The Physiological Impacts of Soil Alkalising Agents on Legumes

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Declaration

I declare that the contents in this thesis are my own work and have not been submitted in the same form for the award of a higher degree elsewhere.

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Abstract

To meet predicted future requirements for food production, sustainable intensive agricultural systems will continue to supply supplementary fertilisers. Furthermore, crop nutrient availability needs to be optimised to maximise output, traditionally achieved by applying lime (calcium carbonate) to target soil pH ranges (6-6.5) that optimise nutrient availability and subsequent yield. Additionally, industrial by-products that typically have been regarded as waste are being increasingly used as alternatives to traditional fertiliser sources. One of these, cement kiln dust (CKD), is a potential liming agent and potassium fertiliser. In a field trial, CKD equally increased soil pH and available potassium when compared to agricultural lime and a commercial potassium fertiliser. However, pod yield of field bean (*Vicia Faba* L. cv Fuego) was reduced by ~30% by liming with equivalent rates of lime or CKD. In subsequent pot trials, recommended liming rates (targeting pH 6.5) consistently reduced stomatal conductance by 26-35% and 20-59% and photosynthesis by 11-20% and 17-30% in dwarf bean (*Phaseolus vulgaris* L. Nassau) and tall pea (*Pisum sativum* L. cv. Alderman) respectively. Consequently, mean shoot dry weights were reduced by 13% and 17% in *Phaseolus* and *Pisum* respectively. Although lime significantly increased rhizospheric calcium concentrations, xylem sap calcium concentrations (a potential antitranspirant) were only increased in *Phaseolus* and not *Pisum* and thus did not cause stomatal closure. Xylem sap and tissue analysis of *Pisum* suggests that reduced gas exchange is caused by an increase in the plant hormone abscisic acid (ABA) in response to a lime-induced phosphorus deficiency. When synthetic ABA was supplied to detached pea leaves at the same concentrations induced by liming, stomatal conductance decreased. Furthermore, the ABA deficient mutant pea *'wilty'* showed an attenuated stomatal response to liming, apparently confirming that increased ABA is mediating some legume responses to low phosphorus availability under recommended rates of liming. This research contributes to a mechanistic understanding of the physiological processes limiting gas exchange and growth when lime application limits crop yields, and raises questions about the suitability of current liming recommendations.

Publications arising from this work:

Rothwell S.A. and Dodd I.C. (2014) Xylem sap calcium concentrations do not explain liming-induced inhibition of legume gas exchange. *Plant and Soil*, 382, 17-13.

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Chapter 1: General Introduction

1.1 Introduction

It is estimated that global food production will need to increase by approximately 50% over the next 30 years (The Royal Society, 2009) to meet rising demand from an expanding population that is forecast to reach nine billion people by 2050 (United Nations, 2008). However, any increase in food production has to be set against a backdrop of mitigating factors that include climate change; limitations on agricultural inputs - primarily water and nutrients; and increased competition for land from urbanisation (Government Office for Science, 2011).

Current thoughts are that in order to meet this challenge, global agricultural systems will need to adopt approaches, appropriate to regional, cultural and crop requirements, that facilitate the 'sustainable intensification' of food production (The Royal Society, 2009). This approach to agriculture recognises that inputs of chemical fertilisers to optimise crop yields will be important if future targets for food production are to be met. However, the impact of the previous Green Revolution that relied so heavily on chemical fertiliser inputs cannot go unrecognised. Indeed, the environmentally detrimental impact of high nitrogen (N) and phosphorus (P) fertiliser input, and subsequent leaching, on both terrestrial and aquatic ecosystems means continuation of such agricultural practices is commonly deemed to be no longer acceptable (Tilman et al., 2002). In meeting this challenge, many agricultural systems that are currently heavily reliant on excessive nutrient input need to be

optimised with regard to their nutrient balance to maximise output from existing available land without the continued detrimental effects observed during the previous Green Revolution and for the past 50 years (Scherr and McNeely, 2008). Central to this issue is the fact that many agricultural systems in developed nations are currently maintained at a 'saturated state' with regard to their macro-nutrient status, principally to ensure continued availability of bio-available plant nutrients that facilitate high crop yields. These situations are indicative of 'leaky' systems where excess nutrients may be leached from the soil; not only is this a waste of expensive and valuable resources but is also responsible for the associated environmentally detrimental effects. Organic approaches to agriculture that typically use manures, crop residues and legume rotations to manage soil fertility (Watson et al., 2002) and restrict the use of chemical fertilisers could help alleviate this issue (Gomiero et al., 2011). However, 34% lower yields when compared to conventional agriculture (see Seufert et al., 2012 for review), raise doubts as to whether organic agriculture could deliver future food requirements. Approaches to soil nutrient management that optimise growth from carefully managed fertilizer input are, therefore, central to sustainable agricultural approaches. Legumes were historically considered important in crop rotations to supply N for subsequent crops via their symbiotic relationship with N fixing bacteria (Stinner et al., 1992). More recently, legume rotations have become less popular as growers have instead relied on relatively cheap and abundant chemical N fertilisers (Crews and Peoples, 2004). However, with the increased pressure on fertiliser supplies (see Section 1.5 for more details) legume crops are likely to become more important in improving N nutrition in crops (Pretty, 2008). Hence research that seeks to better understand legume crop

physiology is justified. However, regardless of the source, without adequate nutrition plants will inevitably grow less and (from an agricultural perspective) yield less.

1.2 Plant responses to reduced nutrient availability

Plants require 14 essential elements in varying concentrations to maintain normal growth and development (Table 1.1, Marschner, 2012). Deficiency in one or more of these nutrients may impair biochemical and physiological processes that will reduce the plants ability to achieve their full developmental potential. Hence plants have developed adaptive physiological strategies that allow them to survive nutrient deficiency and maintain their lifecycle.

Table 1.1: Plant elements considered essential and average shoot dry matter concentrations required for adequate growth. (Source: adapted from Marschner, 2012).

Element	Typical concentration ($mg\ g^{-1}$)
Nitrogen (N)	15
Potassium (K)	10
Calcium (Ca)	5
Magnesium (Mg)	2
Phosphorus (P)	2
Sulphur (S)	1
Iron (Fe)	0.1
Manganese (Mn)	0.05
Boron (B)	0.02
Zinc (Zn)	0.02
Copper (Cu)	0.006
Molybdenum (Mo)	0.0001

A common response to many nutrient stresses is impaired shoot growth while maintaining root proliferation, hence typically increasing root:shoot ratio. This adaptive response can maintain soil resource (commonly nutrients and water) acquisition while reducing the demand from the above ground parts of the plant. Specifically, in response to N (Zhang et al., 1999) and P (Lopez-Bucio et al., 2002) deficiency plants will promote lateral root proliferation thus assisting uptake from nutrient rich upper soil layers. In contrast, root development is not altered by potassium (K) or magnesium (Mg) deficiency (Cakmak et al., 1994).

Plants may also directly influence nutrient availability in the immediate vicinity of the roots by release of root exudates into the rhizosphere (Dakora and Phillips, 2002), a response that can be particularly well exploited by legume crops for P acquisition (Raghothama, 1999). The carboxylates malate, citrate and malonate were variously exuded by *Triticum aestivum*, *Brassica napus* and 11 legume species (including *Cicer arietinum*, *Pisum sativum*, *Lens culinaris*, *Lupinus* spp. and *Vicia faba*) in response to 3 µM P as opposed to 300 µM in controls (Pearse et al., 2006). In this experiment, *Lupinus*, *P. sativum* and *C. arietinum* species all showed the highest increases in low P induced carboxylate exudation, whereas *T. aestivum, B. napus, V. faba* and *L. culinaris* showed little or no change. Furthermore, modelling approaches have demonstrated that the exudation of carboxylates increases mobility and root uptake of P (Gerke et al., 2000).

Nutrient deprived plants commonly exhibit reduced rates of gas exchange (stomatal conductance (g_s) and photosynthesis (Pn)). Stomatal conductance and Pn were 67%

and 53% lower respectively in rice plants grown for 20 days in an N free nutrient solution compared to N sufficient plants, the reduced Pn was thought to be caused by impaired photochemical efficiency (Huang et al., 2004). Similar observations in bean supplied with only 0.5 mM N and 0.5 μM P were also attributed to biochemical limitations in Pn (Lima et al., 1999). Furthermore, increased intracellular CO_2 concentration implied that reduced g_s in P deficient (P availability described as trace amounts) soybean was mediated by reduced photochemical efficiency (Nian et al., 2003). However, in these examples plants were either deprived of nutrients or exposed to a severe nutrient stress that might be expected to limit biochemical processes involved in Pn, rather than more subtle nutrient deficiencies that may elicit different physiological responses.

Many of these described physiological responses to nutrient deficiency are commonly regulated by the action of plant hormones. Reduced g_s under P deficiency was correlated with increased root xylem concentrations of abscisic acid (ABA), a potent antitranspirant (Jeschke et al., 1996). Furthermore, stomata of P deficient cotton exhibited greater stomatal sensitivity to ABA than P replete plants when fed ABA in a detached-leaf transpiration assay (Radin, 1984). Similarly, lower g_s under N deficiency in cotton were attributed to ABA/cytokinin (CK) balance (Radin et al., 1982). However, ABA did not regulate reduced g_s in N deficient pea (Dodd, 2003a).

With respect to growth responses, inhibited primary root elongation and enhanced lateral root proliferation under P deficiency in *Arabidopsis* were attributed to the action of auxin (Al-Ghazi et al., 2003). Furthermore, auxin is important for root hair initiation and elongation (Bates and Lynch, 1996, Lopez-Bucio et al., 2005). Plant root

responses to auxin may be regulated by redistribution within the root (Nacry et al., 2005) or increased auxin sensitivity (Perez-Torres et al., 2008).

The plant hormone ethylene is known to regulate plant adaptations to a range of nutritional deficiencies (Lynch and Brown, 1997). Although the specific effects of ethylene on root architecture can vary considerably (Peret et al., 2011) inhibiting ethylene production with aminoethoxyvinyl-glycine or action with 1-methylcyclopropene decreased root elongation rates in *Arabidopsis* thus demonstrating its importance in maintaining root growth under P deficiency (Ma et al., 2003).

Plant CK concentration decreased in response to N and P deficiency (Salama and Wareing, 1979), and has been attributed to inhibiting shoot but promoting root proliferation under P deficiency (Martin et al., 2000). Furthermore, the relatively recently discovered plant hormones strigolactones are also thought to be involved with root developmental response to a range of nutrient deficiencies possibly by interacting with the action of auxin and CK (Brewer et al., 2013).

By moderating shoot behaviour in response to an edaphic (nutrient) stress it could be argued that some of these plant hormones must be acting as root-to-shoot signals (Jeschke and Hartung, 2000). To be defined as a root-to-shoot signal, a compound (hormone) must move acropetally via the apoplast and/or symplast and act remotely from the site of synthesis (root) on shoot behaviour (Dodd, 2005). There is strong evidence that plant responses to other abiotic stresses are co-ordinated by root-to-shoot signalling of plant hormones. In particular, increased root synthesis of ABA and subsequent transport to the shoot via the xylem is known to regulate plant stomatal

responses to reduced water availability (Hartung et al., 2002, Wilkinson and Davies, 2002). However, given that a wide range of nutrient deficiencies can increase root and shoot ABA concentrations (Battal et al., 2003) the role of ABA in mediating shoot responses to nutrient deficiency has received surprisingly little attention.

Many of these described physiological responses are regarded as important ecological adaptations that help plants cope with living in challenging environments (Chapin et al., 1993). However, from an agricultural perspective, limited nutrient availability and subsequent plant adaptive responses that inhibit shoot growth will typically result in reduced yield. Therefore, to maximise yield, agricultural soils must be managed appropriately to ensure the adequate supply of nutrients. One of the principle factors affecting the availability of plant nutrients is soil pH. Furthermore practices commonly employed in intensive agriculture (for example, overuse of mineral N fertilisers) can exacerbate soil acidification (Johnston et al., 1986).

1.3 Soil pH and nutrient availability in agricultural soils

Soil acidification is a gradual process, most prevalent in soils where rainfall exceeds evapotranspiration, driven by the release of H^+ ions into the soil solution from a range of natural processes. The predominant processes that produce H^+ ions include the photosynthetic assimilation of CO_2 and subsequent root deposition of H^+ ions following plant balancing of internal pH; oxidation and mineralisation of both N and sulphur (S) from organic or mineral sources; and plant uptake of cation nutrients, with resulting exudation of H^+ ions to maintain charge balance (Bolan et al., 2003a).

Further processes that may exacerbate the acidification of soil are the leaching of basic cations at a rate greater than mineral weathering processes can re-supply (White, 2006) and the deposition of acid compounds, predominantly nitric and sulfuric acids, from precipitation (Rodhe et al., 2002). Intensive agricultural practise may compound some of these issues by increased N and S oxidation processes resulting from application of additional chemical fertilisers (Matsuyama et al., 2005). Additionally, plant uptake and subsequent removal at harvest of soil neutralising basic cations can exacerbate the issue (Guo et al., 2010).

The implications of soil acidification on food production should not be underestimated and, unless remediated, soil acidity can decrease crop yields. The primary factors that impact on crop production in low pH soils are the decreased availability of some plant nutrients (principally calcium (Ca), molybdenum (Mo), Mg, K, P) and the phytotoxic effects of increased aluminium (Al) and manganese (Mn) availability (Sumner and Yamada, 2002). Additional agronomic issues associated with increased soil acidity include negative influences on many natural soil microbial communities that are important to nutrient mineralisation and cycling processes (Kemmitt et al., 2006). Classically, the optimal relationship between soil pH, soil nutrient availability and Al/Mn toxicity has been understood to be in the range of pH 5.5 to 7 (Figure 1.1, Brady and Weil, 2008).

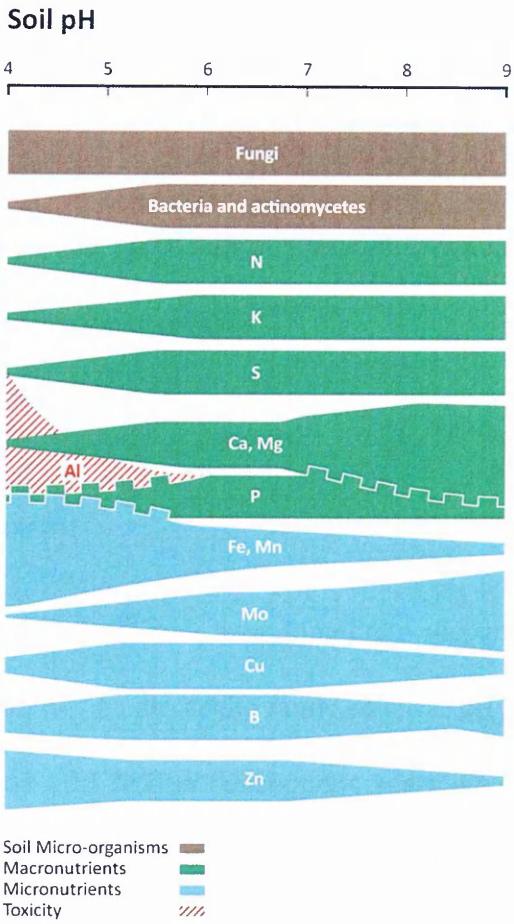

Figure 1.1: Diagrammatic representation of the relationship between soil pH and nutrient availability and the activity of soil microorganisms. (Source: Adapted from Brady and Weil, 2008).

It is estimated that nearly 40% of global arable soils are affected by acidity (Vonuexkull and Mutert, 1995). Therefore, careful manipulation of soil pH should be an intrinsic component in the management of sustainable agricultural soils and optimisation of resource use and crop production. Traditionally this has been achieved through the application of basic materials such as lime ($CaCO_3$) (Bolan et al., 2003a) at levels aimed to target pHs in the range of 6.5-7 depending on crop type

(Goulding et al., 2008). When lime is applied to acidic soils, $CaCO_3$ reacts with the excess H^+ ions, producing Ca^{2+}, H_2O and CO_2. Additionally, Al^{3+} will react with OH^- ions to produce $Al(OH)_3$ (the insoluble mineral gibbsite) thus helping to alleviate Al toxicity. Consequently, increased yields associated with alleviation of soil acidity are mostly explained by improved plant nutrient relations, reduced Al and Mn toxicity and improved soil mineralisation processes.

1.4 Liming to manage soil pH

The benefits of liming agricultural soils have long been recognised. Applying lime at the equivalent of 2 tonne ha^{-1} to an 'acidic sandy soil' (no pH value given) improved soybean yields by 50, 67 and 78% in subsequent years which was thought to be achieved through improved P availability (Whitson, 1913). Similarly, in classical long-term liming experiments at Rothamsted and Woburn, liming two low pH soils (pH 4.8 and 5.4) to pH 6.5-7 was optimum for field bean and barley yields when supplied with P and K fertiliser (Bolton, 1970). Increasing soil pH from 4.6-5 to 5.7-6.2 with lime increased yield of bean by 1.7- to 3.1-fold (Buerkert et al., 1990). Similarly, increased soil pH from <4.5 to 6 improved yields of wheat, barley and faba bean by 70% (Farhoodi and Coventry, 2008). However, more acid tolerant potato and oat showed no yield benefit to liming when supplied with standards rates of NPKMg fertilisers in the long-term Rothamsted and Woburn trials (Bolton, 1977). Similarly, although liming three low pH soils (initial pH 4.32, 4.43, and 4.51) at an equivalent rate of 2 tonne ha^{-1} in pot trials increased potato yield by 40%, liming did not

increase yield in five other soils with an initial pH above pH 4.9 (Vanlierop et al., 1982a).

Liming responses are not always positive, and yield reductions can occasionally occur at moderate lime applications. Liming to increase soil pH from 5.6 to 6.8 reduced yield of white clover by 40% in pot trials despite daily watering with a complete nutrient solution and was attributed to a change in Ca:Mg ratios (Carran, 1991). Similarly, yield of six clover species showed a negative correlation with liming rates that raised soil pH from 5.2 to 5.7, 6.3 and 7.2 and was thought to be driven by reduced P availability (Maxwell et al., 2012). Furthermore, a review of liming responses suggested that Mg fixation into insoluble minerals with silica, chlorite or Al may cause some negative yield responses (Sumner et al., 1978).

Clearly, soil pH amendments that mitigate the deleterious impacts of soil acidification are an important feature of most sustainable agricultural systems. However, the percentage area of UK tillage crops receiving lime has declined by a fifth and for legumes, halved in the last 20 years (Figure 1.2). This implies that managing soil pH is not as prevalent as it was, perhaps demonstrating a reliance on the excessive use of additional mineral fertilisers. However, careful soil pH management through liming does not entirely negate the need for supplementary fertilisers and chemical inputs will still be necessary in order to maximise yield potential. Nonetheless, with correct soil pH management, chemical inputs can be limited to appropriate levels that improve yield while minimising wastage and associated environmental impacts.

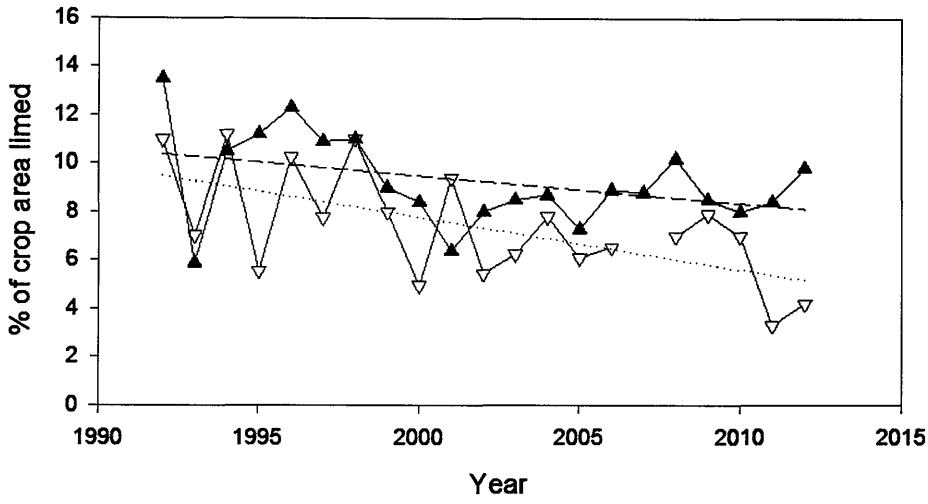

Figure 1.2: Percentage of UK crop area receiving a liming treatment for legume (open triangles) and all tillage crops (filled triangles) from 1992 to 2012. The overall trend is indicated by the dotted (legume) dashed (all tillage) lines. Data is plotted from The British Survey of Fertiliser Practice annual surveys from 1992 to 2012. Liming typically occurs every 3-5 years, hence the maximum % of crop area limed in any one year would be 20%. (Source: http://archive.defra.gov.uk/evidence/statistics/foodfarm/enviro/fertiliserpractice).

1.5 Pressures on fertiliser supply

In addition to the environmental cost of fertiliser input, the manner of resource acquisition and processing has implications if chemical fertilisers are to fit into a sustainable agricultural approach. Nitrogen fertiliser, though sourced from unlimited atmospheric N via the Haber-Bosch process (Follett et al., 1981), is energy intensive and heavily reliant on fossil fuels for processing (Dawson and Hilton, 2011), which themselves are not only a finite resource but contribute greatly to climate change that currently threatens agriculture and food production. Potassium and P are exclusively sourced and processed from mined mineral deposits which are obviously finite in their availability. The future limitations on P supply has received much attention (Cordell et al., 2009) and is recognised, quite correctly, to be a potential

serious limiting factor in future food production. However, modelling approaches to nutrient audits (Sheldrick et al., 2002) that take account of crop off take and fertiliser inputs may suggest that, globally, it is the supply of K fertilizer that is of most concern with global agricultural arable soils having an average K deficit of -20kg ha^{-1}. Though not as limiting a resource as P (Dawson and Hilton, 2011), it is concerns over the nearness of demand to production and supply (Sheldrick et al., 2002) that has driven unprecedented global K price increases in the last six years (Chaize, 2010). In fact market prices for N, P and K all increased substantially during 2008; however K prices (which was traditionally the cheapest fertiliser) have remained high at levels three-fold in 2013 when compared to prices a decade ago (Figure 1.3). With the increasing pressure on natural mineral deposits of fertilisers and high global prices, alternative sources of obtaining K fertiliser for agriculture are of increasing interest (Manning, 2010).

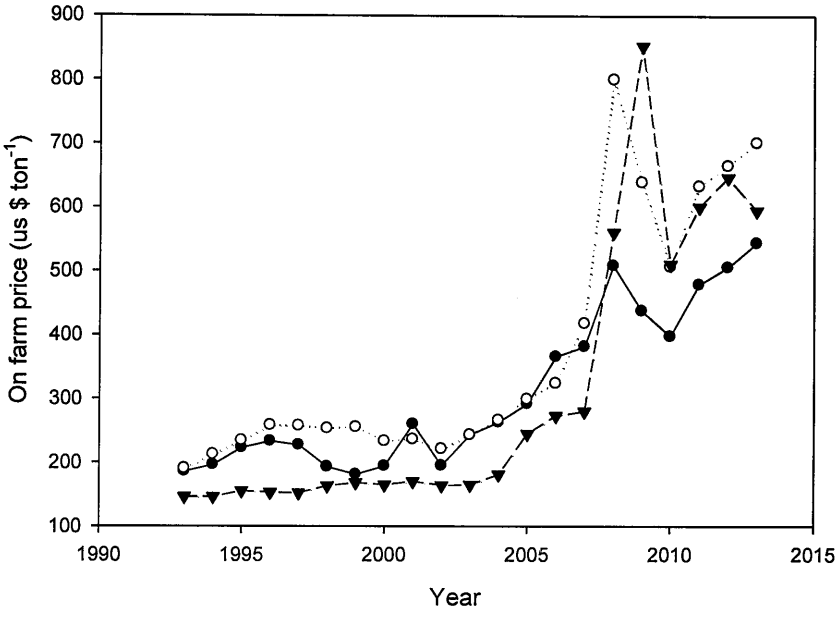

Figure 1.3: On farm US fertiliser price for ammonium nitrate (filled circles), superphosphate (open circles) and potassium chloride (filled triangles) from 1993 to 2013 (Source: www.ers.usda.gov/data-products/fertilizer-use-and-price.aspx).

1.6 Alternative nutrient sources

That future sustainable agricultural systems will require input from chemical fertilizers to maximise crop growth is undoubted. However, an important issue is the economic and environmental pressures associated with the procurement of such fertilizers and how they are then efficiently translated to crop yield in the field. Thus, novel approaches to sources of additional plant nutrition could potentially provide alternatives to traditional, expensive and increasingly limited nutrient resources. Increasingly, by-products and wastes from a range of industrial processes are being identified as potential alternative sources for cheap, accessible and valuable plant nutrients and base pH soil amendments (Huang and Lu, 2000). Examples of such by-products recognised as having value in agricultural systems include slags from metal

refining processes (Bhat et al., 2010, Li et al., 2010); fly ashes from combustion processes, primarily fossil fuels for electricity production (Seshadri et al., 2010, Singh et al., 2010); gypsum, a by-product of the phosphate fertiliser production process (Illera et al., 2004); and sludges from the paper manufacturing industry (Camberato et al., 2006). All of these have varying degrees of nutritional and/or pH amendment benefits. However, of particular interest to the present study is the agricultural usefulness of by-products of the cement manufacturing industry, namely cement kiln dust and cement by-pass dust, herein collectively referred to as cement kiln dust (CKD).

1.7 Cement kiln dust: Production and properties

CKD is a waste product derived during the production of Portland cement. The cement manufacturing process involves the firing of raw materials at high temperatures in large rotator blast kilns. Raw kiln feed comprises of approximately 80% calcium carbonate ($CaCO_3$), usually limestone or chalk, and 20% silica (SiO_2), usually sourced from shale, clay or sand, with small amounts of alumina (Al_2O_3) and iron oxide (Fe_2O_3). Temperatures of 800-900°C are required to initiate the calcining of $CaCO_3$ to produce calcium oxide (CaO), followed by higher temperatures of 1450°C to instigate a sintering stage to form the reaction products, namely, calcium silicates, aluminates and aluminoferrite, the primary constituents of cement. Once cooled a clinker is formed that is then ground with gypsum to produce a final cement product.

Depending on manufacture process, waste dusts are filtered and collected from either the main kiln exhaust or a raw feed preheater/precalciner that uses residual

heat from exhaust gases to calcine raw feed before entering the rotary kiln, thus making the process more efficient (Figure 1.4). The precalcifying process also has the added advantage of removing vaporised alkali products such as K_2SO_4, KCL, NaCl and Na_2SO_4 that may adversely affect the quality of the final product.

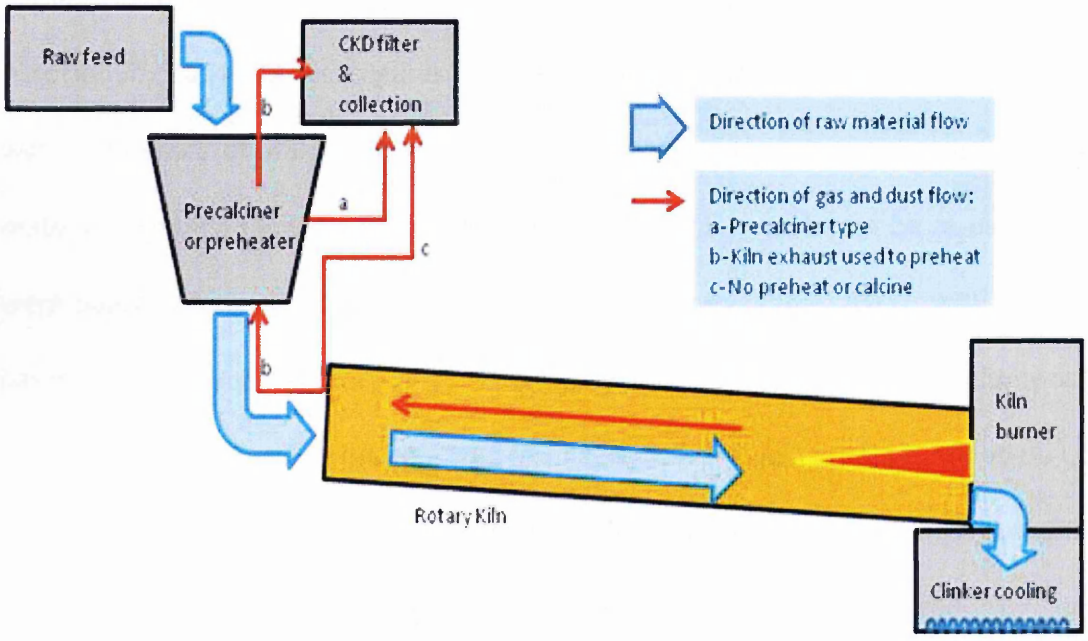

Figure 1.4: Diagrammatic representation of cement kiln and differing dust collection process. Dusts are collected either from (a) precalciner with separate heat source (by-pass dust); (b) preheater that uses kiln exhaust gases to heat raw materials; (c) direct from kiln exhaust.

The dusts are comprised mainly of partially calcined and un-reacted kiln feed, dust from the fully formed cement clinkers and ash from the kiln fuel. Typical components of CKDs are difficult to quantify owing to site to site variability arising from differing manufacturing process, raw feed, fuel source and dust collection method (Adaska and Taubert, 2008), however, its primary constituents are silicates, calcium oxide, potassium oxide, sulphates, chlorides, sodium oxide and various other metal oxides

(US EPA, 2011). Being composed of essentially raw feed in various stages of reaction, much of the dust can be re-fed into the kiln and further reacted to form cement. However, production technicalities in older facilities, and often undesirably high alkali salt content, mean a proportion material collected cannot be re-fired and is therefore removed, generating significant quantities of waste material.

Cement production in the UK for 2013 was 11.3 million tonnes (Mineral Products Association, 2014). Similar figures for 2009 produced nearly 1.3 million tonnes of waste materials of which 1.2 million tonnes were recovered and re-used as raw materials. Some 45,000 tonnes of waste cement products could not be re-used and were subsequently disposed of (Mineral Products Association, 2011), with landfill being the common option (US EPA, 1993). Pressures to reduce the volume of industrial wastes reaching landfill come from a European level. The UK is signed up to both the EU Waste Framework Directive 2008 (DEFRA 2011) and the Landfill Directive 1999 (Environment Agency, 2011) with the overall aim of reducing the volume of waste products entering landfill either by reduction or reuse. Furthermore, the financial penalties for utilising valuable landfill capacity are an increasing burden on industry sectors that generate significant volumes of non-recyclable waste materials. Although modern cement manufacturing processes are facilitating the increased re-cycling of cement dust (Ghorab et al., 2004), clearly significant volumes are still reaching landfill, therefore, sustainable alternative uses for waste CKD are of interest not only to the cement manufacturing industry itself but also to government.

Motives behind the use of all industrial by-products as agricultural inputs are, therefore, not only due to their potential agricultural value but also economic and political. Should industrial by-products be suitable as agricultural amendments, re-directing industrial by-products from landfill to farmer's fields could help meet the 'sustainability agenda' on two fronts, both from the perspective of sustainable food production and of waste management. Significant levels of calcium oxide, potassium oxide and sulphates within CKD have generated interest from agronomists as a potential source of valuable K fertilizer and furthermore, its high inherent pH has seen it used as a soil acidity amendment in lieu of agricultural lime.

1.8 Cement kiln dust: Use in agriculture

Existing studies that have investigated the role of CKD as an agricultural input are few, however, available data does highlight the potential for CKD as an alternative source of agricultural liming material and/or fertiliser. CKD was equally effective at increasing extractable K than comparable rates of commercial K fertiliser in a field trial of potato (Lafond and Simard, 1999). Similarly, CKD increased yields of potato, barley and alfalfa equally to those seen by the addition of a commercial K fertilizer, potassium chloride (KCl) or potassium sulphate (K_2SO_4) in a range of K deficient soils (Vanlierop et al., 1982b). In a comparison of natural and industrial liming products, Lalande et al., (2009) found that CKD significantly increased soil extractable K by 50% in a sandy loam soil.

Other studies have noted the effectiveness of CKD as a soil pH amendment. Surface applied CKD had a similar neutralising effect to agricultural lime on a forage crop

system (Rodd et al., 2004). CKD incorporated into the top 10 cm of soil increased soil pH similarly to that of agricultural lime in a newly created pasture system (Dann et al., 1989). CKD amendments increased bioavailability and plant accumulation of the nutrients Ca, K, and Cu (copper) (and consequently yield of a grass/clover forage system) to a greater extent than lime additions (Rodd et al., 2010) although this may again be explained by differences in material fineness, and therefore reactivity.

It is apparent from the literature that CKD is currently being successfully utilised in many agricultural systems as both a soil pH amendment and K fertiliser and if applied appropriately can be a cheap alternative to commercially produced agricultural inputs. So successful in fact that this once waste product is increasingly being re-dug from some existing landfill deposits as awareness of its economic and potential agricultural value grows (Adaska and Taubert, 2008). The potential of CKD for use within agricultural systems is, therefore, apparently not in doubt, although inherent product variability may make broad statements about specific agricultural applications difficult. Therefore, field trials investigating CKD mediated soil pH adjustments and K nutrition are required to demonstrate the potential of CKD use in the UK.

1.9 CKD and heavy metals

CKD is clearly a potentially useful agronomic input as both a pH amendment and/or K fertiliser and research into the effects of such inputs on plant physiology are well justified. However, in common with many industrial by-products, there may be some

concern regarding potential heavy metal contamination, or potentially toxic elements (PTE's), with CKD amended soils.

CKD can contain varying levels of heavy metals including mercury (Hg) , Cu, nickel (Ni), lead (Pb), cobalt (Co) and cadmium (Cd) (US EPA, 1993) all of which may be phytotoxic if present at sufficient levels (Alloway, 1995). Furthermore, bioaccumulation of potentially zootoxic elements from heavy metal contaminated soils may also have implications higher up the food chain, either from consumption of crop species or meat from livestock fed with heavy metal contaminated grazings or fodder (Nicholson et al., 2006). However, whether CKD contains PTE's present in sufficient levels to affect crop (or human) health, or are indeed any different to levels already associated with existing agricultural inputs, is a questionable issue. Further information regarding heavy metal loading of soils specific to CKD and agriculture is reviewed in Chapter 2, however, a meta-analysis of data regarding heavy metal concentrations in CKD and other agricultural inputs such as N and P fertilisers and agricultural lime (Figure 1.5) would indicate that potential Pb and arsenic (As) levels in CKD may far exceed those found in other commonly used agricultural inputs.

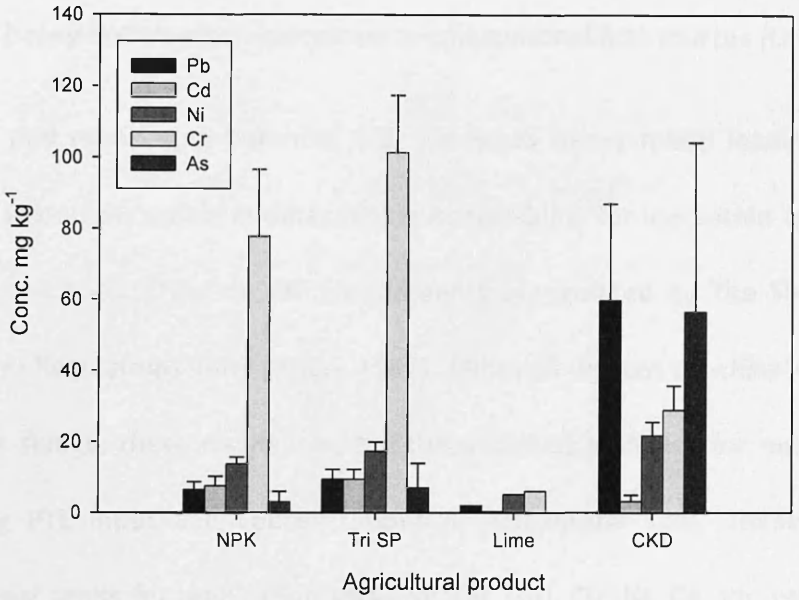

Figure 1.5: Concentration of selected heavy metals in CKD, agricultural lime and two fertiliser types: NPK and triple superphosphate (Tri SP). Data are means ± SE of 4 and 3 replicates respectively (NPK and Tri SP from US EPA (1993)); 3 replicates (CKD from Lafond and Simard (1999), Dinel *et al* (2000) and US EPA (1993)) ; lime data from Nicholson *et al*. (2003).

Clearly an inherent problem with assessing the potential content and bioavailability of heavy metals in CKD and subsequent agricultural implications is inter-product variability. This occurs between differing manufacturing sites but also from batch to batch. Factors that affect this variability include raw feed materials, method of manufacture and dust collection and especially the fuel used to fire the kiln. This final point is of particular interest as cement kilns in many countries, including the UK, are now commonly co-fired with fuel derived from hazardous waste materials (Guo and Eckert, 1996) such as waste oils, toxic solvents and used tyres (Reijnders, 2007). The extreme temperatures involved (>1480°C) mean that cement kilns are increasingly seen as a cost effective method for disposal of hazardous wastes leading to concerns from some sectors as to the agricultural suitability of cement dusts derived from this

fuel source. Kilns fired or co-fired with hazardous wastes can contain elevated levels of certain heavy metals when compared to conventional fuel sources (US EPA, 1993).

Assessing and monitoring potential CKD mediated heavy metal loading of soils is clearly an important aspect in determining its suitability for use within agriculture. In the UK, application PTE's via soil amendments is regulated by The Sludge (Use in Agriculture) Regulations 1989 (Anon, 1989). Although written specifically for the use of sewage sludge, these regulations are the accepted standard for monitoring and controlling PTE input and concentrations in agricultural soils. These regulations provide clear limits for application rates of zinc (Zn), Cu, Ni, Cd, Pb, chromium (Cr) and Hg based on a ten year average annual application and also maximum total acceptable levels for PTE's within the soil. The Department of the Environment document 'Code of Practice For Agriculture Use Of Sewage Sludge' (DEFRA, 2006) provides additional guidance for levels of Mo, selenium (Se), As and fluoride (F), thus there is clear guidance on acceptable application rates for a wide range of PTE's. Experiments assessing the levels of PTE's in CKD and subsequent bioavailability are clearly required in assessing the suitability of CKD as an agricultural input.

1.10 Conclusion and Future Directions

This review has aimed to understand the potential use of CKD within future sustainable agricultural approaches. It is apparent that alternative sources of additional plant nutrition are required if future food requirements are to be met and, unless constrained by issues around heavy metal toxicity, industrial by-products such as CKD could offer a partial solution. In addition, materials that are base in their

composition could improve the efficient use of existing nutrient supplies through alleviating the deleterious effects of soil acidification caused by intensive agricultural practices. However, investigations into the physiological impacts of soil pH change and nutrition availability and how these interact with plant hormone relations, could further our understanding in this area and inform appropriate future agronomic practice.

Consequently, Chapter 2 of this thesis compared physiological responses of several legume crops to both CKD and agricultural lime to establish the suitability of CKD as an alternative liming agent and K fertiliser. However, since there was a consistent reduction in leaf gas exchange, plant growth and yield, in both pot and field trials, in response to applying recommended rates of liming, further studies aimed to determine whether these deleterious physiological responses could be attributed to excessive Ca (Chapter 3) and/or changes in nutrient uptake (Chapter 4). The role(s) of plant hormones and plant water status in mediating physiological responses to a lime-induced P deficiency were further investigated (Chapters 4, 5). To our knowledge, this study represents a first example where a standard nutrient management treatment adversely affects crop growth and development by altering root-to-shoot signalling of a specific plant hormone.

Chapter 2 – Both agricultural lime and cement kiln dust decrease growth and gas exchange of three legume species

2.1 Introduction

Legume crops are considered important in some crop rotation systems, principally through their ability to fix atmospheric N, thus reducing the need for chemical N fertilisers (Pretty, 2008). Most legume crops are relatively intolerant of soil acidity (Graham and Vance, 2003) therefore when growing such species liming to raise soil pH to recommend levels (pH 6.5) would be considered normal agricultural practice. As discussed in Chapter one, alternative liming materials and/or nutrients are being increasingly utilised due to limited availability and increased cost of conventional sources. Of particular interest to the present study is cement kiln dust (CKD) which typically has a high pH (12.6-13) and significant K levels (3%-9%) (data supplied by 4R Recycling Ltd) making it a possible alternative liming and K source.

Globally, there are few existing studies that have investigated CKD as a potential agricultural input. Furthermore data for UK agriculture is either non-existent or unpublished (S Dudman 2010, pers. comm.). However, available data does highlight the potential for CKD as an alternative agricultural liming material for pH management. CKD at application rates of 4 and 6 tonnes ha^{-1} incorporated into the top 10 cm of a soil with an initial pH of 4.9 increased soil pH to 5.5 and 5.7 respectively after two months, values comparable to those generated by the same rates of agricultural lime (Dann et al., 1989). Though there was no significant effect

on pasture herbage yield in the first year of application, after two further years pasture yields were approximately 3- and 4-fold higher when compared to an unamended control. A surface application of CKD on a forage crop system neutralised soil pH to a greater extent than agricultural lime supplied at equivalent neutralising rates at depths measured from 3 cm to 20 cm (Rodd et al., 2004). However, 2 tonne ha^{-1} applications of CKD at two sites gave no significant change in soil pH with mean values being -0.37 and +0.11 pH units different from initial values (Lafond and Simard, 1999) though potato tuber yield was increased by approximately 45% when compared to an unamended control. This lack of pH change is not surprising given the low application rate which was chosen principally to supply K rather than moderate soil pH.

Since CKD is commonly high in K, several studies have investigated the suitability of CKD as a K fertiliser. Treating soils with 100, 150 or 200 kg ha^{-1} CKD and equivalent additions of potassium chloride (KCl) or potassium sulphate (K_2SO_4) fertiliser produced identical increases in extractable K (Vanlierop et al., 1982b). Furthermore, these fertilisers had similar effects on yields of potato, barley and alfalfa in pot trials using a range of K deficient soils. Similarly, CKD applied at 2 tonnes ha^{-1} was equally effective at increasing extractable K than comparable rates of commercial K fertiliser (50% KCl and 50% K-Mg sulphate) at two different field sites, when assessed 15 days after planting (Lafond and Simard, 1999). CKD significantly increased soil extractable K by around 50% when compared to control in sandy loam soil (Lalande et al., 2009). Furthermore, surface application of CKD increased bioavailability and plant accumulation of K (and consequently yield of a grass/clover forage system) to a greater extent than lime additions (Rodd et al., 2010).

Although clearly a potential liming substitute and/or K fertiliser (as discussed in Chapter 1), a possible concern of CKD (and other industrial by-products) are levels of Potentially Toxic Elements (PTE's) and subsequent plant availability. However, the literature on PTE's supplied by CKD is scarce. Soil extractable Cd, Cr, Ni (nickel) and Pb levels were not significantly increased after two field applications of CKD at rates of 0.5, 1 and 2 tonnes ha^{-1} (Lafond and Simard, 1999). Further, potato tuber uptake of these metals was similar to comparable levels of commercial K fertiliser and lime. Furthermore, extractable Cu, Zn, Cr, Ni, Pb, Co and As were similar in biosolids stabilised with CKD or lime, implying that CKD did not contribute PTE's to any greater extent than agricultural lime (Dinel et al., 2000), results similar to those found for Pb, Cr, Ni and Cd (Simard et al., 1999). Though CKD is known to contain PTE's (Reijnders, 2007), it is apparent that they may not be present at levels that inhibit its use in agricultural soils.

It is clear from existing data that CKD has potential to be agronomically useful, however the lack of data from the UK, and possible differences in chemical composition caused by differing parent materials and kiln fuel source (Guo and Eckert, 1996), highlights the need for trials using UK sourced CKD and soils. Therefore this chapter will investigate the capacity of CKD to change soil pH and supply plant available K compared to traditional agricultural lime and K fertiliser in field and pot trials. Additionally, the liming substrates used will be characterised to assess their neutralising and nutritional value and levels of PTE's measured to ensure compliance with current guidance on application of PTE's to agricultural land. Furthermore, the performance (biomass, yield, gas exchange and nutrient uptake) of three commonly grown legume crops (*Vicia faba, Phaseolus vulgaris* and *Pisum sativum*) will be

assessed under the above treatments. *Vicia faba* was chosen for the field trial owing to its common use in the UK as a forage legume. *Phaseolus vulgaris* and *Pisum sativum* were chosen as their rapid growth and morphology made them particularly suitable for pot trials.

2.2 Materials and methods

2.2.1 Characterisation of liming materials

2.2.1.1 Nutrient and PTE analysis

To prepare liming substrates for analysis of macro-nutrients and PTE's, samples were subject to microwave assisted acid digestion (Mars-5 Xpress microwave accelerated reaction system, CEM Corporation, Matthews, North Carolina, US). A CEM standard method was adopted for digestion of kiln dust, where 0.5 g samples of liming substrate were accurately weighed on an analytical balance and transferred to a pre-washed Teflon pressure vessel. To this, 10 ml of concentrated trace metal grade HNO_3 (Sigma-Aldrich, Dorset, UK) was added and the vessels allowed to stand open in a fume hood while the initial reaction subsided (approximately 20 minutes). The Teflon vessels were then sealed with a pressure release screw lid and uniformly distributed in the microwave carousel. Ten samples (each liming material in triplicate and one blank) were then digested for 10 minutes at a maximum temperature of 175°C. Once cooled, the pressure vessels were carefully opened and 5 ml of the digestate was decanted into a 50 ml centrifuge tube, diluted to 20% HNO_3 with 20 ml Millipore water and stored awaiting analysis.

To prepare samples for analysis, 1 ml of the 20% acid digestate was further diluted ten times with Millipore water to a final 2% HNO_3 concentration and filtered through a 0.45 micron syringe filter. Macro-nutrients (Ca, K, Mg, P, and S) were analysed using Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES; iCAP 6300, Thermo Scientific, Massachusetts, USA). PTE's were analysed using Inductively Coupled Plasma – Mass Spectrometry (ICP-MS; 7500a, Agilent Technologies, California, USA). In both the ICP-OES and ICP-MS analysis, samples were compared against standards of a known range of concentrations and corrected, if required, using determinations from blank samples run in the microwave digestion.

2.2.1.2 Determination of neutralising value

The neutralising value (NV) as calcium oxide equivalent (CaO%) of the two liming substrates used in the field and pot trials was determined by titration following the method described by Faithfull (2002). Briefly, 1 g of agricultural lime or 0.5 g CKD was weighed to the nearest 0.001 g with an analytical balance and transferred to a 250 ml conical flask with 50 ml of 0.5 M HCl in a fume cupboard. After the initial reaction had finished (approximately 20 minutes), the conical flask was covered with a watch glass and the mixture gently boiled for five minutes on a hot plate then cooled to room temperature. Three drops of phenolphthalein indicator (0.25 g phenolphthalein dissolved in 150 ml 95% ethanol and diluted to 250 ml with distilled water) were added and then the solution titrated with 0.5 M NaOH to the indicator end point. An initial quick titration was undertaken using a 50 ml glass burette to determine the approximate end point. Three subsequent more accurate titrations

determined the exact end point which was indicated by a bright pink colouration. NV was calculated from the following equation:

$$NV = y \times 0.01402/x \times 100$$

Where y = volume of titre and x = sample weight.

The mean value of the three titrations was taken to be the CaO equivalent NV of the liming substrates.

2.2.2 Broad bean (*Vicia faba*) field experiment

A field scale experiment was carried out at Lee Farm, Myerscough College, Lancashire, UK on a site previously established as pasture. The site was chosen as a low pH sandy loam soil (pH 5.6, 46% sand, 32% silt, 9% clay, 13% organic matter) that allowed application of agronomically significant levels of liming materials. Initial soil pH was determined from sixteen 20 cm soil cores collected from across the field site that were homogenised, air dried and analysed in triplicate using the method described in section 2.2.2.4 of this chapter. This initial soil pH was used to determine liming treatment rates.

Treatments were calcium carbonate ($CaCO_3$) based agricultural lime (J. Arthur Bowers Ltd Coarse Screened Limestone, William Sinclair Horticulture Ltd, Lincoln, UK) that had a neutralising value (CaO equivalent) of 57%, at an application rate of 7 tonnes ha^{-1}. A second liming agent used was CKD with a neutralising value (CaO equivalent) of 53%, at an application rate of 6 tonnes ha^{-1}. Application rates were

calculated to meet the DEFRA (2010) recommended target soil pH of 6.5 using the Rothlime online liming calculator (McGrath, 2002) that allowed inclusion of neutralising value and Ca speciation ($CaCO_3$ for lime and CaOH for CKD) in generating the recommendations. In addition to the above treatments and an unlimed control, sulphate of potash fertiliser (SOP; Yara UK Ltd, Grimsby, Lincolnshire, UK) was applied to half the plots at a rate of 226 kg ha^{-1} which was the equivalent K supplied by the CKD. This gave six treatments in a three by two factorial experiment that was arranged in a randomised complete block design with four repeats as detailed in Figure 2.1 below. Plot size was 5 x 3 meters.

The treatments were applied by hand at the beginning of February to previously ploughed plots and incorporated into the top 10-15 cm of the soil profile using a tractor mounted rotavator. A crop of field bean (*Vicia faba* L. cv Fuego) was drilled two months later at a rate of 25-30 seeds m^2. The crop was then managed using standard agronomic crop establishment and protection practice which included an application of Bentazon selective herbicide (at recommended rates supplied by the manufacturer) one month after drilling to control weeds.

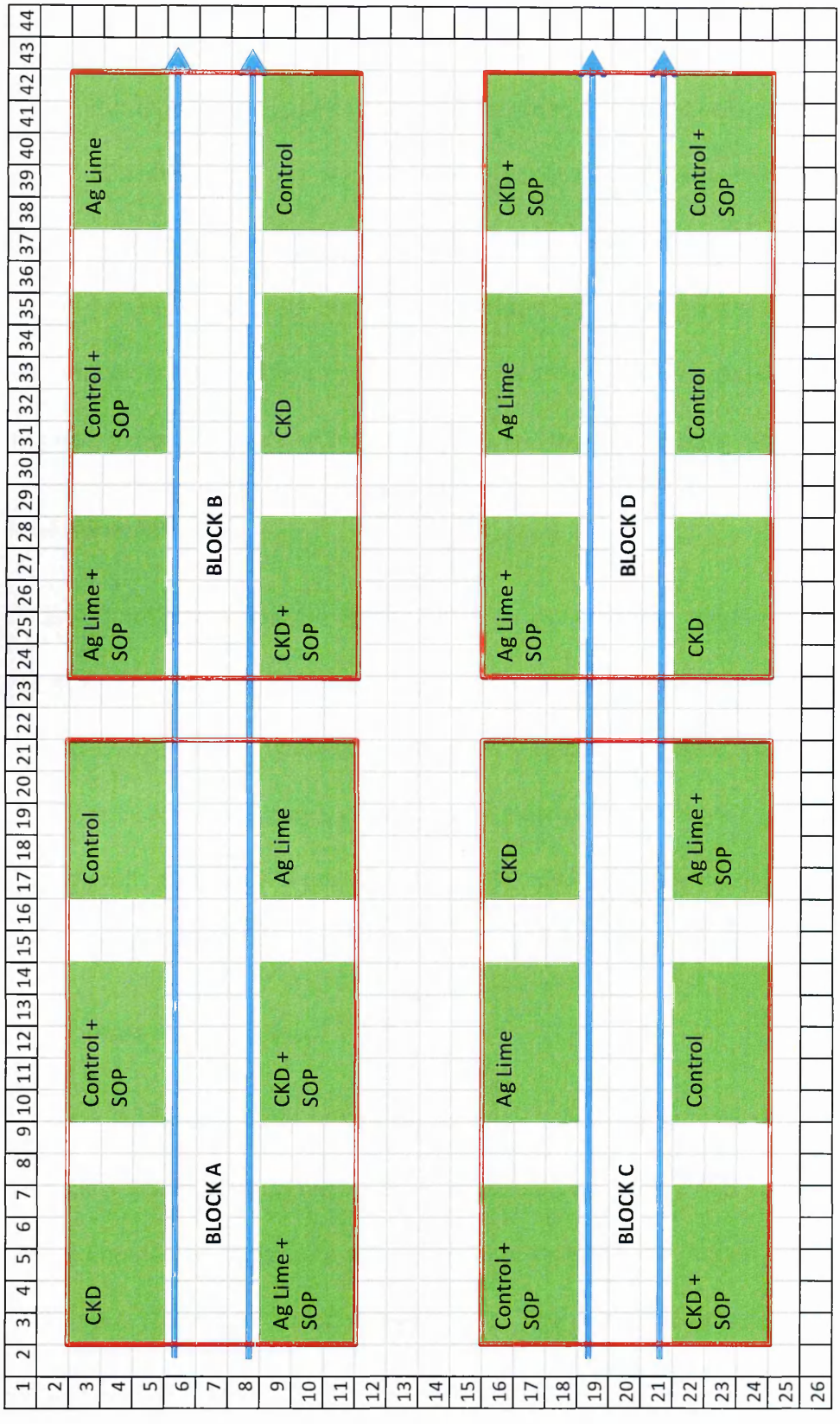

Figure 2.1: Schematic of field trial design. Treatments were arranged in four blocks (A to D) each containing six treatments in randomised 5 x 3 m plots indicated by green rectangles. Total planted crop area was 44 x 26 m, blue lines indicate tractor tram line position.

2.2.2.1 Crop growth and development

To monitor crop growth, plant height was recorded periodically through the growing season. Quadrats of 1 m^2 were randomly selected and stem density and height recorded using a meter rule, three replicates within each treatment plot were used. Density was recorded to account for effects on plant height owing to poor germination rates. Final bean pod yield was recorded in two randomly selected 1 m^2 quadrats per plot and pod count, stem count and total pod weight recorded.

2.2.2.2 Gas exchange

On two occasions during the growing season, gas exchange measurements (g_s and Pn) were recorded on the newest fully expanded leaf using infra-red gas analysis (6400xt Li-Cor Portable Photosynthesis System, Lincoln, Nebraska, USA). CO_2 was set at ambient levels (390 ppm), radiation was matched to ambient conditions on the day (typically 600 to 1000 µmol m^{-2} s^{-1} PPFD), cuvette temperature of 22°C was set and ambient humidity used. Three replicates were taken per treatment plot.

2.2.2.3 Plant tissue analysis

For plant tissue nutrient and PTE analysis, three fully expanded leaves were collected from each treatment plot two weeks prior to harvest, oven dried at 80°C for seven days, combined and ground to a fine powder using a ball mill (Retsch MM400, Retsch UK Limited, Castleford, West Yorkshire, UK). The dried and ground samples were then subject to microwave assisted acid digestion (Mars-5 Xpress microwave accelerated reaction system, CEM Corporation, Matthews, North Carolina, US). The method used was adapted from a CEM recommended protocol, where 0.25 g

samples of plant material were accurately weighed on a four point analytical balance and transferred to a pre-washed Teflon pressure vessel. To this, 5 ml of concentrated trace metal grade HNO_3 (Sigma-Aldrich, Dorset, UK) was added and the vessels allowed to stand open in a fume hood while the initial reaction subsided (approximately 20 minutes). The Teflon vessels were then sealed with a pressure release screw lid and uniformly distributed in the microwave carousel. Samples were then digested for 30 minutes at a maximum temperature of 200°C. Once cooled the pressure vessels were carefully opened and 1 ml of the digestate was decanted into 15 ml centrifuge tubes, diluted to 20% HNO_3 with 4 ml Millipore water and stored awaiting analysis.

To prepare samples for analysis, 1 ml of the 20% acid digestate were further diluted ten times with Millipore water to a final 2% HNO_3 concentration and filtered through a 0.45 micron syringe filter. Macro-nutrients (Ca, K, Mg, P, and S) were analysed using Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES; iCAP 6300, Thermo Scientific, Massachusetts, USA). PTE's were analysed using Inductively Coupled Plasma – Mass Spectrometry (ICP-MS; 7500a, Agilent Technologies, California, USA). In both the ICP-OES and ICP-MS analysis, samples were compared against standards of a known range of concentrations and corrected, if required, using determinations from blank samples run in the microwave digestion.

2.2.2.4 Soil sampling and analysis

Soil samples were taken from each treatment plot using a soil auger in the top 20 cm of the soil profile immediately prior to treatment application and three further times through the growing season with the final samples being taken two weeks after harvest. Five samples were randomly collected within each plot and homogenised for analysis. Soil pH was measured in air dried soil that had been crushed and passed through a 2 mm sieve using the DEFRA recommended (MAFF, 1986) method of a ratio of 1:2.5 soil to distilled water. A 20 g soil sample was mixed in small plastic containers with 50 ml distilled water, thoroughly stirred and left for one hour. Soil pH was determined by re-mixing and immediately measuring the suspension with a pH electrode (Orion Sure Flow, Fisher Scientific, Loughborough, UK) and meter (Denver Instruments, Bohemia, New York, USA).

Major cations were analysed using an ammonium acetate extraction using the following method:

- Weigh 2 g of air dried soil, crushed to pass through a 2 mm sieve, into a 50 ml centrifuge tube.
- Add 30 ml of 1 M ammonium acetate solution and extract on an end over end mechanical shaker for 15 minutes.
- Centrifuge at 3000 rpm for 15 minutes and filter the supernatant through Whatman No 1 filter paper into a 100 ml volumetric flask.
- Repeat two further extracts from the same sample with 30 ml of ammonium acetate so an approximate total of 90 ml extract is collected, make up to 100 ml with 1 M ammonium acetate.
- Add 2.5 ml of 10% w:v lanthanum chloride to all samples as a releasing agent.

- For Ca and Mg determine by atomic absorption spectroscopy (AAS; AAnalyst 200, PerkinElmer, Massachusetts, USA) in the above ammonium acetate extract and compare against a range of standards of known concentration. Absorption determined at 423 nm for Ca and 285 nm for Mg.
- For K, determine by flame photometry (Sherwood Model 410, Sherwood Scientific Ltd, Cambridge, UK) calibrated using standards with a known range of concentrations and using the same ammonium acetate extract as above.

Phosphorus was determined colorimetrically (Jenway 6315 Colorimeter, Bibby Scientific Ltd, Stone, UK) in an Olsen bicarbonate extraction using the following method:

- Weigh 1 g of air dried and 2 mm sieved soil sample into 50 ml centrifuge tubes.
- Extract on a mechanical end over end shaker for 30 minutes using 20 ml of Olsen reagent (0.5 M $NaHCO_3$ adjusted to pH 8.5 with 10 M NaOH solution).
- Centrifuge samples at 3000 rpm for 5 minutes and filter supernatant through Whatman No 2 filter paper into a clean centrifuge tube.
- Decant 2.5 ml of extract into a clean centrifuge tubes, add 0.5 ml of 1.5 M H_2SO_4 and swirl to release the carbon dioxide.
- Sequentially add 10 ml of 0.15% w/v ammonium molybdate solution and 2.5 ml of 85 mM ascorbic acid solution.
- Stand for 30 minutes to allow colour to develop, record absorbance at 880 nm and compare to a calibration curve generated from a range of standards at known phosphorus concentrations.

2.2.3 Broad bean (*Vicia faba*) pot trials

2.2.3.1 Plant material

Seeds of broad bean (*Vicia faba* L. cv Longpod) were sown directly into 1.5 litre pots (140 mm high, 150 mm diameter) containing a 2:1 (v:v) mixture of a low pH sandy loam field soil and horticultural grit sand (DA30, Boughton, Kettering, UK). Two seeds were sown per pot and one plant selected for uniformity after emergence. The field soil was collected from the top 20 cm of the same research site (Lee Farm, Myerscough College, Lancashire, UK) used for the field trial described above. Grit sand was used to aid drainage. The soil/sand combination was homogenised in a cement mixer for 5 minutes, passed through a 10 mm sieve, and sterilised (Camplex 68 l, Thermoforce Ltd, Cockermouth, UK) at a minimum temperature of 82°C to prevent infection from imported soil borne pests and diseases.

Lime treatments were the previously described agricultural lime and CKD at rates of 3 and 2.5 g l^{-1} respectively. All liming rates were targeted to reach a final soil pH of 6.5 as recommended by DEFRA (2010) and converted from tonnes ha^{-1} to g l^{-1} with the assumption that soil pH is measured in the top 20 cm soil profile and 1 ha contains 2000000 litres of soil at 20 cm depth. Field soil, grit sand and lime combinations were thoroughly homogenised in 15 litre batches for five minutes in a cement mixer before incubation in black plastic bags for a minimum of four weeks prior to planting.

In addition to the liming treatments, prior to potting, half the pots received an application of sulphate of potash fertiliser (SOP; Yara UK Ltd, Grimsby, Lincolnshire, UK) at a rate of 0.157 g l^{-1} which supplied 0.075 g l^{-1} K, the same as provided by the

CKD liming agent, the SOP was homogenised with the soil/sand/lime combinations for 5 minutes with a cement mixer.

All pots were watered to run off and weighed after 24 hours to establish weight at drained capacity and maintained well watered by replacing full evapotranspiration (determined gravimetrically) daily and maintained in a semi-controlled naturally lit greenhouse with supplementary lighting (supplied by Osram 600 w daylight bulbs) for 12 hours and 22°C/16°C minimum day/night temperature at the Lancaster Environment Centre.

2.2.3.2 Plant measurements

Stomatal conductance was recorded on three week old plants between 11:00 am and 13:00 pm on the abaxial leaf surface using an AP4 diffusive porometer (Delta-T Devices, Cambridge, UK). Measurements were made 24 hours prior to harvest on one leaflet of the third leaf pair numbered from the base, two readings were taken per plant and the mean taken as being the value for that plant.

Shoot fresh weight was determined when the plants were 4 weeks old, along with leaf area using a leaf area meter (Li-3050A, Li-Cor, Lincoln, Nebraska, USA). Roots were collected, washed of soil and checked for nodulation. Both shoot and root material was then dried at 80°C for one week to record dry weight and stored in air tight containers to provide samples for nutrient analysis.

2.2.3.4 Soil and plant tissue analysis

Both soil and plant tissue nutrient analysis was undertaken using the same procedures as described in Sections 2.2.2.3 and 2.2.2.4 for the field trial above. Leaf

tissue analysis was undertaken on a homogenised oven dried sample of all leaves present at time of harvest.

2.2.4 Dwarf bean (*Phaseolus vulgaris*) and tall pea (*Pisum sativum*) pot trials

2.2.4.1 Plant material

In separate experiments, seeds of dwarf bean (*Phaseolus vulgaris* l. cv. Nassau) and tall pea (*Pisum sativum* l. cv. Alderman) were pre-germinated on tissue paper dampened with distilled water and kept in the dark. On emergence of the radicle (2-3 days), uniformly germinated seeds were individually transplanted into 0.8 litre pots (110 mm high, 120 mm diameter) containing the same sterilised 2:1 (v:v) mixture of a low pH sandy loam field soil and horticultural grit sand as described previously. Pots were weighed when filling to ensure equal volumes of soil.

To establish that *Phaseolus* and *Pisum* responded similarly to previous observations in *Vicia*, a dose response experiment was undertaken using liming rates that spanned the recommended application rate. Treatment was agricultural lime (J. Arthur Bowers Ltd. Coarse Screened Limestone, William Sinclair Horticulture Ltd, Lincoln, UK) or CKD at application rates of 0, 1, 2, 3, 4 g l^{-1} soil. Recommended application rates to target a final soil pH of 6.5 (DEFRA, 2010) would be 3 g l^{-1} and 2.5 g l^{-1} for lime and CKD respectively. Field soil, grit sand and lime combinations were thoroughly homogenised in 15 litre batches for five minutes in a cement mixer before incubation in black plastic bags for four weeks prior to planting.

All plants were watered to run off and weighed after 24 hours to determine drained capacity, kept well watered by replacing full evapotranspiration (determined gravimetrically) daily and maintained in a semi-controlled naturally lit greenhouse (with supplementary lighting supplied by Osram 600 w daylight bulbs for 12 hours and 22°C/16°C minimum day/night temp) at the Lancaster Environment Centre.

2.2.4.2 Plant biomass and gas exchange measurements

Gas exchange (g_s and Pn) was measured on the middle leaflet of the fully expanded first trifoliate leaf (*Phaseolus*) or one leaflet of leaf pair four (*Pisum*; youngest fully expanded leaf) using infra-red gas analysis (6400xt Li-Cor Portable Photosynthesis System, Lincoln, Nebraska, USA). CO_2 was set at ambient levels (390 ppm), radiation at 600 µmol m^{-2} s^{-1} PPFD, cuvette temperature of 22°C was set and ambient humidity was used.

Shoot fresh weight was recorded at time of harvest, along with leaf area using a leaf area meter (Li-3050A, Li-Cor, Lincoln, Nebraska, USA). Roots were collected, washed of soil and checked for nodulation. Shoot and root material was then dried at 80°C for one week to record dry weight.

2.2.5 Statistical analysis

Two-way Analysis of variance (ANOVA) was used to determine significant treatment effects and interactions of lime and SOP on soil nutrient status and leaf tissue macro-nutrients (Tables 2.4-2.7) and gas exchange and yield parameters (Figures 2.3, 2.4, 2.6, 2.8, 2.10 and 2.11) in *Vicia faba* field and pot trials. Regression analysis was used

to determine dose response relationships between application rate of liming agent and soil pH, gas exchange and yield parameters in *Phaseolus vulgaris* and *Pisum sativum* pot trials (Figures 2.12-2.14). All analyses used Minitab v16.

2.3 Results

2.3.1 Characterisation of liming materials

The PTE analysis of the liming materials used in the field and pot trials is shown in Table 2.1. CKD contained all assayed elements, however, at the spreading rate used in the field trial, levels of most PTE's were <5% of the maximum application rates stated in the 'Code of Practice For Agriculture Use Of Sewage Sludge' (DEFRA, 2006) except Cd (18%), Mo (12%) Pb (11%) and Se (40%). The agricultural lime contained only trace concentrations of all PTE's.

Table 2.1: PTE levels determined by ICP-MS within liming materials used in field and pot trials and the levels of PTE's supplied at the rate used in the field trial (7 tonnes ha^{-1} for agricultural lime and 6 tonnes ha^{-1} for CKD). Data are means of three replicates. [A] Mean data from 17 CKD samples provided by 4R Group as ICP-MS did not support analysis of Hg and F. [B]As stipulated in the 'Code of Practice For Agriculture Use Of Sewage Sludge' (DEFRA, 2006).

PTE	PTE levels in liming material mg kg^{-1} (± SE)		PTE supplied @ field trial rate(kg ha^{-1})		Max allowable av. Annual PTE application rate (kg ha^{-1})[B]
	Lime	CKD	Lime	CKD	
Zn	0	124 ± 6.90	0	0.618	15
Cu	0	79.5 ± 3.40	0	0.397	7.5
Ni	4.77 ± 0.19	18.2 ± 1.06	0.029	0.091	3
Cd	0.21 ± 0.10	5.46 ± 0.26	0.001	0.027	0.15
Pb	0.26 ± 0.03	345 ± 18.7	0.002	1.725	15
Hg[A]	n/a	0.04 ± 0.01	n/a	0	0.1
Cr	3.74 ± 0.14	27.3 ± 1.48	0.022	0.137	15
Mo	0.70 ± 0.05	4.82 ± 0.35	0.004	0.024	0.2
Se	0.31 ± 0.05	8.73 ± 0.28	0.002	0.044	0.15
As	0.18 ± 0.02	5.39 ± 0.17	0.001	0.027	0.7
F[A]	n/a	68.3 ± 12.2	n/a	0.342	20

The nutrient analysis of the liming substrates shown in Table 2.2 reveal that, as expected, both lime and CKD are predominantly Ca based. Both contain trace levels of Mg, P and S, and CKD also has some trace Na. The lime contains no measurable K, however, the CKD contains approximately 4% elemental K. The CaO% equivalent neutralising value of lime and CKD, as determined by titration was 57% and 53% respectively (Table 2.3).

Table 2.2: Macronutrient levels of the liming substrates used in the field and pot trials as determined by ICP-OES. Data are means of three replicates.

Nutrient	Nutrient concentration in liming material in mg kg^{-1} (± SE)	
	Lime	CKD
K	0	37748 ± 1628
Mg	2610 ± 88	5601 ± 233
Na	0	3592 ± 155
P	208 ± 135	513 ± 27
S	541 ± 33	1781 ± 486
Ca	449259 ± 15971	424410 ± 18064

Table 2.3: Calcium oxide equivalent (CaO%) neutralising value of the liming substrates use in the field and pot trails as determined by titration. Data are means of three replicates.

Liming material	Neutralising value CaO% (± SE)
Lime	56.7 ± 0.28
CKD	52.7 ± 0.43

2.3.2 Broad bean (*Vicia faba*) field experiment

Sixteen weeks after application, both agricultural lime and CKD increased soil pH to 6.2 (Figure 2.2B), slightly less than the pH 6.5 target. However, pH was increased by 1 pH unit by both liming treatments which was hoped for (Figure 2.2A). Samples taken

later in the growing season (26 and 32 weeks after application) showed that pH had dropped slightly in both treatments to values between 5.8 and 6.

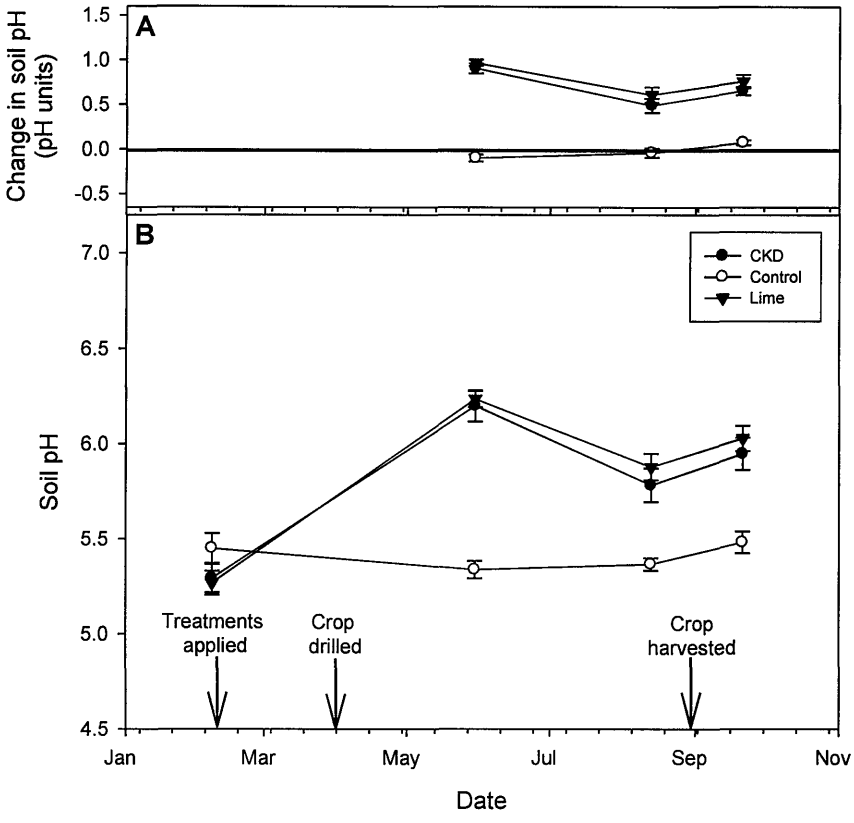

Figure 2.2: Soil pH measured through the growing cycle of the *Vicia faba* field trial (panel B) and change in pH from initial pre-treatment values (panel A). Data are means ± SE of 4 replicates.

The results of soil nutrient analysis on samples taken 16 weeks after application are shown in Table 2.4. Ammonium acetate exchangeable K was increased by 50% with CKD, 75% with lime plus SOP, 90% with SOP alone and 108% with the CKD plus SOP treatment. Exchangeable Ca was increased by 17% and 15% and Mg reduced by 20% and 24% by lime and CKD respectively, though neither were statistically significant. Olsen extractable P remained unchanged with all treatments.

Table 2.4: Soil exchangeable cations and extractable phosphorus from *Vicia faba* field trial measured 16 weeks after treatment application. Data are means of 4 replicates. P values from two-way ANOVA are indicated in the right hand side of the table with significant ($p<0.05$) values highlighted in bold.

	Soil exchangeable Ca, Mg, K and extractable P (mg kg^{-1})(± SE)						ANOVA		
Element	Control	Control SOP	Lime	Lime SOP	CKD	CKD SOP	Liming agent	SOP	Liming* SOP
Ca	2697 ± 390	2772 ± 1108	3169 ± 786	3217 ± 343	3105 ± 754	3283 ± 501	0.74	0.85	0.99
Mg	146 ± 17	169 ± 60	116 ± 36	118 ± 10	111 ± 21	125 ± 19	0.30	0.58	0.93
K	189 ± 24	358 ± 41	194 ± 24	319 ± 23	282 ± 33	394 ± 41	**0.04**	**<0.001**	0.66
P	38.6 ± 5.4	48.5 ± 3.1	37.7 ± 1.6	39.8 ± 4.8	41.1 ± 3.9	41.5 ± 3.6	0.58	0.19	0.42

Gas exchange data taken 13 and 15 weeks after drilling (Figures 2.3 and 2.4) revealed no significant change in g_s or rate of Pn in response to any treatment.

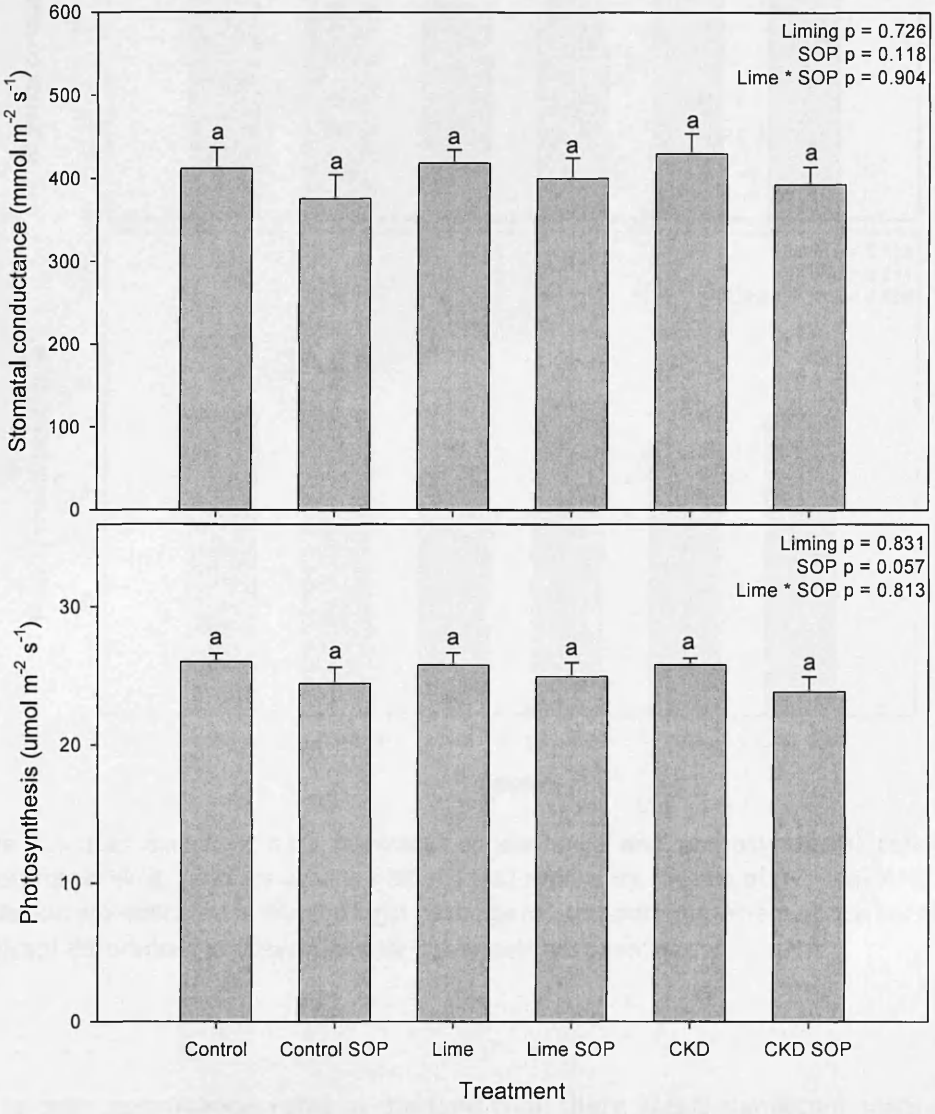

Figure 2.3: Gas exchange data (stomatal conductance and photosynthesis) collected 13 weeks after drilling. Data are means ± SE of 11-12 replicates. Results of two-way ANOVA with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

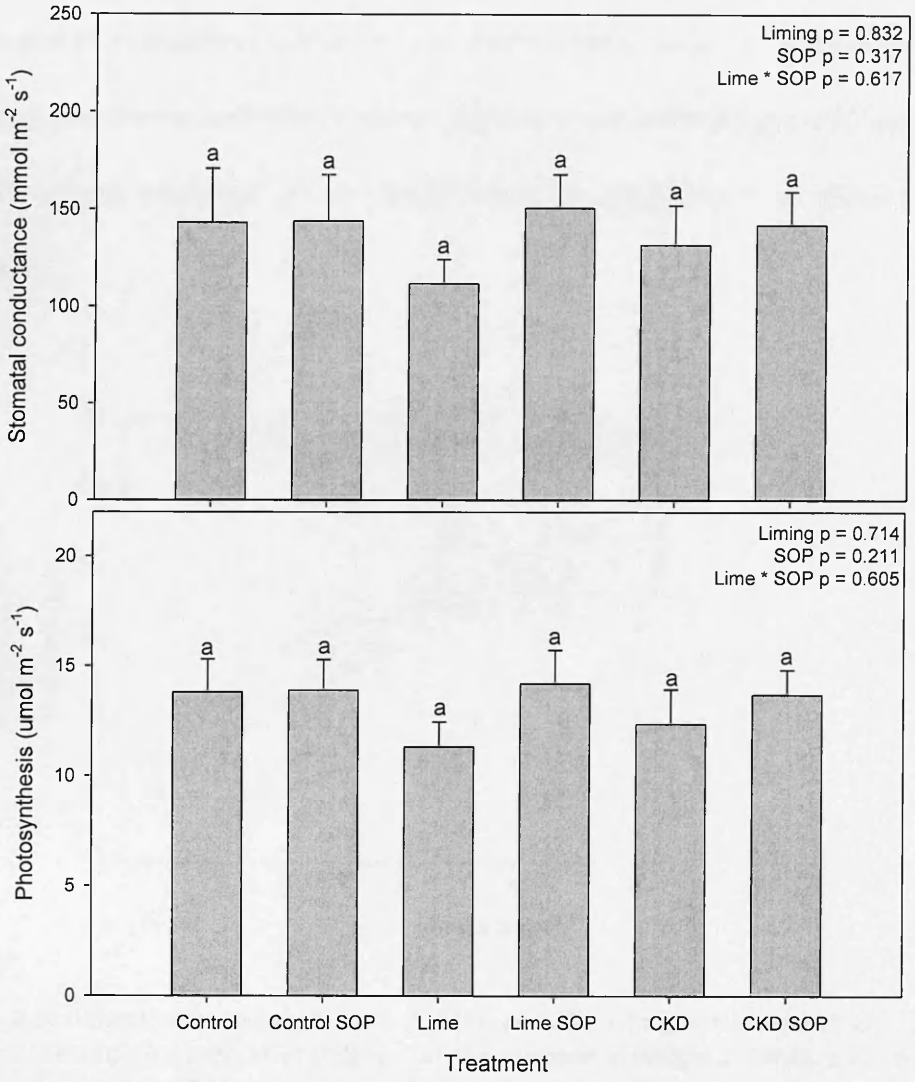

Figure 2.4: Gas exchange data (stomatal conductance and photosynthesis) collected 15 weeks after drilling. Data are means ± SE of 11-12 replicates. Results of two-way ANOVA with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Due to poor germination rates in the field trial, there was a significant stem density effect on plant height. To account for this, only quadrats with a stem count above which density ceased to be statistically significant were included in the analysis of plant height measured on both occasions (see Figures 2.5 and 2.7 for explanation). ANOVA revealed a significant (p=0.003) liming effect on plant height measured 14

weeks after drilling (Figure 2.6). Liming and CKD treatments decreased plant height by 5% and 10% respectively. Plant height measurements taken 19 weeks after drilling revealed that liming inhibition of plant height was sustained (Figure 2.8). Application of SOP had no treatment or interaction effect on plant height on either sampling occasion.

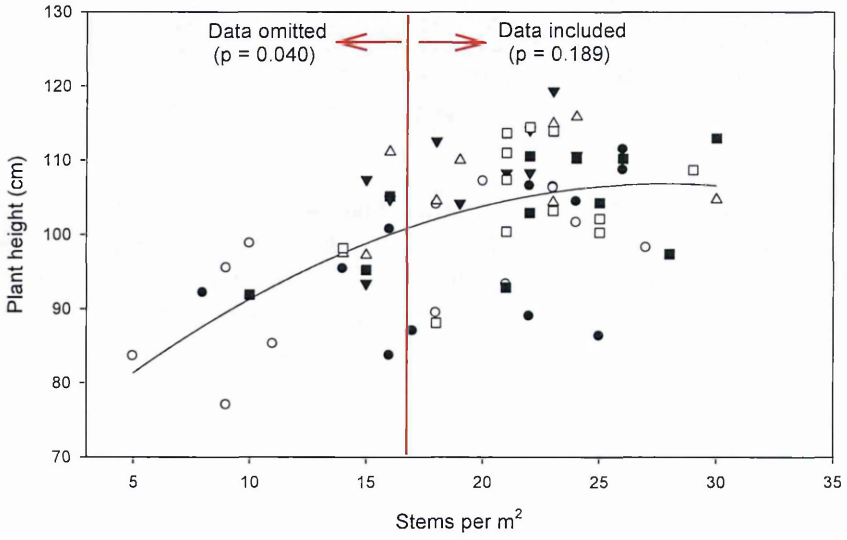

Figure 2.5: Relationship between stem density and plant height for data in all treatment groups collected 14 weeks after drilling. For the purpose of height analysis, plots with stem densities fewer than 17 stems per m^2 were omitted as covariate analysis revealed significant density effect (P values with full data set and without densities below 17 per m^2 indicated in parenthesis). Treatments are lime (squares), CKD (circles) or control (triangles), with (open symbols) or without (closed symbols) SOP fertiliser.

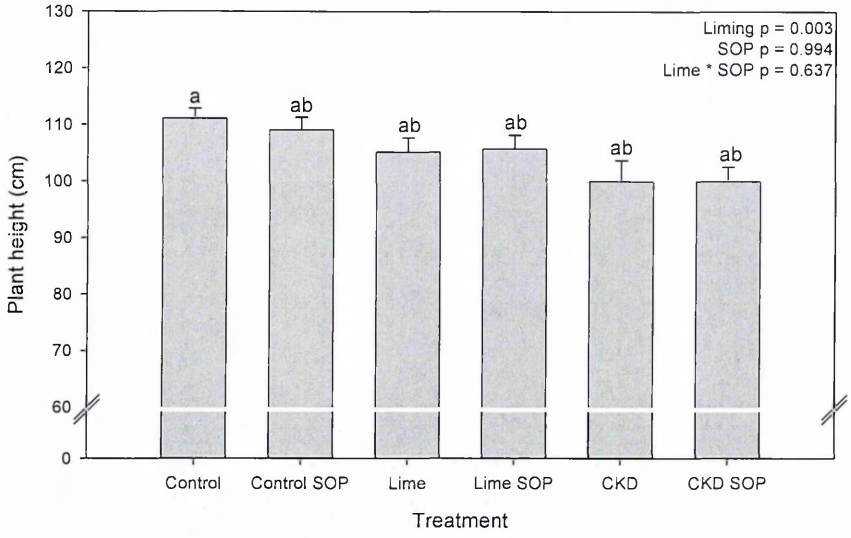

Figure 2.6: Plant height recorded 14 weeks after drilling. Data are means ± SE of four plots with three 1 m^2 quadrats sampled per plot, only plots with stem densities of 17 and above stems m^2 are included. Results of two-way ANOVA with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

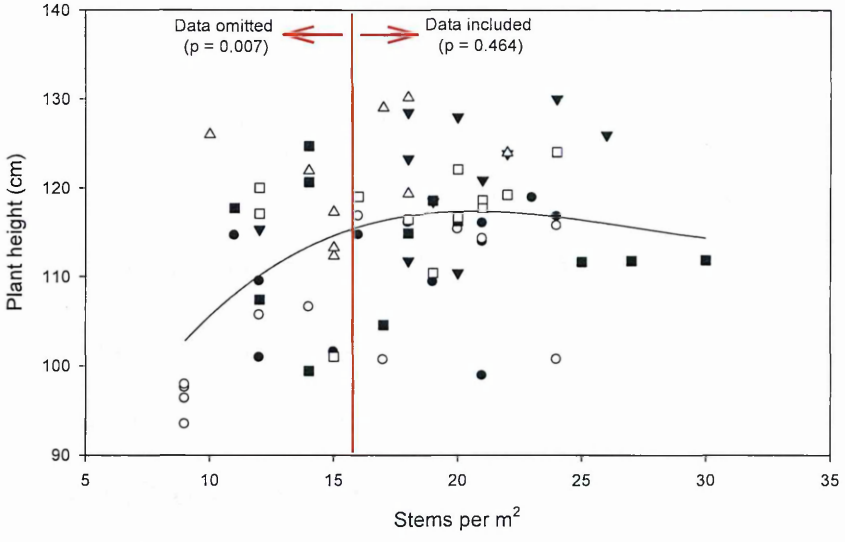

Figure 2.7: Relationship between stem density and plant height for data in all treatment groups collected 19 weeks after drilling. For the purpose of height analysis, plots with stem densities fewer than 16 stems per m^2 were omitted as covariate analysis revealed significant density effect (P values with full data set and without densities below 16 per m^2 indicated in parenthesis). Treatments are lime (squares), CKD (circles) or control (triangles), with (open symbols) or without (closed symbols) SOP fertiliser.

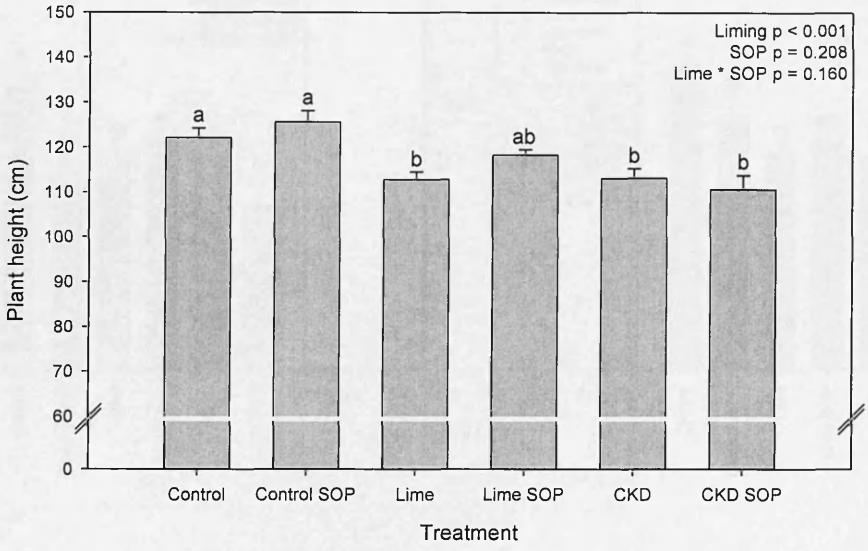

Figure 2.8: Plant height recorded 19 weeks after drilling. Data are means ± SE of four plots with three 1 m^2 quadrats sampled per plot, only plots with stem densities of 16 and above stems m^2 are included. Results of two-way ANOVA with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Total fresh pod yield was recorded 21 weeks after drilling and GLM analysis revealed a significant block effect (Figure 2.9A) which could be explained by the unlimed plots in one of the blocks having a pH below 5.5 (see Figure 2.9B for explanation). These plots were removed for further analysis without the influence of the block effect (Figure 2.9C). Stem density did not significantly influence pod yield. Figure 2.9C shows that treatment with lime and CKD reduced fresh pod yield by 28% and 30% respectively and CKD plus SOP reduced yield by 45% when compare to control. SOP did not significantly affect pod yield though reduced yield by 15% in the control group.

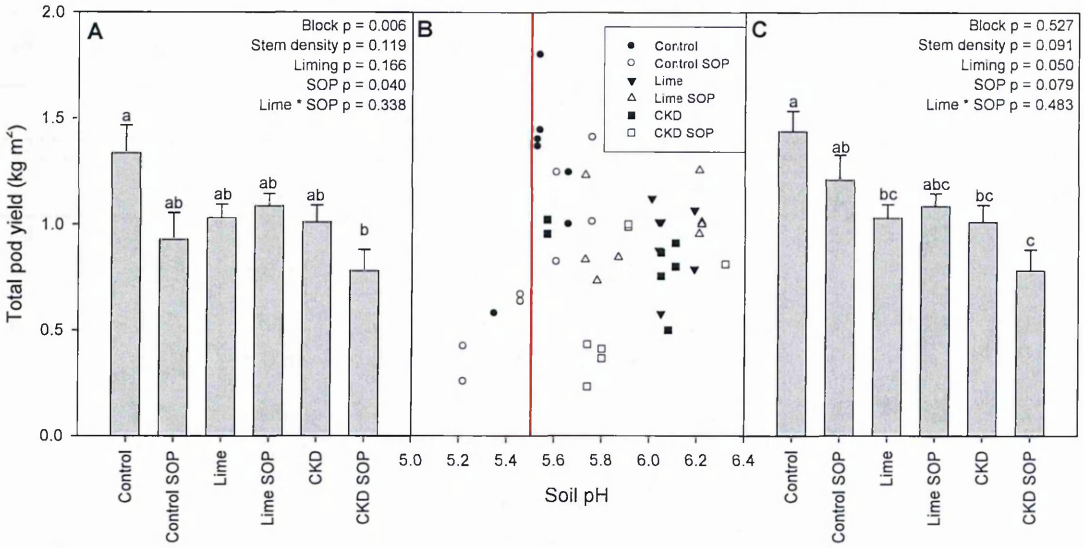

Figure 2.9: Total fresh pod yield recorded 21 weeks after drilling (panel A). The red line in panel B indicates a soil pH of 5.5, plots with a pH below this were omitted from further analysis in panel C which accounted for the significant block effect. Data are means ± SE of four treatment plots with two 1 m^2 quadrats sampled per plot. Results of GLM with block effect, covariate (stem density) and treatment effects with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Table 2.5 shows the results of leaf tissue macro-nutrient analysis. Leaf K level was increased equally by ~14% in the control SOP, lime SOP and CKD treatment groups, CKD plus SOP increased leaf K by 19%. Leaf tissue Ca was increased by 19% and 23% by lime and CKD respectively. Leaf Mg and P were unchanged by any treatment.

Table 2.5: Leaf tissue macro-nutrient analysis from the *Vicia faba* field trials. Data are means of 4 replications. P values from two-way ANOVA are indicated in the right hand side of the table with significant ($p<0.05$) values highlighted in bold.

	Leaf tissue concentration (g kg^{-1})(± SE)						ANOVA		
Element	Control	Control SOP	Lime	Lime SOP	CKD	CKD SOP	Liming agent	SOP	Liming *SOP
Ca	8.64 ± 0.95	8.61 ± 0.47	10.3 ± 0.77	10.1 ± 0.47	10.7 ± 0.75	10.1 ± 0.92	0.06	0.67	0.94
K	24.9 ± 1.86	28.4 ± 0.86	25.5 ± 2.02	28.5 ± 1.79	28.1 ± 1.09	29.6 ± 0.91	0.33	**0.04**	0.80
Mg	2.45 ± 0.23	2.68 ± 0.14	2.66 ± 0.14	2.79 ± 0.19	2.67 ± 0.18	2.61 ± 0.13	0.65	0.50	0.70
P	3.74 ± 0.43	3.70 ± 0.26	3.77 ± 0.20	3.74 ± 0.15	3.88 ± 0.17	3.47 ± 0.20	0.96	0.45	0.70

Leaf tissue PTE levels are shown in Table 2.6. Leaf Zn, Cu, Cd, Pb and As levels were all significantly reduced under liming with either agricultural lime or CKD and Ni, Cr, Mo and Se remained unchanged when compared to the unamended control. Application of SOP significantly reduced leaf Ni and Cu content across all liming treatments including control, though all other PTE's analysed were unaffected by the SOP application.

Table 2.6: Leaf tissue PTE analysis from the *Vicia faba* field trials. Data are means of 4 replicates. P values from two-way ANOVA are indicated in the right hand side of the table with significant ($p<0.05$) values highlighted in bold.

	Leaf tissue concentration (mg kg^{-1}) (± SE)						ANOVA		
Element	Control	Control SOP	Lime	Lime SOP	CKD	CKD SOP	Liming agent	SOP	Liming *SOP
Zn	176 ± 6.49	214 ± 22.9	121 ± 11.9	103 ± 9.79	151 ± 15.8	135 ± 11.5	**<0.001**	0.95	0.08
Cu	17.2 ± 0.68	14.7 ± 0.79	12.1 ± 1.09	9.71 ± 1.18	13.9 ± 1.28	12.1 ± 1.00	**0.001**	**0.01**	0.81
Ni	3.13 ± 0.23	2.20 ± 0.23	2.50 ± 0.19	1.84 ± 0.20	3.07 ± 0.51	2.20 ± 0.18	0.17	**0.01**	0.86
Cd	0.22 ± 0.02	0.23 ± 0.02	0.07 ± 0.03	0.04 ± 0.00	0.16 ± 0.02	0.14 ± 0.05	**<0.001**	0.88	0.73
Pb	0.43 ± 0.09	0.53 ± 0.06	0.28 ± 0.01	0.25 ± 0.05	0.37 ± 0.07	0.23 ± 0.04	**0.01**	0.69	0.19
Cr	1.97 ± 0.33	2.29 ± 0.48	2.37 ± 0.40	1.95 ± 0.22	1.65 ± 0.24	2.21 ± 0.81	0.91	0.57	0.71
Mo	2.21 ± 0.94	0.75 ± 0.16	1.35 ± 0.51	1.59 ± 0.40	1.78 ± 0.24	1.83 ± 0.24	0.74	0.34	0.19
Se	1.30 ± 0.36	1.92 ± 0.11	1.01 ± 0.20	1.10 ± 0.21	1.55 ± 0.35	1.57 ± 0.19	0.16	0.33	0.41
As	0.57 ± 0.09	0.80 ± 0.06	0.38 ± 0.06	0.38 ± 0.06	0.34 ± 0.06	0.45 ± 0.06	**<0.001**	0.08	0.25

2.3.3 Broad bean (*Vicia faba*) pot trials

Table 2.7 details soil exchangeable cations and extractable P. Exchangeable Ca was increased by ~70% by both lime and CKD and unaffected by the SOP application. Exchangeable K was significantly affected by both liming agent and SOP, in increasing order of magnitude, soil K was increased above control by 50% (lime), 90% (control SOP), 114% (CKD), 200% (lime SOP) and 256% (CKD SOP). Olsen extractable P was

reduced by ~24% by the lime application and ~15% by the CKD treatment. Exchangeable Mg was unaffected by either liming agent or SOP.

Table 2.7: Soil exchangeable cations and extractable phosphorus from *Vicia faba* pot trial. Data are means of 3 replicates. P values from two-way ANOVA are indicated in the right hand side of the table with significant ($p<0.05$) values highlighted in bold.

	Soil exchangeable Ca, Mg, K and extractable P (mg kg^{-1})(± SE)						ANOVA		
Element	Control	Control SOP	Lime	Lime SOP	CKD	CKD SOP	Liming agent	SOP	Liming *SOP
Ca	913 ± 46.5	1025 ± 48.1	1580 ± 72.4	1543 ± 103	1522 ± 145	1831 ± 133	**<0.001**	0.14	0.26
Mg	60.9 ± 2.47	65.7 ± 3.85	63.4 ± 3.03	61.7 ± 4.34	60.4 ± 7.82	73.2 ± 8.12	0.71	0.26	0.43
K	303 ± 7.48	572 ± 19.8	460 ± 15.0	909 ± 45.5	647 ± 13.0	1081 ± 71.4	**<0.001**	**<0.001**	0.06
P	23.8 ± 1.03	22.6 ± 0.52	17.5 ± 0.59	18.7 ± 0.54	20.9 ± 0.30	19.8 ± 0.31	**<0.001**	0.50	0.12

Stomatal conductance of pot grown *Vicia* recorded 24 hours prior to harvest was reduced by 60% and 50% in the lime and CKD treatments respectively when compared to control (Figure 2.10), the addition of SOP had no effect in the control or liming treatment groups. Shoot fresh weight was reduced by 35% in the lime treatment group and by 16% with CKD compared to the unlimed control, again SOP had no additional effect in any treatment group (Figure 2.11).

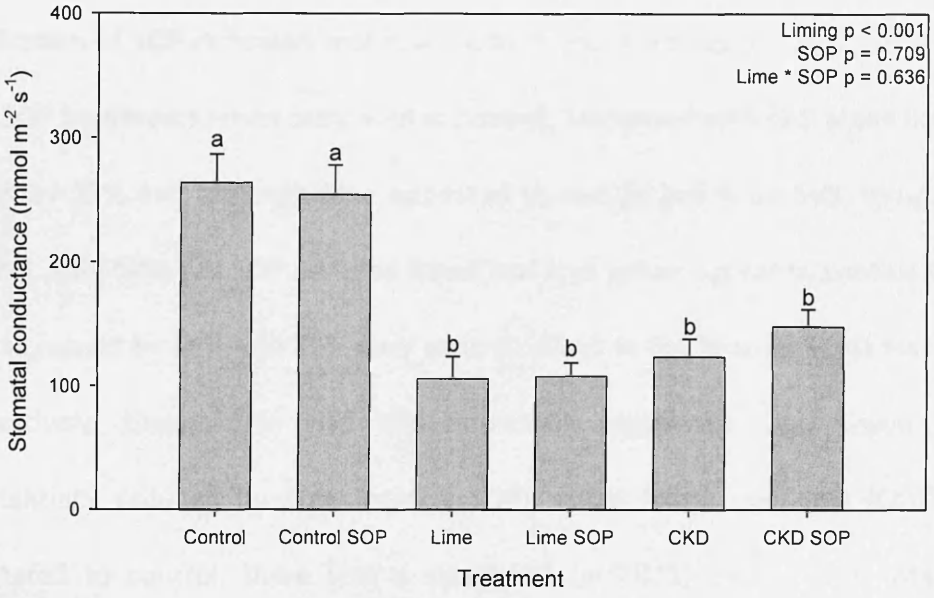

Figure 2.10: Stomatal conductance recorded 24 hours prior to harvest. Data are means ± SE of 11-12 replicates. Results of two-way ANOVA with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

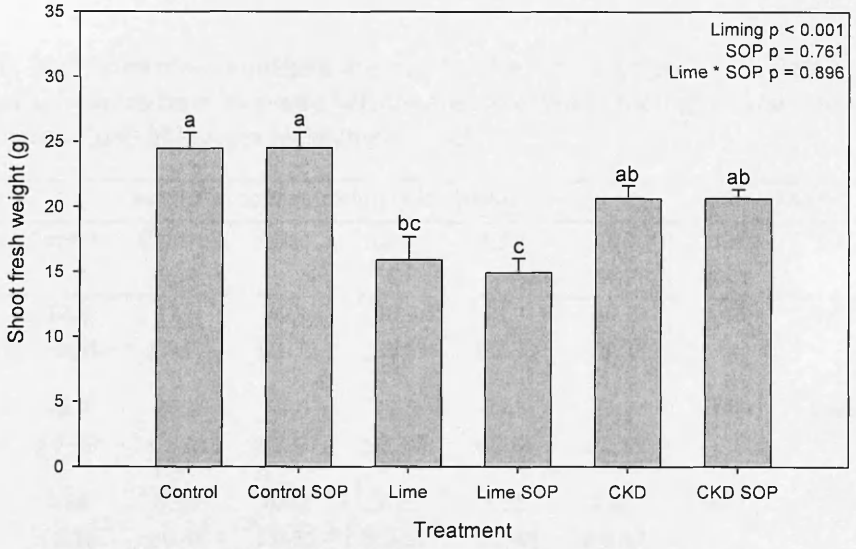

Figure 2.11: Shoot fresh weight of *Vicia faba*. Data are means ± SE of 11-12 replicates. Results of two-way ANOVA with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Results from leaf tissue analysis in the *Vicia* pot trials are shown in Table 2.8. Application of SOP increased leaf tissue K by ~35% in the control plus SOP and CKD plus SOP treatments when compared to control. Treatment with CKD alone increased leaf K by 22% and although lime appeared to reduce leaf K by 14% compared to control, application of SOP restored limed leaf K to values similar to control. Leaf Ca was increased by 10% and 20% over control values in the lime and CKD treatments respectively, though this was not statistically significant. Leaf tissue P was substantially reduced by 30% (control SOP), 50% (lime) and 40% (CKD) when compared to control, there was a significant (p=0.011) Lime * SOP interaction meaning that leaf P was reduced by SOP treatment in the control group but remained unaffected by SOP in both of the liming treatment groups. Leaf tissue Mg was unaffected by any of the treatments.

Table 2.8: Leaf tissue macro-nutrient analysis for the *Vicia faba* pot trials. Data are means of 5 replicates. P values from two-way ANOVA are indicated in the right hand side of the table with significant ($p<0.05$) values highlighted in bold.

	Leaf tissue concentration (g kg^{-1})(± SE)						ANOVA		
Element	Control	Control SOP	Lime	Lime SOP	CKD	CKD SOP	Liming agent	SOP	Liming *SOP
Ca	17.6 ± 0.54	17.5 ± 0.97	19.1 ± 1.72	20.7 ± 1.15	21.1 ± 2.59	19.7 ± 1.96	0.18	0.92	0.68
K	26.7 ± 2.03	36.2 ± 1.08	23.0 ± 2.67	27.1 ± 1.88	32.5 ± 2.57	36.3 ± 2.79	**0.001**	**0.01**	0.37
Mg	4.86 ± 0.18	5.39 ± 0.45	4.92 ± 0.55	5.12 ± 0.21	4.58 ± 0.48	4.82 ± 0.47	0.57	0.35	0.91
P	3.24 ± 0.18	2.29 ± 0.16	1.62 ± 0.08	1.69 ± 0.15	2.01 ± 0.21	1.89 ± 0.17	**0.001**	**0.02**	**0.01**

2.3.4 Dwarf bean (*Phaseolus vulgaris*) and tall pea (*Pisum sativum*) dose response pot trials

The soil pH dose response to application of lime and CKD is shown in Figure 2.12. Both liming agents showed a near identical linear relationship between application rate and pH with a soil pH of 6.4 occurring at a rate of 3 g l^{-1} in both treatments. For both species and both liming agents, treatment application rate was significantly negatively correlated with gas exchange (g_s and Pn) (Figure 2.13). At the 3 g l^{-1} application rate (which generated the soil pH nearest the DEFRA recommendation of 6.5) lime reduced g_s by 35% and 20% in *Phaseolus* and *Pisum* respectively and CKD reduced g_s in *Pisum* by 60% compared to control (Figure 2.13A, C, E). Similarly, Pn was reduced by 20% and 17% under liming in *Phaseolus* and *Pisum* respectively and by 30% with CKD treatment in *Pisum* compared to the zero application rate (Figure 2.13B, D, F).

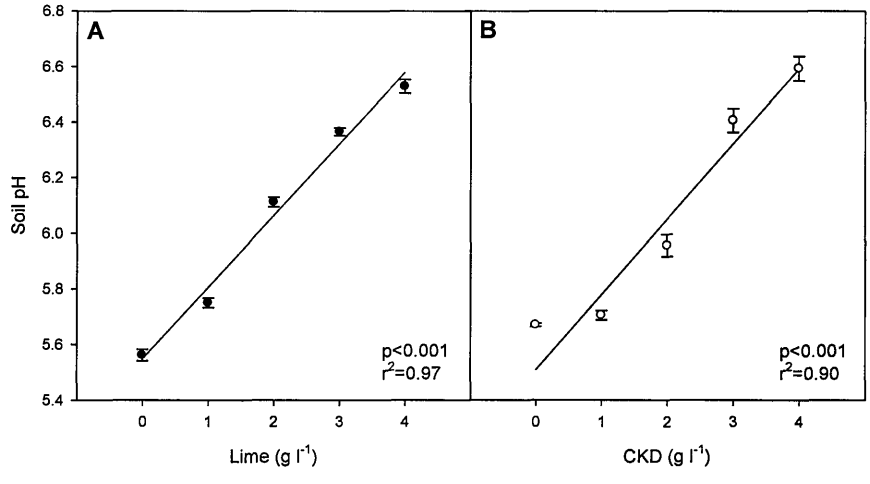

Figure 2.12: Relationship between additions of agricultural lime (panel A) or CKD (panel B) and soil pH in the pot trials. Data points are mean values ± SE of 6 replicates, linear regression line fitted and p values for regression analysis reported in the bottom right of each panel.

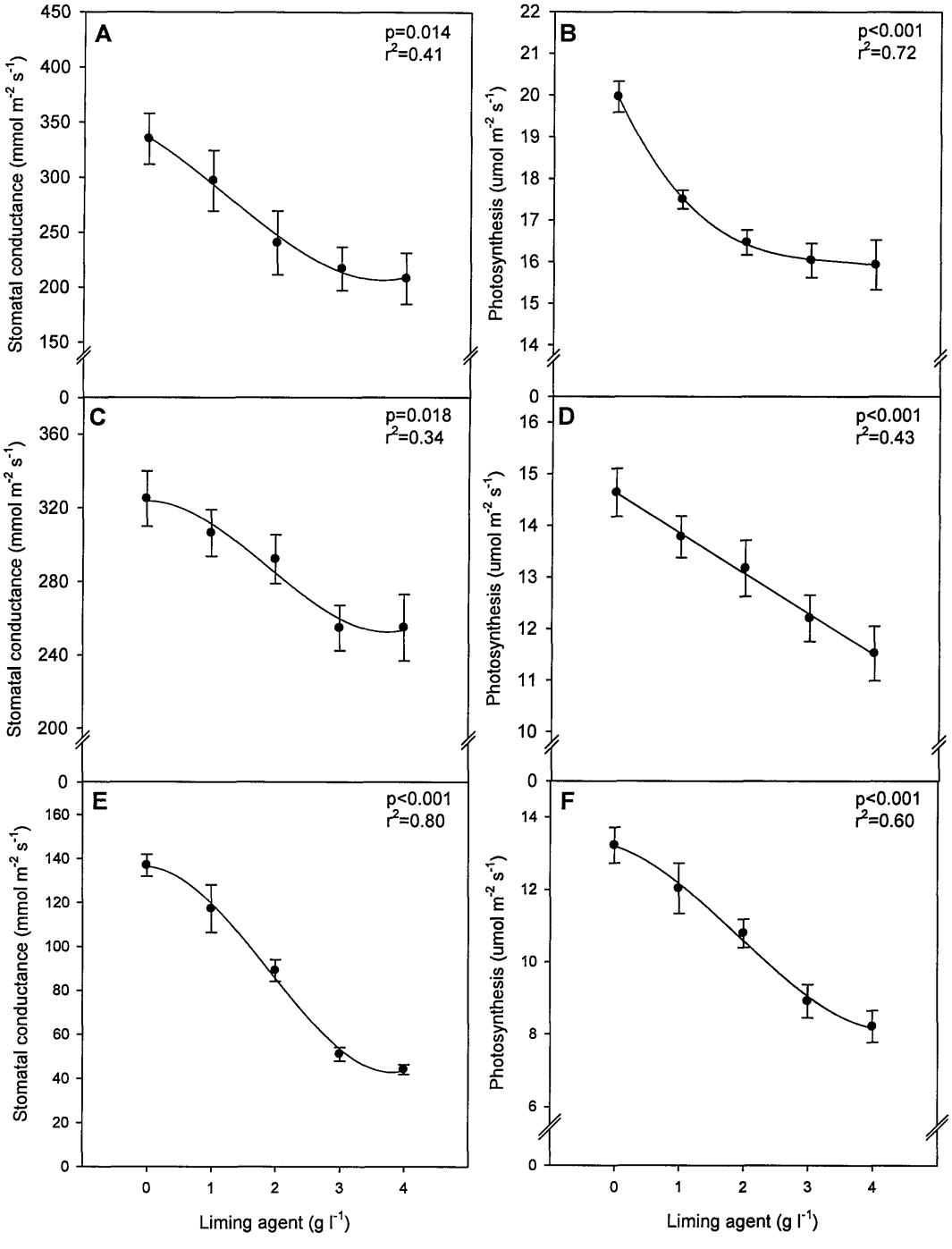

Figure 2.13: Relationship between additions of agricultural lime and gas exchange data in *Phaseolus* (panels A and B) and *Pisum* (panels C and D) or CKD and *Pisum* (panels E and F). Data points are mean values ± SE of 6-10 replicates. Third order polynomial regression lines fitted in panels A, B, C, E and F, liner regression line fitted to panel D and P values for regression analysis reported in the top right of each panel.

The yield data (shoot dry weight and leaf area) shown in Figure 2.14 again revealed significant negative correlations between *Phaseolus* and *Pisum* and application rates of both lime and CKD. Lime at the 3 g l^{-1} application rate reduced both *Phaseolus* shoot dry weight and leaf area by 25% and 28% respectively. Shoot dry weight and leaf area reductions of 14% and 13% were observed in *Pisum* under lime and 17% and 30% with the CKD treatment.

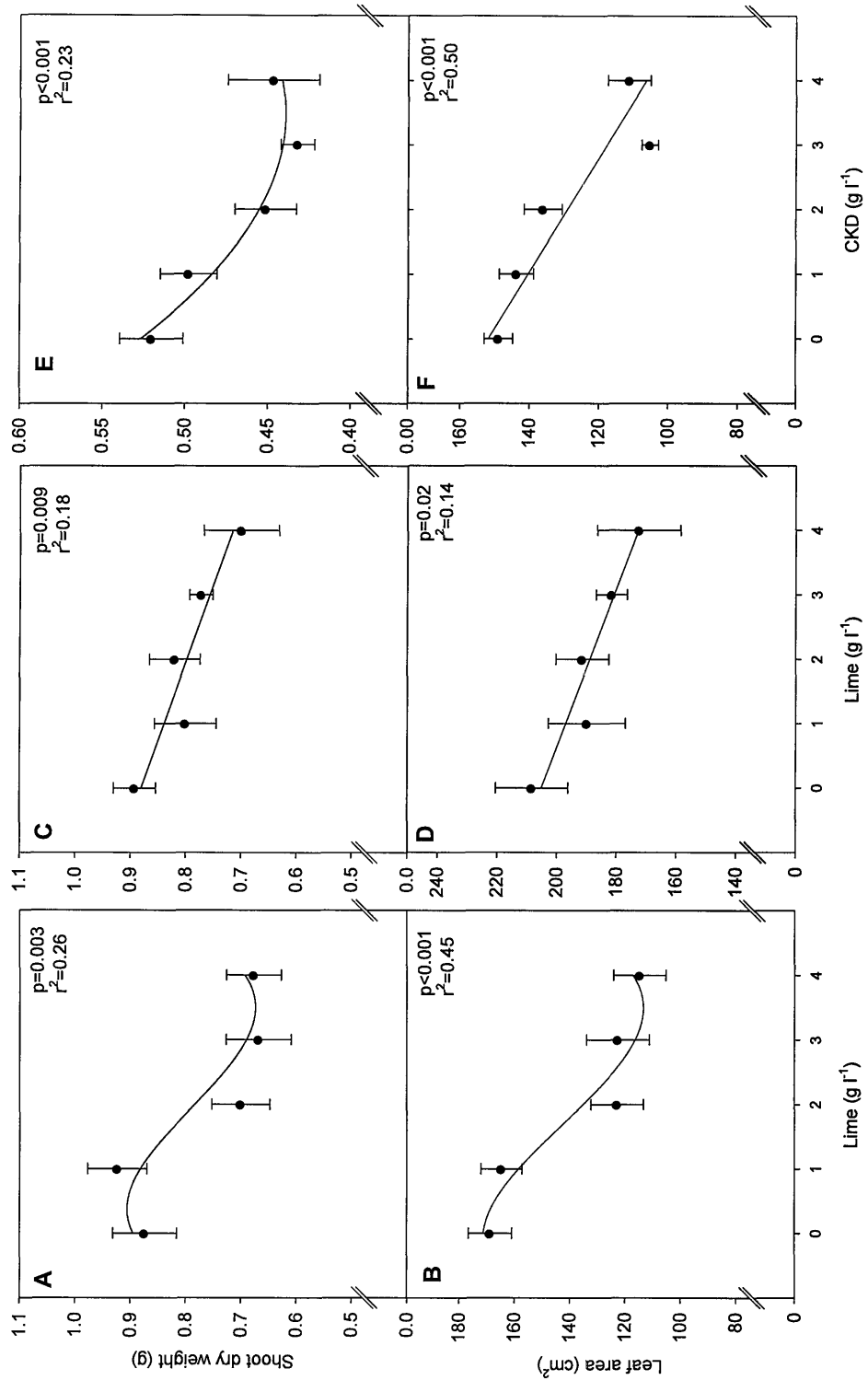

Figure 2.14: Relationship between additions of agricultural lime and harvest data in *Phaseolus* (panels A and B) and *Pisum* (panels C and D) or CKD and *Pisum* (panels E and F). Data points are mean values ± SE of 6-8 replicates. Linear regression lines fitted in panels C, D and F, second order polynomial in panel E and third order polynomial in panels A and B with P values and r^2 reported in the top right of each panel.

2.4 Discussion

This chapter sought to understand if CKD could be used as a suitable alternative to agricultural lime to raise soil pH and supply plant available K in field and pot trials. As far as the author is aware, these are the first such trials in the UK.

Analysis of CKD (Table 2.1) and *Vicia* leaf tissue collected 16 weeks after sowing (Table 2.6) indicates that PTE's are not an issue with this particular source of CKD. Levels of PTE's added at the application rate used in the field trial (6 tonnes ha^{-1}) fall well below the regulatory guidance for application to agricultural land (DEFRA, 2006). Furthermore, despite consistently higher levels of PTE uptake than lime, CKD leaf PTE uptake was always similar or lower than the control treatment (Table 2.6). In fact both liming agents significantly reduced leaf tissue uptake of Zn, Cu, Cd, Pb and As when compared to the control (unamended) treatment. The chemistry of adsorption (and thus immobilisation and reduced bioavailability) of heavy metals to soil colloid surfaces is well understood (Bradl, 2004) and is driven principally by soil pH (Martinez and Motto, 2000, Sauve et al., 2000). This process is often exploited by applying lime or alkali soil amendments to reduce the bioavailability of PTE's in agricultural soils (Bolan et al., 2003b). For example, the application of the basic industrial by products phosphogypsum, red gypsum and dolomitic residue increased sorption (and thus bioavailability) of Cd, Cu and Pb in a low pH (4.7-5.0) soil (Illera et al., 2004). Furthermore, CKD successfully reduced availability of Zn in a Zn contaminated soil (Moon et al., 2010), and the high inherent pH of CKD was utilised to stabilise PTE's contained within biosolids prior to land application (Dinel et al., 2000).

Whether repeated applications of CKD and thus continued loading of the soil with PTE's might cause future issues with heavy metal uptake, in particular Cd (McLaughlin et al., 1996) is uncertain and would need further investigations. Furthermore, it is important to note that the CKD and leaf tissue uptake PTE values should only apply to this particular source of CKD. Variation in chemical properties between batches seems inherent with CKD (Adaska and Taubert, 2008) driven by variations in parent material and, more importantly, the increasing use of cement kilns as disposal routes for toxic waste material (Guo and Eckert, 1996, Reijnders, 2007). This not only means that different sources of CKD may have differing chemical properties but product variation may occur within the same production facility. Thus regular analysis of CKD for PTE's is needed if the product is to be applied safely to agricultural soils (Adaska and Taubert, 2008).

The respective applications of 7 and 6 tonnes ha^{-1} of both lime and CKD generated mean soil pH values after 16 weeks of ~6.2 which was short of the target pH of 6.5 (Figure 2.2B). This can be explained by an unexpectedly low starting pH in some of the trial plots that was not identified in the initial soil sample used to determine pH and application rates. Initial sampling used the recognised standard method of homogenised sample cores taken in a W fashion across the field (DEFRA, 2010), but it is apparent that there were substantial gradients in soil pH even across the small area of the field site (Figure 2.15) which were not identified in the initial sampling. Thus, when soil pH is analysed as a change from initial values, both treatments increased pH by approximately 1 pH unit (Figure 2.2A) as intended. Therefore CKD appears equally capable of raising soil pH as agricultural lime. This is supported by the data from the dose response curves in the pot trials (Figure 2.12) where

agricultural lime and CKD showed a near identical linear relationship between application rate and pH. This is in agreement with Dann et al. (1989) who found a similar linear relationship between application rate and soil pH of both agricultural lime and CKD. This is perhaps unsurprising given that the CKD used in this trial had a neutralising value similar to the agricultural lime (Table 2.3).

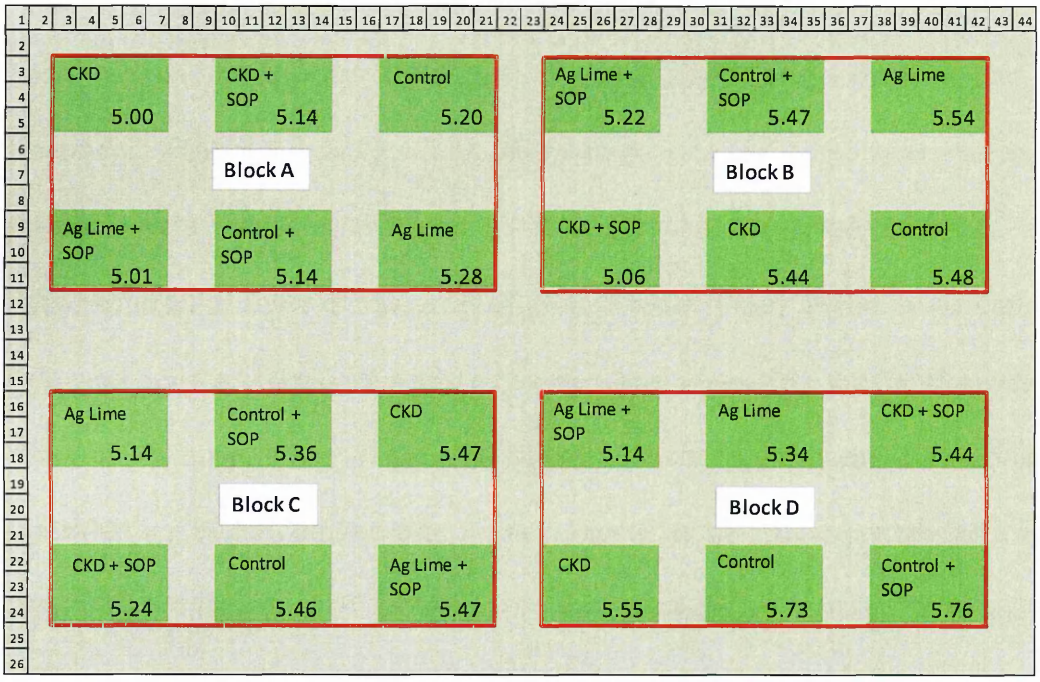

Figure 2.15: Schematic detailing soil pH (value at bottom right of each plot) of individual plots in the *Vicia* field trial taken immediately prior to application of treatments.

In the *Vicia* field trial, CKD increased soil exchangeable K to levels equivalent to the lime/SOP treatment which supplied K at the same rate (Table 2.4) and consequently leaf tissue K was increased comparably suggesting that the soil K was freely available for plant uptake (Table 2.5). In the pot trial, CKD did not appear to increase exchangeable K to the same degree as the lime/SOP treatment, though still increased

over control, (Table 2.7) but leaf tissue K values were higher in the CKD treatment (Table 2.8). Similarly, CKD has consistently been found to provide plant available K in a range of soil and crop types (Vanlierop et al., 1982b, Lafond and Simard, 1999, Lalande et al., 2009, Rodd et al., 2010). Therefore CKD appears to be a suitable alternative to conventional K fertiliser, though only when a substantial raise in soil pH is required at the same time.

The growth response to liming in the field trial was not expected. Both plant height and fresh pod yield were significantly reduced under both the lime and CKD treatments (Figures 2.6, 2.8 and 2.9). Interestingly, a soil pH of 5.5 appeared to be a threshold value below which pod yield significantly decreased, which occurred (unexpectedly) in some of the unlimed control plots. Crops grown in mineral soils below pH 5.5 are likely to be inhibited by increased Al and Mn toxicity (Sumner and Yamada, 2002) and is one of the principle reasons for managing soil pH with liming (Bolan et al., 2003a). Highest pod yields occurred at pH values just above this 5.5 threshold and appeared to decrease with increasing pH in response to liming with either agricultural lime or CKD (Figure 2.9B). Shoot biomass results from the *Vicia*, *Phaseolus* and *Pisum* pot trials (Figures 2.11 and 2.14) support those of the field trial, where all species exhibited a negative growth response to liming.

Yield penalties can occur when soils are over-limed (usually generating soil pH values >8) typically owing to unavailability of P (Bornman et al., 1998) and boron (Bartlett and Picarell, 1973). However, based on soil pH values, over-liming was seemingly not an issue in either the field or pot trials, as soil pH values which were always near the recommended pH of 6.5.

Negative yield responses to moderate or recommended rates of liming are not unknown. Such responses have been attributed to fixation of exchangeable Mg (Sumner et al., 1978) and in the *Vicia* field trial exchangeable Mg was reduced by 20-24% (though not significantly) with all liming treatments. Precipitation of Mg with soil Al and consequent unavailability is a known phenomenon with liming (Myers et al., 1988). However, field trial soil exchangeable Mg remained at levels that would be regarded as sufficient (DEFRA, 2010). Furthermore, leaf tissue Mg was unchanged in all treatments (Table 2.5), indicating that available Mg levels remained adequate regardless of the liming effect. Also, Mg availability was unaffected by lime or CKD in the pot trial (Table 2.7) and consequently leaf tissue Mg concentrations were unchanged (Table 2.8). Therefore it seems unlikely that Mg fixation (and consequent plant Mg deficiency) caused the observed growth or yield reduction.

Increased exchangeable Ca:Mg ratios rather than Mg deficiency *per se* were thought to cause a negative yield response of *Trifolium repens* to liming (Carran, 1991). It was suggested that an exchangeable Ca:Mg >20 was a threshold value where yield was negatively impacted. Soil exchangeable Ca and leaf tissue Ca were increased by agricultural lime and CKD application in both the field and pot trials (Tables 2.4, 2.5, 2.7 and 2.8). This is perhaps not unexpected given that both liming substrates are Ca based (Table 2.2). Similarly, Ca tissue concentration in a grass and clover forage sward was increased by both agricultural lime and CKD though CKD increased leaf tissue Ca to a greater extent (Rodd et al., 2010). This is in agreement with the *Vicia* pot trials where CKD mediated leaf tissue Ca increase (20%) was twice that of lime (10%) (Table 2.8). Rodd et al. (2010) attributed this to the speed of the liming reaction with CKD owing to its fineness and higher CaO concentration, which is more

reactive than $CaCO_3$. As a consequence of increased Ca availability, in both the field and pot trials liming with agricultural lime or CKD increased Ca:Mg ratios above this 20:1 threshold level (Table 2.9) and thus cannot be discounted as a possible cause of growth inhibition observed in the current experiments.

Table 2.9 Effect of liming substrate on the ammonium acetate extractable Ca:Mg ratios from the *Vicia* field and pot trials.

Treatment	Field trial Ca:Mg	Pot trial Ca:Mg
Control	18.5	15.0
Lime	27.3	24.9
CKD	28.0	25.2

Other examples of growth inhibition in response to liming have been attributed to reduced P availability (Haynes, 1982). Shoot P concentration and consequently shoot dry biomass of six clover species were significantly reduced with lime applications above 2 tonnes ha^{-1} generating a soil pH >5.7 (Maxwell et al., 2012). This is in agreement with the *Vicia* pot trial where both Olsen extractable and leaf tissue P were significantly reduced by liming (Table 2.7 and 2.8). Similarly, liming to target pH 6.5 decreased Olsen extractable P in five out of six acidic soils (Curtin and Syers, 2001), a process thought to be driven by precipitation of calcium phosphates (Delgado and Torrent, 2000). In contrast, the field trial revealed no apparent change in P availability or leaf deficiency (Tables 2.4 and 2.5), however, this does not preclude the possibility of a P deficiency early in the crop cycle (when measurements

were not made). If P availability was limited by the application of liming agents within the upper soil profile (depth to 10-15 cm) then sample soil cores of 20 cm depth may have misrepresented P availability. Alternatively, P availability may have been improved during the growing season by the action root exudates (Dakora and Phillips, 2002) which is known to improve P mobility in the soil and hence uptake, particularly in legume crops (Raghothama, 1999). Furthermore, P status of leaf tissue samples collected 16 weeks after drilling may not reflect a temporary limitation of plant P status early in the crop development (Bell, 2000). However, early inhibition of growth by P deficit could have a residual inhibitory effect on further plant development (Thomson et al., 1992). Reduction in P availability cannot therefore be discounted as a possible cause of growth and yield penalties in both the field and pot trials.

Growth inhibition in the pot trials might be explained by the observed reduction in gas exchange under liming with either lime or CKD (Figures 2.10 and 2.13). Stomatal limitation of photosynthetic rate is likely to inhibit growth through reduced carbon assimilation (Lawlor, 1995). However, existing data on the effects of liming on plant gas exchange appears limited. Grape grown in a naturally high pH (8.4) soil had reduced photosynthetic rate but no impairment of transpiration compared to a more neutral soil (Bavaresco and Poni, 2003). This was thought to be caused by leaf chlorosis and thus leaf chlorophyll concentration, however this seems unlikely at the slightly acidic soil pH values generated in the present trial (Mengel, 1994). Although no significant treatment effect was observed on gas exchange in the field trial (Figures 2.3 and 2.4), similar to plant P status, it is possible that gas exchange was limited early in the crop growth cycle. Attempts to measure gas exchange during

early crop development were hampered by the detrimental effect of the Bentazon herbicide used to control weeds on Pn (Mine and Matsunaka, 1975). Thus any inhibitory effects of liming agent on gas exchange in the early growth stages on the plant may have been missed. Given the consistency of the response across all three species sampled in the pot trials, the causality of this reduction in gas exchange is worthy of further investigation and will be addressed in Chapters 3, 4 and 5 in this thesis.

In conclusion, the neutralising value and capacity of CKD to change soil pH suggest that it could be a suitable alternative to agricultural lime for managing soil pH. Additionally, its ability to supply plant available K make it particularly suitable if K fertilisation is also required. Furthermore, substrate and leaf tissue PTE analysis suggest that PTE's are not problematic with this particular source of CKD. However, negative effects of recommended rates of liming with agricultural lime or CKD on plant growth, yield and gas exchange were surprising. Since the cause(s) of these responses are unknown, this thesis will aim to determine the physiological mechanism(s) by which liming reduced both gas exchange and growth in legumes.

Chapter 3 - Xylem sap calcium concentrations do not explain liming-induced inhibition of legume gas exchange

3.1: Introduction

In Chapter 2, liming (to recommended rates) of *Vicia*, *Phaseolus* and *Pisum* reduced gas exchange, which could limit growth. Since lime is usually applied as calcium carbonate or calcium hydroxide, one possibility is that increased Ca uptake, and thence xylem sap Ca^{2+} concentration, may decrease g_s.

Calcium ions (Ca^{2+}) are known to be an important signalling component involved in regulating guard cell turgor and therefore stomatal opening and closure. It is well established that increased guard cell cytoplasmic Ca^{2+}concentration causes stomatal closure (McAinsh et al., 1990) and experiments using calcium-channel blockers at least partially attribute this to flux across the plasma membrane (McAinsh et al., 1991). Thus elevated apoplastic Ca^{2+}concentrations can influence this signalling mechanism and induce stomatal closure. In experiments using isolated epidermal strips of *Commelina communis*, a change in concentration from 0.1 mM to 0.5 mM $CaCl_2$ in the bathing medium (representing the apoplast) significantly reduced stomatal aperture by approximately 50% (De Silva et al., 1985). Similarly, McAinsh et al (1995) found *Commelina communis* epidermal strips bathed with concentrations of 0.1 mM and 1 mM $CaCl_2$ significantly reduced stomatal aperture by 10% and 36% respectively when compared to epidermal strips incubated in the absence of Ca^{2+}. This evidence suggests that quite small increases in apoplastic Ca^{2+} concentrations could inhibit stomatal opening, therefore, regulation of Ca^{2+} uptake and delivery to the shoot may be important in stomatal behaviour.

Calcium uptake into the root is primarily via the mass flow of soil water driven by transpiration (McLaughlin and Wimmer, 1999). Apoplastic movement of Ca^{2+} through the root was classically thought to be impeded by the presence of the casparian band (Clarkson, 1984) where solutes would be forced to move symplastically to bypass this impermeable barrier, thereby providing a regulatory mechanism over delivery of Ca^{2+} to the xylem. However, the absence of expected signatures of protein-catalysed membrane transport provides evidence for the existence of both apoplastic (regulated by transpirational flux) and symplastic (regulated by active transport) pathways (White, 2001). The existence of an apoplastic pathway for Ca^{2+} delivery to the xylem suggests that some plants may not have full regulatory control over delivery of Ca^{2+} to the shoot.

Further, there is evidence to suggest that elevated rhizospheric Ca concentration can increase xylem sap Ca^{2+} concentration. Increasing nutrient solution Ca^{2+} concentration from 1 mM to 8 mM gave a threefold increase in root xylem sap Ca^{2+} concentration (from 0.75 mM to 2.32 mM) after 15 hours in *Commelina* (Atkinson et al., 1990). Therefore, depending on the plants ability to regulate Ca^{2+} uptake, elevated rhizospheric Ca can lead to increased Ca^{2+} concentrations within the xylem transport fluids. If delivered to the apoplast in the vicinity of stomata, this elevated Ca^{2+} level might interfere with normal stomatal function. *Commelina* grown in a high Ca^{2+} (15 mM) nutrient solution exhibited a 15% reduction in g_s after 13 days when compared to plants grown at 4 mM Ca^{2+} (Ruiz et al., 1993). Therefore, it is not unreasonable to suppose that plants grown in soil with elevated Ca availability may exhibit similar responses.

Calcium deficiency is uncommon in most agricultural soils and it is typical for Ca to be abundant in the rhizosphere at concentrations higher than those required for adequate plant or crop nutrition. Exchangeable Ca was 291 μg (± 291) in a podzolic soil and 6958 μg/g (± 472) in a calcareous soil (Lee, 1999), though mean exchangeable Ca across 11 different UK agricultural soils was 1954 μg/g (± 261) (Chambers and Garwood, 1998). Common to most liming materials is a high inherent Ca concentration (typically as calcium carbonate or calcium hydroxide) which, dependent on solubility, will increase the amount of Ca available to plants in the soil (Tyler and Olsson, 2001a). Therefore, liming with Ca based substrates is likely to increase plant available Ca to levels far exceeding those required for adequate nutrition. Liming increased shoot Ca concentrations of both *Lupinus angustifolius* and *Lupinus pisolus* by 41% after 44 days in pot trials (Tang and Turner, 1999). Similarly, shoot Ca concentrations increased by a maximum of 88% in pot grown *Agrostis capillaris* in response to a range of lime rates up to 15 g l^{-1} (Tyler and Olsson, 2001b). If Ca^{2+} uptake is not strongly regulated by the plant, increased Ca^{2+} concentration in the xylem transport fluids can be expected.

Since liming will have increased plant available Ca in the soil used during these experiments (Table 2.7), this chapter tests the hypothesis that elevated xylem sap Ca^{2+} concentration is responsible for the reduction in gas exchange observed in Chapter 2. Using a whole plant pressure chamber (Gollan et al., 1986) or Scholander pressure chamber (Liang and Zhang, 1997) xylem sap can be collected from leaf apoplast and/or root xylem, assayed for Ca^{2+} and compared with gas exchange data taken immediately prior to sampling. Secondly, feeding different artificial xylem saps (comprising ions at the concentrations found in xylem sap, and altered only in their

Ca^{2+} concentrations) via the transpiration stream to detached leaves/shoots in a transpiration bioassay can determine if any observed changes in Ca^{2+} concentrations in response to liming are likely to have affected stomatal behaviour.

3.2 Materials and methods

3.2.1 Plant material

In separate experiments, seeds of dwarf bean (*Phaseolus vulgaris* l. cv. Nassau) and tall pea (*Pisum sativum* L. cv Alderman) were pre-germinated on tissue paper dampened with distilled water and kept in the dark. On emergence of the radicle (2-3 days), uniformly germinated seeds were individually placed in a 2:1 mixture (v:v) of a low pH sandy loam field soil and horticultural grit sand (DA30, Boughton, Kettering, UK). The field soil was collected from the top 10 cm of a research site located at Lee Farm, Myerscough College, Lancashire, UK (soil description provided in Section 2.2.2). Field soil and grit sand combinations were thoroughly homogenised in 15 litre batches for five minutes in a cement mixer and sterilised by heating to a minimum of 82°C (Camplex 68 l, Thermoforce Ltd, Cockermouth, UK) to prevent infection from imported soil borne pests and diseases. Treatment consisted of agricultural lime as calcium carbonate ($CaCO_3$) (J. Arthur Bowers Ltd Coarse Screened Limestone, William Sinclair Horticulture Ltd, Lincoln, UK) that had a neutralising value (CaO) of 57%, and a fineness of a minimum 15% total passing through 150 micron sieve, at an application rate of 3 g l^{-1}. This represented the DEFRA (2010) recommended application rate of 6 tonnes ha^{-1} to target a final soil pH of 6.5 converted from tonnes ha^{-1} to g l^{-1} with the assumption that soil pH is measured in the top 20 cm of the soil

profile and 1 ha contains 2000000 litres of soil at 20 cm depth. Sterilised soil and lime treatments were then homogenised in a cement mixer and left to incubate in plastic bags for a minimum of four weeks for the lime reaction to occur (see Section 1.3 for details of lime reaction), unlimed control soil was mixed and treated the same.

The pre-germinated *Phaseolus* seeds were grown into pots made from cylindrical plastic tubing (69 mm diameter x 240 mm height) with a steel mesh base designed to fit into a Scholander type pressure chamber. On emergence, plants were maintained in the dark for 2-3 days by covering with a large plastic container to encourage hypocotyl extension to facilitate fitting into the pressure chamber. Plants were removed from the dark when the stem had reached approximately 8 cm in length. All pots were watered to run off and re-weighed after 24 hours to determine weight at drained capacity and the top of the pots were taped using fabric-backed duct tape (Advance Tapes, Leicester, UK) to reduce evaporation from the soil surface (see Figure 3.1).

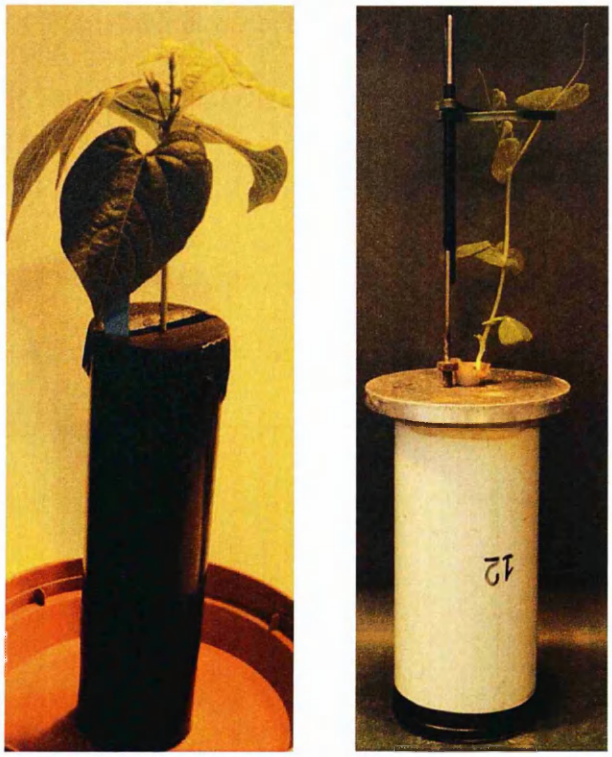

Figure 3.1: Detail of *Phaseolus* plant in plastic tubing pot with tape covering soil surface (left image) and *Pisum* plant established through aluminium capping plate of whole plant pressure chamber pot (right image).

The pre-germinated *Pisum* seeds were grown in pots made from cylindrical plastic tubing (90 mm diameter x 200 mm height) capped with a circular aluminium plate designed to fit into a whole plant pressure chamber to allow sampling of leaf apoplastic xylem sap from intact plants. The seeds were established on small plastic funnels, filled with the treatment soil, which directed the developing root system through a small (5 mm diameter) hole in the centre of the aluminium capping plate (see Figure 3.1).

Initially (2-3 days) the seed/funnel was kept moist and in the dark by covering with a plastic cup containing saturated filter paper. Once established (7-10 days after transplanting) the funnel and soil were carefully removed and any lateral roots that

had developed in the funnel were removed with a razor blade. The exposed root was then washed free of adhering soil particles and the top of the pot sealed with a two-part silicone elastomer (Sylgard 184, Dow Corning, Midland, USA).

Plants in both experiments were weighed daily to record evapotranspirational (ET) losses and had full ET replaced daily using tap water. They were maintained in a semi-controlled naturally lit greenhouse (with supplementary lighting supplied by Osram 600 w daylight bulbs for 12 hours and 22°C/ 16°C minimum day/night temp) at the Lancaster Environment Centre.

3.2.2 Gas exchange measurements, root xylem sap collection and harvest

3.2.2.1 *Phaseolus*

Immediately prior to harvest, gas exchange (g_s and Pn) was measured on the middle leaflet of the fully expanded first trifoliate leaf using infra-red gas analysis (6400xt Li-Cor Portable Photosynthesis System, Lincoln, Nebraska, USA). CO_2 was set at ambient levels (390 ppm), radiation at 600 µmol m^{-2} s^{-1} PPFD, a cuvette temperature of 22°C and ambient humidity (typically 40% to 50%) was used. Plants were then immediately de-topped below the cotyledons (see Figure 3.2 for detail of sampling positions), leaving 8 cm of stem protruding above the soil, and the whole pot sealed in a Scholander type pressure chamber.

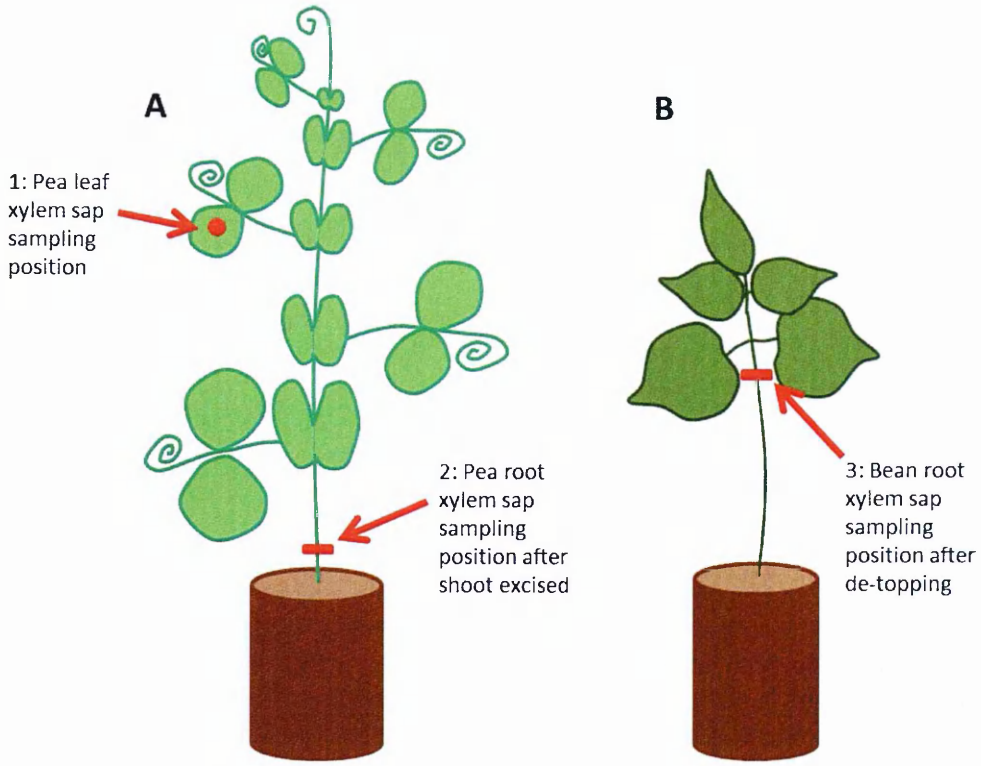

Figure 3.2: Detail of sap sampling methodology. In *Pisum* (A) sap was first sampled at position 1 from the cut mid-rib of the youngest fully expanded leaf in a plant sealed in a whole plant pressure chamber. The plant was then excised at position 2 to allow sampling of root xylem sap. In *Phaseolus* (B) root xylem sap was sampled from position 3 in plants de-topped immediately below the cotyledon leaving 8 cm clear stem and then sealed in a Scholander pressure chamber.

The cut surface of the de-topped shoot was rinsed three times with distilled water and dried with filter paper to remove contamination from damaged cells. Sap was collected using an over-pressure that matched actual *in vivo* flow rates, as a previous experiment had established a diluting effect of artificially increased flow rates (Figure 3.3).

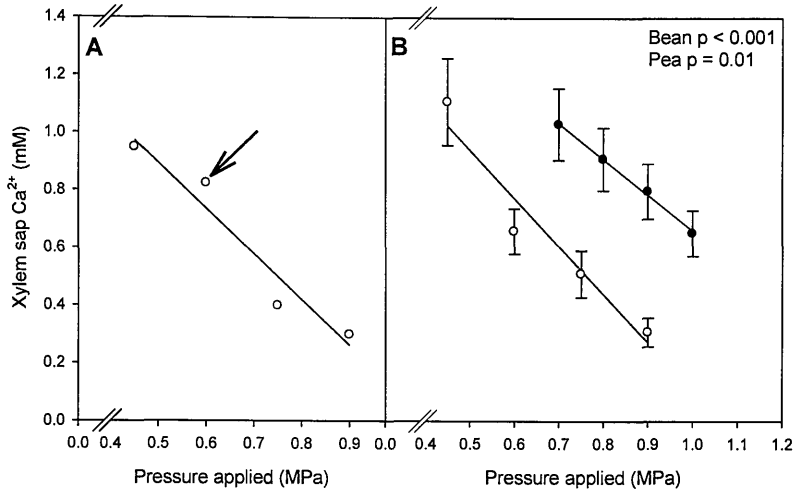

Figure 3.3: Relationship between incrementally increased root pressure and Ca^{2+} concentration in xylem sap samples collected from *Phaseolus* root (open circles) and *Pisum* leaf (closed circles). Panel A shows the relationship for an individual *Phaseolus* plant with the pressure generating the nearest match to *in vivo* sap flow (47.05 µl min^{-1} generated flow vs 53.05 µl min^{-1} *in vivo* flow determined gravimetrically) indicated with an arrow. Data points in panel B are means ± SE of 6 (*Pisum*) and 7-10 (*Phaseolus*) replicates, P values from regression analysis are reported in the top right of panel B.

Whole plant transpiration rate (sap flow rate *in vivo*) was determined gravimetrically one to two hours prior to harvest by sealing the top and base of the pot with aluminium foil and dividing the weight loss by time to determine an average sap flow rate per 20 seconds. When collecting sap in the pressure chamber, sequential increases in pressure of between 0.05 and 0.1 MPa were applied to the de-topped plant and sap samples collected for 20 seconds at each pressure and weighed until generated flow rates approximately matched actual flow rates. On reaching the appropriate over-pressure, 50-60 µl samples of xylem sap were collected and immediately assayed for Ca^{2+} using a micro ion-selective electrode (B-751, LAQUAtwin, Horiba Instruments Ltd, Northampton, UK). The ion-selective electrode was compared against a flame photometer (Sherwood Model 410, Sherwood

Scientific Ltd, Cambridge, UK) using solutions of known Ca concentration to ensure accuracy.

3.2.2.2 *Pisum*

Gravimetrically determined sap flow rates were recorded for the *Pisum* one to two hours prior to harvest and gas exchange measurements made, as described above, on one leaflet of leaf pair four (the youngest fully expanded leaf). The plant was then carefully sealed into the whole plant pressure chamber and a small v shaped section cut from the mid-rib (Figure 3.4) of the same leaflet used for gas exchange.

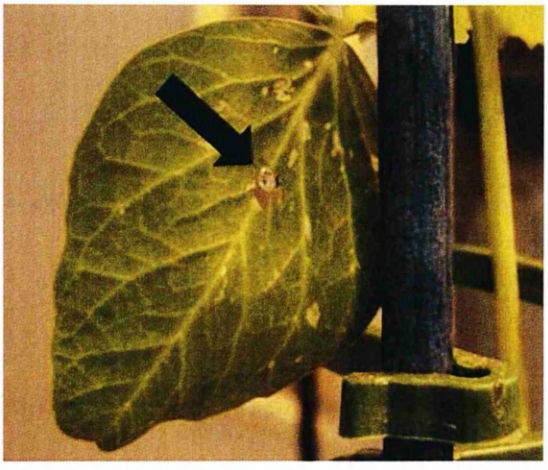

Figure 3.4: Detail of section cut from *Pisum* leaf mid-rib exuding xylem sap (indicated by arrow).

The cut surface was then rinsed with distilled water, dried with filter paper, then pressure gradually applied (0.1 MPa increments) to the root system until sap started flowing from the cut surface. Leaf xylem sap samples were collected (timed and weighed to determine flow rate) and a further four to five sap samples were then collected at sequential 0.1 MPa increased pressures in order to obtain a sample closely matching *in vivo* flow rates when normalised for leaf area of sampled leaf.

After releasing the applied pressure, the entire shoot was excised approximately 3 cm above the pot cap, the cut surface cleaned and dried and root xylem sap samples collected again using sequential 0.1 MPa pressure increases from the initial pressure when sap appeared on the cut surface (see Figure 3.2 for detail of sampling positions). All sap samples were collected using a pipette and immediately assayed for Ca^{2+} using the above described micro ion-selective electrode.

Sampling in both experiments occurred when plants were three weeks old. Immediately after sap sampling, shoot fresh weight and individual leaf area (using a leaf area meter - Li-3050A, Li-Cor, Lincoln, Nebraska, USA) were recorded. Roots were collected, washed of soil and checked for nodulation, though no nodules were found on either species in any of the experiments. Shoot and root material was then dried at 80°C for one week to record dry weight.

3.2.3 Detached leaf transpiration bioassay to determine effects of calcium on *Pisum* and *Phaseolus* transpiration

Seeds of tall pea (*Pisum sativum* L. cv. Alderman) and dwarf bean (*Phaseolus vulgaris* l. cv. Nassau) were pre-germinated on tissue paper dampened with distilled water and kept in the dark. On emergence of the radicle (2-3 days), uniformly germinated seeds were potted, (*Pisum* 8 per pot, in 5 litre pots and *Phaseolus* 15 per tray, in seedling trays, 38 cm L x 24 cm W x 6 cm D) into a 2:1 mixture of a sandy loam field soil and horticultural grit sand as described earlier. All plants were grown in an unlimed control soil except for one *Pisum* assay where a control soil and limed soil were compared. For the *Pisum*, fully expanded leaf pairs including petioles were

detached four to five hours into the photoperiod from two to three week old shoots using a razor blade and immediately re-cut under distilled water to prevent embolism. Maintaining a meniscus of water on the cut petiole surface, the leaflets were then placed in a 1.5 mL eppendorf tube containing an artificial xylem sap solution varying only in its Ca^{2+} concentration (and appropriate counter ion). The eppendorf tubes had the lids removed and the tops covered with parafilm with a small hole for inserting the petiole. The artificial xylem sap contained: 3 mM KNO_3, 1 mM KH_2PO_4, 1 mM K_2HPO_4, 0.1 mM $MnSO_4$ and 0.1 mM $MgSO_4$ (as in Dodd et al., 2003). Calcium was supplied at 0.5, 2.5 and 5 mM concentration as either $CaCl_2$ or $Ca(NO_3)_2$ to determine possible effects of the chosen counter ions.

For the *Phaseolus*, four days after emergence of the first tri-foliate leaf the primary leaves were removed to leave a single shoot/tri-foliate leaf combination and three to four days later the whole shoot was excised just above the soil surface using a razor blade and re-cut under distilled water as above. The shoots were placed in 10 ml glass vials covered with parafilm and containing the same artificial xylem sap as described above.

In separate experiments, *Pisum* leaflets (placed in small glass vials to allow them to sit upright) or *Phaseolus* shoots were randomly placed in a controlled environment growth chamber with fan assisted air flow at a temperature of 24°C with a relative humidity of around 60%. Vials were weighed on a four point analytical balance initially then every fifty minutes over a five hour period to determine transpiration rates gravimetrically. At the end of the assay leaf area of the leaflets was recorded

using a leaf area meter (Li-3050A, Li-Cor, Lincoln, Nebraska, USA) to normalise transpiration rate for leaf area.

3.2.4 Statistical analysis

Treatment differences in gas exchange and xylem sap Ca^{2+} concentration and delivery rates were distinguished with a students *t*-test (Figures 3.6, 3.7, 3.8). Two-way Analysis of Covariance (ANCOVA) was to determine any interaction between treatment and g_s (Figures 3.9 and 3.10). When no interaction effects were found, linear regression lines are fitted where significant. One-way ANOVA was used to determine treatment effects of Ca^{2+} concentration on transpiration (Figures 3.11 to 3.14) and two-way ANOVA was used to determine treatment effect and interaction in Figure 3.15. All analyses used Minitab v16.

3.3 Results

Liming increased soil pH to with ± 0.2 pH units of target pH and increased soil EC_p by ~0.1 mS cm^{-1} in both species (Table 2.1). Liming also increased soil exchangeable Ca by ~50% and ~100% in the *Phaseolus* and *Pisum* experiments respectively (Table 2.1).

Table 2.1: Ammonium acetate exchangeable calcium (Ca), pH and EC_p of soils used for both *Phaseolus vulgaris* and *Pisum sativum* experiments reported in this study. Data are means ± SE of 3 replicates (Ca) and 5-12 replicates (pH and EC_p).

	Exchangeable Ca ($mg\ kg^{-1}$)		Soil pH (1:2.5 H_2O)		Soil EC ($mS\ cm^{-1}$)	
Species	Control	Lime	Control	Lime	Control	Lime
P. vulgaris	1055 ± 11	1564 ± 11	5.78 ± 0.01	6.37 ± 0.02	0.46 ± 0.01	0.55 ± 0.01
P. sativum	896 ± 83	1843 ± 37	6.00 ± 0.04	6.67 ± 0.02	0.76 ± 0.02	0.83 ± 0.02

The root xylem sap collection methodology described above for *Phaseolus* plants generated mean pressure induced xylem sap flow rates for control and limed plants at 90% (± 3.6, n=13) and 86% (± 6.8, n=14) of *in vivo* flow rates respectively (Figure 3.5A) (a percentage significantly higher or lower that 100% would indicate over-estimated or under-estimated flow rates respectively). Using the sap collection methodology described for *Pisum*, the lowest pressure used to generate sap flow and sample leaf xylem sap over-estimated *in vivo* transpirational flow by approximately two-fold (control 179% ± 12.2, n=5, limed 187% ± 17.9, n=6) though the relationship between pressure and flow was constant for both treatments (Figure 3.6C). However, *Pisum* root xylem sap sampling closely matched measured *in vivo* flow rates (control 101% ± 3.8, n=5, limed 98% ± 5.8, n=6) (Figure 3.5B).

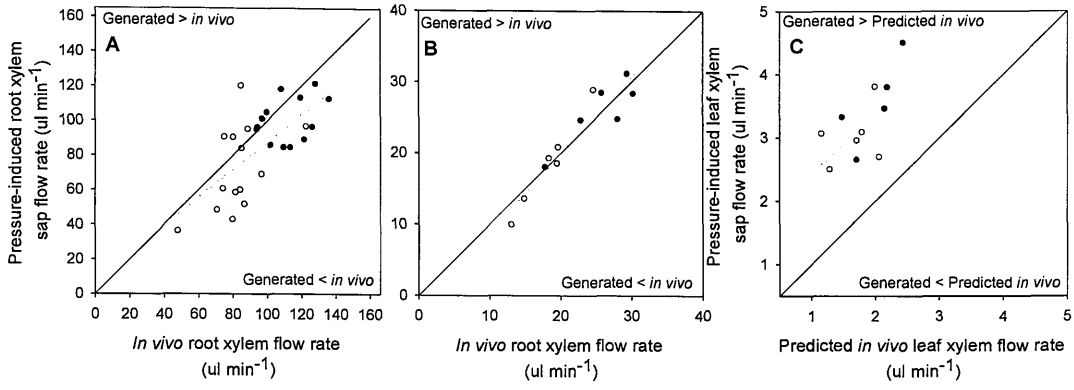

Figure 3.5: Relationship between *in vivo* root xylem flow rates and pressure-induced xylem flow rates in bean (panel A) and pea (panel B) and between predicted *in vivo* leaf sap flow rates and pressure generated flow rates in pea (panel C) for control (filled circle) and limed (open circles) plants. Each point is a separate xylem sap sample, solid lines indicate the 1:1 relationship and dotted lines indicate the regression between all samples.

The effect of liming on gas exchange parameters shown in Figure 3.6 is in agreement with those seen in Chapter 2. Liming reduced g_s by 26% and 59% in *Phaseolus* and *Pisum* respectively. Similarly, liming inhibited Pn by 11% and 30% in *Phaseolus* and *Pisum* respectively when compared to unlimed control plants.

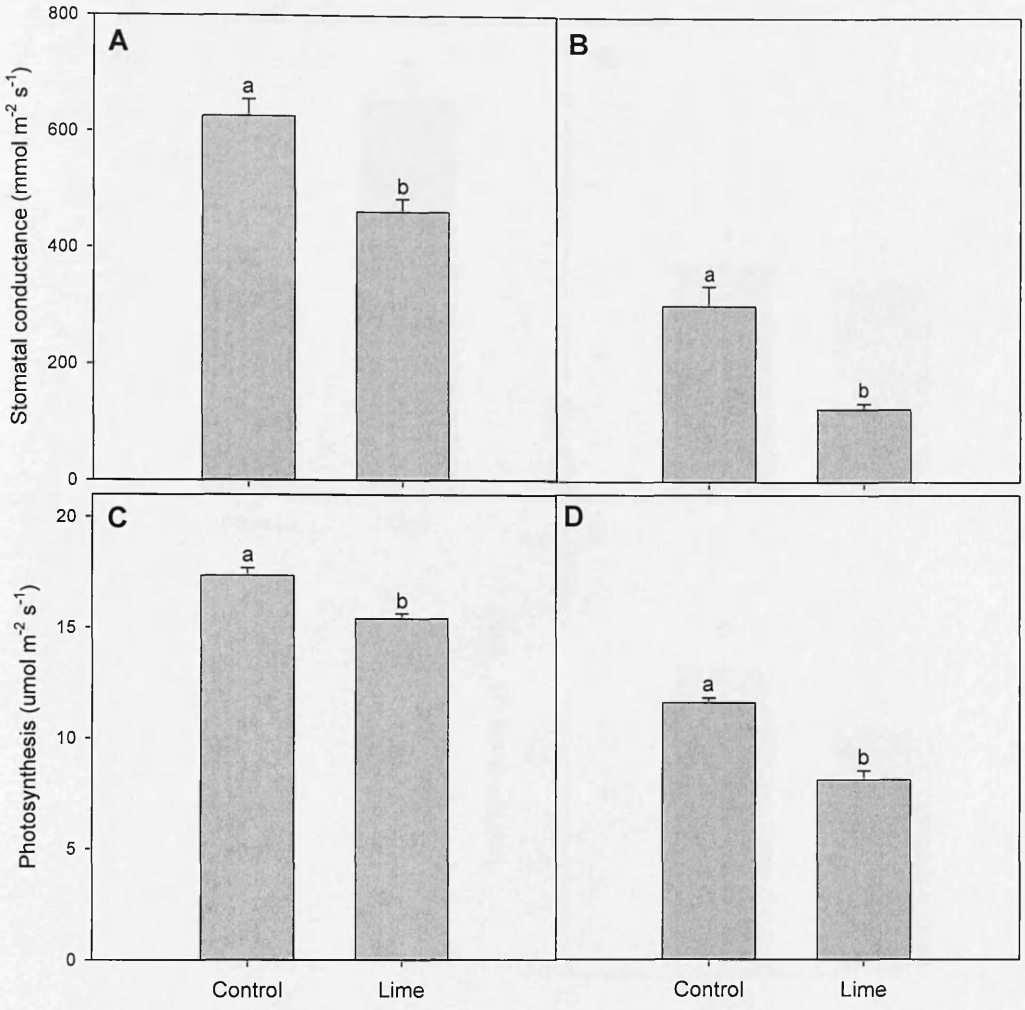

Figure 3.6: Gas exchange (stomatal conductance and photosynthesis) taken immediately prior to sap collection in limed and unlimed (control) bean (panels A and C) and pea (panels B and D). Data are means ± SE of 12 (bean) and 5-6 (pea) replicates. Differing letters indicate significant differences within each panel according to a students *t*-test ($p<0.05$).

In root xylem sap samples collected from *Phaseolus*, liming increased measured Ca^{2+} concentration by 83% from a mean of 0.9 mM in control plants to 1.7 mM in the limed treatment (Figure 3.7A). In contrast, *Pisum* root and leaf Ca^{2+} concentrations remained unchanged in response to liming (Figure 3.7B and C). Increased root xylem sap Ca^{2+} was correlated with decreased g_s in *Phaseolus* (Figure 3.8A), though no such relationship was found in *Pisum* (Figure 3.8B and C).

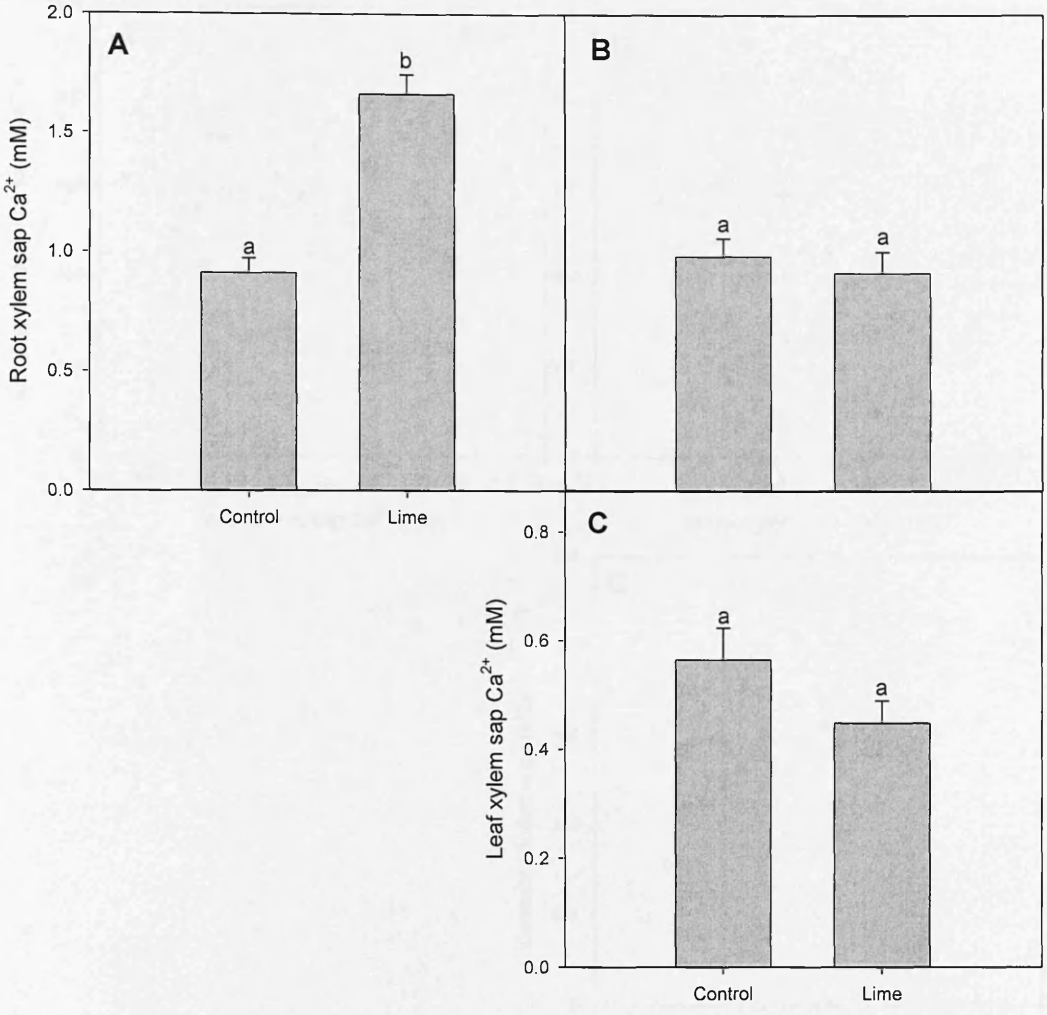

Figure 3.7: Xylem sap Ca^{2+} concentration in samples collected from bean root (panel A), pea root (panel B) and pea leaf (panel C). Data are means ± SE of 12 (bean) and 5-6 (pea) replicates. Differing letters indicate significant differences within each panel according to a students *t*-test ($p<0.05$).

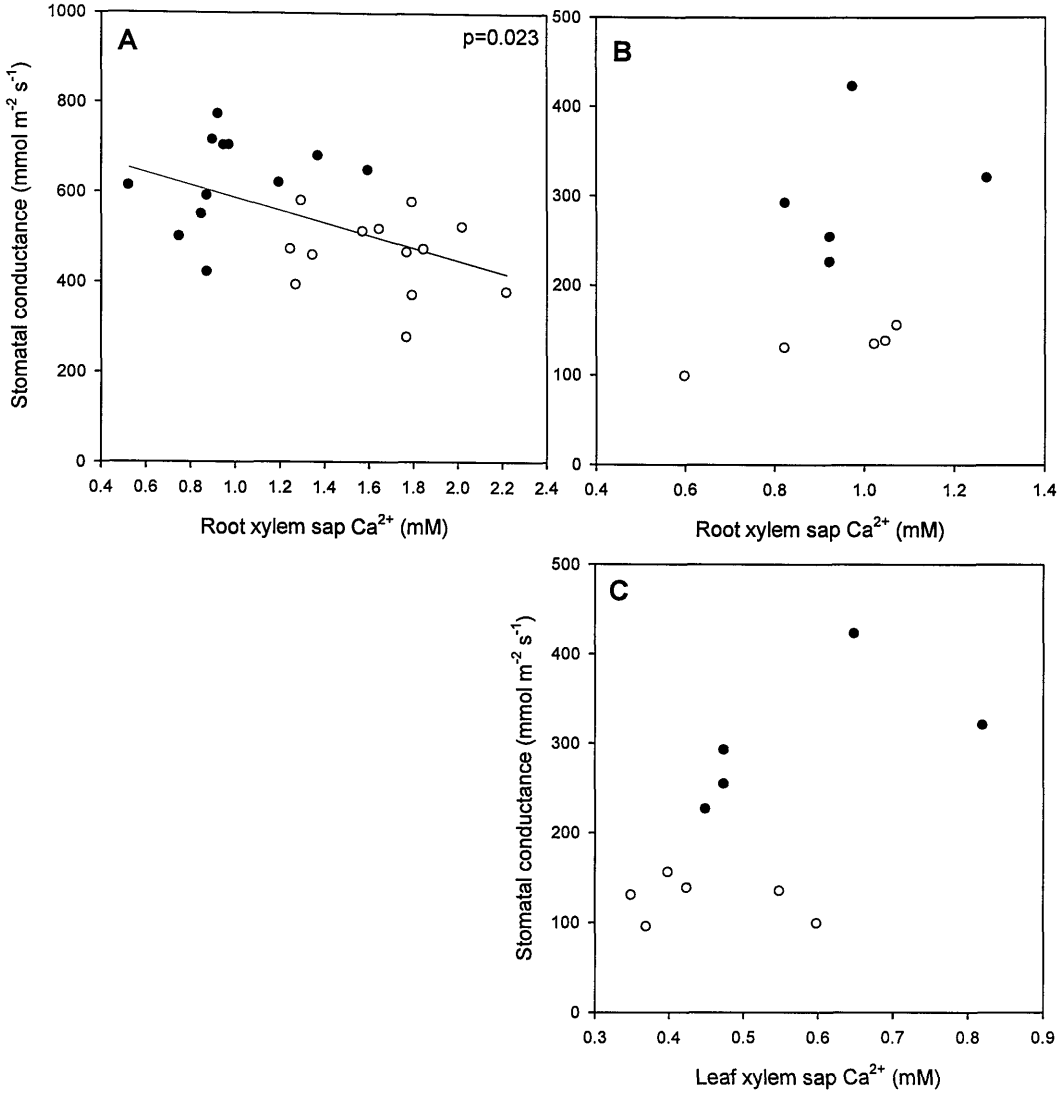

Figure 3.8: Relationship between Ca^{2+} concentration in xylem sap samples collected from control (filled circles) or limed (open circles) bean root (panel A) and pea root (panel B) and pea leaf (panel C) and stomatal conductance. Data points are individual samples, single regression lines fitted where significant, P values reported in top right of panel.

Changes in xylem sap Ca^{2+} delivery rates (the product of Ca^{2+} concentration and generated sap flow rate), are shown in Figure 3.9. Delivery rate of Ca^{2+} to the site of *Phaseolus* root sampling was increased by 26% (Figure 3.9A), though delivery to the middle leaflet on the first tri-foliate leaf (used for gas exchange measurements), which was estimated assuming equal distribution of xylem transport fluids to all of

the plant leaf area, was unchanged (Figure 3.9B). In contrast, Ca^{2+} delivery rates to both root and leaf of *Pisum* were reduced in the limed treatment by 42% and 29% respectively (Figure 3.9C and D) (though this was not significant to the leaf).

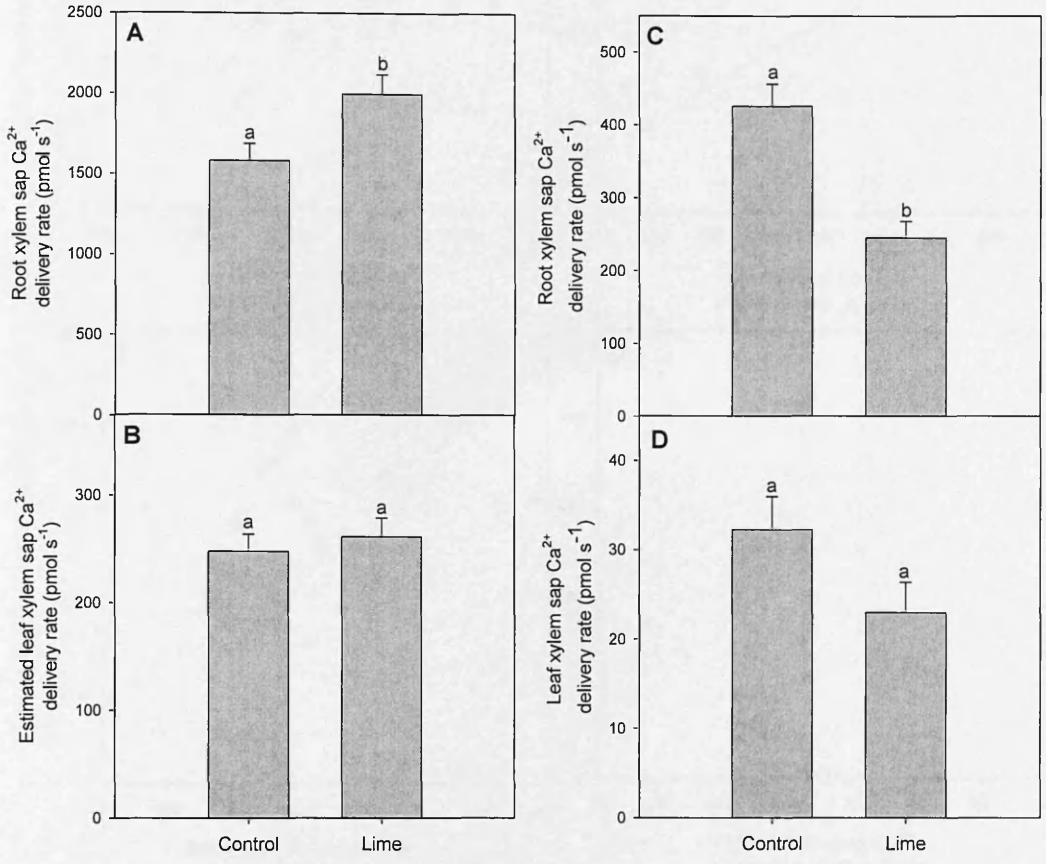

Figure 3.9: Root and leaf xylem sap Ca^{2+} delivery rates calculated from sap flow rate and Ca^{2+} concentration in samples collected from bean root (panel A), pea root (panel C) and pea leaf (panel D) or estimated (assuming equal distribution of root xylem transport fluids to whole leaf area) in bean leaf (panel B). Data are means ± SE of 12 (bean) and 5-6 (pea) replicates. Differing letters indicated significant differences according to a students *t*-test ($p<0.05$).

Increased root xylem Ca^{2+} delivery rates were correlated with increased g_s in *Pisum* (Figure 3.10B) though no such relationship was found with delivery rates to the leaf (Figure 3.10D). Similarly, no relationship was found between root or estimated leaf xylem Ca^{2+} delivery and g_s in *Phaseolus* (Figure 3.10 A and C).

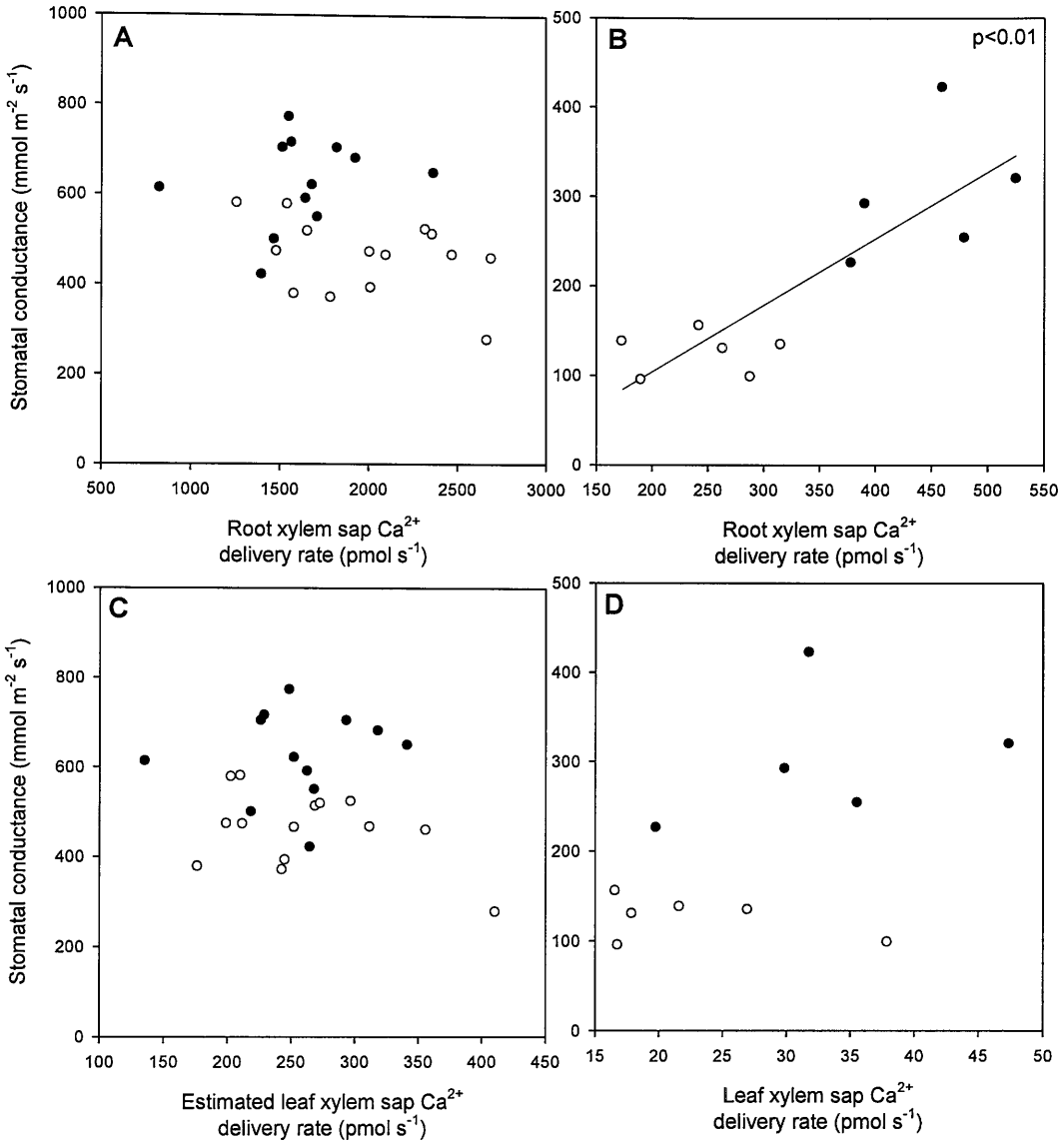

Figure 3.10: Relationship between root and leaf xylem sap Ca^{2+} delivery rates calculated from sap flow rate and Ca^{2+} concentration in samples collected from control (filled circles) or limed (open circles), bean root (panel A), pea root (panel B), pea leaf (panel D) or estimated (assuming equal distribution of root xylem transport fluids to whole leaf area) in bean leaf (panel C) and stomatal conductance. Data points are individual samples, single regression lines fitted where significant, P values reported in top right of panel.

In the detached leaf bioassays, transpiration rates of *Pisum* leaflets grown in limed soil were initially lower than those of control plants but recovered after 200 minutes to levels of control plants when fed a low (0.5 mM) Ca^{2+} artificial sap (Figure 3.15).

However, limed and unlimed leaflets showed equal sensitivity to a high (5 mM) Ca^{2+} artificial sap as demonstrated by the absence of a lime * calcium interaction (Figure 3.15B), thus justifying the use of unlimed plants in the dose response assays. Artificial xylem sap containing 5 mM Ca^{2+} as either $CaCl_2$ or $Ca(NO_3)_2$ inhibited transpiration rates of detached *Pisum* leaflets and *Phaseolus* shoots when compared to Ca^{2+} at 0.5 mM or 2.5 mM (Figures 3.11, 3.12, 3.13 and 3.14). The similarity of response with both chloride and nitrate suggests that it is the Ca^{2+} concentration that is limiting transpiration and not the counter ion used. Mean values from when the assays had stabilised after 200 minutes reveal that 5 mM Ca^{2+} reduced transpiration in *Phaseolus* shoots by around 20% when compared to 0.5 mM Ca^{2+}. Similarly, 5 mM Ca^{2+} decreased transpiration of *Pisum* leaflets by 26%.

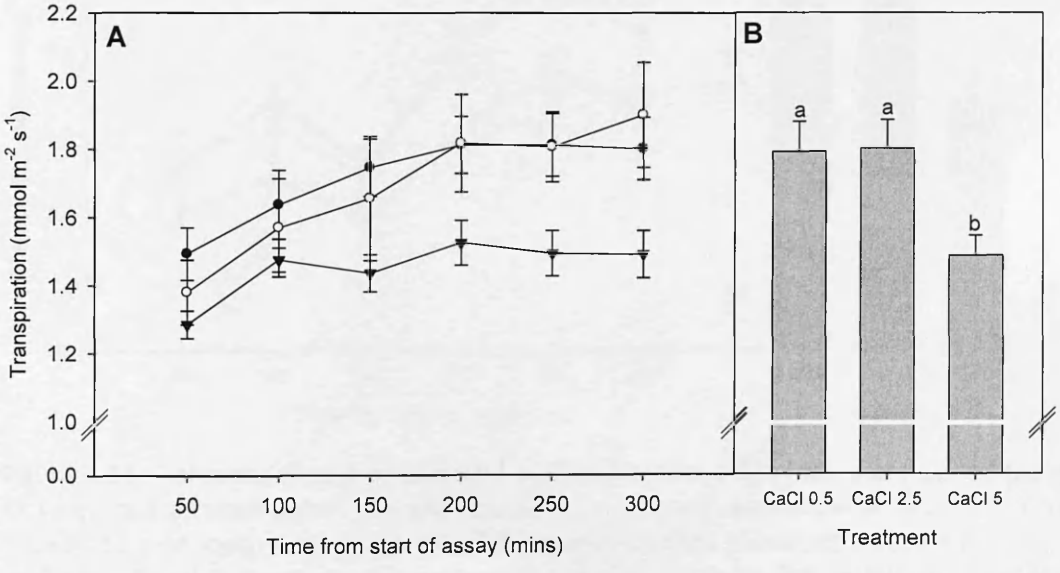

Figure 3.11: Transpiration rate of detached bean shoots over a five hour assay period (panel A) when fed artificial xylem sap with calcium chloride concentrations at 0.5 mM (filled circles), 2.5 mM (open circles) or 5 mM (filled triangles). Data points are means ± SE of 8-12 replicates. Panel B shows mean transpiration rates (± SE) from 200 to 300 minutes with differing letters indicating significant differences ($p<0.05$) as determined by one-way ANOVA.

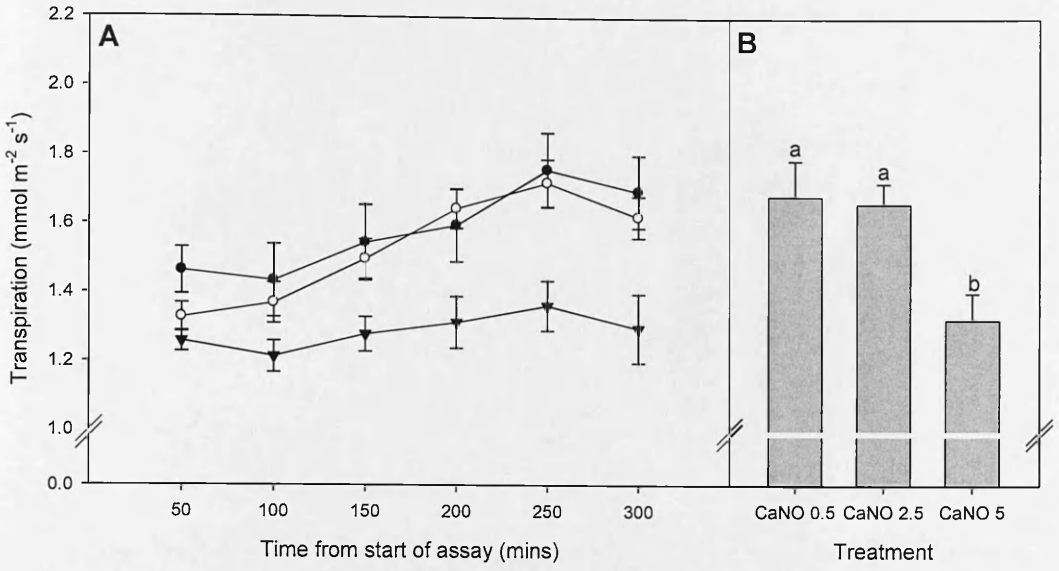

Figure 3.12: Transpiration rate of detached bean shoots over a five hour assay period (panel A) when fed artificial xylem sap with calcium nitrate concentrations at 0.5 mM (filled circles), 2.5 mM (open circles) or 5 mM (filled triangles). Data points are means ± SE of 12 replicates. Panel B shows mean transpiration rates (± SE) from 200 to 300 minutes with differing letters indicating significant differences ($p<0.05$) as determined by one-way ANOVA.

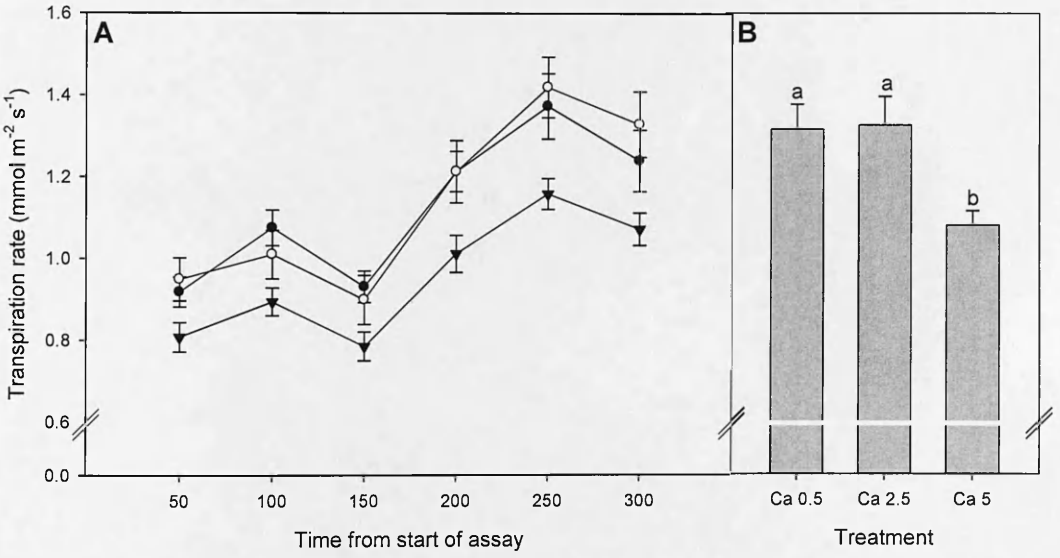

Figure 3.13: Transpiration rate of detached pea leaflets over a five hour assay period (panel A) when fed artificial xylem sap with calcium chloride concentrations at 0.5 mM (filled circles), 2.5 mM (open circles) or 5 mM (filled triangles). Data points are means ± SE of 9-10 replicates. Panel B shows mean transpiration rates (± SE) from 200 to 300 minutes with differing letters indicating significant differences ($p<0.05$) as determined by one-way ANOVA.

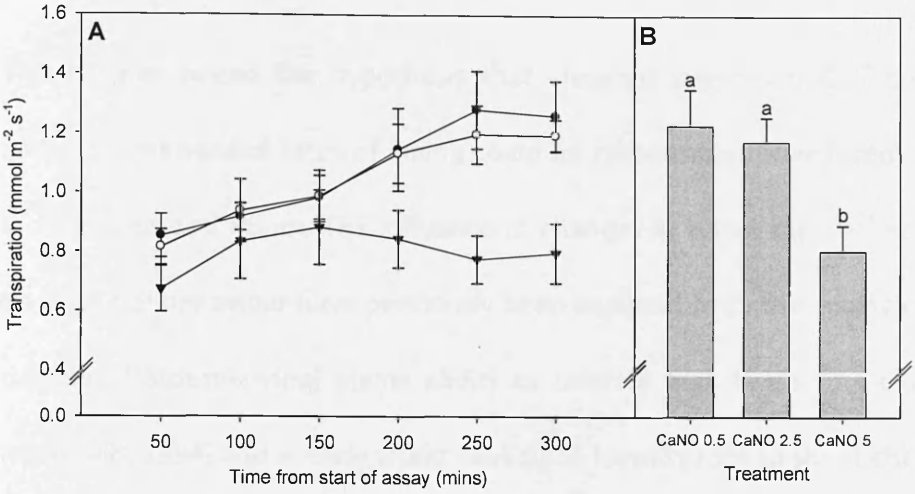

Figure 3.14: Transpiration rate of detached pea leaflets over a five hour assay period (panel A) when fed artificial xylem sap with calcium nitrate concentrations at 0.5 mM (filled circles), 2.5 mM (open circles) or 5 mM (filled triangles). Data points are means ± SE of 5 replicates. Panel B shows mean transpiration rates (± SE) from 200 to 300 minutes with differing letters indicating significant differences ($p<0.05$) as determined by one-way ANOVA.

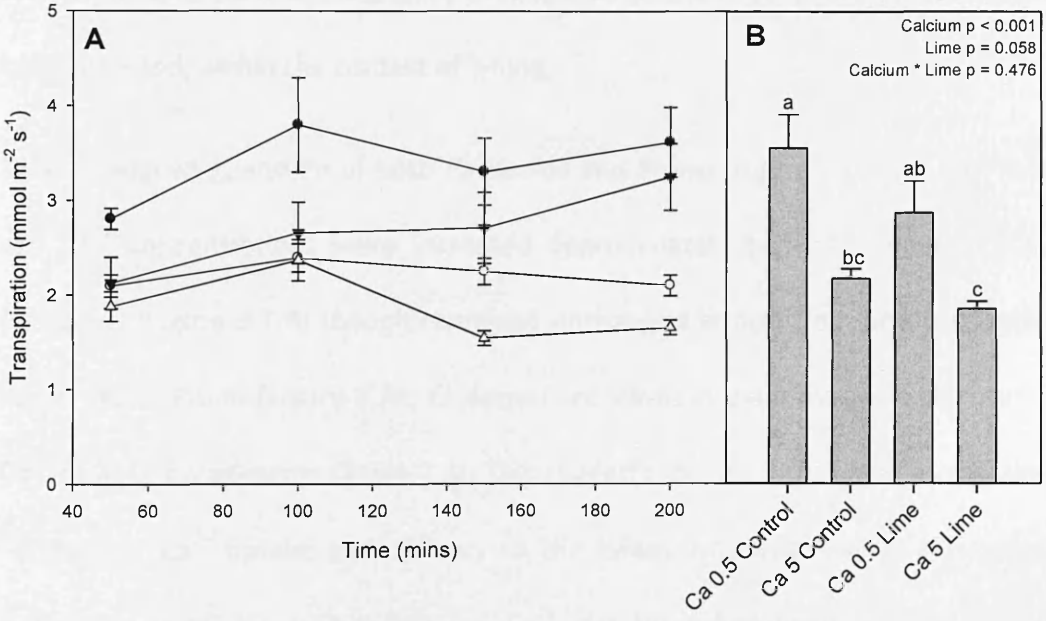

Figure 3.15: Transpiration rate of detached pea leaflets grown in limed (triangles) or control (circles) soil over a three hour assay period (panel A) when fed artificial xylem sap with calcium chloride concentrations at 0.5 mM (filled symbols) or 5 mM (open symbols) Data points are means ± SE of 7-8 replicates. Panel B shows mean transpiration rates (± SE) from 100 to 200 minutes. Results of two-way ANOVA with interaction are presented in the top right of panel B with differing letters indicating significant differences ($p<0.05$) as determined by Tukey pair-wise comparison.

3.4 Discussion

This chapter tested the hypothesis that elevated xylem sap Ca^{2+} concentrations under recommended rates of liming could be responsible for reduced gas exchange in *Phaseolus* and *Pisum*. The influence of changes in xylem sap Ca^{2+} concentrations on stomatal behaviour have previously been explored from the ecological context of calcicole (calcium-loving) plants ability to tolerate high levels of Ca (De Silva and Mansfield, 1994) and in early works seeking to identify root to shoot chemical signals responsible for regulating stomatal aperture (Mansfield and Atkinson, 1990), particularly in response to soil drying (Gollan et al., 1992). However, as far as the author is aware, this is the first time that the physiological impact of adding substantial quantities of potentially available Ca to the rhizosphere on stomata has been assessed, within the context of liming.

Liming reduced g_s and Pn in both *Phaseolus* and *Pisum* (Figure 3.6) and root xylem sap Ca^{2+} concentrations were increased approximately two-fold under liming in *Phaseolus* (Figure 3.7 A) though remained unchanged in both root and leaf derived xylem sap in *Pisum* (Figure 3.7B, C) despite increases in plant available rhizospheric Ca^{2+} in both experiments (Table 2.1). This suggests species differences in the ability to regulate Ca^{2+} uptake and delivery to the xylem independently of rhizospheric availability. Similarly, root xylem sap Ca^{2+} was three-fold higher across three *P. vulgaris* cultivars than three *Pisum* cultivars grown in the same rhizospheric Ca concentrations (Atkinson et al., 1992), suggesting greater regulation of Ca^{2+} uptake in *Pisum*, perhaps caused by species differences in endodermis permeability to Ca^{2+} (Chino, 1981). However, differences in regulation of root Ca^{2+} uptake does not

account for the classification of species as calcicoles and calcifuges (De Silva and Mansfield, 1994); instead it has been suggested that sequestration of Ca^{2+} in the leaf mesophyll is important in regulating apoplastic Ca^{2+} concentrations around the guard cells (Ruiz and Mansfield, 1994). Epidermal peels from both calcicoles and calcium-neutral plants showed a similar stomatal response to apoplastic Ca^{2+} concentrations (De Silva and Mansfield, 1994). Furthermore, both calcicole species used in this study (White and Broadley, 2003) and the calcifuge *Lupinus luteus* (Atkinson, 1991) showed a similar stomatal response to xylem sap Ca^{2+} independent of calcicole/calcifuge classification. Since xylem sap Ca^{2+} concentration was unrelated to stomatal closure of limed plants (Figure 3B, C), classification of plants as calcicoles and calcifuges is unlikely to inform species differences in sensitivity to lime.

Furthermore, increased root xylem sap Ca^{2+} concentration was significantly correlated with decreased g_s (Figure 3.8 A). Although it could be concluded that elevated xylem sap Ca^{2+} decreased g_s of *Phaseolus*, g_s declined similarly in *Pisum* (Figure 3.7A and B) but xylem sap Ca^{2+} concentrations were not elevated, (independently of whether xylem sap was collected from the root or the leaves). There are two possible explanations for these divergent responses: (a) the sensitivity of stomata to Ca^{2+} in response to liming differs between the species, (b) the negative correlation observed in *Phaseolus* (Figure 3.8A) is not causative.

The evidence from the transpiration bio-assays where detached shoots/leaflets were fed artificial xylem sap with differing Ca^{2+} concentrations (Figures 3.11-3.14) suggests that *Phaseolus* and *Pisum* exhibited similar sensitivity to Ca^{2+}. Both species showed no change in transpiration in response to 0.5 or 2.5 mM Ca^{2+} and significantly

reduced transpiration (by 20-26%) in response to 5 mM Ca^{2+}, regardless of the counter ion (nitrate or chloride) used. Furthermore, ANOVA analysis of combined data from all the CaCl bioassays reveal no Species * Calcium interactions (P=0.835) (data not shown) thus demonstrating equal sensitivity of both species to Ca^{2+} fed in the artificial xylem sap.

Since the mean Ca^{2+} concentration observed in the sap collected from limed *Phaseolus* plants was 1.7 mM, and feeding 2.5 mM Ca^{2+} via the transpiration stream to detached leaves did not decrease transpiration rate, it seems unlikely that this concentration could have influenced stomatal behaviour. Similarly, increasing total xylem sap Ca^{2+} concentration from 0.75 mM to 2.32 mM by exposing *Commelina* to a nutrient solution containing 8 mM Ca^{2+} was not enough to close stomata (Atkinson et al., 1990). However, a xylem sap Ca^{2+} concentration of 3.76 mM was high enough to inhibit g_s by 15% in *Commelina* when compared to 0.87 mM (Ruiz et al., 1993) suggesting a possible threshold xylem Ca^{2+} concentration is needed to induce stomatal closure. Since 5 mM xylem Ca^{2+} reduced transpiration rates of both *Phaseolus* and *Pisum* in the bio-assays, this raises the question whether Ca^{2+} concentrations in the collected xylem sap are truly representative of *in vivo* concentrations? If xylem Ca^{2+} concentration(s) are underestimated, Ca^{2+} may still be causing stomatal closure.

The accuracy of ionic or chemical concentrations measured in xylem sap has often caused debate (Schurr, 1998), owing primarily to the diluting and/or concentrating effect of differing (pressure-generated) flow rates on ionic (Munns, 1985, Else et al., 1995) or phytohormonal (Else et al., 1995, Dodd et al., 2004) constituents. The

plasma membrane of xylem parenchyma cells is more permeable to water than to ions (White, 2012a) meaning loading of constituents into the root xylem sap is typically more constant than water flux. Thus a decreased sap flow rate will generally increase xylem constituent concentrations, therefore correctly reproducing *in vivo* sap flow rates is critical to assess whether root-to-shoot signals cause stomatal closure (Jackson, 1993).

Many different approaches to xylem sap sampling have been employed, not all of which make allowances for the above described flow rate/concentration effect. Exudation of sap from excised shoots and/or roots by applying fixed pneumatic pressures in Scholander type pressure chambers is commonly used (for example: Atkinson et al., 1992, Ruiz et al., 1993). However, this approach does not necessarily produce samples representative of *in vivo* conditions and may be of limited use in assessing the causality of root-sourced signals.

Approaches to help match pressure generated flow to *in vivo* flow have included using shoot water potential to indicate the appropriate rate of pneumatic pressure to apply (Liang and Zhang, 1997), however, this may lead to over estimation of sap flow (Tiekstra et al., 2000). More intricate techniques have employed careful control of transpiration via manipulation of humidity around, or exclusion of light to, plants grown in pressurised whole plant chambers to initiate sap flow and generate representative xylem sap flow rates (Gollan et al., 1992, Schurr and Schulze, 1995). A simpler approach is to ensure that the rate of pressure generated xylem sap flow can be recorded and checked against *in vivo* flow rates measured gravimetrically. This is typically achieved through multiple sample collections at incrementally higher or

lower pneumatic pressures (for example, Netting et al., 2012), as applied to *Pisum* in the whole plant pressure chamber in this study. Though likely to produce a sample closely matching *in vivo* sap flow rates, this approach is both time consuming (possibly causing changes in xylem sap composition as a result of change rhizosphere gas concentrations) and potentially wasteful if large volumes of sap are required for multiple analyses. The approach developed in this study for *Phaseolus* using short 20 second samples at sequentially higher pressures to match flow rates before a larger representative sample is taken for analysis not only makes the sampling procedure much quicker but reduces waste of valuable sap samples.

A further complication in xylem sap sampling is possible interference from recycling of the compound of interest from the shoot back to the root via the phloem. Various approaches have been taken to nullify this including stem girdling (Vernieri et al., 2001) or empirical flow modelling that collects both phloem and xylem saps to determine ion uptake, transport, and utilization within the plant (Peuke et al., 2002). However, as Ca^{2+} is known to be relatively immobile in the phloem (Clarkson, 1984), the rate of Ca^{2+} efflux into the phloem is a small fraction of the xylem influx (Schurr, 1998) and thus is not expected to make a significant contribution to measurements made in the xylem.

Given the accuracy by which pressure generated flow rates were matched to *in vivo* rates in the current experiment (Figure 3.5), and the precision of the ion-selective electrode used, xylem Ca^{2+} concentrations likely reflect those found *in vivo*. Thus it is unlikely that *in vivo* Ca^{2+} concentrations were sufficiently high to influence stomatal response to liming.

Instead, the observed relationship between increased xylem sap Ca^{2+} and g_s in *Phaseolus* could result from decreased xylem sap flow as a result of stomatal closure (caused by another xylem-borne antitranspirant). It was observed previously (and as discussed above) that increased xylem sap flow generated artificially by the pressure chamber diluted Ca^{2+} concentration (Figure 3.3). Therefore the lower transpirational flux in the limed plants could concentrate xylem sap Ca^{2+} (or conversely the higher flux under control may dilute) and could explain the negative correlation between Ca^{2+} and g_s regardless of causality (Dodd, 2005).

It has been suggested that stomatal responses to ionomic or chemical signals can be better assessed in terms of delivery rates (delivery rates are a function of concentration and transpirational flow rate) rather than concentration alone (Jackson, 1993). Gowing et al, (1993) could more accurately explain inhibition of g_s by the antitranspirant plant hormone abscisic acid (ABA) from the amount of the compound fed (delivery rate) rather than concentration alone in detached cherry leaves, though both factors were significant. Conversely, concentration of ABA exerted stronger stomatal control than flux in *Commelina* (Trejo et al., 1995). In the current experiment, delivery rates of Ca^{2+} in the *Phaseolus* root xylem sap were increased by 26% (Figure 3.9A), presumably as a function of increased concentration. However, there was no significant relationship between g_s and root xylem or estimated leaf xylem sap Ca^{2+} delivery rates. Indeed, the opposite relationship was observed in *Pisum* where g_s of un-limed plants was higher at increased Ca^{2+} delivery rates (Figure 3.10), most likely explained by increased transpirational flow rate at the same Ca^{2+} concentration. Since there is no correlation between Ca^{2+} delivery and g_s, stomata in *Phaseolus* or *Pisum* are likely not influenced by delivery rates of Ca^{2+}.

It is well established that concentration gradients of mineral nutrients, including Ca^{2+}, normally exist between plant root and the xylem and between the xylem and leaf (Peuke, 2010), however, less is understood of gradients existing within the xylem itself. Xylem Ca^{2+} concentration apparently increased approximately three-fold between root and shoot xylem sap in two cultivars of *Pisum* (Atkinson et al., 1992). Conversely, xylem Ca^{2+} concentration was reduced 2.5-fold between leaves 2 and 6 (counting acropetally) in *Solanum lycopersicum* when xylem sap was collected at transpirational flow rate using a whole-plant pressure chamber (Tiekstra et al., 2000). Furthermore, leaf xylem sap Ca^{2+} concentration in *Zea mays* was only half that of the root (Goodger et al., 2005). This agreed with the present study where leaf xylem sap Ca^{2+} concentration was approximately half that of the root (cf. Figure 3B, C), perhaps explained by deposition of Ca^{2+} on exchange sites along the xylem vessels (Clarkson, 1984). Whether these apparent disparities in xylem sap Ca^{2+} gradients are a function of sap collection methodology or genotypic differences is unclear. Although gradients in xylem sap Ca^{2+} concentration clearly exist, it is uncertain whether they may regulate the effects of xylem sap Ca^{2+}. Since xylem sap was collected in *Pisum* leaves as close as possible to the site of action of any antitranspirant (the stomata), it seems that stomata of limed plants of *Pisum* are not influenced by gradients in Ca^{2+} concentration.

Although the evidence presented here suggests that elevated xylem sap Ca^{2+} does not decrease leaf gas exchange of limed plants, a possible limitation in this understanding is the use of artificial xylem sap to assess sensitivity to Ca^{2+}. Xylem sap is a highly complex matrix of mineral elements and organic compounds (White, 2012b) which could interact to produce antitranspirant effects (Zhu and Zhang,

1997). Experiments comparing the dose-response curves of antitranspirants in artificial xylem sap and actual xylem sap have delivered mixed results. Although the antitranspirant effect of xylem sap collected from droughted wheat plants could not be explained by its ABA concentration (Munns and King, 1988) removal of ABA from maize xylem sap decreased antitranspirant effect (Zhang and Davies, 1991). However, an artificial xylem sap matched in ABA concentration and pH could not replicate *in vivo* transpiration reductions in nitrate deprived *Capsicum* (Dodd et al., 2003) or flooded tomato (Else et al., 2006). Therefore bio-assays using actual xylem sap collected from limed and unlimed plants, perhaps using chelators such as EGTA to inhibit the bio-activity of Ca^{2+}, may offer further insight into the sensitivity of stomata to *in vivo* xylem sap Ca^{2+}.

In conclusion, this study provides only limited evidence that increased xylem Ca^{2+} concentration or delivery rates causes stomatal closure of limed plants. Although root xylem Ca^{2+} concentration was correlated with decreased g_s in Phaseolus (Figure 3.8A), it can be argued that this results from the concentrating effect of decreased transpirational flux, probably due to another as yet unidentified xylem borne antitranspirant. Further, xylem sap Ca^{2+} concentrations greater than those observed *in vivo* were required to decrease transpiration of detached *Phaseolus* and *Pisum* leaves. Together with the fact that *Pisum* exhibited reduced g_s in the absence of elevated Ca^{2+} suggest that Ca^{2+} is not responsible for the observed stomatal behaviour. Furthermore, the fact that leaves detached from limed plants showed lower transpiration rates (Figure 3.15A) suggest the existence of another antitranspirant that persists in the leaves for at least three hours after detachment. Therefore it must be assumed that an alternative ionic, chemical or hydraulic signal is

responsible for reduced gas exchange under recommended liming in *Phaseolus* and *Pisum*.

Chapter 4 – Reduced leaf gas exchange in limed *P. sativum* is mediated by the phytohormone abscisic acid

4.1 Introduction

One possible explanation for the reduced growth of limed plants (discussed in Chapter 2) is decreased soil P availability (Haynes, 1982), principally through the formation of insoluble calcium phosphates (Delgado and Torrent, 2000). Consequently, foliar P concentrations of *Vicia faba* plants grown in limed soil were 50% lower than in control plants (Table 2.8). Phosphorus is an essential plant nutrient that is necessary for many plant processes including membrane stability, energy transfer and enzyme activation (Hawkesford et al., 2012), therefore inadequate P availability is a major limitation on plant growth and development (Schachtman et al., 1998) and consequently global crop production (Raghothama and Karthikeyan, 2005). It is estimated that 30-40% of global agricultural soils are limited by P availability (Vance et al., 2003) and it is second only to N in limiting agricultural productivity (Holford, 1997).

Although P deficiency might explain reduced growth under liming, it is not clear how low P availability may influence plant gas exchange. Phosphorus deprived plants showed reduced g_s in two cultivars of *Capsicum annum* (Davies et al., 1999) and *R. communis* (Jeschke et al., 1997) and reduced transpiration in *Camellia sinensis* (Nagarajah and Ratnasuriya, 1978). Conversely, transpiration rate in *Trifolium repens* was negatively correlated with plant P status suggesting a possible lack of stomatal

control under P deficiency (Singh et al., 2000). Direct effects of tissue P concentration seem unlikely, as P, N and S deficiency all elicited stomatal closure, suggesting that these responses are not caused by tissue nutrient levels *per se* (Clarkson et al., 2000) but by a common or centralised response to those deficiencies (Chapin, 1990).

It has been suggested that P and other nutrient deficiencies may limit g_s via hydraulic mechanisms caused by reduced root hydraulic conductance (Clarkson et al., 2000). However, maintaining leaf water status by root pressurisation failed to prevent reduced g_s in response to soil drying in *Triticum aestivum* and *Helianthus annuus* (Gollan et al., 1986) or salt stressed *T. aestivum* and *Hordeum vulgare* (Termaat et al., 1985) suggesting that hydraulic signals may not regulate stomatal responses. Alternatively, increased root biosynthesis of a chemical signal, the phytohormone ABA, which is known to be a potent antitranspirant, was suggested to reduce g_s in response to P deficiency (Jeschke et al., 1997). Thus it is apparent that the mechanism(s) by which P deficiency may influence stomatal behaviour is still uncertain.

There are many examples in the literature where plant nutrient deficiencies are associated with alterations in phytohormone concentrations. For example, N deficiency is associated with increased concentration of ABA (Wilkinson and Davies, 2002), and decreased CK concentrations (Takei et al., 2002, Argueso et al., 2009); K deficiency with increases in endogenous ABA (Peuke et al., 2002); S deficiency was associated with increased CK and reduced ABA content of leaves and roots (Honsel et al., 2012). More relevant to the current experiment, low P supplied plants with a leaf tissue P concentration of 2.3 mg g^{-1} showed a two-fold increase in leaf ABA

concentration in response to soil drying compared to high P status plants (leaf tissue 6.8 mg g^{-1}) in cotton (*Gossypium hirsutum*) (Radin, 1984). Similarly, a three-fold reduction in root P concentration from 2.7 to 0.9 mg g^{-1} (and consequent two-fold reduction in leaf P from 3.7 to 1.6 mg g^{-1}) lead to a six-fold increase in root xylem sap ABA and near two-fold increase in leaf tissue ABA concentration in well watered castor bean (*Ricinus communis*) (Jeschke et al., 1997). Furthermore, this increase in root xylem sap ABA concentration showed a good correlation with reduced g_s under P deficiency.

Increased ABA synthesis is a typical plant response to many edaphic stresses including reduced water availability (Schachtman and Goodger, 2008), salinity (Chaves et al., 2009) and soil compaction (Mulholland et al., 1999). Elevated endogenous levels of ABA, if delivered to the apoplast in the vicinity of the stomatal guard cells, can instigate stomatal closure (Hartung et al., 2002) thus forming a critical mechanism in the adaption of plants to changing soil water status to avoid fatal dehydration (Wilkinson and Davies, 2002). As ABA is known to increase in response to P deficiency, it is likely that limed plants may exhibit elevated ABA synthesis in response to lime-induced reduced P availability possibly explaining the reduction in gas exchange observed in Chapters 2 and 3.

A further mechanism by which stomata may be influenced is by changes in xylem sap pH, in particular alkalinisation (Wilkinson, 1999). Alkalinisation of xylem sap is known to act as a stomatal signal in response to nutrient deprivation (Dodd et al., 2003) and drought (Wilkinson and Davies, 1997) principally by redistributing ABA from mesophyll cells to the leaf apoplast thereby enhancing stomatal closure (Hartung et

al., 1998). Root xylem sap pH is affected by substrate conditions (water or nutrient availability) and can influence leaf apoplastic sap pH and thus stomata (Jia and Davies, 2007). Furthermore, the root apoplast pH of *Zea mays* was increased by 0.5 units in response to increased external pH from pH 5 to 8 (Felle, 1998), therefore, it is plausible that raising bulk soil pH through liming might increase root xylem sap pH and thus leaf apoplastic pH and could offer an additional mechanistic explanation for the effects of liming on gas exchange.

Chapter 3 tested the hypothesis that increases in root xylem sap Ca^{2+} concentration or delivery rate decreased leaf gas exchange in limed legumes. It was concluded that an alternative antitranspirant must be regulating stomatal behaviour. In this chapter, the wider xylem ionome was investigated to establish the cause of decreased gas exchange in limed pea plants and the following hypotheses were tested:

- Reduced phosphorus availability under liming treatment causes reduced gas exchange by increased root-to-shoot signalling of the plant hormone ABA.
- Raising bulk soil pH by liming increases xylem sap pH thus directly causing stomatal closure.
- Lime induced increase in xylem sap pH are mediating the response of stomata to ABA.

4.2 Materials and methods

4.2.1 Plant material

Seeds of tall pea (*Pisum sativum* L. Alderman) were pre-germinated on tissue paper dampened with distilled water and kept in the dark. On emergence of the radicle (2-3 days), uniformly germinated seeds were individually transplanted into 0.8 litre pots (110 mm high, 120 mm diameter) containing the same control or limed (3 g l^{-1}) sterilised 2:1 (v:v) mixture of a low pH sandy loam field soil and horticultural grit sand as described in Section 2.2.3.1. An additional treatment of superphosphate fertiliser (J. Arthur Bowers Ltd., William Sinclair Horticulture Ltd, Lincoln, UK) at a rate of 0.59 g l^{-1} (to represent a 200 kg ha^{-1} application rate) was added to each control or limed soil immediately prior to potting to give a 2 x 2 factorial experimental design. All pots were watered to run off and weighed after 24 hours to establish weight at drained capacity and maintained well watered by replacing full evapotranspiration (determined gravimetrically) daily and maintained in a semi-controlled naturally lit greenhouse with supplementary lighting (supplied by Osram 600 w daylight bulbs) for 12 hours and 22°C/16°C minimum day/night temperature at the Lancaster Environment Centre.

4.2.2 Xylem sap analysis

Leaf and root xylem sap analysis was determined on samples collected from the *Pisum* plants described in Section 3.2.1. Leaf and root xylem sap pH was measured using a micro pH electrode (B-212 LAQUAtwin, Horiba Instruments Ltd, Northampton, UK). Ionomic analysis (Ca, K, Mg, P and S) was by Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES; iCAP 6300, Thermo Scientific,

Massachusetts, USA), where 250 µl samples of root xylem sap were diluted 20x in 4.75 ml of 2% HNO_3 and filtered through a 0.45 micron syringe filter prior to analysis. Samples were compared against standards of a known range of concentrations. ABA analysis was by radioimmunoassay as described in section 4.2.5 below.

4.2.3 Gas exchange measurements

Stomatal conductance, Pn, and intercellular CO_2 (Ci) was measured on one leaflet of leaf pair four (youngest fully expanded leaf) numbering from the base of the plants, using infra-red gas analysis (6400xt Li-Cor Portable Photosynthesis System, Lincoln, Nebraska, USA) as described in Section 2.2.4.2.

4.2.4 Leaf water potential

Leaf water potential (Ψ_{leaf}) of one leaf of leaflet pair four was measured by thermocouple psychrometry. Leaf discs of 8 mm diameter were punched from the mid-lamina, placed immediately on clean sample holders and then wrapped in aluminium foil to minimize water loss. When all samples had been collected they were unwrapped and loaded into C52 sample chambers (Wescor Inc., Logan, UT, USA), incubated for ~3 hours then voltages were read with a microvolt meter (model HR-33T; Wescor Inc., Logan, UT, USA). Voltages were converted into water potentials based on calibration with salt solutions of known osmotic potential.

4.2.5 ABA radioimmunoassay (RIA)

Determination of xylem sap, leaf and root tissue ABA concentration was by competitive radioimmunoassay (RIA) (Quarrie et al., 1988) using radiolabelled ABA (DL-*cis/trans* [3^H] ABA) and the antibody MAC 252 (Dr Geoff Butcher, Babraham

Institute) that has high specificity for the free acid of (+)-2-*cis*-ABA (Barrieu and Simonneau, 2000). At harvest, whole leaflets were taken from leaflet pair 4 (the same leaf as gas exchange measurements), and root samples taken, washed to remove adhering soil, placed in pre-weighed eppendorf tubes and immediately frozen in liquid nitrogen and stored at -80°C to await analysis. To prepare for analysis, the samples were freeze-dried and ground to a fine powder using dissecting scissors. Both leaf and root tissue samples were extracted at a ratio of 1:25 in distilled water by shaking overnight at 4°C on a mechanical shaker. Leaf and root xylem sap samples were analysed as collected.

Leaf and root tissue extracts were centrifuged for 4 minutes to remove any plant fragments held in suspension which may interfere with the assay. The RIA was then undertaken using the following protocol:

- Add 200 µl of 50% phosphate buffered saline (PBS; 50 mM sodium dihydrogen phosphate, 50 mM disodium hydrogen phosphate and 100 mM sodium chloride adjusted to pH 6) to 2 ml micro-centrifuge tubes held in a foam rack.
- Add 50 µl of ABA standard (0, 62.5, 125, 250, 500, 1000, 2000 pg 50 ul^{-1} and 3 mM ABA) or sample (leaf or root tissue extract or xylem sap).
- Sequentially add 100 µl of [3^{H}] ABA and 100 µl MAC 252.
- Centrifuge for 1 minute, replace in sequential order in the foam rack and incubate for 45 minutes at 4°C.
- Add 500 µl of saturated ammonium sulphate solution to precipitate the ABA-antibody complex.
- Mix by turning over the capped tubes in the rack 6 times and incubate for 30 minutes in the dark at room temperature.
- Centrifuge for 4 minutes to precipitate pellet and discard the resulting supernatant by removing the caps and gently turning the vials over in the

foam racks to poor out the supernatant and remove excess moisture by blotting on tissue paper.

- Add a second wash using 1 ml of 50% ammonium sulphate and re-suspend to remove excess unbound radioactivity.
- Centrifuge for 5 minutes and remove the supernatant as above.
- Dissolve the remaining pellet in 100 µl of deionised water and mix by gentle vibration from a bench-top whirl-mixer.
- Add 1.5 ml of scintillant (Ecoscint H, National Diagnostic, NJ, USA) to each tube to allow radioactivity to be visualised as fluorescence by a scintillation counter (Packard TriCARB 1600TR Liquid Scintillation Analyser; Canberra, CT, USA).
- Data are converted from counts per minute (CPM) to ABA concentrations by comparison with the standard curve produced.

A spike dilution test assessed whether the xylem sap or aqueous extracts of *Pisum* leaf and root tissue contained significant immunoreactive contamination (Bacon, 2001). Serial dilutions (1; 0.5; 0.25) of sap or tissue samples were spiked with known amounts of ABA to generate a series of regression lines: parallel lines indicate the absence of immunoreactive contamination (Figure 4.1).

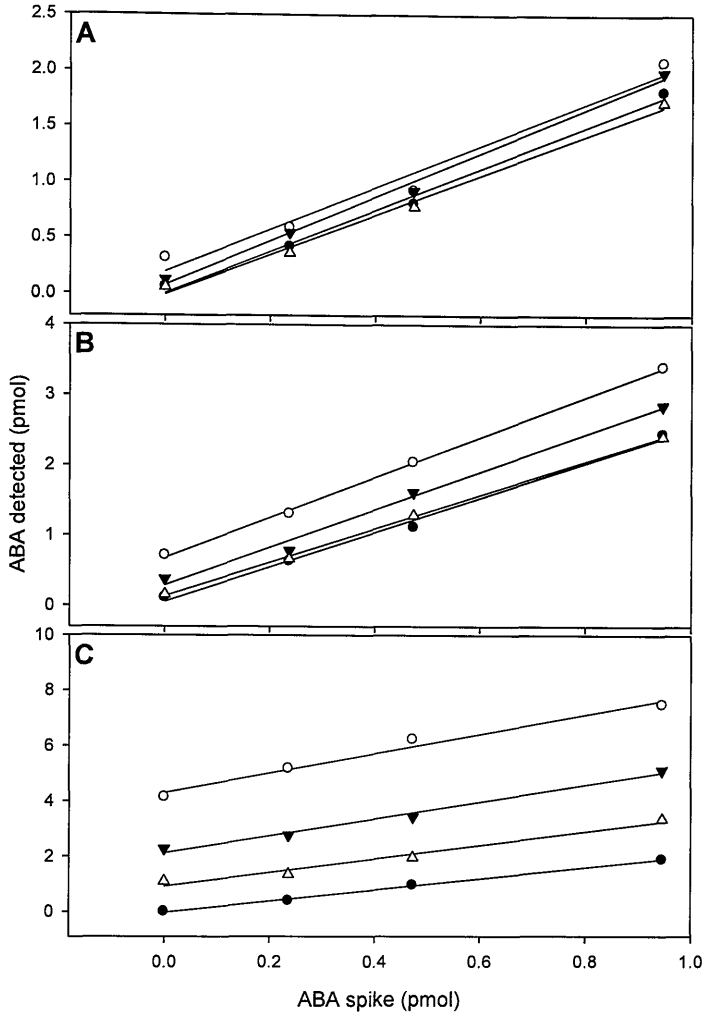

Figure 4.1: ABA spike dilution test for *Pisum* xylem sap (panel A), root (panel B) and leaf (panel C) tissue. Samples of full (open circle), half (filled triangle) or quarter (open triangle) strength sample and water (filled circle) were spiked with known amounts of ABA. Parallel regression lines indicate that none of the substrates assayed contained compounds that may interfere with the RIA.

4.2.6 Soil and plant tissue analysis

Plant tissue nutrient analysis was undertaken using the same procedures as described in Section 2.2.2.3. Tissue nutrient analysis was undertaken on a homogenised oven dried sample of all leaves present at time of harvest and root tissue that had been washed of adhering soil particles and rinsed in deionised water

prior to drying. Soil pH and Olsen extractable P were determined in triplicate from homogenised soil samples in each treatment group as described in Section 2.2.2.4. Soil pore water electrical conductivity (EC_p) was recorded in each individual pot at drained capacity using a WET sensor (Delta-T Devices, Cambridge, UK).

4.2.7 Plant harvest

All plants were harvested at 3 weeks old, shoot fresh weight was recorded at time of harvest along with individual area of leaflet and stipule pairs at each node using a leaf area meter (Li-3050A, Li-Cor, Lincoln, Nebraska, USA). Shoot material was then dried at 80°C for one week to determine dry biomass.

4.2.8 Detached leaf transpiration bioassays

Tall pea plants (*Pisum sativum* L. cv. Alderman) were established to allow detached leaf transpiration bioassays undertaken as described previously in Section 3.2.3 with the exception that a dose response to ABA was established by feeding artificial xylem sap with ABA concentrations of 0, 5, 50 and 100 nM. Transpiration was recorded over a 5 hour period and mean values used from 200-300 minutes when transpiration had stabilised.

4.2.9 Statistical analysis

Treatment differences in xylem sap ionomic and ABA concentration were distinguished with a students *t*-test (Table 4.1, Figure 4.2). Two-way ANOVA with Tukey pair-wise analysis was used to determine treatment and interaction effects on growth (Figure 4.3), tissue P concentration (Figure 4.5), gas exchange (Figure 4.7), tissue ABA concentration (Figure 4.8) and Ψ_{leaf} (Figure 4.12). Regression analysis was

used to determine correlative relationships and single linear or second order regression lines were fitted as appropriate where significant (Figures 4.6, 4.9-4.11, 4.13, 4.14). All analyses used Minitab v16.

4.3 Results

4.3.1 *P. sativum* xylem sap analysis

Ionomic analysis by ICP-OES revealed a significant 45% reduction in root xylem sap P concentration in limed plants when compared to unlimed controls (Figure 4.2A). Liming did not significantly change the concentration of other measured plant nutrients (Ca, Mg, Na, S and K) in the root xylem sap. Similarly, xylem sap pH collected at the leaf or root was unchanged by liming and there was no apparent difference between root and leaf samples within each treatment (Table 4.1). However, leaf and root xylem sap ABA concentrations were increased approximately two-fold by the liming treatment but again there was no apparent gradient in concentration between root and leaf sap samples in either treatment (Table 4.1).

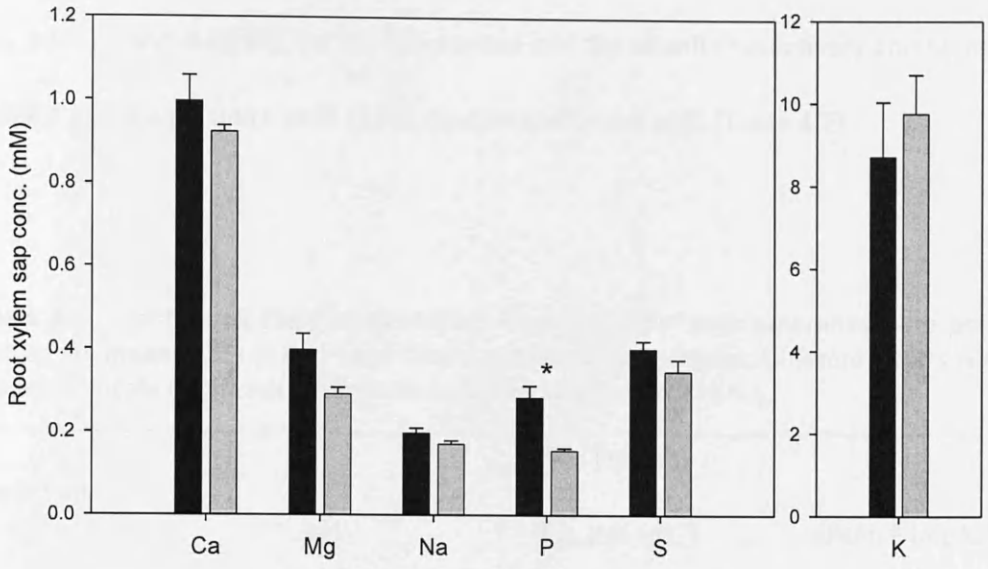

Figure 4.2: Root xylem sap nutrient analysis of control (black bars) and limed (grey bars) *Pisum*. Data are means ± SE of 3 replicates, * indicates significant difference ($p<0.05$) between treatments.

Table 4.1: Root and leaf xylem sap ABA concentration and pH collected from control and limed *Pisum* plants grown in whole plant pressure chambers. Data are means (± SE) of 5-6 replicates. Different letters within a column indicate significant differences according to a students *t*-test ($p<0.05$).

Treatment	Root xylem sap ABA (nM)	Leaf xylem sap ABA (nM)	Root xylem sap pH	Leaf xylem sap pH
Control	5.09 ± 0.6 a	3.97 ± 0.3 a	5.62 ± 0.03 a	5.65 ± 0.02 a
Lime	9.21 ± 1.4 b	10.4 ± 0.8 b	5.68 ± 0.07 a	5.68 ± 0.03 a

4.3.2 *P. sativum* liming and superphosphate experiment

Liming increased control soil pH by 0.7 units from pH 5.77 to pH 6.43, increased soil EC_p by ~0.1 mS cm^{-1} and reduced Olsen extractable P by 7% (Table 4.2). Application of superphosphate fertiliser increased Olsen extractable P in both limed and unlimed

control soil by 19% and 24% respectively. Superphosphate also slightly increased soil EC_p by 0.11 and 0.05 mS cm^{-1} in the control and limed soil respectively and increased soil pH by ~0.2 pH units both in the control and limed soils (Table 4.2).

Table 4.2: Selected soil chemical properties from the *Pisum* lime/superphosphate pot trial. Values are mean (± SE) of 3 (pH and Olsen) or 8-10 (EC_p) replicates. Different letters within a column indicate significant differences according to one-way ANOVA.

Treatment	Soil Property		
	pH	EC_p (mS cm^{-1})	Olsen P (mg kg^{-1})
Control	5.77 ± 0.03 a	0.49 ± 0.02 a	34.4 ± 0.3 a
Control + P	5.97 ± 0.03 a	0.60 ± 0.02 b	41.0 ± 0.9 b
Lime	6.43 ± 0.07 b	0.57 ± 0.02 b	32.1 ± 1.8 a
Lime + P	6.67 ± 0.07 b	0.62 ± 0.01 b	39.7 ± 1.1 b

Growth analysis showed that liming significantly reduced both shoot dry biomass (Figure 4.3A) and total leaf area (Figure 4.3B) by 38% and 31% respectively. The application of superphosphate fertiliser increased shoot dry biomass of control plants by 15% and limed plants by 23%. Similar significant effects were observed on total plant leaf area with 13% and 18% increases respectively (Figure 4.3A, B) suggesting that the control plants were also slightly P limited. Analysis of individual leaf areas at each node (Figure 4.4) suggest that leaf area development was most limited from node 4 (third emerging leaf) and remained apparent in subsequent emerging leaves at nodes 5 and 6.

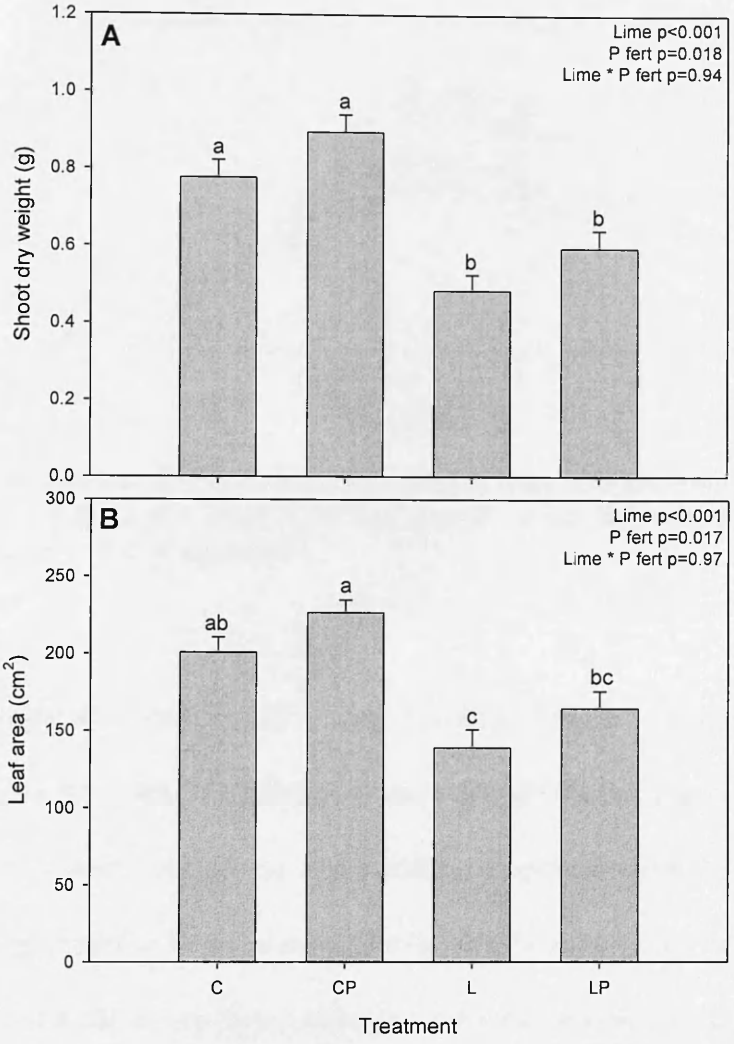

Figure 4.3: Shoot dry biomass (panel A) and leaf area (panel B) of control (C), control + P fert (CP) limed (L) or limed + P fert (CP) treated *Pisum*. Data are means ± SE of 8-10 replicates. Results of two-way ANOVA with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

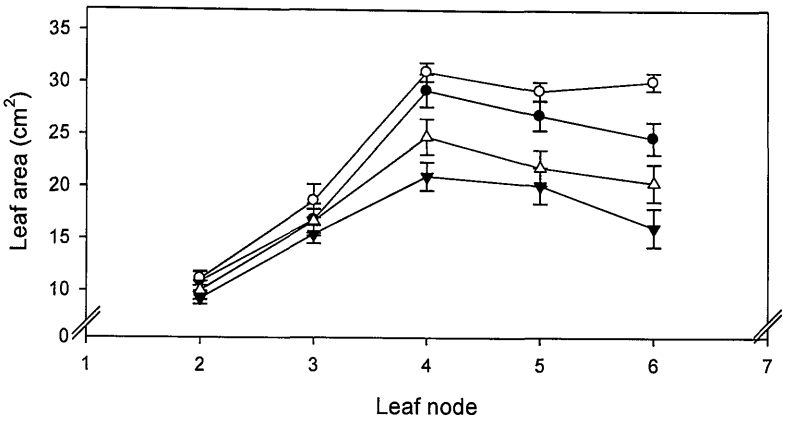

Figure 4.4: Total leaf area of *Pisum* leaflet pairs at each node of limed (filled triangle), lime + P fert (open triangle), control (filled circle) and control + P fert (open circle) treated plants. Data are means ± SE of 8-10 replicates.

Liming approximately halved leaf tissue P concentration compared to unlimed controls (Figure 4.5A) and the addition of superphosphate fertiliser increased leaf P concentration in both control and limed plants by approximately 25% (though not statistically significant in limed plants). Similar effects were observed in root tissue samples (Figure 4.5B) where liming reduced root tissue P concentration by 28% and superphosphate fertiliser increased root tissue concentration by 28% and 10% in control and limed plants respectively (again not significantly in limed plants). There was a significant correlation between leaf tissue P concentration and both shoot dry biomass and total plant leaf area (Figure 4.6A, B).

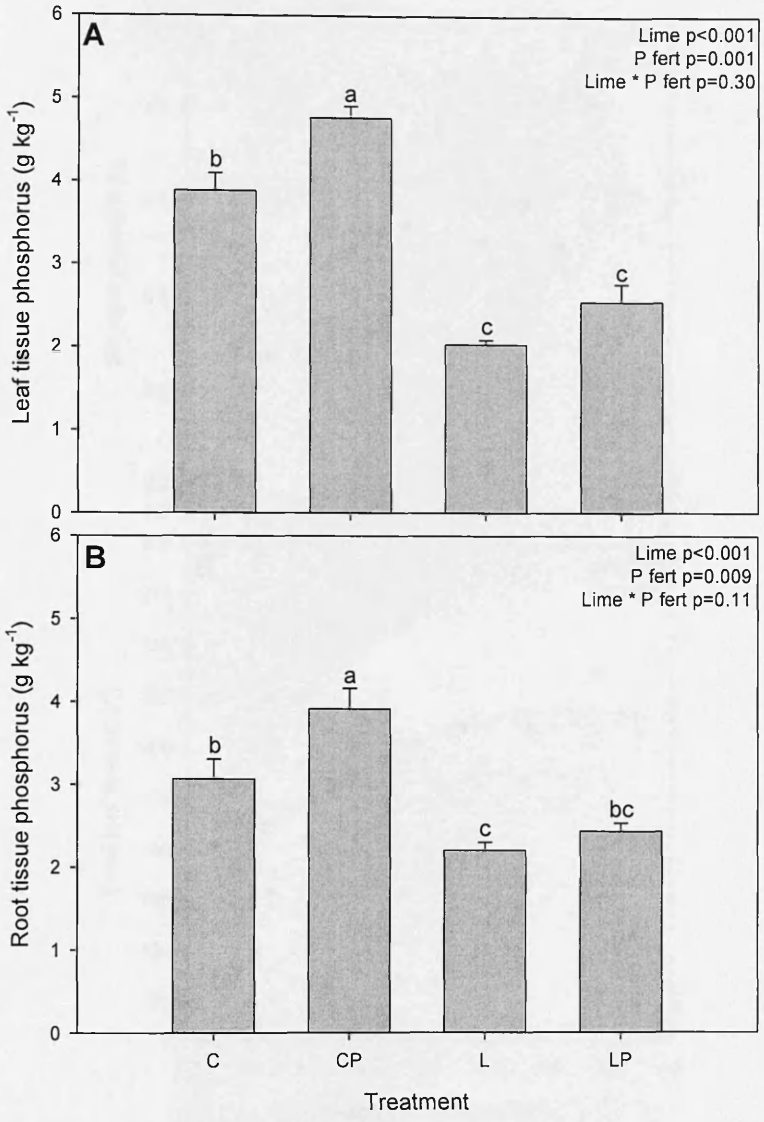

Figure 4.5: Leaf (panel A) and root tissue (panel B) total phosphorous concentration. Data are means ± SE of 6 replicates. Results of two-way ANOVA with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

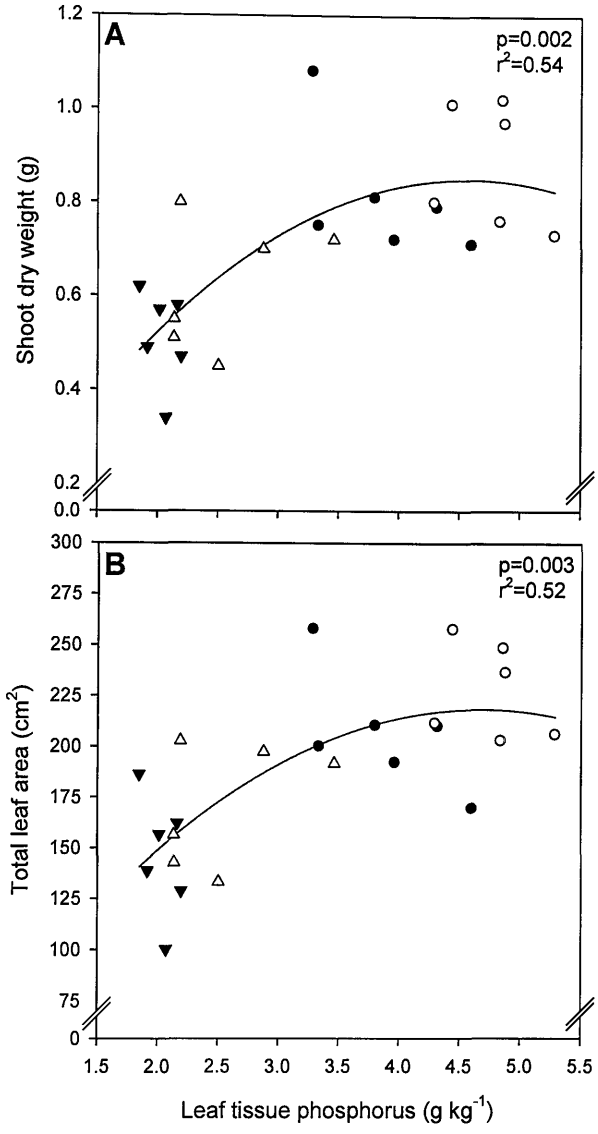

Figure 4.6: Relationship between leaf tissue phosphorous concentration and shoot dry biomass (panel A) and total leaf area (panel B) of limed (filled triangle), lime + P fert (open triangle), control (filled circle) and control + P fert (open circle) treated plants. Data points are paired samples from individual plants, single second order polynomial regression lines fitted where significant, P values and r^2 reported in top right of panel.

Gas exchange data illustrates that the liming treatment approximately halved g_s when compared to the unlimed controls (Figure 4.7A). Applying superphosphate fertiliser partially restored g_s in limed plants by ~50%, but had no significant effect on controls, as signified by the Lime * P fert interaction (P=0.02). Similar effects were

noted with Pn (Figure 4.7B), where liming inhibited Pn by 32% compared to unlimed controls but superphosphate fertiliser restored Pn in the limed plants by 30% to near control levels. Again there was a significant Lime * P fert interaction ($p=0.046$) indicating that superphosphate fertiliser only enhanced Pn in the limed plants and not in the control group. Intercellular CO_2 concentration (Ci) was also reduced under liming by 12% (Figure 4.7C) when compared to controls and the application of superphosphate restored Ci in limed plants to control levels.

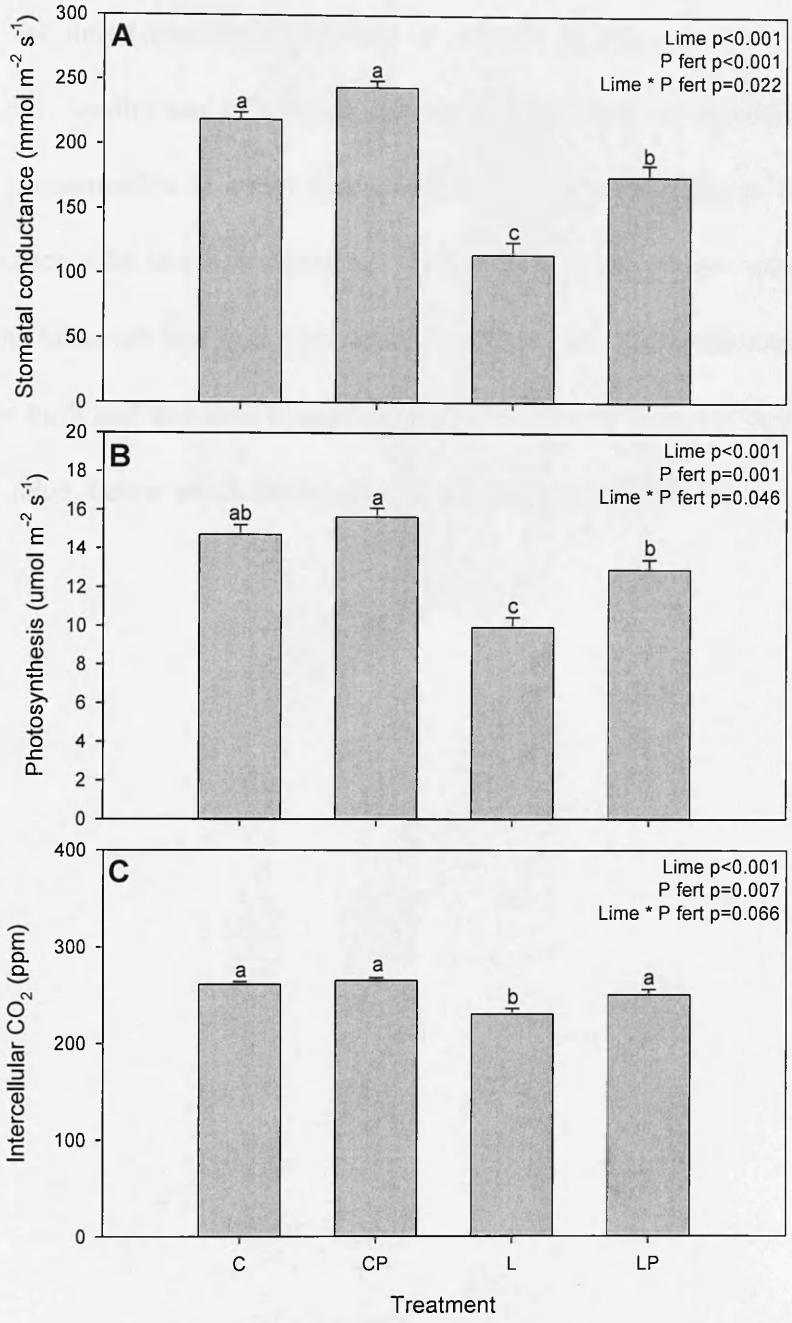

Figure 4.7: Stomatal conductance (panel A), photosynthesis (panel B) and intercellular CO_2 (panel C) data collected two days prior to harvest. Data are means ± SE of 8-10 replicates. Results of two-way ANOVA with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Liming increased leaf and root tissue ABA concentration by 31% and 62% respectively (Figure 4.8A, B). There was a significant Lime * P fert interaction with leaf tissue ABA,

indicating that superphosphate fertiliser restored leaf tissue ABA levels to control values in the limed treatment but had no significant effect in the control group (Figure 4.8A). Application of superphosphate fertiliser had no significant effect on root ABA concentration in either control or liming treatments though did appear to slightly reduce ABA concentrations by ~12% (Figure 4.8B). There were significant correlations between leaf and root tissue P status and ABA concentration (Figures 4.9A, B). In both leaf and root tissue a P concentration of ~2.5 g kg^{-1} appears to be a threshold value, below which tissue ABA values increase (Figure 4.9).

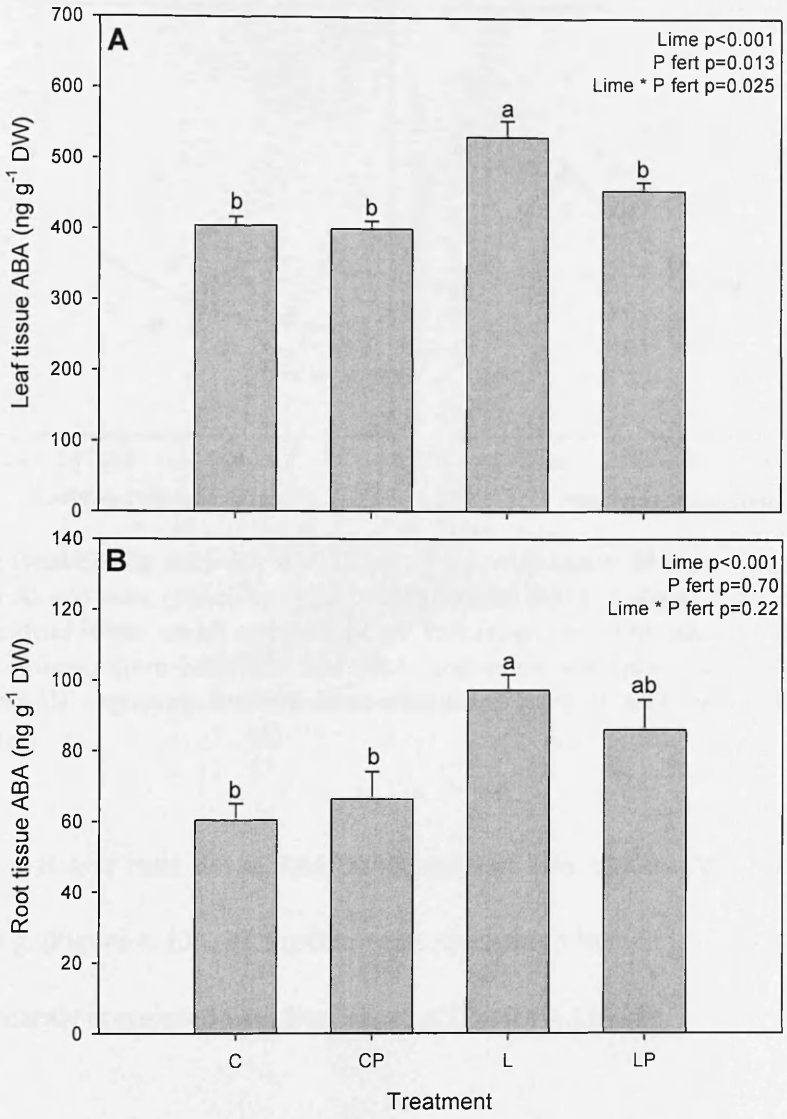

Figure 4.8: Leaf (panel A) and root (panel B) tissue ABA concentration. Data are means ± SE of 8-10 replicates. Results of two-way ANOVA with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

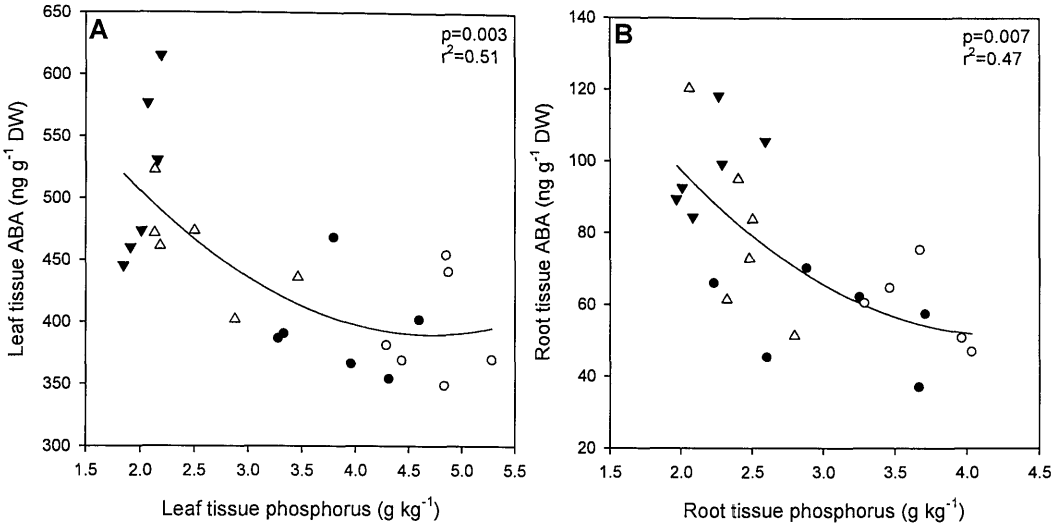

Figure 4.9: Relationship between leaf (panel A) and root (panel B) tissue phosphorous and leaf (panel A) and root (panel B) tissue ABA of limed (filled triangle), lime + P fert (open triangle), control (filled circle) and control + P fert (open circle) treated plants. Data points are paired samples from individual leaf (ABA) and whole leaf (phosphorus). Single second order polynomial regression lines are fitted where significant, P values and r^2 reported in top right of panel.

Increased leaf and root tissue ABA concentration was significantly correlated with decreased g_s (Figure 4.10A, B). Furthermore, decreased leaf and root P concentration was significantly correlated with decreased g_s (Figure 4.11A, B).

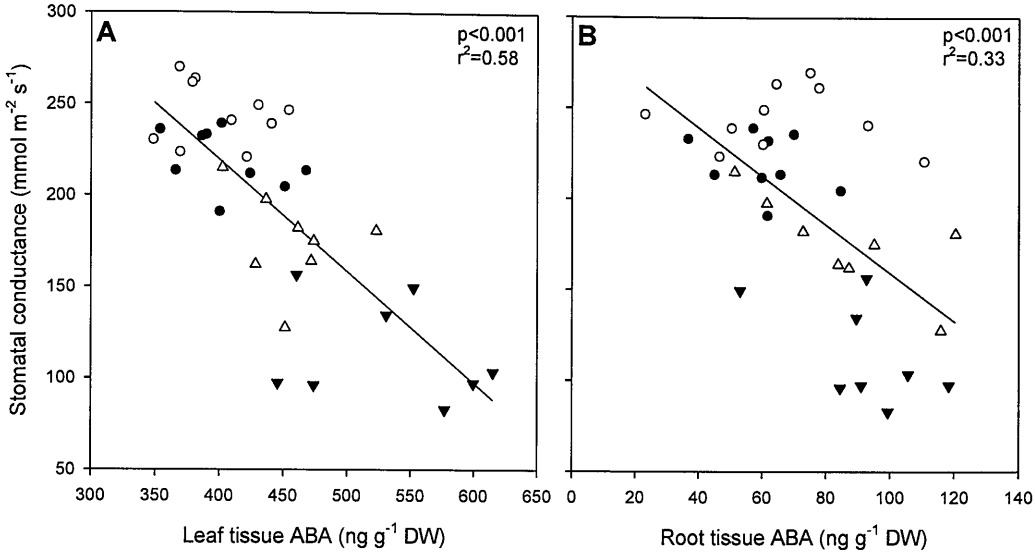

Figure 4.10: Relationship between leaf (panel A) and root (panel B) tissue ABA concentration and stomatal conductance (g_s) of limed (filled triangle), lime + P fert (open triangle), control (filled circle) and control + P fert (open circle) treated plants. Data points are paired samples of g_s and ABA taken from the same leaflet. Single linear regression lines are fitted where significant, P values and r^2 reported in top right of panel.

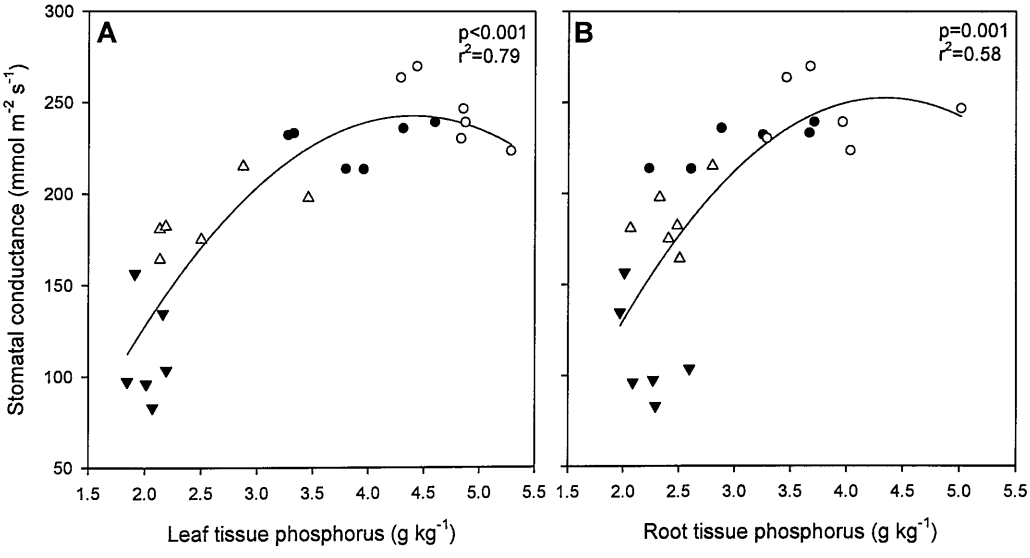

Figure 4.11: Relationship between leaf (panel A) and root (panel B) tissue phosphorous concentration and stomatal conductance (g_s) of limed (filled triangle), lime + P fert (open triangle), control (filled circle) and control + P fert (open circle) treated plants. Data points are paired samples taken from individual plants. Single second order polynomial regression lines fitted where significant, P values and r^2 reported in top right of panel.

Liming reduced Ψ_{leaf} by -0.27 MPa when compared to unlimed controls (Figure 4.12). Superphosphate fertiliser partially restored Ψ_{leaf} to control values by -0.14 MPa and had no effect in the unlimed control group, though there was no apparent Lime * P fert interaction. Leaf tissue ABA was significantly correlated (p=0.004) with Ψ_{leaf} (Figure 4.13A) though the relationship was quite weak (r^2=0.22). Similarly, a weak correlation between Ψ_{leaf} and g_s was also observed (Figure 4.13B).

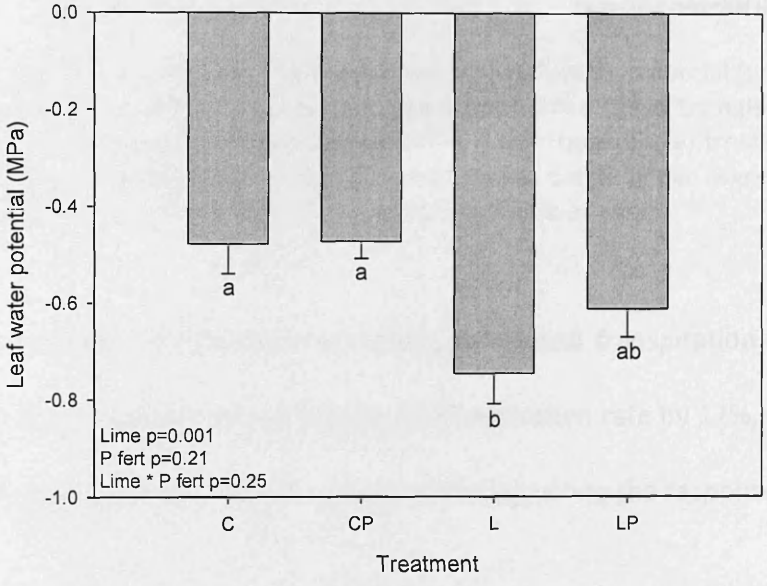

Figure 4.12: Leaf water potential data collected over two consecutive days. Data are means ± SE of 7-9 replicates. Results of two-way ANOVA with interaction are indicated in the bottom left of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

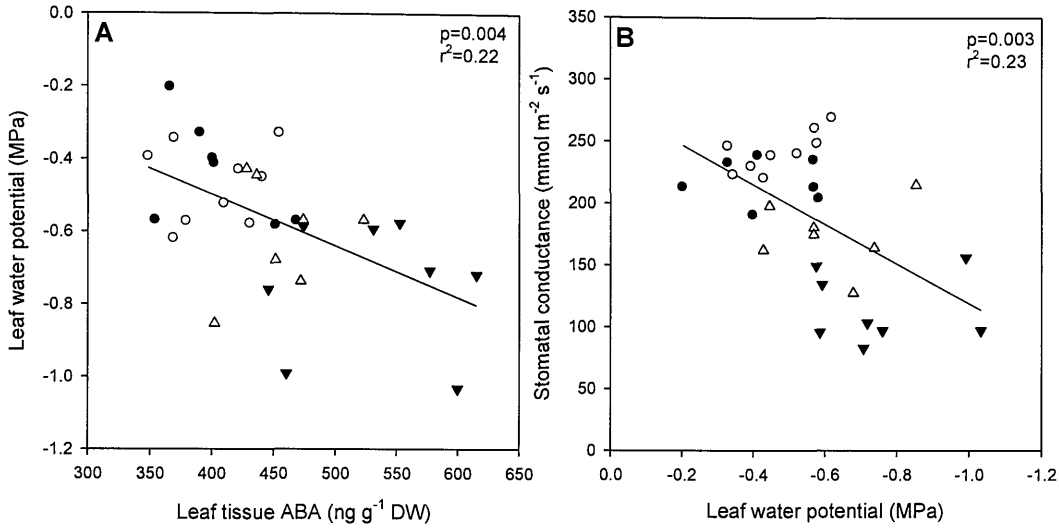

Figure 4.13: Relationship between leaf tissue ABA and leaf water potential (panel A) and leaf water potential and stomatal conductance (panel B) of limed (filled triangle), lime + P fert (open triangle), control (filled circle) and control + P fert (open circle) treated plants. Data points are paired samples taken from individual plants. Single linear regression lines are fitted where significant, P values and r^2 reported in top right of panel.

Supplying synthetic ABA to detached leaves decreased transpiration (Figure 4.14). ABA at a concentration of 10 nM decreased transpiration rate by 17%, while both 50 and 100 nM approximately halved transpiration suggesting the response saturated.

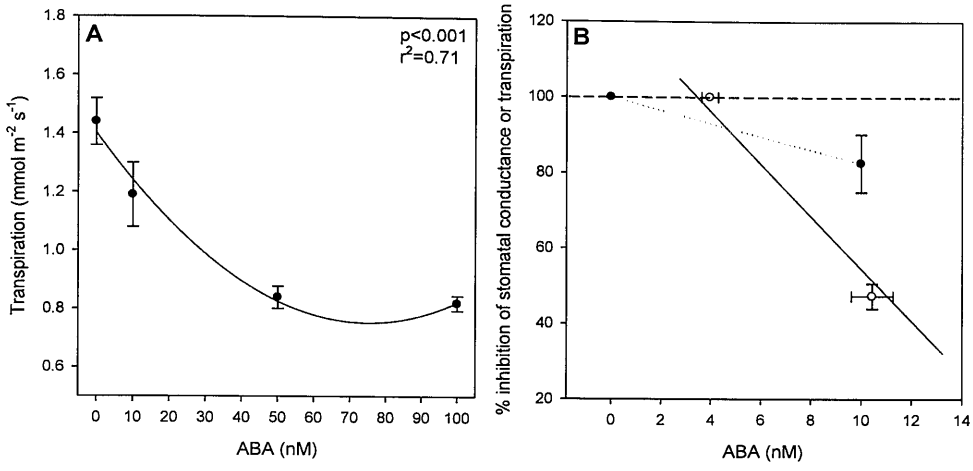

Figure 4.14: Transpiration rate (Tr) of detached *Pisum* leaflets fed artificial xylem sap containing ABA at 0, 10, 50 and 100 nM concentration (panel A) and % inhibition (panel B) by *in vivo* ABA of stomatal conductance (g_s) in limed plants compared to unlimed control (hollow circles) or Tr of detached leaflets fed artificial xylem sap containing 10 nM ABA (filled circles). The dashed line in panel B represents g_s of unlimed plants or Tr of 0 ABA treated leaflets. Data are means ± SE of 6-8 (panel A) or 5-8 (panel B) replicates. Second order polynomial regression line fitted in panel A with P value and r^2 reported in the top right.

4.4: Discussion

This chapter sought to further understand the mechanisms by which liming decreased gas exchange in *Pisum* and tested the hypotheses that increased root-to-shoot signalling of the plant hormone ABA, or alkalinisation of xylem sap pH were responsible for these observations.

Consistent with observations in Chapters 2 and 3, both g_s and Pn were reduced by ~50% and 32% respectively in limed plants when compared to controls (Figure 4.7). The application of superphosphate fertiliser partially restored this response and a significant Lime * P fert interaction indicated that increasing plant P status improved gas exchange in limed but not control plants (Figure 4.7). This apparent effect of plant P status on stomatal behaviour is consistent with other observations where P

deprived plants showed reduced g_s or transpiration (Nagarajah and Ratnasuriya, 1978, Jeschke et al., 1997, Davies et al., 1999). As root and leaf tissue P concentration was significantly correlated with g_s (Figure 4.11) this could suggest a direct effect of P deficiency as causing or acting as a signal for stomatal closure.

It has been proposed that changes in ionomic sap composition might act as root-to-shoot signals that influence stomatal behaviour (Bahrun et al., 2002). Increased root xylem sap sulphate concentration was thought to enhance the antitranspirant effect of ABA in *Zea mays* exposed to soil drying (Ernst et al., 2010). However, no correlative relationship was observed between a range of measured cation and anions (including phosphate) and g_s at the onset of soil drying (Gollan et al., 1992), suggesting that changes in xylem sap ion concentration are unlikely to directly regulate stomatal responses (Schurr et al., 1992). More generally, a deficiency of certain ions might be expected to directly influence stomatal behaviour, especially K (Fournier et al., 2005, Benlloch-Gonzalez et al., 2008) which is known to act as an osmoticum in the guard cells during stomatal opening and closure. Furthermore, a P deficiency might affect stomata via a decreased CO_2 fixation rate caused by impaired photochemical efficiency (for example, Jacob and Lawlor, 1991, Jacob and Lawlor, 1993) though in these experiments P is excluded from the plant, hence photosystem impairment may be expected and consequently Ci values increase. However, in the current experiment Ci values were not elevated under liming (Figure 4.7C) therefore it is likely that decreased photosynthetic rate is a reflection of down regulation of photosynthetic capacity in response to a stomatal limitation (Chaves et al., 2002).

It seems unlikely, therefore, that a direct effect of P deficiency on stomata will have caused the observed reduction in gas exchange under liming. Given the previously mentioned (see Introduction, this Chapter) commonality of stomatal responses to some ionomic deficiencies in well watered plants it seems more plausible that any signal affecting stomatal behaviour would be part of a centralised response (Chapin, 1990). Hence, the significant correlation between P and g_s (Figure 4.11) may simply reflect plant P status and may not be causative. A possible signal common to many nutrient stresses is the plant hormone ABA (Vysotskaya et al., 2008).

Liming increased ABA concentration in both root- and leaf-derived xylem sap by approximately two-fold to a mean of 10 nM (Table 4.1) and root and leaf tissue ABA by 62% and 31% respectively (Figure 4.8). This appears to be in response to the reduction in P availability cause by liming (Figure 4.9), further demonstrated by the significant ABA * P fert interaction (Figure 4.8A). Indeed, a root and leaf tissue P concentration ~2.5 mg g^{-1} appeared to be a threshold value below which tissue ABA concentrations increase. Similarly, a leaf P concentration of 1.6 mg g^{-1} compare to 3.7 mg g^{-1} in control plants gave a two-fold increase in leaf ABA concentration and six-fold increase in xylem sap ABA concentration in *R. communis* (Jeschke et al., 1997). Furthermore, a threshold tissue P value in the region of 2.5 mg g^{-1} might explain why low P supplied well watered *G. hirsutum* plants with a leaf P concentration of 2.3 mg g^{-1} did not show elevated ABA concentrations compared to control plants containing 6.8 mg g^{-1} (Radin, 1984). However, when exposed to soil drying the low P plants did show increased leaf ABA concentration compared to high P plants.

In the current study, both leaf and root ABA tissue concentration was significantly correlated with g_s (Figure 4.10). Furthermore, the transpiration rate of detached leaves fed an artificial xylem sap containing 10 nM ABA was reduced by 17% when compared to a sap with no ABA (Figure 4.14A) suggesting that the concentration of ABA in the root and leaf xylem sap of limed *Pisum* plants (Table 4.1) was sufficient to close stomata. However, the scale of inhibition observed *in vivo* with 10 nM ABA was much greater (50%) (Figure 4.14B) indicating a disparity between the effectiveness of ABA *in vivo* and that fed via the transpiration stream to detached leaves. This could be explained by an apparent increased sensitivity of P deficient leaves to ABA. The transpiration rate of detached *G. hirsutum* leaves fed 100 nM ABA was inhibited by an additional 35% in low P supplied compared to P sufficient leaves (Radin, 1984). Furthermore, stomatal sensitivity to ABA is increased at lower Ψ_{leaf} (Tardieu and Davies, 1992) and limed plants in the current experiment exhibited a Ψ_{leaf} -0.27 MPa lower than control plants (Figure 4.12). It is plausible therefore, that the endogenous levels of ABA measured in limed plants (10.4 ± 0.8 nM; Table 4.1) were high enough to elicit stomatal closure.

Although Ψ_{leaf} was reduced by 0.27 MPa under liming (Figure 4.12), it was weakly correlated with g_s (Figure 4.13B) in comparison to the much stronger relationship between g_s and leaf ABA (Figure 4.11A). Furthermore, there are other instances where g_s of *Pisum* plants is not closely linked to leaf water status. Flooded *Pisum* plants exhibited reduced g_s without any change in leaf water status (Zhang and Zhang, 1994). Soil drying increased Ψ_{leaf} by ~0.2 MPa, which was thought to be

caused by stomatal closure in *Pisum* (Belimov et al., 2009). Thus it seems unlikely that reduced Ψ_{leaf} of *Pisum* caused stomatal closure of limed plants.

It is apparent that ABA is increased in both root and leaf tissue (Figure 4.8) and root and leaf xylem sap (Table 4.1) in response to a lime-induced P deficiency and this may mediate stomatal behaviour. However, the source of the ABA and whether the ABA is acting as a root-to-shoot signal is uncertain. Synthesis of ABA, stimulated by changes in tissue water status, can occur in the root (Zhang and Davies, 1987), or leaf (Zeevaart and Creelman, 1988). Although leaf tissue ABA and Ψ_{leaf} were weakly correlated (Figure 4.13A), significant leaf synthesis of ABA is not thought to occur until leaf water status reaches zero turgor (Pierce and Raschke, 1981), therefore supplementary ABA delivered via the xylem would most likely be required to influence stomatal behaviour (Hartung et al., 2002). Flow modelling techniques to determine the synthesis, long distance transport and metabolism of ABA indicate that the root becomes a major site of ABA synthesis under P deficiency, contributing 82% of ABA exported in the xylem as opposed to being a moderate sink for ABA metabolism in P replete plants (Jeschke et al., 1997). In this experiment, newly synthesised ABA was loaded into the xylem and transported to the leaf, but foliar ABA concentration was similar to P replete plants, suggesting rapid ABA metabolism in the leaf mesophyll (Jeschke et al., 1997).

If root tissue is the most likely source of increased ABA synthesis under a lime induced P deficiency, an important question is why does this occur? Phosphorus deficiency is associated with decreased root hydraulic conductivity (Radin and Eidenbock, 1984), thought to be mediated by a decrease in abundance and/or

activity of aquaporins (Carvajal et al., 1996, Clarkson et al., 2000) which are important in regulating plant water status (Chaumont and Tyerman, 2014). Furthermore, accumulation of root tissue ABA increases linearly with decreasing root water potential (Simonneau et al., 1998). Thus, a probable mechanism is that increased root synthesis of ABA under P deficiency, and subsequent transport to the shoot as a xylem borne signal, is stimulated by an aquaporin mediated reduction of root water potential. In the current study, measurement of root water potential proved technically difficult owing to positive root pressures of de-topped plants using Scholander and whole-plant pressure chamber measurement approaches. Measurement of root water potential via psychrometry (for example Puertolas et al., 2013) coupled with measurements of root P and ABA xylem sap concentrations and ideally aquaporin expression would resolve this question.

Substantial evidence is required to convincingly establish the physiological significance of any plant hormone in a given response, according to Jackson (1993), who established several criteria for hormone action. **Correlation** between response and hormone concentration has been established *in vivo* (Figure 4.10), and **duplicated** in an isolated system (Figure 4.14). Further evidence can be established by seeking to manipulate the endogenous concentration of the hormone of interest by using plant mutants that are either impaired in their ability to synthesise the hormone of interest or are insensitive to its action (Jones et al., 1987, Nagel et al., 1994, Dodd, 2003b). Furthermore, reciprocal grafting of wild-type and mutant rootstocks and scions can help determine the source of the hormone of interest (Holbrook et al., 2002, Dodd et al., 2009). Experiments using ABA deficient legumes

are therefore required to fully establish the significance of ABA on physiological responses to liming and will be addressed in Chapter 5.

Xylem sap pH derived from either roots or leaves of *Pisum* grown in whole-plant pressure chambers was unchanged by liming (Table 4.1) despite a 0.7 unit increase in bulk soil pH (Table 4.2) implying effective regulation of sap pH in response to external pH changes. Maintenance of an apoplast/cytoplast pH gradient is important for cellular processes involving substrate exchange and hence close control of apoplastic pH is critical (Felle and Hanstein, 2002). Active proton pumping across plasma membranes is important in regulating both internal and external pH compartments (Raven, 1985). Furthermore, apoplastic sap has an intrinsic buffering capacity that will resist short term changes to pH (Felle, 1988, Oja et al., 1999). Additionally, metabolism of organic acids such as malate may also be important in long term regulation of pH (Felle, 2001). Although changes in xylem sap pH (and hence apoplastic pH) occur in response to environmental stresses and are established as an important signalling mechanism in adaption to those stresses (Wilkinson, 1999), in the current experiment the plants tightly regulated internal pH and prevented any changes due to increased external pH. As xylem sap pH was not affected by external soil pH, it seems unlikely that reduced gas exchange of limed plants is mediated by xylem sap alkalinisation.

In conclusion the evidence established in this chapter suggests that reduced gas exchange may be mediated by the plant hormone ABA in response to a lime-induced P deficiency. However, to fully ascertain the physiological role of ABA in the

response, experiments that alter the endogenous levels of ABA are required. This will be addressed in the next chapter using the ABA deficient mutant pea '*wilty*'.

Chapter 5 – Lime-induced ABA accumulation restricts leaf gas exchange, but is required to maintain root growth of pea: Evidence from the ABA-deficient *wilty* pea mutant

5.1 Introduction

Chapter 4 proposed that reduced gas exchange of peas grown in limed soil was mediated by the antitranspirant plant hormone ABA in response to a lime-induced P deficit, as evidenced by significant correlations between both leaf and root tissue P and ABA concentrations (Figure 4.9A, B) and leaf tissue ABA and g_s (Figure 4.10). Furthermore, the putative effect of increased liming-induced xylem sap ABA concentration (Table 4.1) on stomata could be duplicated by physiologically relevant ABA concentrations in a detached leaf transpiration assay (Figure 4.14 and discussed in Section 4.4). However, to convincingly establish the physiological significance of any plant hormone, further experimental evidence may be sought by determining the physiological responses of plants in experiments that delete (in as far as is possible) the levels or action of that hormone (Jackson, 1993). As discussed earlier in Chapter 4, this can be demonstrated with the use of hormone-deficient or -insensitive mutants (Dodd, 2005).

The ABA-deficient tomato mutant *flacca* is typified by higher g_s than wild-type plants and an inability to close stomata at night (Tal, 1966) or in response to water stress (Neill and Horgan, 1985). Partial restoration of these phenotypes by exogenous ABA indicates the relevance of ABA in regulating stomatal behaviour (Imber and Tal, 1970). Furthermore, ABA-deficient mutants demonstrated the importance of

endogenous ABA in preventing xylem alkalisation from closing stomata (Wilkinson et al., 1998). However, some ABA-deficient mutants retain the capacity to respond to some environmental stresses, as nitrate deprivation induced similar stomatal closure of the ABA-deficient *wilty* pea mutant and its wild-type (Dodd, 2003a), indicating that ABA may not regulate g_s in response to this stress.

Similarly, ABA-deficient mutants are useful in elucidating the role of ABA in different growth processes. Experiments with ABA-deficient mutants demonstrated the requirement for endogenous ABA to maintain shoot growth in well watered *Lycopersicon* (Sharp et al., 2000) and in *Hordeum* subject to soil compaction (Mulholland et al., 1996a, Mulholland et al., 1996b). Furthermore, *Zea mays* mutants deficient in ABA revealed that increased endogenous ABA production is required to maintain root growth at low water potentials (Saab et al., 1990, Sharp et al., 1994).

While mutants are clearly a useful experimental tool, their use is not without caveats. Firstly, mutants deficient in ABA will often display altered endogenous levels of other phytohormones, in particular increased ethylene concentration (Sharp et al., 2000, Dodd et al., 2009), which may confound interpretation of specific effects of ABA on shoot behaviour. Furthermore, even in reciprocal grafting studies, the translocation of leaf sourced ABA via the phloem and subsequent xylem re-circulation and can make comparison of the relative contribution of root- or shoot-sourced ABA difficult (Holbrook et al., 2002). Finally, ABA-deficient mutants show decreased Ψ_{leaf} and turgor (Cornish and Zeevaart, 1988, Fambrini et al., 1994) that may confound any treatment or hormone effects or interactions and make interpretation difficult. The latter point can be addressed in experimental systems

that moderate the genotypic effect on leaf water status. Approaches to help maintain Ψ_{leaf} include growing plants in root pressure chambers where appropriate pneumatic pressure can be applied to the roots to keep the leaves at full turgor (Termaat et al., 1985, Dodd et al., 2002). Alternatively, plants can be grown in a high humidity environment which can be achieved through shoot misting (Dodd et al., 2009) or by growing plants in controlled environment chambers (Sharp et al., 2000).

The ABA-deficient pea mutant *wilty* (*Pisum sativum* L.) has been used previously to assess the role of ABA in stomatal (Dodd, 2003a) and growth (Dodd, 2003b) responses to nitrate deficiency. Although the exact mutation of *wilty* has yet to be fully elucidated (Taylor et al., 2000) it is known to be impaired in the early stages of ABA biosynthesis (Duckham et al., 1989). Although stomata of *wilty* show similar sensitivity to ABA (Donkin et al., 1983), the mutation is characterised by reduced foliar ABA concentrations under both well watered and water stressed conditions and thus an impaired ability to regulate water loss from the leaves and maintain leaf water status (Wang et al., 1984). This impaired ability to synthesise ABA makes the *wilty* pea a suitable tool to assess the physiological significance of lime-induced ABA accumulation on both the reduced gas exchange and shoot growth observed.

Phosphorus deficiency impairs leaf area development (for example, Rao and Terry, 1989, Rodriguez et al., 1998a), possibly mediated by reduced hydraulic conductance of the root system and a subsequent decreased leaf water status and hence cell turgor that is required for cell expansion (Radin and Eidenbock, 1984). Perturbation of root hydraulic conductance is thought to occur through lowering of aquaporin expression and activity in response to nutrient deprivation (Clarkson et al., 2000).

However, in some circumstances, increased ABA synthesis in response to external environmental stresses is thought to limit leaf expansion (Dodd et al., 1996, Tardieu et al., 2010). Additionally, ABA is thought to improve plant adaptation to low water availability by differentially affecting shoot and root growth and thus increasing root to shoot ratio (Saab et al., 1990). Similarly, plants experiencing P deficiency typically maintain root growth while shoot growth is inhibited (Fredeen et al., 1989, Cakmak et al., 1994), an important adaption for enhanced P acquisition in P limited environments (Nielsen et al., 2001). If ABA synthesis is increased in limed plants (Figure 4.8) it may offer an explanation for the previously observed inhibition of shoot growth (Figure 4.3).

To assess the potential roles of ABA and leaf water status on leaf area development in limed *Pisum* an experimental approach is required where Ψ_{leaf} can be moderated regardless of any treatment or genotypic effect. Therefore, in addition to glasshouse trials, this chapter raised WT and ABA-deficient plants in controlled environment growth chambers at high and low relative humidity's (as in Sharp et al., 2000) in lime/superphosphate factorial experiments to test the following hypotheses:

- The ABA-deficient *wilty* pea will exhibit an attenuated gas exchange response to lime-induced P deficiency.
- The ABA-deficient *wilty* pea will show improved leaf growth under a lime-induced P deficiency.
- Reduced leaf growth under liming is mediated by reduced leaf water status.

5.2 Materials and methods

5.2.1 Screening different pea genotypes for their stomatal responses

5.2.1.1 Plant material

Near isogenic seeds of the ABA-deficient pea (*Pisum sativum* L.) *wilty*, its wild-type (WT) (De Bruijn et al., 1993), Alderman (Ald), Progress No. 9 (Prog) and Hurst Greenshaft (Hurst) were pre-germinated on dampened tissue paper and kept in the dark. Alderman was chosen as it was used in previous experiments (Chapters 2 and 3) and both Progress No. 9 and Hurst Greenshaft were selected as two commonly used commercial *Pisum* genotypes. On emergence of the radicle (2-3 days), uniformly germinated seeds were individually transplanted into 0.8 litre pots (110 mm high, 120 mm diameter) containing the same control or limed (3 g l^{-1}) sterilised 2:1 (v:v) mixture of a low pH sandy loam field soil and horticultural grit sand as described in Section 2.2.3.1. All plants were well watered by replacing evapotranspirational losses (determined gravimetrically) daily and maintained in a semi-controlled naturally lit greenhouse with supplementary lighting (supplied by Osram 600 w daylight bulbs) for 12 hours and 22°C/16°C minimum day/night temperature at the Lancaster Environment Centre.

5.2.1.2 Stomatal conductance

Stomatal conductance was recorded between 11:00 am and 13:00 pm two days prior to harvest on the abaxial leaf surface on both leaflets of leaf 4 (youngest fully expanded leaf) using an AP4 porometer (Delta-T Devices, Cambridge, UK). Mean data from both leaflets was taken as the g_s for the individual plant.

5.2.2 Wilty pea liming/superphosphate experiment

5.2.2.1 Plant material

Seeds of *wilty* and WT were established and maintained as above though half of the pots received an additional treatment of superphosphate fertiliser (J. Arthur Bowers Ltd., William Sinclair Horticulture Ltd, Lincoln, UK) at a rate of 0.59 g l^{-1} (to represent a 200 kg ha^{-1} application rate) as described in Section 4.2.1.

5.2.2.2 Stomatal conductance

Stomatal conductance was measured on the abaxial leaf surface on both leaflets of leaf 4 (youngest fully expanded leaf) using an AP4 porometer as described in Section 5.2.1.2 above.

5.2.2.3 Leaf water potential

The Ψ_{leaf} of one leaf of leaflet pair four was measured on three occasions in the week prior to harvest by thermocouple psychrometry as described in Section 4.2.4.

5.2.2.4 Plant tissue analysis

Plant tissue nutrient analysis was undertaken using the same procedures as described in Section 2.2.2.3. To provide enough sample material, tissue nutrient analysis utilised homogenised oven dried samples of all leaves present at time of harvest and root tissue that had been washed of adhering soil particles and rinsed in deionised water prior to drying.

5.2.2.5 ABA radioimmunoassay (RIA)

Leaf tissue ABA concentration was sampled on leaflet pair four, the same leaf sampled as g_s, and root tissue ABA from a representative sample of washed roots. Both were determined by competitive radioimmunoassay (RIA) (Quarrie et al., 1988) as described in Section 4.2.5.

5.2.2.6 Plant harvest

Shoot fresh weight was recorded at time of harvest, along with leaf area of individual leaves and stipules at each node using a leaf area meter (Li-3050A, Li-Cor, Lincoln, Nebraska, USA). Roots were collected, washed of soil and checked for nodulation. Shoot and root material was then dried at 80°C for one week to record dry weight.

5.2.3 Wilty pea high/low humidity experiment

5.2.3.1 Plant material and growth conditions

A second set of ABA-deficient *wilty* pea and WT, established as above, only received the liming treatment and were grown in two separate controlled environment growth chambers (Snijder Microclima 1750, Snijder Scientific, Tilburg The Netherlands). Both chambers were set for a 12 hour photoperiod with 24°C/20°C day/night temperature with Phillips daylight and red/far red fluorescent bulbs supplying ~300 µmol PAR at canopy height. One chamber had a relative humidity set at 50% to replicate typical greenhouse conditions, the second chamber had a high relative humidity of 90/95% day/night to moderate any effect of liming and/or plant

ABA status on Ψ_{leaf} (Figure 5.1). This generated a mean vapour pressure deficit (VPD) values of 1.45 (±0.02) kPA and 1.15 (±0.01) kPA for day and night in the 50% humidity chamber and 0.33 (±0.01) kPA and 0.12 (±0.00) kPA in the 90/95% humidity chamber. All plants were maintained well watered by replacing daily evapotranspiration (determined gravimetrically).

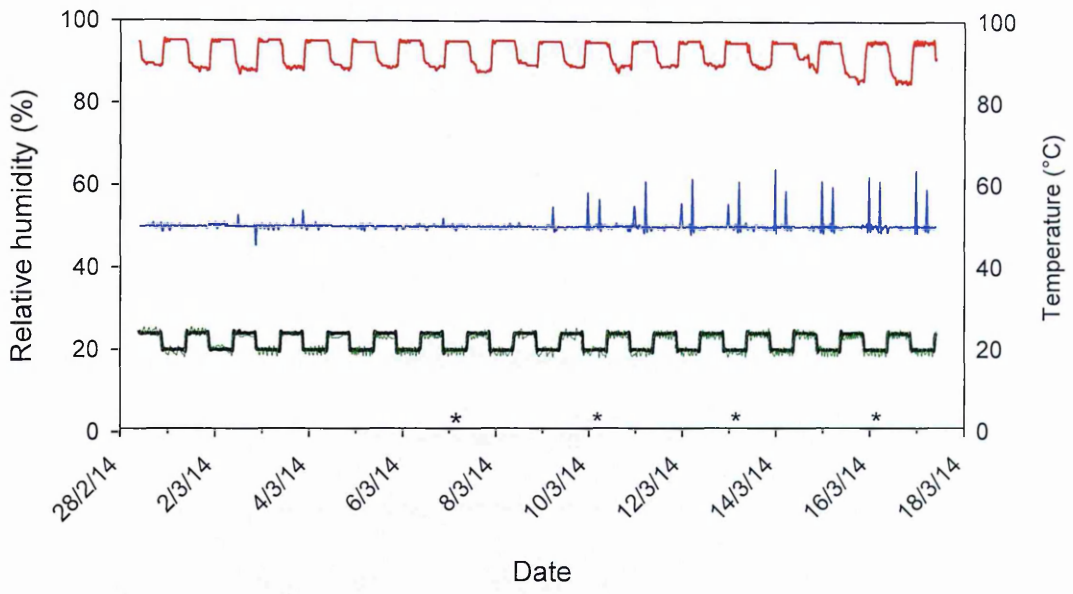

Figure 5.1: Relative humidity (RH) and temperature (T) of growth chambers recorded every 30 minutes during experimental period of Section 5.2.3. Red (RH) and black (T) lines represent high humidity chamber, blue (RH) and green (T) line represent low humidity chamber. * indicates days when pre-dawn and mid-day leaf water potential were recorded. Plants were transferred to the chambers on 28/2/14.

5.2.3.2 Leaf water potential

Leaf water potential was measured on 4 occasions (as indicated in Figure 5.1) both 1 hour before the photoperiod began (pre-dawn) and in the middle of the photoperiod (mid-day) by thermocouple psychrometry as described in Section 4.2.4. Samples for

Ψ_{leaf} were taken initially from leaf 3 and then leaf 4 (counting from the base) as the plants developed.

5.2.3.3 Plant harvest

Shoot fresh weight was recorded at time of harvest, along with leaf area of individual leaves and stipules at each node using a leaf area meter (Li-3050A, Li-Cor, Lincoln, Nebraska, USA). Roots were collected, washed of soil and checked for nodulation. Shoot and root material was then dried at 80°C for one week to record dry weight.

5.2.4 Statistical analysis

Genotype and liming effects were determined by two-way ANOVA in Figure 5.2. Three-way ANOVA with Tukey pair-wise and interactions was used to determine liming, superphosphate and genotype effects in Tables 5.1-5.4 and Figures 5.3, 5.5 and 5.9. One-way ANOVA determined treatment effect on leaf initiation in Table 5.5 and a students *t*-test was used to compare leaflet areas in Figure 5.6. and relative effects of genotype in Figure 5.15. General Regression Model with interaction was used to determine treatment effects in Figures 5.4, 5.7, 5.8 and 5.10. Separate regression lines were fitted where genotype or interaction was significant, and a single line fitted when only x axis effect was significant. Four-way ANOVA with Tukey pair-wise analysis determined lime, genotype, humidity and sampling time effects in Figure 5.11 and three-way ANOVA with Tukey pair-wise analysis determined liming, genotype and humidity effects with interaction in Figures 5.12 and 5.13. All analysis used Minitab v16.

5.3 Results

5.3.1 *Pisum* Genotype screen

The mean g_s of control (unlimed) *wilty* was approximately two-fold that of WT, Alderman and Progress No. 9 and ~25% higher than Hurst Greenshaft which exhibited a higher control g_s than other genotypes that were considered to have normal ABA biosynthesis (Figure 5.2). When limed, all the *Pisum* genotypes used (including WT) exhibited a reduced g_s of between 26% and 50%. However, *wilty* did not show reduced g_s as highlighted by the significant Lime * Genotype interaction (P=0.02) (Figure 5.2).

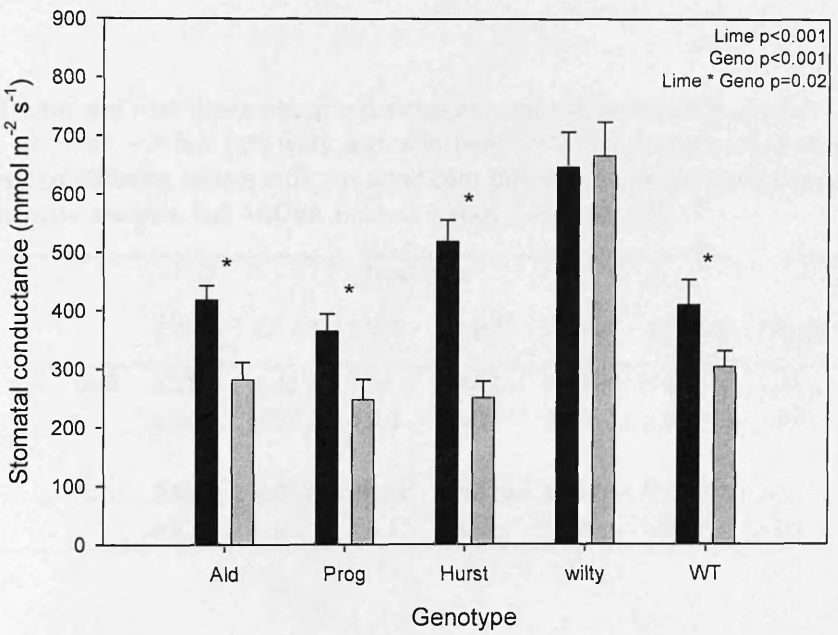

Figure 5.2: Stomatal conductance of five genotypes of limed (grey bars) or unlimed control (black bars) *Pisum* recorded between 11:00 am and 13:00 pm. Data are means ± SE of 7-8 replicates, * indicates significant difference (P<0.05) between treatments, results of two-way ANOVA with interaction are shown in the top right of the panel.

5.3.2 Wilty pea liming/superphosphate experiment

Liming reduced root and leaf tissue P concentration in both WT and *wilty* plants by ~40% when compare to the unlimed controls (Table 5.1). The addition of superphosphate fertiliser increased root tissue P by ~70% in the controls and ~15% in the limed treatment in both the WT and *wilty* plants respectively (Table 5.1). Leaf tissue P concentration increased by ~35% in both the control and limed WT plants with the addition of superphosphate and by 47% and 60% in the control and limed *wilty* plants respectively (Table 5.1). However, there was no significant P fert * Genotype interaction (P=0.08) (Table 5.2), suggesting that leaf tissue P concentration of both genotypes responded similarly to the addition of superphosphate.

Table 5.1: Leaf and root tissue phosphorus concentration in control (C), control + P fert (CP), limed (L) or limed + P fert (LP) *wilty* and wild-type (WT) *Pisum.* Data are means ± SE of 5 replicates and differing letters indicate significant differences within each row according to Tukey pair-wise analysis. Full ANOVA analysis is shown in Table 5.2.

		Treatment							
		C WT	CP WT	L WT	LP WT	C wilty	CP wilty	L wilty	LP wilty
Phosphorus ($mg\ g^{-1}$)	Leaf	3.31 b ± 0.2	4.43 a ± 0.1	1.98 d ± 0.1	2.68 c ± 0.1	3.12 bc ± 0.1	4.6 a ± 0.2	1.72 d ± 0.1	2.73 c ± 0.1
	Root	3.55 bc ± 0.2	6.01 a ± 0.1	2.25 d ± 0.1	2.63 cd ± 0.1	3.57 b ± 0.1	6.27 a ± 0.4	2.16 d ± 0.1	2.37 d ± 0.2

Table 5.2: Three-way ANOVA for leaf and root tissue phosphorus data shown in Table 5.1. P values are presented for each main effect or interaction.

Treatment or interaction	Leaf tissue phosphorus	Root tissue phosphorus
Lime	<0.001	<0.001
P fert	<0.001	<0.001
Genotype	0.53	0.98
Lime * P fert	0.02	<0.001
Lime * Genotype	0.62	0.24
P fert * Genotype	0.08	0.82
Lime * P fert * Genotype	0.89	0.42

Lime reduced g_s in limed WT plants by 27% compared to unlimed controls, though this effect was attenuated in the *wilty* plants as demonstrated by the significant Lime * Genotype interaction (P=0.01) (Figure 5.3). However, limed *wilty* still showed a small (though non-significant) reduction in g_s compared to unlimed controls. Applying superphosphate fertiliser to limed plants restored the g_s of both genotypes but had no significant effect on g_s in unlimed plants, as demonstrated by the significant Lime * P fert interaction (P=0.035).

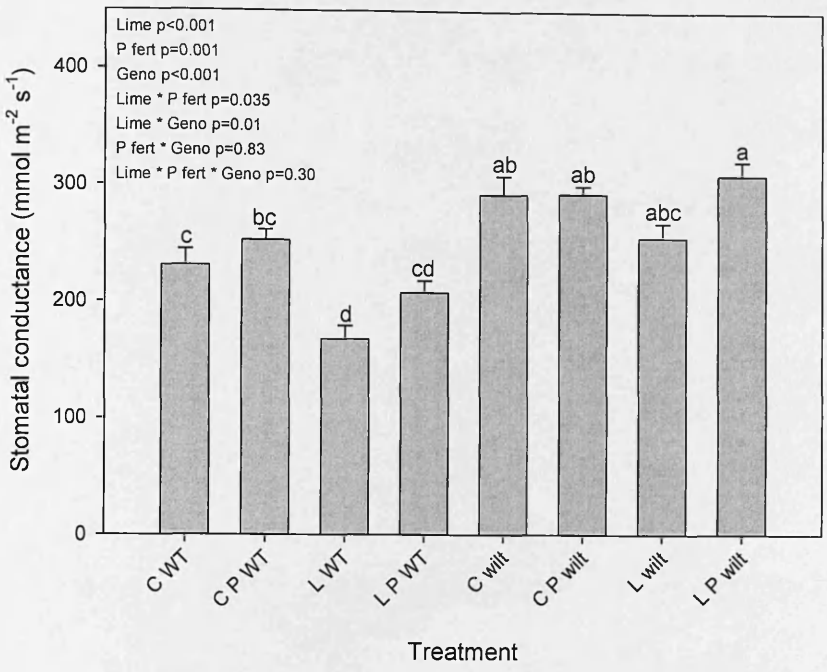

Figure 5.3: Stomatal conductance of control (C), control + P fert (CP), limed (L) or limed + P fert (LP) *wilty* (wilt) and wild-type (WT) *Pisum.* Data are means ± SE of 5-8 replicates. Results of three-way ANOVA with interaction are indicated in the top left of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Combined across all treatments, ANOVA demonstrated that leaf ABA concentration of *wilty* was ~20% less than its wild-type. Although leaf tissue ABA concentration of *wilty* was 13% less than WT in the unlimed controls (Table 5.3), this result was not statistically significant (Table 5.4). Liming increased leaf tissue ABA by 33% and 31% in WT and *wilty* respectively when compared to controls. This effect was partly attenuated by applying superphosphate fertiliser, more noticeably in the *wilty* plants (Table 5.1) although there was no significant P fert * Genotype interaction (Table 5.4). There appeared to be no significant treatment or genotype effect on root tissue ABA concentration despite *wilty* having ~30% less ABA than the WT in control plants (Table 5.4).

Table 5.3: Leaf and root tissue ABA concentration in control (C), control + P fert (CP), limed (L) or limed + P fert (LP) *wilty* and wild-type (WT) *Pisum.* Data are means ± SE of 5 replicates. Data are means ± SE of 5-8 replicates and differing letters indicate significant differences within each row according to Tukey pair-wise analysis. Full ANOVA analysis is shown in Table 5.4.

		Treatment							
		C WT	CP WT	L WT	LP WT	C wilty	CP wilty	L wilty	LP wilty
ABA ($ng\ g^{-1}$ DW)	Leaf	81.0 bc ± 4.3	73.4 c ± 3.5	108.3 a ± 7.3	95.8 ab ± 4.0	70.2 bc ± 5.7	67 bc ± 6.0	92.7 ab ± 7.0	69.3 bc ± 10.0
	Root	37.0 a ± 4.1	32.7 a ± 3.9	31.2 a ± 3.3	34.9 a ± 1.7	25.2 a ± 5.0	29.8 a ± 3.4	29.3 a ± 4.7	17.6 a ± 15.0

Table 5.4: Three-way ANOVA for leaf and root tissue ABA data shown in Table 5.3. P values are presented for each main effect or interaction.

Treatment or interaction	Leaf tissue ABA	Root tissue ABA
Lime	<0.001	0.86
P fert	0.007	0.65
Geno	0.001	0.20
Lime * P fert	0.14	0.68
Lime * Geno	0.14	0.51
P fert * Geno	0.70	0.60
Lime * P fert * Geno	0.76	0.51

Leaf tissue P concentration was correlated with g_s in both WT and *wilty* but a significant Leaf P * Genotype interaction (P=0.043) indicates that this relationship differed between genotypes (Figure 5.4B). WT had a linear relationship between leaf tissue P concentration and g_s whereas *wilty* showed a threshold response with only a slight reduction in g_s at leaf tissue P concentration <0.25 mg g^{-1}. Leaf tissue P concentration was correlated with leaf tissue ABA concentration in both WT and *wilty* though *wilty* had lower leaf ABA concentrations overall (Figure 5.4A). Leaf tissue ABA concentration was correlated with g_s in both genotypes (Table 5.4C).

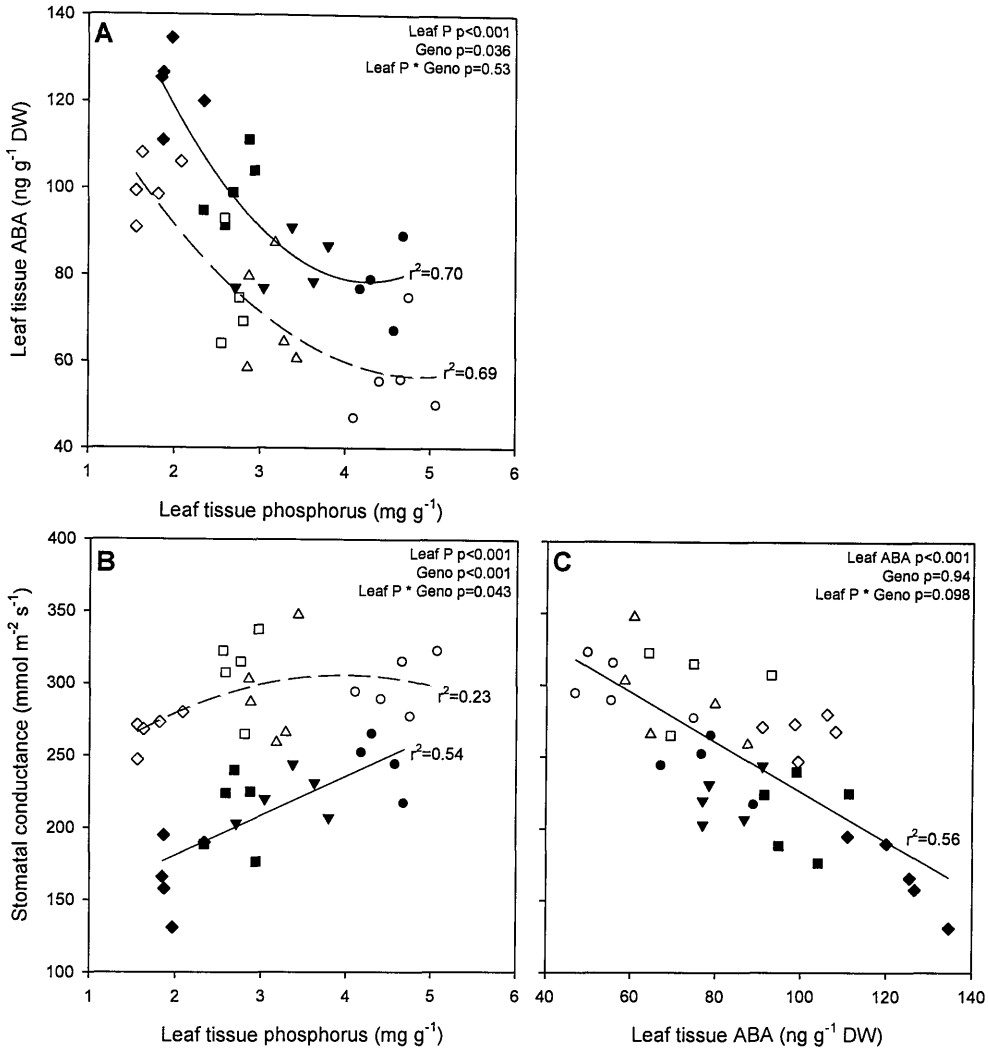

Figure 5.4: Relationship between leaf tissue phosphorus and leaf tissue ABA concentration (panel A) and stomatal conductance (g_s) (panel B) and leaf tissue ABA and g_s (panel C) in wild-type (WT) (filled symbols) or *wilty* (open symbols) *Pisum*. Treatments are control (triangles), control + P fert (circles), lime (diamonds) and lime + P fert (squares). Results of General Regression Model with interaction are shown in top right of each panel. Separate second order polynomial regression lines fitted in panels A and B (solid line WT, dashed line *wilty*), single linear regression line fitted in panel C, r^2 values are shown within each panel.

Liming reduced total leaf area (leaflets and stipules) by ~40% regardless of genotype (Figure 5.5A) and superphosphate fertiliser partially restored leaf area in both WT and *wilty*. Superphosphate also increased leaf area in the control treatments by ~30% in both WT and *wilty*. A significant Lime * P fert interaction (P=0.02) suggests

that the effect of superphosphate on leaf area was more noticeable in unlimed controls. The *wilty* genotype had a lower total leaf area across all treatments. Liming reduced leaf initiation by one node in both WT and *wilty* though *wilty* typically had an additional leaf at time of harvest (Table 5.5). Lime induced inhibition of fully expanded leaflet area was apparent from nodes 1-6 in WT with the exception of node 4 (Figure 5.6A) and in *wilty* from nodes 4-6 (Figure 5.6B).

Liming reduced dry shoot biomass by ~40% in both the WT and *wilty* genotypes when compared to unlimed controls and this response was partially restored by the application of superphosphate fertiliser (Figure 5.5B). Superphosphate also increased dry shoot biomass in the WT and *wilty* control treatment by ~30% suggesting that the unlimed plants were P limited. There was no significant genotype effect on shoot dry weight.

Effects of liming on root dry weight were less noticeable (Figure 5.5C). Liming only reduced root dry weight of the WT plants by 7% but the *wilty* plants showed a 17% reduction compared to unlimed controls. This induced a significant Lime * Genotype interaction (P=0.008) on root:shoot ratio (Figure 5.7D). The ratio of root to shoot dry weight was increased by 60% by liming in the WT plants but only by 32% in *wilty*. Superphosphate fertiliser reduced root:shoot ratios in both control and liming treatments in both genotypes, but this response was most prevalent in the liming treatment as indicated by a significant Lime * P fert interaction (P=0.004).

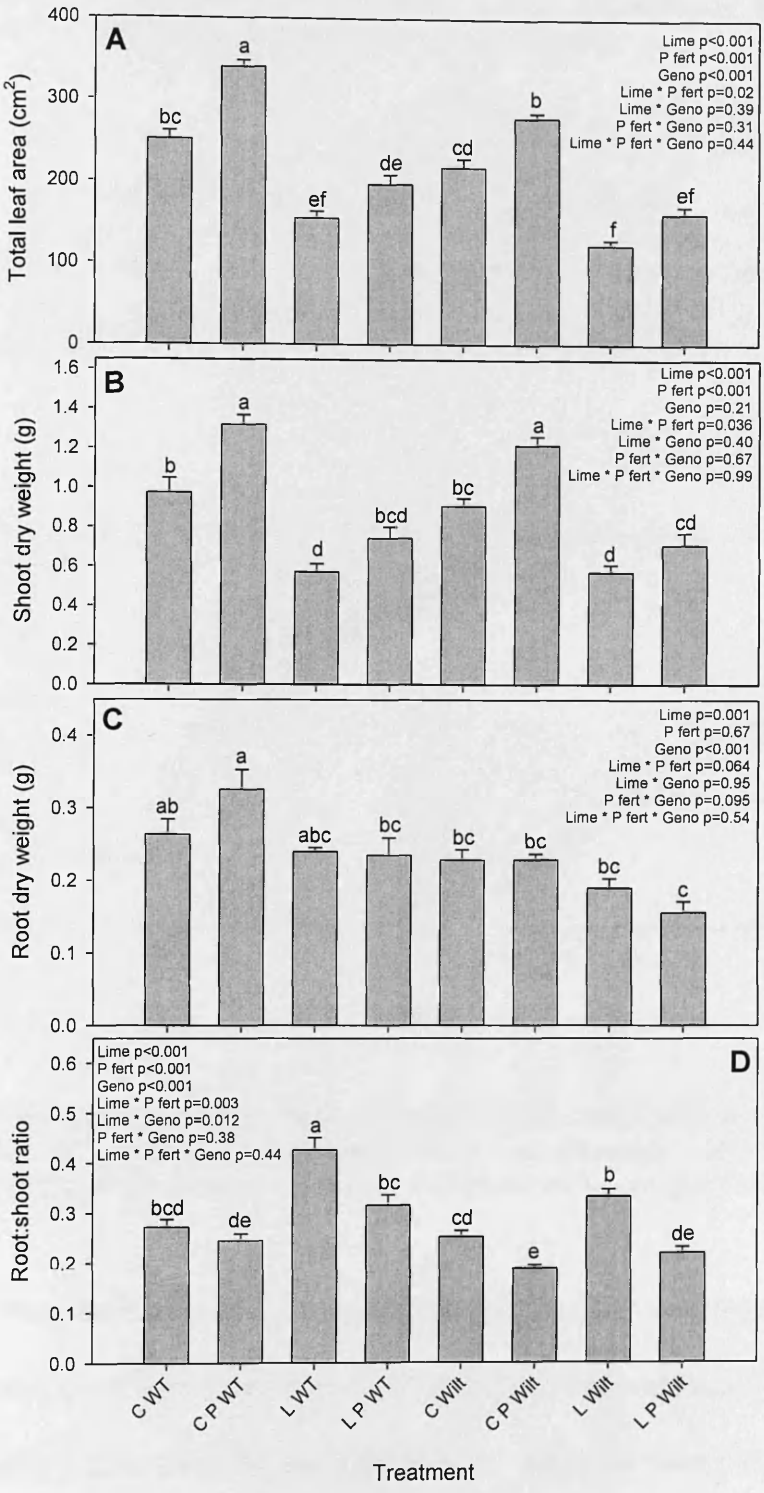

Figure 5.5: Total leaf area (panel A), shoot (panel B) and root (panel C) dry weight and root:shoot ratio (panel D) of control (C), control + P fert (CP), limed (L) or limed + P fert (LP) *wilty* (wilt) and wild-type (WT) *Pisum*. Data are means ± SE of 5-8 replicates. Results of three-way ANOVA with interaction are indicated in the top right (panels A, B, C) or left (panel D) of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Table 5.5: Leaf initiation in control (C), control + P fert (CP), limed (L) or limed + P fert (LP) *wilty* and wild-type (WT) *Pisum*. Data are means ± SE of 5-8 replicates, differing letters indicate significant differences as determined by one-way ANOVA.

	Treatment							
	C WT	CP WT	L WT	LP WT	C wilty	CP wilty	L wilty	LP wilty
Leaf no.	7.9 ab ±0.1	8.0 ab ±0.0	7.2 b ±0.2	7.6 ab ±0.2	8.4 a ±0.2	8.8 a ±0.2	7.6 ab ±0.4	7.6 ab ±0.7

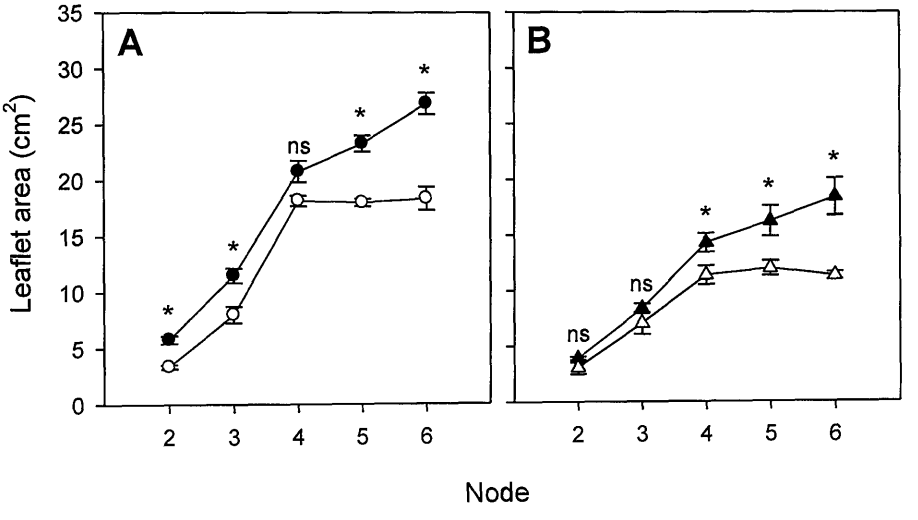

Figure 5.6: Fully expanded leaflet area at different nodes of control (filled symbols) or limed (open symbols) WT (panel A) or *wilty* (panel B) *Pisum*. Data are means ± SE of 5-8 replicates, * indicates significant differences at each node as determined by a students *t*-test.

Leaf tissue P concentration was correlated with both total leaf area (Figure 5.7A) and dry shoot biomass (Figure 5.7B) in both WT and *wilty*, however, significant Leaf P * Genotype interactions (P=0.015 leaf area, P=0.037 shoot dry biomass) suggest that the relationship between leaf P and growth was different between the genotypes. This was most apparent at the higher leaf P concentrations where WT performed better than *wilty* with this difference becoming less apparent at low leaf P

concentrations. Root tissue P was correlated with root dry biomass in both WT and *wilty* but again a significant Root P * Genotype interaction (P=0.007) shows that WT produced more root biomass at higher root tissue P concentrations than *wilty*. Both root and leaf tissue P concentration was correlated with root:shoot ratio in WT and *wilty* (Figure 5.7C, E). Root:shoot ratios were lower in *wilty* than WT across the full range of root and leaf tissue P concentrations though the relationship between root and leaf P and root:shoot ratio was the same for both genotypes as demonstrated by the non-significant interactions (P=0.21 leaf P, P=0.46 root P). Leaf tissue ABA concentration was correlated with both total leaf area and shoot dry weight (Figure 5.8).

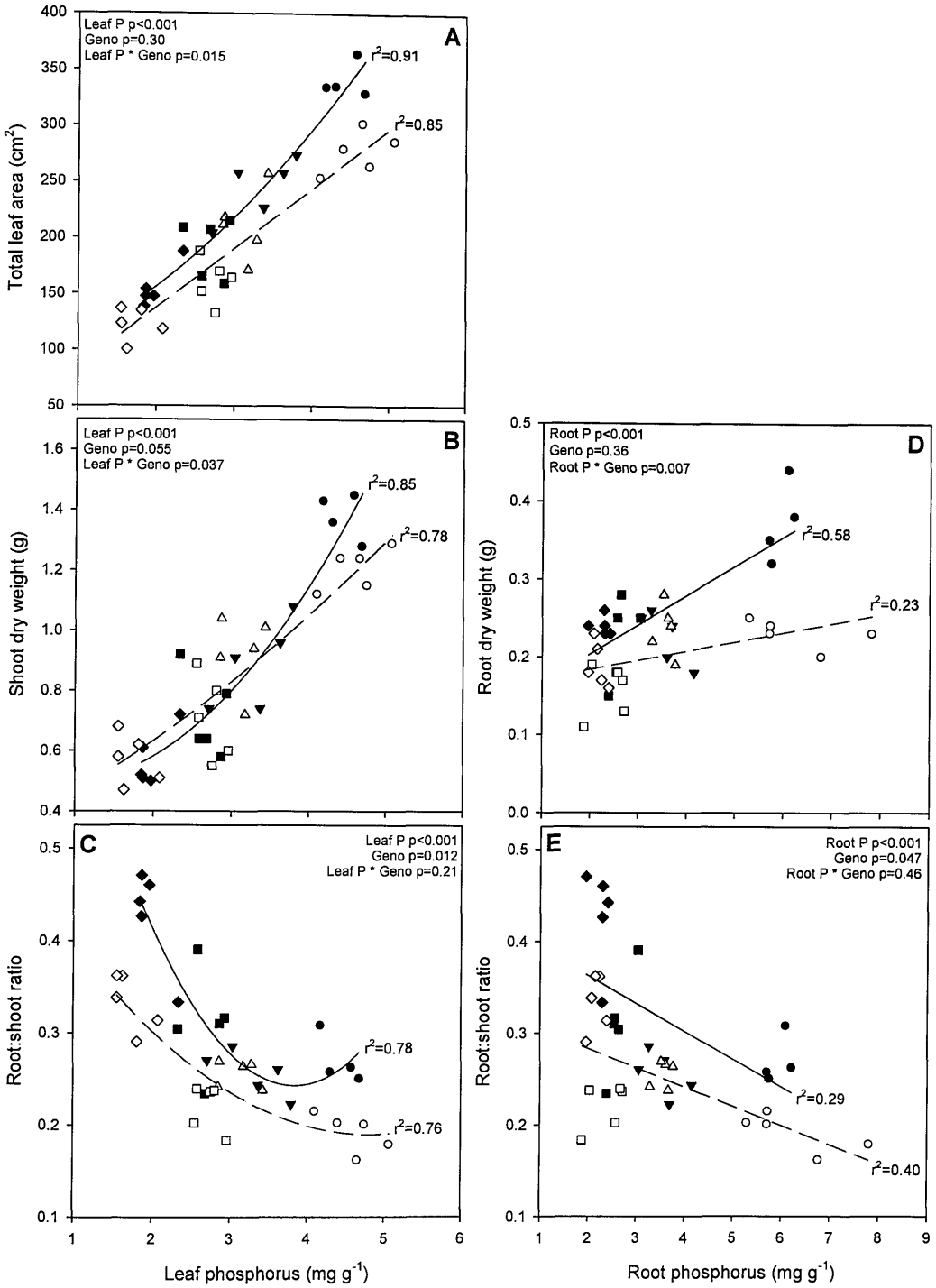

Figure 5.7: Relationship between leaf tissue phosphorus concentration and leaf area (panel A), shoot dry weight (panel B) or root:shoot ratio (panel C); root tissue phosphorus concentration and root dry weight (panel D) or root:shoot ratio (panel E) in wild-type (WT) (filled symbols) or *wilty* (open symbols) *Pisum*. Treatments are control (triangles), control + P fert (circles), lime (diamonds) and lime + P fert (squares). Results of General Regression Model with interaction are shown in top right or left of each panel. Separate second order polynomial (panels A-C) or linear (panels D, E) regression lines are fitted (solid line WT, dashed line *wilty*), r^2 values are shown within each panel.

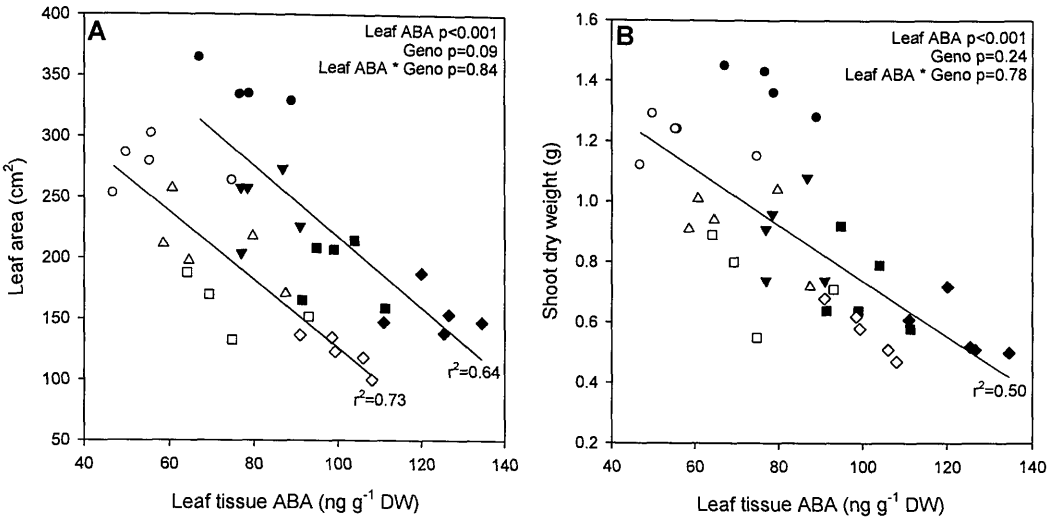

Figure 5.8: Relationship between leaf tissue ABA concentration and total leaf area (panel A) and shoot dry weight (panel B) in wild-type (WT) (filled symbols) or *wilty* (open symbols) *Pisum*. Treatments are control (triangles), control + P fert (circles), lime (diamonds) and lime + P fert (squares). Results of General Regression Model with interaction are shown in top right of each panel. Individual linear regression lines are fitted in panel A, single line fitted in panel B and r^2 values are shown within each panel.

Liming reduced Ψ_{leaf} by 0.12 MPa and 0.09 MPa in the WT and *wilty* genotypes respectively and *wilty* had Ψ_{leaf} 0.26 MPa and 0.22 MPa lower than the WT plants in the control and limed group respectively (Figure 5.9). Superphosphate had no apparent significant effect on Ψ_{leaf} in any treatment (Figure 5.9). Although leaf tissue P concentration was weakly correlated (r^2 0.12) with Ψ_{leaf} (Figure 5.10A), Ψ_{leaf} was not correlated with leaf area in either genotype (Figure 5.10B).

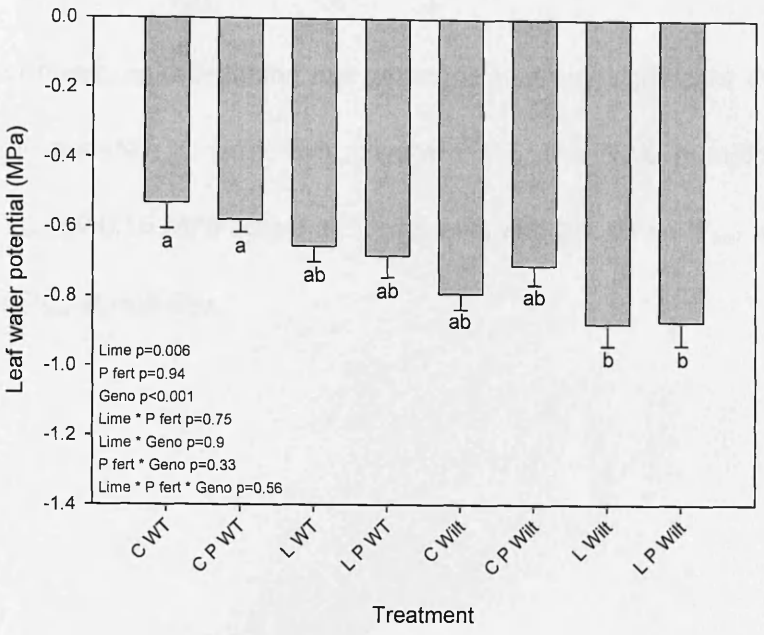

Figure 5.9: Leaf water potential of control (C), control + P fert (CP), limed (L) or limed + P fert (LP) *wilty* (wilt) and wild-type (WT) *Pisum.* Data are means ± SE of 6-9 replicates. Results of three-way ANOVA with interaction are indicated in the bottom left of the panel and differing letters below bars indicate significant differences as determined by Tukey pair-wise analysis.

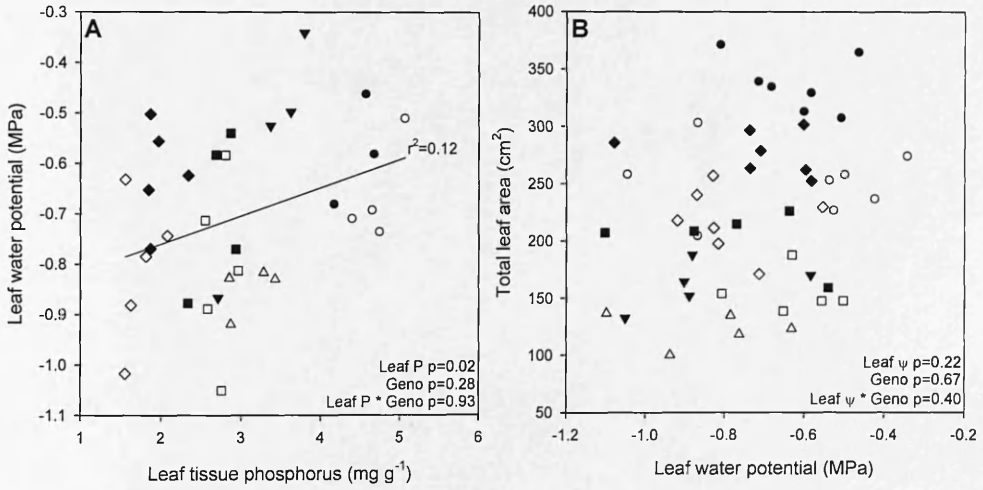

Figure 5.10: Relationship between leaf tissue phosphorus concentration and leaf water potential (panel A) and leaf water potential and total leaf area (panel B) in wild-type (WT) (filled symbols) or *wilty* (open symbols) *Pisum*. Treatments are control (triangles), control + P fert (circles), lime (diamonds) and lime + P fert (squares). Single linear regression fitted in panel A with r^2 value shown in the panel. Results of General Regression Model with interaction are shown in the bottom right.

5.3.3 Peas grown at different humidities

In this experiment, neither liming nor genotype had any significant effect on Ψ_{leaf} measured at mid-day or pre-dawn (Figure 5.11). The 90% humidity treatment increased Ψ_{leaf} by 0.16 MPa across all treatments and pre-dawn Ψ_{leaf} was 0.18 MPa higher than Ψ_{leaf} at mid-day.

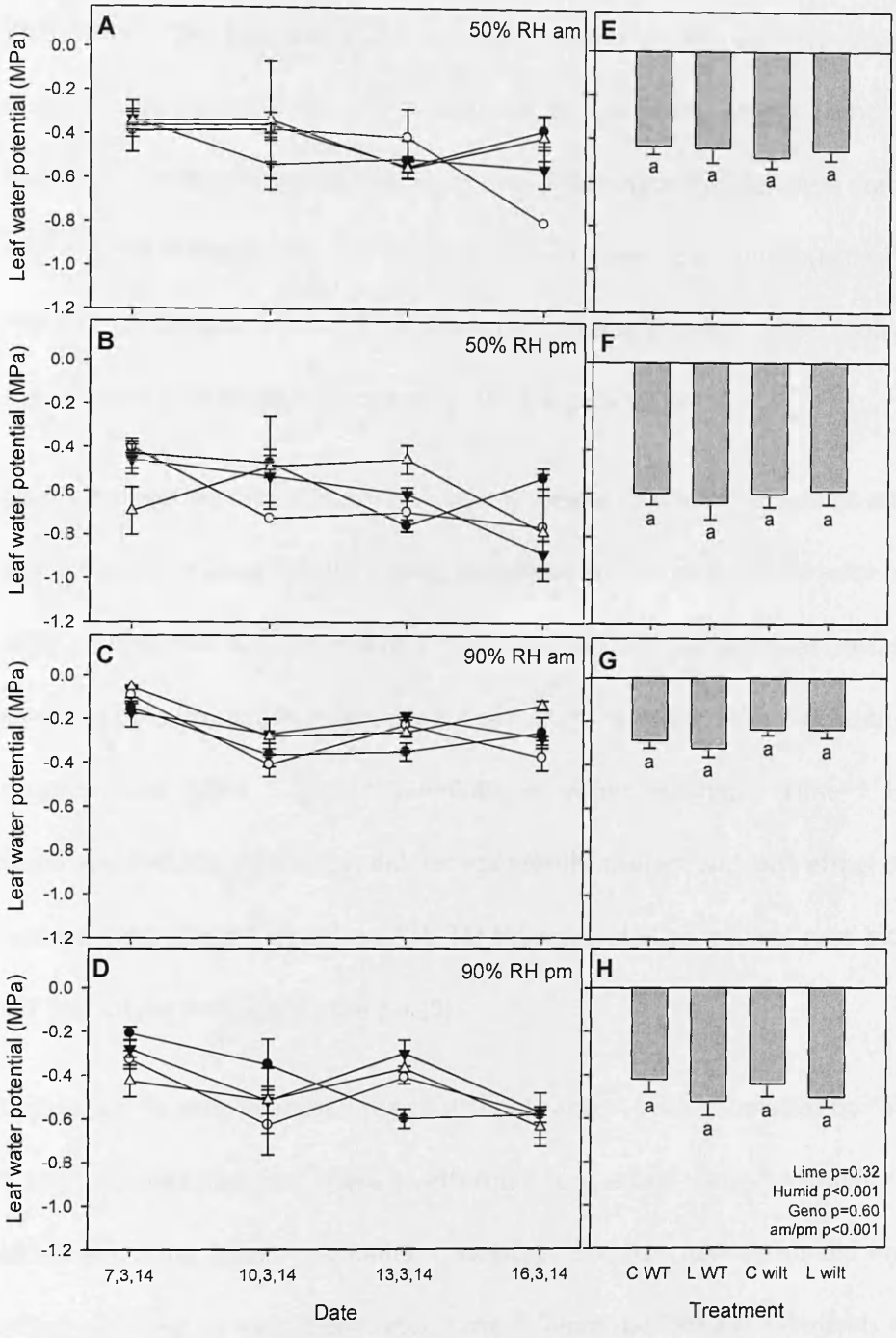

Figure 5.11: Leaf water potential of control wild-type (filled circles, C WT), limed wild-type (open circles, L WT), control *wilty* (filled triangles, C wilt) or limed *wilty* (open triangles, L wilt) *Pisum* taken pre-dawn (panels A, C) or mid-day (panels B, D) at 50% (panels A, B) or 90% (panels C, D) relative humidity. Data are daily means ± SE of 2-3 replicates (panels A-D) or means ± SE over the entire experimental period of 7-11 replicates (panels E-H). Results of four-way ANOVA shown in bottom right of panel H, interaction terms not shown but none were significant, differing letters below bars in panels E-H indicate significant differences as determined by Tukey pair-wise analysis.

Liming reduced shoot dry weight of WT plants by 35% and 33% and of *wilty* by 46% and 40% in the low and high humidity treatments respectively (Figure 5.12A). Humidity did not alter the effect of liming on shoot dry weight (Lime * Humidity P=0.54) in either genotype (Lime * Humidity * Genotype P=0.68). ABA status (*wilty* vs WT) did not interact with the liming effect on shoot dry weight (Lime * Genotype P=0.28). Similarly, *wilty* and WT had similar shoot dry weight when grown at low or high humidity (Humidity * Genotype P=0.89; Figure 5.12A).

Liming did not significantly affect root dry weight in the WT plants at either low or high humidity (Figure 5.12B). Liming decreased root dry weight of *wilty* by 16% and 14% at low and high humidity respectively but this effect was not statistically significant (Figure 5.12B). Humidity did not alter the relationship between liming and root biomass (Lime * Humidity P=0.80) in either genotype (Lime * Humidity * Genotype P=0.95). ABA status did not apparently interact with any effect of liming on root biomass (Lime * Genotype P=0.31) though *wilty* had a lower root biomass than WT (Genotype P<0.001; Figure 5.12B).

Liming significantly increased the root:shoot ratio in both genotypes by ~50% (Figure 5.12C). Humidity did not interact with this liming effect (Lime * Humidity P=0.55) in either genotype (Lime * Humidity * Genotype P=0.51). ABA status did not alter the effect of liming on root:shoot ratio (Lime * Genotype P=0.29), however, *wilty* had a lower root:shoot ratio than WT (Genotype P<0.001; Figure 5.12C).

Total leaf area of WT plants was reduced by ~36% by liming at both humidity treatments (Figure 5.13A). Total leaf area of *wilty* was reduced by 47% at the low humidity and 38% in the high humidity treatment (Figure 5.13A) though there was no

significant interaction between the effects of lime and humidity (Lime * Humidity P=0.08) in either genotype (Lime * Humidity * Genotype P=0.52). The high humidity treatment did significantly decrease total leaf area (P<0.001) and this effect was similar for both genotypes (Humidity * Genotype P=0.96). ABA status did not influence the effect of lime (Lime * Genotype P=0.82) though overall *wilty* had a lower total leaf area than WT (Figure 5.13A).

More detailed analysis shows that the high humidity inhibition of leaf area occurred only in the leaflets (P<0.001) and not the stipules (P=0.21; Figure 5.13B, C). However, liming inhibited size of both leaflets and stipules (Figures 5.13B, C). The inhibitory effect of liming on leaflet area was partly attenuated by growth at high humidity (Lime * Humidity P=0.009) and this was not affected by genotype (Lime * Humidity * Genotype P=0.40).

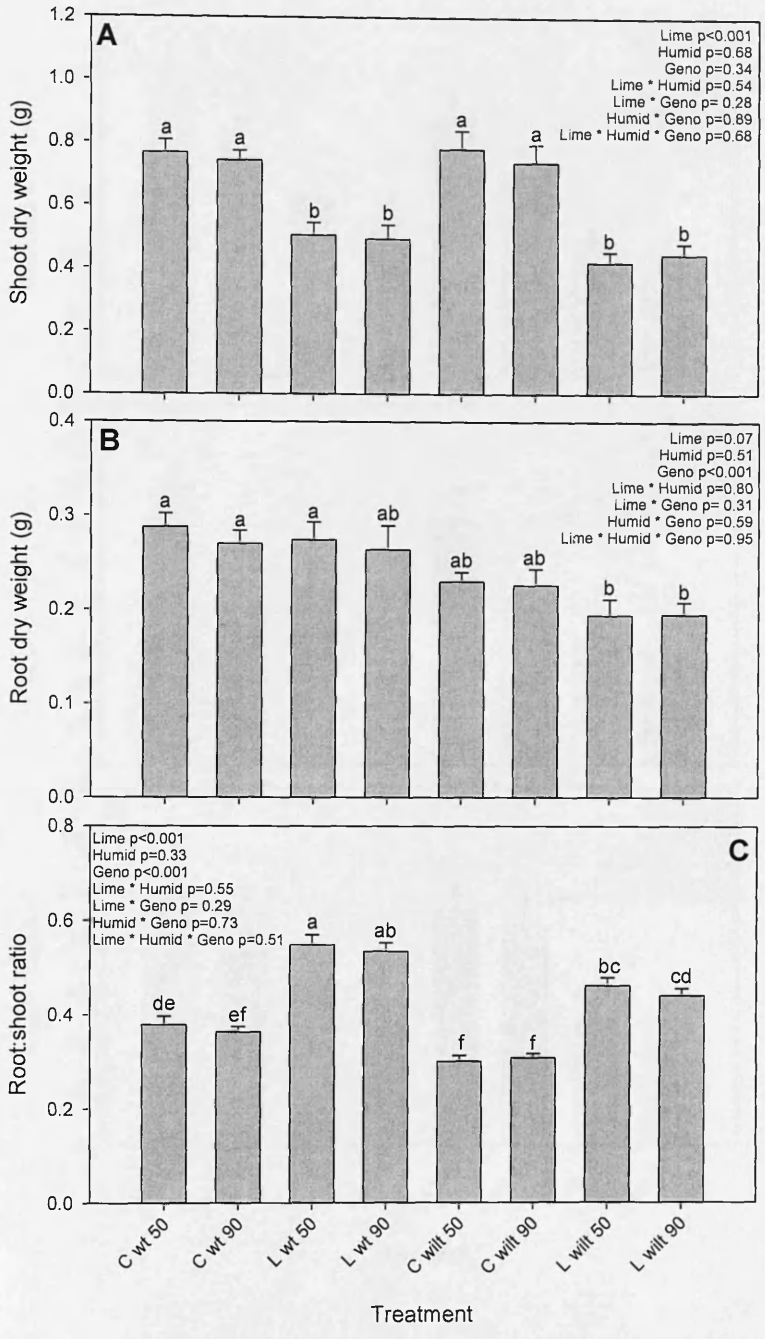

Figure 5.12: Shoot (panel A) and root (panel B) dry weight and root:shoot ratio (panel C) of control (C) or limed (L) *wilty* (wilt) and wild-type (WT) *Pisum* grown at 50% or 90% relative humidity. Data are means ± SE of 6-9 replicates. Results of three-way ANOVA with interaction are indicated in the top right (A,B) or left (C) of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

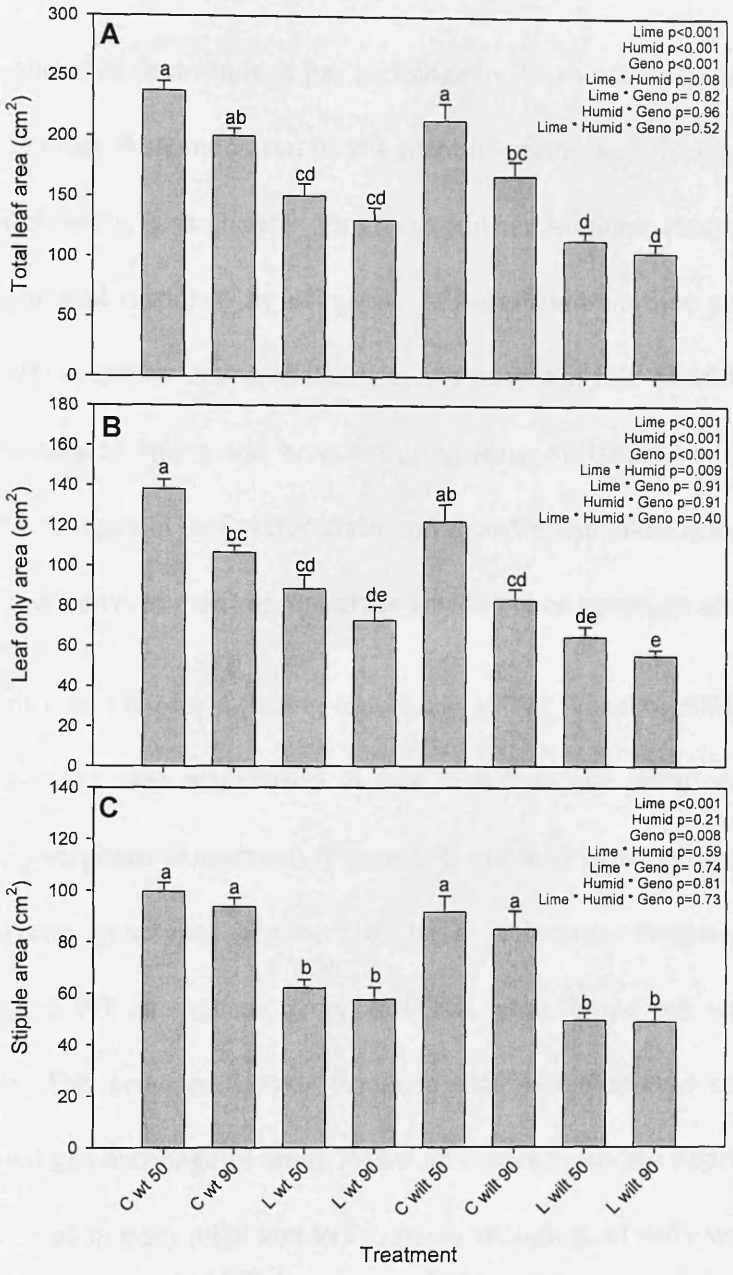

Figure 5.13: Total leaf area (panel A) leaf only area (panel B) and stipule area (panel C) of control (C) or limed (L) *wilty* (wilt) and wild-type (WT) *Pisum* grown at 50% or 90% relative humidity. Data are means ± SE of 6-9 replicates. Results of three-way ANOVA with interaction are indicated in the top right of the panel and differing letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

5.4 Discussion

Chapter 4 concluded that reduced gas exchange in *Pisum* under recommended rates of liming was most likely mediated by the plant hormone ABA in response to a lime-induced P deficiency. This chapter sought to further evaluate evidence for an ABA mediated stomatal response by using the ABA-deficient mutant *wilty* pea and its wild-type (WT) in similar liming trials. Also, the potential role of ABA in moderating growth responses to liming was assessed using *wilty*. Furthermore, the influence of lime-induced changes in leaf water status on growth was investigated using a high humidity growth environment to moderate any liming or genotype effect on Ψ_{leaf}.

In agreement with Chapter 4, liming reduced g_s in WT *Pisum* by 27%, however, this stomatal response was attenuated in the ABA deficient *wilty*, not only in the liming/superphosphate experiment (Figure 5.3) but also when compared in a wider screen of *Pisum* genotypes (Figure 5.2). Here, Alderman, Progress No. 9, Hurst Greenshaft and WT all reduced g_s by 26%-50% when limed but *wilty* showed no change in g_s. This provides further evidence that ABA-mediated stomatal closure decreased leaf gas exchange of limed *Pisum*. In contrast, nitrate deprivation induced similar decreases in both *wilty* and WT g_s (even though g_s of *wilty* was ~25% higher across both N treatments) demonstrating that ABA was not mediating stomatal responses to nitrate deprivation (Dodd, 2003a).

However, whether the site of increased ABA synthesis is the root or the shoot is still unclear, particularly as root tissue ABA concentrations did not apparently alter in response to liming (Table 5.3, 5.4). This is in contrast to experiments in Chapter 4 (Figure 4.8B; 4.9B) where root tissue ABA concentration increased in response to a

lime-induced P deficiency. However, increased root ABA biosynthesis is not necessarily associated with measurable increases in root tissue ABA concentration, particularly under P deficiency. Newly root synthesised ABA accounted for 82% of a six-fold increase in xylem ABA export under P deficiency but this ABA was rapidly transported into the xylem stream and not accumulated within the root tissue (Jeschke et al., 1997). This could explain the lack of root tissue ABA accumulation in response to liming in the current experiment and the discrepancy with experiments in Chapter 4.

However, explaining the similarity between WT and *wilty* root tissue ABA concentration is more difficult. Differences between WT and ABA deficient mutant root ABA concentrations are typically less than those seen in the shoot (for example, Netting et al., 2012) and although not statistically significant, *wilty* root tissue concentration was ~30% lower than WT (Table 5.3) therefore, non statistical differences may be due to low repetition (n=5). However, no data for *wilty* and WT ABA root concentrations have been previously published, hence this may be typical of the *wilty* mutation. Careful analysis of root and leaf tissue and xylem and phloem transport fluids using flow modelling approaches that determine sites of ABA biosynthesis and metabolism (Jeschke and Pate, 1991, Jeschke et al., 1997) in limed/unlimed WT and *wilty* plants would determine the site of ABA production under liming and elucidate the true root ABA phenotype of *wilty*.

Stomatal conductance declined with leaf tissue P concentration in both genotypes (Figure 5.4B) although the relative insensitivity of g_s to tissue P concentration in *wilty* pea suggests that ABA is needed to mediate lime-induced stomatal closure. In both

genotypes, leaf tissue ABA concentration increased as leaf tissue P concentration decreased (Figure 5.4A), even though leaf ABA concentrations of *wilty* were ~20% less. Since g_s declined linearly with increasing tissue ABA concentration in both WT and *wilty* (Figure 5.4C), it seems likely that tissue ABA (and not P) status is the principal regulator of g_s. Interestingly, there was a single relationship between leaf tissue ABA and g_s in both WT and *wilty* (Figure 5.4C) confirming that though impaired in its ability to synthesise ABA, *wilty* stomata are still sensitive to its action (Donkin et al., 1983). Similarly, the ABA deficient barley mutant *Az34* showed a comparable relationship between xylem sap ABA concentration and g_s to its wild-type. In agreement with the current study, g_s was consistently higher and ABA concentration lower in the mutant (Mulholland et al., 1996a).

Although leaf tissue ABA concentrations between unlimed WT and *wilty* were statistically similar (Tables 5.3; 5.4), leaf tissue ABA concentrations were consistently ~20% lower in *wilty* than WT across a range of leaf P concentrations (Figure 5.4A). In contrast, previous studies demonstrate that *wilty* had ~50% (Dodd, 2003b) or 90% (Wang et al., 1984) less leaf tissue ABA concentration than its WT under well watered conditions. This discrepancy may be explained by different analytical approaches i.e. GC-MS (Wang et al., 1984, Dodd, 2003b) compared to the radioimmunoassay used in the current study. However, despite differences in the level of the ABA deficiency, in the current experiment, *wilty* had a g_s 25-50% (Figure 5.2) or 35% (Figure 5.3) higher than genotypes considered normal in their ABA biosynthesis, confirming that the *wilty* mutation altered stomatal behaviour. Similarly, g_s was ~60% (Donkin et al., 1983) and 33% (Dodd, 2003a) higher in *wilty* than WT plants.

Previous experiments have used ABA deficient mutants to effectively demonstrate the requirement for ABA in mediating stomatal closure (for example, Wilkinson et al., 1998). This current experiment using the ABA deficient *wilty* pea provides evidence that by partially **excluding** ABA from limed *Pisum* plants, stomatal closure can be partially or fully restored. This, when considered in conjunction with the **correlation** between ABA and g_s (Figures 4.10, 5.4C) and **duplication** *in vitro* using detached leaf transpiration bioassays (Figure 4.14), provides strong evidence that reduced gas exchange in legumes in response to liming is mediated by ABA.

The second hypothesis tested in this chapter examined the role of increased ABA concentration in limiting shoot and leaf growth under liming. In contrast to g_s, there was no significant Lime * Genotype interaction with leaf area (P=0.39; Figure 5.5A) or shoot dry biomass (P=0.40; Figure 5.5B), thus it seems unlikely that endogenous leaf ABA levels are moderating leaf and shoot growth under liming. Similarly, *wilty* exhibited a comparable reduction in leaf area to WT when subject to nitrate deficiency (Dodd, 2003b) implying that leaf tissue ABA concentration was not regulating growth responses to this stress. Furthermore, there were no apparent genotypic differences in leaf area (Figure 5.8A) or shoot dry biomass (Figure 5.8B) at lower leaf tissue P concentrations (<3 mg g^{-1}) associated with increased ABA concentration (Figure 5.4A). Significant Leaf P * Genotype interactions (leaf area, P=0.015; shoot dry weight P=0.037) seem to be accounted for by increased growth of WT over *wilty* only at higher leaf tissue P concentrations (>3 mg g^{-1}; Figure 5.7A, B). Therefore, ABA status is likely not regulating leaf or shoot growth responses to lime.

However, in the current experiment, leaf tissue ABA concentrations increased as leaf area (Figure 5.8A) and shoot dry biomass (Figure 5.8B) decreased regardless of genotype possibly identifying a role for ABA in mediating shoot growth. Similarly, increased ABA concentration correlated with inhibited leaf expansion under nutrient (Palmer et al., 1996) or salinity (Cramer and Quarrie, 2002) stress, most likely due to ABA-mediated reductions in cell wall extensibility (Van Volkenburgh and Davies, 1983). Interpretation of the effects of ABA on growth may be confounded by other effects of the *wilty* mutation on leaf area development. Increased leaf ethylene evolution was correlated with reduced leaf growth in the ABA deficient tomato *flacca* when compared to WT (Sharp et al., 2000). To distinguish effects of ABA deficiency from enhanced ethylene production on leaf area, a double mutant was created in *Arabidopsis thaliana* that was both deficient in ABA and insensitive to ethylene (LeNoble et al., 2004). Ethylene insensitivity in the double mutant recovered 55% of the leaf area losses caused by the ABA mutation alone thus demonstrating the significant contribution of ethylene to limiting leaf growth in ABA mutants. Unusually (for an ABA-deficient mutant), increased ethylene evolution was not detected in *wilty* (Dodd, 2003b), suggesting that its leaf growth phenotype may help determine if ABA is regulating shoot growth without possible confounding effects of other phytohormones.

Many authors have attributed reduced leaf growth of ABA deficient mutants, including *wilty*, to impaired leaf water status (Bradford, 1983, De Bruijn et al., 1993, Nagel et al., 1994). However, in the current experiment, *wilty* had a lower leaf area when compared to WT (Figure 5.13) despite equal Ψ_{leaf} when grown at a high humidity (Figure 5.11). Similarly, shoot development of the ABA-deficient tomato

mutants *flacca* and *notabilis* remained impaired under high humidity, even though leaf water potentials were equal to or greater than wild-type plants (Sharp et al., 2000). These results seemingly confirm that reduced leaf area development in *wilty* is mediated by non-hydraulic effects.

Similarly, with respect to the third hypothesis tested in this chapter, lime-induced inhibition of shoot biomass and leaf area still occurred at high humidity despite equalisation of Ψ_{leaf} between liming and controls and a higher (0.2 MPa) mid-day Ψ_{leaf} than low humidity grown plants (Figure 5.12A). Furthermore, this lime-induced inhibition of leaf area and shoot biomass was comparable to that observed in the greenhouse (at much higher VPDs), where liming decreased Ψ_{leaf} (Figure 5.5A, B; 5.9). Additionally, although superphosphate increased leaf area of control and limed plants (Figure 5.6A) this was independent of changes in Ψ_{leaf} (Figure 5.9). This suggests that inhibition of leaf and shoot growth in *Pisum* by a lime-induced P deficiency is not mediated by hydraulic effects. This is in contrast to the widely held belief that inhibition of leaf expansion under P deficiency is hydraulically mediated (Radin and Eidenbock, 1984). There was a slight attenuation (3.7%) of the lime effects on leaflet only area at the highest humidity (Figure 5.13B). However, the inhibition of total leaf area by lime at high humidity (-38%) in the absence of treatment differences in Ψ_{leaf} was again comparable to greenhouse grown plants (-40%). Furthermore, despite a weak correlation (r^2=0.12) between leaf tissue P concentration and Ψ_{leaf} (Figure 5.10A), Ψ_{leaf} was not correlated with leaf area (Figure 5.10B).

Interestingly, treatment or genotype Ψ_{leaf} differences were not apparent in the 50% humidity treatment (Figure 5.11). Analysis of vapour pressure deficit (VPD) of the growth environment at the time of Ψ_{leaf} sampling would suggest that the humidity experienced in the 50% humidity chamber was enough to moderate any treatment or genotype effects (Figure 5.14). Taken together, these results suggest a limited role for Ψ_{leaf} in mediating growth responses to either lime, P status or ABA deficiency, which is not surprising given that in many comparisons (particularly those involving ABA-deficient mutants), Ψ_{leaf} is regulated by g_s such that high g_s causes low Ψ_{leaf} (for example, Kudoyarova et al., 2007, Belimov et al., 2009, Dodd et al., 2009).

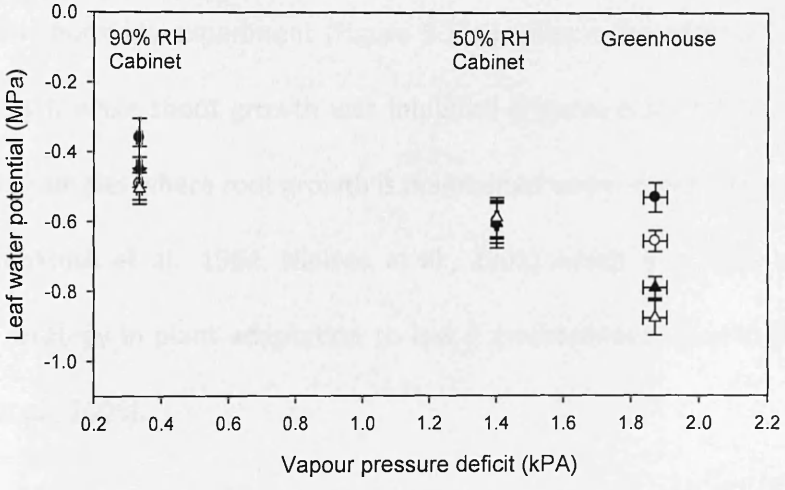

Figure 5.14: Effect of vapour pressure deficit on leaf water potentials in greenhouse and growth chambers at 50% or 90% relative humidity of control (filled symbols) or limed (open symbols) WT (circles) or *wilty* (triangles).

Some authors have suggested that reduced leaf area of P deprived plants is caused by reduced leaf initiation (Rodriguez et al., 1998b). However, in the current study, although leaf initiation of limed plants was typically one node less than controls (Table 5.5), a lime-induced inhibition of fully expanded leaflet area was still apparent

at the individual nodes (Figure 5.6). Thus reduced leaf area under liming must be caused by reduced leaf expansion. There is some disagreement as to whether reduced leaf expansion under P deficiency is caused by impaired cell division (Assuero et al., 2004) or reduced cell elongation (Radin and Eidenbock, 1984). However, the non-responsiveness of leaf area to increased Ψ_{leaf} when grown at high humidity (Figure 5.13) would suggest that it is not turgor-mediated cell expansion that is limiting leaf area development under P deficiency. Techniques measuring leaf cell number and area (for example, Gonzalez et al., 1998) would determine if cell division or expansion processes are mediating leaf growth.

The lime-induced increase in root:shoot ratios in both the greenhouse (Figures 5.5D) and high/low humidity experiment (Figure 5.12C) reflects the relative maintenance of root growth while shoot growth was inhibited (Figures 5.5C, 5,12C). This agrees with many examples where root growth is maintained under P deficiency (Fredeen et al., 1989, Cakmak et al., 1994, Nielsen et al., 2001) which is considered to be an important strategy in plant adaptation to low P environments (Raghothama, 1999, Hermans et al., 2006).

In the greenhouse experiment, the significant Lime * Genotype interaction with root:shoot ratio (P=0.012; Figure 5.6D) would suggest that ABA is important in moderating biomass partitioning under P deficiency. However in the high/low humidity experiment (Figure 5.12C) this statistical interaction was not apparent. Lower root:shoot ratios are characteristic of *wilty* (De Bruijn et al., 1993) as observed here (Figures 5.6D, 5.12C), and in other studies using ABA-deficient mutants (for example, Bradford, 1983) apparently confirming a role for ABA in root:shoot

partitioning. The difference in statistical interactions observed in the two experiments here are difficult to reconcile as the apparent impact on shoot growth was similar in both examples, as discussed previously. However, liming reduced root biomass of *wilty* by 17% and 16% in the greenhouse and high low humidity experiments respectively without affecting root biomass of WT plants (Figures 5.6C; 5.12B). Although this effect was not statistically significant, clearly *wilty* could not maintain root development under liming in either experiment whereas WT did. Furthermore, combined data from plants grown in the greenhouse and low and high humidity treatments reveals that the relative impact of liming on root biomass was significantly more severe (-16%) in *wilty* than WT (-5%) (Figure 5.15). Thus ABA is likely important in maintaining root proliferation under a lime-induced P deficiency and consequently increasing root:shoot ratios. Similar root:shoot ratio increases in nutrient deficient wheat were attributed to increased leaf ABA accumulation caused by reduced leaf turgor and subsequent re-distribution to the roots via the phloem (Vysotskaya et al., 2008). However, in the current experiment, plants grown at high humidity that would be unlikely to have lower leaf turgor still exhibited increased root:shoot ratios.

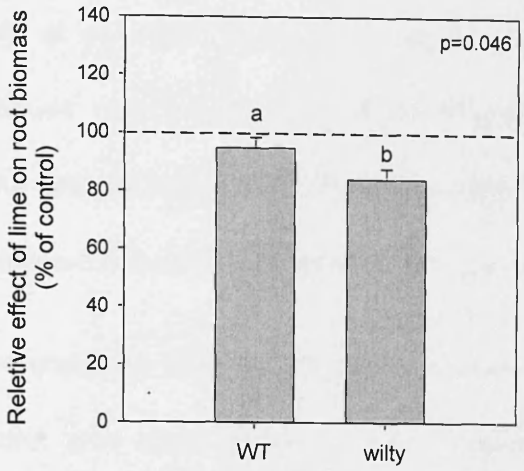

Figure 5.15: The relative effect of liming on root dry biomass in WT and *wilty* combined across plants grown in the greenhouse and at 50% and 90% relative humidity. Data are means ± SE of 17 (WT) and 18 (*wilty*) replicates, differing letters above bars indicate significant differences according to a students *t*-test, with P value reported in the top right of the panel.

The role of phytohormones in mediating root growth responses to P deficiency is well established (Chiou and Lin, 2011, Niu et al., 2013). Auxin has been attributed to inhibited primary root growth but increased lateral root proliferation under P deficiency, probably by changes in auxin sensitivity (Perez-Torres et al., 2008) or root distribution (Nacry et al., 2005). Root and shoot concentration of CK decrease in response to a P deficiency (Salama and Wareing, 1979) and since CKs are often inhibitory to root growth (Werner et al. 2001) have been implicated in increased root:shoot ratios under P deficiency (Martin et al., 2000). Although the role for ethylene in modulating root architecture under P deficiency is still unclear (Niu et al., 2013) it is important for maintaining root elongation (Borch et al., 1999, Ma et al., 2003). However, a possible role for ABA in mediating root P deficiency responses has only been speculated (Chiou and Lin, 2011). Similarities with increased root:shoot

ratios observed under reduced water availability, which is thought to be ABA-mediated (Creelman et al., 1990, Saab et al., 1990), may suggest that ABA is important in continued root development under P deficiency. More detailed examination of *wilty* and WT roots might identify possible specific ABA mediated changes in root elongation or architecture under a lime-induced P deficiency.

Two interesting observations from the high/low humidity experiment are the impairment of leaflet area when grown at high humidity across both liming treatment and genotype (Figure 5.13B) and the differential effect on stipules (Figure 5.13C). Plants growing at high relative humidities usually experience Ca deficiency (Torre et al., 2001) which might directly affect growth and is typically apparent in young expanding leaves (White and Broadley, 2003) which may explain the difference between leaflets and stipules. Alternatively *Vicia faba* stomata exhibited an enhanced sensitivity to CO_2 in growth chambers at a relative humidity >85% compared to those grown at 55% (Talbott et al., 2003), thus a CO_2 mediated stomatal limitation of Pn was possible at the higher humidity which could explain the leaf area reduction if leaf growth was carbohydrate limited. However, loss of stomatal control at high humidity (Torre et al., 2003) would suggest that this last explanation is unlikely.

In conclusion this chapter demonstrates that stomatal closure in response to liming was attenuated, or absent, in the ABA-deficient pea *wilty*. This finding apparently confirms that reduced gas exchange under a lime-induced P deficiency is mediated by ABA and thus has furthered our mechanistic understanding of the processes involved. The negative growth responses to liming are clearly not mediated by

hydraulic effects though the possible role of ABA in effecting shoot and root growth is less clear. The absence of any Lime * Genotype interactions with shoot biomass (Figures 5.6B; 5.12A) or leaf area (Figures 5.6A; 5.13A) suggest that ABA is not mediating shoot growth responses to liming. However, a significant Lime * Genotype interaction with root:shoot ratio in the greenhouse experiment implies that ABA may be important in mediating biomass partitioning (Figure 5.6D). Though this interaction was not apparent in the high/low humidity experiment (Figure 5.12C), liming still reduced *wilty* root biomass equally in both experimental systems while WT root biomass remained unaffected (Figures 5.6; 5.12), thus demonstrating a possible role for ABA in maintaining root proliferation under a lime-induced P deficiency.

Chapter 6 – General Discussion

Dwindling natural P resources (Cordell et al., 2009), the reliance on limited fossil fuel reserves for N production (Dawson and Hilton, 2011) and concerns over K supply and demand (Sheldrick et al., 2002) mean the continued adequate supply of supplementary nutrients is a potential limiting factor in maintaining future crop production (White and Brown, 2010). Therefore, an important challenge facing plant and crop scientists is not only to ensure the continued supply of suitable fertilisers, but also to ensure that any fertilisers applied are used as efficiently as possible.

Increasingly, by-products from industrial processes, typically disposed as waste, are being considered as alternatives to conventional fertilisers for use in agriculture (for example, Camberato et al., 2006, Li et al., 2010, Seshadri et al., 2010, Singh et al., 2010). The potential of one of these, cement kiln dust (CKD) (Vanlierop et al., 1982b, Dann et al., 1989, Lafond and Simard, 1999, Lalande et al., 2009) was evaluated in Chapter 2. Having a high pH and commonly being high in water soluble K, CKD is now commonly applied to UK agricultural soils as both a soil pH amendment and K fertiliser (C Rudd 2012, pers. comm.). The CKD tested and trialled in Chapter 2 was equally capable of raising soil pH and both soil available and plant tissue K levels when compared to commercially available lime and K fertiliser (Figure 2.2; Tables 2.4, 2.5). Furthermore, levels of potentially toxic elements (PTEs) that could be of concern with using CKD in agriculture (Adaska and Taubert, 2008) were found to be well below recommended thresholds in the sample analysed (Table 2.1). However, possible variation in levels of PTEs between different cement production sites means

the findings here should only apply to this particular dust used. Therefore it is appropriate to recommend that, providing PTEs are regularly monitored and do not exceed regulatory levels, CKD is a suitable alternative to agricultural lime and K fertiliser.

Careful management of soil pH by liming, typically targeting pH values around pH 6.5, has traditionally been used to optimise nutrient availability and crop productivity. However, in the field trial (Chapter 2), liming at DEFRA recommended rates (DEFRA, 2010) with CKD or lime unexpectedly suppressed *Vicia* pod yield by ~30% (Figure 2.9). Subsequent pot trials revealed that liming consistently inhibited leaf gas exchange (Figures 2.10; 2.14) and shoot biomass (Figures 2.11; 2.14) of *Vicia*, *Phaseolus* and *Pisum.* Since the cause of this effect was not clearly understood, the focus of the thesis changed from an assessment of the agricultural usefulness of CKD to investigate the physiological mechanisms causing yield suppression following liming.

The understanding generated in this thesis of the physiological processes involved in a lime-induced reduction of gas exchange and growth in legumes is summarised in Figure 6.1. The principle mode of action is a lime-induced reduction in P availability, most likely by formation of calcium phosphates, for example apatite (Moody et al., 2013). This triggered an increase in both root and shoot concentrations of the plant hormone ABA, which is a well-established antitranspirant known to regulate stomatal behaviour in response to many environmental stresses (Wilkinson and Davies, 2002). Exactly how plant roots can sense a change in nutrient availability and subsequently increase synthesis of ABA is currently unknown. However, a probable

mechanism is an aquaporin mediated lowering of root water potential (Chaumont and Tyerman, 2014) which is known to stimulate ABA production (Simonneau et al., 1998). Aquaporins are membrane water channel proteins that actively facilitate the transport of water in plants (Maurel et al., 2008) that are known to show decreased expression and activity under nutrient deficiency (Clarkson et al., 2000) and have been implicated in mediating root hydraulic properties under nutrient deficiency (Cabanero and Carvajal, 2007). Further examining the relationship between nutrient stress, aquaporin expression and activity and ABA would be beneficial to understand plant adaption to low nutrient stresses.

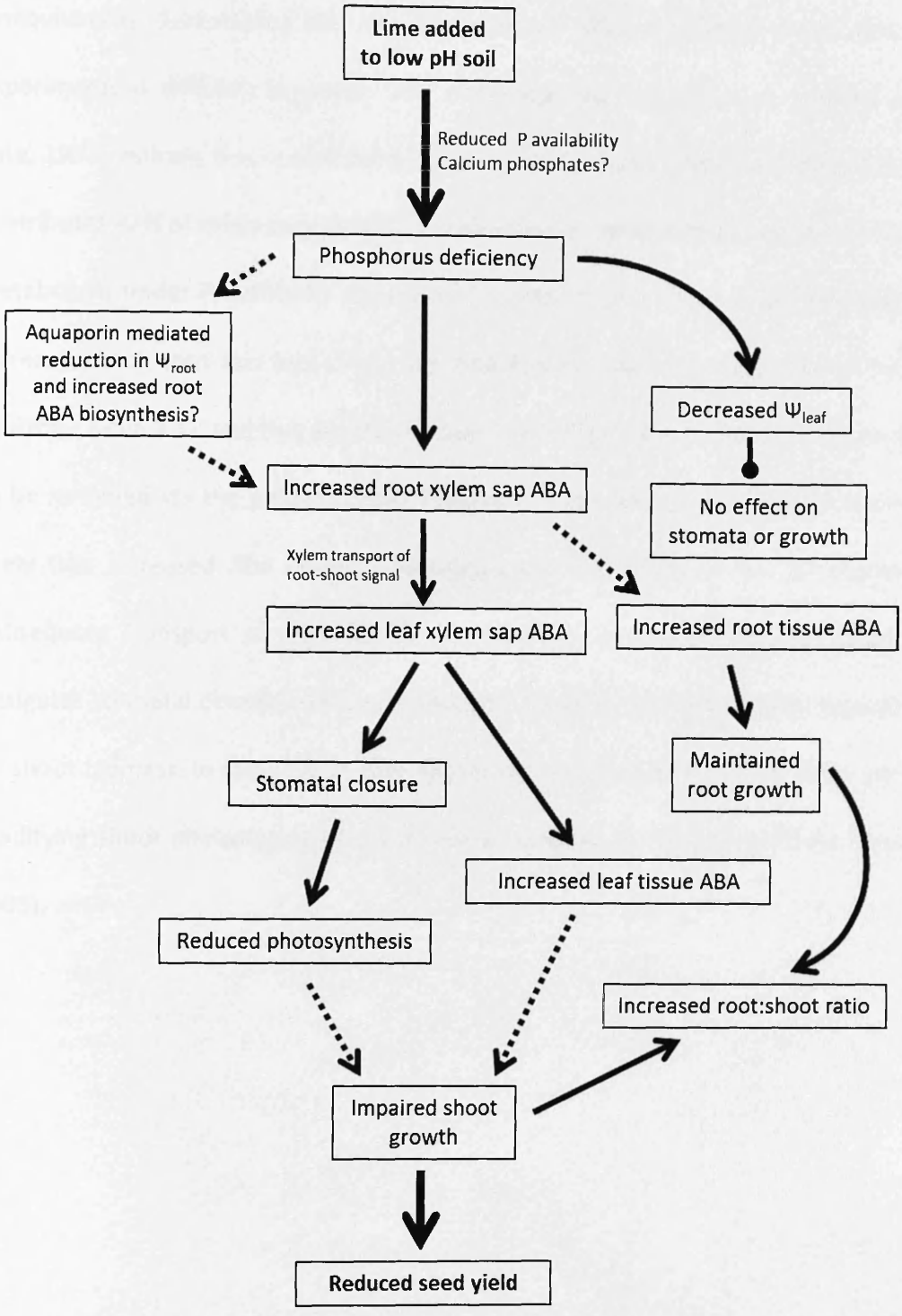

Figure 6.1: Conceptual diagram detailing the physiological processes involved in reduced gas exchange and growth under a lime-induced phosphorus deficiency in legumes. Solid arrows indicate processes established in this thesis, dotted arrows represent possible mechanisms.

Unequivocally determining the site of increased ABA production from current experiments is difficult. However, flow modelling techniques (*sensu*, Jeschke and Pate, 1991) indicate that in well watered plants under P deficiency, root biosynthesis contributes 82% of xylem exported ABA as opposed to being a moderate sink for ABA metabolism under P sufficient conditions (Jeschke et al., 1997). Given that liming increased both root and leaf xylem sap ABA concentrations equally over unlimed controls (Table 4.1); and that significant quantities of leaf synthesised ABA is unlikely to be re-cycled via the phloem under P deficiency (Jeschke et al., 1997), it is most likely that increased ABA under a lime-induced P deficiency is root synthesised. Subsequent transport of ABA via the transpiration stream to the leaf apoplast instigates stomatal closure and hence limits Pn (Figure 6.2), likely directly impacting on shoot biomass. In this respect ABA appears to be acting as a root-to-shoot signal modifying shoot physiological responses to an edaphic (P deficiency) stress (Dodd, 2005).

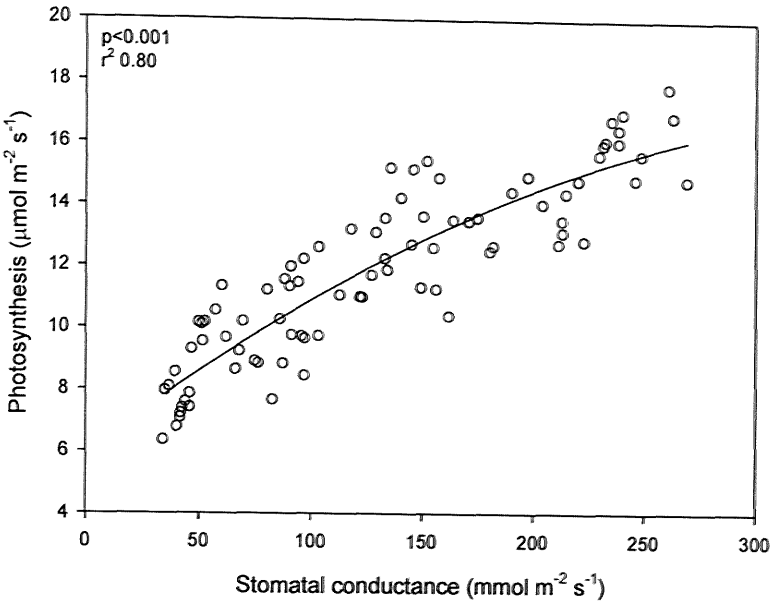

Figure 6.2: Relationship between stomatal conductance and photosynthesis in *Pisum*. Data are combined from Chapters 2 and 4, second order polynomial curve fitted, P value and r^2 shown in top left of panel.

The conventional understanding that impaired leaf development under a P deficiency is hydraulically mediated (Radin and Eidenbock, 1984) is challenged by the findings of this thesis. Limed (P deficient) plants grown in a high humidity to abolish any effect of P deficiency on Ψ_{leaf} (Figure 5.11) still exhibited similar inhibitions in leaf and shoot growth as plants grown in more moderate humidity (Figures 5.5, 5.12, 5.13), implying that shoot development is not limited by hydraulic mechanisms. Alternatively, it was suggested that a chemical signal might be limiting growth. Since ABA was clearly moderating stomatal responses, it was an obvious candidate as it has previously been implicated as an inhibitor of leaf growth independently of any hydraulic effects (Tardieu et al., 2010). Furthermore, mutants deficient in or insensitive to the action of ABA have been used to probe a role for ABA in mediating growth to abiotic stresses (for example, Saab et al., 1990). However, no significant leaf or shoot growth

interactions between liming and genotype in comparing responses of WT and the ABA-deficient mutant pea *wilty* (Figure 5.5) would suggest that this chemical signal is not ABA.

However, increased ABA concentration appeared important in regulating biomass partitioning, as demonstrated by a significant genotype * liming interaction affecting root:shoot ratio when comparing WT and *wilty* peas (P=0.012; Figure 5.5), possibly caused by better root proliferation under a lime-induced P deficiency in WT plants (Figure 5.15). Similarly, ABA was required to maintain root growth at low water potential (Saab et al., 1990, Sharp et al., 1994). Further investigations into the role of ABA in regulating root development and architecture, particularly its interaction with known regulators of root growth and architecture under P deficiency, i.e. ethylene (Borch et al., 1999), auxin (Perez-Torres et al., 2008) and CK (Martin et al., 2000), would be useful.

While a role for ABA in mediating stomatal and growth responses to P deficiency has been identified, a wider understanding of the function of ABA in response to nutrient deficiencies is not clear. ABA has been shown to increase in response to general nutrient deficiencies in wheat (Vysotskaya et al., 2009), N, P, K, S and Fe deficiencies in maize (Battal et al., 2003), K deficiency in castor bean (Peuke et al., 2002) and N deficiency in maize (Schraut et al., 2005) so is clearly a common plant response to nutrient deprivation. However, a full understanding of the role this ABA takes in helping plants adapt to nutrient deficiency is not apparent. Though ABA is mediating stomatal behaviour under P deficiency in the current experiment and has been similarly implicated previously (Jeschke et al., 1997) it did not apparently explain

reduced g_s under N deficiency (Coleman and Schneider, 1996, Dodd, 2003a) and increased ABA did not affect g_s under K deficiency (Peuke et al., 2002). However, the influence of ABA on stomatal behaviour can vary considerably according to environmental conditions or interactions with other hormones (Dodd et al., 1996, Dodd, 2003a, Wilkinson et al., 2012).

Similarly, with regard to developmental responses, ABA was suggested to modulate growth between roots and shoots in nutrient deprived wheat (Vysotskaya et al., 2009), as seen in the current experiment. However, increased ABA production was suggested to occur in the leaf in response to reduced leaf turgor which was unlikely in the high humidity plants grown in the present study that still increased root:shoot ratios under liming (Figure 5.12). Conversely, ABA was not thought to be regulating root:shoot ratios under N deficiency in tomato (Coleman and Schneider, 1996).

While ABA clearly has some physiological role in plant adaptions to nutrient stressed environments, disparities such as those demonstrated above only highlight the lack of understanding of this role. Further research examining the site of ABA biosynthesis and metabolism, using the flow modelling approaches of Jeschke and co-workers, in reciprocally grafted ABA-deficient and WT plants could help elucidate specifically how ABA influences plant adaption to low nutrient stress.

It is clear that the negative yield response in the current study is caused by a lime-induced P deficiency, demonstrating that current UK liming recommendations (DEFRA, 2010) may not be appropriate for all agricultural soils. Elsewhere, liming has been variously demonstrated to increase (Holford et al., 1994, Barman et al., 2014, Jaskulska et al., 2014) or decrease (Bartlett and Picarell, 1973, Curtin and Syers, 2001,

Maxwell et al., 2012) P availability. Considering the importance of P management within the context of dwindling resources (Cordell et al., 2009), a better understanding of the relationship between liming and P availability in different soils is clearly important in generating more appropriate liming recommendations.

Current UK guidance on managing soil P levels is based on maintaining available P (usually determined by Olsen extraction) within an indices range of 16-25 mg l^{-1} (Index 2) which is considered to be optimal for crop production (DEFRA, 2010). Although levels of 'critical P' (the point at which increased soil available P will no longer provide a yield benefit) can fall outside this range (Johnston et al., 2013), field trials typically agree with the DEFRA recommendations (Tang et al., 2009, Bai et al., 2013, Poulton et al., 2013). The control soil used in the *Pisum* pot trials (Chapter 4) had an initial Olsen extractable P of 34 mg kg^{-1} (Table 4.2) which would be considered an Index 3 soil and thus would not be expected to deliver increased crop yield to P fertiliser additions. Therefore, the recommendation is for a low level maintenance application of P (35 kg ha^{-1}) to account for crop offtake (DEFRA, 2010). However, attempts to attenuate the liming-induced P deficiency in *Pisum* by application of superphosphate fertiliser at 5 times (200 kg ha^{-1}) the recommended rate failed to fully restore plant P status and growth in limed plants (Figure 4.5) indicating that most of the applied P was rendered unavailable, again presumably by precipitation as calcium phosphate. Therefore, accurately predicting the effect of liming on P availability is clearly important if more appropriate liming recommendations are to account for the effects of lime on P availability.

To accurately predict crop P availability, soil P tests must measure only the P that will be accessed and utilised by that crop (Moody et al., 2013). This is ultimately determined by P available in solution which is in turn determined by processes that variously make available/unavailable different pools of soil P (mineral, organic and sorbed) (Holford, 1997, Nash et al., 2014). However, in the current experiment, liming reduced Olsen P by only 2 mg kg^{-1} compared to control soil but approximately halved leaf tissue P concentration. Furthermore, applying superphosphate to the limed soil increased Olsen P to 40 mg kg^{-1} but failed to restore leaf tissue P to control levels (Figure 6.3). Clearly Olsen P is not an appropriate measure for assessing the impacts of liming on P availability. Furthermore, the suitability of Olsen P extraction in soils with high calcium carbonate concentrations has previously been questioned (Mallarino, 1997).

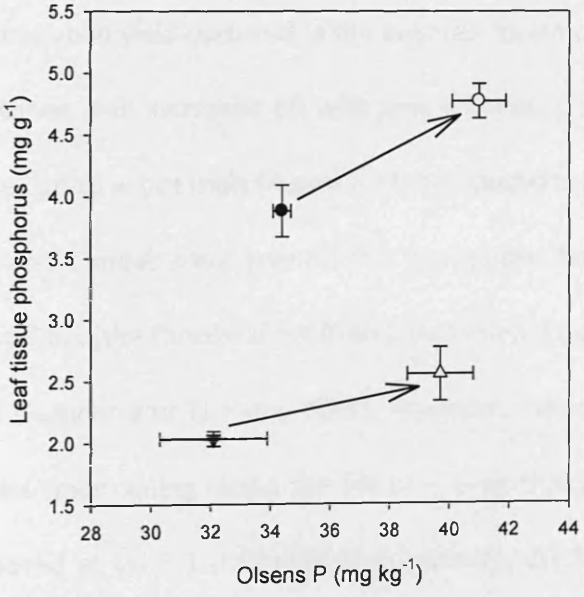

Figure 6.3: Relationship between Olsen extractable and leaf tissue phosphorus concentration in control (circles) and limed (triangles) soil with (open symbols) or without (closed symbols) superphosphate fertiliser in *Pisum*. Data are means ± SE of 4 (Olsen) or 6 (leaf P) replicates.

Therefore, to accurately assess the likelihood of a soil exhibiting a negative yield response to liming via decreased P availability, alternative measures of P availability may be more appropriate. One approach might be Diffusive Gradients in Thin Films (DGT) that was highly correlated (r=0.89) with solution P (Moody et al., 2013) and accurately predicted the dry matter accumulation, and hence critical P thresholds, of wheat in response to P fertiliser (Mason et al., 2010). Furthermore, DGT accurately predicted P availability in soils with a range of pHs from 5.5 to 8.6 including two calcareous soils with high Ca content (24 and 26%) (Mason et al., 2013) and thus may not be influenced by the effects of liming. Further investigations into the accuracy of existing soil P measurements in limed soils would be useful in generating more accurate liming recommendations.

If liming limits yield through reduced P availability perhaps the most appropriate recommendation is not to apply lime to soils exhibiting this response. In the field trial (Chapter 2), maximum pod yield occurred in the unlimed treatments at a soil pH just above 5.5 and declined with increased pH with lime addition (Figure 2.9). Similarly, lime dose response curves in pot trials (Figure 2.14) demonstrate liming at any rate in this particular soil could impair plant growth. This implies that liming to raise soil pH much higher than pH 5.5 (the threshold for Al and Mn toxicity) may not be necessary to maximise yield (Sumner and Yamada, 2002). However, maximum yields of *Vicia faba* were achieved when liming raised soil pH to 6 even though the effects of Al toxicity were removed at pH 5.2 (Farhoodi and Coventry, 2008). Though no plant nutrient analysis was undertaken in this study, it was thought the additional yield response above the Al toxicity threshold was mediated by improved nutrient availability thus possibly demonstrating the potential benefits of liming above Al

toxicity thresholds. Therefore, if P status of the crop could be maintained, liming to higher pH values (i.e. 6.5) might still be an important agronomic strategy in optimising yield, even in lime sensitive soils.

One thing that is uncertain from the current experiments is how long any negative impact of liming will last. Time constraints only allowed for a single year of field trials, so although the negative effects of liming are clearly apparent within the same growing season as lime application, longer term field trials are required to understand the impacts on crop P nutrition in subsequent rotations. Intriguingly, should restoration of plant tissue P status occur at the end of the growing season due to root exudates increasing P availability (see Dakora and Phillips, 2002 for review); legumes might actually be the most appropriate crop to grow in the first season of a rotation cycle after liming by mobilising available P for subsequent crops (Nuruzzaman et al., 2005).

Another aspect of legume physiology that has not been directly addressed in this thesis and one that is perhaps relevant to soil pH management is root nodulation. Within the pot trials nodulation was checked at harvest but was consistently absent, most likely owing to the age of the plants (<3 weeks) and that the soil was routinely sterilised before use. Therefore nodulation was not considered to be an important factor mediating the observed gas exchange and growth responses. Nodulation will most have likely occurred in the *Vicia* field trial, though this was not assessed. However, symbiotic N fixation is affected by soil pH (Bordeleau and Prevost, 1994) and legume nodulation occurs best at neutral to slightly acidic soils (Brockwell et al., 1991, Zahran, 1999). Therefore liming is generally considered to be beneficial to

nodulating bacteria (Buerkert et al., 1990, Grewal and Williams, 2003) and is perhaps an additional argument for why liming may still be important in lime sensitive soils.

In conclusion, this thesis has added new insights into the previously unexplored physiology of yield suppression following liming and provided a better mechanistic understanding of the processes regulating gas exchange and growth under a lime-induced P deficiency. In elucidating some of these mechanisms, questions have been raised about the appropriateness of current guidance for soil pH management, particularly with respect to effects on soil P availability. While not unknown globally (Haynes, 1982), there appears to be little understanding of this phenomenon in UK soils, and if more prevalent than the example presented here, may represent a hidden yield penalty in UK agriculture. In light of additional future pressures on crop production from limited supplies of supplementary nutrients, liming recommendations that limit crop yield are clearly not acceptable. Further research is therefore needed to better understand the relationship between liming and nutrient availability in a wider range of soils and crop species so that soil pH management in future sustainable agricultural approaches can be more appropriately applied.

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