace quantities of various sulphates and aryl sulfatase activity has been shown to be significantly correlated with soil organic matter content (Tabatabai and Bremner, 1970). Therefore the alternative explanation is that chitin directly involved by stimulating the activity of aryl sulfatase as a result of the increased organic matter with chitin amendment.

Substrate mix addition did not have a significant effect on acid phosphatase, alkaline phosphatase activities and phosphodiesterase activity was only affected in the non inoculated plants at 20-35cm and 35-50cm. It was expected to find an increase in activity with the addition of a large amount of organic phosphate, however, the readily available soluble form that was added would have been broken down into an inorganic form rapidly in the soil environment. Increasing available inorganic soluble phosphate is known to have an inverse effect on phosphatase production (Tabatabai 1982; Tadano *et al.*, 1993). Data from other experiments (unpublished) showed that glycerophosphate addition alone causes an overall decrease in alkaline phosphatase activity. This means that the other substrates added masked the actual effect of the phosphate addition by stimulating microbial activity and overall enzyme production.

It was expected that an input of nutrients would increase the size of the rhizosphere community, but substrate mix addition actually decreased the biomass as measured by ATP. A possible reason for this anomaly is that substrate mix additions caused a change in the physiological state or the constituents of the microbial community, with a higher proportion of microbes with a smaller ATP content in the substrate mix treatments than in the treatments without substrate mix addition. This explanation is made plausible by the observations of Karl (1980) who compiled data of the ATP content of several micro-organisms and found a range of 0.5 to 18 ηmol ATP per mg^{-1} dry weight organism.

Inoculation with bacteria (without substrate mix addition) had a significant effect on chitobiosidase, urease, and alkaline phosphatase at 5-20 cm depth only (where activity is at its highest). Most of the enzymes show a mean difference between the inoculated and the uninoculated treatments, but these differences could not be shown to be statistically significant. The lack of statistical significance may be due to the low replication number in the experiment, and thus the sensitivity could be improved with a higher number of replicates. There was no obvious effect of treatment on the plant-associated acid phosphatase activity and the effect on phosphodiesterase activity was variable. There was an apparent reduction in alkaline phosphatase activity, which can be attributed to a direct or indirect effect of the inoculum, resulting in a displacement of the rhizosphere communities that produce larger amounts of this enzyme.

Microbial inoculation in the presence of the substrate mix additions, showed significant differences in chitobiosidase, alkaline phosphatase, aryl sulfatase, and urease activities, as well as in biomass. All of the effects on the enzymes were the opposite to those seen with microbial inoculation without the substrate mix addition, i.e. aryl sulfatase, chitobiosidase and urease activities were significantly lower with the inoculation in the presence of substrate mix additions than without inoculation, whereas alkaline phosphatase activity was significantly higher. Therefore the microbial inoculation had a 'buffering effect' on the rhizosphere ecosystem, i.e. there was a reduction in the response of enzyme activities to the change in conditions presented by the substrate mix additions.

A possible mechanism for this reduction in response is that the inoculant inhabited niches of other microbes in the community that would respond in a more dynamic fashion to the change in conditions. However, the mechanisms of these perturbations are far from certain in the complex rhizosphere ecosystem. There are several possible ways in which the microbial inoculation could initiate changes in the microbial community. The first possibility is, that the inoculant is competitively excluding certain microbial populations. This would be a direct effect of the large inoculum size, giving the introduced microbe a 'head start' over the soil's resident microflora and was highlighted by De Leij *et al* (1995), who found a large population of the same organism in the rhizosphere of wheat several weeks after inoculation. Other possible effects of the inoculant could be in the

strain's metabolic activity, that might have directly affected the indigenous microbial community (Keel *et al*, 1992) or modified root secretions (Mozafar *et al* 1992).

The data presented in this paper indicated that soil biochemical properties can be a useful tool for use as indicators of perturbations caused by microbial inoculation and other soil treatments as was found by Mawdsley and Burns (1995). It also highlights the importance of soil biochemical properties in such studies, as they give an indication of ecosystem function rather than just measure perturbation. The traditional population methods De Leij *et al* (1994a, 1995) which have indicated population perturbations but do not indicate an effect upon the ecosystem function.

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Fig. 1. Urease activity in a silty loam soil as influenced by depth and six treatments: Se, presence of seedling; Mi, microbial inoculation; Su, substrate mix addition; None, none of the former added. Significant effects, depth, $p < 0.01$; substrate mix addition, $p < 0.01$; seedling and substrate mix addition versus seed, microbial inoculation & substrate mix addition, $p < 0.01$; 5-25 cm depth seedling versus seedling & microbial inoculation, $p < 0.05$.

Fig. 2. N acetyl glucosaminidase activity in a silty loam soil as influenced by depth and six treatments: Se, presence of seedling; Mi, microbial inoculation; Su, substrate mix addition; None, none of the former added. Significant effects, depth, $p < 0.01$; substrate mix addition, $p < 0.05$.

Fig. 3. Chitobiosidase activity in a silty loam soil as influenced by depth and six treatments: Se, presence of seedling; Mi, microbial inoculation; Su, substrate mix addition; None, none of the former added. Significant effects, depth, $p < 0.01$; substrate mix addition, $p < 0.01$; interaction between depth and substrate mix addition, $p < 0.01$; seedling and substrate mix addition versus Seed, microbial inoculation and substrate mix addition, $p < 0.05$; 5-25 cm depth seedling versus seedling and microbial inoculation, $p < 0.01$.

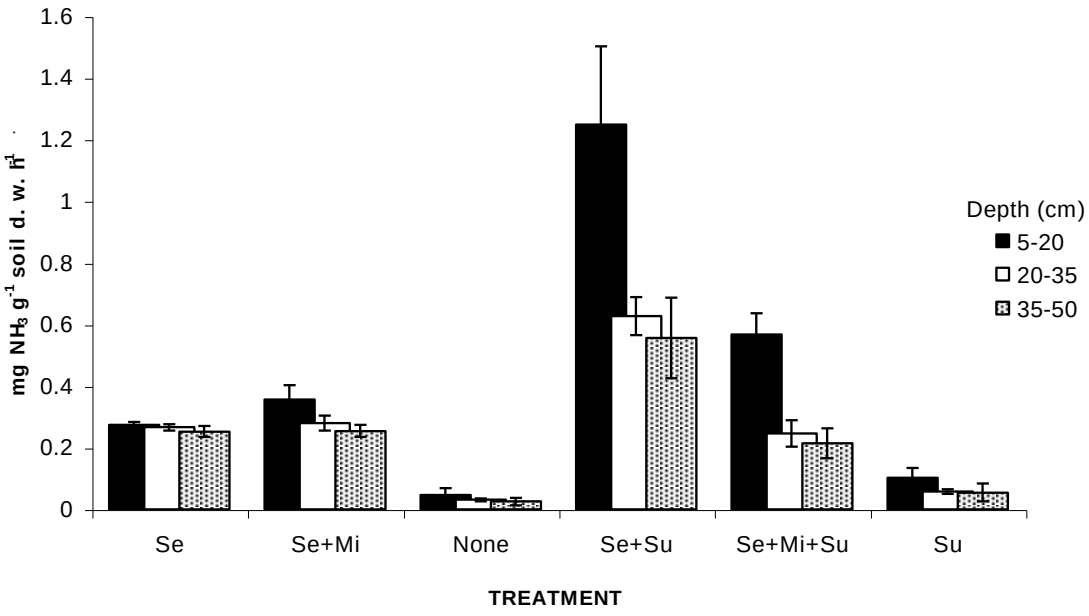
Fig. 4. Aryl sulfatase activity in a silty loam soil as influenced by depth and six treatments: Se, presence of seedling; Mi, microbial inoculation; Su, substrate mix addition; None, none of the former added. Significant effects, depth, $p < 0.01$; substrate mix addition, $p < 0.05$; interaction between depth and substrate mix addition, $p < 0.05$; seedling and substrate mix addition versus seed, microbial inoculation and substrate mix addition, $p < 0.05$.

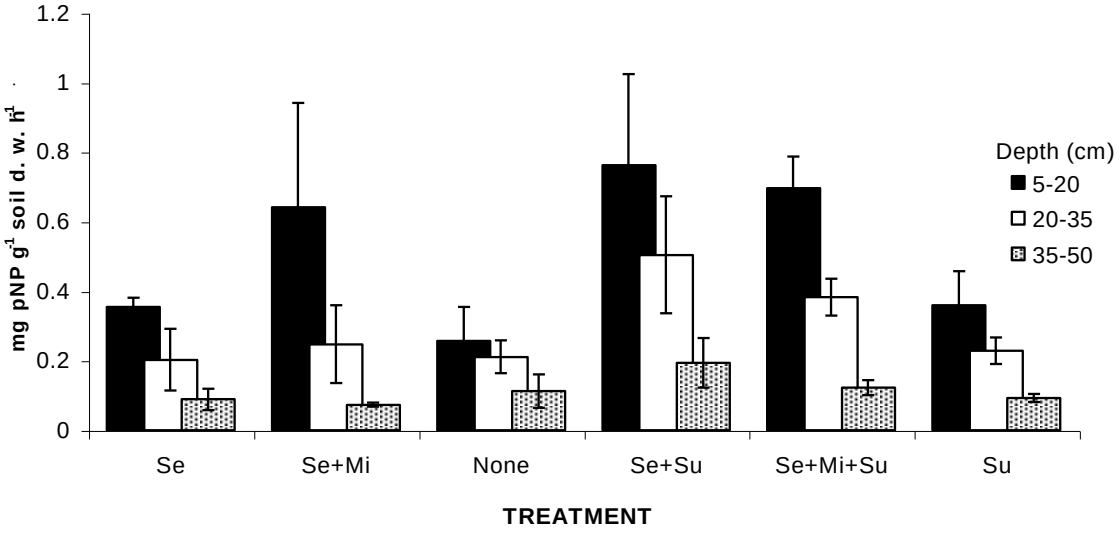
Fig. 5. Phosphodiesterase activity in a silty loam soil as influenced by depth and six treatments: Se, presence of seedling; Mi, microbial inoculation; Su, substrate mix addition; None, none of the former added. Significant effects, depth, $p < 0.01$.

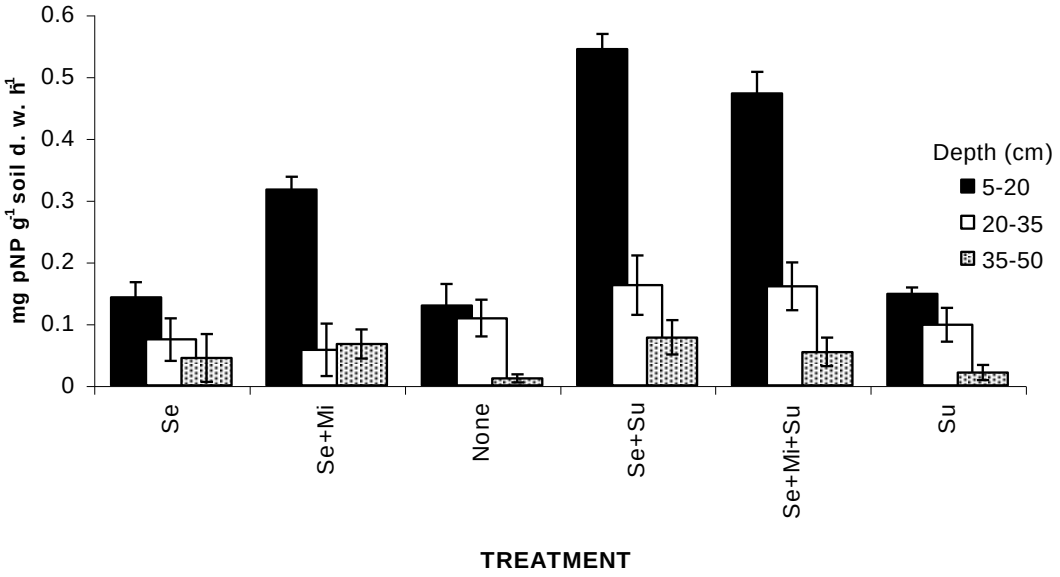
Fig. 6. Alkaline phosphatase activity in a silty loam soil as influenced by depth and six treatments: Se, presence of seedling; Mi, microbial inoculation; Su, substrate mix addition; None, none of the former added. Significant effects, depth, $p < 0.01$; 5-25 cm depth seedling and substrate mix addition versus Seed, microbial inoculation and substrate mix addition, $p < 0.01$; 5-25 cm depth seedling versus seedling and microbial inoculation, $p < 0.01$.

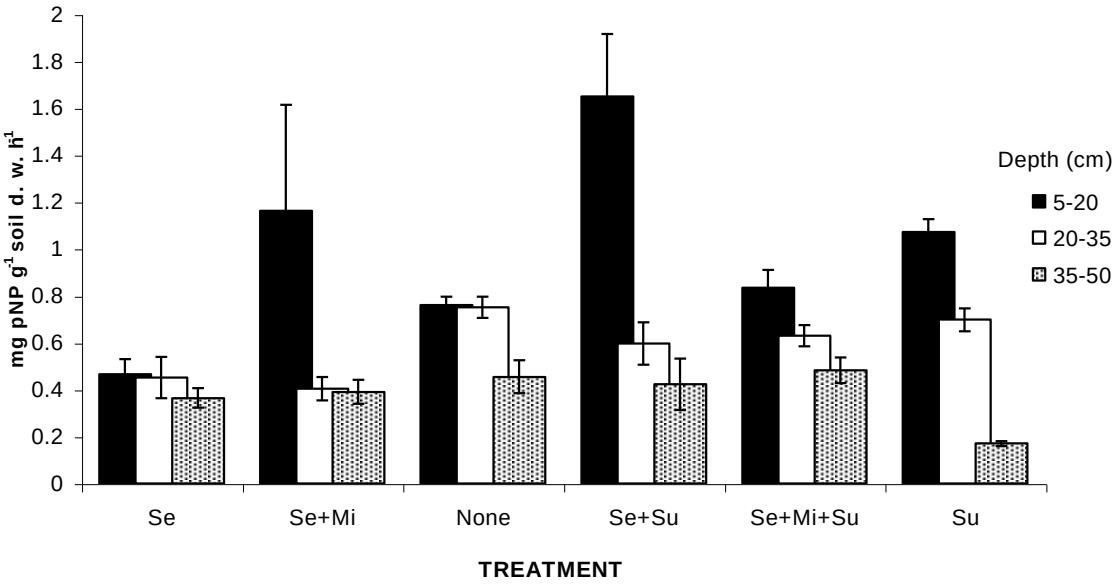
Fig. 7. Total biomass carbon in a silty loam soil as influenced by depth and six treatments: Se, presence of seedling; Mi, microbial inoculation; Su, substrate mix addition; None, none of the former added. Significant effects, depth, $p = 0.01$; substrate mix addition, p

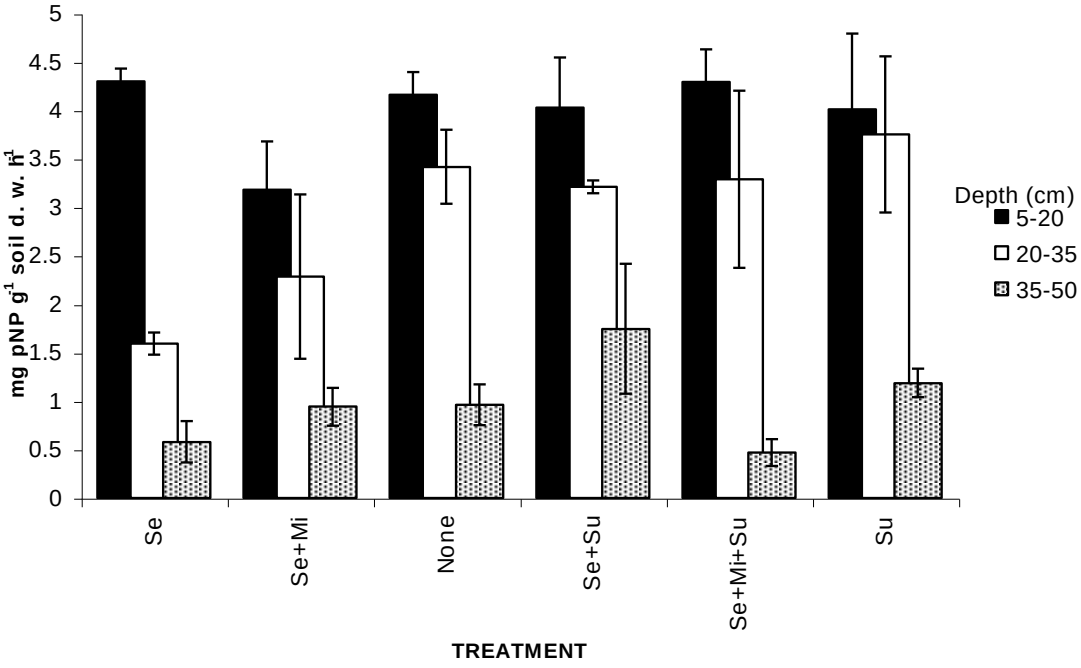
<0.05; seedling and substrate mix addition versus seed, microbial inoculation and substrate mix addition, p <0.01.

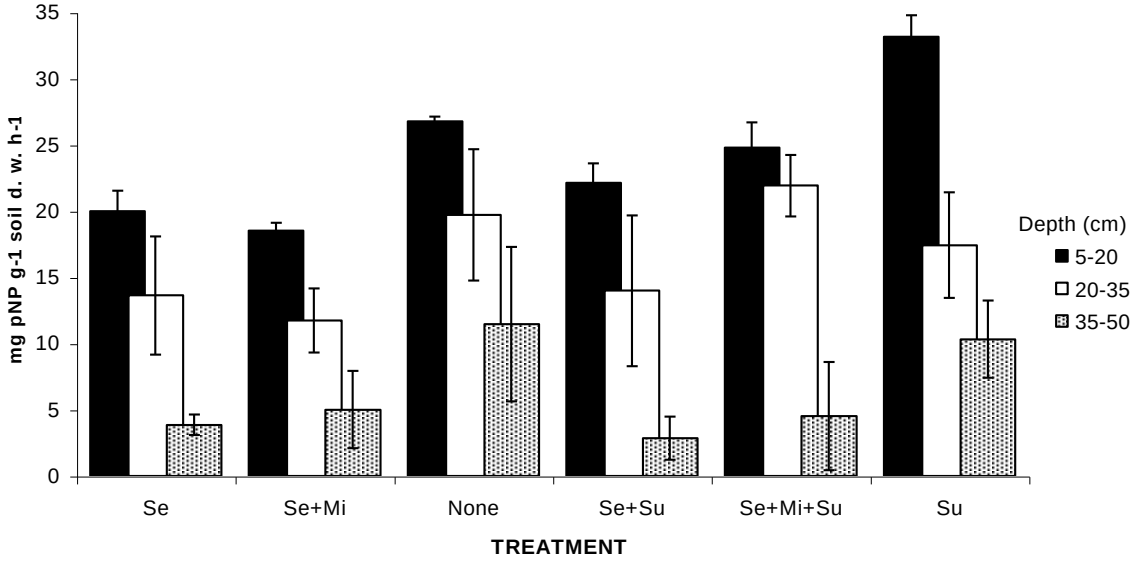

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
mg NH3 g-1 soil d. w. h-1
Depth (cm)
5-20
20-35
35-50
Se
Se+Mi
None
Se+Su
Se+Mi+Su
Su
TREATMENT

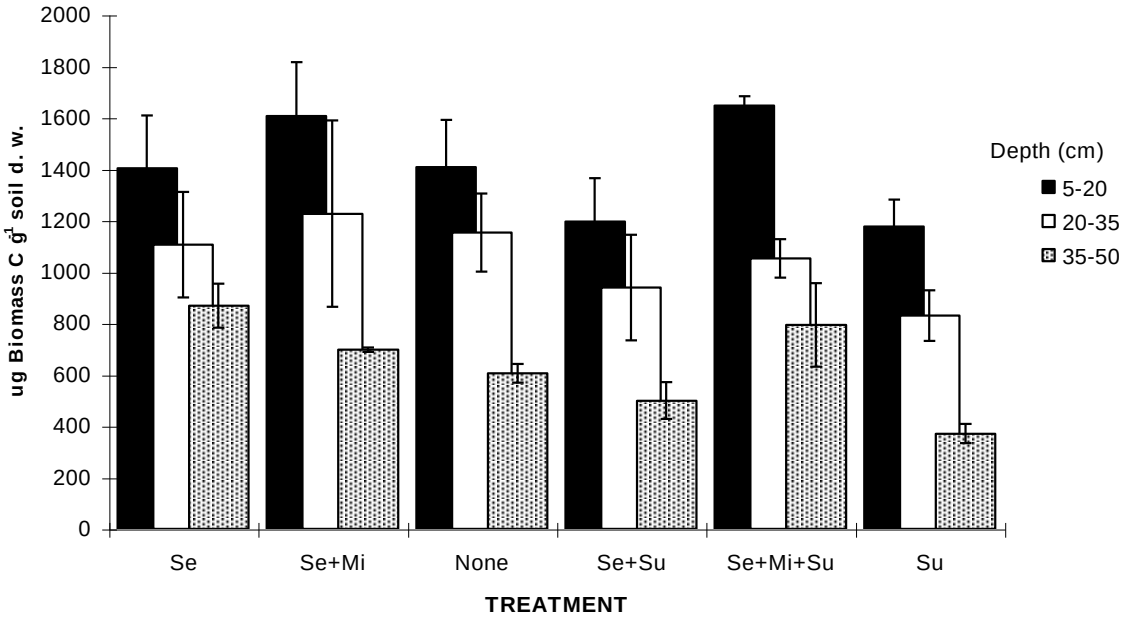

1.2
1
0.8
0.6
0.4
0.2
0
mg pNP g-1 soil d. w. h-1
Depth (cm)
5-20
20-35
35-50
Se
Se+Mi
None
Se+Su
Se+Mi+Su
Su
TREATMENT


0.6
0.5
0.4
0.3
0.2
0.1
0
mg pNP g-1soil d. w. h-1
Depth (cm)
5-20
20-35
35-50
Se
Se+Mi
None
Se+Su
Se+Mi+Su
Su
TREATMENT


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
mg pNP g-1 soil d. w. h-1
Depth (cm)
5-20
20-35
35-50
Se
Se+Mi
None
Se+Su
Se+Mi+Su
Su
TREATMENT


5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
mg pNP g-1 soil d. w. h-1
Depth (cm)
5-20
20-35
35-50
Se
Se+Mi
None
Se+Su
Se+Mi+Su
Su
TREATMENT


35
30
25
20
15
10
5
0
mg pNP g-1 soil d. w. h-1
Se
Se+Mi
None
Se+Su
Se+Mi+Su
Su
TREATMENT
Depth (cm)
5-20
20-35
35-50


2000
1800
1600
1400
1200
1000
800
600
400
200
0
ug Biomass C g-1 soil d. w.
Se
Se+Mi
None
Se+Su
Se+Mi+Su
Su
TREATMENT
Depth (cm)
5-20
20-35
35-50