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Enhanced phosphate cycling using Tithonia diversifolia

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Enhanced phosphate cycling using *Tithonia diversifolia*

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Summary

The green manure plant, *Tithonia diversifolia* (Tithonia) has been identified as having the potential to significantly enhance soil phosphorus (P) cycling in high P fixing, acid tropical soils, farmed by resource-poor smallholders. It has been hypothesized that Tithonia has the potential to exploit soil P pools that are largely unavailable to crop plants such as rice and maize.

To support agronomic research, controlled environment experiments were conducted. Aspects of phosphorus uptake by Tithonia and the influence of Tithonia residues on crop P uptake were investigated.

The kinetics of the P uptake transporters were determined for whole Tithonia root systems using a solution culture P depletion method. At a whole root system level the P uptake kinetic parameters were similar to those determined for a range of crop plants by the same method.

Radiotracers were used to investigate P translocation in Tithonia. High concentrations of P (ca. 3.5g P kg^{-1}) have been reported in Tithonia shoots, of plants growing in low-available-P soils. The results of this study indicated that P accumulates in the vacuoles of green tissues, with P acquired by roots being supplemented by P from rapidly senescing lower leaves.

The uptake of P by roots and associated mycorrhizal fungi was investigated using radiolabelled substrates. Tithonia plants were grown in rhizotrons with or without root access, but always with hyphal access, to radiolabel P placed in a subcompartment. The presence of Tithonia roots contributed to improved uptake of a relatively labile Po source, but hyphae were able to take up inorganic P, even when bound to iron or calcium in similar quantities to when roots were also present.

Tithonia has been reported as possessing allelochemicals that inhibit seed germination and root development. To determine if this might constrain use of Tithonia as a green manure, allelopathic effects of Tithonia applications were determined. Root and shot growth of maize, but not wheat, was reduced 10 days after being placed in soil in which Tithonia shoots had been applied, at rates similar to those used by farmers growing crops with Tithonia as a green manure.

Low plant recovery of mineral P fertilizer discourages farmers with limited cash resources from investing in P fertilisation. Small mineral P applications, applied with Tithonia residues have been reported to improve total P recovered by the subsequent crop. In an experiment in which wheat was grown in rhizotrons with subcompartment radiolabelled P applications, it was shown that to improve P uptake and plant growth, Tithonia and mineral P fertiliser had to be combined. This indicates that the effect is not strongly associated with improved general crop nutrition, but is due to microbial P immobilisation, followed by mineralisation at rates synchronous to crop P demand.

Although these experiments and those of other workers suggest that expectations for Tithonia in enhancing P cycles in P limiting soils may be without solid foundation, the research has advanced understanding of the issues involved in the use of green manures to increase crop available P in tropical soils.

Plate 1. (Top) Tithonia growing on a hillside in Costa Rica. (Below) Close up of a Tithonia flower.

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Chapter One

Enhanced phosphate cycling using *Tithonia diversifolia*: Literature review.

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Summary
Phosphorus deficiency, resulting in poor crop yields, has been identified as a key constraint to agronomic and social development in tropical areas where oxic soils predominate. The high phosphate fertilizer input required to rectify this situation is unaffordable to small-scale farmers. This review considers recent research into the potential of using *Tithonia diversifolia* green manure applications, to maintain enhanced levels of biologically active phosphorus, within the context of current understanding of soil phosphorus cycles and plant uptake and utilisation.

1. Introduction

1.1. Phosphate as a constraint on development

The number of people in absolute poverty (those subsisting on per capita incomes of < US$1.00 per day) in the world has continued to grow despite many years of development aid, capital loans and economic management aimed at stimulating economic growth in the world's least developed economies. Most of the economies in this category are in sub-Saharan Africa with regions of countries in South America and Southeast Asia being similarly affected. A common feature of some of the poorest areas of the tropics is poor soil fertility (World Bank 1996). It is noted by Fairhurst *et al.* (1999) that in Europe and North America a substantial investment in soil phosphorus (P) was made prior to the availability of mineral nitrogen (N) fertiliser. By contrast, in much of the tropics, N fertilisers were introduced without balanced applications of P and potassium (K), with the consequence that these minerals were effectively mined from agricultural soils.

The trade in agricultural products associated with urbanisation and exports has created a drain on soil fertility with, for example, 21000 Mt P yr^{-1} being exported from Vietnam and Thailand in rice (Fairhurst *et al.*, 1999) and an estimated 75 kg P ha^{-1} lost from cultivated land in Africa (Sanchez *et al.*, 1997). There has been a 17-fold increase in P fertiliser consumption in the developing world between 1960 and 1995, which in 1995 constituted 60% of a global annual P fertiliser consumption of 14 million Mt P yr^{-1} (International Fertilizer Association statistics quoted in Fairhurst *et*

al., 1999). Most of this increase in P fertiliser applications has been in East Asia and the Pacific, with little increase in sub-Saharan Africa. It has become evident that it is particularly small farmers on very low incomes and particularly those without access to distribution infrastructure that are most unable to invest in P fertiliser (Sanchez *et al.*, 1997). As a result there is a pattern of declining yields, with slow closure of the crop canopy contributing to leaching and soil erosion and creating an increased pressure to bring remaining forest into cultivation (Fairhurst *et al.,* 1999).

1.2. N_2 fixing species and P

The use of tree and herbaceous N_2 fixing species to provide N-rich organic residues for incorporation into soil has proven an effective means of increasing soil organic matter (SOM), while addressing the primary soil nutrient constraint to improved yields (Sanchez *et al.,* 1997). Technologies based on this principle have been successfully incorporated into farmers' crop husbandry in many areas and have been supported by an increasingly sophisticated understanding of the residue quality and soil parameters influencing synchronised N mineralization with crop demand (Heal *et al.,* 1997). However P, deficiency affects the growth and N_2 fixation of legumes, undermining the potential of N_2 fixing species to address soil N deficiency (Giller *et al.,* 1994). Low soil N and P are also associated with a decline in SOM leading to increased leaching and soil erosion and a decreased cation exchange capacity (CEC; Weischet and Cariedes, 1993).

Most plant species adapted to tropical conditions with low levels of available P, including most legumes, have a high internal P efficiency and low P concentrations in the leaves. Mineralisation studies on a range of soils have established that to achieve net P mineralisation from organic residues, requires an initial tissue P concentration >0.25% (Singh and Jones, 1976; Blair and Boland, 1978). This is greater than the leaf P concentrations of most tree legumes currently in use as green manures in the tropics (Palm, 1995).

It has long been recognised that the addition of organic matter to soils can increase plant available P through a decrease in soil acidity (Oades *et al.,* 1989; Haynes and Mokolobate, 2001), the adsorption of organic anions to mineral P sorption sites (Iyamuremye and Dick, 1996), an increase in microbial activity (Gressel and McColl, 1997) and improved general mineral nutrition, especially for N and K, leading to improved root size and function. Vegetation suitable for use as a green

manure for enhanced P cycling needs to optimise these functions and must therefore also posses the characteristics of a quality green manure for N cycling. In addition to high mineral nutrient concentrations and the potential for rapid net P mineralisation, the organic material should have a low-to-moderate lignin and polyphenol content if microbial immobilisation of N is to be avoided (Melillo *et al.,* 1982; Palm and Rowland, 1997).

1.3. History of Tithonia Research

Swidden farmers in Indonesia and the Philippines working on P limiting soils, were observed to encourage 'Daisy Fallows' where *Asteraceae* species, notably, the Mexican Sunflower, *Tithonia diversifolia* (Hemsley) A. Gray, (subsequently referred to as tithonia) were valued as soil improvers (Cairns *et al.,* 1998 and references therein). In the mid 1990's these studies and the work of Nagarajah and Nizar (1982) who had reported good results from using tithonia green manure with rice in Sri Lanka, were followed up in a series of on farm experiments in the highlands of western Kenya (Gachengo, 1996; Jama *et al.,* 2000). The results of these experiments indicted that an application of 5 tonnes dry weight ha^{-1} of tithonia leaf and soft stem biomass containing 14 kg P ha^{-1} P could supply the P equivalent of 19.6 kg P ha^{-1} of fertilizer (Palm *et al.,* 1999). The implication of this work was that the rapid mineralisation of P from tithonia, combined with the broader nutritional benefits and changes in P sorption characteristics, made P from tithonia more available to plants than triple super phosphate (TSP). Furthermore, when tithonia was mixed with TSP there was a greater crop uptake of phosphate than when the same total P was added solely as TSP (Nziguheba *et al.,* 1997).

Adding to the advantages of using tithonia as a potential model species for examining the mechanistic basis of enhanced P cycling in green manure amended soils was its distribution. Tithonia is now widely distributed beyond its native Mexico and is common throughout the humid and sub-humid tropics of central and South America, Africa and Asia (La Duke, 1982), where it forms dense clumps on hillsides, grows along road sides and is frequently found in farmers hedgerows (Buresh and Niang, 1997). In addition, tithonia appeared to be growing in soils with low available P, while analysis of leaves indicated high P concentrations (ca. 3.5 g P kg^{-1} DW), double the concentration of most species. This series of observations led to

speculation that tithonia was accessing soil P pools not available to most plants (Buresh and Niang, 1997).

The opportunity to develop a technology, comparable to the utilisation of N_2 fixing species, to allow the poorest farmers in the tropics to manage their soils sustainably while exploiting reserves of soil phosphate not available to plants or gaining added benefits from added P fertilizer, seemed within grasp. The wide availability of tithonia throughout the target areas pointed to the possibility of a rapid dissemination of research recommendations without access to tithonia germplasm being a constraint on uptake by farmers.

1.4. Research Priorities

No single species can provide a high P use efficiency (yield per unit of P), high external P use efficiency (uptake of sparingly available P) and provide a large quantity of biomass for use as a P source (Buresh, 1999). There is, however, the possibility of integrating these functions within the farming systems through the use of a number of different species arranged in a temporal and spatial pattern compatible with species ecophysiological requirements and the economic utilisation of farming system land and labour resources. Within this context the utilisation of tithonia is dependent on knowledge of a number of parameters.

The concentrations of P in tithonia leaves have been found to be in the range of 0.22% in a P depleted Kenyan field soil, to 0.7% in a rock phosphate quarry (George *et al.,* 2001; Jama *et al.,* 2000), with most reports of tithonia P in non-cultivated soils being in the range of 0.3% to 0.4% (Jama *et al.,* 2000). These concentrations are in the upper range for species growing in the prevailing conditions (Palm,1995) and suggest that tithonia may exhibit a particular enhancement of one or more known P acquisition mechanisms: rhizosphere pH modification, exudation of organic ligands, exudation of phosphatases, increased specific root area, enhanced P uptake transporters and or beneficial mycorrhizal associations (Buresh, 1997). It should be noted that tithonia leaf nutrient concentrations appear to increase with decreased soil moisture (Drechsel and Reck, 1998). Enhancement of mechanisms associated with crop available Pi (inorganic phosphate) pools would suggest that tithonia is unsuitable for growing *in-situ*, and would require biomass transfer from land not under cultivation. The enhancement of mechanisms associated with less

available Po (organic phosphate) and mineral bound Pi would provide the counter-indication (George *et al.,* 2001).

The timing of crop establishment following the application of biomass to soil needs to be synchronised with nutrient release to ensure the maximum rate of uptake and to minimise leaching losses (Myers *et al.,* 1994). Knowledge of the decomposition patterns under differing moisture and temperature regimes for both mulch and soil incorporation applications are essential. Understanding of the qualitative characteristics of the green manure that determine optimum decomposition and mineralisation rates is also essential for the identification of other possible species for use as a green manure (Palm *et al.,* 2000).

The addition of organic matter to soil alters the P sorption characteristics of the soil by a number of mechanisms (Iyamuremye and Dick, 1996). Where P fertilizer or rock phosphate is also being added an understanding of the mechanisms by which a green manure is altering P availability is the basis for predicting the quantity of the green manure required to make the optimal economic contribution (Lyasse *et al.,* 2001). Other qualitative characteristics of a green manure may also be important insofar as they alter the functional soil ecology, with possible effects on Po hydrolysis, mineralisation and the establishment of mycorrhizas associated with a subsequent crop.

2. Tithonia (*Tithonia diversifolia*)

Tithonia is a woody shrub capable of attaining a height of up to 4 m (Cairns *et al.,* 1999), but more often reported to reach 2 m (Ayeni *et al.,* 1997). It has alternate palmate leaves 7 to 20 cm in length. These are slightly hairy, strongly aromatic and leave a bitter tasting residue on the fingers when touched. Yellow daisy flowers about 7 cm diameter are produced under declining day length followed by the formation of seed heads carrying several hundred seeds. The viability of seeds appears to be affected by growing conditions and seed heads are susceptible to fungal attack under moist conditions. Propagation can be achieved both from seed and vegetative cuttings. In natural stands, mature stems fall to the ground and take root, the original stem decaying as new plantlets are formed (Ayeni *et al.,* 1997). It is thought to have travelled from its native Mexico as seed in maize and in animal fodder of transported cattle. It may also have been deliberately transported as an ornamental, as an animal fodder species and for its medicinal qualities (Jama *et al.,* 2000).

Tithonia is encouraged to grow in farm and field boundaries in East Africa and also grows beside roads and in clumps on open land (Drechsel and Reck, 1998). Its applications in Africa are reviewed in Jama *et al* (2000) and include apiculture, medicine, animal fodder, and termite control (Adoyo *et al.,* 1997). In South America tithonia is grown close to coffee fields, where it attracts beneficial insects involved in biological pest control (Kato, 1999). In addition to its nutrient value as a fodder, tithonia also appears to play a role in control of parasites in the digestive tract of livestock (Kato, 1999) and when used as a supplementary feed for poultry increases production (Odunsi *et al.,* 1996).

The medicinal uses for tithonia are extensive and include: cancer (Mungarulire, 1993; Gu *et al.,* 2002), HIV (Cos *et al.,* 2002), hepatitis (Kuo and Chen, 1997), as an anti-inflammatory (Lin *et al.,* 1993; Rungeler *et al.,* 1998), liver complaints (Lin *et al.,* 1993), as an antidiarrhoeal (Tona *et al.,* 1993; Tona *et al.,* 2000), as an antiamoebic (Tona *et al.,* 2000) and the treatment of chickenpox (Lamaty *et al.,* 1991) and malaria (Lamaty *et al.,* 1991).

In addition there has been interest in the potential of tithonia as an insect anti-feedant (Hongsbhanich *et al.,*1979; Dutta *et al.,* 1986; Sarma *et al.,* 1987; Dutta *et al.,* 1993; Adoyo *et al.,* 1997) and in the control of soil nematodes (Tiyagi *et al.,* 1985; Nisar *et al.,* 1989; Tiyagi and Wani, 1992).

Production of above ground biomass by tithonia grown in field conditions is in the region of 2 to 4.5 t ha^{-1} (dry weight) six months after establishment, with production levels reflecting moisture and soil fertility (Rutunga *et al.,* 1999; Jama *et al.,* 2000; Sherchan, 2001). This compares with 23 t ha^{-1} for another common green manure *Sesbania aculeata* growing in the same conditions in Nepal (Sherchan, 2001). Tithonia appears to grow more slowly than leguminous green manures, with which it has been compared, during the first two months after establishment, and then grows rapidly until flowering starts at about eight months. Regrowth after cutting may be at a slightly faster rate (Jama *et al.,* 2000). Hedgerow tithonia biomass production under favourable conditions is about 1 kg m^{-1} yr^{-1} when cut twice per year (Drechsel and Reck, 1998). The N and P concentrations in tithonia leaves (see *Table 1*) are at the upper limits of other green manure species and appear to be less dependent on soil fertility parameters than total shoot biomass. This suggests low internal use efficiency for nutrients, but more importantly, suggests that the high mineral concentrations found in tithonia leaves may be due to the translocation of nutrients from senesced

leaves to growing shoot tissues. Although senesced leaves are a highly visible feature of the growth habit of tithonia, there is little comment on this in the literature and the differences between gross and net green leaf biomass production between tithonia and other green manure species has not been considered to account for the relatively high mineral concentrations in tithonia leaves. Drechsel and Reck (1998) found P and N concentrations in leaves to be greater during the dry season, supporting of the hypothesis that translocation from senesced leaves may be a key factor responsible for the observed leaf N and P concentrations in tithonia. The use of tithonia in two-year fallows in Indonesia is based on observed improvements in soil quality (Cairns *et al.*, 1999), but the impact of senesced leaf litter on soil ecology has not been specifically considered.

Table 1. Published values for key mineralisation parameters for leaves (unless otherwise stated) of Tithonia diversifolia as per cent of dry weight. PP denotes polyphenol content. Csol and Psol represent the soluble C and P content of the tissues. Tithonia is a fast growing annual, with a high moisture content (85%), low carbon (39-40% dry weight), low lignin (12%), and low polyphenol content (1.84%-3.8%), but with relatively high N (3.6%), P (0.36) K (3.3%), Ca (0.7-1.5%) Mg (0.29%)

Origin	C	N	P	PP	Lignin	Csol	Psol	Data source
	(percentage of dry weight)							
Shri Lanka	39.9	4.3		2.3	12			Senevirantne *et al.*, 1998
Kenya		3.7	0.50	3.7	12	3.58	0.3	Nziguheba *et al.*, 2000
Philippines		1.8	0.23					Cairns *et al.*, 1999
Indonesia	38.5	2.1	0.30	3.3	9.8			Hairiah *et al.*, 2000
Nepal	39.7	3.6	0.36	3.8	12			Sherchan, 2001
Nepal (stem)	38.7	2.7	0.29					Sherchan, 2001
Rwanda (wet season)		2.4	0.17					Drechsel and Reck, 1998
Rwanda (dry season		3.3	0.29					Drechsel and Reck, 1998

2.1. Tithonia Research.

All field trials, comparing tithonia with other fertilizer regimes, with application rates based on dry weight mineral nutrient concentrations, have indicated that tithonia is a high quality fertiliser capable of providing high yields of maize (Jama *et al.*, 2000), wheat (Sherchan, 2001) and rice (Saha, 1986; Sherchan, 2001). Decomposition studies in Kenya, Nepal and Colombia, using the litterbag technique (Kwabiah *et al.*, 2001; Sherchan, 2001; Cobo *et al.*, 2002) have shown that tithonia decomposes rapidly, with net mineralisation of NPK within 2 days of application. Where decomposition studies have compared surface application with incorporation into the soil, decomposition has been more rapid when incorporated (Sherchan, 2001). Sequential P fractionation of soils after application of tithonia and other P sources has

indicated that in moderately P adsorbing soils there is a greater increase in plant available P following application of tithonia than following applications of some other green manures, or inorganic P fertiliser (Nziguheba *et al.,* 2000). Furthermore, tithonia in combination with triple super-phosphate (TSP) increased the availability of TSP (Nziguheba *et al.,* 1997).

In work to identify the litter quality parameters that result in rapid P mineralisation, Nziguheba *et al* (2000) compared a range of green manures in a Kenyan Nitosol under maize and concluded that in addition to N, P, lignin and polyphenol contents in residues, the ratio of soluble carbon to soluble P constituted a fifth key parameter describing P mineralisation. This hypothesis, proposed by Palm and Rowland (1997), assumes that P immobilisation occurs when there is insufficient residue P to meet microbial demand and that soluble C constitutes the initial C pool available to microbes. A soluble carbon-to-soluble P ratio of 30 is proposed as a parameter capable of explaining 90% of the variation in resin P seven days after the application of a range of green manures with comparable N, P, lignin and polyphenol contents. This work cannot yet be regarded as definitive as it does not consider the role of organic anions (Hue, 1991) and other dissolved organic matter (Ohno and Crannell, 1996) in decreasing P adsorption. Factors concomitant with narrow soluble C: soluble P ratios need to be considered. Interestingly, Nziguheba *et al* (2000) found net P mineralisation from *Calliandra calothyrsus* Meissner, containing 0.16% P, well below the immobilisation threshold identified by Singh and Jones (1976) and Blair and Boland (1978). This supports the view that factors other than decomposition characteristics and total P content are involved in determining P availability following organic additions to soil.

Field studies have given little consideration to moisture and precipitation during decomposition, and although moisture can be limiting in the tropics and precipitation events often create conditions for leaching, most controlled decomposition studies have used a moisture regime optimal for microbial activity. When tithonia mulch was applied to a greenhouse soil under a range of moisture regimes, heavy precipitation resulted in rapid decomposition and N leaching (Seneviratne *et al.*, 1998). This raises the possibility that the characteristic of green manure species for optimal N and P cycling may not be the same and that green manures may need to be blended *in situ* to meet crop nutrient requirements under the prevailing soil and moisture conditions.

The important questions relating to P acquisition by tithonia, have by contrast received little attention. George *et al.* (2002a) studied the depletion of P from pools of differing availability by tithonia and other agroforestry and crop species growing in the field but could not show conclusively that tithonia was accessing pools unavailable to crop plants. In a laboratory study of soil phosphatase activity associated with 4 agroforestry species, *Tithonia diversifolia*, *Crotalaria grahmiana*, *Tephrosia vogelii* and *Zea mays*, tithonia and crotalaria induced significant increases in acid phosphatase at the rhizoplane, decreasing exponentially with distance from roots (George *et al.,* 2002b). As alkali phosphatases, exclusively associated with soil bacteria and fungi, had relatively low activities, it was suggested that the agroforestry species were the source of most of the acid phosphatase and that this was of an iso-form adapted to remain active in the soil environment. A larger root system developed in maize and there were higher levels of alkali phosphatase activity in the associated rhizoplane soil, indicative of increased microbial activity arising from root exuded carbon.

In a study of mycorrhizal infection of tithonia roots of plants collected from Central and South America, Africa and South Asia, it was found that the proportion of roots infected by VA mycorrhizas was not unusual, although infection was almost entirely limited to *Glomus* species as determined by PCR amplification using fungal-specific primers (Sharrock *et al., in press*). There was no evidence of ectomycorrhizal infection and vesicles were rarely present. These results could suggest a specific relationship with *Glomus* species. No data on hyphal length or density in soil was presented. The apparent high specificity of *Tithonia diversifolia* for *Glomus* species may reflect host-symbiont compatibility to facilitate high P acquisition.

Exudation of low molecular weight organic acids has been shown to increase aluminium tolerance in plant roots and possibly increase P availability (Jones, 1998). The only published study of organic acid exudation from roots of tithonia in response to Al toxicity used very extreme Al concentrations likely to damage roots, however, oxalic acid was observed in the growth solution (Olivares *et al.,* 2002). Other findings of these authors that substantial quantities of calcium oxalate are present in leaves of tithonia are not in agreement with Hairiah *et al.* (2000) who found no oxalic acid, but that citric acid constituted 1.5% of shoot biomass in tithonia shoots from Java. Ca concentrations in tithonia leaves are typically in excess of 1.4% of dry weight and Olivares *et al* (2002) suggest that tithonia is calciophobic, with calcium

oxalate forming to avoid calcium toxicity, however, the production of oxalate has been shown to be associated with nitrate reduction in plants grown in solution culture (Rinallo and Modi, 2002). Certainly though, the presence of calcium oxalate would not be surprising as it is common in the vacuoles of angiosperms as one of the principal binding forms for excess calcium (Marschner, 1995). If most of the oxalate in tithonia were in the calcium oxalate form, it would precipitate and not be present in the liquid extracts used for organic acid determination. Alternatively, calcium oxalate may form during sample preparation for organic acid analysis, but either way oxalic acid would not be observable by HPLC analysis as used in Hairiah *et al* (2000). Although it has been shown that oxalate can compete for mineral P sorption sites in soils (Violante *et al.,* 1991), little consideration has been given to the impact of the calcium oxalate in additions of green and senesced leaves to acid soils.

3. Phosphorus deficient tropical soils

Acid soils with a high phosphorus fixation capacity characterize many of the areas of the tropics where small-scale low-input farming is practised (Buol *et al.,* 1980). This smallholding agriculture is in large part due to the generally low fertility of many of these soils mitigating against the development of larger commercial holdings. Three major soil types are associated with medium to high P fixation; Oxisols and Ultisols, which account for 23% and 20% of tropical soils respectively (Sanchez and Salinas, 1981), and Andosols. The Andepts, or Andosols are derived from volcanic ash and have a clay fraction dominated by x-ray amorphous colloids and can fix large quantities of P on the surface of the Al mineral, allophane (Brady 1990). Andosols are generally otherwise highly fertile and productive and do not constitute a major contribution to poverty or pose a constrain to development. The distribution of these soils is defined by the geological faults associated with volcanic activity.

Of greater importance in this context are the more highly weathered tropical soils classed as Oxisols and Ultisols in which total native soil P is typically in the region of 100-200 mg kg^{-1} compared with 3000 mg kg^{-1} in some younger temperate soils (Sanchez and Salinas, 1981). A generalised description of Oxisols is provided by Buol *et al.* (1980). Oxisols are the dominant soils of the interior of the South American and African continents (Sanchez and Salinas, 1981) and are mostly under forest or savannah vegetation. They are thought to have formed predominantly from

weathered alluvial material, with further weathering *in situ*, with desilication giving rise to an oxic horizon characterised by >15% 1: 1 kaolinite clays and sesquioxides. These soils have a low cation exchange capacity (<100 meq kg clay^{-1}) and as a consequence are very low in nutrients. They have almost no weatherable primary minerals or 2:1 clays apart from Al interlayered vermiculite type 2:1 and 2:2 clays low in exchangeable bases. Nutrient minerals are predominantly present within the organic cycle and turnover rapidly, especially under hot humid forest conditions. Under cultivation, organic matter rapidly declines and the limited nutrients are quickly removed in harvested material or are leached. The degree of P fixation varies depending upon the soil's pH and clay content. Fixation is lower when the sesquioxides are more crystalline and increases with the content of fine clays as surface area increases (Sanchez and Uehara, 1980). Exchangeable Al associated with these soils is low, with surface A horizon Al-P minerals being at concentrations around 0.8 mg kg^{-1} compared with Fe-P concentrations around 10 mg kg^{-1} (Sanchez and Salinas, 1981). Oxisols have however proven productive where P fixation has been addressed though the addition of large amounts of P fertiliser and measures to reduce P fixation such as the addition of silica. Such measures though, require a high level of capital investment as P fixation is in the range of 300 to 1000 mg P kg^{-1} (Sanchez and Uehara, 1980). Traditionally, oxisols have been used for shifting agriculture and low intensity grazing. With applications of lime, phosphate and other mineral fertilizers, however, oxisols have been brought into commercial production, with their associated level topography, resilient soil structure and low vulnerability to erosion favouring large highly mechanised plantations. In such high input systems, oxisols have proven highly effective for coffee, bananas, pineapples, wheat, maize and soya beans production (Buol *et al.,* 1980).

A description of Ultisols is also provided by Buol *et al* (1980). These soils are characterised by originally being low base status forest soils in which clays have leached to form an argillic horizon, under a fine textured surface horizon, with bases deposited at depth being available to deep rooting plants. This soil classification includes some laterites, and the red-yellow tropical podzolic soils. Ultisols contain predominantly kaolinite clays, but with bases available to deep rooting trees, under forest conditions these soils can build up fertility associated with SOM that turns over less rapidly than with Oxisols. Once the forest cover is cleared, base cations can be leached resulting in savannah grassland with roots unable to reach these nutrients.

Ultisols often have good water retention capacity, favouring irrigation and paddy rice. While the surface horizons are not usually strongly P adsorbing, if not managed carefully the fine particulates in the surface soil are removed by surface erosion to expose P adsorbing clays and sesquioxides in the B horizon. The high levels of exchangeable Al decrease with liming and if well fertilised or managed with biocyling green manure plants, even degraded Ultisols can be highly productive (Sanchez and Salinas, 1981). In many areas, Ultisols have been used for shifting agriculture, but have often proven productive in permanent low input systems with careful management. Ultisols are the dominant soils of South-East Asia and are common in the upper Amazon basin, parts of West Africa and in the region surrounding Lake Victoria (Sanchez and Uehara, 1980).

Where rainfall is variable and irrigation potential is constrained by topography, maintaining SOM levels compatible with the soil fertility requirements of crops can be problematic even under high input systems (Weischet and Cariedes, 1993). It is therefore of some concern that tropical basin Oxisols with very rapid mineralisation rates, and upland plateaux Oxisols and Ultisols constitute the principal reserves of cultivatable land to feed anticipated increased human populations (Weischet and Cariedes, 1993).

Clay and sesquioxide rich soils Alfisols and oxic Inceptisols can also fix substantial amounts of P under acid conditions (Sanchez and Uehara, 1980), but do not generally posses the combination of constraints limiting production associated with Oxisols and eroded Ultisols.

4. Systems and mechanisms of the phosphate cycle.

4.1. The global P cycle

The phosphorus cycle has been extensively reviewed (e.g. Stephenson, 1986) and is discussed here in detail only where green manure practices are considered to have a significant influence on the cycle.

Total global phosphorus content is about 8.4×10^{15} tonnes, making it the eleventh most abundant element in the lithosphere, however, 99.97% of this is contained in ocean sediments. This distribution reflects the geological phosphorus

cycle in which ocean sediment P returns to the land mass in basic igneous rocks. The distribution of the 1.47×10^{11} tonnes of terrestrial P is presented in *Figure 1*.

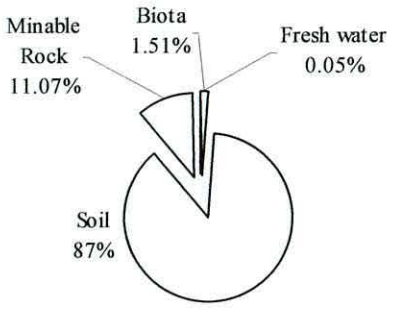

Figure 1. The relative distribution of global terrestrial phosphorus (from Richey 1983)

Human activity is accelerating parts of the geological cycle, with 14×10^6 tonnes of P present in P rich sedimentary rocks being converted into soluble form for agricultural and industrial use each year. A further 4 to 7×10^6 tonnes of P is thought to be transferred to aquatic systems through human activity. These transfers constitute the depletion of the mineable reserves 5×10^9 tonnes and depletion of the most available soil P pools, respectively (Richey, 1983). Effective management of P cycles is therefore likely to become a critical aspect of long-term agricultural sustainability. In practice, this entails maintaining phyto-available soil P close to the economic optimum for crop production while minimising leaching and harvest P transfers. The economic optimum for phyto-available P is described by a combination of agro-economic and soil factors. In moderately to high P fixing soils, small P additions are adsorbed and do not increase phyto-available P and at higher P addition levels the benefit in terms of crop yield is spread over several years. Low-income small farmers must therefore contend with a decrease in liquidity as part of the cost of maintaining adequate available P.

4.2. The nature of phosphorus and phosphorus in nature

The physical nature of elemental phosphorus determines its chemical interactions in both soil and biota and determines its roles in organic systems. Phosphorus is strongly ionised with 5 electrons in its outer ring (P^{5+}). Under atmospheric conditions it is not stable in this form and forms the tetrahedral centre for strongly associated

oxygen atoms as phosphate, PO_4^{3-}. This physical structure provides phosphate bonds with high kinetic stability under physiological conditions, with phosphoric acid dissociation constants (pk_a) at 2.2 and 6.2 (dissociating from H_3PO_4, to $H_2PO_4^-$, to HPO_4^{2-}). At pH 6.0 the ratio of the mono-valent to the bi-valent form is 15: 1, but drops to 1.5: 1 at pH 7.0 (Stevenson, 1986). Inorganic P (Pi) is therefore always ionised at physiological pH. This is significant in cellular chemistry as Pi is able to remain ionised while forming diesters including DNA and RNA, molecules that need to be very kinetically stable and anhydrides, such as adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP), that are required to be thermally unstable and therefore hydrolysable by enzymes. As phosphate is trivalent these diesters remain ionised and are therefore contained in cells as salts that cannot pass through cell wall lipids (Westheimer, 1987). The charge chemistry of phosphates is a critical feature in their role in a range of other organic molecules including phospholipids, phosphate esters such as phenol esters and metabolic pathway intermediates such as glucose-6-phosphate.

As with other elements, the role of phosphorus in biochemistry is a consequence of its physical attributes and the structure and charge status of the molecules it forms under biotic conditions. These same qualities describe the interactions of phosphorus in the soil environment where ionised P molecules associate with both the mineral and organic soil components with transformations between pools and states of availability being driven by P quantity, intensity and biotic intervention under the influence of temperature, moisture, pH, electrolyte composition and biological demand. These interactions in turn determine the patterns of the phosphate cycle.

The complexities of the biogeochemistry of the phosphorus cycle inevitably give rise to methodological constraints on the development of an understanding of the system. This however, is a prerequisite to the development of phosphorus cycle management as an applied science. The current state of knowledge is considered in this section in the context of the green manure biomass transfer model in *Figure 2*.

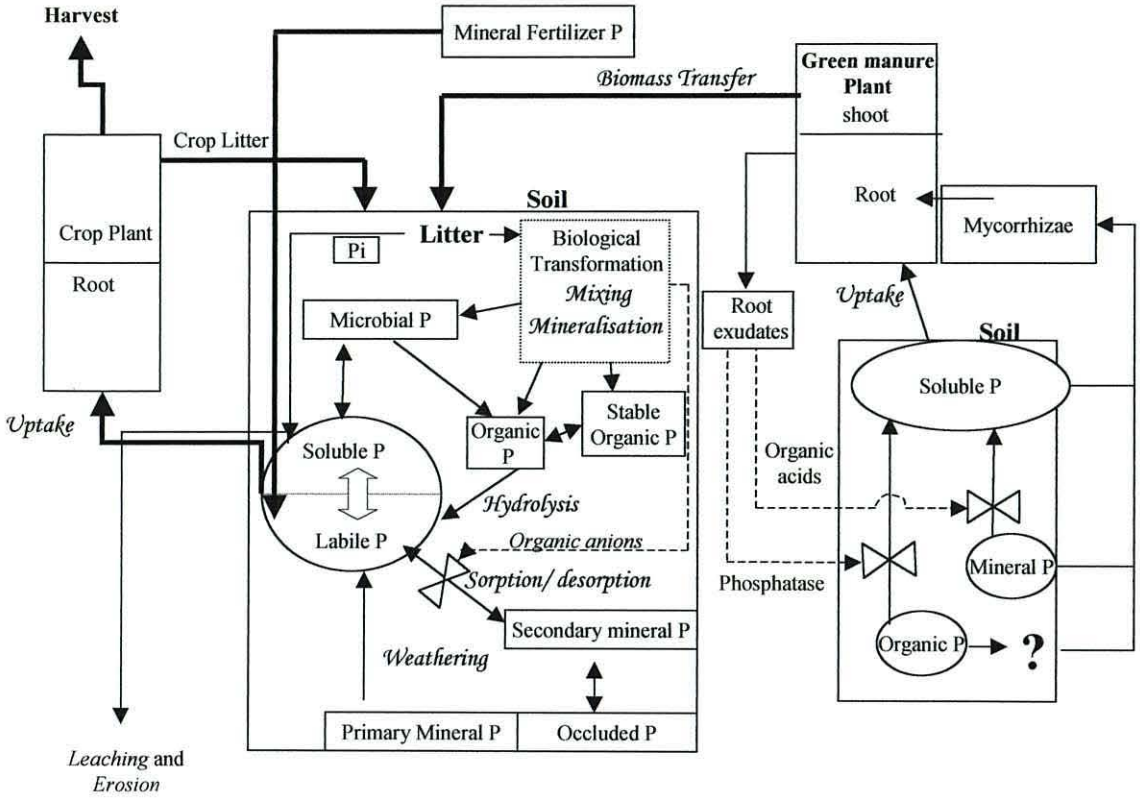

Figure 2. A conceptual model of the phosphate cycle used to describe a green manure biomass transfer to arable crop system, emphasising processes, transformations and transfers within and through the system.

4.3. Reactions of P in Soil

The phyto-availability of phosphate in soils is described by plant uptake characteristics, root morphology, P mobilisation strategies and the reactions of phosphate in both its organic and inorganic forms with the biological and physio-chemical soil environment (*Figure 3*).

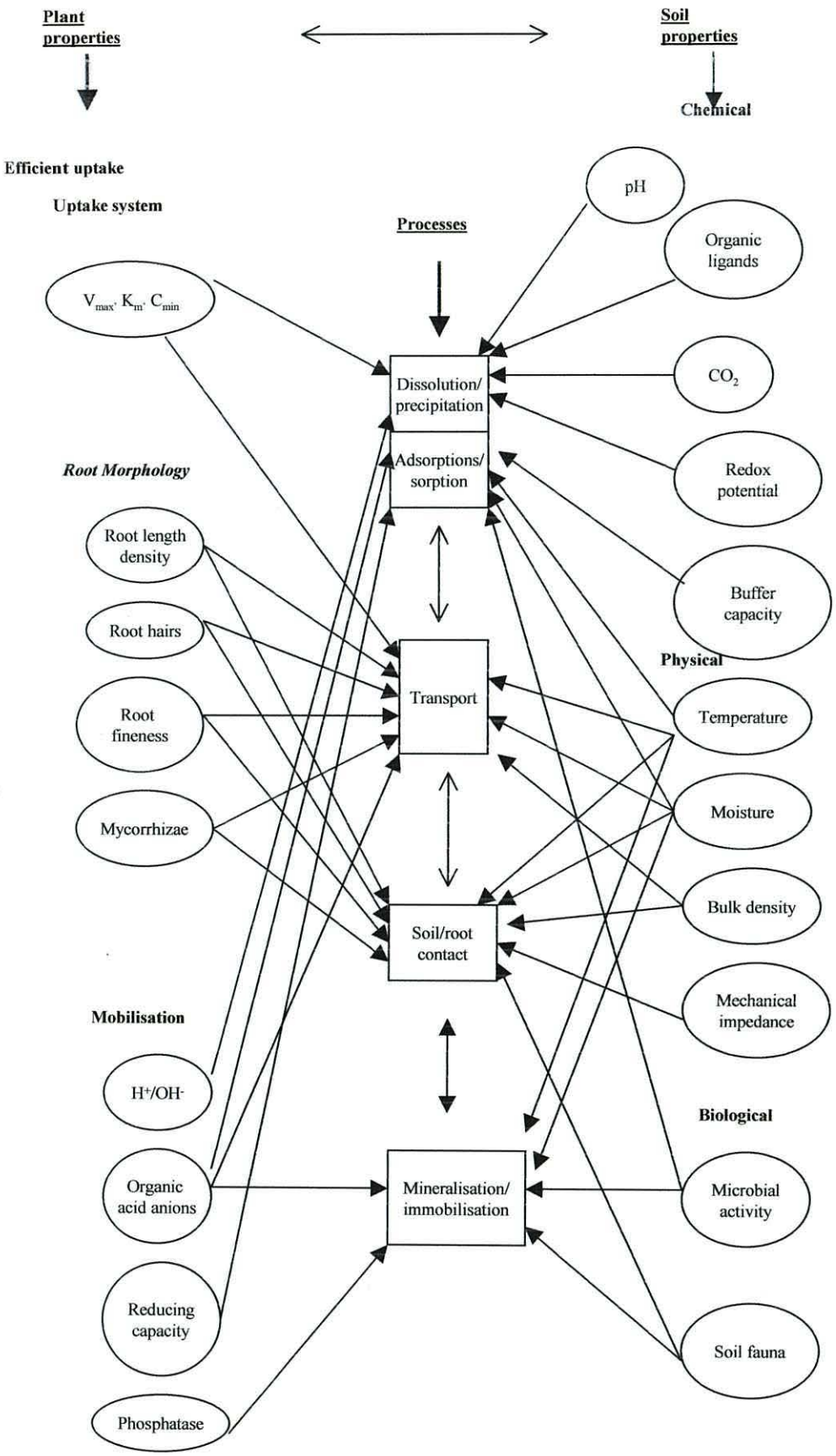

Figure 3. Processes governing acquisition of soil and fertiliser P by crops as affected by plant and soil properties. (Horst *et al.*, 2001)

The scope of this thesis includes the uptake of phosphate by green manure plants and the effects on phosphate availability to subsequent crops following the incorporation of the green manure into the soil. Since it is the enhanced cycling of phosphate in acid soils that is being considered the following discussion emphasises the reactions of phosphate in acid rather than calcareous soils.

4.4. Interactions of inorganic phosphate (Pi) with acid soils.

P in the soil solution readily reacts with the dominant strong cations in three principal reactions: specific adsorption, non-specific adsorption and precipitation (Sposito, 1986). As described previously, Pi carries a strong negative charge and dissociates from the $H_2PO_4^-$ form to HPO_4^{2-} at around pH 7, the reactions relevant to this discussion are therefore predominantly with the mono-valent form.

4.5. Specific adsorption

Specific adsorption or ligand exchange occurs when OH_2^τ groups on the crystal surface of hydrous Al or Fe structures become displaced by an anion that is then specifically adsorbed to the charged surface. At higher pH values, hydroxyl ions replace water at the surface and displacement of the OH ion requires initial protonation, this being enhanced if the anion is protonated, e.g. $H_2PO_4^-$. The new ligand then becomes part of the charged surface (Mott, 1988). So long as the species replacing the oxyanion can be accommodated on the atomically non-smooth surface both in terms of its bulk and interactions with other surface groups then the process is energetically favoured. Reactions of Pi with Fe and Al on soil surfaces are similar, although both the rate and quantity of adsorption is also dependent on the availability of sorption sites within the geometry of the surface. When Pi is adsorbed to a surface, the remaining negatively charged surfaces repulse further Pi from adsorption to adjacent surface cations, unless the negative charge is satisfied by a divalent cation e.g. $Ca^{2\tau}$ (Helyar *et al.,* 1976). The increase in negative surface charge has to be balanced by a cation, with increases in CEC of 0.8 meq $mmol^{-i}$ of P added being observed in an Oxisol (Sample *et al.,* 1980). This also changes the anion status of the bulk solution. As the surface becomes more negatively charged further adsorption is inhibited (Mott, 1988).

Adsorption can be to either one or two cations, with Pi frequently creating bridges between positively charged surfaces, contributing to soil aggregation.

Phosphate often forms a bridge ligand, particularly in the iron oxide, goethite, with both protons being exchanged for Fe. This can effectively form an iron-phosphate skin on the iron oxide particle changing the surface charge of the iron oxide, but also reducing the solubility of the phosphate (Mott 1988).

The pH dependence of the rate of reaction arises from both the hydration state of the cation and its charge state. With increasing temperatures the rate of adsorption of anions decreases and the rate of cation adsorption increases (Barrow, 1992). Where the soil has a variable charge, the effect of the strength of the electrolyte in solution will increase the rate of sorption where the net charge of a surface is negative (at high pH). Conversely, at low pH where the net charge is positive, the strength of the electrolyte will not affect the rate of sorption (Barrow, 1983).

4.6. Non-specific adsorption.

At lower solution Pi concentrations, non-specific adsorption occurs. When protonation of the Al or Fe hydroxyl ion creates a positive surface charge, Pi is attracted, but does not displace the OH_2^+ groups on the mineral surface. This reaction is rapid and is complete with a few days with the rate determined by pH, temperature and the strength of the electrolyte. P sorbed in this way is readily available and constitutes the 'labile P pool', but may participate in more specific adsorption over time.

4.7. 'Fixed' P.

The amount of P becoming adsorbed to a surface decreases as sorption sites become occupied. The amount P adsorbed that becomes firmly held (non-labile) is linearly related to the saturation of surface sorption sites. This creates a three-compartment sorption model in which P can be either in solution, weakly sorbed or labile, or 'fixed' within the crystal lattice. The process by which P becomes firmly held is a slow reaction with the adsorbing surface in which adsorbed P continues to react with imperfect crystal structures, becoming embedded within the crystal. This occurs at a rate that is described by temperature (activation energy) and the nature of the surface, in a process conforming to solid-state diffusion kinetics (Barrow, 1983), with the rate of diffusion being described by pH, P concentration, the soil: solution ratio, temperature and the time of contact. The induction of a low surface concentration therefore results in desorption from the solid phase. If this is correct,

the term 'fixed' is not appropriate although a sustained diffusion gradient away from the solid would be required for a substantial time to mobilize P imbedded in a disturbed crystal structure.

4.8. Precipitation.

As the soil solution must always remain charge neutral (Mott, 1988), the presence of ions with a strong charge will always precipitate a reaction. Under very acid conditions the abundance of H^+ ions causes the dissolution of Al and Fe as hydrous oxides (e.g. $FeOH_2^+$), with hydroxyl ions replacing water as pH increases. Pi in solution readily participates in ligand exchange with Al^{3+} and Fe^{3+} with the associated water or hydroxyl ions being displaced by O^- in the phosphate group. Precipitation of Al phosphates as amorphous variscite ($AlPO_4 \cdot 2H_2O_{(s)}$), or Fe phosphates as strengite ($FePO_4 \cdot 2H_2O_{(s)}$) can occur, but is rare as it depends on a high Pi and cation concentration in solution and involves a very slow reaction (Talibudeen, 1981). More typical is the formation of amorphous aluminium phosphate or organised phases such as sterretite, with Fe phosphate precipitating as tinticite or griphite, depending on solution and surface conditions (Frossard *et al.,* 1995). The infrequent observation of crystalline precipitates in soil following P application suggests that the amorphous Al and Fe phosphates prevail in most situations (Wang *et al.,* 1991) although formation of the ultimate products mentioned above will occur with time (Sample *et al.,* 1980). Dissolution of phosphate precipitates is dependent on the availability of OH^- ions, usually from soil microbes, and is very slow.

4.9. Analysis of inorganic P fractions

The phosphorus pools described above are not discreet, but are part of a continuum dependent upon soil conditions. Analytical methods that define P pools in terms of availability are not precise, but reflect the release of P from the soil by different extractants. In the sequential fractionation procedure developed by Hedley *et al.,* (1982) and modified by Tiessen and Moir (1993), the P fractions are interpreted as follows: Ion exchange resin extractable Pi represents Pi in the soil solution or weakly adsorbed on hydroxides or carbonates (Mattingly, 1975), $NaCO_3$ (0.5M at pH 8.5) also extracts weakly adsorbed Pi (Hedley *et al.,* 1982), but also easily hydrolysable organic P such as RNA and glycerophosphate (Bowman and Cole, 1978); NaOH (0.5M) extracts Pi associated with amorphous Al and Fe hydroxides and

clay minerals, but also extracts Po associated with fulvic and humic acids; Pi associated with precipitated minerals is extractable with HCl (1 M) and hot acid extraction recovers Pi and Po in very stable forms. Determination of Pi in each extract is generally by the molybdate blue method (e.g. Murphy and Riley, 1962) in which Po is non-reactive with the reagents. Determination of Po in each fraction is achieved by subtracting Pi determined prior to carbon oxidation of the extract from Pi following oxidation. In a similar process, microbial Pi can be determined by subtracting determined Pi in the $NaCO_3$ extract from Pi in a duplicate sample in which bacterial cells have been lysed with chloroform. This fractionation technique has been found useful in the study of P transformations under changing land use and following fertilizer additions.

The reactions of Pi with soil minerals, described above are dependent on Pi intensity and time, in addition to soil qualities. The phosphorus radioisotopes ^{32}P and ^{33}P have been usefully employed to trace the movement of added P between pools and to determine exchangeable Pi by measuring changes in specific activity over time in P extracts following the addition of carrier-free isotopes (Di *et al.,* 1997).

In the work reported in chapters 5 and 6, the sequential fractionation procedure is used to study the transformation of organic matter and mineral P additions to soil in the presence of plant roots and mycorrhizas.

5. Organic Soil Phosphorus

The reactions of inorganic P with soil minerals are quite well understood, and allow for extraction procedures that reflect plant available Pi. By contrast, the organic phosphorus (Po) present in soils is both difficult to characterise and the processes involved in its turnover difficult to elucidate (Dalal, 1977). The significance of our limited understanding of organic soil phosphorus fractions is expressed by the proportion of total soil P in organic forms. In the surface soil layers this can range from 20% in cultivated soils to 80% in some forest and pasture soils (Dalal, 1977).

Sequential fractionation techniques that compare molybdate reactive P prior to, and following oxidation (e.g. Hedley *et al.,* 1982), have a limited usefulness that has allowed the patterns of Po accumulation in soils to be understood in general terms. While there is an increase in Po under vegetation, especially in more acid soils high in clays, higher temperatures and moisture conditions favourable to microbial activity

decrease Po. Most Po appears to be associated with the fulvic acid SOM, with relatively small amounts in the humic acid fraction (Dalal, 1977).

While in general terms organic phosphorus cycles are reflective of soil carbon cycles (Stevenson, 1986), where P availability limits biotic activity, soil carbon inputs and cycles are constrained. Thus Walker (1965) considered P availability as a rate limiting mineral in pedogenesis. Gressel and McColl (1997) proposed a nutrient cycle model in which Po cycling is independent of N, C, S, O and H cycles in SOM, with extra cellular phosphatases hydrolysing Po when available Pi is low. In this model, Po increases through pedogenesis and then declines in older soils as available P decreases. This is supported by narrow C: Po ratios observable in P limited soils. It is commonly observed that the addition of Pi to P limiting soils results in a rapid increase in the organic P fractions, supporting this hypothesis (McLaughlin 1988; Oberson *et al.,* 2001; Buehler *et al.,* 2002).

^{31}P nuclear magnetic resonance (NMR) was first applied to the study of organic phosphorus by Tate and Newman (1980) and since then a general picture of Po compound turnover and persistence in soil has begun to emerge using this technique. Achieving substance specific peaks has proven difficult, although this may improve with better gel separation techniques (Turner and McKelvie, 2002). Ideally, analytical techniques would be sufficiently informative to allow the fluxes in organic P forms and pools to be measured as a response to soil husbandry. What has become clear from the range of techniques to study Po is that the organic phosphorus forms are as susceptible to the influence of pH, soil mineralogy, soil texture, temperature and soil moisture as inorganic P, though not always for the same reasons. When Pi limits mineralisation of organic carbon, Po depletion increases, but where there is a continual input of organic carbon, as in established pasture and forestry, there is a turnover of Po and relatively little labile Pi. Under cultivation, where carbon inputs are lower and degradation is enhanced by aeration and exposure to ultra-violet light, Po declines (Daroub *et al.,* 2001).

In Ao soil horizons the Po species are predominantly present as orthophosphate diesters, phosphonates, phospholipids and to a lesser extent as choline and partially hydrolysed phospholipids (Condron *et al.,* 1985). The orthophosphate diesters are predominantly nucleic acids and appear to turnover rapidly, with RNA being particularly rapidly hydrolysed. Teichoic acids, that can constitute up to 85% of the cell walls of gram positive bacteria are an additional source of sugar phosphates

with rapid turnover in the organic horizon, but little observed in mineral layers and cultivated soils (Condron *et al.,* 1990).

In the mineral horizons there is both a change in the predominant carbon and Po forms. Whereas in the litter layer, alkyl C and aryl C predominate and are associated with labile Po forms, in the mineral horizons carbonyl and O-alkyl carbon species are the dominant forms and the more recalcitrant orthophosphate mono-esters, notably, inositol hexaphosphate (IP_6), are the main Po species observed (Moller *et al.,* 2000). It would appear that in mineral soils diester P and aryl C are not effectively stabilised and decay rapidly. Under anaerobic conditions there is evidence of an accumulation of orthophosphate diesters, both in Gley soils (Condron *et al.,* 1990), in paddy rice soils (Mahieu *et al.,* 2000) and cold, wet, acid soils (Makarov *et al.,* 2002).

Although orthophosphate monoesters are a small proportion of the total P cycled through soil, a consistent feature of Po speciation studies is the accumulation phytate recovered in alkali soil extracts from mineral soils (Stevenson, 1986) where it can typically constitute over 50% of total Po and 25% of total soil P (Anderson, 1980). The number of substituted phosphate groups on the inositol ring can vary between 1 and six. The free acid is referred to as phytic acid and the salt as phytate. The six-phosphate group form, inositol hexaphosphate (IP_6) is synthesised by plants as the principal P storage form in seeds and pollen (Hubel and Beck, 1996). IP_6 from this and other plant tissues is often concentrated in animal manures before passing to the soil (He and Honeycutt, 2001). There is also evidence of the microbial synthesis of phytate in soil (Cosgrove, 1964). IP_6 is strongly negatively charged, with net charge being pH dependent, but ranging from $IP6^{6-}$ to $IP6^{8-}$ in normal soil conditions. As such, it participates strongly in ligand exchange with polyvalent cations, binds to clays and forms part of the high molecular weight soil organic acids through the formation of complexes with proteins (Anderson, 1980). Phytate forms salts with decreasing solubility in the order: Na, Mg, Ca, Fe, and Al, and therefore IP_6 is less available in P fixing soils (Anderson, 1980). As with inorganic P, in acid tropical soils, Fe and Al are the principal adsorbing minerals for inositol phosphates.

The stereochemistry of specific adsorption of IP_6 to goethite and clays was studied by Celi *et al* (1999), in order to understand the resilience of phytate to enzymatic hydrolysis. These authors concluded that adsorption of IP_6^{6-} occurs on four of the six negatively charged sites with goethite, with adsorption to clays being on two sites. Adsorption to type 1:1 clays is greater than on type 2:1 clay, reflecting the

increased positive charge in the former. The effect of the presence of adsorbed phytate is therefore to increase the net negative charge of the soil and so increase the CEC. This change in soil electrochemical qualities is obviously greater with clays than goethite. As with Pi adsorption, there is an increase in IP_6 adsorption to Fe(OOH) and Al(OOH) in the presence of Ca^{2+} (De Groot and Golterman, 1993). IP_6 has a greater affinity for P sorption sites than Pi, and can displace adsorbed Pi and reduce further Pi adsorption (De Groot and Golterman, 1993). Significant amounts of inositol phosphates are associated with both the humic and fulvic acid fractions (Borie *et al.*, 1989), but the aggregate nature and importance in tropical acid soils of the resulting organo-mineral complex has been given little consideration. Desorption of inositol phosphates from Fe and Al surfaces appear to require high concentrations of a stereo-specific organic ligand such as citrate (Hayes *et al.,* 2000). Accumulated phytate in acid tropical soils therefore constitutes a P pool of minimal bio-availability and may play an important role in soil aggregation (Anderson, 1980).

Much attention has been given to the enzymatic hydrolysis of organic P by extra-cellular enzymes of plant, fungal and bacterial origin, often with the consideration that it would be advantageous if, either through soil bacterial soil inoculation (Rodriguez and Fraga, 1999), plant breeding (Yun and Kaeppler, 2001) or genetic modification (Richardson *et al.,* 2001), crops could be enabled to access more organic P. This approach is based on the unproven assumption that Po mineralisation is limited by insufficient soil phosphatase with specificity for the Po species present. The evidence supporting this hypothesis is not conclusive. Under P limiting field conditions, litter Po is in more recalcitrant forms and oxidation of the carbon structures occluding Po can be a greater constraint on P mineralisation than phosphatase activity (Gressel and McColl, 1997). Where Po substrates are added to soils, mineralisation of up to 20 times plant requirements have been observed (Tarafdar and Claassen, 1988). That Po mineralisation is primarily limited by available substrate is supported by the observation that Po decreases under cultivation (Daroub *et al.,* 2001) where carbon oxidation is enhanced. Po increases in fine textured acid mineral soils relative to similar soils with decreased sorption surfaces (Soloman *et al.,* 2002), indicating that surface adsorption stabilises Po.

Studies of phosphatase in soil have concentrated on determining the activity of the enzyme for different substrates (Macklon *et al.,* 1997; Tarafdar *et al.,* 2001) or identifying the production of different isoforms from different soil organisms

(Rodriguez and Fraga, 1999; Joner and Johansen, 2000) including plants (Hübel and Beck, 1996; Asmar and Gissel-Nielsen, 1997). These studies have been constrained by methodological limitations from elucidating answers to the fundamental questions that describe the ecological importance of Po in soil P cycles. In particular, the role of extra-cellular enzymes of plant origin in hydrolyzing Po has been an enticing area of research, but has been hampered by difficulties in identifying the origin and activity of phosphatases in non-sterile conditions. Although genes associated with phosphatase secretion under P deficiency stress in plants have been identified (Liu *et al.,* 2001; Miller *et al.,* 2001), it remains unclear if increased root phosphatase secretion is an evolved P acquisition strategy or leakage of intra-cellular phosphatase resulting from insufficient phospholipid P to retain cell membrane integrity (Yun and Kaeppler, 2001).

Enzymes are subject to different states of ionisation depending on pH and the ionisation constants of the various reactive groups. Optimal functionality of phosphatase in soils is therefore dependent on the binding and catalytic sites being in their proper ionisation states (Frankenberger and Johanson, 1982). The soil is a hazardous environment for enzymes, with variable pH, proteolytic organisms, low substrate concentrations, denaturing substances and adsorption to humic and clay surfaces (Burns, 1982). In studies of the effects of adsorption of acid phosphatase on to soil surfaces, it has been found that about 50% of the enzymes become inactivated, but that, depending on the chemo-physical qualities of the adsorbing surface the enzymes can show increased thermal and kinetic stability (Rao *et al.,* 2000). It has been suggested that adsorption can increase the pH optima of acid phosphatase, either where the adsorbing surface is negatively charged and therefore locally more acid (Rao *et al.,* 2000) or through structural alteration of the enzyme (Staunton and Quiquampoix, 1994).

Under P deficient conditions plants can secrete acid phosphatase (Yadav and Tarafdar, 2001), but the rates of activity associated with soil Po substrates are low relative to rates associated with fungal phosphatase for hydrolysis of the same substrates (Tarafdar *et al.,* 2001). This supports the hypothesis that the primary function of plant root secreted phosphatase is not so much to acquire soil Po, but to recover organic phosphates lost from roots as a result of root cell turnover and cell leakage (Barrett-Lennard *et al.,* 1993). Only acid phosphatase, capable of hydrolysing orthophosphate diesters, glycerophosphate, glucose-1-phosphate and with limited

activity for phytate, and with an optimal pH of about 5, appears to be produced by plants (Tarafdar and Claassen, 1988). This is compatible with a rhizosphere root organic P recovery function.

Mineralisation of labile Po is a function of labile Pi availability (Harrison, 1982) and the primary source of phosphatase, especially phytase is microbial in origin, with *Pseudomonas* species (Richardson and Hadobas, 1997) and *Aspergillus niger* being particularly important in the mineralisation of phytate (Richardson *et al.,* 2001). Chen *et al* (2000) observed that Po depletion under conifers appeared to be associated with low available Pi combined with high leaf litter. This suggests that a close C:P ratio combined with low soil Pi is the principal driving force in the depletion of labile Po. These conditions prevail in the rhizosphere and would account for the frequently observed decrease in phosphatase activity away from the rhizoplane and would seem more probable than phosphatase diffusion, given the size and charge of the phosphatase molecules (48 to 160 kDa; Brinch-Pedersen *et al.,* 2002). Furthermore, as DOP is hydrophilic (Kaiser *et al.,* 2001) it is inherently more mobile in soil than Pi (Haunapel *et al.,* 1964) and it is reasonable to assume that depletion of rhizosphere Pi and DOP would result in increased diffusion of DOP toward the rhizoplane. It should be noted, however, that solution Po, increases if a soil has been air dried (Bartlett and James, 1980), possibly due to tearing of organic molecules, possibly resulting in DOP overestimates.

Enzymatic assays of labile Po have shown that HCO_3^- extracted Po does not constitute a labile pool available to phosphatase (Hayes *et al.,* 2000), but Po extracted with citrate is 80% hydrolysable. This points to a possible role for root exuded organic acids in the desorption of Al from DOP, prior to de-phosphorylation. Jones and Kochian (1995) found that enzymatic cleavage of Pi from ATP was inhibited in the presence of Al. In acid lake water, competitive inhibition of phosphatase by Al has been demonstrated (Bittl *et al.,* 2001). It can be hypothesized that where an extractant is required to desorb Po prior to hydrolysis that Al associated with the organic P molecule needs to be disassociated by the extractant. It is probably not coincidental that *Aspergillus niger* increases exudation of citrate when supplied with phytate (Wang and Liu, 1998). The decline in the utilization of Po by plants and soil organisms in the presence of adequate Pi might be then at least partly due to a reduction in organic acid exudation under internal P sufficiency. Equally, it has not been established that the release of extra-cellular phosphatases is not always due to

cell leakage by P deficient organisms and that the feedback mechanism reducing Po hydrolysis could also be related to the resumption of membrane integrity (Joner *et al.*, 2000).

6. Microbial P

P in the microbial biomass constitutes 1 to 2% of total soil P (Stevenson, 1986) and is directly correlated with microbial biomass carbon. Saprotrophic microbial activity is limited by the least available essential nutrient with allocation of resources to extra-cellular enzymes facilitating the optimal level of activity (Sinsabaugh and Moorhead, 1994). Thus when Pi is limiting, microbial phosphatase activity increases. Conversely, when Pi is sufficient, Po rapidly accumulates through microbial turnover (McLaughlin *et al.*, 1988). Microbial biomass P therefore constitutes an immobilised P pool, with turnover defined primarily by the availability of suitable carbon substrate and P availability. Predation of soil microbes by meso-fauna only results in a net release of microbial P once microbial populations are substrate limited (Anderson *et al.*, 1981).

7. Organic additions to acid tropical soils.

Although it is not appropriate to deviate into a detailed discussion of soil organic matter management in the tropics, the addition of green manures constitutes a general SOM conservation strategy as well as a fertilizer addition with more narrowly definable impacts on the soil P cycle. It is therefore important to emphasize the role of SOM, particularly in weathered tropical soils, in maintaining soil fertility. Nor should the broader nutritional impacts on plant P uptake arising from improved mineral nutrition be overlooked.

Fertile soil as a medium for higher plants is an emergent quality of dynamic interactions between detritus and soil minerals mediated by soil organisms under the influence of temperature and moisture. While lacking the coherent self-organising mechanisms of living organisms, the intensity of diverse soil biotic activity during organic matter decomposition gives rise to soil qualities that optimise the physical and chemical conditions for plant growth, and hence further organic inputs.

Cultivation disturbs these processes, altering soil physical and ecological structure through direct disturbance, exposure to sunlight and mixing. Damage to hyphal networks, destruction and incorporation of plants, the release of soil CO_2 and

oxidation, provide a short lived increase in available minerals, but decreases the functional diversity of the soil organisms. Perhaps most pertinently, the quantity and quality of organic inputs to the soil are drastically reduced.

A key feature of Oxisol and eroded Ultisol soils of the humid and sub-humid tropics is the combination of weathered clays of low CEC (type 1:1 clays) and rapid turnover times for SOM due to the combination of high temperatures and high humidity. Under forest, these soils have high litter inputs and most of the CEC is associated with humic and fulvic acids and soil structure is maintained by polysaccharides. Once brought into cultivation there is a rapid loss of the less recalcitrant SOM fractions that can result in a weakening of soil structure, Al toxicity, increased P sorption and leaching of bases. N, P and S that was previously incorporated into the organic fraction and was sparingly available becomes leached or removed through erosion or are immobilised. The loss of surface area and weakening of soil structure associated with declining SOM reduces the available water potential resulting in a further loss of fertility.

In a superficial sense, the resulting constraints on crop production can be described in terms of inadequate mineral nutrition and water and a liming requirement (Weischet and Cariedes, 1993). In commercial systems where mineral fertilisers, lime and irrigation are available, the resulting increase in plant biomass can provide adequate organic residues to maintain functional SOM (Martius *et al.,* 2001). In low input systems, SOM management practices are essential to maintain the fertility of cultivated soils, but may require mineral inputs to reflect diminished ecosystem function relative to forest or pasture as well as removal of minerals through harvest (Sanchez *et al.,* 1997). The addition of either green or animal manure to soil can significantly enhance the nature of the soil with respect to plant growth. Depending upon the nature of organic additions, the activity and community structure of the soil biota can be affected, particularly through the addition of carbohydrates, which in turn influences mineralisation rates and the formation of stable metabolic products that in turn influence soil biogeochemistry. Through an understanding of the mechanisms regulating the flows and cycles of mineral nutrients, and the processes that describe the development of soil structure, it is theoretically possible to design a soil amendment regime that provides optimal fertility in a given soil with nutrient release synchronized to meet plant demand (Heal *et al.,* 1997).

As described above, the soil P cycle is complex and dynamic. Without intervention, plants, soil fungi, bacteria, actinomycetes and meso and macro soil fauna create a permanent state of nutrient flux, producing constant alterations to the geo-chemical and physical soil environment. Photosynthesizing organisms input chemical energy to a system that is otherwise constrained by the availability of mineral nutrients, in a physical environment described by the soil organo-mineral complex, moisture and temperature. These constraints create negative feedback loops at a molecular level, the cumulative effects of which describe the characteristics of bulk soil.

The uptake of P by plants is discussed in detail in section 9, but it should be noted that in the current context by providing the other minerals required for enhanced plant growth, N and K in particular, P uptake and demand are also increased through increased plant size, including the size of the root. This is also associated with an increase in root exudates, which consequently increases microbial activity in the rhizosphere resulting in enhanced localised soil enzymatic and geo-physical modifications that are thought to increase mineral nutrition including P availability. The increase in photosynthate also favours mycorrhizal infection and extraradical hyphal proliferation that can also enhance P availability.

In cultivated soils with minimal organic inputs the populations of earthworms and other beneficial macro-fauna decline, however, these can be enhanced by the addition of organic residues to soil (Lavelle *et al.,* 2001). The activity of these 'soil engineers' result in improved drainage, soil aggregation and aeration, creating conditions favourable for root exploration of the soil. Earthworms feed selectively, resulting in casts with lower clay content than the bulk soil, but with increased organic matter content (Chapuis-Lardy *et al.,* 1998). The casts contain lower Po, but increased alkali extractable Pi, presumably reflecting microbial phosphatase activity in the earthworm gut.

Whereas the specific mechanisms implicated in altering P sorption in soils following organic additions can be studied under laboratory conditions, the stimulatory effects of organic additions on the dynamic processes of the soil ecosystem can confound attempts to quantify mineral specific nutritional impacts in agronomic trials under field conditions. The favourable field results for crops grown with tithonia need to be viewed from a system perspective and attempts to accredit results to narrow quality parameters should be considered with caution.

7.1. Some specific effects of green manures on the soil P cycle

The addition of organic matter to acid oxic soils can increase plant available P by four principal direct mechanisms. The addition of P as Pi (Iyamuremye *et al.*, 1996a) or labile Po (Iyamuremye *et al.*, 1996b) can directly reduce P adsorption potential, while ligand exchange and van der waals bonding of organic anions to Al and Fe surfaces has similar effects (Haynes and Mokolobate, 2001). Changes to soil pH (Hoyt and Turner, 1975) can also influence a range of processes affecting P cycling and plant growth conditions. The addition of carbon substrates in water saturated organic matter can lead to the development of localised reducing environments that can release adsorbed P (Scalenghe *et al.*, 2002).

Green leaf manures used in biomass transfer, are qualitatively different from organic materials that have undergone partial decomposition, or crop residues as they have the enzymes and secondary metabolites associated with growing shoots as well as high levels of available carbon and mineral nutrients especially N and P. Mineral concentrations and lignin and polyphenol contents can be expected to vary greatly between species. When green material is added to the soil, the plant cells will release inorganic minerals, organic acids from the Krebs cycle and secondary metabolites such as flavonoids into the soil, together with a substantial amount of water. Initially, unlike with applications of partially decomposed material, there are no higher molecular weight organic anions, these being mainly formed as secondary decomposition products. When the green material is added to a soil possessing a high P sorption capacity, a substantial proportion of the cellular Pi is likely to become rapidly adsorbed (Iyamuremye *et al.*, 1996a).

7.2. Organic acids and P sorption

Low molecular weight carboxylic acids released from plant residues when added to soil may reduce Pi sorption during the early stages of decomposition. Citrate, malate, and oxalate in particular, compete with Pi for sorption sites on the surface of Al and Fe oxide and hydroxide minerals (Iyamuremye and Dick, 1996). Individual organic acids have specific complexing abilities for polyvalent cations which is dependent upon the number of carboxyl groups and their arrangement relative to other carboxyl and hydroxyl moeties (Jones, 1998). This metal complexing ability significantly affects their sorption behaviour in soil (Jones, 1998). Competition for P sorption sites has not been found to be significant at organic acid concentrations

arising from realistic organic additions to soil, but where organic acids are added prior to the addition of P their effectiveness at decreasing sorption of added P increases as pH decreases (Violante and Gianfreda, 1993). The competition for P sorption sites by oxalate varies with the affinity of the sorption site for the organic acid (Violante and Gianfreda, 1993). Organic acid adsorption reactions are concentration dependent and tend to increase with decreasing soil pH (Jones and Brassington, 1998). The significance, if any, of the residue derived organic acids, on maintaining the availability of residue Pi, is likely to be short lived, due to the uptake and mineralisation of the organic acids by soil microbes which is known to be rapid (Jones, 1998) and an increase in soil pH (see below). It has also been suggested that non-specific soluble particles of a larger molecular size from residues are involved in occluding P sorption sites (Ohno and Crannell, 1996).

A broad range of low molecular weight organic acids are produced as by-products of microbial degradation (Stevenson, 1967; Hue *et al.,* 1986; Iyamuremye *et al.,* 1996), including: citric, malic, oxalic, acetic, malonic, succinic, lactic, formic, fumaric, propionic, butyric and phthalic. Only the dicarboxylic and tricarboxylic acids, however, are thought to directly effect P sorption through complexation reactions with metals (Jones, 1998). Studies, primarily related to root exuded organic acids, have shown that the biodegradation of charged organic molecules is highly dependent on the amount and type of sorption to soil particles, with Al and Fe hydroxide citrate and oxalate bonds being apparently particularly resistant to microbial degradation (Jones, 1998; Jones and Edwards, 1998). With suitable organic substrates and conditions, *Aspergillus niger* can produce and excrete massive quantities of citrate (Wang and Liu, 1998), and a number of fungi, particularly the wood rotting basidomycetes, produce oxalate (Dutton and Evans, 1996). The importance of both the plant residue low molecular weight organic acids and those produced by microorganisms in enhanced phosphate cycling with green manures, is not well understood. The conditions for their production, synchronous to other critical processes, have not been investigated. It has however been shown that the decline in adsorbed oxalate over time in an Oxisol is related to an increase in P adsorption (Afif, 1995).

Carboxylic acids synthesized by the green manure plant may have an initial impact on P sorption through the mechanisms described above, however, as decomposition progresses, negatively charged decomposition products increasingly

constitute the organic particles forming complexes with metals. These include aliphatic acids, amino acids, phenolic acids, hyroxamate siderophores, 2-ketogluconic acid and polymeric phenols (Stevenson, 1994). The ability of these compounds of the fulvic acid (FA) SOM fraction to form stable complexes is attributed to their high number of oxygen functional groups (Iyamuremye and Dick, 1996). Particles of greater stability are subsequently formed as phenols are oxidised to form quinones, which form the complex molecules of the humic acid (HA) SOM fraction in conjunction with amino compounds, sugars, and modified lignin (see *Figure 4* Stevenson 1994). The adsorption of FA, HA and other organic compounds onto Al and Fe hydrous oxides and other clay minerals can lead to competition for Pi adsorption sites (Iyamuremye and Dick, 1996). This effect is more pronounced in oxic soils than in volcanic soils with very high levels of exchangeable Al. The decrease in P sorption as FA and HA mineral complexes form is attributable to both a direct decrease in available adsorption sites and the development of an unfavourable electrostatic field around the adsorbed organic molecules (Iyamuremye and Dick, 1996).

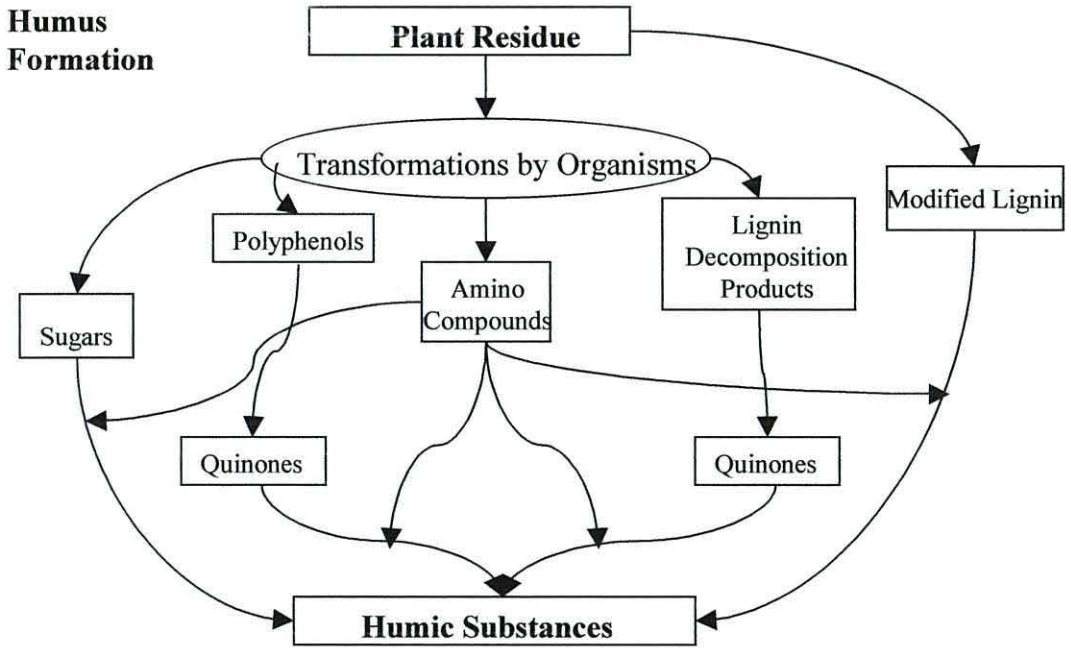

Figure 4. Diagrammatic illustration of the processes involved in the transformation of plant residues to humic substances (Stevenson, 1994).

7.3. Changes in soil pH

Green plant litter tends to contain more cations than anions, with the ionic balance being achieved through low molecular weight organic acids (Marschner, 1995). As the contents of plant cells in the organic additions are released, the organic acids oxidise with a consumption of H^+ or the release of OH^- (Tang *et al.,* 1999). This effect is then followed by a further increase in pH due to the microbial decomposition of the organic acids during which H^+ is consumed and CO_2 is produced (Tang *et al.,* 1999, Yan, *et al.,* 1996). The P sorption capacity of soils with variable charge decreases with an increase in pH due to deprotonation of Al and Fe hydroxyl groups, resulting in a decreased net positive charge or at higher pH, a net negative charge (Oades *et al.,* 1989). An additional increase in pH is driven by the ammonification of organic N, with the release of OH^- (Hoyt and Turner, 1975), but both these effects can be short lived, with pH declining as NH_4^+ is nitrified to NO_3^-, with the release of two protons for each ammonium molecule nitrified (Haynes and Mokolobate, 2001). The net liming effect of organic additions is dependent on the pH buffer capacity of the soil and the excess of base over acid forming elements. It should be noted that in biomass transfer, there is a removal of bases from one site to another and alkalinity is being transferred not created (Pocknee and Sumner, 1997). Although the proton imbalance described above are reversed within one to two months of green manure application, a rise of 0.5 pH units or greater, during initial crop establishment and simultaneous green manure decomposition, could be expected to both improve crop growth in acid soils and decrease exchangeable Al.

While in general the increase in pH decreases P sorption, there is a decrease in the rate of P diffusion as soil pH increases above pH 4.5 (Barrow, 2002). This decreases the P buffer capacity of the soil, although the effect may be concealed by increases in P buffering capacity due to increased P. Where the effect of adsorbed phosphate, adsorbed organic matter and a decrease in protons significantly increases the surface negative charge following organic additions, the strength of the electrolyte has a positive effect on the rate of Pi adsorption (Barrow *et al.,* 1980).

7.4. Reducing reactions

A further phenomena thought to occur in acid soils receiving green manure is the anaerobic reduction of Fe and Mn oxides in anaerobic microsites within

aggregates (Iyamuremye and Dick, 1996). This results in both an increase in pH and a decrease in P sorbing oxides through the following reactions:

$$MnO_2 + 2H^+ + 2e^- \leftrightarrows Mn^{2+} + 2OH^-$$

$$FeO(OH) + e^- + H_2O \leftrightarrows Fe^{2+} + 3OH^-$$

Furthermore, P, predominantly from low affinity sorption sites, adsorbed to Mn and Fe oxides undergoing reduction reactions, can become desorbed (Scalenghe *et al.,* 2002). Reduction of organic carbon can also affect P release from both the decomposing green manure and soil organic matter (NaOH extractable Pi) (Scalenghe *et al.,* 2002). The occurrence of this phenomena during the rapid decomposition of residues may be of significance where it also coincides with the release of Pi from the residues, creating a patch of high solution Pi available to plant roots or soil microbes (Hue and Amien, 1989).

7.5. Changes in exchangeable Al

The addition of green manure can decrease the concentration of exchangeable Al and monomeric Al in the soil solution (Haynes and Mokolobate, 2001). An increase in pH will cause Al in both pools to precipitate as hydroxy Al species (Pocknee and Sumner, 1997). Al can be complexed to decomposing residues in the solid phase and Al in solution can complex dissolved organic matter. However, at higher salt solutions that may arise near residues, the formation of Al complexes is inhibited by competing cations (Mokolobate and Haynes, 2002). This inhibition could be expected to decrease with time through the leaching of excess salts, increased CEC as humic acids form and uptake of cations by plants and soil organisms.

7.6. Microbial P immobilisation

A major factor in the synchronisation of mineralisation with plant uptake of mineral nutrients is microbial immobilisation. The microbial biomass is primarily limited by C substrate and then by N (Barrow, 1960), creating a strong sink for P, with Pi being transformed to microbial Po (McLaughlin *et al.,* 1988). Since the N: P ratio of plant material is always near 10, the decomposition of plant residues is limited by N rather than P in most situations. However, decomposition experiments that

exclude plants as a sink for P, and meso-fauna to cycle microbial P, can fail to observe net mineralisation of plant residue P, even when the initial C: P ratio is less than 150 (Birch, 1961) when it is generally accepted that a C: P <100 leads to mineralisation (Iyamuremye and Dick, 1996). Re-utilisation of residue P by crop plants therefore constitutes a more pertinent parameter than mineralisation as measured by soil P extraction (Blair and Boland, 1978). This is supported by recent work by Nziguheba *et al.* (2000) in which soil samples from trial plots under maize receiving a range of green manures were analysed by sequential P extraction (Hedley *et al.,* 1982). The results contradicted previous studies that had attempted to define a threshold minimum residue P concentration associated with net mineralisation (Singh and Jones 1976; Dalal, 1979; Palm *et al.,* 1999). In this work, of two green manure species with a P content 0.16% of dry weight, below the threshold variously placed between 0.2% and 0.25%, one displayed an initial immobilisation of P and the other did not as described by resin available Pi. These authors considered the determining factor to be the ratio of the more available C and P fractions.

As has been discussed previously, hydrolysis of organic P compounds appears to be driven by demand for Pi. Understanding the mechanisms that drive the P cycle following green manure additions to field soils in order to synchronise P availability with plant demand, necessitates the inclusion of all aspects of the system and should include the soil trophic cycles. The role of carnivorous nematodes in the mineralisation of P is described in *Figure 5.*

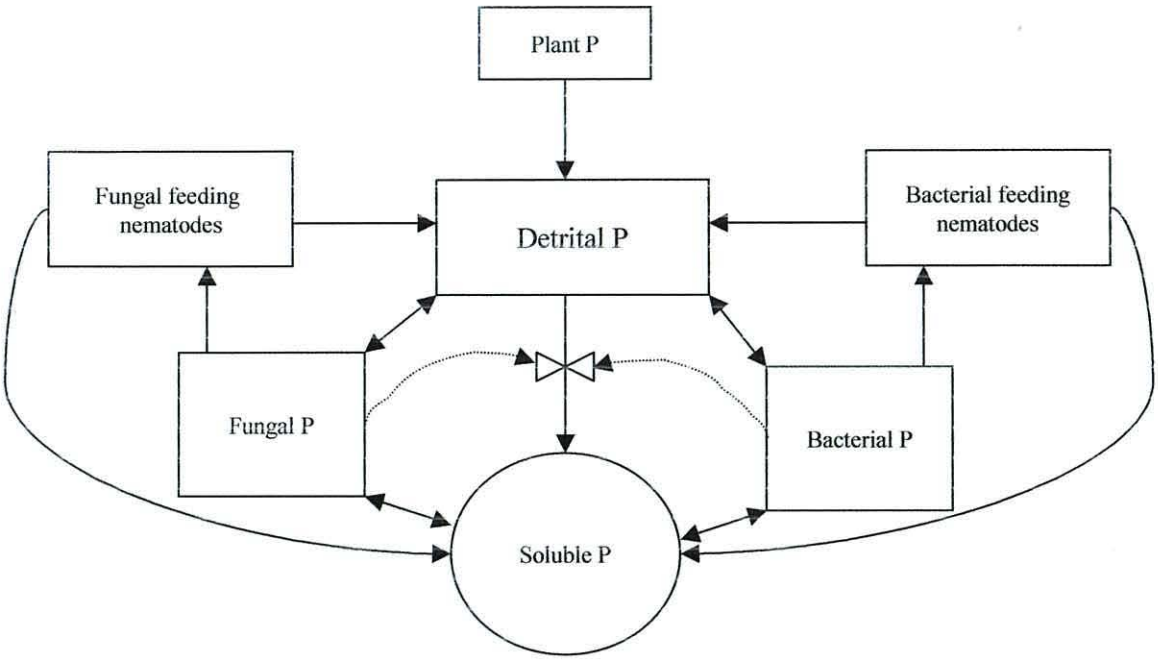

Figure 5. Diagram showing the cycles of P during mineralisation of organic residues and the role of nematodes as the dominant meso-fauna in most soils. After Ingham et al., 1985.

The decomposition of organic matter in soil is primarily performed by bacteria, actinomycetes and fungi, with fungi being the dominant decomposers in acid soils (Killham, 1994). Both fungi and bacteria are susceptible to predation by protozoa (Killham, 1994) and microbial feeding nematodes graze bacteria, fungal hyphae and protozoa (Ingham, 1985; Killham 1994). Carnivorous mites predate nematodes and protozoa (Osler, 2000), with mites and other arthropods including springtails (*Collembola*) graze both saprophytic fungi (Killham, 1994) and mycorrhizas (Larsen and Jakobsen, 1996). At low levels of predation of decomposer species and mycorrhizas, the effect would appear to stimulate bacterial and fungal activity (Ingham, 1985; Larsen and Jakobsen, 1996; Osler, 2000). Low-level predation increases the proportion of bacteria in log phase through increased rates of N and P cycling (Andersen *et al.,* 1981) and compensatory growth of both fungi and bacteria (Lussenhop, 1993).

Both nematodes and mites have low nutrient use efficiency in the range of 15-40% (Andersen *et al.,* 1981) and their excreta constitutes a source of Pi and easily hydrolysable Po that is potentially available to plants and mycorrhizal fungi, during the period when plant less decomposition studies would indicate P as being immobilised by the microbial biomass. Soil micro-arthropods are effectively the system regulators (Yeates, 1987), but if their population is too great as can occur in

leaf litter, mineralisation rates can be decreased due to the reduction in nematodes (Osler, 2000) and excessive fungal grazing (Larsen and Jakobsen, 1996). Functional soil ecology is poorly understood in this context and the impact of manure attributes on soil population dynamics needs to be considered, particularly in the context of synchronised nutrient release and the establishment of crop mycorrhizal symbionts.

8. Integrated P management

It is generally recognised that the addition of green manure P should compliment applications of mineral fertiliser P in P limiting soils (Sanchez *et al.*, 1997;' Nziguheba *et al.*, 1998; Horst *et al.*, 2001). In the context of replenishment of P capital on small farms there is some discussion of the merits of using expensive soluble P fertilisers such as triple super phosphate (TSP) and diammonium phosphate (DAP) or more locally available rock phosphates (PR), with or without some processing to increase reactivity in soil (Buresh *et al.*, 1997). The more soluble P fertilisers are more expensive, but are cheaper to transport and increase yields in P limiting conditions in the crop to which they are applied. Less reactive PR gradually becomes soluble over a number of years and has a greater residual benefit (Buresh *et al.*, 1997).

It would appear that combined applications of a high quality green manure such as tithonia can increase the yield benefit of small soluble P applications (Nziguheba *et al.*, 1998), but it is unclear if this is a result of changes in P sorption characteristics, increased soil microbial activity or a general enhancement of plant nutrition. The limited studies of the effect of green manures of differing qualities on changes in the release of P from PR suggest that the dissolution of low reactive PR increases with green manure addition (Zaharah and Bah, 1997). It is likely that all the effects of green manure addition discussed previously interplay, but the net result probably only serves to emphasize the importance of maintaining soil biological activity to maintain/enhance soil fertility.

9. The acquisition of phosphorus by plants.

9.1. P uptake efficiency

Among the traits required of a green manure plant to enhance P cycling is a high P uptake efficiency, described as the ability of the root system to acquire P from soil and accumulate it in the shoots (Föhse *et al.,* 1988). The relationship between the rate of P uptake, the length of roots and the concentration of P in shoots has been described by the equation:

$$\%P = I_n(L/W)t \times 100 \qquad (\textit{Equation 1})$$

Where I_n = uptake rate per unit of root length (influx), L/W = root-shoot ratio, L = root length and W =shoot dry weight and t = the average period of time the roots absorb P (Claassen and Jungk, 1984 in Föhse *et al.,* 1988). There is a wide range of phenotypic variation in the traits that define these parameters, but in a study of a range of species with differing P uptake efficiencies, species had either high values for I_n or high root: shoot ratios, but never both (Föhse *et al.,* 1988). I_n is described by uptake kinetics and strategies to enhance P availability by mechanisms including rhizosphere pH modification, organic acid exudation, phosphatase secretion and mycorrhizal symbiosis. For plants grown in soil, the root length relative to shoot dry weight is a factor that needs to include root architectural characteristics such as the proliferation of roots within the soil profile sensitive to P availability (Lynch and Brown, 2001) as well as the production of root hairs.

9.2. The availability of P to plants

Movement of phosphate in solution to uptake sites on the root is primarily dependant on diffusion (Barber, 1962) with movement by mass flow accounting for 1-4% of P taken up by plants, depending on plant species, P concentration and water status (Claassen, 1990; Marschner, 1995). Soil solution P concentrations in fertile agricultural soils are in the range 2-10 μmol l^{-1} (Bieleski, 1973) while cytoplasmic concentrations are in the millimolar range. In low P soils the initial solution P concentration can be significantly lower and is rapidly depleted close to the uptake site as replenishment proceeds typically at a diffusion rate (D_{soil}) of $<10^{-12}$ m^2 s^{-1}, providing an effective diffusion distance of 0.03-0.3 mm per day (Bieleski, 1973;

Tinker and Nye, 2000). A consequence of the slow diffusion of P and its reactivity with other minerals is that it is the most immobile, inaccessible and unavailable of all plant nutrients (Holford, 1997).

The process of P uptake is discussed in detail in section 9.3. Plants can only take up phosphate ions (Bieleski, 1973) and continued uptake requires replenishment of solution Pi from labile pools by processes driven by diffusion (Tinker and Nye, 2000). Thus both the movement of Pi in solution and its desorption from the solid phase are diffusion driven processes and as such are rate dependent on a combination of P sorptivity soil factors, soil texture and structure, pH, soil moisture content, and temperature as well as the quantity of Pi in the labile Pi pools (Tinker and Nye, 2000). The P sorption characteristics of a soil define the rates of diffusion and desorption where all other parameters are equal, with the replenishment of Pi in solution being a function of the quantity of labile Pi and an inverse function of the sorptivity of the soil (Holford, 1997) (*Figure 6*). Sorptivity, as described by P sorption isotherms, therefore constitutes a measure of the P buffer capacity of a soil (Fox and Kamprath, 1970).

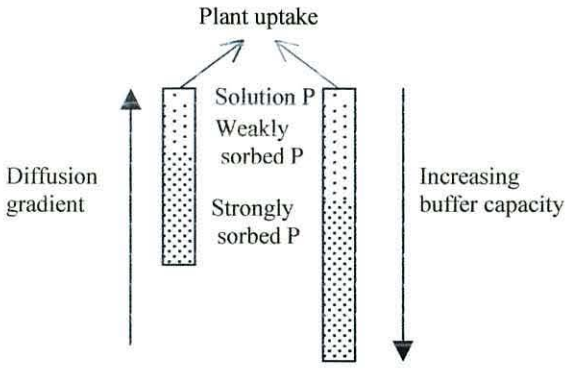

Figure 6. An illustration of the P buffer capacity concept for soils with a lower (left column) and higher (right column) buffer capacity. Although an increased buffer capacity increases the potential to replenish solution P, it increases the resistance to diffusion into solution as the disequilibria between the solution P and the strongly sorbed P increases.

The P buffer capacity concept has proven a useful tool, both in the mathematical modelling of P uptake and in predicting P fertilizer requirements. As described previously, P sorption may involve both specific and non-specific sorption.

Langmuir equation to describe single-phase surface sorption kinetics has been modified to take this into account (Holford *et al.,* 1974), giving the equation:

$$X = \frac{k' xm' C}{1+k' C} + \frac{k'' xm'' C}{1+k'' C} \qquad (Equation\ 2)$$

Where x is adsorption, k is the adsorption/desorption equilibrium constant, xm is monolayer adsorption capacity and the superscripts ' and " refer to high and low energy adsorption surfaces, respectively. The buffer capacity describes the relationship between adsorption and the concentration of P in solution. The above equation has proven applicable for predicting available P (the quantity of P that will come into solution and be taken up by a crop during its lifecycle (Holford, 1997)) although it does not describe the nature of the surfaces involved in adsorption (Sposito, 1982). The P sorption isotherm is calculated from a plot of external P concentration against P adsorbed, with the buffer capacity being described by the initial slope of the isotherm (Holford, 1979). Barrow (1978) proposed use of the Freundlich equation on the basis that it better suited to the current understanding of surface chemistry, but this equation does not allow prediction of maximum P sorption and is therefore less frequently applied (Holford, 1997).

It has been suggested that P fertilizer applications should be sufficient to satisfy high-energy adsorption plus the crop P requirements (Holford *et al.,* 1974), but this may be an impractical recommendation in the context of resource constrained farmers with oxic soils for reasons previously described. Determination of P buffer capacity is nevertheless valuable in the context of the current work as it provides a measure of the P sorption constraints to P availability for a particular soil and allows changes in P buffer capacity arising from organic additions or root exudates to be measured. The diffusion rate for a soil can be calculated using the equation:

$$D_e = D_L \times \theta \times f \times P_b \qquad (Equation\ 3)$$

Where D_e is the effective diffusion coefficient, D_L is the diffusion coefficient in free solution, θ is the fraction of the soil volume occupied by solution and f is the impedance factor. In the consideration of integrated strategies involved in plant P acquisition it is interesting to note that available P is underestimated when soil P desorption rather adsorption is used in calculations, suggesting the uptake of non-

exchangeable P. This has been explained by hysteresis effects which delay desorption in laboratory soil tests (Holford, 1997). The secretion of organic acids and bicarbonate ions by plants would be expected to minimise these effects under plant growth conditions (Nye, 1984). This is supported by predictions of plant P uptake based on cation-anion exchange resin P extractions, with simultaneous reduction in the electrolyte strength, simulating plant cation-anion uptake and replacement with counter ions (Dalal, 1985). It remains to be determined if there is an effective, albeit short lived, reduction in P buffer capacity in rhizosphere soils following secretion of organic anions or *in situ* microbial synthesis of anions that enables plants to extend the root P depletion zone by increasing the zone with an effective diffusion gradient.

9.3. P uptake and transport into the xylem

P ions can be either taken up through the plasmalemma of epidermal cells, and once in the symplasm pass via plasmodesmata through the cortical cells to the stele, or enter the apoplasm. The cell walls of the root epidermal and cortical cells, which together with intercellular spaces make up the apoplasm, carry a net negative charge that confines the movement of strong anions such as phosphate to the larger pore spaces (Smith, 2002). The interlaced fibres that constitute the cell walls of undifferentiated cortical cells further impede movement within the apoplasm. Movement of P by the apoplasmic route can be further constricted by microbial uptake with the result that the concentration at uptake sites on the plasmalemma of endodermal cells can be further reduced (Smith, 2002). The ability to take up P from very low concentration solutions can therefore be considered essential, but it is unclear if the variation between species in this respect is associated with benefits arising from creating a steep diffusion gradient.

It was established in the 1950s that an active transport system described by Michaelis-Menten enzyme kinetics was involved in surmounting this concentration gradient (Epstein and Hagen, 1952; Epstein, 1972). This research also suggested a two-phase transport system, with a high affinity transporter operating at low external concentrations and a low affinity transporter operating at higher external concentrations. In the last few years the application of molecular techniques has allowed an improved understanding of phosphate transport proteins and their regulation. The deduced structure of plant and fungal Pi transport proteins is 12 hydrophobic membrane-spanning domains in a '6+6' configuration, each composed

of 17-25 amino acids arranged in a helix that crosses the membrane (Smith, 2002). It is envisaged that the transporter is doughnut-shaped, allowing $H_2PO_4^-$ ions and protons to pass simultaneously across the membrane. Internal pH is maintained and sufficient co-transport protons provided by the active 'pumping' of protons out of the cell in an active process requiring one molecule of ATP per H^+ exuded. Uptake is thought to be predominantly of $H_2PO_4^-$ (Bieleski, 1973). Ion uptake kinetic studies have shown that high-affinity Pi transport activity is inducible during Pi starvation; whereas the low-affinity transport remains constitutive (Daram *et al.,* 1999) with Pi transport across the tonoplast presumed to occur through the low-affinity transport system (Muchhal and Raghotharma, 1999).

The kinetic parameters of plant Pi transporters have been determined for a range of species (e.g. Silberbush and Barber, 1983; Jungk *et al.,* 1990) and found to follow a modified Michaelis-Menten enzyme kinetic equation that accounts for root efflux of P:

$$V = \frac{V_{max}(C - C_{min})}{K_m + (C - C_{min})} \qquad \textit{(Equation 4)}$$

Where V_{max} equates to I_{max} and describes the substrate saturation concentration. Changes in V_{max} with changes in the plant P status are thought to describe the number of Pi transporter proteins synthesised in the plasmalemma of root epidermal cells (Muchhal and Raghotharma, 1999). K_m, the affinity of the transporter for the substrate at concentrations 0.5 X V_{max} has been found to remain constant, with uptake rates being defined by the number of the transporters (Raghotharma, 1999). It remains uncertain that the variability in the kinetics of P uptake transporters observed across a wide range of species is important in determining the relative efficiency of plant P uptake (Tinker and Nye, 2000) and plant P concentrations. P uptake rates respond to the overall P status of the plant (Drew and Saker, 1984; Muchhal and Raghotharma, 1999) and depend on plant growth stage (Jungk *et al.,* 1990; Tinker and Nye, 2000) as well as broader mineral nutrition (Smith, 2002). Comparisons of uptake kinetics between species and cultivars are further complicated by the variation in measurements arising from the method of determination that can give rise to variations in K_m of a factor of two (Jungk *et al.,* 1990). This is discussed at length by Tinker and Nye (2000).

Sensitivity analysis of the Barber-Cushing P uptake model (Silburbush and Barber, 1983) indicated that neither an increase in the number of transporters nor an increase in their substrate affinity, within the realms of realistic variation, would alter P uptake. The shortcoming of this work was that it was based on P uptake studies from homogenised soil, assumed a simple 'high-low' affinity double transporter system, and defined the transporter kinetics using a whole root system P depletion method. Jackson *et al.* (1990) demonstrated that the number of transporters in a patch of root exposed to high P in a P stressed plant increases in response to high P, with higher external P resulting in an increase in localised uptake by up to 80% compared with the same area of root exposed to low P. Hoffland *et al.* (1989) showed that roots of rape exude organic acids as a specific response in sections of root in contact with rock phosphate, and it would be realistic to expect that such a specific response to external stimuli might also be associated with up regulation of Pi transporters within the same root section. The differential expression of Pi transporter genes or localised post-transcriptional regulation is suggested.

There is little information on the structure/function relationship of the identified transporters (Smith, 2002), but it is likely that their expression in roots is associated with specific physiological responses to P stress and P acquisition from spatially and qualitatively heterogeneous soil P pools. As yet, molecular experimental work has been primarily focussed on whole plant regulation of Pi transporters in response to P deficiency (e.g. Muchhal and Raghotharma, 1999; Kai *et al.,* 2002), rather than addressing the issue of upregulation of transport in response to localized external high P patches. Increased Pi transporter expression in roots of plants with sub-optimal P has confirmed the earlier findings of Clarkson and Scattergood (1982) that uptake can occur along the length of the root (Muchhal and Raghotharma, 1999). It has, however, been shown that the MtPT4 Pi transporter from *Medicago truncatula* is only expressed in the periarbuscular membrane formed in the symbiotic association with arbuscular mycorrhizal (AM) fungi (Harrison *et al.,* 2002). It would be useful to know if this is a widespread adaptation and if similar specific proteins are associated with other P acquisition strategies.

Of the nine Pi transporters identified in *Arabidopsis* (Daram *et al.,* 1999), two, AtPT1 and AtPT2, have been identified as high affinity transporters expressed in roots

as well as other parts of the plant (Karthikeyan *et al.,* 2002). AtPT1 appears to be expressed initially as plant P reaches a sub-optimal level, with AtPT2 being expressed as P stress continues (Karthikeyan *et al.,* 2002). Similar findings for two high affinity transporters activated under increasing levels of P deficiency were found in tobacco (Kai *et al.,* 2002), suggesting that system upregulation is at least in part described by multiple transporters induced by separate signal transduction pathways (Karthikeyan *et al.,* 2002). There is also a suggestion that there is post-transcriptional modification of Pi transporters (Smith, 2002) involved in fine-tuning of the process. Acidification of the cytoplasm, associated with H^+ co-transporters, has been hypothesised as a possible allosteric inhibition of transporters when Pi uptake exceeds plant demand, accounting for the rapid decrease in uptake once Pi supply is restored (Mimura, 2001). A high concentration of transport proteins has been observed in root hairs and secondary roots (Karthikeyan *et al.,* 2002).

Slowly the pieces of the jigsaw are falling into place, but at this stage, with limited knowledge of signal transduction pathways, of the function of the amino acids in Pi transporters and of localised regulation, it remains uncertain if there is a differential performance of the uptake mechanism associated with plant strategies for the mobilisation of soil P and exploitation of high P patches. It is likely that the physiological differences in roots grown in soil and in solution (McCully, 1995) will present further challenges in defining the significance of variability in plant Pi transporters. Analysis of Pi transporters across a range of species suggests that large areas of the genetic code are highly conserved in plants, allowing the development of techniques that combine either the application of reporter genes (Karthikeyan *et al.,* 2002) or northern blot (RNA) analysis,with in-situ single cell tissue sampling from the roots of plants in soil. A full understanding of the role of the Pi transporter system in determining the efficiency of other P acquisition strategies would be useful in breeding plants for low P soils and designing systems for enhanced P cycling.

9.4. Root architecture and changes in root: shoot ratio - one trait to rule them all

Competition for edaphic resources from a heterogeneous soil environment creates adaptive advantages for plants displaying traits for root morphological plasticity. However, presumably due to the need to adapt to a niche, there is a high degree of variability between and within species in the degree, nature and direction of responses to nutrient constraints to growth. Plants have been frequently observed to

increase the proportion of fixed carbon allocated to roots as a response to N, P and S constraints to growth (Chapin, 1980; Marschner, 1995). K deficiency has not been shown to produce this response, but in common with other macronutrients has been shown to alter the pattern of root growth in favour of greater soil exploration and an increase in specific root area. This suggests that increases in the root: shoot ratio resulting from P constraints to growth are not driven by source-sink relationships, but are specific regulated responses involving internal and probably external P sensing and associated signal transduction pathways (Forde and Lorenzo, 2001).

The factors determining plant root architectural responses to environmental heterogeneity constitute a 'higher order organismic trait' within which other traits involved in P acquisition strategies operate at the organ, tissue and cellular levels (Lynch and Brown, 2001). It is perhaps surprising then that so little work has been done to describe root plasticity responses to edaphic conditions for a broader range of species and develop experimental methods that can separate out the effects of plant age and ontogenetic drift from edaphic responses. The area of root involved in nutrient uptake and its distribution within the soil is a critical determinant of uptake of immobile ions (Barber, 1984; Fitter *et al.,* 2002) and mobile ions where there is interplant competition (Fitter *et al.,* 2002). Understanding the systems regulating root architecture is therefore an essential pre-requisite to determining the significance of other nutrient acquisition traits operating at lower levels.

The architecture of the root describes the distribution of plant P uptake transporters and associated traits within the soil. In most soils P concentration is higher near the surface, so P acquisition is enhanced by the growth of adventitious roots and shallow growing basal roots and both traits can be stimulated by P deficiency (Lynch and Brown, 2001).

Although there is good evidence to suggest that some aspects of root plasticity are partially regulated in response to localised nutrient conditions, no mechanism has been fully characterised. Down-regulation of nutrient uptake and an increase in shoot: root ratio is however a universal response to adequate nutrition in split root studies (Drew, 1977; Robinson, 1994) indicating regulation at a whole plant level. An increased exploration of the surface soil by basal and adventitious roots is a frequently observed response to P deficiency (Lynch and Brown, 2001), and is only responsive to whole plant P nutrition. By contrast, regulation of lateral root and root hair initiation appears to be a response to local nutrient availability when not down

regulated at a whole plant level. It remains quite unclear what signal transduction pathways are involved in whole plant responses. A possible role for ethylene, auxin and other phytohormones has been suggested by some studies and disputed by others. Phloem ion concentration has also been suggested as a mechanism for systemic regulation, but this is not supported by correlations between shoot and phloem ion concentrations. An alternative hypothesis involves a nutrient sensory apparatus comparable to phytochrome in phyto-morphological responses of shoots. Zhang and Forde (1998) identified a NO_3^- inducible MADS-box transcription factor encoded in the *ANR1* gene in *Arabidopsis* that was shown to be required for stimulation of lateral root elongation. Forde and Lorenzo (2001) have subsequently proposed a system for the regulation of 'trophomorphogenesis' involving inter- or, more probably, extra-cellular nutrient ion sensor proteins in root cell plasma membranes. These proteins would be capable of providing signals to initiate localised nutrient acquisition strategies, but would be ultimately controlled by systemic chemical signals emanating from the shoot. Identification of plant nutrient sensor proteins comparable to those found in some bacteria is required to support this hypothesis.

9.5. Root hairs

Root hairs are tubular shaped tip-growing cells that emerge perpendicular to the root axis just behind the zone of root elongation from specialised root epidermal cells called trichoblasts. Both formation and elongation of root hairs are dependent on phenotype and are regulated by phytochemicals in response to plant nutrient status (Jungk, 2001). Although functional for only about two days, root hairs serve to increase the volume of soil exploited by increasing the surface uptake area. This is particularly significant in the case of P, for which transport in the soil is dependent on the diffusion gradient and on root hair elongation (Hinsinger, 2001). An increased expression of Pi transporter genes has been shown under P deficiency in tomato (Liu *et al.*,1998). In a study of *Lolium perenne* L. (perennial ryegrass), a species with a very high external P efficiency (Breeze *et al.*, 1984), it was found that 63% of total Pi uptake was by root hairs (Gahoonia and Nielsen, 1998). Root hairs are also thought to be involved in the uptake of most other plant mineral nutrients from soil (Gilroy and Jones, 2000). In addition to simply increasing the adsorbing area of the root, root hairs are also the primary site for root exudation (Jungk, 2001) and can penetrate voids, cracks and pores in soil aggregates. Forming behind the root cap, the site of mucilage

secretion, root hairs can effectively become glued to the soil. The 'injection' of exudates into the soil may then be followed by diffusion of the reaction products back toward the root for uptake (Jungk, 2001).

The length and density of root hairs is highly variable; species with low external P efficiencies, such as onions and other members of the Liliaceae, produce very few, short root hairs whilst those with high efficiencies such as rape produce relatively long root hairs at high densities (Jungk, 2001). The diameter of root hairs has been found to be in the range of 5–17 μm, the length in the range of 0.11–4 mm, and the density in the range from nearly zero to 180 per mm of root (Jungk, 2001). Although plants without root hairs have been shown to have poor P uptake characteristics, there is no clear length/density relationship with P uptake in P efficient genotypes, pointing to the role of root exudates and other P acquisition traits.

In a study of cereals adapted to low P conditions, abundant longer root hairs were a feature of the P efficient genotypes (Gahoonia and Nielsen, 1999). As root hairs provide a greater return per unit of carbon invested, genotypes with long root hairs may be able to allocate more photosynthate to the shoots (Care and Caradus, 1999). Recognition of the importance of root hairs may make a significant contribution to the development of low P adapted crop varieties.

The sites of root hair initiation may, however, constitute a vulnerable area in which the root may be vulnerable to pathogens (Cambell and Greaves, 1990) and while crop plants may have not been bred for P efficiency, they have been selected for disease tolerance. The development of crop varieties with increased root hair function as part of adaptation to low P conditions may need to include the development of increased disease resistance.

The implication of the research in this area is that it may not be necessary or economic to select species for use as a green manure on the basis of their ability to acquire P, but rather, in the context of enhanced P cycling, to concentrate on understanding the potential to favourably manipulate the biogeochemical conditions that favour crop P uptake.

9.6. Mycorrhizal associations

An estimated 80% of terrestrial plant species can form associations with the 149 species arbuscular or endomycorrhizal (AM) fungi belonging to the six genera of

fungi in the order Glomales of the Zygomycetes (Morton and Benny, 1990). AM fungi are obligate plant symbionts thought to have co-evolved with terrestrial plants. Sugars from the plant, predominantly in the form of glucose (Smith *et al.,* 2001), are transferred to the fungi and mineral nutrients, most importantly P, are acquired by the fungi and transferred to the plant. The details of mycorrhizal interactions remain an area of considerable scientific ignorance, due to the variable nature of the interactions and the difficulties of studying mycorrhiza. The importance of AM associations in plant nutrition and plant soil interactions is however extremely significant for plants growing in soils of low fertility and researchers working in this field are extremely concerned that root systems should be considered as symbiotic units (Smith *et al.,* 2001), especially in the context of possible genetic manipulation to enhance P uptake (Cavagnaro *et al.,* 2001).

The spores of AM fungi germinate in response to volatiles including CO_2. Flavonoids and phenolics exuded from plant roots stimulate rapid hyphal branching (Harrison, 1999). Hyphae penetrate the root between epidermal cells or though the epidermal cell walls. They can also enter the root cortex through senescing root hairs (Harrison, 1999). Although the hyphae enter the cells of the cortex, they remain apoplasmic as the hyphae are surrounded by the plasma membrane. Very fine fungal cells create a large common surface area, or arbuscule, for the exchange of nutrients, with the nature of the hyphal structures being variable depending on the interactions between the particular plant and fungal species involved. The vacuole becomes fragmented and there is an increase in the number of organelles. The peri-arbuscular membrane is characterized by high concentrations of ATPase, indicative of active transport processes across the membrane. Some AM species form lipid filled vesicles within the root that are thought to have a storage function (Harrison, 1999). The fungal structures within a cortical cell are short lived and remain active for about two days, after which they decay, leaving the cell intact and available for further infection. Extra-radical mycelia grow rapidly into the soil, extending beyond the nutrient depletion zone created by root uptake, growing from behind the root apex. Mineral nutrients are transferred via the hyphae to the root and transported across the fungal root interface, probably from arbuscules (Smith *et al.,* 2001).

The AM plant fungal symbiosis requires a coordinated cellular differentiation of both plant and fungi and the molecular signal pathways involved are not yet clearly understood. The extent to which plant species are dependent on AM for uptake of P is

highly variable, but has been found to be inversely related to the size of the root system and to root hair growth (Schweiger, *et al.,* 1995; George, 2000; Smith *et al.,* 2001), although exceptions to this general observation have been made in tropical species growing on P limiting soils (Guissou *et al.,* 1998). There is some evidence to suggest that P starvation responses by plants are down regulated by mechanisms other than the supply of adequate P by the fungi (Burleigh *et al.,* 2002) including the plant production of extra-cellular phosphatases (Dodd *et al.,* 1987).

Between 4 and 20% of carbon fixed by the plant may be transferred to the AM fungi as they produce a hyphal mat of 2–27 metres per g soil (Douds *et al.,* 2000). This constitutes a significant cost to plants, but provides plants with an indirect increase in surface area for nutrient uptake of up to 100 fold relative to roots (George, 2000). This is particularly significant in the case of uptake of minerals with slow diffusion in soil such as P and Zn. The K_m of AM Pi uptake transporters has been estimated as 0.17 μM and their I_{max} as 255 nmol m^{-2} s^{-1} (Schweiger and Jakobsen, 1999). This constitutes a potential influx rate two orders of magnitude greater than that observed in plant roots (Smith *et al.,* 2001) and is associated with the density of transporters and the lack of feedback inhibition associated with regulation of plant P uptake.

Smith *et al.,* (2001) present an integrated model of P uptake by plants in which roots grow into soil undepleted of P, with subsequent adsorption by the root apices and root hairs creating a P depletion zone. AM fungi colonize the older regions of the root behind the root hairs and absorb P from beyond the depletion zone. However, in soils with a high plant available P status, plants do not benefit from this association as no depletion zone forms. In this instance the AM association can result in reduced growth as photosynthate is transferred to the fungi with no net benefit to the plant. Plant species with low or minimal AM dependence for P acquisition appear to be able to inhibit colonization and so avoid growth depression under adequate P (Douds *et al.,* 2000).

The narrow diameter of AM fungal hyphae (3-10 μm) facilitates penetration of smaller soil pores than roots (Smith *et al.,* 2001) and so increases access to P within soil aggregates. Working with ectomycorrhizae, Van Breemen *et al.* (2000) observed hyphal penetration of rocks. Although this phenomena has yet to be observed with AM fungi, the ability of hyphae to access Pi within aggregates could be significant in

low P status, high P fixing soils. Hyphae with high affinity transport systems are well positioned to be able to exploit localised P availability events, in competition with other soil microbes and P sorption sites.

The saprophytic potential of AM fungi has been demonstrated (Hepper and Warner, 1983) with both carbon and other nutrients being taken up from decaying organic matter. The transfer of P from the dying roots of one plant to the roots of another has also been observed (Ritz and Newman, 1985).

In addition to P uptake, AM fungi have been demonstrated to transfer Zn, Cu, K, NH_4^+, and NO_3^- as well as small quantities of Fe, and Ca, and may also possibly transfer Mn, Mg and SO_4^{2-} in some instances (George, 2000). AM fungi have been shown to alter microbial population structures in the rhizosphere and to reduce root pathogens (Meyer and Linderman, 1986; Gryndler, 2000), but these interactions are complex as root exudation of carbon compounds also influences microbial diversity in the rhizosphere (Grayston *et al.,* 1998) whilst are themselves influenced by AM fungal infection (Gryndler, 2000).

The cumulative benefits of mycorrhizal associations to plants are reflected in an increased viability of seeds (Koide, 2000), as well as by improved nutrition and resistances to herbivory and disease. Thus, after an estimated 450 million years of co-evolution, most plants are susceptible to AM colonization, although there would appear to be significant variation both within and between plant species in the nature of the relationship with different AM fungal species. This makes investigation of AM symbiosis problematic. If gene expression in both plant and fungi is under the influence of signals from the symbionts and responding to other environmental influences, then inconsistent results of experiments to describe P uptake by AM fungi are expected. For example, the proportion of root colonised has not been shown to be related to P uptake by hyphae, but the length of hyphae has, but not consistently (Jakobsen *et al.,* 2001).

In the context of P uptake from weathered, low P status tropical soils, in which a substantial proportion of total P is in stable Po forms, the hydrolysis of phytate by AM fungi has presented an attractive possibility. Koide and Kabir (2000) showed uptake of phytate by *Glomus intraradices*, but this work did not avoid the pitfalls of so much work in this area described by Joner *et al.* (2000), who cautioned against experiments using unrealistic substrate concentrations, especially in sterile or near sterile soil conditions in which extra cellular protease degradation of phosphatase

enzymes would be reduced. It was shown early on that the acquisition of P by mycorrhizal hyphae could be accounted for by increased surface area (Sanders and Tinker, 1971) and attempts to invoke other mechanisms need to be regarded critically.

Despite the importance of AM symbiosis in P uptake from less fertile soils, little work has been undertaken to understand the impact of organic additions on rhizosphere ecology and the establishment of AM symbiosis. The increase in microbial populations following organic additions to soils results in a rapid increase in mesofaunal populations, especially micro-arthropods and nematodes feeding on fungi, protozoa and bacteria. An increased root: shoot ratio and a reduction in AM hyphae and plant P concentration have been associated with high collembola populations (Harris and Boerner, 1990). At lower densities, however, fungal grazing stimulates compensatory hyphal growth (Kaiser and Lassenhop, 1991). Some fungal species antagonistic to AM fungi, such as *Aspergillus niger* (Gryndler, 2000) are likely to proliferate in green manures incorporated into soil. These aspects need to be investigated further and the influence of secondary metabolites in the green manure on subsequent AM fungal growth considered.

10. Conclusion

The intention of this introductory chapter was to bring together in a single document the principal literature that describes the context for the current research. The P constraints to crop production, described in the introductory section, constitute a practical problem that requires a practical solution, namely mineral phosphate enrichment of those tropical soils that are P deficient either due to pedogenic processes or inappropriate husbandry. Fallow periods and the application of organic matter are an intrinsic necessity to the provision of sustainable fertility in many weathered soils. If, however, water, land and labour resources are to be allocated to soil improvement, the use of N_2 fixing species can constitute a more economically advantageous choice than green manure species unable to 'fix' N. Although some N_2 fixing species are constrained by P in low fertility soils, this does not apply universally, and adequate N nutrition can contribute to improved P uptake by both green manure plants and crops (Sprent, 1999).

The 'Tithonia approach' to enhanced P cycling rests on the concept of a net transfer of P from non-labile P pools, to which crop plants have limited access, to a labile P pool that mineralises at a rate synchronous to crop P demand. This must be

recognised as an experimental concept, supported in the main by a theoretical understanding of P uptake and organic matter transformations in soil. Much of the basic science underlying theory remains incomplete due to the complexity of the interactions between plants, soil organisms and soil organic and inorganic chemistry. Mathematical models appropriate for the prediction of P uptake under adequate P conditions lack adequate detail for the prediction of P uptake by plants dependent on interactive mechanisms, often involving symbiotic and other organisms. Although a great deal is known, sensitivity analysis is extremely difficult. Experimental techniques that can describe the significance of particular plant traits or plant-soil interactions have been slow to evolve.

The work reported in the following chapters attempts to address some of the key issues involved in P uptake by Tithonia and its utilisation in integrated P management of tropical soils. Each of the areas of work constitutes a separate area of expertise and the sheer breadth of the subject, at the resolution of detail required, has perhaps resulted in a lesser contribution to our understanding of the potential of wild species to enhance P cycling in acid soils, than might have been achieved by a narrower focus.

Chapter Two

Phosphate uptake kinetics of *Tithonia diversifolia* and *Sesbania aculeata*

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Summary

Many tropical soils contain relatively high levels of phosphate (P) but paradoxically low levels of phyto-available P, due to adsorption and largely irreversible fixation by Fe or Al oxides and hydroxides. Crop P deficiency has been identified as a major contributing factor to rural poverty in some regions where P fertilisers are unaffordable or not available. Organic soil amendments are necessary in these soils to maintain fertility and soil quality. When livestock manure is in limited supply, shoots of non-crop plants can be applied as a green manure. This practice has a high land and labour requirement and therefore the species used need to provide significant yield improvements for it to be economic. Green manure species need to be evaluated in terms of their potential to take up P from recalcitrant soil P pools and their potential to subsequently release this P into the labile pool upon soil incorporation. This study compared the P uptake kinetics of two species currently being used as green manures and purported to be efficient soil P mobilizers, *Tithonia diversifolia (Hemsley) A. Gray* and *Sesbania aculeata (Wild.) Pers.* Their uptake characteristics were then compared with those of conventional crop species. The results showed that the uptake characteristics of both species could be adequately described by a single Michaelis-Menten kinetic equation. The kinetic parameters for both species ranged from 6.1 to 7.9 μM for K_m and 3.7 to 5.7 nmol m^{-2} s^{-1} for V_{max} which are similar to those of crop plants. The results suggest that the relatively high P concentrations measured in tissues of Tithonia and Sesbania grown on P deficient soils were probably due to other mechanisms rather than the presence of unusually high affinity root transporter proteins.

Key words: Ion transport, Kinetics, Nutrient uptake, Phosphorus, Sesbania, Tithonia

1. Introduction

Phosphorus (P) is an essential nutrient for plant growth and development, participating in most metabolic pathways and acting as a structural component of nucleic acids, coenzymes, phosphoproteins and phospholipids. It is, however, a growth limiting nutrient in most soils and is particularly poorly available in highly weathered soils such as ultisols and oxisols due to adsorption and largely irreversible fixation by Fe or Al oxides and hydroxides (Sample *et al.*, 1980). Such soils dominate in all regions of the tropics (Buol *et al.*, 1980). The low agricultural yield from resource poor small farms located in these areas is a major cause of rural poverty and an obstacle to development (Sanchez *et al.*, 1997). Declining soil fertility and the

unavailability of fertilisers has been frequently identified as a major constraint to increased crop production throughout South East Asia and Sub-Saharan regions (e.g. Sanchez and Uehara, 1980; Buresh and Niang, 1997). Total phosphorus in these soils can contain significant quantities of P present in forms unavailable to plants. Fertility would be therefore enhanced by a transformation of P from recalcitrant soil P pools (e.g. Fe-P) to more labile soil P pools (e.g. organic-P).

The adoption by farmers of green manure technologies, utilising species able to access P not available to crop plants, would allow an active transfer of P from recalcitrant soil P pools into labile organic P pools in a manner compatible with sustainable crop production. Suitable species need to be high biomass yielding and with mineral and decomposition characteristics appropriate to the requirements of cropping systems. Previous work has suggested that *Tithonia diversifolia* (Hemsley) A. Gray (Tithonia) may possess the desired qualities (Buresh and Niang, 1997).

Tithonia, a shrub of the Asteraceae family, is thought to have originated in the uplands of southern Mexico and is now widely distributed throughout the humid and sub-humid tropics. It produces large quantities of leaf biomass and is identified as a high potential green manure plant for enhanced phosphate cycling in soils with low phosphorus bio-availability (Buresh and Niang, 1997). Tithonia foliage from plants growing in areas of low fertility has been found to contain P concentrations of the order of 2.2 g kg^{-1} dry weight of shoot biomass in a P depleted Kenyan field soil (George *et al.*, 2001) to over 4 g kg^{-1} dry weight of shoot biomass in some circumstances (Cairns *et al.*, 1998). This contrasts with the dry weight leaf P concentrations of less than 2 g kg^{-1} dry weight in most crop plants grown under adequate P conditions (Bieleski, 1973). Analysis of samples of foliage from Tithonia and another common green manure, the N-fixing shrub Sesbania aculeata (Wild.) Pers (Sesbania) grown in Nepal, show a leaf tissue P concentration of 3.5 g kg^{-1} for Tithonia and 2.3 g kg^{-1} for Sesbania (Table 1). Tithonia foliage also contained macro and micronutrients at elevated concentrations compared with most crop and green manure plants grown under similar conditions (see Chapter Five: Table 1).

Table 1. P content (g kg^{-1} dry weight) of a range of plant materials typically applied as green manures by Nepalese lowland farmers.

Green manure species	P content (g kg^{-1})
Tithonia diversifolia (leaf and soft stem)	3.5
Dalbergia species (leaf & soft stem)	2.9
Melia (leaf)	2.7
Sesbania aculeata (leaf)	2.3
Sesbania aculeata (stem)	1.6

There is a high degree of variability in the external P efficiency, described by Föhse *et al.* (1988) as the ability of the root system to acquire P from soil and accumulate it in the shoots, both between and within species, with species often demonstrating adaptation to particular soil conditions. Recommendations for the use of Tithonia as a green manure in cropping systems needs to be based on a scientific understanding of the impacts of its utilisation and in particular the impact on transformations of P pools of differing availability to crops.

To develop a predictive model for changes in soil P fractions and crop yields resulting from the use of potential of green manure species to enhance P cycling, it is necessary to evaluate the key plant and soil parameters that describe the rate of P uptake from a given soil. The Claassen-Barber model for predicting P uptake from a growing plant root system (Claassen and Barber, 1976) uses the following parameters: the rate of root growth, the initial soil P concentration in solution, root radius, the buffer capacity, the diffusion coefficient for the soil and the Michaelis-Menten kinetic parameters for the root P uptake transporters. Other plant factors that affect uptake of P include root morphology, root exudates and mycorrhizal associations.

Up-regulation of the P uptake transport system in roots is often associated with the exploitation of high P patches in soil. In a P stressed plant, Jackson *et al.* (1990) demonstrated that the number of transporters in a region of root exposed to high P increased, resulting in an increase in P uptake by 80% compared with the same area of root exposed to low P. Based on field observations, Cairns *et al.* (1998) hypothesised that the observed high concentration of P in Tithonia tissues was due to the plants' ability to effectively scavenge plant available soil P. Here we hypothesise that P acquisition by Tithonia may be associated with an enhanced ability to up-regulate P uptake transporters in the roots and that these transporters possess a higher than usual substrate affinity. To address this issue, and to obtain data that could be used in a

The study used the mineral nutrient uptake kinetic determination method of Claassen and Barber (1974), in which the progressive removal of P from solution by a whole plant is monitored and the Michaelis-Menten equation fitted to the experimental data. As discussed later, this method has limitations, but is appropriate for comparison with other species where kinetic parameters have been determined by the same method. For purposes of comparison, kinetic parameters were also determined for the green manure Sesbania. The results are compared with the kinetic parameters determined for a range of crop species by other authors.

2. Materials and methods

2.1. Plant material

Tithonia seeds of a South African origin (Natal) and Sesbania seeds originating from Nepal were imbibed for 12 h in aerated distilled water and then placed onto moist filter paper at 20°C to germinate. After 5 d, individual seedlings (ca. 3-5 cm tall) were transferred to 300 cm^3 pots containing half-strength Long-Ashton nutrient solution (Hewitt, 1966) modified to provide NaH_2PO_4 at a concentration of 330 μM.

Tithonia stem cuttings taken from plants of a Colombian provenance were propagated in John Innes No 1 compost for 10 days. The stems had diameters ranging from 5 to 7 mm and had four axial buds. The rooted stems were then washed carefully in tap water to remove soil adhering to the roots before being placed into half-strength Long Ashton growth solution modified as for the seed grown plants. The stem cuttings were supported so that the base of the stem cutting was suspended in the top 10 mm of solution. The plants growing in hydroponic culture were placed in a climate-controlled growth room with light intensity of 300 μmol m^{-2} s^{-1} PAR, day length of 16 h and day/night temperatures of 21/18°C. The nutrient solutions were changed every four days. All pots were aerated by a single air tube delivering about 100 ml min^{-1} throughout the experiment.

2.2. Determination of P depletion curves

Measurement of the rate of depletion of solution P by Sesbania roots was made after 24 days growth in solution and the measurement of the rate of depletion of solution P by Tithonia roots was made after 30 days growth in solution. Prior to the measurement of the rate of depletion of solution P, plants were pre-treated by transfer

to a half-strength Long Ashton growth solution modified to provide zero-P (0 μM P) as described in Table 2.

Immediately prior to performing the kinetic studies, the seedlings/cuttings were placed in half-strength Long Ashton nutrient solution containing zero-P for 5 min to remove surface and apoplastic P from the roots. The root systems of seed-grown plants were then transferred into acid washed 300 cm^3 pots containing half-strength Long Ashton growth solution modified to provide 30 μM $NaH_2{}^{33}PO_4$ with an initial specific activity of 40 kBq l^{-1}. Cutting-grown plants were transferred into solutions containing 50 μM $NaH_2{}^{33}PO_4$ with an initial specific activity of 50 kBq l^{-1}. Three of the seed grown Tithonia plants (pre-treated with zero-P solution for 1 day) were placed in a nutrient solution buffered at pH 5.5 with 5 mM MES-Tris buffer during the P depletion experiment.

Table 2. Matrix indicating the number of Sesbania plants and seed and cutting grown Tithonia plants pre-treated in zero-P growth solution in each treatment (number of days pre-treatment in a zero-P solution). – indicates no plants in this treatment.

	Days pre-treatment in zero P solution				
	0 days	1 day	2 days	3 days	6 days
Sesbania	-	$n = 3$	$n = 3$	$n = 3$	-
Tithonia seed	-	$n = 6$	-	-	-
Tithonia Cuttings	$n = 1$	$n = 3$	-	$n = 1$	$n = 1$

The root-bathing solution was sampled at 15 min intervals with 0.5 ml of solution removed for colorimetric analysis of total P content and 0.5 ml of solution removed for measurement of ^{33}P by liquid scintillation counting. At each P sampling event, 1 ml of distilled water was replaced into each pot to maintain a constant solution volume. The P removed by this progressive sampling procedure was estimated to be <0.3% of that absorbed by plants and so was subsequently ignored in calculation of the kinetic parameters K_m and V_{max}. Samples of root-bathing medium were collected until the P concentration estimated from ^{33}P scintillation counting indicated that the solution concentration had stabilised at a very low level. ^{33}P concentrations were measured using a Wallac 1404 Liquid Scintillation Counter (EG&G Ltd., Milton Keynes, UK) with Wallac Optiphase 3 liquid scintillation fluid (EG&G Ltd., Milton Keynes, UK) with all readings corrected for ^{33}P decay. Colorimetric analysis of ^{31}P in nutrient solutions was performed by the molybdenum

blue procedure of Murphy and Riley (1962). An identical container containing no plant was run in all the experiments to estimate losses of ^{33}P due to evaporative/aerosol loss, sorption to vessel walls, precipitation etc. The volume of the residual nutrient solution was measured to estimate evapotranspiratory losses and the root-bathing P concentrations corrected accordingly (assuming a linear water loss over the course of the experiment).

2.3. Root measurements

At the end of the experiment, the plants were harvested. Root length and root area were estimated by the following procedure. The roots from each plant were placed into an aqueous solution containing an iodine stain, before being transferred to a glass bottomed tray containing tap water. A fine mesh was placed on top of the roots to hold them in position. The tray was placed on a Hewlett-Packard image scanner and a digital image of the root obtained. Roots from each plant were divided into between four and six sections to provide a suitable quantity of root for image scanning. The images of each root section were analysed by GS Root 5.1 automated root measurement software, calibrated using the software's facility for calibration against a scanned image of a line known length. After root measurement the stained roots and the shoots were dried (60°C, 24 h) and the dry weight of the plants determined.

2.4. Statistical analysis

The rate of plant uptake was determined at each time point and plotted against the external solution concentration at each step. The Michaelis-Menten kinetic equation (Equation 1) was then fitted to this experimental data to determine values for the kinetic parameters V_{max} and K_m using the Enzyme Kinetics Module for Sigma Plot 8.0 (SPSS Inc., Chicago, ILL).

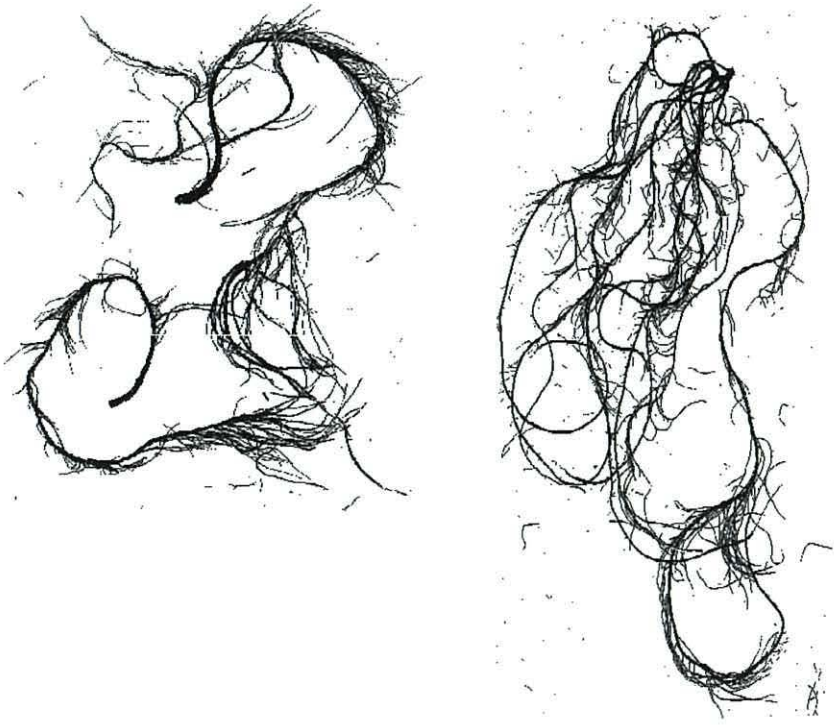
3. Results

3.1. Growth of plants in solution

Observations indicated that the growth and morphology of the Tithonia cuttings in solution was different from seed-grown plants, with cutting-grown shoots having reduced internode length, curling of immature leaves and pale coloration. The roots of both Tithonia cuttings and seed-grown plants were predominantly red; suggesting high levels of the stress reporter compound anthocyanin. Soil grown roots are normally white and visual inspection suggested that the red roots associated with plants grown in solution had a reduced production of fine laterals in comparison to plants grown in soil (*Figure 1*). The shoots of seed grown Tithonia appeared normal and healthy.

Sesbania plants appeared to grow normally in solution culture and grew faster than the Tithonia seedlings. P depletion measurements for Sesbania were carried out 24 days after transfer to solution culture when the root system appeared to be a similar size to Tithonia plants at 30 days.

Figure 1. Photographic images of the root systems of seed-grown (right image) and cutting-grown (left image) Tithonia plants.



Root dry weight, root length and root area data for Tithonia seed- and cutting-grown plants and for Sesbania plants used in the experiments is presented in *Table 3*. The Tithonia plants propagated from cuttings had root systems that were variable in all the parameters measured. The age and radius of roots are known to be key variables in nutrient uptake (Marschner, 1995) and this variability may account for some of the differences in the V_{max} parameters determined for the different treatments. Tithonia plants grown from seed were more homogenous and had a lower dry weight, but a similar root length and root area to the cuttings, indicating an increased development of fine roots. Sesbania root were smaller than the Tithonia root systems and the lower dry weight-to-length ratio suggests that there was less investment in structural tissues in Sesbania than in Tithonia. The root area of Sesbania was also lower than Tithonia, both in absolute terms and as related to dry weight and root length, indicating that all Tithonia plants had a greater proportion of small diameter roots.

Table 3. Root dry weight, root length and root area data per plant for Tithonia seed-grown and cutting-grown plants and for Sesbania plants used in the ^{33}P deletion experiments. Where treatments were replicated the values represent means ± SEM (n = 3).

Plant type	P starvation time (days)	Root dry weight (g)	Root length (m)	Root area (m^2)
Tithonia seed	1	0.70 (± 0.01)	31.6 (± 0.6)	1.91 (± 0.04)
Tithonia cuttings	0	1.04	33.6	2.42
	1	1.50 (± 0.11)	28.6 (± 2.4)	1.92 (± 0.32)
	3	2.00	29.1	2.11
	6	1.26	27.0	1.84
Sesbania	1	0.15 (± 0.01)	21.9 (± 0.9)	1.02 (± 0.15)
	2	0.13 (± 0.01)	23.6 (± 2.0)	0.80 (± 0.06)
	3	0.14 (± 0.01)	22.3 (± 1.2)	0.86 (± 0.09)

3.2. Root P uptake kinetics

There was a good general agreement between solution P concentrations measured by both ^{33}P isotope and ^{31}P colorimetric methods. The colorimetric P concentrations, however, reflected possible exudation of P and showed small fluctuations in concentration between sequential measurements (*Figure 2*).

Colorimetric estimation of nutrient solution P concentrations at each time interval were consistently greater than that estimated by the ^{33}P radioisotope. Furthermore, the colorimetric data appeared to be more scattered. These results are consistent with a simultaneous root efflux of ^{31}P during the uptake of ^{33}P. This consequently led to a continual dilution of the external ^{33}P pool and was particularly evident with cutting-grown Tithonia plants (*Figure 2*). At concentrations below 0.75 μM P, the colorimetric measurements lacked sensitivity and therefore determination of the uptake kinetic parameters was made using only the ^{33}P isotope data. It was not possible to obtain an accurate measurement of C_{min} from the colorimetric measurement of P remaining in solution. ^{33}P was greater than colorimetric P at very low P concentrations, indicating that C_{min} was below the limits of detection for colorimetric P measurement. The basic Michaelis-Menten kinetic equation (Equation 1) was therefore used to determine values for the kinetic parameters V_{max} and K_m.

The effects of P starvation on the rate of depletion of ^{33}P in solution were clearly demonstrated for Tithonia cuttings (*Figure 2*) and Sesbania (*Figure 3*). The Tithonia cutting treatments, where plants were starved of P for zero, three and six days were not replicated, but indicate that the rate of uptake is strongly influenced by the initial plant internal P status. The depletion of solution ^{33}P by the Tithonia cuttings not P starved and starved of P for one day took about six hours, whereas the plants starved for three and six days removed solution ^{33}P in about half that time. The rate of depletion by all P starved plants decreased as the external P concentration declined. This was not evident in the Tithonia cutting not exposed to P starvation.

The Sesbania depletion curves (*Figure 3*) show slow root uptake of P after 1 day without P, compared to Sesbania plants starved for two and three days, indicative of a high initial internal P concentration.

Figure 2. Phosphorus depletion from the external root-bathing solution by Tithonia stem cuttings measured by ^{33}P radioisotope (upper panel) and ^{31}P colorimetric (lower panel) methods after varying periods of P starvation (0 to 6 days). Symbols represent experimental data points.

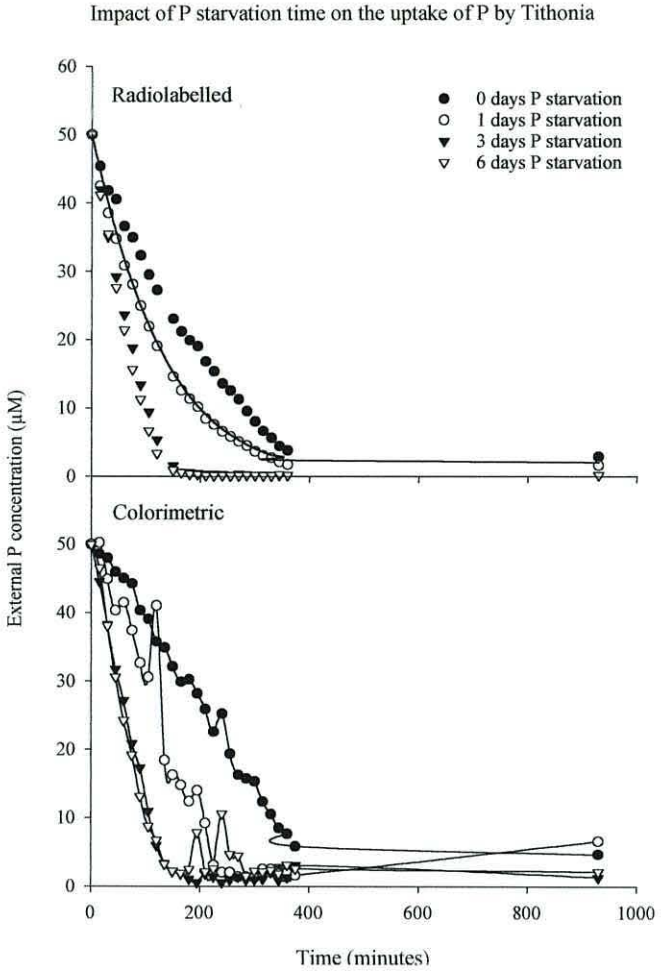
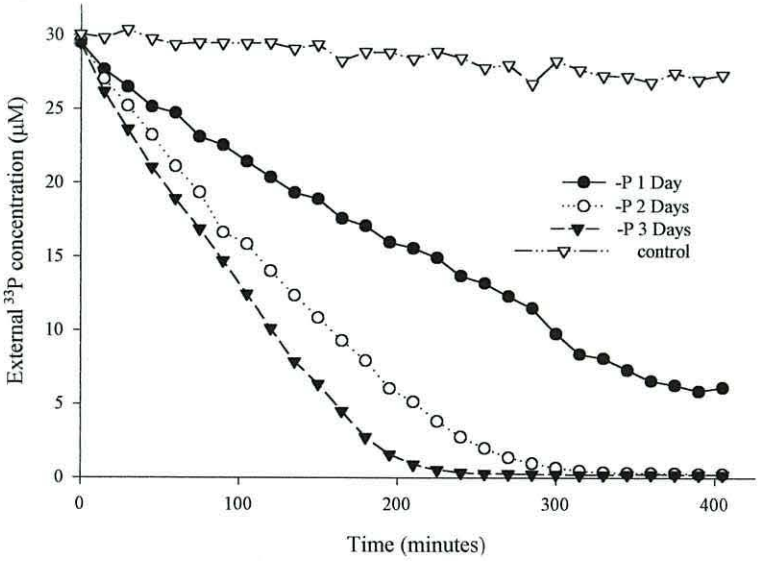
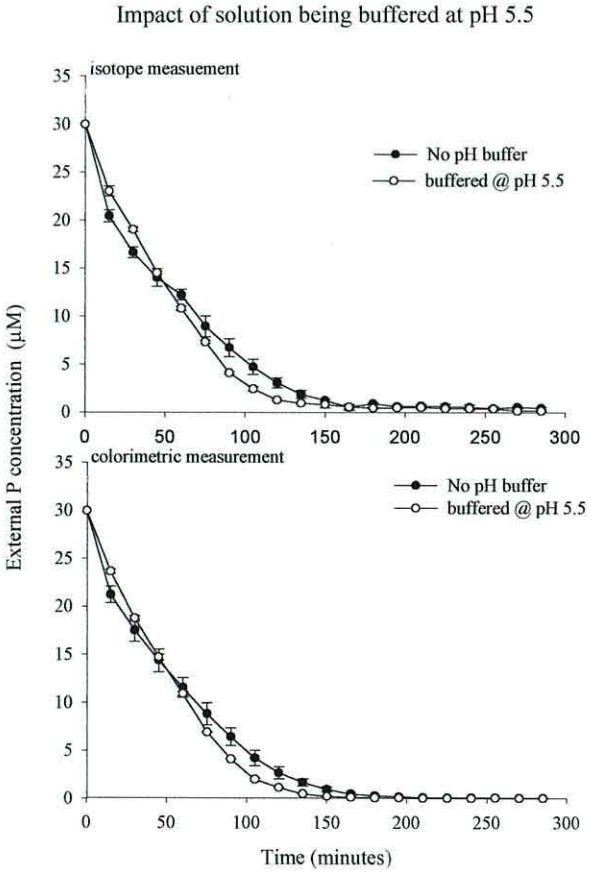


Figure 3. ^{33}P depletion from the external root-bathing solution by Sesbania after varying times of P starvation (1 to 3 days without P). The control had no plant and indicates ^{33}P depletion due to dilution. Values represent means (n = 3, except control where n = 1).



Half-strength Long Ashton solution has an initial pH of 6.3, but during plant culture the solution pH gradually rises as protons are taken up due to the uptake of nitrate, the predominant N source in the nutrient solution. The second dissociation constant (pk_a) for phosphoric acid is pH 6.2 (dissociating from $H_2PO_4^-$, to HPO_4^{2-}). At pH 6.0 the ratio of the mono-valent to the bi-valent form is 15: 1, but drops to 1.5: 1 at pH 7.0 (Stevenson, 1986). Plant P uptake is predominantly in the form of $H_2PO_4^-$ and P uptake is highest between pH 5.0 and pH 6.0 (Schachtman *et al.*, 1998). The effect of buffering the solution at pH 5.5 on the depletion of ^{33}P by Tithonia grown from seed was to both increase the rate of uptake and to reduce the variability of solution concentrations at each sampling time between replicates (*Figure 4*).

Figure 4. Phosphorus depletion of the root-bathing solution by seed-grown Tithonia, measured by ^{33}P radioisotope (upper panel) and by colorimetric method (lower panel), either in a pH un-buffered or pH buffered nutrient solution. Values represent means ± SEM ($n = 3$).



Compared to cutting-grown plants, seed-grown Tithonia provided less evidence of P root exudation into solution and there was reduced variability between replicates, especially in the pH buffered treatments. There was little difference

between the isotopic and colorimetric P measurements, except at low P concentrations where the total P (^{31}P +^{33}P) concentration was below the limits of detection of the colorimetric method. This suggests that root P exudation was minimal.

The experimental data was fitted to the integrated rate equation for Michaelis-Menten kinetics to describe the rate of ion transport by the roots as a function of the change in concentration over time. The values obtained for V_{max} were calculated as nmol m^{-2} s^{-1} using the root area data presented in *Table 3.* The plots for the rate of uptake predicted by the Michaelis-Menten regression are presented in *Figure 5* for Tithonia and in *Figure 6* for Sesbania. P uptake at low external P concentrations was increased by both the time of pre-treatment with a zero P solution (i.e. duration of P starvation) and by buffering at pH 5.5. Sesbania after three days in zero P solution and Tithonia cuttings after six days had the highest rate of P uptake.

Figure 5. Michaelis-Menten curves describing Tithonia root P uptake kinetics as determined by the ^{33}P depletion method. Plants were grown by two propagation methods (seed and cutting grown) and exposed to a range of P starvation treatments (0 to 6 days –P). The rates of P depletion under seed grown plants were determined with and without the solution being pH buffered (pH 5.5).

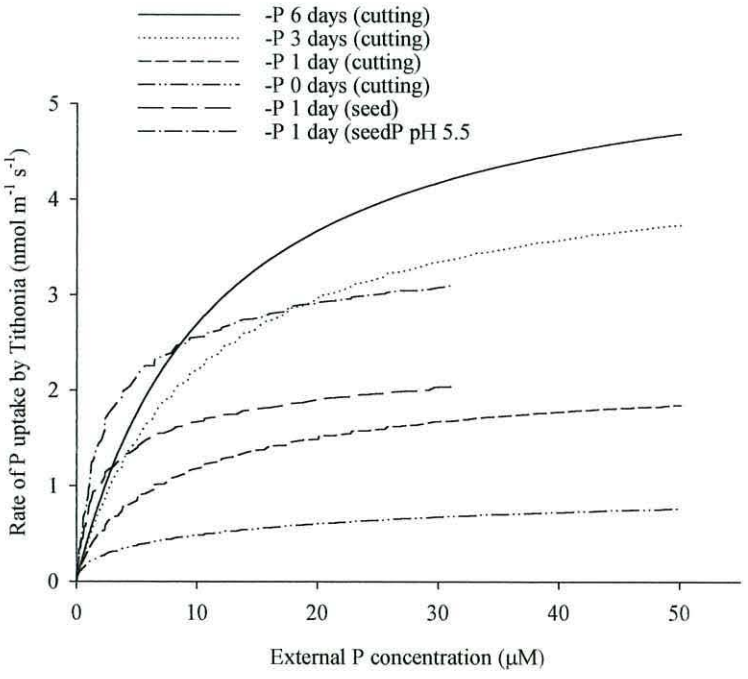
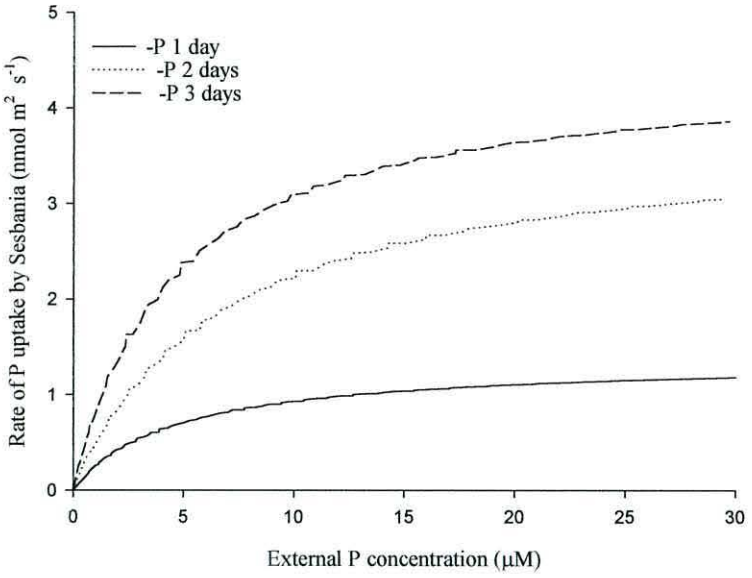


Figure 6. Michaelis-Menten curves for Sesbania root P uptake kinetics, determined by the ^{33}P depletion method. Plants were exposed to a range of P starvation treatments (i.e. P withheld for 1 to 3 days).



The values of V_{max} (maximum possible P influx rate) and K_m (the solution P concentration where P influx is one half V_{max}) are shown in *Table 4*. The calculated values for α which is defined as V_{max}/K_m and describes the relative uptake characteristic at solution P values lower than K_m is also shown. The r^2 values showing the goodness of fit to the Michaelis-Menten equation were lower where the rate of uptake was slower.

Table 4. Michaelis-Menten kinetic parameters (V_{max}, K_m, and α) for root P uptake by either Tithonia plants grown from seed and cuttings or by Sesbania plants. The r^2 value is the goodness of fit of the Michaelis-Menten equation to the experimental data.

	P starvation period (days)	K_m (μM)	V_{max} (nmol m^{-2} s^{-1})	r^2	α (nmol m^{-2} s^{-1})
Tithonia	1 + pH buffer	5.7	3.7	0.978	0.6
(seed)	1 – pH buffer	7.9	2.6	0.962	0.3
Tithonia	0	6.8	1.0	0.424	0.1
(cuttings)	1	8.6	2.2	0.444	0.3
	3	10.8	4.5	0.955	0.4
	6	11.3	5.7	0.959	0.5
Sesbania	1	5.6	1.4	0.590	0.2
	2	6.1	3.7	0.669	0.6
	3	4.1	4.4	0.880	1.1

There was little difference in the predicted maximum velocity of uptake (V_{max}), or the affinity of the transporters (K_m) for phosphate, when cutting and seed-grown Tithonia plants received one day zero P pre-treatment and when the external solution was not pH buffered. However, the lower r^2 for the cuttings indicates the data from the seed grown plants more closely fits the Michaelis Menten model. When the solution was pH buffered V_{max} increased and K_m was lower compared to similar Tithonia plants in an un-buffered solution. This observation is compatible with uptake of $H_2PO_4^-$ being favoured. The kinetic parameter K_m increased slightly with increasing time of zero P pre-treatment in Tithonia, but decreased marginally in Sesbania, while there was little difference in the maximum velocity of uptake between the two species for P starvation up to 72 hours. Based on these kinetic parameters, Sesbania and Tithonia appear to be similarly adapted to take up P at lower external solution concentrations as indicated by the calculated α values.

4. Discussion

Tithonia cutting-grown plants were placed in 50 μM ^{33}P solution rather than 30 μM solution used for Tithonia seed-grown plants and for Sesbania. This was because the shoots were larger than the seed grown plants and constituted a stronger sink for P. It is not possible to separate out the differences in uptake P between the seed-grown and cutting–grown Tithonia arising from the different method of propagation and the differences in the initial solution concentration. The cuttings did not appear to grow normally in solution and this would be expected to influence P uptake. In the field, farmers typically propagate Tithonia from stem cuttings and a comparison between the P uptake characteristics for the seed and cutting propagated plants would be of interest. However, for the reasons described above, a comparison of Michaelis-Menten parameters for seed and cutting grown Tithonia when uptake was determined after one day pre-treatment in zero P solution, does not allow a confident conclusion that seed propagated plants are better adapted to low P soil conditions.

The experimental design was based on the method described by Claassen and Barber (1974) and modified by Drew *et al.* (1984). These authors used a light intensity of 450 μmol m^{-2} s^{-1} PAR, compared to 300 μmol m^{-2} s^{-1} PAR used in the

current study. The significance of this variation in allowing a direct comparison with other results obtained using similar methods should be given consideration. The principal effect of light availability constraining growth would be to reduce plant demand for P and hence the predicted V_{max}.

Some of the results from studies of the kinetics of P uptake transporters by Barber and co-authors using the whole plant P depletion method are presented in *Table 5* for purposes of comparison.

Table 5. Reported values for the Michaelis-Menten kinetics parameters K_m, V_{max} and α for P uptake determined for a range of species after 24 hours of P stress.

Species	Age (days)	K_m (μM)	V_{max} (nmol m^{-2} s^{-1})	α (nmol m^{-2} s^{-1})	Reference
Corn	16	5.8	32.6	5.6	Schenk and Barber (1979)
Soybean	18	2.7	6.5	2.4	Silberbush and Barber (1982)
Wheat	26	5.3	11.1	2.1	Itoh and Barber (1983)
Lettuce	30	2.0	10.6	5.3	Itoh and Barber (1983)
Tomato	26	6.1	49.9	8.2	Itoh and Barber (1983)
Onion	32	3.6	17.0	4.7	Itoh and Barber (1983)
Carrot	33	3.1	12.7	4.1	Itoh and Barber (1983)

The K_m parameters, describing the affinity of the transporters for the substrate at low concentrations, were 5.7 μM for Tithonia (seed grown plants, solution buffered at pH 5.5, P starved for 24 h) and 6.1 μM for Sesbania (P starved for 24 h) as described in *Table 4*. These results are comparable with those presented in *Table 5* and do not suggest that any apparent difference in the external P efficiency of Tithonia is due to an increased transporter affinity for P at low solution P concentrations.

The values for V_{max} determined in this experiment (*Table 4*) are smaller than those reported in *Table 5*. P uptake rates respond to the overall P status of the plant (Drew and Saker, 1984). V_{max} is therefore variable according to changes in the plant P status, as the number of active Pi transporter proteins in the plasma membrane of root epidermal cells is regulated to meet internal P demand (Muchhal and Raghotharma, 1999). Variability in the measured V_{max} is not therefore simply a product of genetic traits associated with an adaptation to a particular P availability environment, but also a product of variability in the initial P status of the plants, the area of root involved in active P uptake and P demand as described by other factors relating to growth conditions including available light, as described above. The

variation between the results obtained for V_{max} and those determined by Barber's group could be due to the initial P status or to the intensity and quality of light used in the experiment. Comparison of the results for V_{max} and for α (V_{max}/K_m), a parameter to describe the relative uptake characteristic at solution P values lower than K_m, can therefore only be made for the results of these experiments and not those reported in the literature.

From various studies of P uptake kinetics it has been observed that as the solution concentration decreases there is no decrease in K_m, although small increases have been observed (Raghotharma, 1999). The Tithonia cuttings placed in zero P for three and six days had a higher K_m value than the less P starved plants. By contrast extended exposure to zero P decreased K_m in Sesbania. In both species this change was associated with an increase in V_{max} with the V_{max} increase in Sesbania being proportionately much greater than with the Tithonia Cuttings.

The slow depletion of the solution P by Sesbania after one day with zero P, suggests the plants had high vacuolar P reserves and that the P uptake transporters remained down regulated after growing at high exogenous P (330 μM P). Since the Sesbania root systems were smaller than for the Tithonia plants, there may have been a greater luxury uptake of P prior to transfer to the zero P solution. With some caution, therefore the kinetic parameters for Sesbania, pre-treated with zero P for two days, may be more appropriately compared with the Tithonia seed plants pre-treated with zero P for 1 day. On this basis, Sesbania and Tithonia would appear to have similar P uptake characteristics, although for a more robust comparison both solutions should be buffered at pH 5.5 and the root volumes should be similar at the time of the depletion experiment.

When the kinetics of seed-grown Tithonia plants were measured with the solution being buffered at pH 5.5, K_m decreased and V_{max} increased, resulting in a doubling in the value for α, relative to measurement in an un-buffered solution. P is primarily taken up as $H_2PO_4^-$, but above pH 6 the proportion of P in the monovalent form decreases rapidly. A $H_2PO_4^-$ transporter has been invoked to account for uptake at higher pH where P concentrations are low (Bieleski, 1973) and the observed fluctuations in uptake where pH was not regulated could be indicative of more than one transporter.

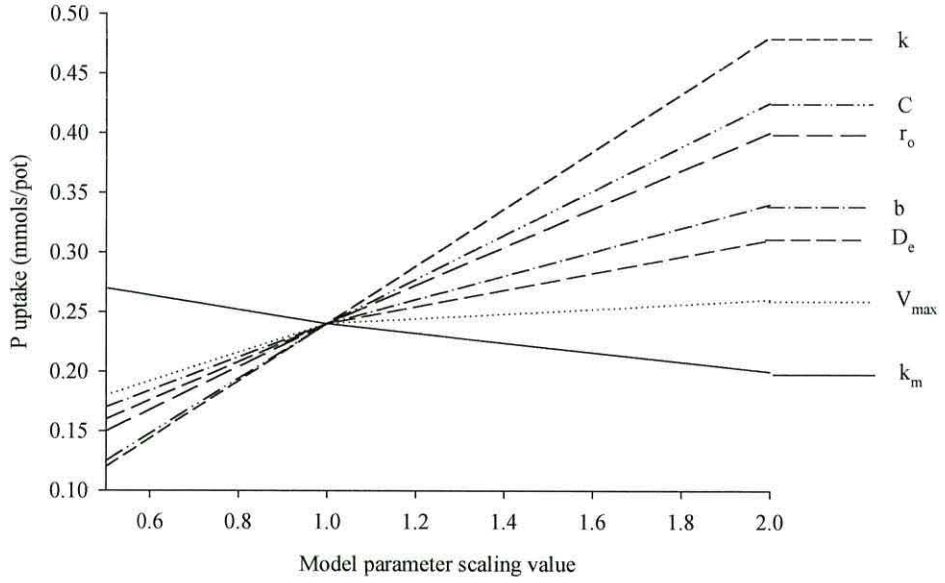
In this context, it is worth noting that the justification for use of the Michaelis–Menten equation [$V = V_{max}.C/(C + K_m)$] is based on the concept of a single enzyme. Recent advances in the study of P uptake transporters have identified nine Pi transporters in *Arabidopsis* (Daram *et al.,* 1999), two of which, AtPT1 and AtPT2, have been identified as high affinity transporters expressed in roots as well as other parts of the plant (Karthikeyan *et al.,* 2002). Comparisons of the kinetics of P uptake transporters between and within species may therefore not be realistic in whole plant studies. Regulation of P uptake transporters is a response to the internal P concentration (Clarkson and Scattergood, 1982; Muchhal and Raghotharma, 1999), and the internal concentrations that upregulate increased root uptake are presumably variable both between species and with ontogeny.

The whole plant depletion method provides an estimation of transport kinetics averaged over the entire area of the root. It is not therefore able to take account of root senescence and other root surfaces not involved in active uptake. For this and other reasons it has been criticised by Tinker and Nye (2000). They argue that when uptake data fits a hyperbola it is an artefact of controlled conditions and fast growing plants with relatively even-aged tissues. The plant P acquisition system is carefully regulated to maintain adequate internal P under the prevailing external P conditions and the depletion method involves a rapid decrease in the external P concentration. This means that there is no balance achieved between the internal and external P concentration.

Robinson (1994) found that an unstirred layer of about 100 μm surrounds individual roots even in a well-stirred solution. This results in a difference between the measured solution concentration and that at the surface of the root, so leading to overestimation of K_m. In this study, a single aeration line was used to stir the solution and some variation in the effectiveness of stirring would have occurred due differences in the size of root systems. To overcome these problems Jungk *et al.* (1990) used continuous flow cultures, in which plants were brought to equilibrium with a range of different solution P concentrations within the range experienced by plants in soils. The K_m values so determined were two orders of magnitude lower than those reported by Barber and observed for Tithonia and Sesbania in the current study. V_{max} was also lower in the same study.

That these differences in determined P uptake parameters have not resulted in problems with mathematical P uptake models is explained by Silberbush and Barber (1983), who made a sensitivity analysis of uptake parameters for soybeans grown in a silt soil (*Figure 7*). Change in uptake kinetic parameters between species was found to be only marginally responsible for the variation in actual P uptake from an homogenous soil. Soil P characteristics and root growth were the determining factors, with root absorptive area being a far more important variable than the number and affinity of P uptake transporters.

Figure 7. A sensitivity analysis showing the impact of independently changing model parameters on the uptake of P by soybeans growing in a silt-loam soil. The parameters are defined as follows; k = rate of root growth, C = initial soil P concentration, r_o = root radius, b = buffer capacity and D_e = the diffusion coefficient. The x-axis scaling factor describes the change in the model output arising from changing one of the parameters from its original value (i.e. 1.0) to either half its original value or to double its value (i.e. 2.0). After Silberbush and Barber (1983).



This does not take account of plant adaptation to the heterogeneous distribution of P in soil, reflected both in root architectural responses (Lynch and Brown, 2001) and the high concentration of Pi transporters in epidermal cells near the root apex (Liu *et al.*, 1999). Regulation of lateral root and root hair initiation appears to respond to local nutrient availability when not down regulated at a whole plant level (Lynch and Brown, 2001) and Forde and Lorenzo (2001) have produced evidence pointing to the external sensing of P concentrations. Jackson *et al.* (1990) demonstrated that the number of transporters in a patch of root exposed to high P in a

P stressed plant, increases in response to high P, with higher external P resulting in an increase in localised uptake by up to 80% compared with the same area of root exposed to low P. Plants are clearly adapted to the heterogeneous distribution of P in soil and the relative unimportance of a change in K_m and V_{max} in the sensitivity analysis in *Figure 7* is dependent on the soil being homogenous. Consideration of the contribution of P uptake transporters to actual P uptake in soils needs to take account of processes in the rhizosphere.

Where P availability is low, most plant P acquisition occurs in growing roots, with the formation of root hairs behind the root apex being responsible for most uptake by roots (Smith et al., 2001), but creating a conical P depletion zone. In the P depleted zone, the P sorption equilibrium favours the release of sorbed P into solution. The extent to which this contributes to a continued re-supply of P to the root is defined by the strength of the resulting P diffusion gradient, the distance desorbed ions are from the root uptake sites, and soil barriers to diffusion (Smith, 2002). Plants can exert an influence on this process by maintenance of an effective P diffusion gradient toward the root, combined with the secretion of organic acids and bicarbonate ions that can occupy P sorption sites in the P depleted zone. This effectively reduces the P buffer capacity of the rhizosphere soil. Within such a rhizosphere model, a change in the P uptake kinetic parameters could be expected to be of greater significance.

Where AM symbiosis is invoked, it is becoming apparent that there are further problems inhibiting the utilisation of kinetic parameters in predictive P uptake models. In a recent study, Burleigh *et al.* (2002) showed that there is altered regulation of the genes involved in the synthesis of plant P transporters in response to mycorrhizal infection. The implication is that P uptake kinetics are not simply under the influence of the relationship between internal and external P concentrations, but are variable in response to the functionality of AM associations and that this variability is itself variable between species.

In conclusion, the data produced in this experiment gives no indication that the inorganic P uptake system in Tithonia is effectively different from the crop species investigated by the same methods. There are, however, justifiable concerns about the limitations of the solution depletion method for determining P uptake kinetic parameters. There are also strong arguments that we should be interested primarily in the transporters in the growing roots of plants under realistic conditions. Such

measurements are technically challenging and may best be achieved using reporter-genes where the kinetics of the individual transporters expressed has been previously evaluated. This would enable the role of P uptake transporters to be considered in the context of integrated plant P acquisition strategies.

Chapter Three

The Significance of Secondary Metabolites of *Tithonia diversifolia*: Allelopathic effects and possible influence on soil ecology and soil physical properties influencing the phyto-availability of phosphorus

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Summary
There has been much recent interest in the potential of Mexican sunflower, *Tithonia diversifolia* (Tithonia) as a green manure to enhance phosphate cycling in P fixing soils. However there are also reports that secondary metabolites from Tithonia can have a negative allelopathic effect on plant growth. The effects of the green manure species, Tithonia and *Sesbania acluleata* on the growth of *Zea mays* and *Triticum aestivum* seedlings were investigated in bio-assays. Tithonia shoots altered the growth of maize relative to controls. These results are considered in the context of other possible impacts Tithonia residues may be having on the ecology and chemistry of soils.

Key words: Allelopathy, Maize, Mycorrhiza, Phosphorus, Sesbania, Tithonia, Wheat.

1. Introduction

Parallel to current research into the potential of using Tithonia diversifolia (Tithonia) as a green manure to enhance phosphate cycling in P fixing soils in the tropics is a broad effort to understand the medicinal and other uses of secondary metabolites produced by the species. It is thought that the plant's role in traditional medicine was important in hastening Tithonia's distribution around the globe. The range of ailments reportedly treated by Tithonia is large and includes cancer (Mungarulire 1993; Gu *et al.*, 2002), HIV (Cos *et al.*, 2002), hepatitis (Kuo and Chen, 1997), inflammatory disorders (Lin *et al.*, 1993; Rungeler *et al.*, 1998), liver complaints (Lin *et al.*, 1993), diarrhoea (Tona *et al.*, 1999; Tona *et al.*, 2000), amoebic infections (Tona *et al.*, 2000), chickenpox (Lamaty *et al.*, 1991) and malaria (Lamaty *et al.*, 1991).

In addition, there has been interest in the potential of employing Tithonia as an insect anti-feedant (Hongsbhanich *et al.*, 1979; Dutta *et al.*, 1986; Sarma *et al.*, 1987; Dutta *et al.*, 1993; Adoyo *et al.*, 1997) and in the control of soil nematodes (Tiyagi *et al.*, 1985; Nisar *et al.*, 1989; Tiyagi and Wani, 1992).

The search for the active ingredients of Tithonia responsible for the reported medicinal benefits and insect and nematode control has focused on constituents of the

essential oils extracted from the leaves, flowers and roots. Lamaty *et al.* (1991) studied the essential oils of Tithonia and found 20 terpenoid substances, of which 87.4% were monoterpene hydrocarbons and 8% were sesquiterpene hydrocarbons, with (Z)-beta-ocimene constituting 40% of the terpene fraction. The sesquiterpene lactones tagitinin A and tagitinin C and a flavonoid, hispidulin, are considered by most investigators to be the active chemicals (Sarma *et al.,* 1987; Rungeler *et al.,* 1998; Gu *et al.,* 2002). Dutta *et al.* (1986) suggest that the same substances are responsible for the insect anti-feedent effects, whilst Sarma *et al.* (1987) focused on tagitinin A. The sesquiterpene lactones and flavonoids from Tithonia are also thought to be allelochemicals, the effects of which have been the subject of a number of studies (Baruah *et al.,* 1994; Tongma *et al.,* 1998).

Tagitinin A and tagitinin C appear not to have an allelopathic effect on radish, cucumber or onion at concentrations of less than 250 μM, but at a similar concentration, hispidulin inhibited their germination and growth (Baruah *et al.,* 1994). Tongma *et al.* (1998) found that rice and sorghum grown in soil with Tithonia as the previous crop showed a suppression of root length of between 40-50%. Reports of yield improvement following applications of Tithonia green manure contradict the agricultural importance of these findings. The issue of potential allelopathic effects deserves consideration in the light of the recommendation of Jama *et al.* (2000) that Tithonia may be best utilised in the production of high value horticultural crops.

In the Chitwan valley of Nepal, an area of recovered lowland marsh with high agricultural potential, yields of paddy rice, wheat and maize grown in annual rotations have declined after twenty years of continuous cultivation with urea as the principal fertilizer (Sherchan, 2001). Analysis of the soil has shown low available K, Ca, Mg, Zn and P, low organic matter, and a low CEC. Yields improved with additions of a range of organic and inorganic fertilisers, with Tithonia producing yields comparable or better than animal manure, *Sesbania aculeata*, or NPK fertiliser (Sherchan, 2001). It was, however, noted that early growth of maize with Tithonia manure was slower than with other fertilisers in the field (Sherchan, 2001).

An experiment was therefore conducted under controlled conditions to confirm the reported allelopathic effects of Tithonia, using treatment methods similar to Tongma *et al.* (1998), but at rates of Tithonia application that are in keeping with

applications of green manures by smallholders. An alternative green manure, the N_2 fixing legume, *Sesbania aculeata* (Wild.) Pers., was included in the assay as a control.

2. Materials and Methods

2.1. Soil

Soil was obtained from the Ap horizon (0-20 cm) of a Eutric fluvisol located in the Chitwan Valley in Bharatpur, Nepal (27°36' N, 84°22' E; altitude 187 m; rainfall of 2500 mm $year^{-1}$) in which *Zea mays* L. and wetland *Oryza sativa* L. had been previously grown in rotation. The soil is a poorly structured sandy loam with a $pH_{(H_2O)}$ of 5.48, total organic C content of 0.51%, total N content of 0.03%, C-to-N ratio of 17 and electrical conductivity (H_2O; 1:1 v/v) of 0.13 mS cm^{-1}. The soil has an anion exchange resin extractable P of 0.12 mmol kg^{-1}, 0.58 mmol kg^{-1} $NaHCO_3$ extractable P and a total P content of 6.5 mmol kg^{-1}. The exchangeable cation content (extracted with 1 M NH_4Cl) in units of $mmol_c$ kg^{-1} was: K, 24; Na, 4; Ca, 84; Mg, 60. After collection, the soil was sieved to pass 5 mm and stored at field moisture levels at 15°C until required for experimentation.

2.2.Pretreatment of soil

Seeds of a South African ecotype of *Tithonia diversifolia* were soaked in aerated distilled water overnight and then transferred to petri-dishes containing moist filter paper for 6 d at 20°C. Two plantlets with fully expanded cotyledons and a main root axis approximately 5 cm in length were then transferred to 5-l pots containing Chitwan soil at a bulk density of 1.3 g cm^{-3}. Soil moisture was brought to 20 % by volume with the addition of dilute Long Ashton nutrient solution (Hewitt, 1966). Seeds of a Nepalese ecotype of *Sesbania aculeata* (Wild.) Pers were germinated by the same method and transferred to similar pots. The plants were placed in a climate-controlled growth room with 22/18°C day/night temperatures, 16-h photoperiod and a light intensity of 350 µmol m^{-2} s^{-1} (PAR). After 10 weeks, when plants of both species had attained a height of about 1 m, and when roots had explored the soil extensively but had not become 'pot bound', plants were destructively harvested. All roots were removed from the soil by hand and the soil retained for the allelopathy assay.

2.3 Preparation of root and shoot material and aqueous extracts

The recovered root and shoot material was dried at 60°C (24 h). 5 g (dry weight) of Tithonia and Sesbania leaves were ground to a fine powder in a coffee grinder (to < 0.5mm diameter), placed in 500 ml distilled water and shaken on a rotary shaker for 16 h at 150 rpm. The aqueous extracts were obtained by sequential filtrations to remove the larger particles followed by vacuum filtration with a Whatman 541 filter paper. Extracts were stored at 4°C.

2.4. Germination test

10 maize (*Zea mays*) seeds and 10 wheat (*Triticum aestivum* cv. Abbott) seeds were placed in separate petri-dishes lined with absorbent paper. The paper was saturated with aqueous extracts of Tithonia and Sesbania, prepared as described above. The lids of the petri-dishes were sealed to avoid evaporation and placed in a temperature controlled cabinet at 22°C for 5 days.

2.5. Allelopathy assay treatments

200 g dry weight of field moist Chitwan soil was treated as described in *Table 1,* packed into 6.5-cm-diameter pots at a bulk density of about 1.3 g cm^{-3}, and brought to 20% moisture content. Tithonia and Sesbania leaves and soft stems, grown in the soil used in the experiment, were cut into 1-cm^2 sections and mixed into the soil. Seeds of maize (*Zea mays*) and wheat (*Triticum aestivum* cv. Abbott) were imbibed overnight and pre-germinated on moist paper. When the seedlings had roots 1–1.5 cm in length (4 d after sowing), they were transplanted into the prepared soil in pots. Four seedlings were placed in each pot and there were three replicate pots for each treatment. Plants were grown in a growth room with 22/18°C day/night temperatures, 16-h photoperiod and a light intensity of 350 μmol m^{-2} s^{-1} for 10 d. Soil moisture was maintained at about 20% with the addition of distilled H_2O. The individual pots were placed at random on a defined surface within the growth room (using random numbers to allocate the initial position) and rotated within the defined surface to reduce positional effects.

Table 1. Treatments applied to 200 g dry weight soil in allelopathy assay.

Treatment	Abbreviation	Soil used	Long Ashton solution (ml)	Aqueous extract (ml)	Shoot /root addition (g wet wt)
Control	C	Untreated	40	0	0
Tithonia extract	TE	Untreated	0	40	0
Sesbania extracts	SE	Untreated	0	40	0
Tithonia soil + shoots	TS S	Tithonia	0	0	2
Sesbania soil + shoots	SS S	Sesbania	0	0	2
Tithonia soil + roots	TS R	Tithonia	0	0	1
Sesbania soil + roots	SS R	Sesbania	0	0	1
Tithonia soil	TS	Tithonia	0	0	0
Sesbania soil	SS	Sesbania	0	0	0

2.6. Harvest procedure

Plants were destructively harvested after 10 d of growth in the treated soils. The total area and length of leaves were measured with a digital leaf scanner prior to leaves and roots being dried at 60°C (24 h). The dry weight of roots and leaves was recorded. Leaf data for individual plants was collected, but roots were treated as roots per pot

2.7. Statistical Analysis

Analysis of variance followed by Tukey's test was used for multiple comparisons between the means where the ANOVA F statistic was significant (Zar 1984).

3. Results

There was a 100% germination of wheat and maize germinated in both the Sesbania and Tithonia extracts. Mean data for growth parameters for maize are presented in *Figures 1* and *2*, and for wheat in *Figure 4*. To determine allelopathic effects of Tithonia on the growth of maize and wheat, multiple comparisons were made between the Tithonia and other treatments for dry weight of the roots and shoots and for leaf measurements including specific leaf area.

Both differences in available mineral nutrients and allelopathic effects are possible sources of variation between treatments. It is assumed that the available minerals in each treatment pair (e.g. Tithonia shoot or Sesbania shoot, Tithonia root or Sesbania root) are sufficiently similar to not cause significant differences. Furthermore it is assumed that neither Long Ashton nor Sesbania have allelopathic effects on maize or wheat.

3.1. Maize

Figure 1. The effect of treatments on (A) the height of tallest leaf, (B) total dry weight of leaves per plant, (C) dry weight roots per plant and (D) the calculated root: shoot ratio. Letters indicate significant differences between treatments ($P < 0.05$). Values represent means ± SEM. [$n = 3$,]. For a detailed explanation of the treatment symbols see *Table 1*.

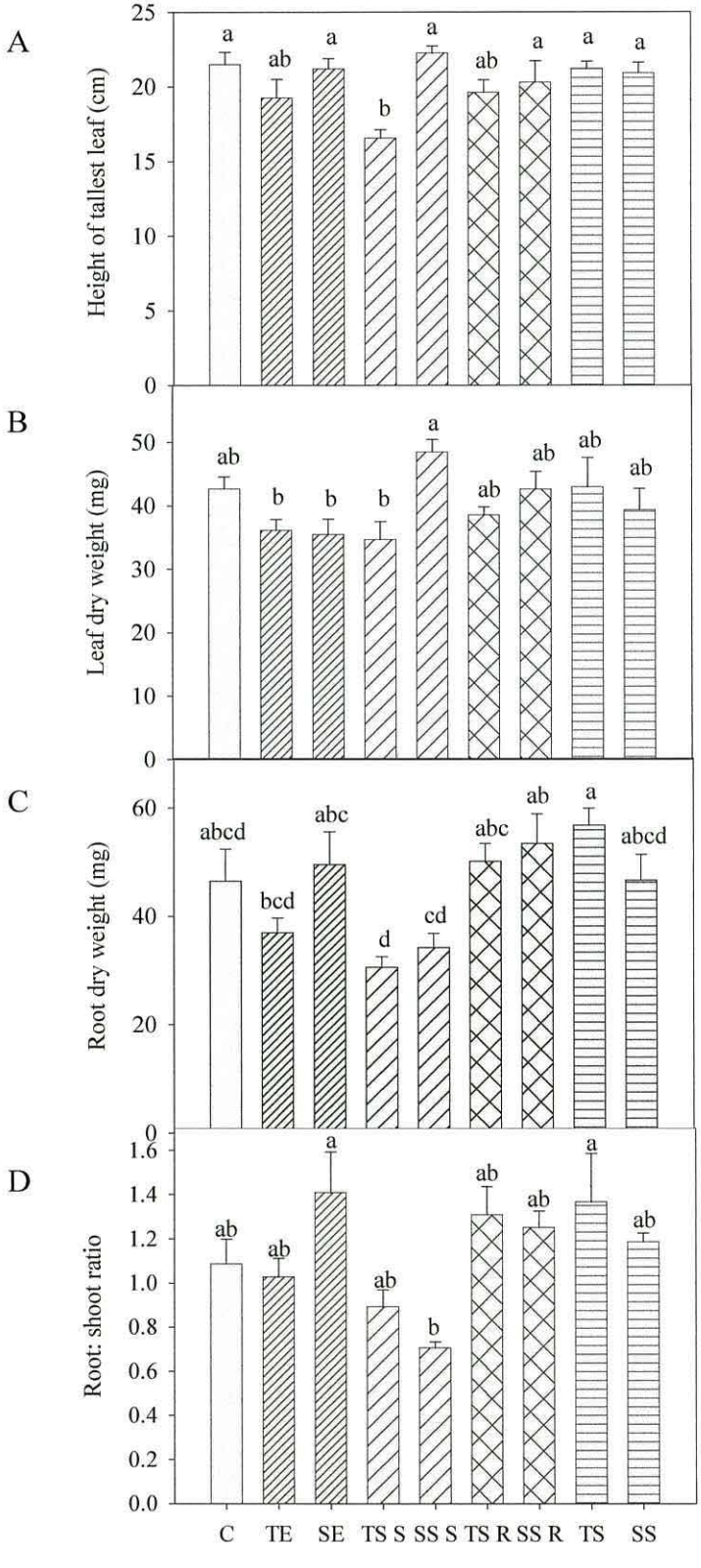
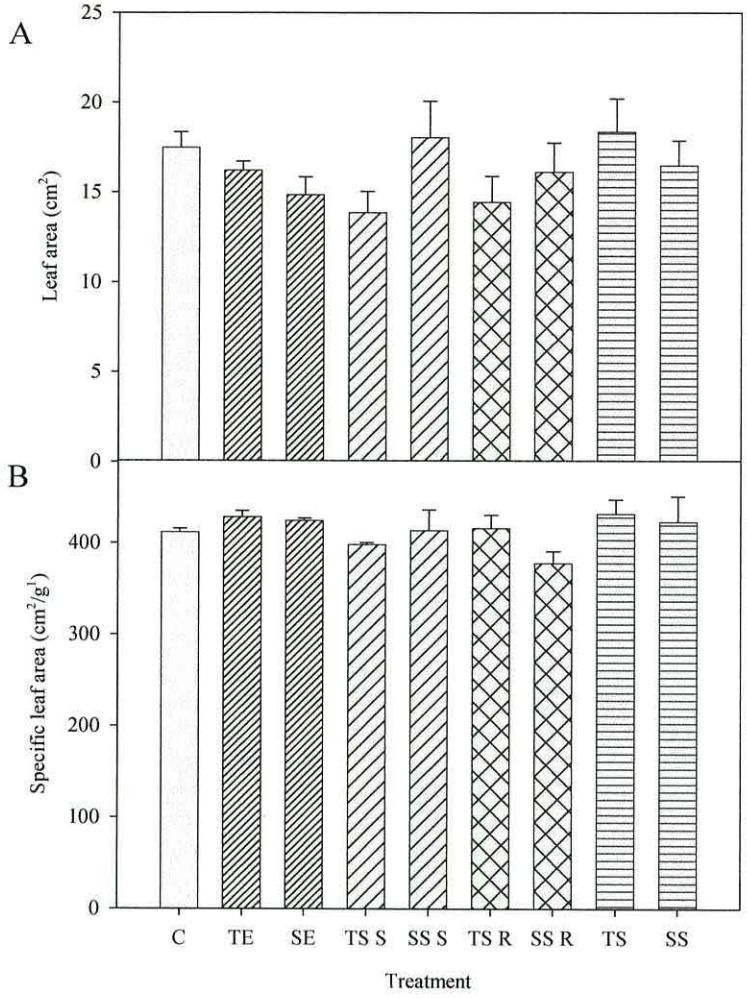


Figure 2. The effect of treatments on (A) leaf area and (B) specific leaf area. There were no significant differences between treatments ($P > 0.05$). Values represent means ± SEM. [$n = 3$,]. For a detailed explanation of the treatment symbols see *Table 1*



All plants had three leaves present at the time of harvest and all shoot material was divisible into separate leaves for measurement purposes. With a harvest at only one time point, the height of the plants is essentially for descriptive purposes only (*Figure 1A*). Maize grown with Tithonia shoots was shorter, but with wider leaves than other treatments. There were no other visible differences between the plants.

Maize plants grown in soil in which either Tithonia or Sesbania had been grown previously did not differ from the control for all parameters, although presumably having a lower mineral nutritional status. This suggests that the previous crop did not have a negative effect on the growth of maize and that mineral nutrition was not a limiting factor for the growth of maize.

With the exception of the addition of shoot martial to the soil there were no differences between the paired treatments for any of the parameter measured or the

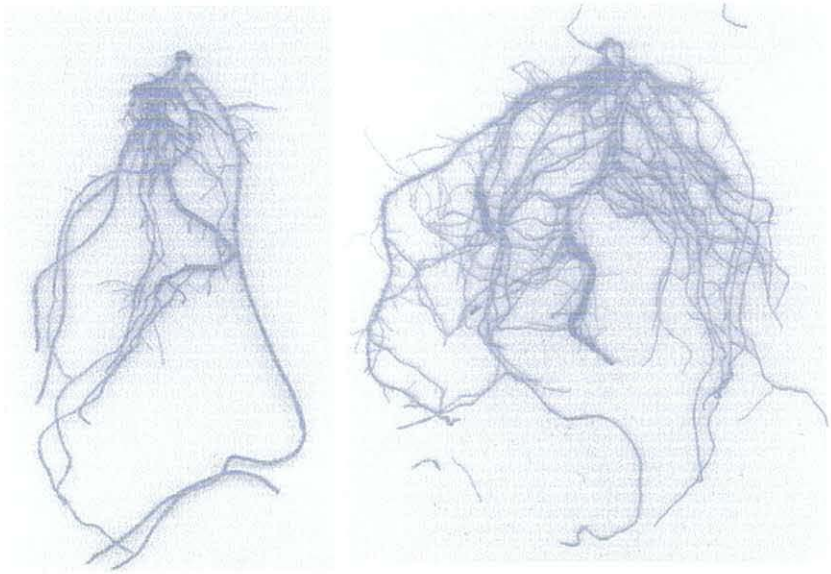
calculated ratios describing physiological characteristics relating to plant growth. The dry weight of maize plants grown with Tithonia shoots was significantly lower ($P<0.05$) than for those grown with Sesbania shoots (*Figure 1B*). This appears to due to both the shoot dry weight of the plants receiving the Sesbania shoot treatment being greater than with other treatments and the shoot dry weight of plants receiving the Tithonia shoot treatment being lower. The root systems of maize plants with these treatments had a similar dry weight, resulting in a similar root: shoot ratio. Visually, maize plants growing in Tithonia soil with Tithonia shoots appeared smaller, but healthier than plants in other treatments. The scanned images of roots of a maize plant grown in soil containing Tithonia shoots and of a plant grown in the fertilised control soil (*Figure 3*) provide qualitative evidence that the Tithonia shoot treatment was influencing the growth of lateral roots. The root system of plants with the Tithonia shoot treatment appear smaller, but also lack the extensive lateral roots evident in the control.

Maize plants treated with the liquid extracts of Tithonia and Sesbania did not differ significantly ($P > 0.05$) from the fertilised control although they appeared to have a lower shoot dry weight and the Sesbania extract resulted in a high root-to-shoot ratio.

Sesbania or Tithonia roots incorporated into the soil did not affect the growth of maize relative to other treatments.

There was a high degree of variability in the leaf area of maize plants within treatments and no significant difference in the specific leaf area was found (*Figure 2*).

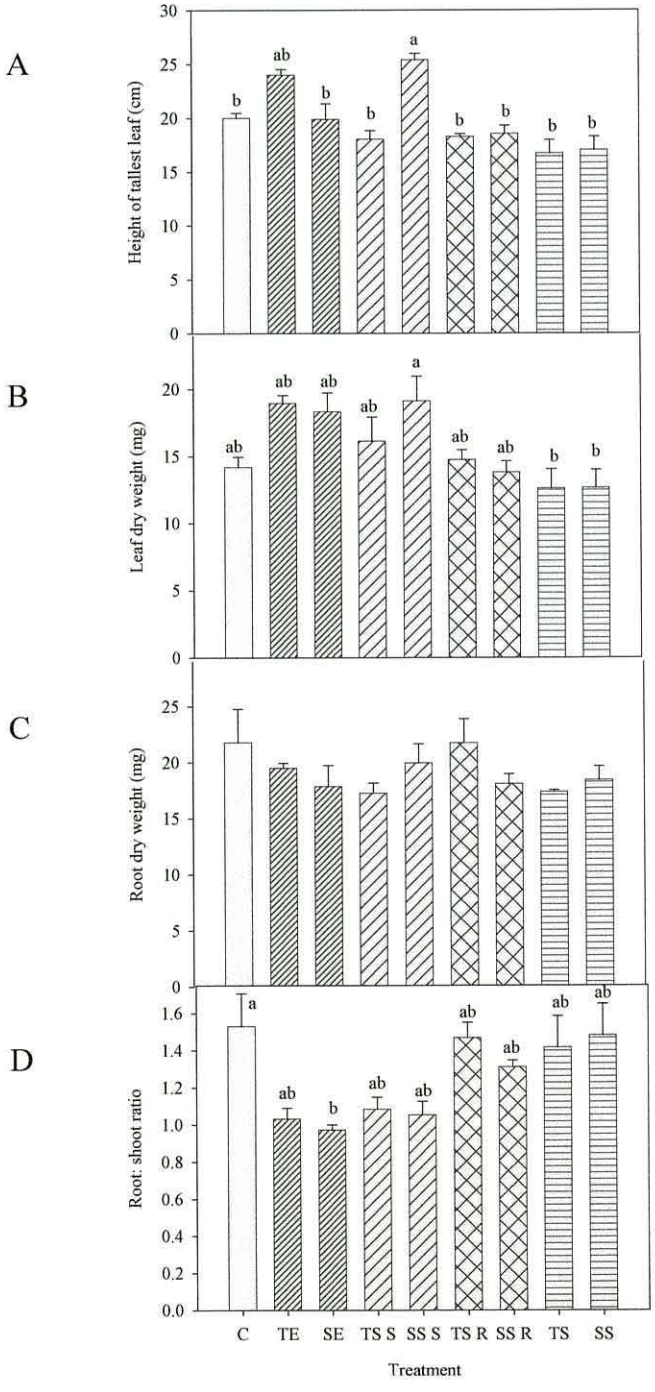
Figure 3. Photographs of maize roots after 10 days growth in a soil containing Tithonia shoots (left panel) and fertilised with half strength Long Ashton growth solution (right panel)



3.2. Wheat

The growth of wheat at the level of light provided was restricted, resulting in the development of very narrow leaves that could not be reliably measured by the equipment used. It was not therefore possible to calculate leaf area and specific leaf area.

Figure 4. The effect of treatments on (A) the height of tallest leaf, (B) total dry weight of leaves per plant, (C) dry weight roots per plant and (D) the calculated root: shoot ratio. Letters indicate significant differences between treatments ($P < 0.05$). Values represent means ± SEM. [$n = 3$,]. For a detailed explanation of the treatment symbols see Table 1.



Wheat grown in soil where either Tithonia or Sesbania had been grown previously had a lower leaf dry weight than where Sesbania shoots were incorporated into soil in which Sesbania had been grown previously (*Figure 4B*). The tallest wheat leaves were also recorded where the Sesbania shoot treatment was applied (*Figure 4A*). There were no significant differences observable between treatments for the root dry weight ($P > 0.05$).

The root: shoot ratio of the wheat plants grown with Sesbania extract was significantly lower ($P < 0.05$) than the control soil with mineral fertiliser addition, while the leaf dry weights were comparable.

There were no significant differences in the growth of wheat plants grown in soils with Tithonia and Sesbania extracts for the parameters measured. Sesbania shoot additions resulted in plants with a taller leaf than where Tithonia shoots had been added to the soil, but the differences in root and shoot dry weight were not significant. The addition of root material from Tithonia and Sesbania to the soil did not result in differences in the growth of wheat relative to either no addition to the soil or the addition of leaf extracts.

Mineral nutrition effects on the growth of wheat appeared to be greater than on the growth of maize in these experiments. Leaf dry weight and the root: shoot ratio of wheat plants grown in soil with either Tithonia or Sesbania extracts or shoot material had a trend toward larger shoots and smaller relative root systems than in other treatments where the added available nutrients could be expected to be lower.

Discussion

The limited literature on the allelopathic effects of Tithonia has been primarily concerned with identifying new phyto-toxic chemicals for use in weed control (e.g. Baruah *et al.*, 1994; Tongma *et al.*, 1998). Such studies have therefore used concentrations of Tithonia-derived substances in excess of those likely to be encountered in green manure applications.

The experiments reported here were modelled on the work of Tongma *et al.* (1998) who germinated seeds and grew seedlings in soil from the Tithonia rhizosphere, with aqueous extracts of Tithonia shoots, or with aqueous extracts of soil from the Tithonia rhizosphere. Tongma *et al.* (1998) used a Tithonia aqueous extract at the same concentration as used here and reported depressions in root growth of radish, rice and sorghum similar to the observed growth depression in maize grown

with Tithonia shoots. At this concentration (10 mg ml^{-1}) both Tithonia and Sesbania aqueous extracts had a fertiliser effect on both wheat and maize and there was no detrimental effect on germination of either species.

Tongma *et al.* (1998) also observed growth depression in plants grown in Tithonia rhizosphere soil, but this work was not representative of field conditions as the Tithonia plants had been grown in 3.4-l pots for six months and would have become severely pot bound. This is supported by the results reported here, with little difference between the fertilised control and the treatments with no additions to soils in which Tithonia and Sesbania had grown previously. The addition of roots appeared to have no influence and both treatments, either with Tithonia soil and roots or Sesbania soil and roots, showed a higher root-to-shoot ratio, indicative of P or N deficiency, compared with more effectively fertilised treatments.

In the this study, the shoot and root additions were designed to reflect the concentrations of Tithonia experienced by crop plants growing either following Tithonia cultivation or receiving Tithonia residues as fertiliser. The treatments where shoot material was added could be expected to have a lower root: shoot ratio due to the nutrient rich hotspot this material creates in the soil, reducing the necessity for root exploration of the soil and this is reflected in the results. This explanation however would suggest that maize plants growing with added Tithonia shoots were not nutritionally deficient. Where the Tithonia aqueous extract was added to the soil there also an indication of depressed growth although the difference relative to the Sesbania extract treatment was not significant ($P < 0.05$).

Only maize growing with Tithonia shoots showed a response that could be considered indicative of allelopathy. Tithonia applied as a green manure has been demonstrated to be an effective fertiliser for maize (Jama *et al.*, 2000; Sherchan, 2001), however an initial slow growth of maize when Tithonia residues had been applied, relative to a range of other organic and inorganic fertilisers, has been reported (Sherchan, 2001). The current study had a single harvest ten days after the transfer of seedlings to soil. While it would appear that Tithonia is affecting the growth of young maize plants, the nature of the affect is unclear. Though smaller, the Tithonia shoot treated maize plants were visibly the most healthy and strong. Allelopathy includes any direct or indirect beneficial or harmful effect of one plant on the other through the release of chemicals into the environment (Inderjit, 1996) and in this instance it is unclear if the effect is beneficial or harmful.

Baruah et al. (1994) gave consideration to the nature of the active chemicals from Tithonia involved in negative effects on growth and describe a range of sesquiterpene lactones, characterised by hydroxyl groups that these authors consider to be playing an active role. They suggest that α-methylene and γ-lactone moieties may serve as biological nucleophiles and disrupt root function. If this were the effect of Tithonia on the maize plants in this experiment, it would have been expected that the root-to-shoot ratio would have been high as resources were allocated to root growth. Furthermore, it would have been expected that the Tithonia leaf extracts would have exhibited a similar effect on maize as the shoot material, as sesquiterpene lactones are in the soluble extracts (Baruah et al. 1994). It is possible though that drying Tithonia residues at 60°C may have resulted in the volatilisation of secondary metabolites during the preparation of the aqueous extracts, reducing the effect of this treatment on the maize plants.

At the present time Tithonia is the subject of research in four unrelated contexts: as a green manure to enhance P cycles, as a source of phyto-medicines, as a source of allelo-chemicals and as a source of natural substances for the control of insects, nematodes and small arthropods. It is beyond the scope of the research reported here to attempt to establish the possible significance of the work on the secondary metabolites to the effects of Tithonia on P cycles. The reported literature does, however give some insight into areas that warrant further investigation.

Sesquiterpene lactones are strongly negatively charged with exposed O^- and OH^- surfaces and therefore may be capable of acting as chelating agents. In which case their direct negative impact on plant growth would be minimal, especially in acid soils (Inderjit, 1996). Picman (1987) reported that sesquiterpene lactones persist in soil for 90 days. Rice (1979) cites research from Yugoslavia pointing to an increase in P availability when similar chemicals were added to soil. Further work in this area would be justified.

Tithonia leaf flavonoids may be increasing the growth and colonisation of crop roots by arbuscular mycorrhiza (AM) fungi. Root-exuded flavonoids are thought to play an important role as allelo-chemical signals in the mycorrhizal symbiotic relationship (Smith and Read, 1997; Vierheilig *et al.*, 1998). Despite the importance of AM symbiosis in P uptake from less fertile soils, little work has been undertaken to understand the impact of organic additions on rhizosphere ecology and the establishment of AM symbiosis. However, one possible mechanism to account for the

allelopathic effect of Tithonia shoots on young maize plants would be that the Tithonia stimulated the germination of AM soil spores resulting in the infection of maize roots. The resulting increased allocation of photosynthate to fungal rather than plant growth would explain the slow initial growth of maize. This explanation would also be compatible with the smaller root system (*Figure 1*) and with the observable health of the plants. In a separate experiment (see *Chapter 7*) to confirm such a stimulatory effect of Tithonia residues on AM fungal activity associated with wheat no effects were observed. Further work with maize may nevertheless prove fruitful.

The secondary metabolites in Tithonia leaves are a pronounced feature of the species and are probably associated with the low investment in structural carbon resulting in little structural protection from either predation or damaging UV-B radiation. The flavonoid hispidulin is found in the leaves of Tithonia (Dutta *et al.*, 1993) and probably is involved in protection from UV-B radiation (Caldwell *et al.*, 1983). As well as providing possible UV-B protection, hispidulin, along with the sesquiterpene lactones also present, has been shown to be an insect anti-feedant (Dutta *et al.*, 1986; Dutta *et al.*, 1993), with nematocidal properties (Tiyagi *et al.* 1985; Tiyagi *et al.* 1992). If flavonoids in Tithonia residues are stimulating AM colonisation, a further associated beneficial on crop P uptake should be considered

The insect anti-feedant and nematocidal effects of Tithonia may affect both AM fungal activity and mineralisation processes through a regulatory effect on soil ecological processes: The decomposition of organic matter in soil is primarily performed by bacteria and fungi (particularly actinomycetes), with fungi being the dominant decomposers in acid soils (Killham, 1994). Both fungi and bacteria are susceptible to predation by protozoa (Killham, 1994), and microbe-feeding nematodes graze bacteria, fungal hyphae and protozoa (Ingham, 1985; Killham, 1994). Carnivorous mites prey on nematodes and protozoa (Osler, 2000), with mites and other arthropods including springtails (*Collembola*) grazing on both saprophytic fungi (Killham, 1994) and mycorrhizas (Larsen and Jakobsen, 1996).

The increase in microbial populations following organic additions to soils results in a rapid increase in mesofaunal populations, especially micro-arthropods and fungi-, protozoa- and bacteria-feeding nematodes. This delays net mineralisation and creates a difficult environment for the establishment of AM symbioses with crop plants. However, at low levels of predation of decomposer species and mycorrhizas, bacterial and fungal activity is stimulated (Ingham, 1985; Larsen and Jakobsen, 1996;

Osler, 2000). Low-level predation increases the proportion of bacteria in log phase through increased rates of N and P cycling (Andersen *et al.,* 1981) and compensatory growth of both fungi and bacteria (Lussenhop, 1993). If it were established that Tithonia residues effectively control the rise in nematode and micro-arthropod populations when applied to soil, compared with alternative residues, it would alter our understanding of the qualities that may be desirable in a green manure to enhance P cycles.

Results from some recent studies suggest that there is an increase in P availability when plant residues with a narrow soluble carbon-to-P ratio are incorporated into soil (Nziguheba *et al.,* 2000; Kwabiah *et al.,* 2001). This conclusion has been based on field studies using residues from a range of species, including Tithonia. The hypothesised role of secondary metabolites on the availability of P from residues would suggest a reinterpretation of this observation, as a narrow soluble carbon-to-P ratio is associated with low structural carbon in fast growing species. If, as with Tithonia that necessitates the production of secondary metabolites and these subsequently affect mineralisation processes, the soluble carbon-to-P ratio may be a coincidental factor.

In conclusion, although Tithonia shoots may decrease growth of maize seedlings, the negative effects are probably short lived and do not appear to undermine the health of the plants, and may be associated with enhanced fitness. Wheat plants were unaffected by Tithonia allelo-chemicals and Sesbania had no allelopathic effects on maize or wheat. The secondary metabolites of Tithonia warrant further investigation in the context of enhanced P cycling. Further work in this area may provide interesting and possibly important insights into the management of soil ecosystems.

Chapter Four

Phosphorus uptake, partitioning and retranslocation by *Tithonia diversifolia*

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Summary
Tithonia diversifolia (Tithonia) has been identified as a species with high potential as a green manure to enhance phosphorus (P) cycling in high P fixing, acid tropical soils. This is supported by studies of Tithonia's decomposition characteristics, agronomic trials and a C-to-P ratio compatible with net mineralisation of P from Tithonia. The relatively high P concentrations in Tithonia shoots can be explained as either due to high uptake from soil or by concentration of P in green tissues. Using radiolabelled P tracers in Tithonia plants grown in hydroponic solution, the retranslocation of P from older to younger shoot tissues was shown to occur with luxury levels of tissue P. The shoot tissue P concentrations of plants grown in soil significantly exceeded normal physiological requirements. Tithonia conserves P in the most photosynthetically active tissues by re-translocating P from rapidly senescing lower leaves, but that cellular P concentrations do not exceed the P storage capacity for Pi.

1. Introduction

Phosphorus (P) is an essential element for the growth and metabolism of all life. The physical qualities of P that contribute to its role in biochemistry are responsible for its sparing availability in soil. Under atmospheric conditions, P is oxidised forming the tetrahedral centre for strongly associated oxygen atoms as phosphate, PO_4^{3-} (Pi). With the phosphoric acid dissociation constants (pk_a) at 2.1 and 7.2 (dissociating from H_3PO_4, to $H_2PO_4^-$, to HPO_4^{2-}), phosphate is always ionised at physiological pH. The physical structure of the molecule provides phosphate bonds with high kinetic stability under physiological conditions. This is significant in cellular chemistry as Pi is able to remain ionised while forming diesters including DNA and RNA, molecules that need to be very kinetically stable and anhydrides, such as adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP), that are required to be thermally unstable and therefore hydrolysable by enzymes. As phosphate is trivalent, these diesters remain ionised and are therefore contained in cells as salts that cannot pass through cell wall lipids (Westheimer, 1987). The charge chemistry of phosphates is a critical feature in their role in a range of other organic molecules including phospholipids, phosphate esters, such as phenol esters, and metabolic pathway intermediates such as glucose-6-phosphate. Reflecting the importance of P in energy metabolism and structural

integrity, P is involved in enzyme regulation, metabolic intermediates and as a component in signal transduction cascades (Rausch and Bucher, 2002).

Perhaps inevitably, the same energy characteristics of the P atom also describe the stability of Pi in soil and its accessibility to plants, with the formation of strong bonds to organic and inorganic substances in the soil. In forest and temperate soils with high organic matter contents, between 30% and 80% of soil P may be bound in organic molecules of different complexity (Dalal, 1977). In many tropical agricultural soils, however, immobilisation of P is more commonly associated with the formation of complexes with iron and aluminium oxides and hydroxides, particularly under leached and acid conditions (Buol *et al.,* 1980).

Although plants have evolved a wide range of mechanisms to enhance P uptake from soils with low available P, P deficiency still represents a major constraint to agricultural production in many regions of the world. The increases in agricultural production that have facilitated the rapid expansion of human population over the last century have been as dependent on the exploitation of phosphate from high quality rock phosphate reserves to soils as on any other agricultural innovation. The global distribution of phosphate fertiliser has however, been socially and geographically inequitable (Fairhurst *et al.,* 1999), and P remains a particular constraint to the productivity of small-scale farmers in much of sub-Saharan Africa and South Asia (Sanchez *et al.,* 1997). Adding to the problem, modern cereal varieties transfer a greater proportion of plant P to the grain than wild relatives. This can lead to up to one third of P taken up by crops being effectively lost from farm systems as farmers sell their produce into urban markets (Sanchez et al., 1997).

In the mid hills of Nepal, P deficiency has been identified as a constraint to crop production and many of the contributing conditions are similar to those affecting resource-poor farmers throughout much of Africa and South East Asia. Increased population has undermined traditional soil management practices. De-forestation makes unfeasible the traditional practice of transferring biomass from hedges and forests to cultivated soils (Thapa, 1994). Due to poor transport infrastructure mineral fertiliser is not economically available and without organic additions the soil loses its inherent structure and so becomes more difficult to cultivate and less productive. The potential of novel species for use as a green manure is the subject of on-farm trials, with particular emphasis on Tithonia diversifolia (Sherchan, 2001).

Previous research has shown that *Tithonia diversifolia* (Tithonia) grown in managed fallows can enhance P availability to plants (Cairns *et al.,* 1998). Field experiments indicted that an application of 5 tonnes dry weight ha^{-1} of Tithonia leaf and soft stem biomass containing 14 kg P ha^{-1} P could supply the yield improvement P equivalent of 19.6 kg P ha^{-1} of triple super-phosphate fertilizer (Palm *et al.,* 1999). When grown in P deficient soils, Tithonia shoots have been found to contain 3.5 g P kg^{-1} dry weight (Cairns *et al.,* 1998).

This suggests that Tithonia may be able to access P not available to conventional crop plant species and that it accumulates P at levels higher than those required for normal physiological function. It is not known if the uptake of P by Tithonia is indicative of a species-specific P storage mechanism or if the elevated tissue P concentrations are in line with general plant physiological principles established for P partitioning, transport and storage (Marschner, 1995).

Studies of phosphate homeostasis in plant cells have shown that the P concentrations in the metabolised pools and Pi in the cytosol are remarkably constant, with Pi in the vacuole constituting a reserve supply at P concentrations up to 25 mmol L^{-1} (Mimura, 1995). Organic P compounds in plant tissues can be determined with ^{31}P NMR (Lee and Ratcliffe, 1993), but this would only be justified in the current context if the P concentrations in Tithonia tissues were in excess of normal physiological limits and the synthesis of storage compounds was invoked. In this study, we conducted controlled environment experiments, using the ^{33}P isotope dilution to observe P partitioning and transfer over time. The results of these experiments were compared to analysis of P partitioning in shoots from more mature pot grown plants. The results are discussed with reference to the literature describing the principles of P transport, utilisation and storage in plants.

2. Material and methods

2.1 Radio tracer experiment

Tithonia seed of South African origin (Natal) was sterilised in a 5% sodium hypochlorite solution and then placed in aerated distilled water in a conical flask for 12 h. The seeds were then germinated on wet paper and after six days, six germinated seedlings were transferred to 300-mL pots with aerated half strength Long Ashton nutrient solution (Hewitt, 1967) modified to provide 333 μmol L^{-1} $NaH_2{}^{33}PO_4$ initial P solution concentration, with a specific activity of 0.8 kBq ^{33}P mg^{-1} P. The root–bathing was changed every 4 days. The P concentration in the hydroponic solution culture is substantially greater than soil solution P concentrations (2-10 μM, Bieleski, 1973), but preliminary studies demonstrated that plants growing in non-continuous flow solution culture require a high concentration to avoid P deficiency. The initial activity of the ^{33}P isotope was selected on the basis of a back calculation, allowing for decay, to achieve a sample with sufficient activity as to be in the optimal range for accurate scintillation counting.

Twelve days after being placed in the ^{33}P labelled hydroponic solution, and when the plants had produced the third pair of foliage leaves, three plants were destructively harvested and divided into root, stem and leaf pairs. The harvested material was dried at 60 °C for 48 h and tissue dry weights for each plant measured. The material was then ashed in a muffle furnace at 500°C for 24 hours and the ash brought into solution with 1 mL of 1 M HCl. The resulting solution was then analysed for ^{33}P using a 1304 liquid Wallac scintillation Counter (EG&G Wallac, Milton Keynes) and 'Optiphase Hisafe 3' Scintillation fluid, (EG&G Wallac, Milton Keynes). Chemo-quench effects arising from reactions between the scintillant and the HCl were minimal when used in a 10-to-1 scintillant-to-sample ratio. The ^{33}P tissue concentrations were calculated after correction for isotope decay.

The remaining three plants were grown on in a non-radiolabelled ^{31}P hydroponic solution at the same P concentration and with the solution being changed every four days. Twenty-four days after being transferred to solution culture these plants had produced 6 pairs of proper leaves and the first leaf pair had senesced. At this stage, plants were also producing axial shoots along the stem. Destructive harvest was carried out as described above. The position and number of axial shoots were variable between plants and were therefore treated as a single tissue sample associated

with each plant. After ashing, sub-samples of the HCl-ash solution were taken for ^{31}P colorimetric analysis using the modified molybdenum blue method of Murphy and Riley (1962). The remaining solution was analysed for its ^{33}P concentration by liquid scintillation counting as described above. The amount of ^{33}P taken up during the plants first 12 days in solution was calculated for each tissue type. Total P was also calculated for each plant tissue enabling an estimation of (1) ^{33}P taken up over the first 12 days, (2) the uptake of non-labelled P between days 12 and 24 and (3) the transfer of ^{33}P between plant tissues after the labelled ^{33}P had been withdrawn.

2.2. Soil grown plants

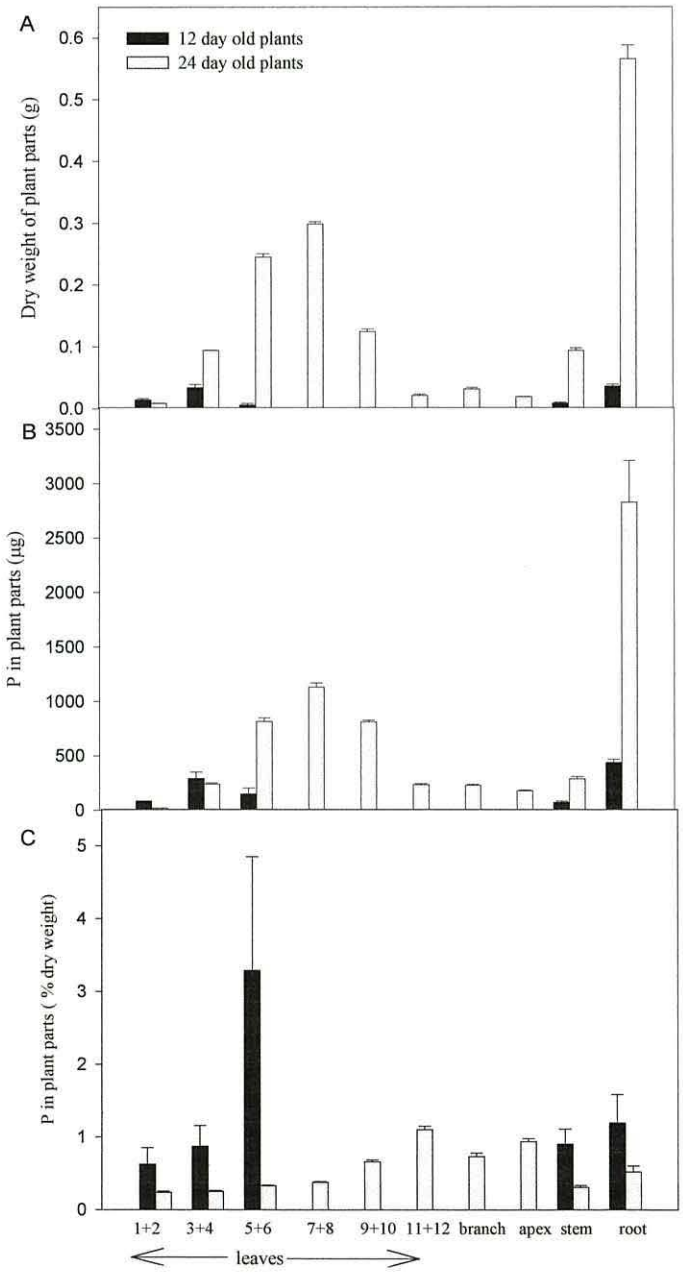
For the study of shoot tissue P distribution in more mature plants, three stem cuttings of a Costa Rican Tithonia provenance were grown in 'John Innes No 1' compost in 10 L pots. The plants were grown in a glasshouse with the temperature regime of 20 ± 2°C and with a minimum daytime photon flux density of 100 μmol m^{-2} s^{-1}. The plants were watered daily and the soil fertilised with a general-purpose garden fertiliser after six weeks to provide mineral nutrients in excess of requirement (Growmore 7-7-7). Three months after being placed in to the soil, the new shoots were destructively harvested. Leaf pairs along each stem were removed for analysis and the stems divided into two parts to allow separate analysis of the stem from the more woody base and the green stem in the upper part. The harvested material was dried at 60 °C for 48 h and tissue dry weights for each plant part measured. The dried tissues were ground to a fine powder using a pestle and mortar and a representative sample taken for colorimetric P analysis using the modified molybdenum blue method of Murphy and Riley (1962).

3. Results

3.1. Radio tracer experiment

At day 12, plants grown in hydroponic solution had 6 leaves, this increased to 12 by day 24, with the dry weight of the largest leaf in each plant increasing more than 8 fold (*Figure 1A*). The distribution of P between tissues remained similar (*Figure 1B*) although the concentration in the apical leaves declined by day 24 (*Figure 1C*).

Figure 1. The dry weight of shoot tissues and the total root system of Tithonia plants grown in hydroponic solution containing 333 μM P at 12 and 24 days (A), the P distribution of the same plants (B) and the data expressed as P content on a percentage of dry weight basis (C). Values represent means ±SEM ($n = 3$).



Significant retranslocation of radiolabelled ^{33}P was observed within the Tithonia plants in the 12 day period after removal of ^{33}P from the root bathing solution (*Figure 2*). The amounts of ^{33}P recovered from the plants at day 24 were only slightly lower than at day 12 suggesting little ^{33}P leakage from the roots and ^{33}P was not found in the nutrient solution at day 24. At the 24 day harvest the lower leaves (leaves 1 and 2) had senesced and 75% of the P present in these leaves at day 12 had been retranslocated. Leaves 3 and 4 also showed a large decline (75%) in ^{33}P between the 12 and 24 day harvests and a 25% decline in total P content despite a 226% increase in dry weight. Leaves 5 and 6 continued to be a sink for P as the leaves matured, with an increase in dry weight of 2600% and an increase in P of 572%, but no apparent retranslocation of ^{33}P. Leaves 7 and 8 were not present at day twelve, but by day 24 were the largest mature leaves and contained 15% of the ^{33}P taken up in days 1-12. In the upper leaves and stem at day 24, ^{33}P was 10% of the total P. ^{33}P concentrations in the roots, lateral shoots and the lower section of stem were similar to leaves 7 and 8 at day 24.

Figure 2. ^{33}P content of Tithonia plants after root exposure to a 333 μM ^{33}P solution for 12 days and then the subsequent distribution of this label over a subsequent 12 day period (day 24), following removal from the ^{33}P solution. At day 12 the Tithonia plants only had 3 leaf pairs. Values represent means ± SEM (n = 3).

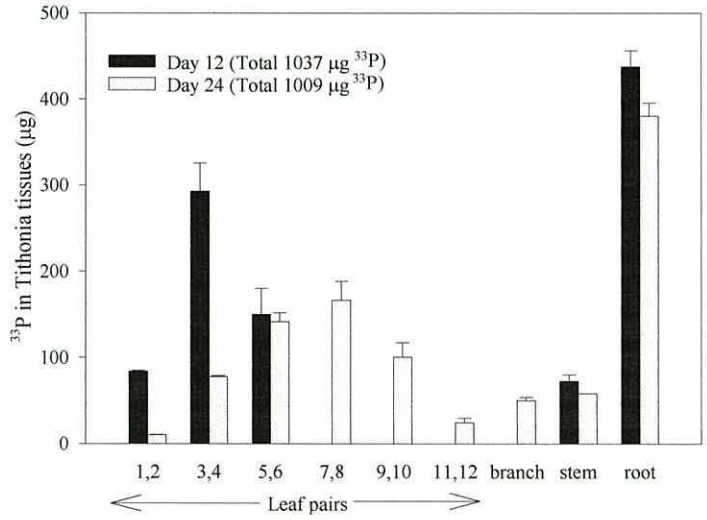
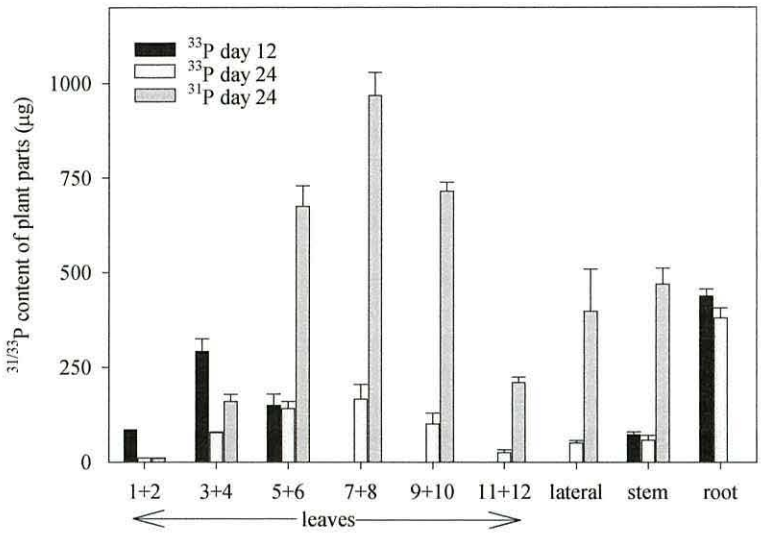


Figure 3. ^{33}P and ^{31}P content of Tithonia leaves over a 24 day growth period. Plants were exposed to ^{33}P in the root-bathing medium from days 1-12 at which point ^{33}P was removed, but the ^{31}P concentration maintained at 333 μM. Bars represent means ± SEM ($n = 3$). The ^{31}P content of roots after 24 days was 3017 μg ± 126.



Only a small proportion of the total P in the plants at day 24 had been taken up during days 1 to 12 (13.5%). The rapid increase in the rate of growth and P uptake between days 12 and 24 was associated with an increase in the root-to-shoot ratio (dry weight) from 0.59 at day 12 to 0.66 at day 24. This change was reflected by a shift in the root P-to-shoot P ratio from 0.73 to 0.83, but ^{33}P in roots was nearly unchanged. Neither was there a change in the stem ^{33}P between harvests, but the ^{33}P distribution in these tissues at day 24 is in proportion to the ^{31}P-to-^{33}P ratio in the plants

3.2. Glasshouse grown plants

Both ontogenetic drift and the growth of plants in solution culture can influence the pattern of growth and the associated retranslocation and compartmentation of P. The results of the solution culture experiment were therefore compared with soil grown plants. Four similar stems from 3 plants were destructively harvested three months after establishment as stem cuttings (*Table 1*). Unfortunately, most of the senesced leaves had fallen from the plants and could not be included, but each stem had shed 5 – 10 leaf pairs.

Table 1. The number of leaves and stems present on Tithonia plants after three months growth in soil. Stem length to the nearest 10 mm. All values are on a per stem basis

Plant	Stem	No.leaf pairs	Stem length (mm)
1	1	15	600
2	2	14	500
	3	14	400
3	4	15	600

The P concentration was greatest in the shoot apex at about 0.7% of dry weight and about 0.45% in the rest of the leaves (*Table 2)*. The older leaves were decreasing in dry weight, but not in P concentration in a pattern commensurate with P and other assimilates being re-translocated. The fully expanded leaf pair (9-10) appeared larger than the older leaves and was starting to senesce. Stem P concentration decreased toward the base of the shoot where the stem was brown woody and hollow, reflective of the increasingly structural role of the older stem (data not shown: the stem P data in *Table 1* is the mean for each whole stem). As with the plants harvested at 24 days after sowing, the pot grown plants had lateral shoots with lower P concentrations than the shoot apex, indicating apical dominance.

The range of tissue P concentrations in leaves along the stems of three-month-old plants suggests that that same pattern of P retranslocation observed in the younger plants continues to be a feature of the growth pattern of Tithonia in mature plants.

Table 2. Dry weight, total P and P concentration of leaves and stems of pot grown Tithonia shoots 3 months after the onset of vegetative growth of cuttings. Values represent means (n = 4) with SEM in brackets.

Shoot part	Dry weight (g)	P in shoot parts (mg)	P concentration (mg g^{-1})
Leaves 1+2	0.29 (0.15)	1.22 (0.36)	4.21 (0.74)
Leaves 3+4	0.61 (0.29)	2.87 (0.85)	4.70 (0.57)
Leaves 5+6	0.91 (0.37)	3.92 (0.94)	4.31 (0.52)
Leaves 7+8	1.17 (0.39)	4.60 (1.25)	3.93 (0.70)
Leaves 9+10	1.48 (0.74)	6.49 (2.08)	4.39 (0.54)
Leaves 11+12	1.36 (0.82)	6.62 (2.14)	4.87 (0.48)
Leaves 13+14	0.73 (0.37)	4.08 (0.89)	5.59 (0.68)
Shoot apex	0.15 (0.11)	1.04 (0.29)	6.93 (0.33)
Stem	2.42 (1.51)	10.37 (3.11)	4.29 (0.67)

4. Discussion

Most studies of phosphate nutrition in higher plants are concerned with the effects of phosphate deficiency on crop plants and there are relatively few studies of plants grown with high levels of phosphate. A number of general principles that affect the uptake, transport, storage and utilisation of phosphate have, however been clearly established (Bieleski, 1973; Marschner, 1995; Mimura, 1995; Rausch and Bucher, 2002).

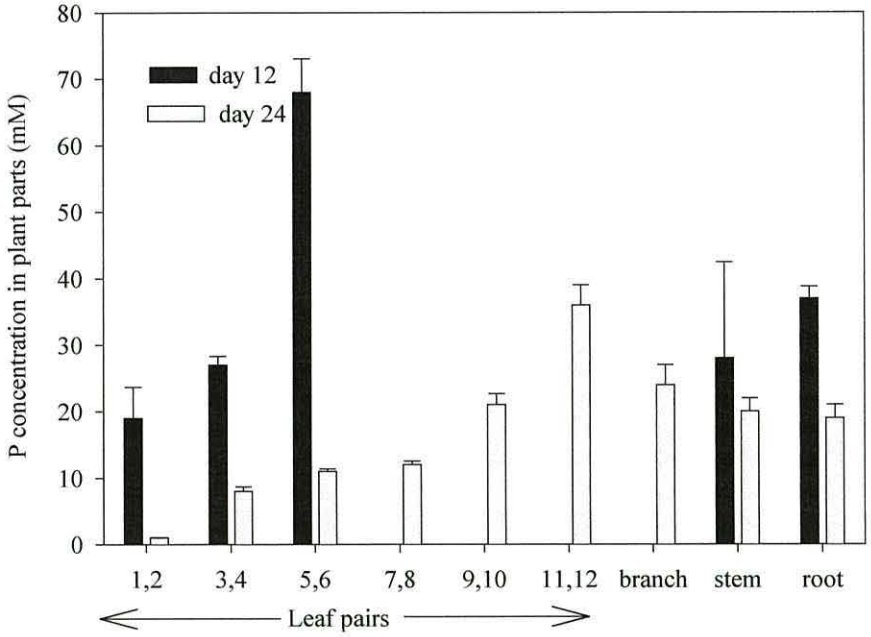
Early studies of the distribution of P into metabolic pools and physical compartments were derived from analysis of isolated organelles and from the partitioning of ^{32}P between different chemical fractions (Bieleski, 1973). These studies established that lipid, nucleic acid and P-ester pools increased under exogenous P supplies up to 650 μM in hydroponic growth solution, but thereafter it was only the vacuolar Pi pool that increased (Marschner, 1995). The mapping of intracellular pools became possible with the introduction of ^{31}P NMR. From these studies it was deduced that the cytoplasmic Pi pool, which constitutes 1-5% of the cell total P, rapidly turns over, but remains at a concentration of 5-10 mM irrespective of vacuolar Pi concentrations (Lee et al., 1990; Lee and Ratcliffe, 1993; Mimura, 1995). In contrast, vacuolar concentrations fluctuate with P availability to the plant, but have rarely been observed above 25 mM (Mimura, 1995).

Homeostatic regulation of P in the cytosol is very finely regulated, as plants utilise P not only as a substrate in energy metabolism, but also as a regulator of the enzymes associated with energy metabolism. Pi allosterically inhibits starch synthesis in the chloroplasts, but starch synthesis is stimulated by triose phosphates, the main product of CO_2 fixation (Rausch and Bucher, 2002). At high P concentrations in the cytosol there is therefore an increased export of sugars to the vacuole. This results in insufficient carboxylation products in the stroma to regenerate the CO_2 acceptor, ribulose biphosphate, and so reduces photosynthesis (Marschner, 1995).

Assuming that Tithonia is not fundamentally different from plants studied previously, there is no need to invoke the synthesis of P storage compounds in tissues other than the apical shoots. To enable comparison with ^{31}P NMR data in the literature, the P concentration of the tissues from the plants grown in solution for 12 and 24 days was calculated on a mM P basis, assuming the fresh plant material has a bulk density of 1 g cm^{-3} and that green tissues had a 90% moisture content (*Figure 4*).

The concentration of P in shoot tissues is greatest in the youngest plants (12 days) reaching a peak concentration of over 60 mM in the youngest leaves. Plants are known to accumulate P during the early stages of growth (Hopkinson, 1964), but this concentration, if stored as Pi, is normally considered toxic (Marschner 1995). Phytate has been found in the young leaves of plants and appears to play a similar storage role in roots and young shoots as in seeds (Campbell *et al.,* 1991). If the P in the emergent leaves (leaves 5 and 6) at day 12 is in excess of the cellular Pi storage capacity of the vacuole and is in the form of phytate, in order to maintain homeostatic regulation of the cytosol, the quantities involved are not significant and this pool is clearly transient as by day 24 the P concentration is well within the range reported in the literature.

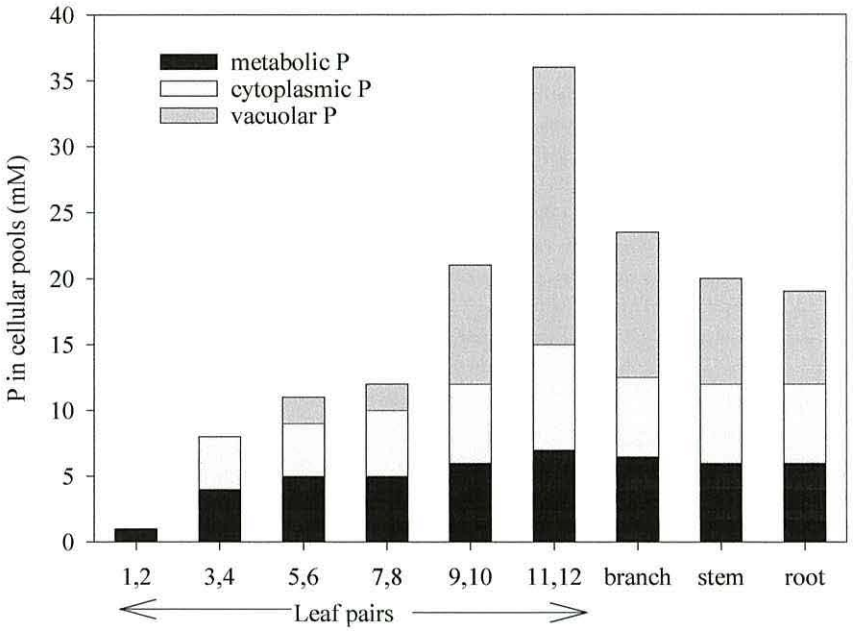
Figure 4. The P concentration of Tithonia tissues on a mM P basis, for plants grown in hydroponic solution culture containing 0.333 mM P calculated assuming 1 cm^3 = 1 g of fresh weight and green tissues contained 90% H_2O. Bars represent means ± SEM (n = 3).



Relatively high concentrations of P can be metabolised into tissue structures, remain in the cytosol in organic forms, as well as be present as Pi in the cytosol and the vacuole. Based on the cellular P compartmentation measurements of Lauer *et al* (1988), *Figure 5* describes the expected P compartmentation of the 24 day old solution culture grown Tithonia plants between metabolised, cytosol and vacuolar P pools in the cell. The concentration of P in the tissues would need to be significantly

greater to attain P toxicity levels and invoke storage of P as phytate as even in the emergent leaves (leaves 11 and 12) vacuolar P is within the 25 mM limit described by Mimura (1995).

Figure 5. An estimated distribution of cellular P in Tithonia plants grown in hydroponic solution culture containing 0.333 mM P, based on the phosphate compartmentation study of Lauer *et al.* (1988).



Although storage of phytate or polyphosphate in the tissues of Tithonia cannot be excluded, the observed high P concentrations are commensurate with vacuolar Pi storage capacities. It should be assumed that a large proportion of the P in Tithonia shoot material is in the form of vacuolar Pi and is available to roots and soil microbes when Tithonia is applied to soil as a green manure.

As Tithonia P concentrations appear to be constitutively higher than in many plant species it suggests that the down-regulation of the systems for P acquisition do not occur in Tithonia at the same internal concentrations as lower P status plants. However, when wheat (*Triticum aestivum*), was grown in solution culture at the same P concentration (data not shown), plant P concentrations were greater than those in Tithonia. Very little is understood about the regulation of P uptake at a whole plant level, although it is thought to involve transcriptional factors (Raghothama, 1999; Rausch, and Bucher, 2002). This observation suggests that the regulation of P at a whole plant level incorporates factors describing the economic cost of P acquisition.

The results of this study indicate that even when there is a high availability of P to the roots, retranslocation of P from older to younger tissues continues (*Figure 2*). In work with *Hordeum vulgare* it was found that P deficient plants retranslocated P from older leaves to maintain the expansion of younger leaves, but plants with high phosphorus accumulated P in mature leaves (Greenway and Gunn, 1966; Mimura, 1995). It is thought that Pi transport from the site of uptake in the root to the shoot is via the xylem, but that retranslocation is via the phloem (Mimura, 1995). The movement of ^{33}P between days 12 and 24 (*Figure 3*) is supportive of this understanding, with ^{33}P taken up in the first 12 days of growth being transferred from mature leaves to younger leaves. There was, however, little evidence of retranslocation of ^{33}P from the roots. In leaf pairs 3 + 4 (*Figure 3*), there is evidence of concurrent xylem import of ^{31}P with phloem export of ^{33}P. Again this is in agreement with the observations for expanding leaves under low P conditions (Greenway and Gunn, 1966).

There is a high degree of species-specific variation in the re-adsorption efficiency for mineral nutrients from senesced leaves (Killingbeck, 1996). If green manure crops are to be grown *in situ* or as an intercrop then a low re-adsorption efficiency for N and P is desirable, but if the plant is to be used in nutrient transfer then a high re-adsorption efficiency raises the concentration in the green biomass. It would appear from these results that the P re-adsorption efficiency of Tithonia is about 75%, compared with a typical recovery of nearer 50% in most species (Aerts, 1996).

Most of the literature of phosphorus in plants has concerned itself with crop plants and cereals in particular. Our knowledge of re-translocation of minerals in shrubby plants is therefore somewhat deficient. To conclude that Tithonia is particularly unusual in re-translocating P from older to younger leaves when P is not limiting would probably be an error. The *Asteraceae* includes a large number of competitive opportunists that need to compete aggressively for light and water in short intense growing seasons. It would seem an effective and probable adaptation, to minimise the proportion of leaf that is in shade, to senesce leaves and re-translocate the minerals to leaves closer to the canopy. Drechsel and Reck (1998) observed that P and N tissue concentrations increased during the dry season. This would be compatible with the re-translocation of N and P from leaves that were senescing to minimise transpiration losses. The hypothesis that Tithonia synthesises P storage

compounds not commonly found in other species is not supported by this study and the observed high tissue P concentrations are explainable as the product of P retranslocation.

Tithonia can be described as a conservative opportunist. There is a low investment in structural tissues and the less photosynthetically active leaves senesce under stress. This allows the maintenance of conservative nutrient supplies within the growing tissues, as well as the accumulation of P and other minerals necessary for the production of viable seeds. As Tithonia grows in clumps, nutrients in senesced leaves that fall to the ground are easily re-cycled as the leaves decay rapidly. Tithonia is fast growing, but biomass accumulation is lower than in less conservative and N_2-fixing species (Sherchan, 2001). The potential role of Tithonia as a green manure in low-input tropical agriculture would therefore appear to be as a concentrator of mineral nutrients that might otherwise be lost from farmland due to leaching, run off or erosion. Tithonia is not easily propagated from seed in field conditions, suggesting that its role in soil fertility management may be as a supplementary source of high quality biomass to be transferred from hedgerows and mixed with residues of N_2-fixing green manures grown *in situ*. Such a practice could help to facilitate net mineralisation of P provided from the combined residues.

Chapter Five

Role of arbuscular mycorrhizas in the exploitation of soil P pools by the green manure *Tithonia diversifolia*

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Summary
The green manure *Tithonia diversifolia* (Tithonia) has been identified as having the potential to significantly enhance soil P cycling in resource poor agroecosystems. It has been hypothesized that Tithonia has the potential to exploit soil P pools that are largely unavailable to conventional crop plants such as rice and maize. The aim of this study was to investigate whether associations between Tithonia and endomycorrhizal fungi may be able to account for the relatively high tissue P concentrations observed in Tithonia grown on low P status soils. Using a mesh exclusion technique, uptake of P by Tithonia plants was measured from a ^{33}P-labelled subcompartment into which either mycorrhizal hyphae or roots and mycorrhizal hyphae could penetrate. In treatments allowing hyphae only access to the ^{33}P-subcompartment, the plants took up 15% less P in comparison to treatments where both roots and hyphae could access the ^{33}P subcompartment. Where roots could access the subcompartment shoot growth was enhanced in comparison to hyphal access only and control treatments. This study demonstrates that endomycorrhizal associations with Tithonia roots can make an important contribution to P uptake.

1. Introduction

Soil fertility depletion in smallholder farms is the fundamental biophysical root cause for declining per capita food production in many areas of the world (Sanchez *et al.*, 1997). This fertility decline is multifaceted and is typically caused by losses of nutrients by leaching, erosion and crop removal, losses of organic matter, increased acidity and salinity, and loss of soil structure. To maintain yields and food security, this agriculturally mediated exploitation of soil resources must be matched by large external inputs. These inputs may be achieved either through the addition of mineral fertilisers or organically based manures. As the availability, price, and transport of mineral fertilizers in many developing countries is often erratic, and the availability of animal manure often scarce (Quiñones *et al.*, 1997), green manures may offer an alternative strategy in some socio-economic settings. In the case of nitrogen, it is well established that nutrient levels can be replenished to some degree by growing species capable of biological N_2 fixation in a range of site specific management strategies including sole crop, fallows, hedgerows, intercrops etc (Giller *et al.*, 1997). However, in the case of other nutrients such as P where biological

fixation is an impossibility and rainfall inputs are small, there is currently a lack of management strategies available to farmers to restore soil P capital.

Within the soil, P can be considered to be contained in three main pools – the organic matter which may hold up to 50% of the total soil P reserves, the insoluble inorganic fraction, and a small, very variable part, that is soluble and can be absorbed by plants (Ozanne, 1980). One way to increase plant available P is to facilitate the transfer of P from previously non–labile soil P pools to the soluble pool. In an attempt to achieve this, many studies have been performed to select crop varieties that possess an enhanced P use efficiency (Rao *et al.*, 1999; Manske *et al.*, 2000). In most cases these have met with little overall success as P accumulation in crops plants is generally conservative (Bieleski and Ferguson, 1983; Lambers *et al.*, 1998). An alternative strategy is to grow non-crop species that are better adapted than crop plants to acquire P from less labile pools and incorporate the residues as a green manure. In tropical soils low in organic matter this approach is compatible with good soil husbandry. When selecting a green manure for soil P mobilization the desired attributes of the plants include high biomass potential and external P use efficiency in a species that can be utilised with appropriate management in terms of labour and space costs (Nziguheba *et al.*, 1997). *Tithonia diversifolia* has been identified as a green manure that may fulfil this requirement (Buresh *et al.*, 1997).

Green manures may raise the concentration of the labile P pool in soil by a number of both direct and indirect ways (Iyamuremye and Dick, 1996). One such hypothesised route is that a simple accumulation of P in labile soil organic matter will accelerate transfer to the labile P pool. The mechanistic basis of this is that upon entering the soil, the green manure will be rapidly mineralised and the P contained within the residues transferred to the soil microbial pool. The microbial decomposer community is considered to be relatively labile with a turnover time of weeks to months and that may suit crop demand to a greater extent than mineral fertilizers (Buresh *et al.*, 1997). It is also conceivable that mycorrhizal fungi associated with crop roots could transfer P directly from these organic residues to the subsequent crop, circumnavigating the typical mineralization-immobilization step. However, if the green manure is simply recycling the labile soil pool this attaches no benefit to a subsequent crop and may be detrimental in that it locks up P in an organic form, some of which will inevitably enter the recalcitrant soil P pool (half life > 10 years). Therefore green manures that can be recommended for broad scale use should have

proven mechanisms to accelerate the transfer of P from the non-labile to labile soil P pools.

Plant roots are equipped with a variety of mechanisms to mobilize soil P including: enhancing the number and length of root hairs, increased root branching, releasing organic acids and phosphatases, upregulating of P transporters (increased V_{max}, decrease K_m), and the formation of endo- and ectomycorrhizal symbioses (Marschner, 1995; Jones, 1998). In a wide range of plant species, mycorrhizas have been shown to actively transfer soil P to the plant, effectively bypassing the slow route of diffusion of P through the soil to the root surface (Tinker, 1980; Marschner, 1995; Smith and Reid, 1997). It is also well established that their greater surface area to volume ratio in soil permits a more effective scavenging of P in comparison to roots (Smith and Read, 1997). The extent to which mycorrhizas are capable of exploiting root unavailable soil P reserves, however, remains controversial (Tinker, 1980). In the case of ectomycorrhizas it has recently been shown that they are capable of tunnelling through soil minerals, whilst roots do not appear to possess this capability (van Breemen *et al.*, 2000). This result and others (Daft and Nicholson, 1966; Ross and Gilliam, 1973) suggests that mycorrhizas may possess the capability to effectively exploit non-root available soil P reserves and that this may be particularly important in fast growing plants with high nutrient demands which have adapted to low available P environments.

Recently, there has been increased interest in the 'Mexican Sunflower', *Tithonia diversifolia* (Tithonia), a shrub belonging to the asteraceae, as a green manure. Tithonia is naturalised across much of the tropics and is easily propagated from cuttings and so is available to many farmers. Once established, Tithonia can produce a large amount of biomass with a high mineral nutrient content, including P (*Table 1*). The P mobilization potential of Tithonia and its apparent ability to accumulate P has recently been the subject of a number of studies in Kenya (Nziguheba *et al.*, 1998; Gachengo *et al.*, 1999; George *et al.*, 2002[b]). The purpose of this study was to assess the role of mycorrhizas in P acquisition by Tithonia. Specifically, the aims were to determine whether mycorrhizas were accessing soil P reserves unavailable to roots and the extent to which they supply plant P in a Nepalese soil where fertility decline is a significant issue (IIED, 1993; Ali, 1996). The soil used in this research was taken from a field site in the Chitwan Valley in lowland Nepal

where an associated field study was researching the potential of Tithonia as a green manure (Sherchan 2001).

Table 1. Mineral nutrient content of some plant tissues from the Bharatpur region of Nepal typically used in green manure practices in Nepal. (Sherchan, 2001)

	P g/kg	N g/kg	K g/kg	S g/kg	Ca g/kg	Zn mg/kg	Mg mg/kg	Cu mg/kg	Fe mg/kg	Mn mg/kg
Tithonia leaf	3.5	37	33	3.07	16	110	5.0	2	353	291
Tithona leaf/stem	3.3	38	15	3.52	8	45	2.7	2	241	161
Dalbergia leaf	2.0	23	13	2.20	13	31	3.5	1	300	156
Dalbergia leaf/stem	2.8	19	13	2.03	15	11	3.4	0	388	54
Sesbania leaf	2.3	40	13	4.54	8	42	1.4	10	364	109
Sesbania stem	1.5	22	14	1.18	9	0	2.3	0	423	24
Sesame leaf	2.7	37	19	4.48	12	23	5.9	10	467	22
Sesame stem	0.6	8	22	4.33	17	25	8.4	3	307	4
Forage grass	2.9	13	9	1.90	3	3	2.0	0	276	24
Maize green leaf	3.1	28	23	3.60	3	9	1.9	0	247	12
Maize browning leaf	3.1	20	37	3.14	5	24	3.5	0	277	17
Rice seeds	2.9	13	3	2.92	1	4	1.2	0	150	18
Rice stems	1.8	8	18	2.43	3	5	3.4	0	294	107
Melia leaf	2.7	34	14	5.48	14	17	2.8	0	153	11
Melia leaf/stem	2.1	12	36	4.72	12	24	3.8	2	232	9
Artemisia leaf	5.3	38	13	3.88	5	50	1.7	18	277	64

2. Materials and Methods

2.1. Soil

Soil was taken from the Ap horizon (0-20 cm) of a field site located in the Chitwan valley in the Bharatpur region of Nepal. After collection, the soil was sieved to pass 2-mm and stored field moist at 15°C until required for experimentation. Details of the soil are provided in *Table 2.*

Table 2.

Soil Details and Characteristics	
Soil origin: Chitwan valley in the Bharatpur region of Nepal (27°36' N, 84°29' E; altitude 250 m)	
Rainfall: bimodal rainfall of 2500 mm per year	
Current annual cropping cycle: *Zea mays* and wetland *Oryza sativa*	
Soil Classification (USDA)	Eutric fluvisol
Texture	Sandy loam
$pH_{(H_2O)}$	5.48
Organic Carbon	0.51%
Total N	0.03%
Electrical conductivity (H_2O 1:1 v/v)	0.13 mS cm^{-1}
C:N ratio	17
Total P	6.5 mmol kg^{-1}
Anion resin P	0.12 mmol kg^{-1}
$NaHCO_3$ P	0.58 mmol kg^{-1}
K	24 $mmol_c$ kg^{-1}
Na	4 $mmol_c$ kg^{-1}
Ca	84 $mmol_c$ kg^{-1}
Mg	60 $mmol_c$ kg^{-1}

2.2. Plant growth

Seeds of a South African ecotype of *Tithonia diversifolia* were soaked in aerated distilled water overnight and then transferred to petri-dishes containing moist filter paper for 6 days at 20°C. Two plantlets with fully expanded cotyledons and a main root axis approximately 5 cm long were then transferred to rhizotrons containing 540 g of soil packed to a bulk density of 1.3 g cm^{-3}. The rhizotrons were similar to that described by Marschner (1995) and consisted of a 150 × 150 × 20 mm box made from clear plexiglass. In the reverse side of the box a 5 cm diameter hole was cut allowing a ^{33}P soil filled subcompartment to be attached (*Figure 1*). The circular subcompartment was composed of a 1 cm long section of nylon pipe which could be divided from the main compartment by means of a mesh inserted between the two. Three mesh sizes were used to selectively prevent mycorrhizal hyphae or roots present in the main compartment from entering the subcompartment containing ^{33}P labelled soil:

A. 2 mm mesh – allow passage of both mycorrhizal hyphae and roots
B. 45 μm mesh – allow passage of mycorrhizal hyphae but not roots
C. 0.4 μm mesh – prevent passage of both mycorrhizal hyphae and roots

The rhizotrons were maintained in a climate controlled growth room with 22/18°C day/night rhythm, 16 h photoperiod and light intensity of 350 μmol m^{-2} PAR. The moisture content of the rhizotrons was maintained at approximately 20% volumetric water content by the daily addition of distilled water to the surface of the rhizotrons, with the rate of water addition calculated by daily weight loss in the rhizotrons. After 4 weeks, the plants were fertilised with N and K at rates equivalent to 75 kg K ha^{-1} and 150 kg N ha^{-1} (based on rhizotron surface area) using KNO_3 and NH_4NO_3.

At 6 weeks, when the plants had two pairs of senesced or senescing leaves, two pairs of fully expanded leaves and 2 leaves not fully expanded and a stem height of 49 ± 1 mm (n = 30), the subcompartment was attached to the rhizotron. $KH_2{}^{33}PO_4$ (specific activity = 100 TBq $mmol^{-1}$; Amersham Pharmacia Biotech, Little Chalfont, Bucks.) was added to the subcompartment soil at a rate calculated to provide the equivalent to a 10 kg P ha^{-1} surface application. The surface area of the rhizotrons was 30 cm^2 and so 3 mg ^{33}P with an activity of 50 kBq was applied in each treatment. The ^{33}P solution was added as 15 × 20 μl drops distributed evenly across the surface of the soil. The ^{33}P labelled soil was packed into the subcompartment to a depth of 0.5 cm and an estimated bulk density of about 1.3 g cm^{-3} (similar to field conditions). To provide a soil buffer zone between the main and sub compartments a further 0.5 cm layer of soil containing no added P was placed on top of the radiolabelled soil layer. The mesh was then placed over the buffer zone, and the subcompartment attached to the back of the rhizotron (*Figure 1*). Each of the treatments was replicated four times with the individual rhizotrons positioned at random in the growth chamber and repositioned daily to avoid effects due to variation in light and temperature. To provide a control, no ^{33}P was added to three remaining available rhizotrons,.

2.3. Rhizotron harvesting

After 3 weeks exposure to ^{33}P, the rhizotrons were harvested. Above ground plant parts were removed, separated based upon developmental stage, dried at 60°C (24 h) to determine dry weight, ashed (500°C, 24 h) and the residue dissolved in 1 M HCl. The ^{33}P content of the HCl solutions was then determined by liquid scintillation counting using HCl compatible scintillation fluid (Optiphase Hisafe 3, EG&G Wallac, Milton Keynes) and a Wallac 1304 liquid scintillation counter (EG&G Wallac, Milton

Keynes). The total P content of shoot material was determined in an aliquot from the 1 M HCl solutions using the colorimetric procedure of Murphy and Riley (1962). The ^{31}P content of shoots was calculated by deducting ^{33}P from total P. Roots contained within the main rhizotron compartment were removed by hand, oven dried, ashed and the ^{33}P content determined as described for the shoots.

After removal of roots (from the 2 mm mesh samples only), soil from the subcompartment was subjected to a sequential P fractionation procedure according to the methods of Sibbesen (1976) and Hedley *et al.* (1982). Briefly, 1 g of soil was placed in a 50 ml polypropylene centrifuge tube containing 30 ml of deionized water and one resin bag containing 4 ml anion exchange resin in the bicarbonate form (Amberlite 420, bead diameter > 30 mesh) and another containing 2.8 ml cation resin (Amberlite 120) in the NH_4^+ form to maintain the balance of the electrolyte. Resins were pre-treated according to the methods of Somasiri *et al.* (1991). The tubes were then shaken at 200 rpm on an end-over-end shaker for 16 h at 20°C, the resin bag removed, washed with deionized water and the P contained on the resin displaced with 30 ml of 0.5 M HCl (16 h, 20°C).

The remaining soil was centrifuged (10,000 *g*, 30 min), the supernatant solution removed and then 30 ml of 0.5 M $NaHCO_3$ added and the soil shaken for 16 h at 20°C. After removal of the $NaHCO_3$ supernatant solution by centrifugation, repeat extractions of the remaining soil were performed with 0.1 M NaOH (×2) and 1 M HCl. In each case, the supernatant solution was retained for P determination. After the HCl extraction, the remaining soil residue was digested with perchloric acid (10 ml; 4 h, 200°C) for the determination of residual soil P. Aliquots of the resin, $NaHCO_3$ and the NaOH extracts (1 ml) were exposed to autoclave oxidation (30 min, 121°C) following addition of 100 mg potassium-persulfate and acidification of the alkali extracts. The P content in each extract was determined by flow injection analysis (Perstorp Flow Injection 3000 Analyzer; Perstorp Analytical, Maidenhead, England) based on the molybdate blue procedure of Murphy and Riley (1962). The ^{33}P content of the extracts was determined by liquid scintillation counting using Optiphase Hisafe 3 scintillation fluid (EG&G Wallac, Milton Keynes) and a Wallac 1304 liquid scintillation counter (EG&G Wallac, Milton Keynes). Colour and chemical quench curves were determined for the different extracts and the ^{33}P measurements corrected accordingly before correction for isotopic decay.

2.4. P sorption isotherms

The concentration dependent sorption of phosphate was determined as described in Jones and Brassington (1998). Briefly, 10 ml of a KH_2PO_4 solution (pH 5.2) containing increasing amounts (0.05-1.0 mM) of P in a constant background of 5 mM KCl was added to 1 g of soil. The soils were then mixed vigorously by vortexing, shaken for 10 minutes at 320 rpm, and high-speed centrifuged (16,000 *g*, 5 mins). The amount of phosphate remaining in the supernatant was then measured as described in Murphy and Riley (1962). The amount of ligand adsorbed was calculated from the difference in initial minus final anion concentration. All experiments were replicated twice.

Freundlich, single Langmuir and double Langmuir equations were fitted to the experimental sorption data. The single Langmuir equation describes sorption kinetics for a single-phase surface and was modified by Holford *et al.* (1974) to take into account specific and non-specific ion adsorption in soils. This provides a two stage (double Langmuir) equation:

$$x = \frac{k' xm' C}{1+k' C} + \frac{k'' xm'' C}{1+k'' C} \qquad \textit{(Equation 1)}$$

where x is adsorption, k is the adsorption/desorption equilibrium constant, xm is monolayer adsorption capacity and superscript ' and " refer to high and low energy adsorption surfaces. Equation 1 was fitted to the data to determine values for k', k", xm' and xm", using the computer program SigmaPlot 4.0 (SPSS Inc., Chicago, ILL). These values were then used to characterise the soil in terms of the high and low energy P sorption for a concentration range.

The solid-solution phase partition coefficient of P in the soil (P_b) was estimated using the buffer power equation where

$$P_b = \frac{DC_L}{DC_S} \qquad \textit{(Equation 2)}$$

and where C_L is the amount of solute per unit volume in liquid phase and C_S is the concentration of diffusible solute.

2.5. Statistical analysis

The experiment had a randomised block design. Significant differences between group means were determined by analysis of variance (ANOVA) and Tukey's test was used for multiple comparisons between the sample means where the ANOVA F statistic was significant.

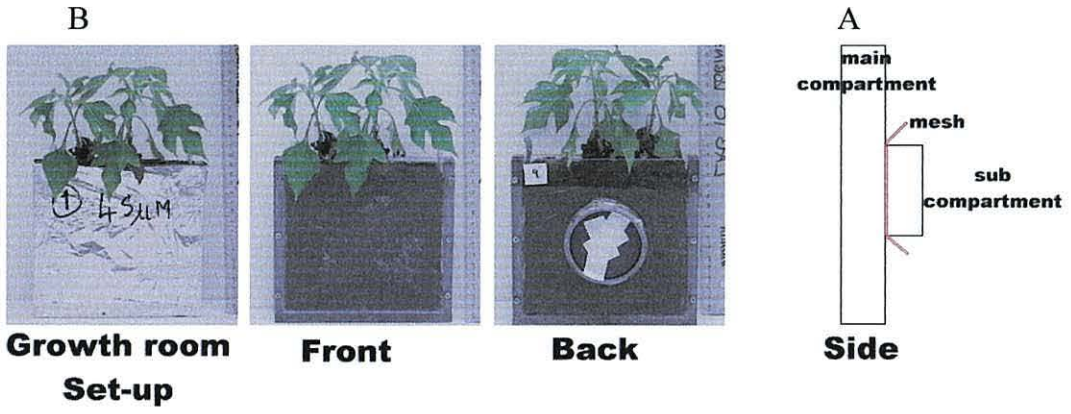
3. Results

Under field conditions Tithonia is characterised by a rapid senescing of the lower leaves as they become progressively shaded or the plant becomes nutrient or water stressed. When grown in the Nepalese soil which possessed an intrinsically low fertility (Eutric fluvisol), Tithonia growth was morphologically identical to that grown in the field with typically only 3 to 4 pairs of leaves photosynthetically active followed by a progressive senescence towards the base.

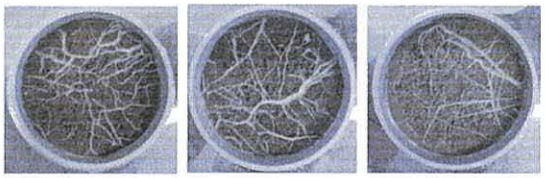
The rhizotron design incorporating the exclusion meshes appeared to operate successfully in all experiments. The 2 mm mesh permitted free passage of roots into the subcompartment while the 45 and 0.4 μm meshes prevented root growth into the subcompartment (*Figure 1*). In addition, in the 45 and 0.4 μm mesh treatments a root mat similar to that found within the 2 mm mesh subcompartment was found to form on the outer mesh surface. The moisture content of the soil within the subcompartment was not significantly different ($P > 0.05$) to that in the main compartment indicating that in all treatments water redistribution within the rhizotrons was not inhibited by the presence of a mesh.

The dry weight distribution of the individual plant parts in each of the three mesh treatments is shown in *Figure 2*, with all the senesced leaves (6 – 7 pairs per plant) pooled as a single 'plant part'. No significant differences were observed in above ground biomass production in the individual mesh treatments ($P > 0.05$). In contrast, the amount of below ground biomass production appeared to increase with a reduction in access to the subcompartment (*Figure 3*), with statistical differences observed between the 2 mm and 0.4 μm meshes ($P < 0.001$). The plants grown in rhizotrons with a 0.4 μm mesh had a greater dry weight than the control, although there is no obvious explanation for this other than it being non-experimental variation.

Figure 1. Schematic (Panel A) and photographic (Panel B) representation of the rhizotron boxes showing the *Tithonia diversifolia* plants at harvest. Panel C shows the build up of roots in the subcompartment with a 2 mm mesh, while Panel D shows the build up of roots at the interface with the subcompartment with the 45 μm mesh.

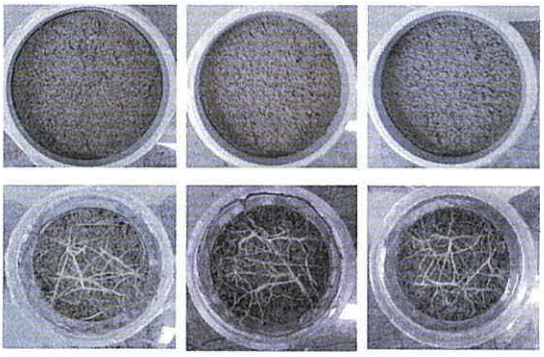


C



3 replicates showing roots which have passed through the 2 mm mesh into the subcompartment

D



3 replicates showing that roots have not passed through the 45 μm mesh into the subcompartment

3 replicates showing that roots are pressed against the 45μm mesh in the main compartment (but have not penetrated)

Figure 2. Dry weight distribution of the above-ground parts of *Tithonia diversifolia* plants at harvest when grown in the rhizotrons with the 2 mm, 45 µm and 0.4 µm exclusion meshes on the subcompartment and with no subcompartment (control). Leaves 1, 2 and 3 represent leaf pairs with decreasing distance from the meristem. Values represent means ±SEM (n = 4, except for control where n = 3).

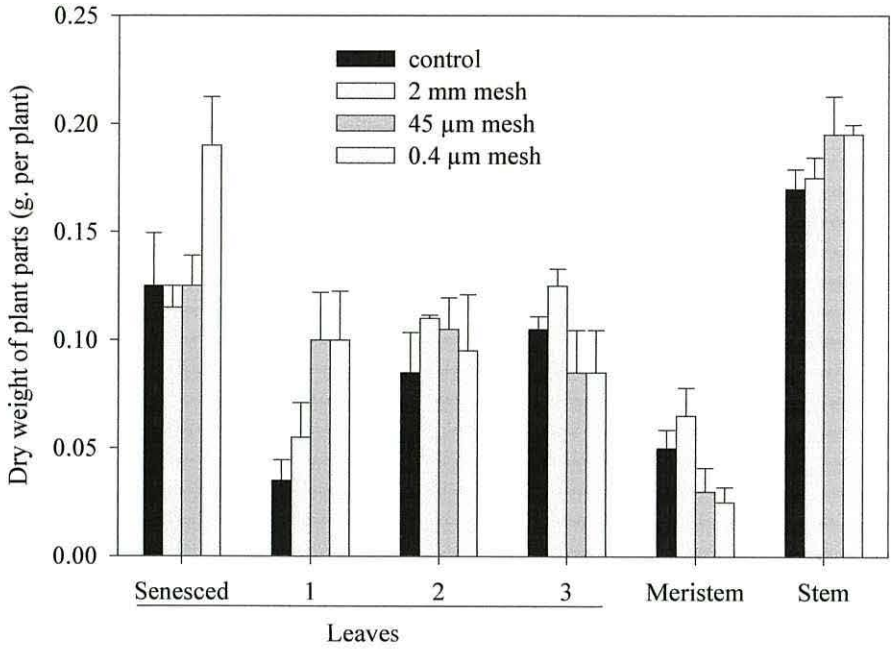
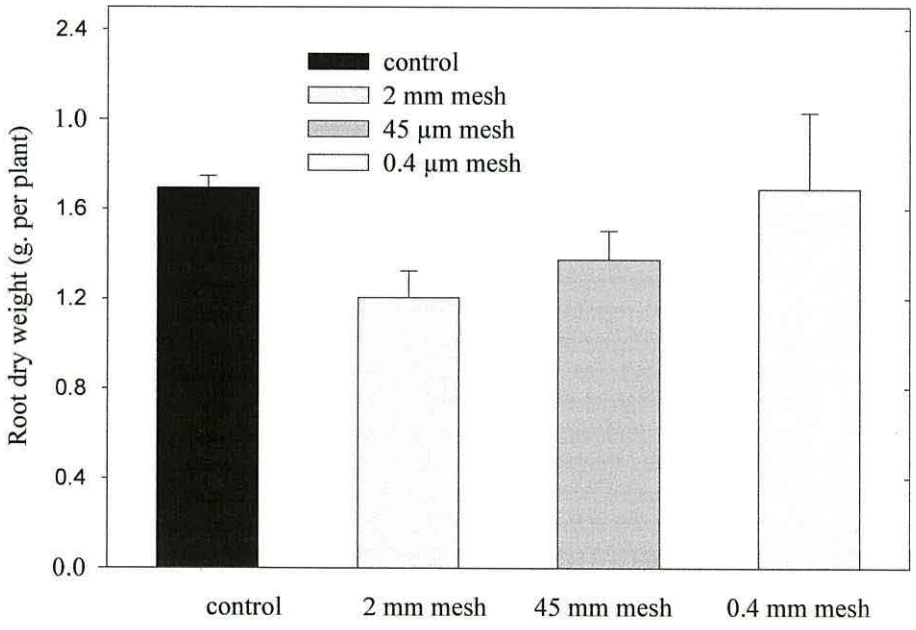
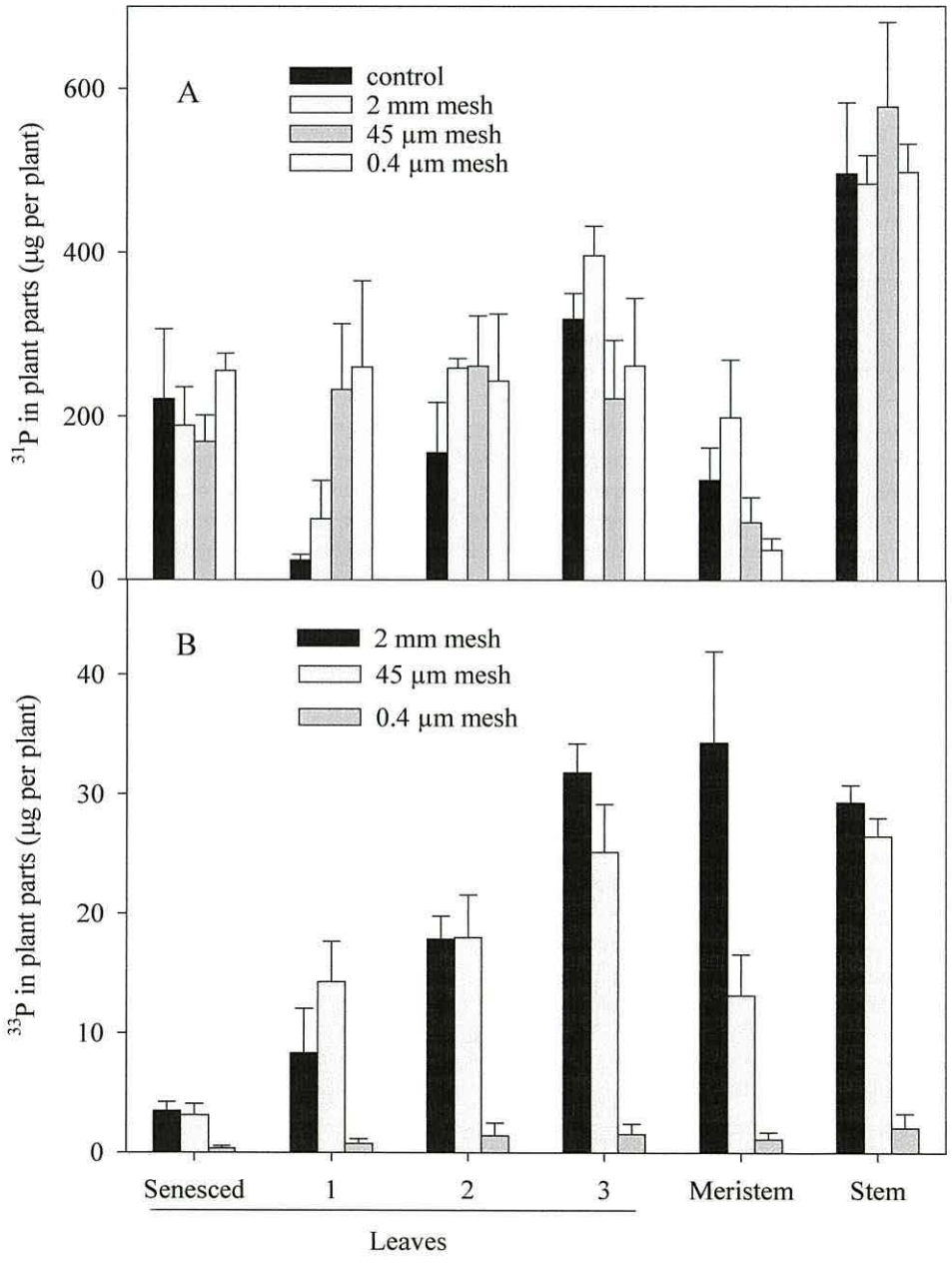


Figure 3. Dry weight of roots of *Tithonia diversifolia* plants at harvest when grown in the rhizotrons with the 2 mm 45 µm and 0.4 µm exclusion meshes on the subcompartment. Values represent means ±SEM (n = 4, except for control where n = 3).



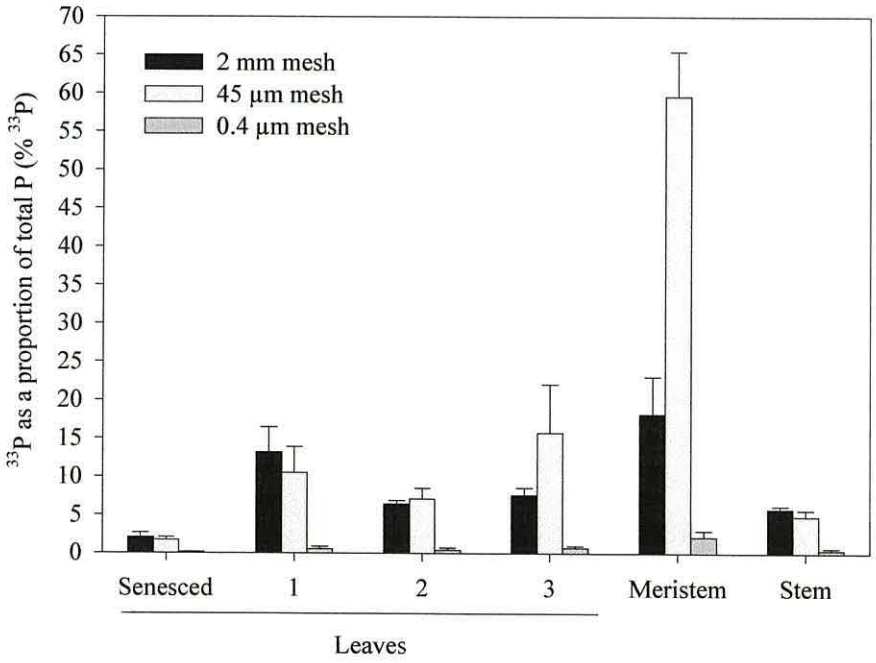
The total P in each shoot part is shown in *Figure 4A*. Calculated P concentrations in the shoot parts were in the range of 0.05% for lower leaves to above 0.4% in the upper leaves. The mean shoot P concentrations were 0.23% of dry weight in the control and 0.4 μm mesh treatments and 0.24% and 0.25% in the 45 μm and 2 mm mesh treatments respectively. The P concentration in the lower senesced leaves was approximately half that in the green fully expanded leaves indicating a P re-translocation efficiency of about 50%.

Figure 4. ^{31}P (A) and (B) ^{33}P in the above-ground parts of *Tithonia diversifolia* plants at harvest when grown in the rhizotrons with the 2 mm, 45 μm and 0.4 μm exclusion meshes on the ^{33}P-labelled subcompartment. Leaves 1, 2 and 3 represent leaf pairs with decreasing distance from the meristem (i.e. 1 being the lowest leaves). Values represent means ±SEM ($n = 4$, except control where $n = 3$).



In the individual exclusion mesh treatments there were no significant differences ($P > 0.05$) in the P concentration in any of the above ground plant parts. Root P concentrations were not determined due to excessive contamination by soil particles that would have invalidated the results. The accumulation of ^{33}P from the radiolabelled $KH_2{}^{33}PO_4$ fertilizer added to the subcompartment is shown in *Figure 4B*. The results indicate that the 0.4 μm mesh, which excluded both mycorrhizal hyphae and roots, almost completely prevented the plant uptake of ^{33}P from the subcompartment and indicates that P transfer out of the subcompartment to the plant must be predominantly biologically mediated. Shoot ^{33}P accumulation in the 2 mm and 45 μm mesh treatments for all plant parts were not significantly different from each other ($P > 0.05$). As expected, there was little accumulation of ^{33}P label in the leaves that had senesced before the addition of the radiolabelled P. In addition, the results clearly show greatest accumulation of ^{33}P in the newly growing regions and particularly in the apical meristem. When the amount of ^{33}P taken up is calculated as a proportion of the total P ($^{31}P + {}^{33}P$) in each plant part a statistically greater proportion of P in the meristem comes from the ^{33}P in the 45 μm treatment relative to the 2 mm treatment ($P < 0.05$; *Figure 5*).

Figure 5. ^{33}P as a percentage of total P in Tithonia shoot parts. Leaves 1, 2 and 3 represent leaf pairs with decreasing distance from the meristem (i.e. 1 being the lowest leaves). Values were calculated from mean measurements of ^{33}P and total P ($^{33}P + {}^{31}P$) for 2 plants per rhizotron with either 2 mm, 45 μm or 0.4 μm exclusion meshes restricting root access to the subcompartment containing 3 mg ^{33}P ($n = 4$).



The impact of root or mycorrhizal penetration of the subcompartment on the distribution of soil P in different extraction fractions is shown in *Figure 6A*. The results indicate that a large proportion of the added P fertilizer entered the labile (resin + $NaHCO_3$) pools causing a marked elevation in available soil P in comparison to that present in the main compartment (control) soil. No significant differences, however, were observed in the amount of $^{total}P$ recovered in each fraction within the subcompartment, indicating that the plants had only accessed small quantities of the added fertiliser (i.e. compare 2 mm and 0.4 μm mesh). In the main compartment soil (bulk soil), most of the P was recovered in the NaOH fraction, which is thought to be associated with Fe and Al oxide/hydroxides, and a perchloric fraction that is generally considered not to be plant available. Very little P was recovered in the HCl fraction, thought to represent Ca-bound P. The impact of the individual mesh treatments on the recovery of ^{33}P in each extraction step is shown in *Figure 6B.* Approximately half of the added P fertilizer remained in a labile form, however, half had become associated with the NaOH fraction and a small component had entered the recalcitrant perchloric fraction. Chloroform fumigation of samples followed by a $NaHCO_3$ extraction indicated that very little of the added ^{33}P had become associated with the soil microbial fraction (data not presented). The 45 μm and 2 mm mesh treatments produced no statistically significant differences in either the ^{33}P or ^{31}P pools, but significantly more ^{33}P was recovered from the 0.4 μm mesh treatment subcompartment soil, presumably due to the lack of plant uptake. The estimated recovery of ^{33}P based on soil and plant measurements was 96% for the 2 mm mesh treatment, 81% in the 45 μm mesh treatment and 110 % in the 0.4 μm mesh treatment. This provides some evidence of the lack of precision in the measurement of soil ^{33}P determination.

The $^{total}P$ data presented in *Figure 6A* can be subdivided into organic and inorganic pools for the resin and NaOH extracts (*Figure 7*) and shows that 15-20% of the P recovered by the anion resin from the subcompartments with either roots or mycorrhizas present was either microbial P or organic P (Po) compounds. The NaOH extraction brings humic material into solution and would have been expected to have had a high Po content. This was not found to be the case, although this may be due to hydrolysis of organic compounds following the acidification of the extract for

colorimetric determination of P. No Po was observable in the $NaHCO_3$ extracts possibly for the same reason.

Figure 6. (A) $^{total}P$ and (B) ^{33}P fractions in the rhizotron subcompartments equipped with the 2 mm, 45 μm and 0.4 μm exclusion meshes at harvest. Values represent means ±SEM ($n = 4$, except control where $n = 3$).

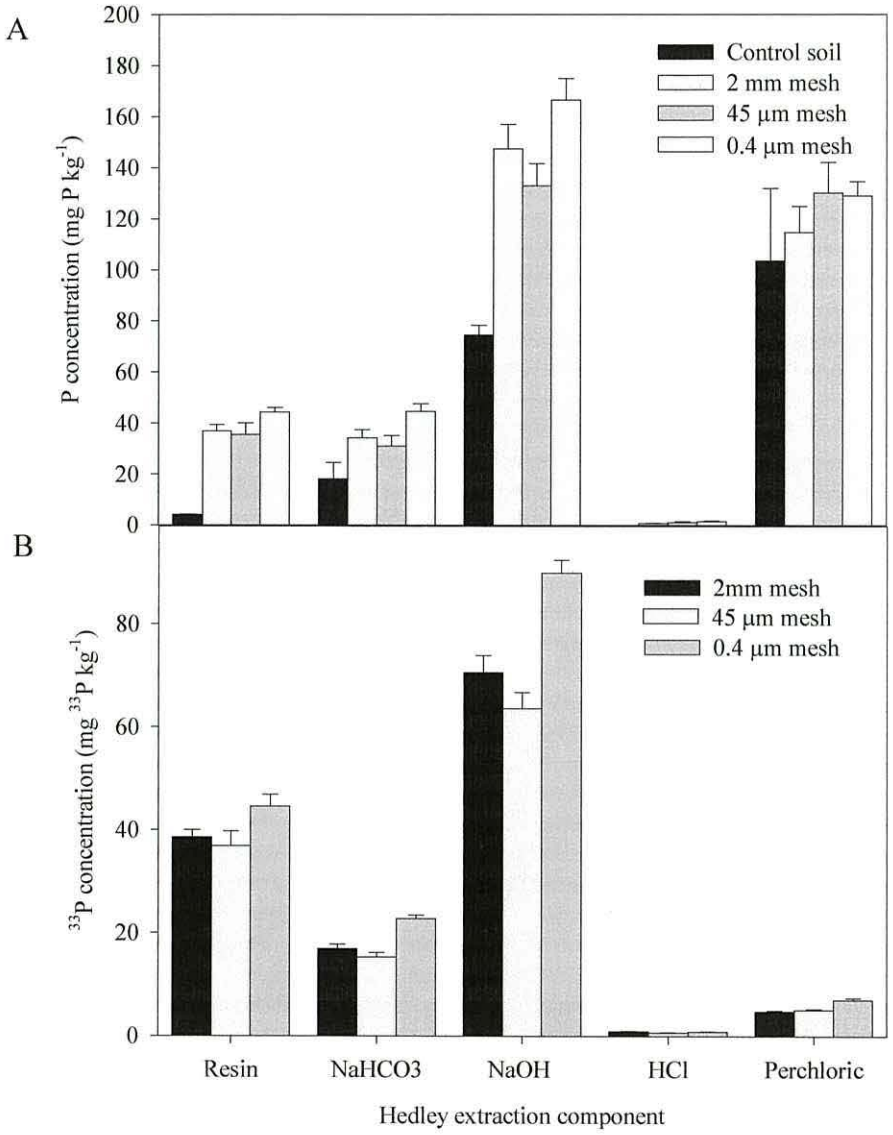
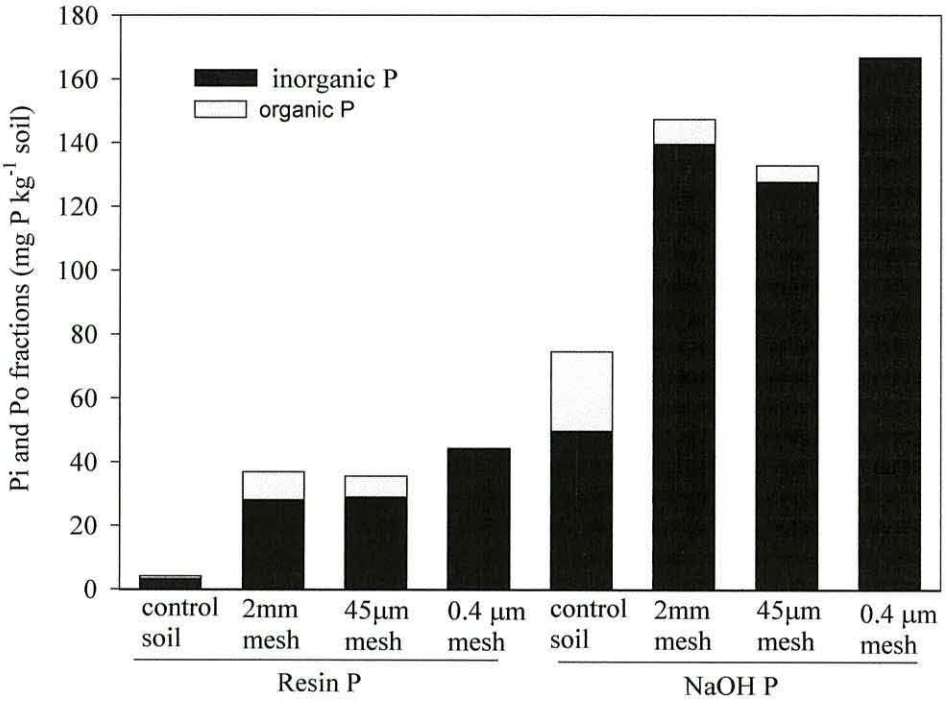


Figure 7. $^{Total}P$ including added ^{33}P, in the resin and NaOH extracts divided into Pi and Po fractions for soils from the rhizotron subcompartments equipped with 2 mm, 45 μm and 0.4 μm exclusion meshes at harvest and soil from control rhizotrons where no P had been added. Values represent means (n = 4, except control where n = 3).



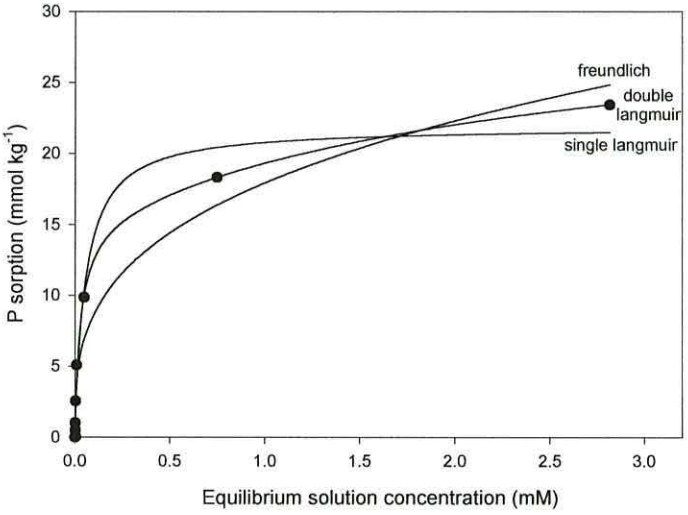
The P sorption characteristics of the Nepalese soil used in this experiment were determined and were plotted using the Freundlich, single Langmuir and double Langmuir equations with the latter best fitting the experimental data (*Figure 8A*). In this experiment, the addition of 3 mg of ^{33}P to the subcompartment soil (20 g at 20% moisture) is equivalent to a concentration of 24.2 mmol P l^{-1} solution. Using the double Langmuir equation (Equation 1) an estimated 0.41 mmol P kg^{-1} soil would remain in solution after P fertilizer addition. The estimated solid-solution phase partition coefficient of P (derived using the buffer power equation (Equation 2)) in the subcompartment soil was 76, indicating a relatively low P-sorbing soil reflective of the low soil clay content (<10 %). The maximum rate of P diffusion in the subcompartment soil was calculated using

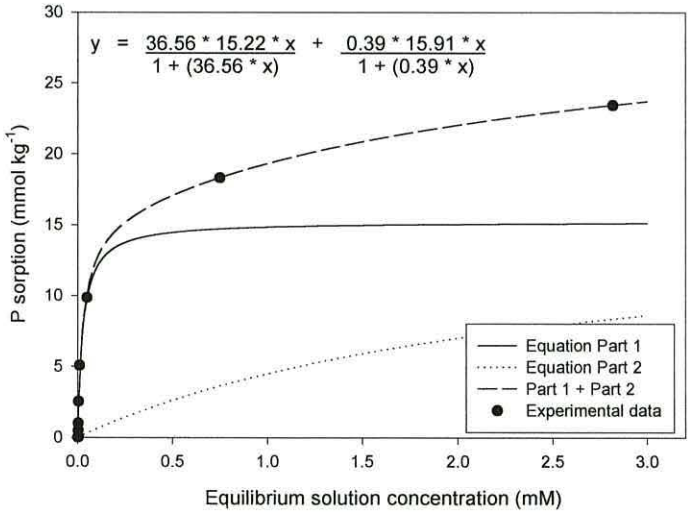
$$D_e = D_L \times \theta \times f \times P_b \qquad \textit{(Equation 3)}$$

Where D_e is the effective diffusion coefficient, D_L is the diffusion coefficient in free solution, θ is the fraction of the soil volume occupied by solution and f is the impedance factor. In making these calculations it was assumed that $D_L = 0.89 \times 10^{-5}$

$cm^2\ s^{-1}$, θ was approximately 0.1 and that the impedance factor was 0.25. This provides an estimate of the effective diffusion coefficient $3.32 \times 10^{-8}\ cm^2\ s^{-1}$ from which it can be calculated that the maximum linear diffusion potential is approximately 0.75 mm d^{-1} (Barber, 1995).

Figure 8. Panel A (top) shows P sorption isotherms for the Chitwan Eutric fluvisol soil at equilibrium P solution concentrations ranging from 0 to 3 mM. The experimental data points are shown by the symbols while the lines represent the fitting of the Freundlich, single Langmuir and double Langmuir equations to the experimental data; Panel B (bottom) shows the component parts (high and low energy sorption) and parameters for the double Langmuir equation for the same soil.





4. Discussion

The Chitwan soil used in this experiment is a sandy silt loam with a low clay content (< 10%) and low organic matter status (organic C = 0.5%). It has very weak structure and is severely depleted of mineral plant nutrients. Although N and K were added, the growth of plants was slow and older leaves senesced, leaving only 3 pairs of photosynthetically active leaves on each plant. The addition of P to the subcompartment soil appeared to only partially alleviate mineral nutrition constraints. This is supported by the slow uptake of the ^{33}P from the subcompartment, despite shoot P concentrations being at the lower end of the range observed in the field (Chapter 1, *Table* 1) and the soil having a low P buffer capacity and the added P therefore being easily available. Allowing for diffusion of ^{33}P into the buffer zone of the subcompartment, slow adsorption reactions and plant ^{33}P uptake, the concentration of ^{33}P added was sufficient to maintain soil solution P concentrations in the subcompartment in excess 10 μM. The residual resin extractable P (*Figure 6*) is substantial, and if, as it is thought, this pool represents P available to plant roots (Somasiri *et al.*, 1991), then the plants were far from depleting the weakly sorbed subcompartment P at the time of harvest.

The concentration of P added to the soil in the sub-compartment was substantially in excess of the sorption capacity of the soil and the calculated maximum daily diffusion rate (0.75 mm day^{-1}) suggests that the 5 mm soil buffer between the subcompartment mesh and the soil receiving the P addition may not have been adequate. However, only very small amounts of ^{33}P were taken up by plants with the 0.4 μm mesh treatment (*Figure 4*), and no ^{33}P was detectable on the inner side of the mesh (data not shown). The P uptake by Tithonia plants accessing subcompartment ^{33}P through the 45 μm mesh was 85% of the uptake by plants accessing subcompartment ^{33}P through the 2 mm mesh. A small proportion of ^{33}P taken up by plants in both these treatments may have been due to diffusion into the main compartment, but this does not detract from the assumption of biologically mediated transport from the 45 μm mesh subcompartment.

Arbuscular mycorrhizal (AM) colonisation of the roots facing the 45 μm mesh was confirmed by the trypan blue staining method (Koske and Gemma, 1989) and root hairs 4 mm long were observed. Root hair density and diameter were not measured. Interpretation of the results of this experiment is based on the assumption

that AM fungal hyphae associated with Tithonia rather than either diffusion across the buffer strip or root hair penetration of the mesh was responsible for mediating P uptake by the Tithonia plants in the 45 μm mesh treatment.

A broad range of circumstantial evidence has been accumulated indicating that the tissue mineral concentrations observed in Tithonia may be due to associations with AM fungi. In work deploying AM-specific PCR primers to identify species colonizing roots of Tithonia, an unusually high specificity for *Glomus* species was observed (Sharrock *et al.,* 2004). Although specificity between plants and AM fungi has been observed elsewhere, it is not clear if this phenomenon is associated with a particularly enhanced interaction (Chanway *et al.,* 1991). However, Tithonia grown in low fertility soil conditions appears to consistently show higher concentrations of those minerals most associated with uptake involving AM species than other species growing in the same conditions. Plant uptake of the minerals analysed in tissues of a range of plants from the Bharatpur region of Nepal (*Table 1*) is thought to be enhanced by AM associations with plants (George, 2000). The concentrations of P, N, Zn, Cu, and Mn observed in the leaves and stems of Tithonia are remarkably high, relative to most of the other species. Uptake of most of these nutrients, particularly P, and Zn is constrained by the combination of low solution concentration and slow rates of diffusion. The principal advantage of the AM symbiosis is thought to be associated with the large surface area of the extrametrical mycelium that allows a more comprehensive exploration of the soil than roots. This is supported by the apparent role of AM in uptake of minerals where mass flow makes only a minor contribution to transport in soil.

There has been substantial speculation that AM fungi are able to hydrolyse organic P through the secretion of phosphatases, however, the literature in this area should be considered with caution due to doubts about methods used in most of the research (Joner *et al.,* 2000). In a study of P under field grown Tithonia in Kenya, George et al. (2002[b]) found that Tithonia appeared to deplete the same soil P pools as *Zea mays*, although there was increased cycling through organic P pools under Tithonia. This finding neither excludes nor implies a key role for AM fungi under field conditions, but rather, suggests that Tithonia stimulates microbial demand for P which it is subsequently able to take up as it is mineralised. It remains unknown if the

phosphatases involved in Po hydrolysis in the rhizosphere of Tithonia are of plant, AM fungal or microbial origin.

The results of the current study suggest that under the experimental conditions tested here AM fungi associated with Tithonia were able to exploit a $^{33}P_i$ hotspot with comparable efficiency to roots plus associated mycorrhiza. There is also an indication that root exclusion in the 45 μm mesh treatment resulted in a decrease in the NaOH extractable P. This pool includes both recalcitrant Po and P associated with Al and Fe oxides and hydroxides. Although the P_i concentration of the soil solution in the subcompartment would have been high, and therefore would not favour desorption reactions by diffusion, the lower $^{total}P$ and ^{33}P in the 2 mm and 45 μm mesh treatments relative to the 0.4 μm mesh treatment indicates a shifting equilibrium between pools of differing availability. The lower observed P concentrations in the NaOH extractable pool in the 45 μm mesh treatment relative to the 2 mm mesh treatment may, however, be due to an increased Po in soils where roots were present. If this were the case, however, it might have been expected that significant microbial P would have been detected in the chloroform treated $NaHCO_3$ extracts. As this was not the case, we conclude that the microbial activity evident in the rhizosphere of Tithonia roots grown under field conditions was not replicated under the experimental conditions.

The growth of plants where only mycorrhizal hyphae could access the ^{33}P remained similar to plants without access to additional P (*Figure 2*). This suggests that the carbon cost of P acquisition was greater than the return from enhanced photosynthetic activity during the time frame of the experiment. However, in the younger shoot tissues of the 45 μm mesh treatment ^{33}P constituted a significantly greater proportion of the tissue P than in the 2 mm mesh treatment (*Figure 5*). This observation is commensurate with slower establishment of an effective extramatrical mycelial hyphal network in the subcompartment relative to roots, but also with a down regulation of plant P starvation responses as Tithonia plants increase their mycorrhizal dependence (Burleigh *et al.,* 2002). By placing a P hotspot where mycorrhizal hyphae, but not roots are able to gain access, an artificially strong pressure is being placed on the plants to commit resources to the symbiont. There has been a general observation that there is an inverse relationship between dependence on AM fungi for P acquisition and plant root hair growth (Schweiger *et al.,* 1995;

George, 2000; Smith *et al.,* 2001). The few exceptions to this have been made in tropical species growing on P limiting soils (Guissou *et al.,* 1998). A more detailed and specific study would be required to determine if Tithonia combines root hair P acquisition with mycorrhizal dependence.

The P availability conditions in the subcompartments in this experiment were designed to allow an assessment of the uptake of P by mycorrhiza relative to roots. Clearly under field conditions, the advantage of the AM association to Tithonia is in enabling access to sparingly available P in soils with high buffer capacities and low solution P concentrations. The results from the current study indicate that Tithonia possesses mycorrhizas which are effective at taking up P and that the experimental methods used provide a useful technique for the further investigation of the effectiveness of mycorrhiza associated with Tithonia to acquire P from less available P sources.

Chapter Six

Endo-mycorrhizas associated with roots of *Tithonia diversifolia* can access mineral bound P not available to roots

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Summary
Enhanced phosphorus (P) cycling using the green manure *Tithonia diversifolia* (Tithonia) assumes Tithonia is able to access soil P pools that are largely unavailable to crop plants such as maize and rice. It was previously established that associations between Tithonia and endomycorrhizal fungi were responsible for substantial uptake of ^{33}P. In the present study, we used a mesh exclusion technique to measure uptake of P by Tithonia plants from ^{33}P-labelled subcompartments into which either mycorrhizal hyphae or roots and mycorrhizal hyphae could penetrate. The subcompartment labelled P was provided as either inorganic ^{33}P (^{33}Pi), microbial ^{33}P or as ^{33}Pi bound to either Ca or Fe. Root access to the subcompartments containing ^{33}Pi associated with added cations did not increase ^{33}Pi uptake relative to mycorrhizal hyphal access only. Uptake of microbial ^{33}P doubled when roots as well as mycorrhizal hyphae were present, indicating that endomycorrhizal fungi associated with Tithonia contribute to uptake of mineral bound P, but do not participate in organic P cycles.

1. Introduction

Attempts to address declining soil fertility in tropical smallholder farms through the use of nitrogen fixing species as green manures has often met limited success due to continued phosphorus (P) constraints to economic crop production. Similar to most crop plants, most leguminous species are conservative in their uptake of P (Bieleski and Ferguson, 1983; Lambers *et al.*, 1998; Palm *et al.*, 1999) and are not able to transfer P to the plant-available pool from otherwise non–labile soil P pools. In consequence, the more soluble plant-available P pools can often become severely depleted, whereas large and potentially valuable reserves of insoluble P and recalcitrant mineral bound organic P often remain. Whilst it is appreciated that P replenishment is required to replace losses due to leaching, erosion and crop removal, in many cases increased farm income is a prerequisite to such inputs. Loss of soil organic matter (SOM) due to erosion and cultivation of vulnerable soils has compounded problems in many instances, resulting in Al toxicity, reduced water retention and decreasing microbial activity. The addition of manure can ameliorate some of the negative impacts of low SOM on P availability. Soluble Al^{3+}, which can reduce the roots ability to take up nutrients, forms complexes with humic substances

rendering it non-toxic (Haynes and Mokolobate, 2001). SOM aids water retention in soils and the rate of diffusion of P in soil increases with increased soil moisture levels while microbial P uptake following manure additions prevents P being locked up by Al and Fe oxides and hydroxides. Microbes are also capable of hydrolysing organic P thereby increasing the biologically active P pool that crop plants can exploit. Net mineralisation of P added in the manure only occurs when the microbial intrinsic requirement has been met, necessitating the application of manures with a P concentration of greater than 2.4 mg P kg dry weight^{-1} of manure (Singh and Jones, 1976; Blair and Boland, 1978). Few N_2 fixing species are capable of providing P at this concentration (Palm *et al.*, 1999) and high P animal manures such as from pig and poultry are rarely available. Furthermore, most manure types encourage high populations of mesofauna that probably exert a detrimental grazing pressure on roots and mycorrhizal hyphae.

Tithonia diversifolia (Tithonia) has been identified as a green manure that appears to meet the requirements for such material by having consistently high tissue concentrations of P, N and other minerals (particularly Zn), whilst having levels of lignin and polyphenols low enough to encourage rapid decomposition and mineralisation (Melillo *et al.,* 1982; Palm and Rowland, 1997). Agronomic trials and decomposition studies have confirmed that P from Tithonia residues is mineralised at a rate compatible with crop demand (Jama *et al.,* 2000; Sherchan, 2001). However it remains unclear whether Tithonia is little more than an effective scavenger taking up P from more labile soil P pools (Cairns *et al.,* 1998), or whether it possesses mechanisms to access less available soil P. If the latter is the case then its utilisation as a green manure can accelerate the transfer of P from the non-labile to labile soil P pools.

In the context of weathered acid soils, significant quantities of recalcitrant P can be associated with clay and amorphous minerals, both as organic and inorganic compounds of varying complexity (Stevenson, 1986). George *et al.* (2002[b]) found that Tithonia litter increased the recovery of labile inorganic P (Pi) by anion exchange resins, and also increased, the recovery of the more recalcitrant NaOH extractable P fraction consisting of inorganic-P (Pi) and organic-P (Po) held by chemisorption (Stewart and McKercher, 1982). The NaOH extractable Pi was also decreased in the presence of Tithonia. Tithonia would therefore appear to affect a transfer of P from

mineral bound forms to possibly more labile organic forms. A number of possible mechanisms to explain this phenomenon may be considered.

Possible mechanism may include the secretion of dicarboxylic and tricarboxylic acids from the rootsthat may mobilize adsorbed P through complexation reactions with metals (Jones, 1998). However, high concentrations of organic acids (> 100 μM for citrate and > 1 mM for oxalate and malate) are required to desorb P through competition for sorption sites (Jones, 1998). Some non-mycorrhizal species and particularly those with proteoid roots, notably *Lupinus albus* and *Brassica napus* (Dinkelaker *et al.,* 1989; Hoffland *et al.*, 1992), appear to mobilize P by allocating considerable amounts of photosynthetic carbon (C) to organic acid synthesis. The functional significance of this mechanism to mobilize P in less specifically adapted species is questionable as biodegradation of organic acids in the rhizosphere can be rapid (Jones, 1998). Olivares *et al.* (2002) studied organic acid exudation from Tithonia roots and observed the production of oxalate only, and at relatively low concentrations. There is therefore no compelling reason to think that Tithonia, a species known to form mycorrhizal associations effective in P acquisition (*Chapter 5)*, and which does not form proteoid roots, uses organic acid exudation to mobilise chemisorbed P.

Acidification of the rhizosphere by Tithonia has been observed in rhizotron experiments and is presumably associated with uptake of NH_4^+ as the principal form of N (Nye, 1984). While this may enhance P diffusion to the root (Nye, 1984; Barrow, 2002) the effect of a decrease in pH on mobilisation of P bound to Fe and Al oxides and hydroxides is predominantly negative. Under field conditions, acidification due to net proton efflux may be counter-balanced by the liming effects of Tithonia leaf litter.

It is conceivable that a combination of plant mechanisms could combine to enable Tithonia roots to exploit the NaOH extractable Pi pool more effectively than crop plants: (1) a decrease in rhizosphere pH to increase desorption of P and its rate of diffusion, (2) root secretion of organic acids to reduce the P buffer capacity in the rhizosphere through ligand exchange with Al and Fe hydroxides, and (3) a very low K_m but high V_{max} for the root P uptake transporter system to drive a sufficiently strong P diffusion gradient away from the solid phase and toward the root. The measurement of P uptake kinetics of Tithonia roots did not indicate that Tithonia possesses

transporters with a low K_m (*Chapter 2*). This hypothesis would therefore probably require that Tithonia produce a high density of long root hairs with the combined mechanisms creating an extended depletion zone. There is currently no root hair data for Tithonia and no reliable organic acid exudation data to enable P uptake by these combined mechanisms to be modelled.

It was previously demonstrated that Tithonia forms mycorrhizal associations that can provide the plant with substantial quantities of P (*Chapter 5*). In the current study, we continue to explore the hypothesis that P acquisition by Tithonia in soils of low available P is achieved through endomycorrhizal symbiosis. Previously a ^{33}Pi enriched subcompartment soil was applied to rhizotrons in which Tithonia plants had been established. Here the same technique was utilised, with mesh separation of the subcompartments to permit only mycorrhizal hyphae or roots (plus associated mycorrhizal hyphae) to access the radiolabelled P. In this experiment ^{33}P was applied to the subcompartments in a range of forms of differing availability: ^{33}Pi, $Fe(OH)_3$ associated ^{33}P, Ca-^{33}Pi, and a ^{33}P labelled bacterial culture. The purpose of this last treatment was to provide an organic radiolabelled P source that was similar to the labile organic soil P fraction.

2. Materials and methods

2.1. Soil

Soil was taken from the Ap horizon (0–20 cm) of a field site located in the Chitwan valley in the Bharatpur region of Nepal (27°36' N, 84°29' E; altitude 250 m; rainfall 2500 mm year^{-1}) in which *Zea mays* and wetland *Oryza sativa* had been grown in rotation. The area receives bimodal rainfall of 2500 mm per year with a main peak in September. The soil is classified as a sandy loam Eutric fluvisol ($pH_{(H_2O)}$ 5.48; organic C, 0.51%; total N, 0.03%; C:N ratio, 17; electrical conductivity (H_2O 1:1 v/v), 0.13 mS cm^{-1}) and has the following exchangeable (1 M NH_4Cl) cations ($mmol_c$ kg^{-1}): K, 24; Na, 4; Ca, 84; Mg, 60. The soil has an anion exchange resin extractable P of 0.12 mmol kg^{-1}, 0.58 mmol kg^{-1} $NaHCO_3$ extractable P and a total P content of 6.5 mmol kg^{-1}. After collection, the soil was sieved to pass 2 mm and stored at field moist at 15°C until required for experimentation.

2.2. Plant growth

Seeds of a South African ecotype of *Tithonia diversifolia* were soaked in aerated distilled water overnight and then transferred to petri-dishes containing moist filter paper for 6 d at 20°C. Two plantlets with fully expanded cotyledons and a main root axis approximately 5 cm long were then transferred to rhizotrons containing 540 g of soil packed to a bulk density of 1.3 g cm^{-3}. The rhizotrons were similar to that described by Marschner (1995) and consisted of a 150 × 150 × 20 mm box made from clear plexiglass. In the reverse of the box a 5 cm diameter hole was cut allowing a ^{33}P soil filled subcompartment to be attached (*Figure 1*). The circular subcompartment was composed of a 1 cm long section of nylon pipe, which could be divided from the main compartment by means of a mesh inserted between the two. Two mesh sizes were used to selectively prevent mycorrhizas or roots in the main compartment from entering the subcompartment containing ^{33}P labelled soil:

A. 2000 μm mesh, allowing passage of both mycorrhizal hyphae and roots
B. 45 μm mesh, allowing passage of mycorrhizal hyphae but not roots

The soil was not inoculated with mycorrhizal spores, but based on previous evidence of mycorrhizal infection of Tithonia plants growing in the same soil (see *chapter 5*) it was assumed that viable spores were present. The rhizotrons were maintained in a climate controlled growth room with 22/18°C day/night temperatures, a 16 h photoperiod and a light intensity of 350 μmol m^{-2} s^{-1}. The moisture content of the rhizotrons was maintained at approximately 20% volumetric water content by the daily addition of distilled water to the surface of the rhizotrons, with the rate of water addition calculated by daily weight loss in the rhizotrons. After four weeks, the plants were fertilised with N and K at rates equivalent to 75 kg K ha^{-1} and 150 kg N ha^{-1}, on a surface area basis, using KNO_3 and NH_4NO_3. At six weeks, when the plants had two pairs of senesced or senescing leaves, two pairs of fully expanded leaves, two expanding leaves and a stem height of 49 ± 1 mm (n = 30), the subcompartment was attached to the rhizotron.

2.3. Treatments

Phosphate was added to the subcompartments as (a) $KH_2{}^{33}PO_4$, (b) a bacterial culture in which $KH_2{}^{33}PO_4$ was fully incorporated into the bacterial tissues, (c)

$KH_2{}^{33}PO_4$ added to sufficient $Fe(OH)_3$ to adsorb all the ^{33}P or (d) $KH_2{}^{33}PO_4$ added to sufficient $CaCl_2$ to adsorb all the ^{33}P. The rate of $KH_2{}^{33}PO_4$ addition was equivalent to 10 kg P ha^{-1} (3 mg or 50 kBq $treatment^{-1}$). Each P source was applied to eight rhizotrons, four with a 2000 μm mesh and four with a 45 μm mesh.

2.4. Preparation of $KH_2{}^{33}PO_4$ sources.

Fe bound $KH_2{}^{33}PO_4$ was prepared by the addition of $KH_2{}^{33}PO_4$ to 1 M FeOH at a Fe-P ratio of 10:1. The solution was shaken for one hour at 150 rpm and centrifuged at 10,000 rpm for 10 minutes. Less than 1% of the ^{33}P remained in the supernatant, which was then removed. For the preparation of Ca bound $KH_2{}^{33}PO_4$, $KH_2{}^{33}PO_4$ was added to 1 M CaCl at a ratio of 1:40. The solution was shaken for one hour and then $NaCO_3$ (0.5 M) added at a ratio of 120 ml for every 100 ml of $KH_2{}^{33}PO_4/CaCl_2$ solution to precipitate $Ca^{33}PO_4$. After centrifugation at 15,000 rpm, less than 1% ^{33}P remained in solution; this was removed and the precipitate recovered.

Microbial ^{33}P was prepared by the incorporation of ^{33}P into a bacterial culture. Approximately 2 mg of the soil used in the experiment was mixed with 250 ml of sterile culture media containing cycloheximide (10 μg ml^{-1}) and the cultures aerated on an orbital shaker at 150 rpm and 25°C. The culture medium was as follows: (in mM) KCl, 8.0; $NaH_2{}^{33}PO_4$, 1.16; $MgSO_4$, 2.0; $CaCl_2$, 0.2; KNO_3, 1.0; glycine, 2.0; and micronutrients (in μM) H_3BO_4, 8.1; $CuSO_4$, 0.25; KI, 0.6; $FeCl_3$, 1.25; $MnSO_4$, 2.6: $NaMoO_4$, 0.97; $ZnSO_4$, 2.5; and vitamins (in μM) biotin, 0.08; Ca-pantothenate, 4.2; folic acid, 0.001; inositol, 55; niacin, 3.3; P-aminobenzoic acid, 1.5; pyridoxine hydrochloride, 1.9; riboflavin, 0.5; thiamine, 1.2. Glucose (50 mM) was added as a C source. The optical density of the solution at 600 nm provides a measure of protein content. After 12 days there was no further increase in the optical density and the incubation was stopped. The culture was centrifuged to separate the culture from the remaining fluid. Less than 1% of the ^{33}P remained in solution.

^{33}P (in the prepared forms) was added to half of the subcompartment soil at a rate equivalent to 10 kg P ha^{-1} (3 mg or 50 kBq $treatment^{-1}$; specific activity = 100 Tbq $mmol^{-1}$; Amersham Pharmacia Biotech, Little Chalfont, Bucks). The ^{33}P was thoroughly mixed with sufficient moist soil for all the replicates of each treatment. The ^{33}P labelled soil was packed into the subcompartment to a depth of 0.5 cm and bulk density of 1.3 g cm^{-3}. A further 0.5 cm layer (buffer strip) of soil containing no added P was placed on top of the radiolabelled soil layer, the mesh placed over the

buffer strip end, and the subcompartment attached to the back of the rhizotron (*Figure 1*). Each treatment was replicated four times. The individual rhizotrons were positioned within the growth cabinet at random (using random numbers to allocate position) and rotated systematically each day to minimise positional effects.

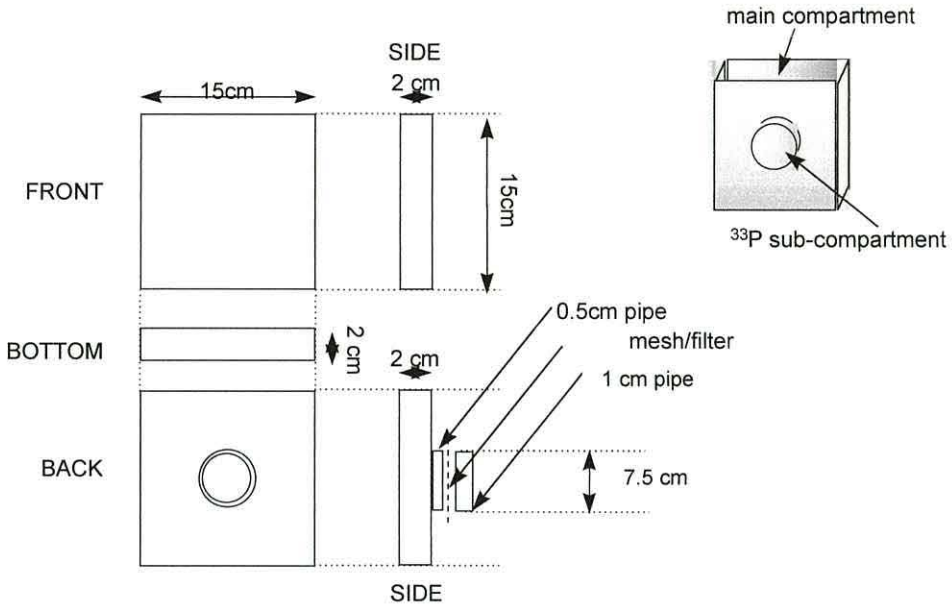


Figure 1. Schematic representation of a rhizotron, showing the assembly of the subcompartment.

2.5. *Rhizotron harvesting*

After four weeks exposure to ^{33}P, the rhizotrons were harvested. Above ground plant parts were removed, dried at 60°C (24 h) to determine dry weight, ashed (500°C, 24 h) and the residue dissolved in 1 M HCl. The ^{33}P content of the HCl solutions was then determined by liquid scintillation counting using an HCl-compatible scintillation fluid (Optiphase Hisafe 3, EG&G Wallac, Milton Keynes) and a Wallac 1304 liquid scintillation counter (EG&G Wallac, Milton Keynes). The P content of shoot material was determined in an aliquot from the 1 M HCl solutions using the colorimetric procedure of Murphy and Riley (1962). Roots contained within the main rhizotron compartment were removed by hand and dried at 60°C (24 h) to determine dry weight.

After removal of roots (from the 2 mm mesh samples only), soil from the subcompartment was subjected to a sequential P fractionation procedure according to the methods of Sibbesen (1977) and Hedley *et al.* (1982). Briefly, 1 g of soil was placed in a 50 ml polypropylene centrifuge tube containing 30 ml of deionized water

alongside one resin bag containing 4 ml anion exchange resin in the bicarbonate form (Amberlite 420, bead diameter > 30 mesh) and another containing 2.8 ml cation resin (Amberlite 120) in the NH_4^+ form to maintain the balance of the electrolytes. Resins were pre-treated according to the methods of Somasiri *et al.* (1991). The tubes were then shaken at 200 rpm on an end-over-end shaker for 16 h at 20°C, the resin bag removed, washed with deionized water and the P contained on the resin displaced with 30 ml of 0.5 M HCl (16 h, 20°C). The remaining soil was centrifuged (10,000 *g*, 30 min), the supernatant solution removed and then 30 ml of 0.5 M $NaHCO_3$ added and the soil shaken for 16 h at 20°C. After removal of the $NaHCO_3$ supernatant solution by centrifugation, repeat extractions of the remaining soil were performed with 0.1 M NaOH (× 2) and then 1 M HCl. In each case, the supernatant solution was retained for P determination. After the HCl extraction, the remaining soil residue was digested with perchloric acid (10 ml; 4 h, 200°C) for the determination of residual soil P. The ^{33}P content of the solutions was then determined by liquid scintillation using a Wallac 1304 liquid scintillation counter (EG&G Wallac, Milton Keynes). The P content in each extract was determined by flow injection analysis (Perstorp Flow Injection 3000 Analyzer; Perstorp Analytical, Maidenhead, England) based on the molybdate blue procedure of Murphy and Riley (1962).

2.6. Statistical analysis

The experiment had a 2 level factorial design, with four treatments (P sources) for each interaction (roots and root exclusion). The significance of interactions between P source and access to the P source were determined by two-way analysis of variance (ANOVA). Tukey's test was used for multiple comparisons between the sample means where the ANOVA F statistic was significant.

3. Results

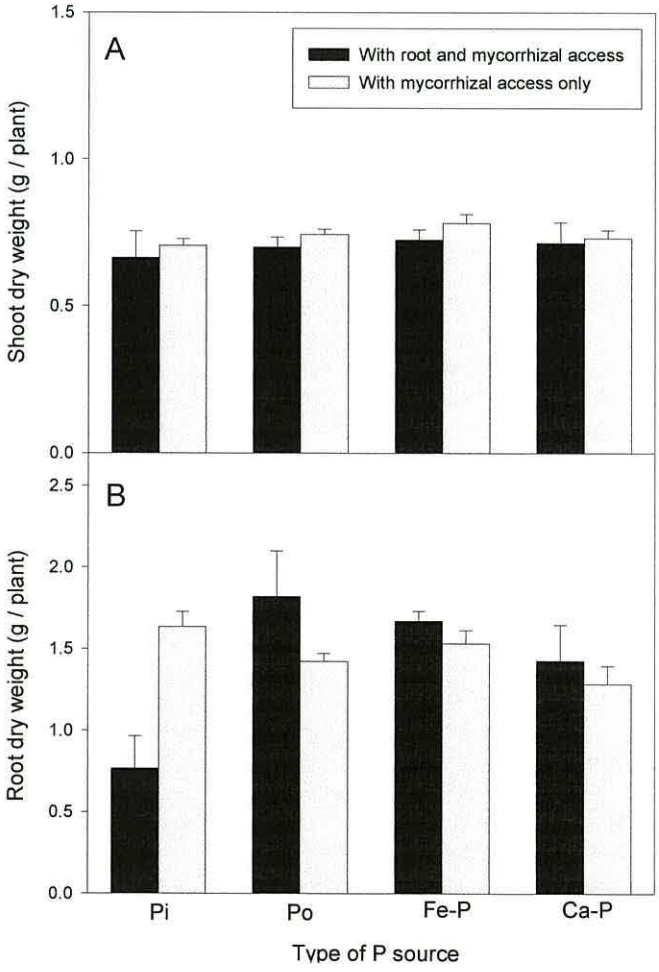
Tithonia is characterised by rapidly senescing lower leaves, probably occurring as a response to shading, stress or ontogenetic drift (Hopkinson, 1964; George *et al.*, 2002[b]). When grown in the Nepalese soil which possessed an intrinsically low fertility (Eutric fluvisol), Tithonia growth was morphologically identical to that grown in the field with typically only 3–4 photosynthetically active pairs of leaves followed by progressive senescence towards the stem base.

The moisture content of the soil within the subcompartments was not significantly different (P>0.05) to that in the main compartment at the time of harvest, indicating that in all treatments water redistribution within the rhizotrons was not inhibited by the presence of a mesh, or altered by the ^{33}P labelled material added.

3.1. P uptake

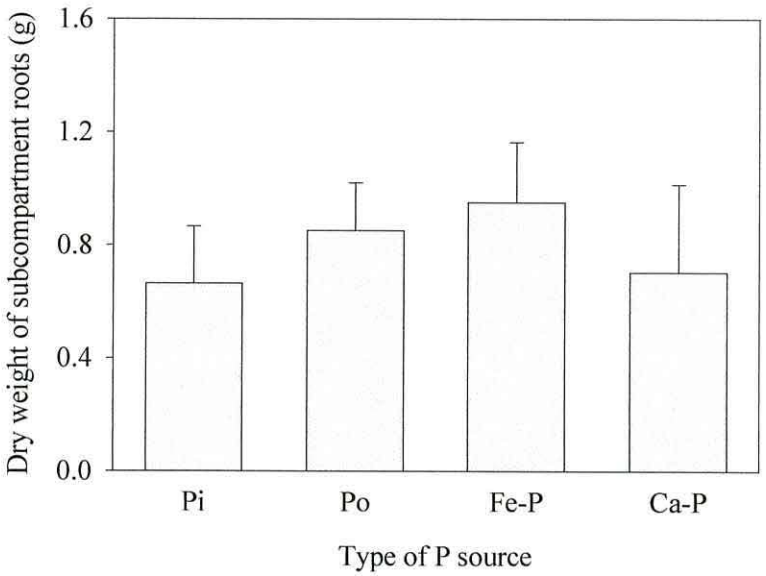
The dry weight distribution between shoots and roots for each of the treatments is shown in *Figure 2*. No significant differences were observed in above ground biomass production (*Figure 2A*), either between mesh treatments or between ^{33}P sources (P>0.05). In contrast, the amount of below ground biomass (*Figure 2B*) of plants with root access to the subcompartment containing ^{33}Pi was significantly less than in all other treatments where either the ^{33}P was in less available forms or only mycorrhizal hyphae were able to access the P source (P<0.05). The high root to shoot ratios of plants without root access to an available P source suggests that the plants in these treatments were P stressed (Lynch and Brown, 2001).

Figure 2. Dry weights at harvest of (A) shoots and (B) roots of Tithonia plants grown in rhizotrons with roots plus mycorrhizal access or mycorrhizal access only to subcompartments containing ^{33}P sources of differing availability. Values represent means ±SEM (n=4).



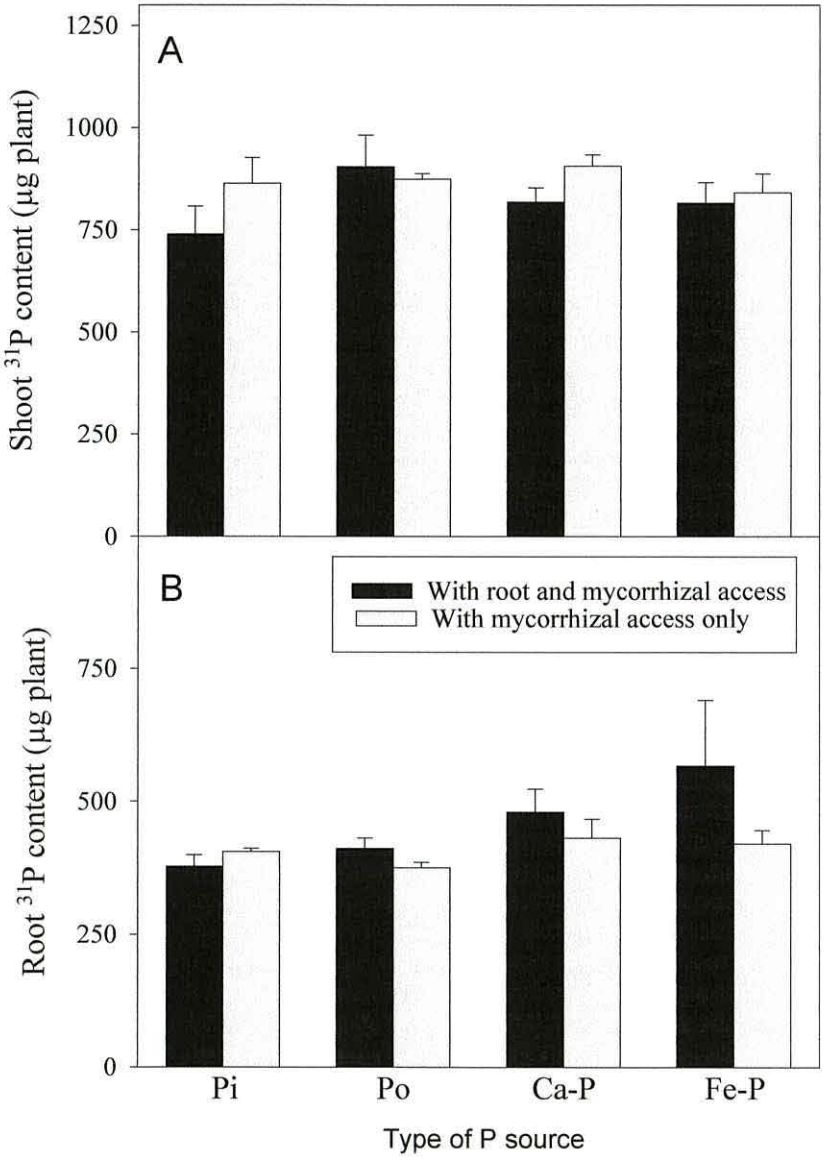
The dry weight of roots colonising the subcompartment soil in the 2 mm mesh treatments were considered separately to asses any significant alteration in root growth as a response to the different P sources (*Figure 3*). No significant differences were observed ($P>0.05$), indicating that differences in ^{33}P uptake due to P source treatment were not due to variable colonisation of the subcompartment by roots.

Figure 3. Dry weight of Tithonia roots in rhizotron subcompartments containing ^{33}P sources of differing availability.



Uptake of 'native' ^{31}P, predominantly from the main compartment soil, was unaffected by treatment (*Figure 4*), both in terms of total uptake and allocation of P between roots and shoots.

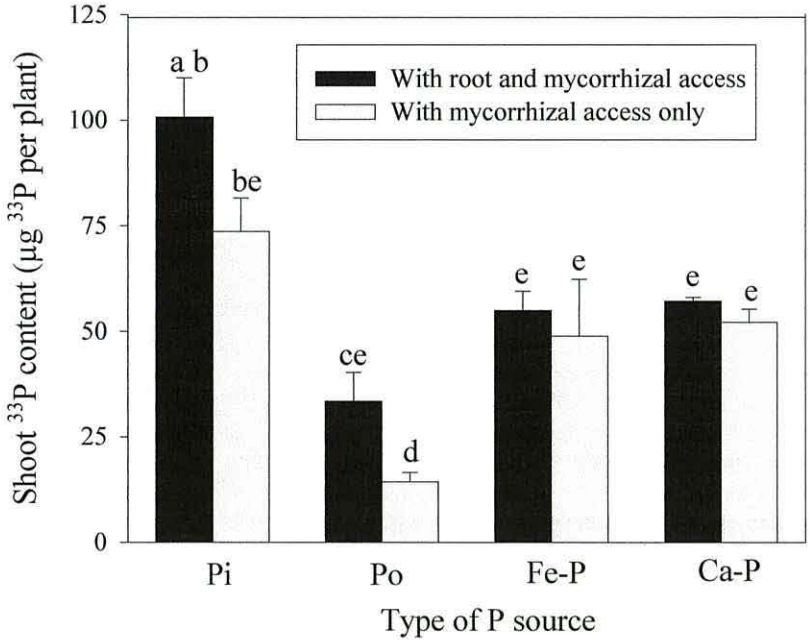
Figure 4. Concentrations at harvest of ^{31}P in (A) shoots and (B) roots of Tithonia plants grown in rhizotrons with root plus mycorrhizal access or mycorrhizal only access to subcompartments containing ^{33}P sources of differing availability. Values represent means ±SEM (n=4).



It was not possible to remove all soil from the roots, and the residue caused severe colour quench interference to the liquid scintillation count measurement of ^{33}P. As a result it was not possible to obtain measurements of the accumulation of ^{33}P in roots. The quantities of ^{33}P taken up and accumulated in the shoots for the different treatments are presented in Figure 5. The restriction of access to mycorrhizal hyphae, but not roots, resulted in significantly less uptake from the ^{33}Pi P source and the microbial ^{33}P source compared with treatments where root access was uninhibited. By contrast, root access did not increase uptake of either of the mineral bound P sources.

Clearly, of the added substrates, microbial P was the least available to either hyphae or to roots. Similar quantities of Fe and Ca associated ^{33}P were taken up by roots (and associated mycorrhiza) and by mycorrhizal hyphae, and transferred to shoots. The quantities of ^{33}P present in shoots at harvest from these treatments were similar to the root excluded ^{33}Pi treatment.

Figure 5. Concentration of ^{33}P in *Tithonia diversifolia* shoots at harvest when grown in the rhizotrons with a range of ^{33}P sources of differing availability. Letters indicate significant differences ($P<0.05$). Values represent means ±SEM ($n=4$).

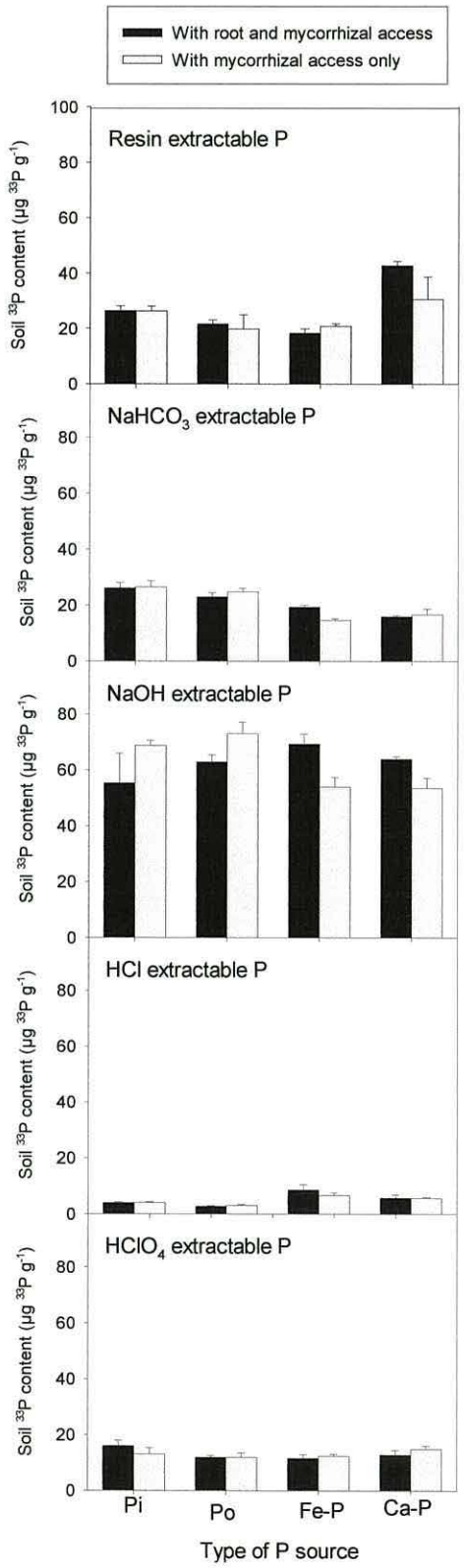


3.2. ^{33}P subcompartment soil fractionation

The results of ^{33}P subcompartment soil fractionation are presented in Figure 6. The purpose of conducting this analysis was to determine if the pattern of ^{33}P depletion in the different soil P fractions was dependant on the nature of the P source and also if the pattern of ^{33}P depletion varied depending on the presence of roots in addition to mycorrhizae.

There were lower levels of resin ^{33}P in the microbial ^{33}P treatments than in the ^{33}Pi treated subcompartment soil ($P<0.05$). Resin ^{33}P was greatest in the Ca associated ^{33}P treatment, but Fe associated ^{33}P in this pool was similar to microbial ^{33}P. Root exclusion resulted in an increased depletion of Ca associated ^{33}P, but there was no variation in depletion of resin ^{33}P for the other ^{33}P sources related to access to the subcompartment.

Figure 6. ^{33}P fractions in the soil recovered from rhizotron subcompartments treated with ^{33}Pi, microbial ^{33}P, FeOH associated ^{33}P or Ca associated ^{33}P following a period of uptake by either Tithonia roots with mycorrhizas or mycorrhizal hyphae associated with Tithonia. Values represent means ±SEM (n=4).



A similar quantity of ^{33}P was recovered in the $NaHCO_3$ extractible pool for each treatment, although the quantities recovered from the ^{33}Pi and microbial ^{33}P treatments were larger than those from the mineral associated ^{33}P treatments. Root exclusion resulted in an increased depletion of Fe associated ^{33}P, but there was no variation in depletion of $NaHCO_3$ ^{33}P for the other ^{33}P sources related to access to the subcompartment.

NaOH extracted more ^{33}P than the other extractants used. A pattern emerged here in which the mineral associated ^{33}P treatments showed a greater depletion with mycorrhizal hyphae access only, while the microbial ^{33}P treatment and probably the ^{33}Pi treatment were accessed more effectively in the presence of roots.

Only low levels of labelled P were recovered in the HCl extract. The amounts were greater in the mineral treatments, but there was no root exclusion effect. There were no differences in the residual perchloric acid digested fraction.

4. Discussion

A number of potential methodological problems require consideration in the interpretation of the results of this experiment and suggest that further work is required.

The diameter of root hairs is in the range 5–17 μm (Jungk, 2001), but their passage through a 45 μm mesh is still thought to be restricted. Other studies have used 30 μm mesh for this purpose (Li et al. 1991), and a 53 μm mesh has been used to create a subcompartment available to root hairs but not roots (Gahoonia and Nielsen, 1998). Further work to confirm that the 45 μm mesh effectively excludes root hairs from Tithonia would add credibility to the current results.

The bacterial ^{33}P culture contained an unknown diversity of organisms and was placed into the subcompartment soil as a living culture. Although the culture had ceased to increase in optical density, it was not known what nutrient was limiting the increase in bacterial populations. It is therefore not possible to predict the rate of decline of bacterial numbers in the soil, or the availability of Po substrates for hydrolysis by extra-cellular phosphatases. Furthermore, it is known that some bacteria and free-living soil fungi are antagonistic to endomycorrhizal fungi (Gryndler, 2000). The low levels of ^{33}P uptake by mycorrhizal hyphae and

subsequent translocation to shoots may be associated with insufficient phosphatase appropriate to the substrate, but equally it may be that the hyphae were not able to establish in this medium. With retrospect, therefore, it would have been advantageous to have subjected the bacterial culture to sonification to lyse the bacterial cells. Had this been followed by anion resin filtration to remove inorganic P, it would be possible to interpret the results with greater confidence.

The ^{33}P microbial culture and the prepared mineral bound ^{33}P additions to the subcompartments appeared to constitute P additions of lower plant availability than the ^{33}Pi addition, based on actual uptake. This however was not supported by the sequential fractionation of ^{33}P from the subcompartments at the end of the experiment (*Figure 6*). The calculated recovery of ^{33}P added to the subcompartments was in the range of 85-95 %, without significant differences between treatments. This suggests that despite a degree of error arising from scintillation counts in soil extracts with high colour and chemical quench effects, that the data is approximately correct. A possible explanation for the apparently similar concentrations in the labile P fractions extracted by anion resin and $NaHCO_3$, is that these extracts include a significant amount of microbial P. Difficulties were encountered in determining the microbial P content of the extracts as the P concentration in the resin and $NaHCO_3$ extracts is sufficiently dilute as to be near the limits of detection for the colourimetric measurement of P. This combined with the hydrolysis of Po compounds by the reagents made it impossible to obtain a realistic measurement of microbial P in fumigated $NaHCO_3$ extracts. An alternative explanation is that the resin extracts microbial P.

The principle of the NaOH extractant is that by raising the pH of the solution, P bound to Al and Fe is brought into solution together with hydrolysed Po. It would therefore have been expected that this extractant would have recovered significantly more ^{33}P in the Fe treatment. That this was not the case suggests that the 0.1 M NaOH solution effectively hydrolysed Po in all treatments and exceeded the pH buffering capacity of the Ca-P treatment, bringing the P into solution. Thus, although the concentrations in the NaOH extracts are similar the processes were different. This explanation is supported by the negligible recovery of Ca-P in the 1 M HCl extract.

The shoot ^{33}P concentrations indicate that the availability of the different P sources was, however, variable (*Figure 5*), suggesting that further work is required to modify the soil P fractionation method to match the synthetic P additions used. Furthermore, if the equilibrium of ^{33}Pi in the soil solution and resin extractable P

fractions, were measured over the time of potential uptake, (without roots or mycorrhizal hyphae being present) it would be possible to assess the comparative availability of the P sources.

The Chitwan soil used in this study is not strongly P adsorbing (see *Chapter 5*), but after over 20 years of continuous cultivation with minimal fertility management the soil has a low cation exchange capacity, low soil organic matter, weak soil structure, and a generally low fertility. Despite N and K additions, the growth of Tithonia plants was slow and the addition of P to the subcompartment soil appeared to only partially alleviate mineral nutrition constraints. This is supported by the slow rate of uptake of ^{33}P from the subcompartment and a low P content in the shoots.

These cautions aside, the differential uptake of ^{33}P described for each treatment in *Figure 5* provides a useful indication of the different roles the roots and associated mycorrhiza play in uptake of P by Tithonia. The data in *Figure 5* suggests that uptake of mineral bound P forms by Tithonia is primarily mediated by AM fungi associated with the roots, while roots alone take up P from more labile and organic pools.

In a study of mycorrhizal colonisation of Tithonia in root samples taken from a wide range of sites across the world, Sharrock *et al.,* (2004) found widespread AM colonisation of Tithonia roots, almost specifically with *Glomus* species. Proponents of the importance of mycorrhizal symbiosis have invoked a broad array of possible mechanisms through which plant health and nutrition can be enhanced (Smith and Read, 1997). However, probably the most significant feature of AM fungi is the extensive extra-mycelial hyphae that provide an indirect increase in surface area for nutrient uptake of up to 100 fold relative to roots (George, 2000). AM hyphae have been found to have P uptake transport kinetic characteristics highly favourable to the creation of a P diffusion gradient away from mineral surfaces, with an estimated K_m of 0.17 μM and an I_{max} of 255 nmol m^{-2} s^{-1} (Schweiger and Jakobsen, 1999). This equates to a potential influx rate two orders of magnitude greater than that observed in plant roots (Smith *et al.*, 2001). Using a mathematical model, the uptake surface area of hyphae has been found to be sufficient to account for the reported levels of uptake of P by AM fungi (Sanders and Tinker, 1971). The results of the current study are compatible with this understanding of the P uptake mechanisms of AM fungi. Where

hyphae only were able to access the mineral bound ^{33}P, uptake to Tithonia shoots was not significantly different from treatments where roots and hyphae had access to the same ^{33}P source. This suggests that in both situations it was hyphae rather than roots that were primarily accessing the mineral bound ^{33}P, as there was no additional benefit due to the presence of roots.

Conversely, the presence of roots in the subcompartment doubled the uptake of ^{33}P from the microbial source relative to the presence of hyphae alone. This supports the arguments of Joner *et al.,* (2000) that AM hyphae are not able to secrete phosphatases in sufficient quantities to hydrolyse soil organic P. George *et al.* (2002[c]) found that acid phosphatase activity increases in the rhizosphere of Tithonia relative to bulk soil. Due to the high levels of protease present in rhizosphere soils, and the low mobility of phosphatase in soil, it is most probable that rhizosphere phosphatase activity is primarily due to microbial secretions. It is conceivable that Tithonia, as has been shown for other species, is able to exert a selective pressure though root exudates to obtain a favourable rhizosphere microbial diversity (Grayston *et al.,* 1998).

Under field conditions Tithonia leaf litter may be creating micro-ecological conditions unfavourable for rapid mineralisation. Under *Lupinus augustifolius,* Osler *et al.* (2000) observed an increase in micro-arthropod populations associated with senesced leaves. Interestingly however, the resulting decrease in mineralisation due to a decline in fungal and bacterial grazers did not occur under lupins but rather under wheat, the succeeding crop, suggesting that lupins were exerting some control on mite populations. It is conceivable that the identified insecticidal phenolics produced by Tithonia (tagitinins) (Dutta *et al.*, 1993) serve to inhibit soil invertebrates in the leaf litter and assist in the development of a balanced ecology conducive to rapid mineralization. High collembola populations in leaf litter have been linked to a decrease in mycorrhizal infection and P uptake due to grazing of both hyphae and fine roots (Harris and Boerner, 1990). This hypothesis may therefore also explain the improved growth in crops grown following Tithonia (Cairns *et al.,* 1998).

Microbial residues can become resistant to mineralisation if adsorbed to clays and oxides (de Neergaard and Magid, 2001). The Chitwan soil, although low in organic matter, is also low in clays and oxides. Further studies should be made using strongly adsorbing soils and characterised microbial residues to investigate the influence of the rhizosphere of Tithonia on the sorption of residues. In particular, the production of citrate in the rhizosphere is of interest as the most effective ligand for

the desorption of organic P (Hayes *et al.,* 2000). This may be produced either as a root exudate or in substantial quantities by soil microbes such as *Aspergillus niger* (Wang and Liu, 1998).

Based on these results and work reported in previous chapters it is now possible to develop a hypothesis to explain the reported high P concentrations in Tithonia residues. The exudation of flavonoids and other compounds from the roots of Tithonia facilitate the rapid development of effective mycelia associated with the roots (Harrison, 1997). AM colonisation of the roots enables Tithonia to establish in soils with high P sorption characteristics, with mineral bound P, not readily available to roots being taken up into the shoots. Feedback inhibition associated with regulation of plant P uptake is reduced as a result of AM colonisation of the roots, resulting in continued uptake despite tissue P concentrations in excess of immediate plant requirements. Ecologically, Tithonia behaves as a conservative opportunist, senescing all but the most photosynthetically active leaves when under stress. Although P is relatively mobile in plant tissues (Marschner, 1995), between 25 and 45% of P taken up into the shoots returns to the soil in senesced leaf litter. Tithonia leaf litter rapidly decomposes and the plant lateral roots, which form a dense mat near the soil surface, reabsorb mineralised nutrients, including P. The results reported here are compatible with other studies that suggest that the roots of Tithonia are probably adapted to facilitating these processes.

Chapter Seven

Phosphorus acquisition by wheat from organic and inorganic P fertilizers: impact of arbuscular mycorrhizas and integrated nutrient management strategies

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Summary

Investment in phosphorus (P) capital in the high P-adsorbing soils of the tropics is constrained by the limited resources of small-scale farmers. Small P additions can provide minimal yield improvements. When applied in conjunction with suitable organic matter, a decrease in the P buffering capacity of the soil, combined with short term microbial immobilisation, can increase the proportion of added P taken up by crops following application. The green manure plant *Tithonia diversifolia* (Tithonia) has been identified as a species well suited to this application due to its high tissue P concentrations and decomposition characteristics. In a controlled environment experiment, wheat (*Triticum aestivum* cv. Abbott) was grown in rhizotrons equipped with dual subcompartments. Radiolabelled ^{32}P fertiliser and ^{33}P Tithonia residues were placed in combinations to determine the interactions between the two P sources and the soil describing P availability to wheat plants. All treatments were performed with root exclusion meshes (45 and 2000 μm) separating the subcompartments from the bulk soil to elucidate the role played by mycorrhizas in combined fertiliser applications. Mycorrhizal $^{32/33}P$ uptake from the subcompartments was similar to roots in all treatments. Uptake of added P was optimised when ^{33}P from Tithonia was mixed with fertiliser ^{32}P and resulted in an increased availability of Tithonia ^{33}P.

1. Introduction

The poverty associated with tropical low-external-input agriculture is considered to be in large part due to fertility constraints (World Bank, 1996). Among the constraints to fertility, phosphorus (P) is among the most recalcitrant. Acid soils with a high phosphorus fixation capacity characterize many of the areas of the tropics where small-scale low-input farming is practised (Buol *et al.,* 1980; Sanchez and Salinas, 1981), with highly P-sorbing oxisols and ultisols accounting for 23% and 20% of tropical soils, respectively (Sanchez and Salinas, 1981). Phosphorus is essential for plant growth, being a constituent of nucleic acids and phospholipids as well as being involved in energy transfer and serving a range of regulatory functions (Marschner, 1995). Where crop plants are grown under P limiting conditions, low yields are associated with slow crop canopy closure, resulting in leaching of mobile mineral nutrients and loss of topsoil by erosion (Fairhurst *et al.,* 1999). Furthermore, the resulting soil degradation undermines the potential of the affected land to be brought into more intensive production in the future (Weischet and Cariedes, 1993).

P constraints are therefore part of a cycle of poverty affecting hundreds of millions of people both now and for the foreseeable future.

Oxisols and some less fertile Ultisols have been used traditionally for shifting agriculture and low intensity grazing. The addition of large amounts of P fertiliser can make oxisols very productive (Sanchez, and Uehara, 1980), and the combined input of fertilisers and green manure plants can make even degraded Ultisols highly productive (Sanchez and Salinas, 1981).

In many areas poor access to agricultural credit and weak transport infrastructure make the cost of P fertilizers prohibitive and their availability minimal (Sanchez *et al.*, 1997). Furthermore, in acid soils, low P fertiliser applications can be ineffective due to a high P sorption capacity, with little of the added P being taken up by plants prior to becoming absorbed to Al or Fe oxides (Barrow, 1983). As a result, use of P fertilisers by resource poor farmers in the tropics is minimal.

The sustainable management of tropical soils necessitates organic additions to replenish soil organic matter (SOM) mineralised during cultivation under the combined influences of high humidity and high temperature. It has long been recognised that the addition of organic matter to soils can increase plant available P (Iyamuremye and Dick, 1996). This arises from a range of effects that depend on the nature of the organic additions and soil conditions. The addition of cations in the organic matter can decrease soil acidity (Oades *et al.,* 1989; Haynes and Mokolobate, 2001). This, and other less direct processes that alter soil pH, influence a broad range of processes affecting P cycling and plant growth (Hoyt and Turner, 1975). Organic anions added directly in the organic matter or formed during decomposition may become absorbed to mineral P sorption sites (Iyamuremye and Dick, 1996) and so reduce the P sorptivity of the soil. The addition of carbon and nitrogen leads to an increase in microbial activity and can stimulate the mineralisation of organic P compounds (Gressel and McColl, 1997). The addition of P as inorganic P (Pi; Iyamuremye *et al.*, 1996a) or labile organic P (Po; Iyamuremye *et al.*, 1996b) can directly reduce P adsorption potential whilst improved mineral nutrition, especially N and K, can lead to improved root growth and functioning. In water-saturated organic matter, the addition of carbon substrates can lead to the development of localised reducing environments that can release adsorbed P (Scalenghe *et al.,* 2002).

Al toxicity is frequently associated with P-deficient acid soils, and is also ameliorated by the various processes associated with additions of organic matter. The

addition of green manure can decrease the concentration of exchangeable Al and monomeric Al in the soil solution (Haynes and Mokolobate, 2001). An increase in pH will cause Al in both pools to precipitate as hydroxy Al species (e.g. $Al(OH)_3$; Pocknee and Sumner, 1997), whilst Al can be complexed to decomposing residues in the solid phase and Al in solution can form complexes with dissolved organic matter. The reduction in Al toxicity directly enhances nutrient acquisition by roots.

In recognition of the benefits of organic additions, tree and herbaceous N_2 fixing species that provide N-rich organic residues for incorporation into soil have been successfully incorporated into farming practices in many areas (Sanchez *et al.,* 1997). This has proven particularly important in areas where population pressures have reduced opportunities for long fallows and the supply of animal manure is constrained by limited grazing. However, for net mineralisation of P from organic residues to occur, an initial tissue P concentration of more than 0.25% is required (Singh and Jones, 1976; Blair and Boland, 1978), and this is higher than the concentration in tissues of most N_2 fixing plants used as green manures (Palm, 1995).

It is a characteristic of weathered acid soils that the discrepancy between total soil P and P pools available to plants is particularly large, with most soil P being either strongly sorbed to mineral surfaces or in recalcitrant organic forms (Anderson, 1980; Stevenson, 1986). Consequently, there has been considerable research interest in identifying plant species that can recover less available soil P so that it may be brought into the more active biotic P cycle of crop systems (Palm *et al.*, 1999). Plants suitable for use as green manures for enhanced P cycling need to have high mineral nutrient concentrations and the potential for net P mineralisation proportional to plant P demand. The organic material should also have a low-to-moderate lignin and polyphenol content if microbial immobilisation of N and P is to be avoided (Melillo *et al.,* 1982; Palm and Rowland, 1997).

The 'Mexican Sunflower', *Tithonia diversifolia*, (subsequently referred to as Tithonia) is a member of the Asteraceae and has been identified as a high potential species for this application (Nagarajah and Nizar, 1982; Gachengo, 1996; Buresh and Niang, 1997; Nziguheba *et al.,* 1997; Cairns *et al.,* 1998; Jama *et al.,* 2000). Tithonia has been found to have P concentrations in the range of 0.3 to 0.4% of shoot dry weight (Jama *et al.,* 2000) when growing in non-cultivated soils. Although biomass production of Tithonia can be significantly lower than fast growing legumes (Sherchan, 2001), it has long been valued as a soil improver in Indonesia (Cairns *et*

al., 1998) and residues decompose rapidly. As Tithonia is now widely distributed beyond its native Mexico and is common throughout the humid and sub-humid tropics of central and South America, Africa and Asia (La Duke, 1982) it is a resource available to small farmers in much of the tropics affected by P constraints to crop production.

There was initial speculation that Tithonia was accessing soil P pools not available to most plants (Buresh and Niang, 1997), but this has yet to be proved. There have, however, been interesting field studies indicating that combined applications of Tithonia and small amounts of soluble P can increase yields of maize (*Zea mays* L.; Nziguheba *et al.,* 1998), although it is unclear if this is a result of changes in P sorption characteristics, increased soil microbial activity or a general enhancement of plant nutrition. It has also been reported that an application of 5 tonnes dry weight ha^{-1} of Tithonia leaf and soft stem biomass containing 14 kg P ha^{-1} P could supply the crop P equivalent to an application of 19.6 kg P ha^{-1} of triple super phosphate (TSP, Palm *et al.,* 1999). These results suggest that the rapid mineralisation of P from Tithonia, combined with the broader nutritional benefits and changes in P sorption characteristics, significantly increases the availability of P to crops.

In particular, the finding that Tithonia mixed with TSP resulted in a greater crop uptake of phosphate than when the same total P was added solely as TSP (Nziguheba *et al.,* 1998) warrants further investigation. It is generally recognised that the addition of green manure P should compliment applications of mineral fertiliser P in P limiting soils (Sanchez *et al.,* 1997; Nziguheba *et al.,* 1998; Horst *et al.,* 2001). Certainly, enhanced depletion of soil P reserves without replenishment constitutes a theft from future generations, but if farmers are to invest in P replenishment there has to be an economic return on investment. If the increased yield from N fertilization is greater than the same monetary investment in P fertilizer, farmers cannot be expected to invest in P when capital is very limited. It is therefore of particular interest to understand the mechanisms involved in enhanced uptake of P from combined mineral and green manure P additions as this suggests a technology that might be able to alter the economics of investment in small mineral P applications. The greater the contribution to increased yield in the year of application the more farmers may be expected to invest in P.

The present study was undertaken to verify the findings of Nziguheba *et al.* (1998) under controlled environment conditions and to determine the dominant processes influencing P uptake by wheat from combined Tithonia and Pi soil treatments. The field studies that showed a benefit arising from combined P fertilizer applications were not able to show whether Tithonia or inorganic Pi was becoming more accessible to plants as a P source. Nor could these studies provide information about the reasons for the benefit of combined treatments. This study considers three hypotheses: (a) increased P uptake is plant-driven, with improved general plant nutrition leading to better plant growth and an enhancement of P acquisition faculties, (b) increased P uptake arises from a reduction in soil P buffering capacity, and (c) decomposing Tithonia stimulates an increase in AM mycorrhiza associated with the crop plant resulting in an enhanced uptake of adsorbed P.

Arbuscular or endomycorrhizae (AM) associations are formed between most angiosperms and about 120 fungal species belonging to six genera of fungi in the order Glomales of the Zygomycetes (Morton and Benny, 1990). They are obligate symbionts that form a dense sheath around lateral roots where exchange of nutrients between plant and fungi occurs (Smith *et al.*, 2001). The fungus obtains carbon from the host plant as simple sugars and grows hyphae that extend out beyond the root nutrient depletion zone where it acquires minerals, predominantly P and others whose availability to plants is controlled by diffusion, and transports them back to the host plant (Smith and Read, 1997). AM fungi are probably the most important and widespread plant-microbe symbiosis, but there is a high degree of interspecific and intervarietal variation in the benefits of AM infection for the host. This is a phenotypic difference overlooked by crop breeders selecting plants for performance under optimal rather than low P conditions (Parke and Kaeppler, 2000). Thus, whilst most US wheat varieties show little or no response to AM infection, UK varieties are moderately responsive (Hetrick, and Wilson, 1992). If biotic factors rather than biogeochemical interactions are describing any enhanced P uptake by combined Tithonia and Pi additions, it would be useful to differentiate between root and AM mediated uptake.

Spore germination, pre-infection hyphal growth and branching are stimulated by signal molecules synthesized and secreted by the root (Hirsch and Kapulnik, 1998). The root-produced molecules are often flavonoids, but it has not been considered that flavonoids in green manures may stimulate AM spore germination and

indeed this would be difficult to establish since CO_2 is also known to have a stimulatory effect (Smith and Read, 1997) and is increased with organic decomposition.

In this experiment two-sub-compartment rhizotrons were used to determine uptake of P by wheat (*Triticum aestivum* L. cv. Abbott) from ^{32}P labelled Pi (KH_2PO_4) or ^{33}P labelled Tithonia residues placed separately, alone or in combination in the sub-compartments. To test the null hypothesis that Tithonia has no effect on the mycorrhizal P uptake of wheat, each treatment was replicated with a modification to exclude root access to the labelled P source

2. Materials and Methods

2.1. Preparation of ^{33}P labelled Tithonia

Tithonia seeds of a Kenyan origin were imbibed for 12 h in aerated distilled water and then placed onto moist filter paper at 20°C to germinate. After 5 d, individual seedlings (3 to 5 cm tall) were transferred to 3.5-l containers containing an aerated (1 l min^{-1}) 80% strength Long Ashton nutrient solution (Hewitt, 1966). The nutrient solution was amended with $NaH_2{}^{33}PO_4$ (specific activity 300 kBq mg^{-1} P; Amersham Pharmacia Biotech Ltd., Little Chalfont, Bucks.) and the plants placed in a climate-controlled growth room with 22/18°C day/night temperatures, 16-h photoperiod and a light intensity of 500 µmol m^{-2} s^{-1}. After 21 d, all the ^{33}P in solution had been removed from the root-bathing solution by the plants. At harvest, the shoots had a water content of 830 g kg^{-1} fresh weight, P content of 2.3 g kg^{-1} dry weight and N content of 25 g kg^{-1} dry weight.

2.2. Soil

Soil was obtained from the Ap horizon (0-20 cm) of a Eutric fluvisol located in Bharatpur, Nepal (27°36' N, 84°22' E; altitude 187 m; rainfall of 2500 mm $year^{-1}$) in which *Zea mays* L. and wetland *Oryza sativa* L. had been previously grown in rotation. The soil is a poorly structured sandy loam with a $pH_{(H_2O)}$ of 5.48, total organic C content of 0.51%, total N content of 0.03%, C:N ratio of 17 and electrical conductivity (H_2O; 1:1 v/v) of 0.13 mS cm^{-1}. The exchangeable cation content (extracted with 1 M NH_4Cl) in units of $mmol_c$ kg^{-1} was: K, 24; Na, 4; Ca, 84; Mg, 60. The soil has an anion exchange resin extractable P of 0.12 mmol kg^{-1}, 0.58 mmol kg^{-1}

$NaHCO_3$ extractable P and a total P content of 6.5 mmol kg^{-1}. After collection, the soil was sieved to pass 2 mm and stored at field moisture levels at 15°C until required for experimentation.

2.3. Rhizotron design

The rhizotrons employed in this experiment were similar to those described by Marschner (1995) and consisted of a 150 × 150 × 20 mm box made from clear plexiglass. On each side of the box a 5-cm-diameter hole was cut to allow a soil filled subcompartment containing ^{33}P or ^{32}P to be attached (*Figure 1*).

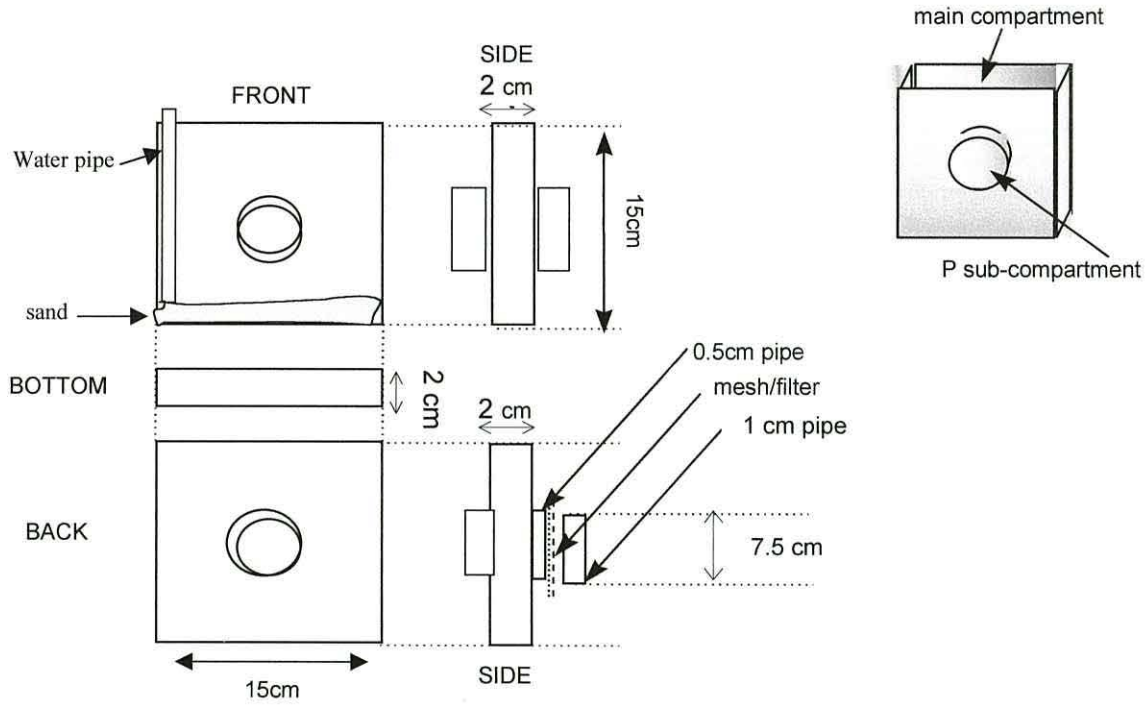


Figure 1. Schematic design of rhizotrons used in the experiments, displaying a double-sided sub-compartment system.

The circular sub-compartments were composed of a 1-cm-long section of nylon pipe that could be isolated from the main compartment by means of a nylon mesh. Two mesh sizes were used to selectively prevent roots from entering the sub-compartment:

A. 2000 μm mesh, allowing passage of both mycorrhizal hyphae and roots

B. 45 μm mesh, allowing passage of mycorrhizal hyphae but not roots

Washed quartz sand (50 g) was placed at the base of the main compartment of each rhizotron and a 10 mm plastic pipe placed vertically at one side so that the soil could be watered from the base.

Soil (500 g) was then packed into the main compartment of each rhizotron to a bulk density of 1.3 g cm^{-3}. The sub-compartment soil was amended with either ^{33}P-labelled Tithonia residues (organic P; P_o) or ^{32}P-labelled KH_2PO_4 (inorganic P; P_i) as outlined in *Table 1*. A total of 3 mg P was added to each set of rhizotron sub-compartments as either Tithonia $^{33}P_o$, $^{32}P_i$ or as a combination of the two (specific activity: 80 kBq mg^{-1} P). The rate of P application was chosen to represent a 10 kg P per ha^{-1} application on a surface area of rhizotron basis. The sub-compartment P application constituted a P 'hotspot', comparable to that which can be created as a result of a fertiliser application.

In Tithonia $^{33}P_o$-only treatments 3.85 g fresh weight of ^{33}P labelled Tithonia shoot was well mixed with 25 g of soil and added to each sub-compartment providing a total of 3 mg ^{33}P per rhizotron. In the $^{32}P_i$-only treatments, 150 µl of 10 mg ^{32}P ml^{-1} was well mixed with 25 g of soil and added to each sub-compartment to again provide a total of 3 mg ^{33}P per rhizotron. In treatments where organic and inorganic P were added separately to sub-compartments, 3.85 g fresh weight of ^{33}P labelled Tithonia shoot was well mixed with 25 g of soil and added to one subcompartment and 150 µl of 10 mg ^{32}P ml^{-1} well mixed with 25 g of soil was added to the other and attached to either side of the rhizotron. In previous experiments utilising a similar experimental system (see *chapters 5 and 6*), 20 g of soil had been placed in the subcompartment. Here this was increased to 25g to ensure that the Tithonia residues were fully incorporated into soil. In treatments where organic and inorganic P were added as a mixture, the P_o (1.925 g of Tithonia) and P_i (75 µl of 10 mg P ml^{-1}) were mixed in 25 g of soil, placed in the sub-compartments and attached to either side of the rhizotron. The $^{33/32}P$-labelled soil was packed into the sub-compartment to a depth of approximately 0.5 cm, providing a bulk density of about 1.3 g cm^{-3}. A further 0.5 cm layer (buffer strip) of soil containing no added P was placed on top of the radiolabelled soil layer, the nylon mesh placed over the buffer strip end, and a sub-compartment attached to each side of the rhizotron. To minimise the broader nutritional effects of adding green manure to the soil (Tithonia residues), mineral nutrients were added to the main compartment of all the rhizotrons as follows (mg kg^{-1}): N, 40; K, 60; Ca, 80; Mg, 24; S, 24; Mo, 1.2; Cu, 2.4; Zn, 2.4; B, 2.4.

Table 1. Summary of treatments applied to the rhizotrons (*Figure 1*). ^{33}P-labelled organic P (P_o) and ^{32}P-labelled inorganic P (P_i) were added to the two sub-compartments in the rhizotron. Further details are provided in the Materials and Methods.

Added P source	Root access to sub-compartment	Sub-compartment content		Abbreviation
		A	B	
Organic	-	P_o	P_o	P_o –R
Organic	+	P_o	P_o	P_o +R
Inorganic	-	P_i	P_i	P_i –R
Inorganic	+	P_i	P_i	P_i +R
Organic plus inorganic	-	P_i	P_o	P_o/P_i –R
Organic plus inorganic	+	P_i	P_o	P_o/P_i +R
Organic plus inorganic	-	$P_i + P_o$	$P_i + P_o$	$P_o + P_i$ –R
Organic plus inorganic	+	$P_i + P_o$	$P_i + P_o$	$P_o + P_i$ +R
None	+	None	None	Control

2.4. Plant growth

Seeds of *Triticum aestivum (*cv. Abbott) were soaked in distilled water for 12 h and then transferred to moist filter paper to germinate. After 2 d, three germinated seedlings were transplanted with equal spacing into each rhizotron. The rhizotrons were maintained in a climate-controlled growth room with 22/18°C day/night temperatures, 16-h photoperiod and a light intensity of 500 μmol m^{-2} s^{-1}. The moisture content of the rhizotrons was maintained at approximately 20% volumetric water content by the daily addition of distilled water. After three and five weeks, the plants were fertilised with 29 mg of NH_4NO_3. Each treatment was replicated four times and the individual rhizotrons placed at random within the growth cabinet (using random numbers to allocate the initial position) and rotated within the cabinet daily to reduce positional effects.

2.5. Rhizotron harvesting

Rhizotrons were harvested after six weeks. The shoots of each of the two plants in each rhizotron were removed and combined for subsequent analysis. The shoots were dried at 60°C (24 h) to determine dry weight. The dried shoots were ground and a representative 40 mg sample ashed (500°C, 24 h) and the residue dissolved in 1 M HCl. The $^{33/32}P$ content of the HCl solutions was then determined by liquid scintillation counting using an HCl-compatible scintillation fluid (Optiphase

Hisafe 3, EG&G Wallac, Milton Keynes) and a Wallac 1304 liquid scintillation counter (EG&G Wallac, Milton Keynes). The ^{31}P content of shoot material was determined in an aliquot from the 1 M HCl solutions using the colorimetric molybdate blue procedure of Murphy and Riley (1962). Roots contained within the main rhizotron compartment and in the sub-compartments where root access was available were removed by hand and dried at 60°C (24 h) to determine dry weight.

2.6. Statistical analysis

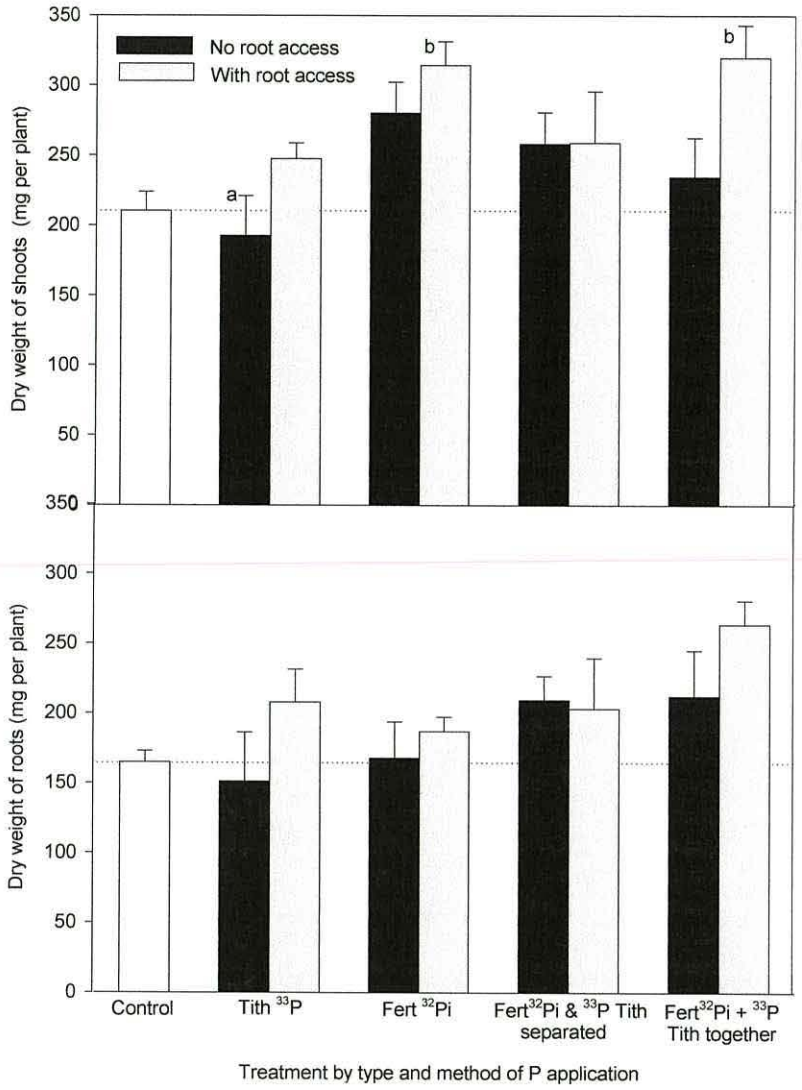
The experiment had a 2 level factorial design, with four treatments (P sources) for each interaction (roots and root exclusion) and a control. The significance of interactions between P source and access to the P source were determined by analysis of variance (ANOVA). Tukey's test was used for multiple comparisons between the sample means where the ANOVA F statistic was significant (Zar 1996).

3. Results

The rhizotron design incorporating the exclusion meshes appeared to operate successfully in all experiments. The 2000 μm mesh permitted free passage of roots into the sub compartment while the 45 μm mesh prevented root growth into the sub-compartment. In the '45 μm mesh' treatments a root mat similar to that found within the '2000 μm mesh' sub-compartment was found to form on the outer mesh surface.

Tillering was observed in all treatments by the time of harvest, but tended to be greatest in one plant in each rhizotron. The dry weights of shoots and roots are presented in *Figure 2*.

Figure 2. Dry weight of wheat shoots and roots for all treatments, with significant differences between treatments indicated by letters, ($P < 0.05$). Bars represent standard errors of the means for each treatment (n = 4).

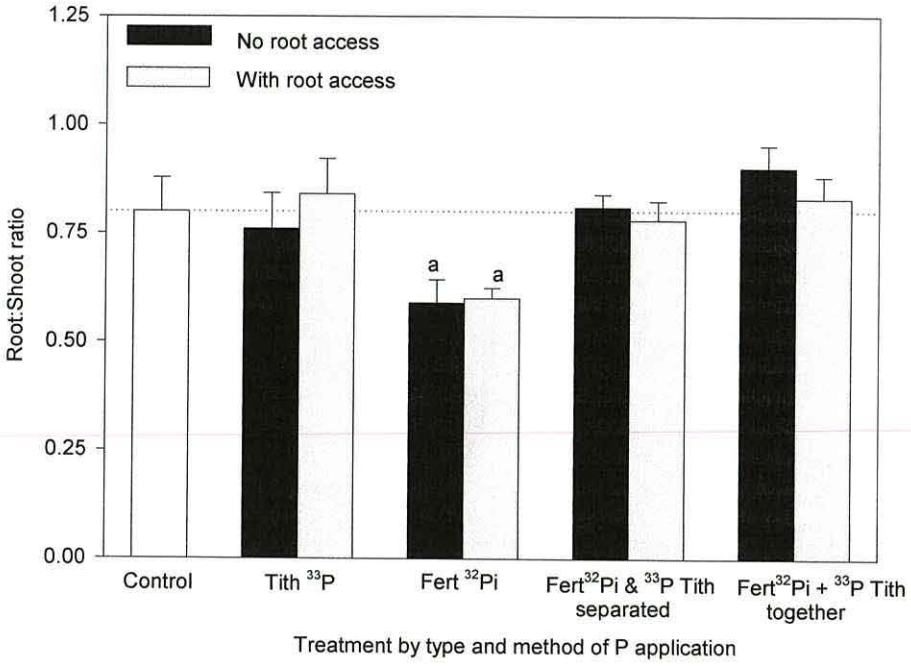


The treatments with root access to 'fertiliser ^{32}Pi' or 'Tithonia and Pi together' had a shoot dry weight significantly greater than the 'Tithonia with roots excluded' treatment ($P < 0.05$). The fact that there were no statistically significant differences between the control and other treatments indicates that the growth of control plants was not severely constrained by soil P availability. The mean root dry weights for each treatment had a similar pattern to the mean shoot dry weights, although the differences between the means were not significant ($P > 0.05$).

Root-to-shoot ratios are shown in *Figure 3*. Despite access to additional P, only when all the added P fertiliser was supplied in the inorganic form was there an

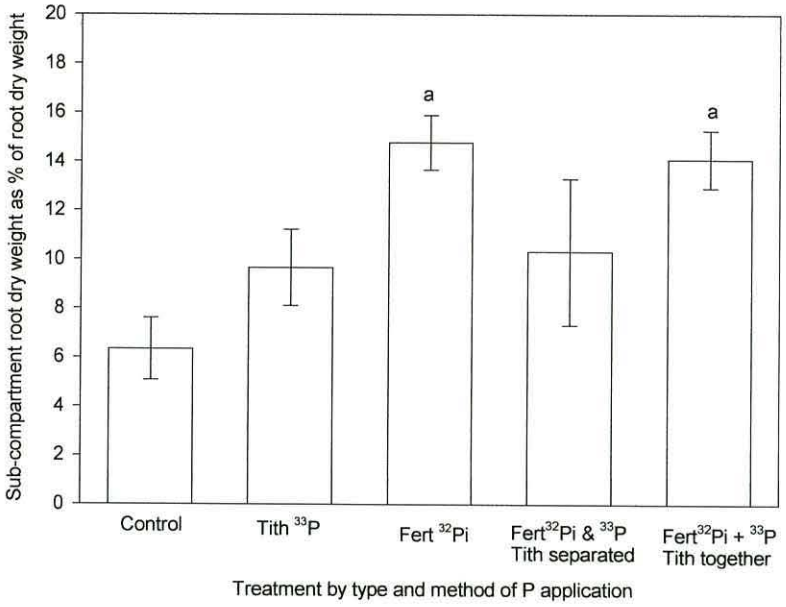
indication of reduced allocation of resources to the roots, indicating that P availability was probably not the major constraint to growth.

Figure 3. Root-to-shoot ratios of wheat plants for all treatments, with significant differences from the control indicated by letters, ($P < 0.05$). Bars are for the standard error of the mean for each treatment ($n = 4$).



The proportion of the root growing in the available P hotspot provides an indication of plant responses to P fertiliser regimes (*Figure 4*). Relative to the control, proportionately more of the root systems of the Pi and 'Tithonia and Pi combined' treatments were present in the P hotspot. When Tithonia and Pi additions were in separate compartments root development in the sub-compartment did not exceed controls.

Figure 4. The proportion of total root dry weight of treatments with access to the sub-compartments recovered from the sub-compartments. Values are expressed as a percentage of the total root biomass. Significant differences from the control are indicated by letters ($P < 0.05$). Bars are for the standard error of the mean for each treatment ($n = 4$).



Total shoot P and total shoot radiolabelled ^{32}P or ^{33}P taken up by three plants in each rhizotron is shown in *Figures 5* and *6* respectively. The amount of 'native' ^{31}P accumulated into the shoots was highly variable. The Tithonia only treatments and the combined 'Tithonia and ^{32}Pi with root access' treatments had a similar total P uptake to shoots as the control, despite access to the radiolabelled P. Uptake of native P in the ^{32}Pi treatments and the combined treatments where only mycorrhizas could access the P hotspot were similar to the control. While increased radiolabelled P uptake was associated with increased native P uptake, this pattern is not reflected in the dry weight data. In particular, the increased dry weight in the 'Tithonia and Pi together' treatment was not related to an increased shoot P concentration.

Figure 5. Total wheat shoot P per plant for all treatments, with significant differences from the control indicated by letters ($P < 0.05$). Bars are for the standard error of the mean for each treatment ($n = 4$).

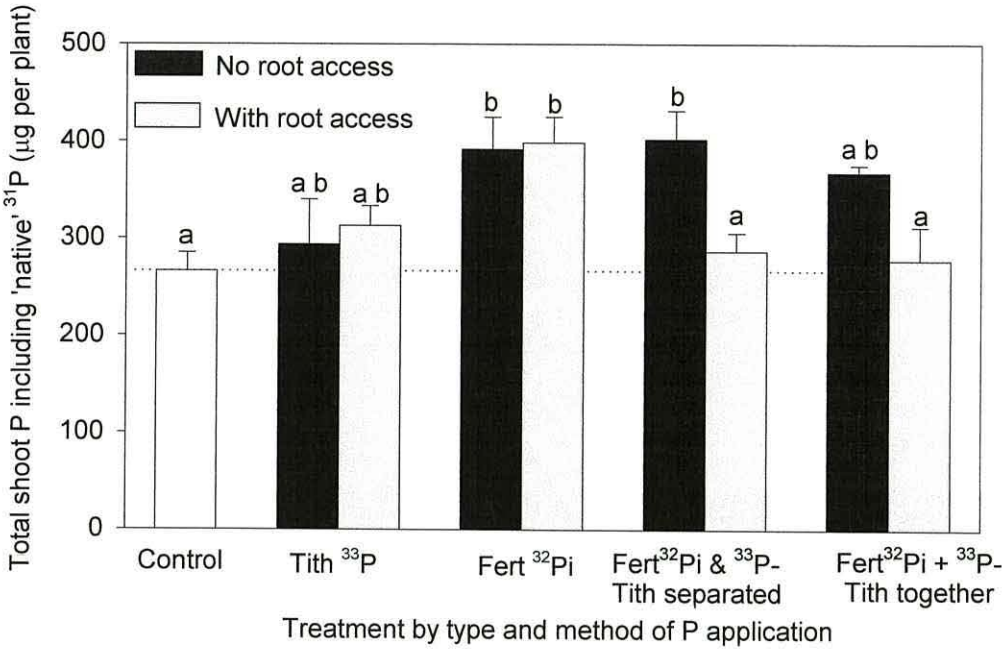
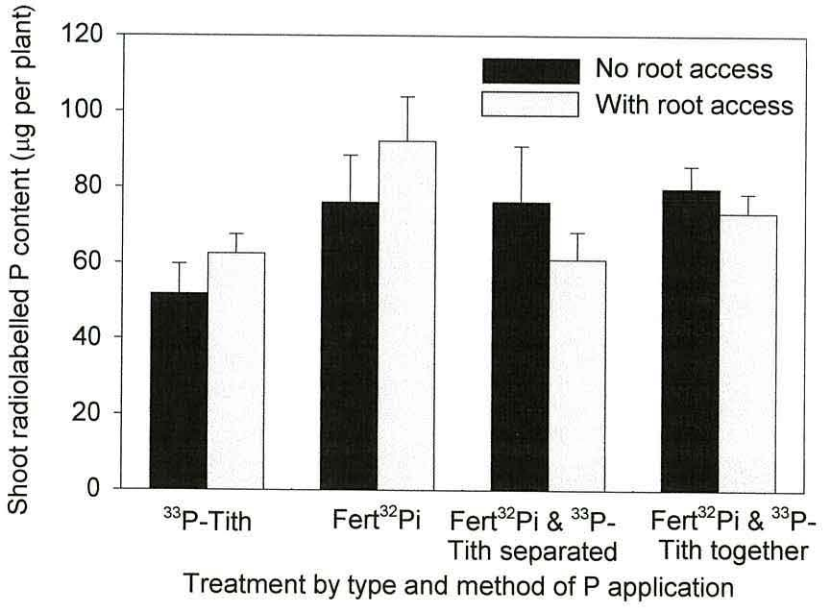


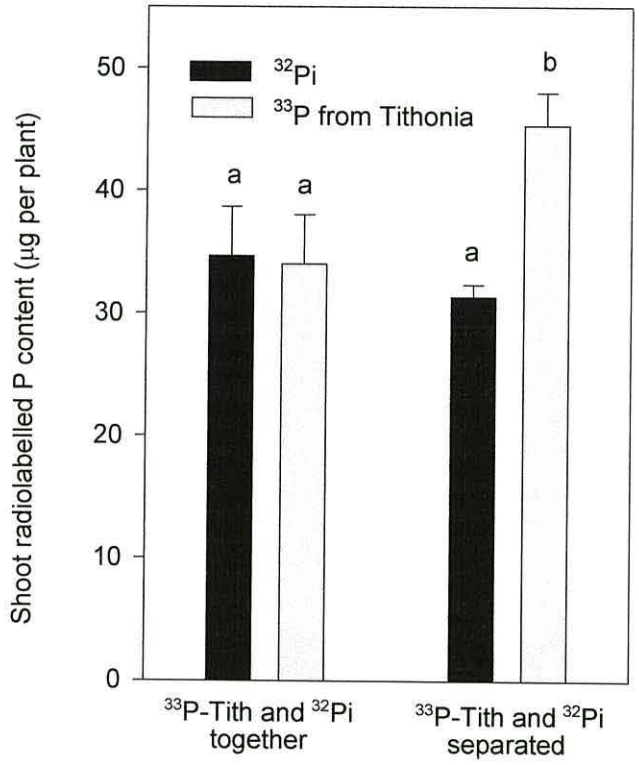
Figure 6. Radiolabelled shoot P per plant for all treatments. Bars are for the standard error of the mean for each treatment ($n = 4$).



In *Figure 7* the labelled P data is broken down into the separate isotopes for the treatments with combined P additions. As no significant interaction between root exclusion and P source could be determined by factorial analysis of the data by two-

way ANOVA, 'the with-roots' and 'without roots' data is grouped together. When Tithonia is combined with ^{32}Pi, it is the Tithonia ^{33}P that becomes more available to wheat plants, but when the two P sources are in separate compartment, the uptake of the P sources is similar.

Figure 7. Radiolabelled shoot P per plant for ^{33}P Tithonia plus ^{32}Pi treatments, by isotope. Root excluded and root with roots data has been combined. Significant differences between treatments are indicated by letters ($P < 0.05$). Bars are for the standard error of the mean for each treatment ($n = 4$).



4. Discussion

There was no significant effect on the amount of P uptake from the sub-compartment by wheat, or on the associated growth parameters arising from the exclusion of roots from the labelled sub-compartments. Wheat is not reported to be strongly mycorrhizal and this result is therefore surprising. The soil was not inoculated with mycorrhizal spores, but based on previous evidence of mycorrhizal infection of Tithonia plants growing in the same soil (see *chapter 5*) it was assumed that viable spores were present. No analysis of the roots to verify mycorrhizal infection of wheat roots was conducted. Therefore the following discussion is based on the assumption that transfer of P from the sub-compartments to the roots of wheat was caused by mycorrhizas rather than by a possible combination of mass flow and diffusion. Since the results presented here suggest that mycorrhizas are as effective in P acquisition as roots plus mycorrhizas, further work would be justified, both to confirm the finding and to determine the relationship of this effect with P status.

Wheat was chosen for use in this experiment, as one of the aims had been to determine if Tithonia residues had a stimulatory effect on mycorrhizas and it was thought that a species with a low mycorrhizal dependence could demonstrate any effect more clearly. As wheat plants where roots were excluded from entering the sub-compartment took up Pi as effectively as P from Tithonia residues, no stimulatory effect on mycorrhizas is suggested.

The significantly lower root-to-shoot ratio in the two treatments with access to inorganic P ($P < 0.05$) is indicative of an absence of P stress throughout most of the period of plant growth. The other treatments caused allocation of more resources to root growth (*Figure 3*). There were no differences in the dry weight of roots growing in the sub-compartments between the treatments with root access (data not shown). This indicates that differences in uptake were not associated with colonisation of the P-hotspot and that the presence of organic matter did not influence the pattern of root growth. P uptake from the labelled sources is therefore related to demand and availability.

In the 'Tithonia only' treatments the relatively small proportion of the total root system occupying the sub-compartments suggests that P and possibly other minerals were immobilised by the microbial biomass (*Figure 4).* Observation of growth and Geiger counter readings during plant growth are compatible with an

interpretation of the results that exploitation of the Tithonia only ^{33}P source was slow, but increasing at the point of harvest.

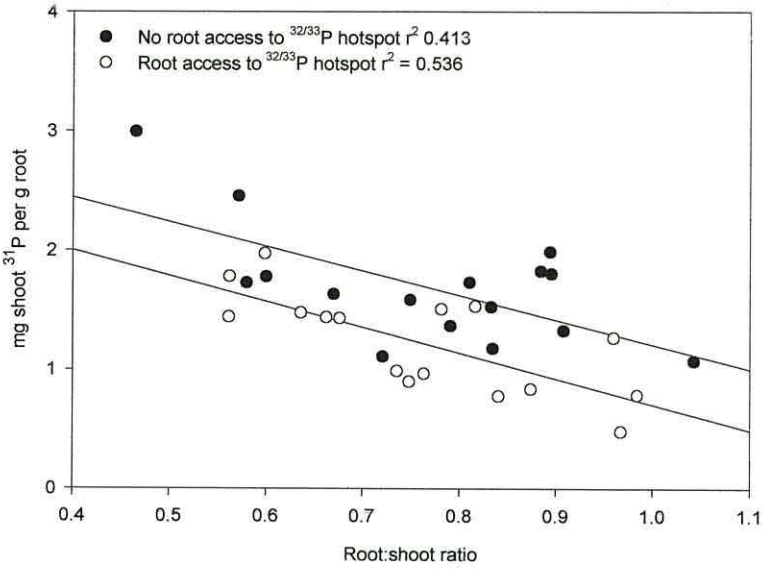
The addition of mineral nutrients other than P to the main compartment soil was supposed to have overcome all other mineral constraints to growth, but the greatest plant growth occurred in plants with root access to combined Tithonia and Pi hotspots. This treatment also resulted in the greatest proportion of the root system to grow in the hotspot. The implication is that while each sub-compartment had only half the amount of Tithonia compared to the Tithonia only treatment, early occupation of this soil by roots foraging for the more available Pi subsequently resulted in enhanced nutrition as other nutrients from the Tithonia became mineralised. This in turn resulted in faster growth, albeit with a lower shoot P concentration.

The ^{32}Pi only treatments with root access developed a greater proportion of the root system within the P-enriched sub-compartment relative to other treatments except for the combined P source treatment with root access. This finding is compatible with the hypothesis of Forde and Lorenzo (2001) who have proposed a system for the regulation of 'trophomorphogenesis' involving inter- or, more probably, extra-cellular nutrient ion sensor proteins in root cell plasma membranes capable of providing signals to initiate localised nutrient acquisition strategies, but with subsidiarity to systemic chemical signals emanating from the shoot. The trophomorphogenic response in these wheat plants appears to require an external Pi concentration greater than that available in the Tithonia only or the separated P source treatments

With a single harvest it is difficult to separate treatment effects on total shoot P from effects due to the growth stage achieved by the plants. The treatments with inorganic P, but without root access to Tithonia had higher total P concentrations in the shoot (*Figure 5)*. If further work was to show that this were not simply due to ontogenetic drift it would suggest that the plants were responding negatively to the presence of Tithonia.

The hypothesis that decomposing Tithonia stimulates an increase in AM mycorrhizas associated with the crop plant is not supported by these results, although mycorrhizal hyphae associated with the wheat plants were as effective as roots in acquiring P from the subcompartments. There were no significant differences ($P < 0.05$) in the uptake of labelled P within treatment pairs, indicating that there is no stimulatory effect of Tithonia on mycorrhizal symbiosis. However, under field conditions the establishment of mycorrhizal associations with crop plants may be

Figure 8. Plots of native P against root shoot ratio for 'with roots' and 'without roots' treatments.



When Tithonia and ^{32}Pi were mixed, there was an increase in the uptake of Tithonia ^{33}P by the wheat plants (*Figure 7*), but where the two P sources were separated, total uptake of the labelled P was lower and the two sources were accessed equally. An interpretation of this result is that the ^{32}Pi constituted a more mobile pool to satisfy the P sorption potential of the soil and meet part of the microbial demand. As Tithonia ^{33}P was either released from cells as vacuolar ^{33}Pi or ^{33}Po in Tithonia tissues was mineralised, the reduced buffer capacity and lower microbial P demand allowed this P source to be more available to the wheat plants.

The soil used in this study has a relatively low P sorption capacity (see *Chapter 5*) and it would be expected that this effect would be more pronounced with a soil with a higher P buffer capacity and low available P. For a limited P addition, the improvement in yield when Tithonia was mixed with TSP as reported by Nziguheba *et al.* (1998) can be therefore explained in terms of an initial stage where much of the added P from both sources is either adsorbed or immobilised by microbes involved in the decomposition of residues. This is then followed by a gradual mineralisation of Tithonia P. This is in line with the work of McLaughlin *et al.* (1988[a]) showing the rapid immobilisation of added P in the presence of residues. As long as crop plants can meet their initial P demand, their root systems can colonise the soil effectively to recover P as it is mineralised. Provided the green manure material decomposes rapidly, mineralisation can be synchronous to plant requirements (Gressel and McColl, 1997).

As has been discussed previously (see *Chapter 1*), Tithonia residues can have high P concentrations. Much of this is probably in the form of Pi stored in the vacuole of cells (see *Chapter 4*) and can be expected to enter the soil very rapidly as cell walls break down (Blair and Boland, 1978). The term 'net mineralisation' is often used in this context and is perhaps misleading. Palm *et al.* (1999) compared Tithonia with TSP as P fertiliser sources for *Zea mays*. These results suggest that in addition to broader beneficial fertilisation effects, the release of vacuolar Pi from Tithonia performed a role similar to the ^{32}Pi in the combined P treatment, reducing the buffer capacity and meeting part of the microbial P demand.

This study was unable to show a clear cumulative benefit from the combined mineral P and Tithonia applications for the recovery of applied P. It did, however provide evidence that when applied with inorganic P fertiliser, Tithonia P is more available. Further studies over a longer time scale would be necessary to establish if other mechanisms can facilitate a greater total recovery from combined P sources.

It is widely recognised that to overcome the fertility and associated poverty problems arising from P deficiency there must be investment in P in tropical soils (Sanchez *et al.*, 1997; Fairhurst *et al.*, 1999). This work supports the concomitant application of adequate P status green manures with an appropriate decomposition characteristic as an effective means of increasing the economic returns from limited P applications provided the green manure is available at low cost. As Tithonia is difficult to establish from seed and is usually cultivated from cuttings, which is inevitably labour demanding, this may not always be the case (Sherchan, 2001).

Chapter Eight

Conclusions and implications for further work

Low yields associated with phosphorus deficient crops pose a major constraint on the development of wealth at household level in much of the tropics. This in turn has a negative affect on other factors influencing development, including health and education. This is a problem that could be addressed by a major investment in mineral fertilisers beyond the resources of the farmers involved. The complexity of the range of social and economic issues that constrain the possible responses to this issue, are beyond the scope of this discussion. However, the use of green manures and other low-external-input technologies to address soil fertility is critical to small farmers' survival in many areas.

If considerable investment was made in enhancing fertilizer availability and the infrastructure to deliver it to farms, there is no doubt that soils could be made more productive. Without investment, however, small-scale farmers who are unable to build soil fertility face an uncertain future as increasing populations create a stronger demand for farm produce. The increasing drive to find sustainable ways to enhance P cycling addresses both a present constraint faced by tropical resource poor farmers and perhaps their longer term security on the land.

There are four principal ways in which phosphorus utilisation can be enhanced within agricultural systems:

1. Facilitating the improved recovery of soil P by crop plants.
2. Improving the availability of added P fertiliser to the crop to which it is applied.
3. The development and use of cereal crop varieties that have lower grain P contents (that are subsequently removed as marketed product).
4. The development and use of crops with a higher internal P use efficiency.

The work reported here considers how aspects of the first two are addressed by the utilisation of *Tithonia diversifolia* (Tithonia) as a green manure. Success by researchers working to respond to points three and four through crop improvement could alter the economics of farming P limiting soils.

Reports of the P mobilization potential of Tithonia, a shrub belonging to the Asteraceae, and its apparent ability to accumulate P (Nziguheba *et al.*, 1998; Gachengo *et al.*, 1999; George *et al.*, 2002[b]) led to speculation that Tithonia was accessing soil P pools not available to most plants (Buresh and Niang, 1997).

8.1. Summary of key findings

In *Chapter 2* the P uptake kinetics of Tithonia were described, and Tithonia was not found to be better adapted to utilising sparingly available P than crop plants. In *Chapter 4* the partitioning of P within Tithonia tissues was described and it was concluded that although tissue P concentrations were higher than found in most conventional crops, there was no evidence of P storage compounds (e.g. polyphosphate). We concluded that Tithonia P concentrations were within the normal physiological range. This work also presented evidence that the high tissue P concentrations found in newly growing regions of the Tithonia plant could be accounted for by re-translocation of P from senescent tissues, rather than by enhanced uptake from the soil.

In Chapter 6 the ability of Tithonia roots to access P from a range of sources of differing availability demonstrated that if Tithonia was accessing P adsorbed to $Fe(OH)_3$ surfaces it was through association with arbuscular mycorrhizas (AM). The same study also showed that Tithonia roots were able to access P from a microbial P source.

In *Chapter 3* an investigation into the allelopathic affects of Tithonia on the growth of maize seedlings found that Tithonia shoots slowed the growth of maize, but in a manner that did not indicate that the health of the plants was being affected. It was hypothesised that the secondary metabolites from Tithonia shoots were stimulating the germination of the spores of AM fungi and that the inhibited growth of maize was due to colonization of the roots by the fungi.

Chapter 7 explored the impact of Tithonia residues incorporated into the soil on the growth of wheat and its AM associations. No evidence of a stimulatory effect on AM symbiosis was found. The same experiment also looked at the effects of combining a mineral P fertilizer with Tithonia shoots and did not find evidence to support the field study results of Palm *et al.* (1999) who reported an additive effect resulting in greater total P uptake relative to the same quantity of P being added as mineral P fertilizer. This research did, however, show that the Tithonia P became

more available to plants as the more labile mineral P became immobilised, either by soil microbes or through sorption to soil surfaces.

Based on these results, it was hypothesised that the exudation of flavonoids and other compounds from the roots of Tithonia facilitate the rapid development of effective AM fungal mycelia associated with the roots. This enables Tithonia to establish in soils with high P sorption characteristics, with mineral bound P, not readily available to roots, being taken up into the shoots. Feedback inhibition associated with regulation of plant P uptake is reduced as a result of AM colonisation of the roots, resulting in continued P uptake despite tissue P concentrations being in excess of immediate plant requirements.

Ecologically, Tithonia behaves as a conservative opportunist, senescing all but the most photosynthetically active leaves when under stress. Although P is relatively mobile in plant tissues (Marschner, 1995), between 25 and 45% of P taken up into the shoots is returned to the soil in senesced leaf litter. This is rapidly decomposed and it was hypothesised that the plant lateral roots, which form a dense mat near the soil surface, reabsorb mineralised nutrients, including P. In the leaf litter, organic P compounds of microbial origin constitute the most rapidly turning over P pool and the ability of Tithonia to access this source of P may be significant.

8.2. Suggestions for future work

When residues of Tithonia shoots are incorporated into soil, mineralisation is rapid and P from the residues is able to make a contribution to crop P requirements. What remains unclear is what secondary effects occur when Tithonia and indeed other green manures are applied to the soil. When large quantities of green shoots are incorporated into soil, the soil flora and fauna populations increase. Tithonia has secondary metabolites that have been found to have a toxic effect on insects, nematodes and arthropods. If this toxicity persists in the soil for any length of time it could be expected to alter the functional soil ecology during the decomposition of the residues.

Similarly it could be expected that the decomposition products of the residues would interact with soil surfaces and that the nature of the residues applied would affect the nature and consequence of such interactions. The research reported in *Chapter 7* found that there was no additive effect of combined organic and inorganic P applications. Additive effects may however occur outside the time frame of the

experiment. Therefore longer experimental trials are required to determine the longer term benefits of green manures. Equally, the lack of agreement with previously published field studies could be due to an effect arising from interactions between the soil and the residues that were not reproduced under the experimental conditions, either due to the nature of the soil or due to the absence of the full natural diversity of soil organisms.

A factor that makes Tithonia an interesting subject to explore the effects of plant secondary metabolites on anion adsorption in soils, is that the chemistry of the active ingredients and their extraction methods has been described and there is abundant data of crop plant growth and mineral concentrations following Tithonia applications on a range of soils. For the same reason Tithonia presents an interesting opportunity to study the effects of secondary metabolites on the population dynamics of soil biota and the impact of changes in these variables on crop growth. Further work to study the changes in the functional soil ecology where Tithonia has been grown or applied as a green manure to soil could provide a useful insight into how P cycles could be enhanced through manipulation of soil ecology during mineralisation.

Research carried out, but not presented here, attempted to trace changes in the concentration of soil solution P and other P pools over time during the decomposition of Tithonia and Sesbania residues in soil and relate the result to a number of parameters including phosphatase activity and the concentration of organic acids in solution. Unfortunately this experiment met with difficulties with sample analysis. Nevertheless the results from such an experiment would allow secondary effects of Tithonia residue application on soil mineralogy to be assessed and the implications for P availability directly ascertained.

Tithonia under field conditions grows in soil that receives a constant input of leaf litter from senesced leaves. The assumption should be that this is a significant factor describing the soil environment in which Tithonia grows. The nature of the senesced leaves is such that the litter decomposes rapidly and this has been observed in the field. This suggests there is a constant recycling of P, but that much of it would be in organic forms and likely to be present as dissolved organic P (DOP). Here there appears to be a gap in the literature, for although DOP is hydrophilic (Kaiser *et al.*, 2001) and is thought to be inherently more mobile in soil than Pi (Haunapel *et al.*, 1964) little consideration has been given to the availability of this P source to roots. Are localized concentrations of DOP sufficient to contribute to plant uptake through a

combination of mass flow and diffusion at rates significantly higher than for Pi? It is difficult otherwise to invoke a P acquisition role for plant secreted phosphatases given that phosphatases are relatively immobile in soil, vulnerable to inhibition by clays and aluminium and exist in a high protease environment. Future work should therefore determine the diffusion coefficients for DOP in soil and the significance of mass flow in the movement of DOP in the soil solution. Characterisation of organic P transformations over time, following the application of ^{33}P labelled Tithonia residues would allow the potential significance of this P pool to meet plant P requirements to be described.

An important omission in the range of experiments conducted was that root hair growth on the roots of Tithonia was never measured. It would appear that for most species root hair development is one of the single most important characteristics describing nutrient acquisition strategies (Jungk, 2001). The observation that the size of the root system and root hair growth are usually inversely related to dependence on AM symbiosis for uptake of P (Schweiger *et al.,* 1995; George, 2000; Smith *et al.,* 2001) suggests root hairs and root architecture should have been studied more closely. Further work would be justified as the presence of root hairs was observed in the course of the study of mycorrhizal infection of Tithonia roots (Sharrock *et al.*, 2004). Confirmation that both root hairs and AM fungal symbionts were simultaneously involved in P uptake in Tithonia would in itself be an interesting finding.

Root hairs are thought to be the principal site for the exudation of organic acids as well as important for the uptake of P. The whole root system study of P uptake transporters (*Chapter 2*) was not able to find discernable differences between crop plants and Tithonia. However, this study and those with which the results were compared used plants that were grown in hydroponic solution, in which roots do not develop root hairs. This suggests that further work is required in which the P uptake kinetic parameters of the growing roots are determined at P concentrations comparable to those in soil and in systems that allow the growth of root hairs. Such a study should also look at the exudation of organic acids and attempt to model the effects of the organic anions on the P buffer capacity in the P depletion zone that forms around the root.

The managed addition of organic matter is critical to the management of weathered tropical soils. These are predominantly forest soils of origin and it is through researching the changes in the biogeochemistry of the soils after conversion

to agriculture, that management decisions can be informed. Particular attention should be given to the decrease in lignin inputs to soil under cultivation and the impact on P sorption arising from the decreased production of oxalic acid by white rot fungi (Dutton and Evans, 1996). Oxalate is of particular interest as it appears to be relatively resistant to microbial activity once adsorbed to Fe and Al oxides and hydroxides (Afif *et al.,* 1995) where it occupies many of the same sorption sites as Pi (Violante *et al.,* 1991). In an Amazonian soil, Afif *et al.* (1995) found that the degradation of adsorbed oxalate was associated with an increase in P sorption. Olivares *et al.,* (2002) found calcium oxalate in Tithonia leaves. This needs to be confirmed and any significant sorption of oxalate to soil minerals, subsequent to Tithonia residue applications, measured, together with the impact on soil P buffer capacity. If organic anions cannot be introduced to soil to fulfil this function, it may need to be recognised that the P management options in the soils being considered are limited. Either applications of sufficient P fertiliser need to be made to satisfy the high-energy soil P sorption potential, or the buffer capacity reduced by the *in-situ* production of fungal oxalate following the addition of high lignin organic matter. This challenges conventional thinking, particularly with regards N immobilization, but N_2 can be biologically fixed; P cannot.

8.3 Conclusion

The results of the research reported in this thesis can be summarized as follows. Both Tithonia roots and mycorrhizas participate in the acquisition of P from soil, but roots appear better adapted to taking up Po. The root P uptake transporters in Tithonia showed similar uptake kinetics to crop species and *Sesbania aculeata.* Tithonia grown in soil has shoots with higher P concentrations in leaves and green shoots than often observed in other species including alternative green manure plants (Palm, 1995), but this is probably due to retranslocation from older leaves, rather than super-efficient uptake. When placed in soil as a fertiliser for wheat plants, uptake of P from Tithonia was enhanced by the addition of soluble fertilizer Pi to reduce the soil P buffer capacity and reduce microbial immobilization of Tithonia P. The presence of secondary metabolites with reportedly insecticidal and nematodicidal qualities in the Tithonia tissue, raises the possibility that under field conditions Tithonia residues may influence the soil ecology. High concentrations of a broad range of minerals

including Zn and Cu were observed in Tithonia leaves and probably contribute to improved crop yields.

Although Tithonia is undoubtedly a high quality green manure, as it is neither N_2 fixing, nor does it appear to be able to access less available organic P reserves. Since propagation from seed is unreliable, Tithonia can not easily be grown *in-situ*. Its role in smallholder agriculture is probably limited to being grown in hedgerows for application by biomass transfer to high value crops (Jama *et al.,* 2000). This conclusion is based on the current research and is in agreement with other researchers.

However, the extensive research on Tithonia to date, both in the context of P cycling and for medical and other interests, suggests that it would be of value to continue research into the use of Tithonia in soil P cycles. There is much that is not clearly understood in this area of research and it will only be through developing our understanding of the processes and mechanism involved that significant progress can be made to enhance P cycling in soils.

Many of the recommendations for further research outlined here call for the development of improved experimental techniques that will require an investment that this area of ecophysiology struggles to attract. Perhaps therefore a key research priority is to describe the economic and social implications for a range of achievable applied objectives to enable a cost benefit analysis that could develop the arguments for improved research funding.

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Appendix 1

Background to the research

The work reported here was conducted as part of a broader programme of research into the potential of Tithonia diversifolia to be utilised as a green manure to enhance phosphorus cycling in tropical low external input agriculture. It was funded by the Department for International Development' Plant Sciences Programme which also funded field research in Nepal. Another associated project funded by the Department for International Development's Forestry Research Programmes investigated the nature and function of mycorrhizal fungi associated with Tithonia. There were other researchers working on the subject elsewhere, who's published work is cited in the literature review.

The research proposal specifically prescribed the work on P uptake kinetics (*Chapter two*), the partitioning of phosphorus (*Chapter four*) and the effects on the mobilisation of Tithonia P and mineral P fertiliser by crops, when the two P sources were combined (*Chapter seven*). The work on the uptake of P sources of differing availability by Tithonia and associated mycorrhizae (*Chapters five and six)* had originally been included in the Forestry Research Programme research proposal, and was taken on as there had been a delay in putting a researcher in place.

It had been originally intended that the controlled environment experiments would be able to investigate questions raised in the course of the field studies in Nepal and the soil used in the controlled environment experiments was therefore taken from the field site in Nepal. In the event neither the need nor the opportunity for close collaboration between the two parts of the project materialised. A consequence of this feature of the project planning was that the soil used in the experiments did not have the P sorption characteristics the use of Tithonia was envisaged to address.

Appendix 2
Soil Details and Characteristics

Soil Details and Characteristics	
Soil origin: Chitwan valley in the Bharatpur region of Nepal (27°36' N, 84°29' E; altitude 250 m)	
Rainfall: bimodal rainfall of 2500 mm per year	
Current annual cropping cycle: Zea mays and wetland Oryza sativa	
Soil Classification (USDA)	Eutric fluvisol
Texture	Sandy loam
$pH_{(H_2O)}$	5.48
Maximum linear P diffusion potential	0.75 mm d^{-1}
Organic Carbon	0.51%
Total N	0.03%
Electrical conductivity (H_2O 1:1 v/v)	0.13 mS cm^{-1}
C:N ratio	17
Total P	6.5 $mmol_c$ kg^{-1}
Anion resin P	0.12 $mmol_c$ kg^{-1}
$NaHcO_3$ P	0.58 $mmol_c$ kg^{-1}
K	24 $mmol_c$ kg^{-1}
Na	4
Ca	84
Mg	60