AGRO-PROCESS INTENSIFICATION USING NANO-STRUCTURED MICRO-POROUS POLYMERS AS SOIL ADDITIVES TO ENHANCE CROP PRODUCTION

A thesis submitted to the University of Newcastle-upon-Tyne
for the Doctor of Philosophy

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AUTHOR'S DECLARATION

This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at University of Newcastle, United Kingdom. All the studies described within are solely my work unless otherwise stated, and were undertaken at the School of Chemical Engineering and Advanced Materials under the guidance and supervision of Professor Galip Akay.

I certify that none of the material offered in this thesis has been previously submitted for a degree or any other qualification at the above or any other university or institute.

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ABSTRACT

Polymerised High Internal Phase Emulsion Polymer (PHP) is a nano-structured micro-porous polymeric material with a variety of applications. PHPs may have a role to play in sustaining or enhancing crop yields in increasingly alien environments. An elastic hydrophilic version has been developed that has been shown to increase crop yields when used as a soil additive. Soil is a natural carrier of water and nutrients as well as bacteria all of which are widely distributed in the soil and inefficiently maintained and utilised by plants. When polymer is added to the soil, water is attracted to the polymer because of its hydrophilic nature, then roots are attracted to the water and nutrients (if present) in the polymer and they become intimately associated with PHP which therefore brings the plant into close proximity with any fertiliser and bacteria loaded into the polymer. Hence the polymer promotes the interactions between water/nutrients/bacteria/plant roots as well as root exudates within microscopic scale acting as a synthetic rhizosphere which benefits the plant in three ways: (1) Efficient water utilisation and conservation by the plant thus allowing plants to grow in drier environments than would otherwise be possible. The dry weight of soybean shoots with PHP added was increased over 100% compared to plants with no PHP. (2) By adding a fertiliser component to the polymer it can act as a slow release fertiliser, releasing the fertiliser in close proximity to the plant roots, so a larger proportion is utilised by the plant rather than being leached away as normally happens when fertiliser is added direct to the soil. By modifying the production method of PHP so that it contained ammonium sulphate, a major component of many fertilisers, the dry weights of soybean shoots and pea shoots were increased by 66% and 48% respectively after 6 weeks growth. (3) By loading the polymer with beneficial bacteria and fungi, in particular nitrogen fixing bacteria and mycorrhizal fungi, it then offers a protective environment for the organisms which then have a competitive advantage over other soil organisms so their numbers can increase enabling them to make a significant contribution to the nutrient requirements of the plants, in particular nitrogen. The addition of PHP soaked with *Azospirillum brasilense* broth produced a dry weight increase in grass shoots of 9.6%, 9.5%, 40% and 145% after 3, 6, 9 and 12 weeks growth respectively compared to plants with no PHP or bacteria.

PUBLICATIONS ARISING FROM THIS THESIS

Akay, G. & Fleming, S. (2011) 'Engineered Ecosystem Development for Agro-Process Intensification', in Villacampa, Y. & Brebbia, C. A. (eds) Ecosystems and sustainable development VIII. WIT Press, Southampton, pp 485-495.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABREVIATIONS

Azo	Azospirillum
Bin.	Bindzil
BNF	Biological Nitrogen Fixation
CFU	Colony Forming Unit
CHN	Carbon Hydrogen Nitrogen
CI	Confidence Interval
Cl.	Clover
DVB	Divinyl Benzene
E	East
EDX	Energy Dispersive X-ray spectroscopy
EHA	Ethylhexyl Acrylate
ENP	Engineered Nano Particles
E. On.	Early Onward (pea variety)
GM	Genetically Modified
HA	Hydroxy Apatite
HIPE	High Internal Phase Emulsion
K	Potassium
Myc.	Mycorrhiza
N	Nitrogen
P	Phosphorus
PAM	Polyacrylamide
PBS	Phosphate Buffer Saline
PD	Potato Dextrose
PDA	Potato Dextrose Agar
Ph	Phoenix (pea variety)
PHP	**P**oly **H**igh Internal Phase Emulsion **P**olymer
PU	Poly Urethane
Rh.	Rhizobium
SAP	Super Absorbent Polymers
SE	South East
SEM	Scanning Electron Microscope
SOM	Soil Organic Matter
VAM	Vesicular-Arbuscular Mycorrhiza
Zyg.	Zygomycete

Chapter 1: Literature Review

1.1 Introduction

Producing enough food to feed a rapidly increasing world population is becoming an urgent problem which requires implementation of diverse strategies to increase crop yields in increasingly alien environments. Global population has grown from 2.5 billion in 1950 to 5.7 billion in 1995 and it is expected to reach 8.3 billion by 2025 (Bockman, 1997), and various estimates suggest it could reach 10 - 11 billion by 2050 (Figure 1) (Parry & Hawkesford, 2010; Bhalla, 2006).

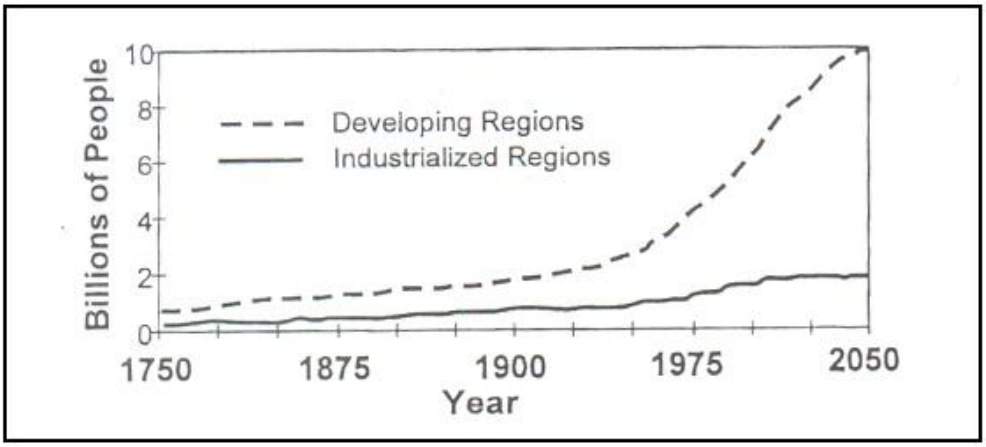


Figure 1: Projected population increase until 2050 (Welch and Graham, 1999)

By far the most rapid increase is in developing countries where the population is estimated to increase by approximately 40% by 2050 but in developed countries it is expected to remain fairly stable at just below 2 billion. This equates to the population expanding by around 100 million/year which, by 2025 will require about 2 billion more tons of grain to feed the extra population (Swaminathan, 2007).

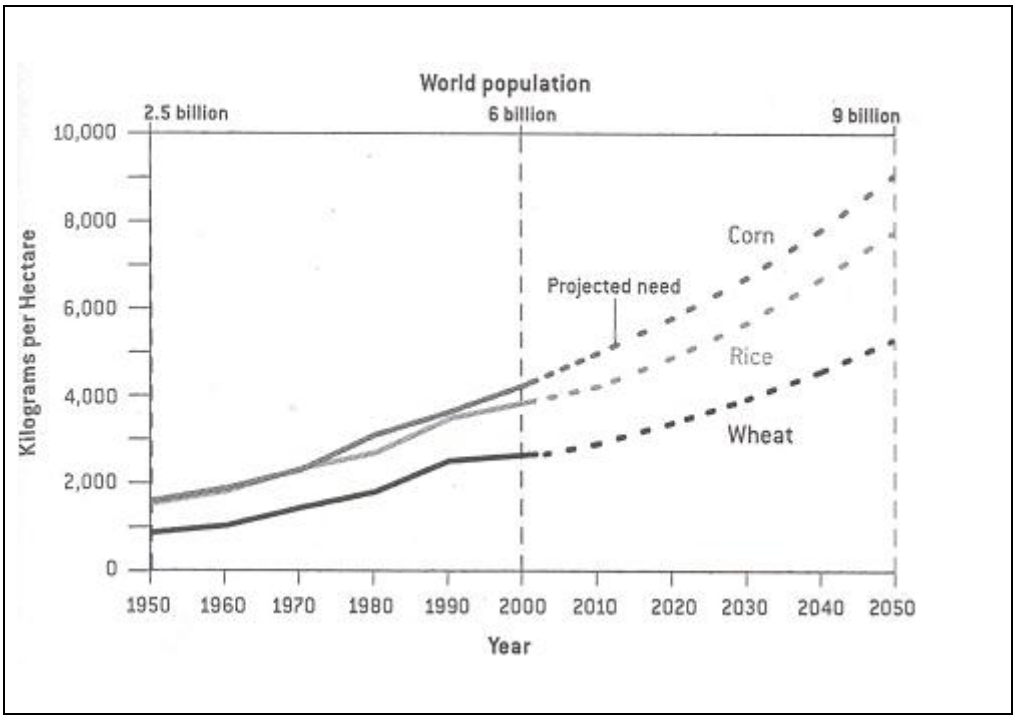


Figure 2: Projected cereal yields required to feed the expanding population (Goff & Salmeron, 2004)

Projected figures for grain yield in 2050 show that it will have to more than double in order to feed the increased population (Figure 2), (Parry & Hawkesford, 2010; Goff & Salmeron, 2004). This will be a difficult target to reach because despite all the advances in agricultural technology and biotechnology, per capita grain production has continued to decrease for the last 20 years. Some estimates suggest that the maximum attainable world grain production in 2050 is likely to be 3300M tons – only 60 % above the 1996 - 2000 average (Gilland, 2002).

Agricultural productivity has increased dramatically over the last 3 centuries, by advances in a combination of factors including artificial fertiliser production, new high yielding strains of cereal grains, powered farm equipment, irrigation, more effective control of insects and diseases, genetic engineering, intensive livestock farming methods and improved strains of livestock. These developments have enabled food supply to keep pace with world population growth (McMichael *et al*, 2007) but as the population continues to grow, more diverse strategies must be found in order to try to continue increasing crop production at an adequate rate to feed the extra mouths.

At the same time as demand for food is increasing, the area of land available for agricultural production is progressively being limited by increased urban development, degradation of land by erosion & salinisation, use of cropland for bioenergy and an increase in the area of marginal, semi arid areas due to the effect of climate change (Godfray *et al*, 2010; Parry & Hawkesford, 2010).

1.2 Climate change

The earth's climate is rapidly changing and the reasons are predominantly an increase in greenhouse gases caused by human activity, mostly as a result of burning fossil fuels, deforestation and intensive agriculture. The temperature has increased by 0.7 ^{0}C since 1900 and the 10 warmest years on record have all been since 1990. It is estimated that the temperature could increase by a further 1-2^{0}C by 2100 (Stern, 2007). Increasing temperature will cause oceans to warm, glaciers to melt, causing sea levels to rise flooding low lying coastal areas and contaminating them with salt water. A warmed atmosphere heats the oceans which lead to more evaporation and more moisture held in the atmosphere so when it rains it frequently produces heavier downpours. Higher temperatures on land also produce more arid areas (Epstein, 2000). Weather patterns will shift and become more erratic with more severe storms more flooding and more droughts (Yuksel, 2008).

Probably the most important contributor to climate change by human activities is the amount of fossil fuel carbon we release into the atmosphere as the greenhouse gas CO_2 (Lenton, 2006). Greenhouse gases move up to the upper atmosphere where they allow the sun rays to pass through but prevent the heat radiation from re-emerging in the same way as the glass of a greenhouse (Yuksel, 2008). Burning fossil fuels – coal, oil and gas provides approximately three quarters of the world's energy at present and at the same time produces greenhouse gases responsible for climate change (Yuksel, 2008). Agriculture is also a contributor with approximately one third of greenhouse gases coming from agricultural activities including the operation of agricultural machinery and also from the production of the fertilisers and sprays which requires large amounts of energy. Nitrous oxide is a potent greenhouse gas which escapes to the atmosphere from nitrogenous fertilisers. Livestock also contribute by producing large volumes of methane which is a potent greenhouse gas. Climate change will result in winners and

losers – by 2020, crop yields could increase by 20% in E & SE Asia, but decrease by up to 30% in central & south Asia and rain-fed agriculture could drop by 50% in some African countries (McMichael *et al*, 2007). This will be mainly due to rainfall patterns changing producing more semi-arid areas unable to sustain production at present levels. Other contributors to loss of productive land are wind and water erosion. Yearly, more than 10 million ha of valuable crop land are degraded and lost because of wind and water erosion of soil (Pimentel and Pimentel, 2006). It has been estimated that about 3.5 billion hectares of land, or almost 30% of the total land surface has now been degraded by human activity (Huttermann *et al*, 2009). Deforestation is extensive in some countries to replace lost land but this also contributes to climate change by increasing the CO_2 levels and it is often only a short term solution as it can lead to extensive soil erosion. It can also contribute to changing weather patterns because the loss of large areas of leaf canopy results in large amounts of evapotranspiration being lost resulting in reduced atmospheric moisture and therefore less rainfall. In large and increasing areas, water is becoming a major constraint on crop yield. Climate change is predicted to continue changing rainfall patterns and it is estimated that by 2050 65% of the global population will live in areas where water is scarce (Parry & Hawkesford, 2010).

Various strategies have been and are being pursued to enhance crop yield in these increasingly alien environments including plant breeding; the use of fertiliser, including biofertilisers; genetic engineering; water management, including irrigation, water recycling, salt water tolerance, desalination and the use of soil conditioners.

1.3 Strategies to enhance crop yield

1.3.1 Plant Breeding

99% of today's agricultural production depends on only 24 different domesticated plant species with rice, wheat and maize accounting for most of it (Goff and Salmeron, 2004). Cereal grains comprise about 80% of world's human food intake and to keep pace with population increase, grain yield will have to increase by 1.5% more every year on a diminishing supply of cultivated land. Ever since our farming ancestors began settling down 10,000 years ago, they have been improving the crops by selectively propagating and crossbreeding plants with desirable traits such as bigger grains, more seeds, plants

that do not drop their seeds, more nutritious seeds, shorter straw, pest & disease resistance, maturation speed, tolerance to drought, more efficient utilisation of fertilisers and higher nutrient content. Maize average yield per acre has increased by nearly 400% since 1950 (Goff and Salmeron, 2004).

Modern methods employed by plant breeders allow the breeding process to be massively speeded up. Several plant genomes have been fully sequenced and tens of thousands of genes have been identified. Large areas of genetic maps in different plants are very similar, so discovery of a gene in one plant species is highly likely to correspond with the same gene in another species. The function of a gene can be ascertained by searching databases of known genes to find a match which allows researchers to predict the gene's function. The function can then be determined by inserting a mutation into the gene which stops its activity and see what effect it has on the plant. Plant breeders produce tens of thousands of seedlings, but instead of having to grow each plant for a whole season to see if a trait has been inherited, a sample of each seedling's DNA is examined to see if it contains genes for the trait being investigated. Only seedlings containing the desired allele will be grown on and crossed with a superior strain with other desired characteristics and the progeny would then be tested in the same way. This speeds up the breeding process massively because many thousands of plants do not have to be grown for whole seasons to find only a few with the desired trait (Goff and Salmeron, 2004).

1.3.2 Fertiliser

Agricultural crops require a wide range of essential nutrients for growth, all of which come from the soil except carbon which comes from CO_2 in the atmosphere. There are 16 essential nutrients for plant growth – carbon dioxide from the atmosphere, hydrogen and oxygen from water and all the others come from the soil. Nitrogen (N), phosphate (P) and potash (K) are primary nutrients and their availability is normally managed through the use of fertilisers. Calcium (Ca), magnesium (Mg) and sulphur (S) are secondary nutrients which can be added in fertilisers if testing indicates they are specifically lacking, but can be available from other sources e.g. liming or manure. Micronutrients or trace elements are only required in small quantities (5-100ppm) and are normally available in sufficient quantities in the soil but can be added if specifically

required. These include boron (B), chloride (Cl), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), zinc (Zn) and cobalt (Co).

One of the main reasons crop yields have increased in the last 50 years has been increased use of artificial fertiliser, but it is expensive to produce, using non-renewable energy and it contributes to global warming by emitting vast quantities of CO_2 estimated to be 1% of all greenhouse gases (Aldhous, 2008). Fertiliser is produced by the Haber-Bosch process which produces ammonia from nitrogen and hydrogen. This reaction is carried out at very high temperatures (400 - 650^{0}C) and pressures (200 - 400 atmospheres) which therefore makes it very expensive.

Nitrogen fertilisation is one of the most expensive inputs in agriculture but a large proportion of the applied fertiliser is never of any benefit to the plant. It has been estimated that up to 65% of all applied mineral nitrogen is lost from the soil without ever benefiting the plant, through surface run off, leaching, denitrification and volatilisation (Bhattacharjee *et al*, 2008; Raun & Johnson, 1999). Nitrogen can be converted to greenhouse gases ammonia (NH_3), nitrous oxide (N_2O) and nitric oxide (NO). Nitrous oxide is a potent greenhouse gas which is 300 times more potent than carbon dioxide (Aldhous, 2008). Nitrogen in its NO_3^- form is a very mobile ion and NO_3^- leaching from the soil is one of the primary sources of contamination in drinking water (Follett and Delgado, 2002). In some areas it has become necessary to limit the amount of fertiliser applied to keep the levels of nitrates in drinking water below acceptable levels. Efforts to reduce this waste are being developed in the form of slow release fertilisers.

1.3.3 Slow release fertiliser

One method to improve the utilisation of fertiliser and reduce waste and pollution is by the use of slow release fertilisers. These can be in several forms including:

- Fertiliser is held in a matrix and must diffuse out through pores to be released into the soil.
- Chemically controlled release products like urea-formaldehyde.
- Inorganic compounds with low solubility such as rock phosphate

- Fertiliser coated with an inert material which can include inorganic material such as sulphur, phosphates or silicates; synthetic organic substances like polyethylene; or natural organic materials like resin, rubber or wax (Liang & Liu, 2006).

The use of artificial fertilisers has one of the biggest effects on crop yields, but it is expensive to produce, using non-renewable resources and they are not available or affordable in developing countries so more affordable, sustainable alternatives must be found to increase production in these places. The need to find a more sustainable, environmentally friendly and less expensive alternative to artificial fertiliser has increased interest in biofertilisers.

1.3.4 Biofertiliser

A strategy to further increase crop yields which is nowhere near its full potential is the use of biofertilisers. Soil is a living environment that contains many and varied organisms. It has been estimated that 1 gram of soil can contain up to 10^{10}-10^{11} bacteria (Horner-Devine *et al*, 2003), 6000-50000 bacterial species (Curtis *et al*, 2002) and up to 200 metres of fungal hyphae (Leake *et al*, 2004). Most of these organisms are harmless, but some have a big impact on plant productivity - either pathogenic organisms that have a detrimental effect, or beneficial organisms that stimulate plant productivity by supplying limited nutrients to the plant (Van der Heijden *et al*, 2008). The beneficial organisms do not normally have a very significant effect on the growth rate of plants because of the competition from all the other organisms, but if the numbers of the beneficial organisms could be enhanced then they could make a significant contribution to the nutrient requirements of the plants. Beneficial organisms can be divided into two main groups: nitrogen fixing bacteria and mycorrhizal fungi.

1.3.4.1 Nitrogen (N) fixing bacteria

The most common nutrient that affects crop yield is nitrogen, which makes up 78% of the earth's atmosphere but it is not available to the plant because they cannot utilise it directly from the atmosphere. However, nitrogen fixing bacteria have evolved the ability to convert atmospheric nitrogen from the air into ammonium nitrogen that can be utilised by the plant and therefore do not require nitrogen to be available in the soil.

Nitrogen fixing bacteria can be divided into two groups: symbiotic nitrogen fixing bacteria which form symbiotic relationships with leguminous plants and free living nitrogen fixing bateria which do not form associations with any specific plants.

Symbiotic nitrogen fixing bacteria

Symbiosis is a relationship between two biological species in which both organisms gain benefits from each other. Leguminous plants and nitrogen fixing bacteria have evolved the ability to form symbiotic relationships in which the bacteria provide the plant with a more sustainable source of nitrogen in the form of nitrates and in return the bacteria obtain energy from the plant. Leguminous plants include peas, beans, lentils, lupins, clover, alfalfa and peanuts. The most important biological nitrogen fixing (BNF) associations occur between leguminous plants and several species of the *Rhizobaceae* family including *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium* and *Mesrhizobium* (Garg, 2007). These associations are very specific - one species of bacteria will only form associations with a specific legume, eg *Rhizobium leguminosarum* with peas, *Bradyrhizobium japonicum* with beans or *Rhizobium trifoli* with clover. The bacteria grow in nodules formed on the roots of leguminous plants. Control of nodulation and N fixing is a complex process involving the coordinated expression of approximately 50 genes from both the leguminous plant and the bacteria (Postgate, 1998). Plant roots secrete exudates which act as chemoattractants for the bacteria. The rhizobia then bind to the plant roots by weak bonds between plant lectins and bacterial exopolysaccharrides followed by tight irreversible bonds with bacterial cellulose which forms bacterial aggregates on the root surface. Flavonoids produced by the plant initiate a series of steps that regulate rhizobial nod genes which then trigger reciprocal signals to the plant root. Small molecules called nod factors released by the bacteria are detected by the plant which triggers the formation of the nodule. Lipo-chito-oligosaccharidic nod-factors are excreted by the rhizobia which are an important factor in root hair curling and infection thread formation (Ladha and Reddy, 2003). The tip of the root hair curls round the bacteria trapping them in a pocket. The root hair wall is then dissolved by enzymes at a localised area allowing the bacteria to enter. An infection thread grows into the cortex of the root which stimulates the cortex cells of the inner root to develop and rhizobia inside the infection thread grow and divide keeping it filled with bacteria. The type of nodule depends on the host plant – soya beans produce nodules with determinate growth

– bacteria are released into dividing cells, so they are distributed through the cell mass of the nodule. Cells then cease to divide and can only increase nodule size by cell enlargement, producing a round shaped nodule. Peas produce nodules with indeterminate growth – they contain a meristem and can continue to grow over long periods producing lobed structures with cells of different ages, the oldest nearest the axis of the root (Dixon and Wheeler, 1986). Nod factors differ between different rhizobia species and it is these differences that to a large extent make them species specific (Garg, 2007; Dixon and Wheeler, 1986). Nod factors induce the legume host to express many genes that initiate the development processes that lead to nodule formation. There are many plant coded genes - nodulin genes involved in nodule development and leghaemoblobins which are structural proteins which must be coordinated in the correct sequence for the correct formation of nodules. Initially nitrogen is reduced to NH_4^+ which then becomes a substrate for ammonia assimilation pathways involving a series of plant enzymes (Shantharam and Mattoo, 1997). Nitrogenases are the enzymes that fix nitrogen. They are composed of two protein sub-units and are sensitive to oxygen so the oxygen concentration must be kept low to allow the nitrogenase to function. This is achieved by a protein called leghaemoglobin in the nodules which is similar to haemoglobin in mammalian blood and gives the nodules a pink colour. It has a high affinity for oxygen, the same as haemoglobin, and by binding to oxygen it keeps the oxygen concentration in the nodules low and allows the nitrogenase to work. The nodules themselves also provide a barrier which slows the diffusion of oxygen into them and therefore keeps the oxygen concentration in the nodule low (Postgate, 1998).

Leguminous plants have a very efficient nitrate uptake system which may be preferred to the energy intensive BNF process performed by the bacteria. So when N is available in a utilisable form in the soil, the plant uses this rather than forming a symbiotic relationship with the bacteria. Legumes consume approximately 10% of the plant's net photosynthesis output for N fixation so N fixing is a physiological burden on the plant. Nitrogenase requires 36 ATP molecules for every N molecule reduced (Shantharam and Mattoo, 1997).

A possible way of enhancing crop yield may be to modify the balance between fertiliser and symbiotically fixed N so the plants still produce nodules and fix N even when some

fertiliser is added. The presence of artificial nitrogen generally inhibits the nitrogen fixing activities of BNF bacteria, because they use the nitrogen from the fertiliser rather than the fixing nitrogen from the air which is more energy intensive. If the bacteria could be modified so that they continue to fix nitrogen even when artificial nitrogen is available, then a combination of the 2 nitrogen sources could substantially reduce the artificial fertiliser requirement (Saikia & Jain, 2007; Graham and Vance, 2000).

The benefits of using leguminous plants as part of a crop rotation have been known since Roman times, with Varro, an early Roman agriculturalist in 37 BC advocating that "legumes should be planted in light soils not so much for their own crop as for the good they do to subsequent crops." (Fred *et al*, 1932). In modern agriculture legumes are still used in crop rotation where the subsequent crop benefits from the decomposition of any crop residues and roots of the legume releasing nitrogenous compounds which have originated from nitrogen from the atmosphere. In some agricultural systems the practice of "green manuring" is applied, where a legume such as clover is grown till flowering stage then cut and left to rot on the land. Nitrates from the decomposing material then become available for the subsequent crop. The process also helps soil and water conservation and weed control so it is of use in organic farming as well as in countries where access to fertilisers and herbicides is not available.

Free living nitrogen fixing bacteria

There are several varieties of non-symbiotic or free living nitrogen fixing bacteria including *Azospirillum, Azotobacter, Bacillus, Klebsiella, Burkholderia* and *Pseudomonas* (Hayat *et al*, 2010). Although *Rhizobium* forms symbiotic relationships with leguminous plants, it can also survive as a free living organism and therefore benefit non-leguminous plants as well (Hayat *et al*, 2010). These organisms can fix nitrogen from the air without any specific associations with plants. They inhabit the region around plant roots which is known as the rhizosphere. The rhizosphere is relatively rich in nutrients caused by the loss of as much as 21% of plant exudates from the roots into the surrounding soil. The rhizosphere supports a large population of microbes which can be 10 – 1000 times higher than the numbers in soil remote from plant roots (Lugtenberg & Kamilova, 2009). A rich flora of nitrogen fixing bacteria can be found in the rhizosphere of all plants. Rhizospheric bacteria live in the rhizosphere around the plant roots. Other species are endophytic which live within the roots of the

plant but are not plant specific and do not form root nodules. Endophytes live in a more protected environment where they are less vulnerable to competition from other soil bacteria, they benefit more directly from nutrients from the plant and the lower oxygen levels enhance nitrogenase activity (Bhattacharjee *et al*, 2008; Vessey, 2003). In return, the plant benefits from the nitrogen fixing activities of the bacteria as well as other growth promoting substances. The important difference between these bacteria and *rhizobia* which form root nodules with specific plants is that they are not plant specific so their presence will benefit any plant including commercially important food crops like wheat, rice and sugarcane so their use can reduce the requirement of synthetic fertiliser. For example, by inoculating rice with *Rhizobium trifoli*, researchers were able to reduce the fertiliser application to one third of normal rate to obtain the equivalent grain yield (Yanni *et al*, 1997). The contribution of nitrogenous compounds to the plants by free living bacteria is less significant than legume-*rhizobium* associations because the lack of direct contact in the form of nodules full of bacteria means the utilisation of any fixed nitrogen is less so the benefit to the plant is less. The bacteria also benefit less from nutrients from the plant because of their less intimate association with the plant compared to nodule forming bacterial associations. But the major benefit of free living bacteria is that they can potentially benefit any plant.

Bacteria can also increase crop yields by other mechanisms as well as nitrogen fixation including production of growth hormones (indole acetic acid, gibberellins and cytokinins), iron sequestering siderophores and phosphate solubilising enzymes, suppression of plant disease by competitive exclusion of plant pathogens & production of antibiotics (Andrews *et al*, 2010; Verma *et al*, 2001). Growth hormones produced by the bacteria enhance the development of lateral roots and increase the density and length of root hairs and therefore improve nutrient uptake from the rhizosphere (Dobbeleare *et al*, 2001).

Rice is the major food crop for nearly half the world population and it requires large amounts of nitrogen to maximise yield. Urea is the most common nitrogen source but only 30-40% of the applied nitrogen is actually utilised by the plant with the rest being lost by denitrification, leaching and volatilisation (Choudhury & Kennedy, 2004). The use of bacterial inoculants including *Azotobacter, Clostridium, Azospirillum, Herbaspirillum, Burkholderia* and *Rhizobium* have been shown to supplement the use of

urea. Inoculation with *Azospirillum* has been shown to increase rice yield by 32-81% in greenhouse experiments although in field conditions the increase was 22% (Choudhury & Kennedy, 2004). Studies using ^{15}N isotope showed that up to 47% of the nitrogen requirement of the rice plants could be met by biological nitrogen fixation (Choudhury & Kennedy, 2004). Recently, *rhizobia* have been shown to enhance yields of wheat, rice, maize and barley without forming root nodules (Mia & Shamsuddin, 2010).

1.3.4.2 Mycorrhizal fungi

Mycorrhizal fungi are present in most soils and form symbiotic associations with 80% of all terrestrial plant species (Smith & Read 1997). Unlike the rhizobium/legume symbiotic relationship which is very specific with one species of bacteria only infecting one specific plant species, the mycorrhizae can infect most plants and therefore can be a benefit to most plants. The fungus increases the surface area of the roots which enhances water and nutrient uptake by the plant and therefore benefits plant growth. In return, the fungus obtains nutrients and carbon compounds from the plant. The fungi obtain nutrients through their extensive fungal network by excreting a wide range of extracelluluar enzymes that can degrade organic matter (Makoi & Ndakidemi, 2009). They can therefore colonise areas with low nutrient availability but where organic matter is available in the form of litter and humus. Figure 3 shows mycorrhiza (white) growing on the roots (brown) of a pine tree seedling. The presence of the fungus increases the surface area of the root system in contact with the soil by many hundred fold and therefore enhances water and nutrient absorption as well as increasing soil stability (Andrews *et al*, 2010).

Figure 3: Mycorrhizae on pine seedling roots (Rosling, 2009)

The mycorrhizae are especially efficient at absorbing phosphate from the soil and can have a big impact on plant growth in low phosphate soils (Bolan, 1991). Some studies have shown that mycorrhizal fungi can enhance P uptake by up to 90% (Van der Heijden, 2008). Uptake of other nutrients can also be enhanced, including copper, zinc and iron. Recent work has indicated that mycorrhizae also have a role to play in the uptake of nitrogen with up to 50% of some plants nitrogen requirements being supplied by mycorrhizae (Miransari, 2011). In some nutrient poor soils, the dry weight of plants inoculated with mycorrhizae have been increased by up to 50 times compared to uninoculated plants (Jeffries, 1987). Inoculation with mycorrhizae may therefore in some soils be more effective than high cost artificial fertiliser. The presence of mycorrhizae also makes plants less susceptible to drought because of the larger surface area of hyphae in close contact with the soil (Jeffries, 1987). The presence of the protective mycelia around the plant roots also confers some resistance to root pathogens. This has been attributed to physical protection of the mycelial barrier, secretion of antibiotics which inhibit the pathogen and surplus nutrients in the root are utilised by the fungus and are therefore not available to the pathogen. There are two types of mycorrhizae that are of agricultural importance – ectomycorrhizae and endomycorrhizae. Ectomycorrhizae are mainly found in temperate forests and the mycelia of these fungi form a dense sheath over the roots (Figure 4), with a few hyphae penetrating the root surface and growing between the cortical cells of the root.

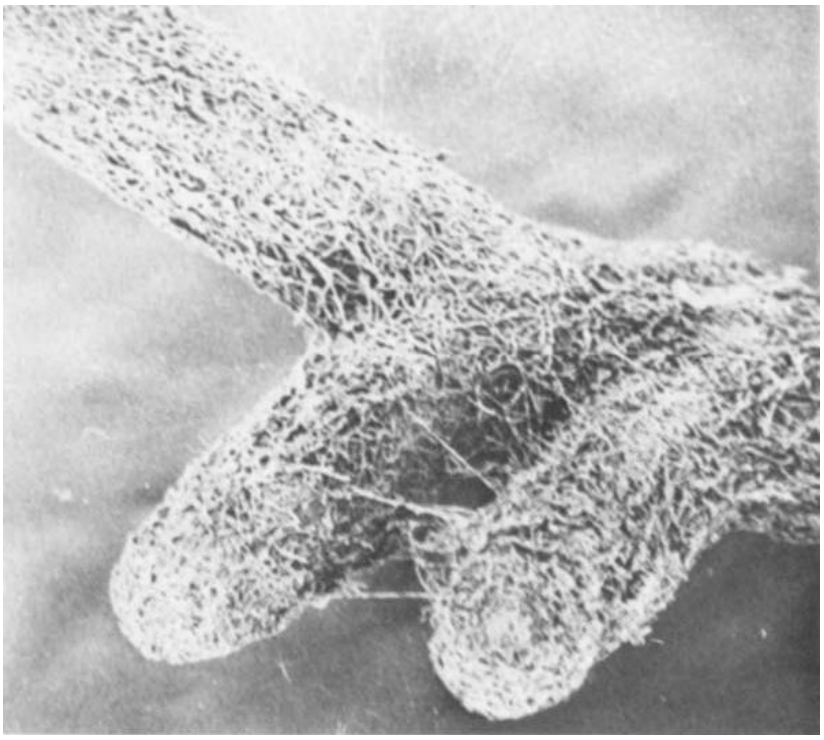


Figure 4: SEM of ectomycorrhizal hyphae covering birch root (mag. X40) (Jeffries, 1987)

Endomycorhizae are the most common type with around 80% of all plants having some association with this group (Peterson & Massicotte, 2004). They do not form a sheath around the plant roots but they penetrate the cortex cells of the roots and form vesicular and arbuscular structures within the root cells (Figure 5).

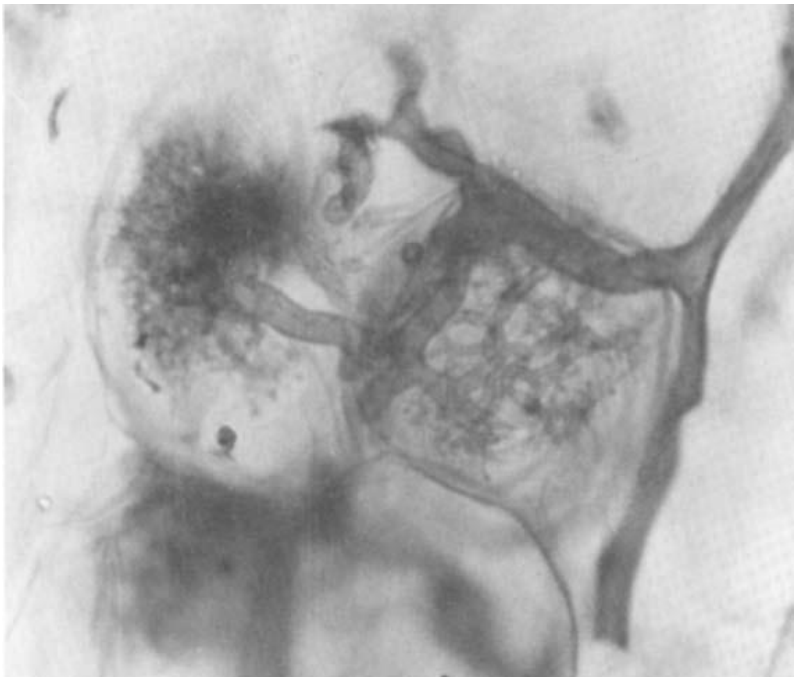


Figure 5: Arbuscule growing inside a root cell (Jeffries, 1987)

They are also known as vesicular-arbuscular mycorrhizae (VAM). Nutrients are absorbed from the plant roots into the fungal mycelia via the arbuscules.

By increasing the numbers of these beneficial bacteria and fungi in the soil, they can act as a biofertiliser, making a positive contribution to the nutrient requirements of the plant. Numbers can be enhanced by inoculating the soil with suitable strains of these organisms.

1.3.5 Inoculation

Nitrogen fixing bacteria and mycorrhizae occur naturally in the soil but competition from large numbers of other bacteria means they do not have a major impact on crop yield. Their numbers can be enhanced by inoculating the soil with appropriate strains of nitrogen fixing bacteria or mycorrhizae. The benefits of inoculation have been known for more than 100 years with the first commercial inoculants of *rhizobium* being introduced in the 1890s (Fred *et al*, 1932). Inoculation is particularly important in dry, tropical areas where the survival of nitrogen fixing bacteria in the soil is poor. An inoculant must provide a suitable environment for the bacteria to survive when introduced into the soil and any inoculated strain must be able to compete successfully with naturally occurring bacteria already in the soil, which is not always the case because native organisms are often better adapted to the local conditions than inoculated organisms. Suitable strains to use as inoculants must be able to compete effectively with indigenous bacteria in the soil.

An effective inoculant must have a suitable carrier that can support viable bacteria of an appropriate strain, and can be effectively stored (Graham & Vance, 2000). Several different types of carriers for the inoculant can be used. An ideal carrier should be a uniform, sterile, stable, non-toxic, easily handled substance with a reasonable shelf life (Bashan, 1998). Peat based inoculants are the most common type of carrier used but they have the drawbacks that the quality can be variable, it gives limited protection to the bacteria and it is not readily available in the tropics.

Polymer based inoculants have been developed which encapsulate the bacteria and therefore offer it more protection against environmental extremes (Fernandes *et al*, 2009). The polymer is degraded by soil microorganisms when it is put into soil. They

have the added advantage that it can be stored over longer periods without refrigeration which is not normally available in many tropical climates. Nutrients can also be added to the capsules which enhance the survival time of the bacteria. The major disadvantage compared to peat carriers is that they are more expensive.

Alternative methods of inoculating include coating the seed with bacteria before planting either with a slurry or powder form, or applying the bacteria as a liquid to the soil at the time of planting but these methods offer no protection against drought, high temperatures or competition from other native bacteria, but they are easier methods for the farmer to apply (Bashan, 1998; Smith, 1992).

Mixed inoculants can produce a synergistic effect where the combined effect of several different organisms produces a higher percentage increase than any of them separately. Govindarajan *et al* in 2008 reported an average over 6 experiments of 14.4% increase in rice yield using a mixed culture of 5 species (*Burkholderia vietnamensis* MGK3, *Azospirillum lipoferum, Gluconacetobacter diazotrophicus, B. vietnamensis* and *Herbaspirillum seropedicae*), compared to increases of 2.6% to 9.36% for each individual strain. *Azospirillum* has also been shown to act as a 'helper' bacterium for *rhizobium*, stimulating greater nodulation and nodule activity and enhancing crop yield. Some commercial inoculants are available containing both *Rhizobium* and *Azospirillum* but have been reported to give varying results (Graham & Vance, 2000; Dobbeleare *et al*, 2001). Co-inoculation with more than one species of bacteria can enhance crop yield where each species provides different benefits, for example nitrogen fixing, phosphate provision, or provision of other minerals (Bashan, 1998). Co-inoculation of nitrogen fixing bacteria with mycorrhizae has also been shown to produce a synergistic effect with enhanced mycorrhizal growth and increased uptake of minerals (Bashan, 1998).

In general, shortly after inoculation, the population of the inoculated bacteria declines progressively, preventing the build up of sufficient numbers to have a positive effect on plant growth (Bashan & Levanony, 1988). When bacteria are inoculated in a solid carrier like peat, they must migrate through the soil for which a continuous film of water is necessary so it is not possible in dry conditions. They also face competition from native microflora (Bashan, 1998). Poly High Internal Phase Emulsion Polymer (PHP) may be able to provide a protective environment where the inoculated bacteria

will be able to become established and compete successfully with the native organisms and therefore make a positive contribution to the nutrient requirements of the plants.

1.3.6 Genetic modification

From 1996-2005 the area of land growing genetically modified crops increased from 4.2 million acres to 222 million acres (Anon, 2006), with the largest proportion (55%) in the United States. Growth has been increasing rapidly in recent years in other countries including Brazil (soybeans, maize), India (cotton), Argentina, Canada, China and South Africa.

Genetic modification is being used to enhance crop production in many ways as well as increasing crop yield:

- Soybeans have been genetically modified to be resistant to glyphosate herbicides which kill all actively growing plants, so all weeds will be destroyed leaving only the soya beans growing (Cerdeira *et al*, 2011).
- Cotton has been produced which is insect resistant and therefore reduces losses from insect predation (Morse *et al*, 2007).
- Rice has been produced with elevated vitamin A levels which will alleviate vitamin A deficiency in millions of people (Tang *et al*, 2009).
- Bananas that produce human vaccines against infectious diseases like hepatitis B (Sharma & Sood, 2011).
- Plants that produce biodegradable plastics (Mooney, 2009).
- Monsanto are engineering maize plants that can tolerate drought conditions (Aldhous, 2008).
- Arcadia from California has produced a GM salt tolerant strain of alfalfa and is working on salt tolerant strains of rice, cotton, tomatoes and oilseed rape (Aldhous, 2008).
- Arcadia has also produced a GM oilseed rape that boosts the ability of the plant to take up nitrate from the soil and claim that the same yields can be obtained using only one third of the fertiliser (Aldhous, 2008). GM crops that require less fertiliser could contribute to making farming more eco-friendly because as well as cutting gas emissions these crops would reduce nitrate pollution of water and reduce production costs.

Opposition to genetic engineered plants is based on the possibility that modified plants may have unforeseen consequences that may be detrimental to the modified plant or to their environment. If herbicide tolerant plants are sprayed with herbicide no other plants will be able to survive so plant biodiversity will be reduced. Or if the gene conferring resistance was to be transferred to a weed then the weed would be uncontrollable. Or plants toxic to insects will result in insect free crops which will consequently reduce the bird population and may affect pollination.

There is hostility towards the introduction of GM crops in Europe due to environmental concerns but climate change, the need to reduce greenhouse gases and increase food production will probably mean GM crops will some time in the future be accepted in Europe. GM crops may have the potential to allow plants to grow in hostile conditions – dry and infertile land where few crops are grown at present.

1.3.6.1 Transfer Nitrogen (N) fixing ability to non-Nitrogen fixing plants

The ultimate goal in BNF research is to succeed in transferring the ability to fix nitrogen to non leguminous plants, including in particular the major food crops rice, wheat and maize. This could be achieved by transferring the nodule forming ability to non legumes so that nitrogen fixing bacteria form nodules on non leguminous plants, or by transferring the actual nitrogen fixing ability from the bacteria into non N fixing plants. By identifying the genes responsible for N fixing and nodule formation and which genes are missing in non N fixing plants such as wheat and rice it will potentially be possible to transfer the N fixing ability to non legume plants (Charpentier & Oldroyd, 2010). This will not be a simple task because a very complex process is involved – it is not only the N fixing genes but also the initiation and control mechanisms that must be transferred. Purified nod-factors do not initiate root hair curling response in rice which may be because of the absence of appropriate receptors to recognise nod-factors or because of degradation of the nod-factors by the rice plant's defence mechanism.

An alternative approach would be to transfer the N fixing (nif) genes into the non N fixing plant. Several nif genes are involved – 3 are responsible for the manufacture of nitrogenase as well as others that encode cofactors and protect nitrogenase from inactivation by oxygen.

A nitrogen fixing cereal would grow slower than a normal cereal because the nitrogenase enzyme requires large amounts of energy to function so this would be a drain on the plant's economy resulting in lower yields, but with much reduced fertiliser requirements it will allow the plants to be grown in areas in developing countries where it is presently impossible.

1.3.6.2 Perennial grain crops

Cereals, legumes and oilseed crops occupy approximately 80% of global agricultural land, with wheat, rice and maize covering more than half of this area, but they are annual plants and have to be resown every year (Glover *et al*, 2007). Annual plants have relatively shallow roots only extending down about 0.3 metres and are therefore more prone to drought than perennial plants. Perennial plant roots extend down at least 2 metres making them much more resilient in the face of environmental stress.

Figure 6: Comparison of roots of annual winter wheat (left of each panel) and its wild perennial relative intermediate wheatgrass (right of each panel) (Glover *et al,* 2010)

Figure 6 shows the roots of the perennial wheatgrass on the right of the panels and the annual winter wheat on the left. The perennial roots extend almost 3 metres deep and can access water and nutrients from a much greater volume of soil than the annual plant.

The more extensive root systems of perennial plants reduces soil erosion compared with annual plants and there can be up to 5 times reduction in water loss and 35 times reduction in nitrate loss from soil planted with perennial grass compared with soil under corn or soya beans. Greater root depths and longer growing seasons allow perennials to boost their sequestration of carbon by 50% or more compared with annual plants because perennial plants continue to photosynthesise for a much longer time, after annual plants have been harvested, drawing on resources deep within the soil (Glover, 2005). Perennial plants can grow on more marginal land with poorer quality soil that will not normally support annual crops because they can access nutrients and water from deeper in the soil that is not available to annual plants.

Development of perennial crops will not solve all agricultural problems – there will be weed, disease, fertility, yield and management problems that still have to be overcome, but they do not require annual planting, tillage and, if they also have the ability to fix nitrogen, substantial quantities of fossil based fertilisers will be saved and fewer passes of machinery will be required which reduces fuel costs and therefore boosts profit as well as benefiting the environment. In developing countries where no machinery is available, farmers spend more than 400hours/hectare hand tilling their fields before planting their crops so a perennial crop would be a big saving here. Yields will be lower at least in the first instance because more of the photosynthate will be required for the enhanced root systems, but once they are established they will be a real benefit both environmentally and agronomically. Annual plants specialise in seed production to enhance their chances of survival in subsequent years, so they produce higher grain yields whereas perennials invest more in themselves rather than in seeds, so the yields are likely to be lower at least in the early years until the plants are properly established.

Plant breeders are developing breeding programmes to produce perennial grain crops although it is a long term programme taking 25 - 50 years to complete (Glover *et al*, 2007). This can be achieved by domestication of wild plants by selection of superior individual plants with desirable traits, or hybridisation of existing annual crops with wild relatives bringing together the best qualities of the domesticated plant and the perennial habit from the wild relative. Breeders are presently developing perennial rice, wheat, sorghum, maize, flax and oilseed crops from sunflower, flax and mustard families (Glover *et al*, 2010).

1.3.7 Drought Resistant plants

Drought resistance is defined as being determined by 'dehydration avoidance' or 'dehydration tolerance' (Levitt, 1972). Plants avoid dehydration by adapting in several ways to increase their resistance to drought. A thick waxy cuticle or dense light coloured hairs on the leaf surface which reflect light can help to reduce water loss from the leaves. Reduced leaf size results in less surface area which therefore reduces transpiration. These adaptations favour survival during a drought but at the expense of reduced photosynthetic rates and therefore reduced yields (Deng *et al*, 2005).

Dehydration tolerance can be achieved in plants that can survive in arid environments by a range of physiological and biochemical responses which enable them to tolerate dehydration including stomatal closure and the repression of cell growth and photosynthesis in dry conditions. These are complex processes involving numerous changes including attenuated growth, activation/increased expression or induction of genes, transient increases in abscisic acid levels, accumulation of compatible solutes, and protective proteins, increased levels of antioxidants and suppression of energy consuming pathways (Bartels & Sunkar, 2005; Cominelli & Tonelli, 2010). Some plants accelerate senescence and abscission of older leaves when exposed to drought – e.g. cotton (Mahajan and Tuteja, 2005). Root growth may be enhanced relative to leaf growth allowing the plant to extract water from deeper layers of soil. Increased temperature or reduced humidity results in increased water loss from the plant by transpiration to which plants respond by closing their stomata to reduce water loss. But stomatal closure reduces the rate of photosynthesis, primarily due to CO_2 deficiency (Mahajan and Tuteja, 2005). Transpiration of water from the leaves is also a means of cooling the plant so it can become temperature stressed by closing the stomata (Deng *et al*, 2005). Plants tend to respond to drought by osmotic adjustment – decreasing their cellular osmotic potential by increasing the accumulation of solutes within the cells. This helps to maintain the cells in a hydrated state and therefore allows cellular functions to continue in drought conditions. These solutes do not interfere with the normal metabolic functioning of the cells. They include proline, glutamate, mannitol, sorbitol, fructans, polyols, trehalose, sucrose, oligosaccharides, inorganic acids and ions such as K^+ (Mahajan and Tuteja, 2005).

1.3.8 Water management

Agriculture is the major user of water worldwide, with over 70% of all available fresh water is used in agriculture. Its availability is one of the most crucial factors for food security in many parts of the world. As the population grows, more water resources will be required to produce enough food to feed them. Water scarcity in some arid regions has become a severe constraint on food production. Some studies have concluded that withdrawals for agriculture will grow by up to 17% by 2025 and total withdrawals including domestic and industrial will grow by up to 32% (De Fraiture *et al*, 2003). In scarce areas, water is being diverted away from agriculture to domestic and industrial use which will decrease food production, and therefore increase prices and affect food security especially in developing countries.

In order to conserve water maybe food production should be concentrated in rain fed areas rather than irrigated areas, but this would mean dry areas would have to rely on food imports. Countries with plenty water should export water intensive crops to water scarce countries, but many of the water scarce countries are the most needy and do not have sufficient exports of other commodities to pay for the imports (De Fraiture *et al*, 2003).

Water shortage can be alleviated by constructing reservoirs, but many that have been built are not big enough to keep a constant supply of water through prolonged dry periods. Another alternative is digging wells, but, as the water table falls, deeper wells must be dug which is more expensive, and some countries have now banned the digging of any more wells.

Monsoon countries have an abundance of rainfall but it all falls in a short time period – approximately 4 months from June – September (Kar *et al*, 2006). A large proportion is lost by runoff and evaporation and is never utilised by agriculture. During the dry winter period the land is left fallow because of water shortage, but, unlike temperate countries, the temperature is high enough to allow plant growth. So by storing the water for use in the winter period double cropping becomes feasible. Other methods of conserving water include field levelling which allows the water to spread more evenly across the ground and not accumulate in the lowest parts or be lost by runoff which increases erosion. Plastic sheeting can be used to cover the soil both before and during

the growing season. This traps moisture below the sheet and has the added advantage that it increases the soil temperature and therefore allows earlier planting of crops and it also reduces weeds. Drought resistant plants have been developed with some varieties producing up to a 10% yield increase over non resistant plants. Retaining stubbles from the previous season's crop over winter rather than ploughing can help reduce evaporation as well as reduce soil erosion. Installing underground piping systems can save up to 30% water compared to open canals by reducing evaporation (Blanke *et al*, 2007).

1.3.8.1 Irrigation

The total cultivated area of the world is approximately 1 billion hectares of which approximately one third is irrigated. Approximately 70% of global water consumption goes into irrigated agriculture. The average yield of cereals can be increased by 30-60% in dry land farming areas by increasing crop water use by 25-35mm (Swaminathan, 2007).

There are several different methods of irrigation available and some are more effective and efficient than others. A rain gun applies large amounts of water in a short time which, although a more convenient application method, is less effective because a large percentage is lost through run off which increases soil erosion and fertiliser leaching. A more efficient method is trickle irrigation where water is applied at low rates over a much longer time period and is much more closely matched to the requirements of the crop (Thorburn *et al*, 2008).

It is not only arid countries that have water shortage problems – because of the high population in China, the water availability per head is among the lowest in the world. China feeds 22% of the world's population with about 9% of the arable land and 6% of the fresh water (Li, 2006). Current farming in China is very intensive and water available for irrigation is being reduced because of pollution from the dramatic progress of industrialisation and urbanisation, shrinking lakes and lowering of the water table. China has pioneered several water saving irrigation techniques in the last 20 years - at the end of the 1980s, irrigation consumed more than 380 billion m^3 but by 2006 it had been reduced to 360billion m^3. Farmers allow the land to become quite dry before irrigating which allows significant savings to be made in the amount of water used without reducing yield (Li, 2006). More water is stored after rainfall for use in dry

periods rather than being lost by run off. In some areas, water price is not constant – when water is in short supply the price increases and the price for extra water is much higher than the standard price therefore encouraging people to use water sparingly.

Irrigation can damage the environment because water withdrawn for agriculture is no longer available for downstream wetlands and forests. In some areas, licences are required to extract water to help conserve water and protect the environment (Li, 2006; Wang *et al*, 2002).

1.3.8.2 Recycle water

Many countries are now realising the potential of water recycling both for economic and water shortage reasons. Recycled water can come from rainwater, domestic waste water or industry. It is usually treated then recycled either within the same organisation or to another organisation, but ultimately a large proportion of recycled water is used for irrigation. Depending on the origin of the water it can contain mineral ingredients which can increase plant productivity but it may also contain toxic heavy metals which accumulate in the soil and can prove hazardous which must be removed before the water is used. One method which has been developed to reclaim water uses algae and aquatic plants to remediate different types of municipal waste water. The plants yield a protein rich biomass which can be used to supplement animal feed or it can be used as a green manure. *Azolla* is a free floating aquatic fern which has proved effective because of its high biomass productivity, high N fixation rate and it is simple and cheap to grow. It can grow in varied environments and it has multiple applications – as a fertiliser, animal feed, biofilter, and heavy metal phytoremediation from floodwater. It removes N and P from water which cause eutrophication. *Azolla* forms symbiotic relationships with N fixing *Anabaena azollae* and is an established N biofertiliser in paddy fields in Asia (Rai, 2007). N fixing by *Azolla* was a major factor in N supply to rice in the 1980s, but its use declined drastically in favour of mineral fertilisers although rising prices now could see a return to these methods (Graham and Vance, 2000).

1.3.8.3 Salt water tolerance

Soil salinisation is one of the most severe causes of yield reduction in modern agriculture. Some estimates indicate that up to 50% of all irrigated land may be salt

affected (Flowers, 1999) and by 2050 more than 50% of all arable land may be affected by salinisation (Wang *et al*, 2003). In addition, 10 million hectares of crop land/year are lost because of salinisation caused by irrigation (Thomas and Middleton, 1993). This can be caused by poor quality of the irrigation water and/or improper management practices which are the principal causes of salinisation in the Mediterranean. The risk is greater if saline water is applied to plant leaves during the daytime when the evaporation rate is high so sprinkler irrigation with saline water may cause more damage than trickle irrigation where the water is applied at the soil surface which keeps the soil moisture level high at the root zone (Paranychianakis and Chartzoulakis, 2005). Excessive groundwater pumping can lower the water table allowing landward penetration of seawater and excessive extraction of water from river systems lower the flow of water below the extraction point and therefore the seawater can flow upstream.

Initially, growth is reduced due to water deficit by osmotic stress, then by the accumulation of salts in the shoot at toxic levels which takes time to develop depending on the resistance of the plant and the intensity of the stress. Growth rate and yield are reduced, but some plants are tolerant to salt water and may therefore potentially be able to grow in salt affected areas (Paranychianakis and Chartzoulakis, 2005). A survival mechanism used by most plants is the production of compatible solutes which accumulate in the plant in response to osmotic stress and therefore maintains cell turgidity even when there is a higher salt concentration in the surrounding environment. Compatible solutes can be amino acids (e.g. proline), quaternary amines (e.g. dimethylsulfoniopropionate) or polyol/sugars (e.g. mannitol) (Wang *et al*, 2003). Transgenic plants which over express these compatible solutes will therefore be more tolerant of higher salt concentrations in the water, but accumulation of these solutes can often cause other detrimental effects in the plants due to disturbances of the metabolic pathways producing them. By accumulating Na^+ and Cl^- ions in old leaves and then shedding the old leaves the plants can become more salt tolerant. This could be due to the rapid growth rate of young leaves and low transpiration rate, or the preferential removal of salts from sap moving to actively growing organs.

1.3.8.4 Desalination

Desalination is emerging as one of the major new sources of fresh water for the developed and some areas of the developing world. Despite high energy demands,

capital costs and environmental concerns desalination can offer an alternative source of fresh water. Desalination can be achieved by distillation or membrane based methods, both of which require large amounts of energy, but solar powered desalination plants may become more feasible in future both because of the escalating cost of conventional power and the reduced cost of solar power (Mittelman *et al*, 2007). Many countries that suffer from a water shortage have abundant solar radiation making this a feasible alternative for water provision. Disposal of the concentrated brine remaining after desalinisation is normally by discharge back into the sea by means of a long pipeline but there are concerns that the extreme concentration may damage the marine environment. This option is not available to landlocked countries with no access to the sea. Desalinated water can supplement urban water supply but the energy requirement rules out its use for crop irrigation unless it is heavily subsidised. However, by first using it in an urban situation then recycling to agriculture, the crops can eventually benefit from the technology.

A new and innovative system is being developed to enable year round crop production to take place on desert land where no production is currently possible in the form of 'seawater greenhouses'. Greenhouses are built on desert land beside the sea and are capable of producing crops using only seawater and sunlight (Figure 7). They use no scarce fresh water or expensive desalination equipment. Mirrors are used to concentrate the sunlight and the heat produced then evaporates the seawater as it trickles down the walls producing a cool, humid environment in the greenhouse. Distilled water is collected in underground storage tanks which is then used to water the plants. Humidity is also increased in the area immediately surrounding the greenhouse which allows plants to grow outside in these areas where they would not normally grow (www.seawatergreenhouse.com, 2010).

Figure 7: Proposed greenhouse complex in the Sahara Desert(www.saharaforestproject.com, 2010).

1.3.9 Soil Conditioning

An important constituent of a well balanced soil is soil organic matter (SOM) which helps improve soil fertility, stability and water retention. SOM can come from the remnants of the previous crop or it can be added in various forms. Soil conditioners can be added to the soil to improve the soil structure, nutrient availability and water retention. Soil conditioners can be several different types, including natural organic, inorganic mineral, biochar and synthetic polymers (Sojka *et al*, 2007).

1.3.9.1 Natural organic conditioners

These include cow manure, compost, peat, sawdust, sewage sludge and green manure. These substances improve fertility by helping to increase water retention, promote aggregation, provide a substrate for microbial activity, improve aeration and resist compaction and erosion (Soffe, 2003).

1.3.9.2 Inorganic mineral conditioners

These include lime (CaO) which raises the soil pH and gypsum ($CaSO_4 2H_2O$) which is a good source of calcium which increases flocculation and stabilises aggregates which helps to reduce erosion (Donahue *et al*, 1983).

1.3.9.3 Biochar

Biochar has been reported to improve soil tilth, nutrient availability and plant productivity. Biochar is a carbon rich product obtained when biomass such as wood is burnt in the absence or at low levels of oxygen (pyrolysis) (Lehmann & Joseph, 2009). This produces a stable residue which allows carbon to be sequestered and stored over longer periods than is possible in untreated biological waste or by depositing it in landfill sites (Atkinson *et al*, 2010). Approximately 50% of the carbon in the original biomass is retained in the biochar and the decomposition rates of biochar are very much slower than uncharred organic material. Addition of biochar to low fertility soils has been shown to produce an increase in cowpea yield of up to 43% (Lehmann *et al*, 2003). Some sources have reported that the addition of biochar can enhance maize yield by 20% as well as improving water quality because reduced fertiliser requirement means there is less nutrient run off in surface water (Renner, 2007). Biochar has been claimed to reduce the emissions of two of the most potent greenhouse gases – nitrous oxide and methane, possibly by altering the structure of the microbial community within the biochar (Renner, 2007). Some studies have shown that biochar acts as an excellent support medium for *Rhizobium* inoculants and the biological fixation rate was significantly increased when biochar was added (Rondon *et al*, 2007). Reduced level of nitrogen available in the biochar compared to the surrounding soil may stimulate the bacteria in the polymer to fix nitrogen from the air rather than obtain their nitrogen requirements from the soil.

Application of biochar has also been shown to stimulate the colonisation of indigenous mycorrhizal fungi. Saprophytic fungi do not colonise the biochar because it does not contain available nutrients. Saprophytic fungi obtain their nourishment from dead or decaying organic matter and therefore cannot survive on an inert substrate like biochar. The mycorrhizae can therefore grow and sporulate in the biochar without competition from other saprophytic fungi and then act as a reservoir of mycorrhizae to grow when conditions are suitable (Saito & Marumoto, 2002; Warnock *et al*, 2007).

Biochar requires biological waste, so it could be available in areas where an excess of biological waste available – for example in regions like the Amazon where vast areas of forest have been cleared for farmland. But in most countries biological waste is already used in one form or another – eg compost, fertiliser, methane production, or stock feed.

The cost of biochar meant it was not competitive with inorganic fertilisers in 2002 (Saito & Marumoto, 2002) but escalating fertiliser costs in recent years may mean it is a more realistic option now.

1.3.9.4 Synthetic polymers

Several synthetic polymers have been developed which can enhance water holding capacity and improve soil stability. Synthetic polymers were first introduced in the 1960s but originally were not very successful because of their low swelling capacity and short life. Starch superabsorbent polymers were developed in the 1970s which had a much higher swelling capacity so less was required per unit of soil and therefore reduced costs. But they were starch based and were therefore a suitable substrate for soil bacteria which resulted in a short lifespan. In the 1980s the first synthetic superabsorbent was introduced which had a high swell rate (100s of times their weight) and a longer life. Superabsorbent polymers need to be inert, non toxic and have a lifespan of many years. They increase water holding capacity; reduce erosion and water run off; enhance soil permeability; reduce compaction, which therefore improves soil aeration and enhances microbial activity; reduce irrigation frequency and reduce fertiliser leaching (Jhurry, 1997). Original products were short lived because of their high cost and difficulty of application and even distribution, but the need to grow agricultural produce in more marginal land and the discovery of new novel materials has renewed interest in soil conditioners (Jhurry, 1997).

Polyacrylamide (PAM) ($-CH_2CHCONH_2-$) is used as a soil stabilising agent which reduces the amount of nutrients in runoff water and reduces soil erosion even at very small application rates of 1-2kg/ha, applied in irrigation water. Approximately 800,000 hectares of irrigated land in US was treated with PAM for erosion management in 2007 (Sojka *et al*, 2007). Linear macromolecule PAMs can be dissolved in irrigation water and stabilises the outer surface of soil aggregates (Sojka *et al*, 2007; Sepaskhah & Mahdi-Hosseinabadi, 2008).

Superabsorbent polymers (SAPs) are strongly hydrophilic gel forming compounds that can absorb up to 2000 times their weight in water (Sojka *et al*, 2007). They can improve water retention in light, sandy soils and therefore reduce the frequency of

irrigation requirement. The most commonly used SAPs in agriculture are polyacrylamide and polyacrylate polymers (Bai *et al*, 2010).

Water absorbent PAMs are cross linked polymer chains which form a three dimensional hydrophilic network structure that can absorb and store large amounts of water. Natural gas is used as the raw material to synthesise PAMs which is becoming more expensive and is not sustainable, so other alternative raw materials are being explored including chitin and starch (Sojka *et al*, 2007). Field trials with starch has shown that it can be almost as effective as PAM at concentrations 8x higher, but it is cheaper to produce than PAM, so as the cost of PAM continues to increase, starch will become a more viable alternative (Orts *et al*, 1999). Although chitin produced similar results to PAM in lab scale experiments, it produced highly variable results in field experiments which were at best only half as effective as PAM, probably because of its lower molecular weight compared to PAM (Orts *et al*, 1999). As well as the cost of the raw materials, the use of conditioners is limited by economics related to transport and application which means their use has been limited to high value crops.

Zeolites are naturally occurring alumino-silicate minerals that have channels in their structure which provides a large internal surface area for cation exchange. Some zeolites have a very high cation exchange capacity and can absorb large amounts of NH_4^+ and K^+ ions and can then potentially act as a slow release fertiliser (Zwingmann *et al*, 2009).

1.3.9.5 Poly High Internal Phase Emulsion Polymer (PHP)

The subject of this research project is PHP which is a synthetic polymer that can be used as a soil conditioner and has the potential to make a contribution to enhancing crop yield in several of the aforementioned categories. It can act as a slow release fertiliser, as a reservoir for water in semi dry environments and as a protected environment for growth of nitrogen fixing bacteria.

Chapter 2: Poly High Internal Phase Emulsion Polymer

2.1 Poly High Internal Phase Emulsion Polymer

Poly High internal phase emulsion Polymer (PHP) is a highly porous, polymeric foam prepared by polymerisation of monomeric continuous phase of a High Internal Phase Emulsion (HIPE). It was developed by Unilever Research Port Sunlight laboratory, Cheshire, UK (Akay, 1995; Barby & Haq, 1982). It is a low density, (approximately 0.1g/cm^3) open structured, polymer which can be manufactured in either hydrophobic or hydrophilic forms. It is made up of large pores connected by smaller interconnecting pores which makes a highly porous substance which allows unrestricted movement of both liquids and gases throughout its structure and also providing a large surface area for support (Figure 8).

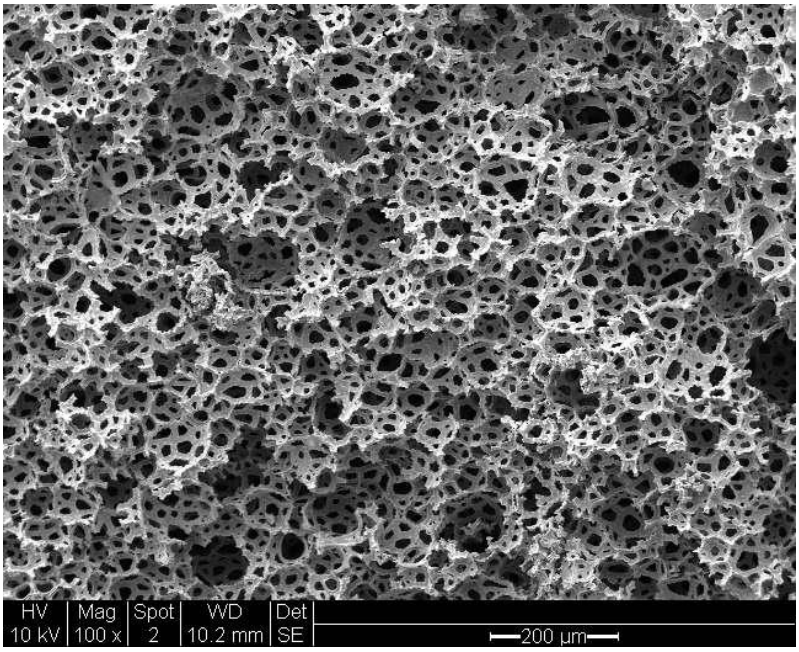


Figure 8: SEM of polymer (x100)

It can be used for a wide variety of applications including gas – liquid separation (Calkan *et al*, 2005); gasification of biomass (Akay *et al*, 2005[1]); intensified demulsification processes (Akay *et al*, 2005[2]); tissue engineering scaffoldings (Bokhari, 2003); membranes (Akay & Wakeman,1994); metal ion removal in water treatment (Wakeman *et al*, 1998); metal foam production (Calkan *et al*, 2005) and as a support matrix for enhanced bacterial growth and antibiotic production (Ndlovu, 2009).

An emulsion is a mixture of two immiscible liquids. A High Internal Phase Emulsion (HIPE) is made up of a dispersed aqueous phase surrounded by a continuous oil phase. A HIPE is defined as an emulsion in which the droplet phase occupies least 74.05% of the emulsion volume. This is the maximum volume that can be occupied by uniform spheres (Akay, 1998). This value can go up to 99% if the droplets are non uniform in size or the spheres are deformed into polyhedra (Cameron, 2005). HIPEs are formed by the controlled addition of the aqueous phase to the oil phase while agitating the mixture, normally by continuous mechanical stirring. The aqueous phase generally consists of sulphuric acid, distilled water and a polymerisation initiator (normally potassium persulphate). The oil phase generally consists of a monomer (styrene), a cross linking agent (divinyl benzene) and a surfactant (sorbitan monoleate - Span 80). The two components of an emulsion would normally be unstable and tend to separate from each other so a stabilising agent or surfactant is used to increase the stability while polymerisation takes place. Surfactants are normally compounds that contain a hydrophobic or water insoluble tail and a hydrophilic or water soluble head. The surfactant must be able to withstand the polymerisation temperature of 60^0C and keep the emulsion stable long enough for polymerisation to take place which is 3 – 8 hours. The most commonly used surfactant is Span 80 (Figure 9).

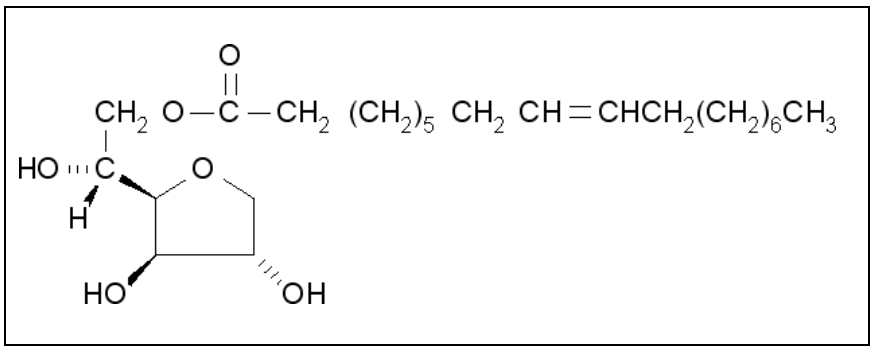


Figure 9: Chemical structure of Span 80

Steric attraction between the tails of the surfactant causes the agglomerations of surfactant molecules at the interface of the two phases, stabilising the emulsion and stopping the components from separating. Once polymerisation is complete, the polymer is washed to remove any unreacted components which also removes the surfactant, leaving small pores or interconnects between the larger pores (Figure 10) (Bhumgara, 1995).

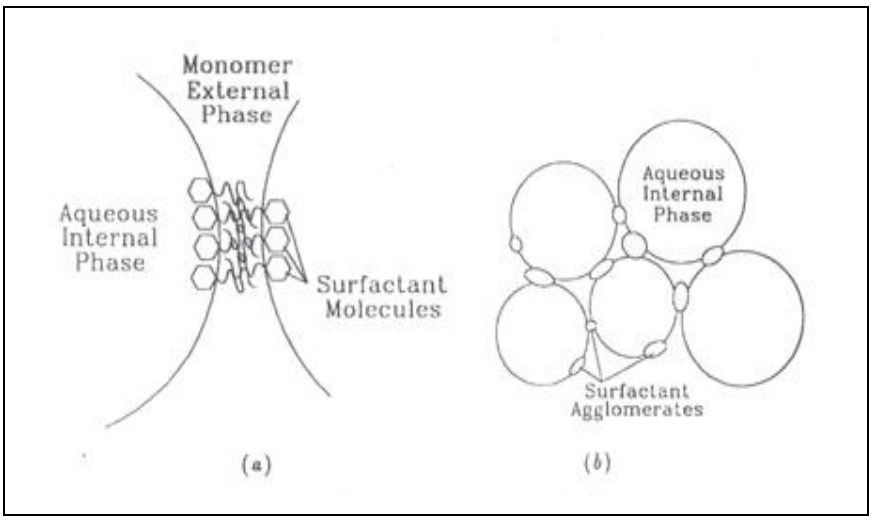


Figure 10: Action of surfactants (Calcan, 2007)

The polymers can be used in many different applications which require them to have different morphology and properties. These can be altered by adjusting the proportions of the ingredients, using different ingredients, adjusting the time of dosing the aqueous phase into the oil phase, the time of mixing after dosing, the temperature of the mixing vessel and the speed of the mixer. More cross linking agent (DVB) produces a more rigid polymer and less makes a spongier polymer (Williams *et al*, 1990; Hainey, *et al*, 1991). Adding 2-ethylhexyl acrylate (2-EHA) to the oil phase produces a more elastic polymer (Normatov & Silverstein, 2008). More potassium persulphate in the aqueous phase leads to smaller pore size (Williams *et al*, 1990). Lauroyl peroxide can be added to the oil phase to act as a polymerisation initiator instead of potassium persulphate in the aqueous phase. More surfactant produces more interconnecting pores. Longer dosing and mixing times and higher mixer speed produces polymer with smaller pores. A wide range of pore sizes (0.5 - 5000μm) and interconnect sizes (0 – 0.5μm) can be produced. Previous work (Burke, 2007) has determined the optimum ingredient proportions and manufacturing conditions for use of the polymer in agricultural roles. The desirable qualities are:

- A structurally stable product which can resist degradation in the soil
- A high level of water absorbency to enhance the water holding capacity of the soil especially in water deficient soils
- A fast rate of water absorbance to collect and store water as efficiently as possible
- A slow rate of water release only releasing it when it is required by plants
- Mixed pore size to allow controlled release rate – large pores will drain water and any nutrients it contains faster and smaller pores will take longer to drain.

Following the appropriate dosing and mixing times, the emulsion is incubated at 60^0C for polymerisation to take place which takes up to 8 hours to be completed. The reaction can be summarised as in Figure 11.

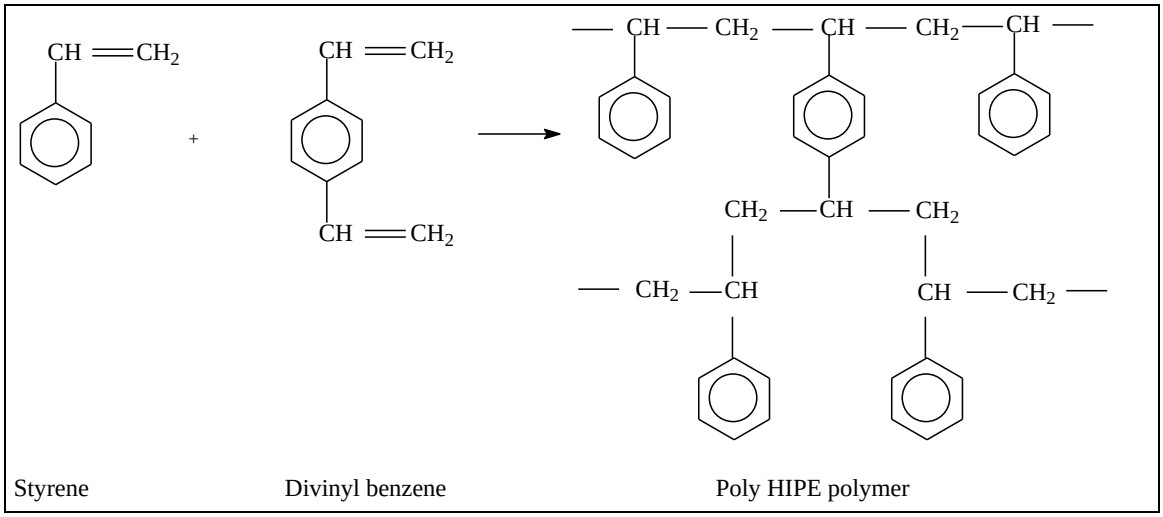


Figure 11: Equation summarising production of polyHIPE polymer

Sulphonation

The polymer produced is hydrophobic but, for many applications, a hydrophilic product is required and the most common way of achieving this is by sulphonation. The process involves the modification of the phenyl rings of the styrene – DVB cross linked polymer to form sulphonic acid functional groups ($SO_3^-H^+$) (Figure 12). Several sulphonating agents can be used including sulphuric acid, oleum, chlorosulphonic acid, fluorosulphonic acid, amidosulphonic acid, free sulphur trioxide and its complexes and halogen derivatives of sulphuric acid (Calkan, 2007).

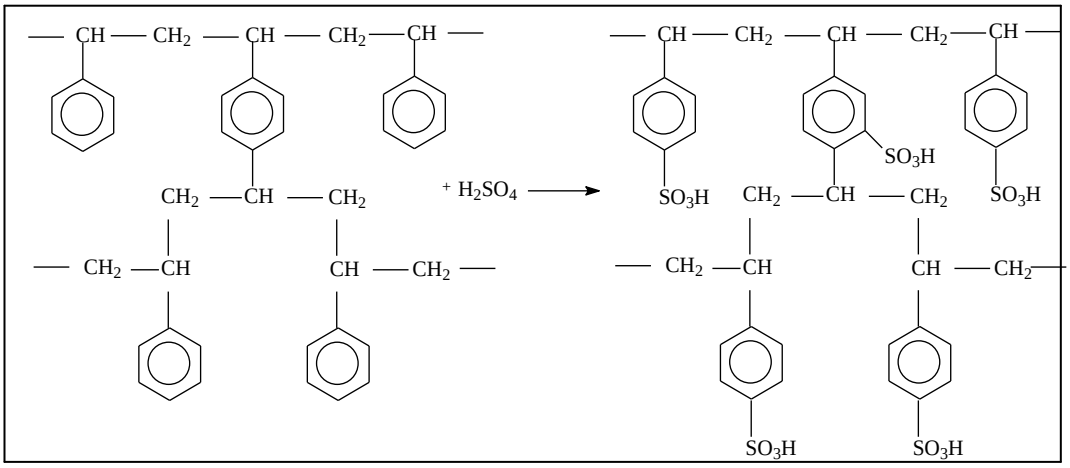


Figure 12: Sulphonation of polyHIPE polymer

Sulphuric acid is a common sulphonating agent although it does produce large quantities of toxic waste so it is not an environmentally friendly technique. The dried

polymer is soaked in sulphuric acid then the reaction is completed by thermal treatment. A microwave oven is used for this purpose as it allows the process to be completed in a much shorter time scale than by using a conventional oven. A hydrophilic product can also be produced by adding vinyl pyridine to the oil phase which makes the product hydrophilic without the need for sulphonation.

2.2 Materials and Methods

Preparation of polyHIPE polymer

Styrene, divinyl benzene, sorbitan monooleate (Span 80), potassium persulphate, concentrated sulphuric acid (98%) were all obtained from Sigma-Aldrich, UK. A 12 cm diameter mixing vessel was used, equipped with a stirrer with two flat paddles 9 cm diameter.

The ingredients are prepared in two phases: the aqueous phase and the oil phase.

Aqueous phase: 5% conc. sulphuric acid
94% deionised water
1% (wt) potassium persulphate

Oil phase: 76% styrene
14% sorbitan monoleate (Span 80)
10% divinyl benzene (DVB)

The total polymer volume of one batch is 250 ml of which 90% (225mls) is aqueous phase and 10% (25 ml) is oil phase. The oil phase was added to the mixer which was set at 300 rpm. The aqueous phase was added with a peristaltic pump at the rate of 45 ml/minute (i.e. dosing time of 5 minutes), followed by a mixing time of 1 minute. The emulsion is then drained from the mixer into 5 x 50 ml plastic tubes, capped, inverted and placed in a conventional oven at 60^0C for 8 hours for polymerisation to take place. The process is summarised in Figure 13.

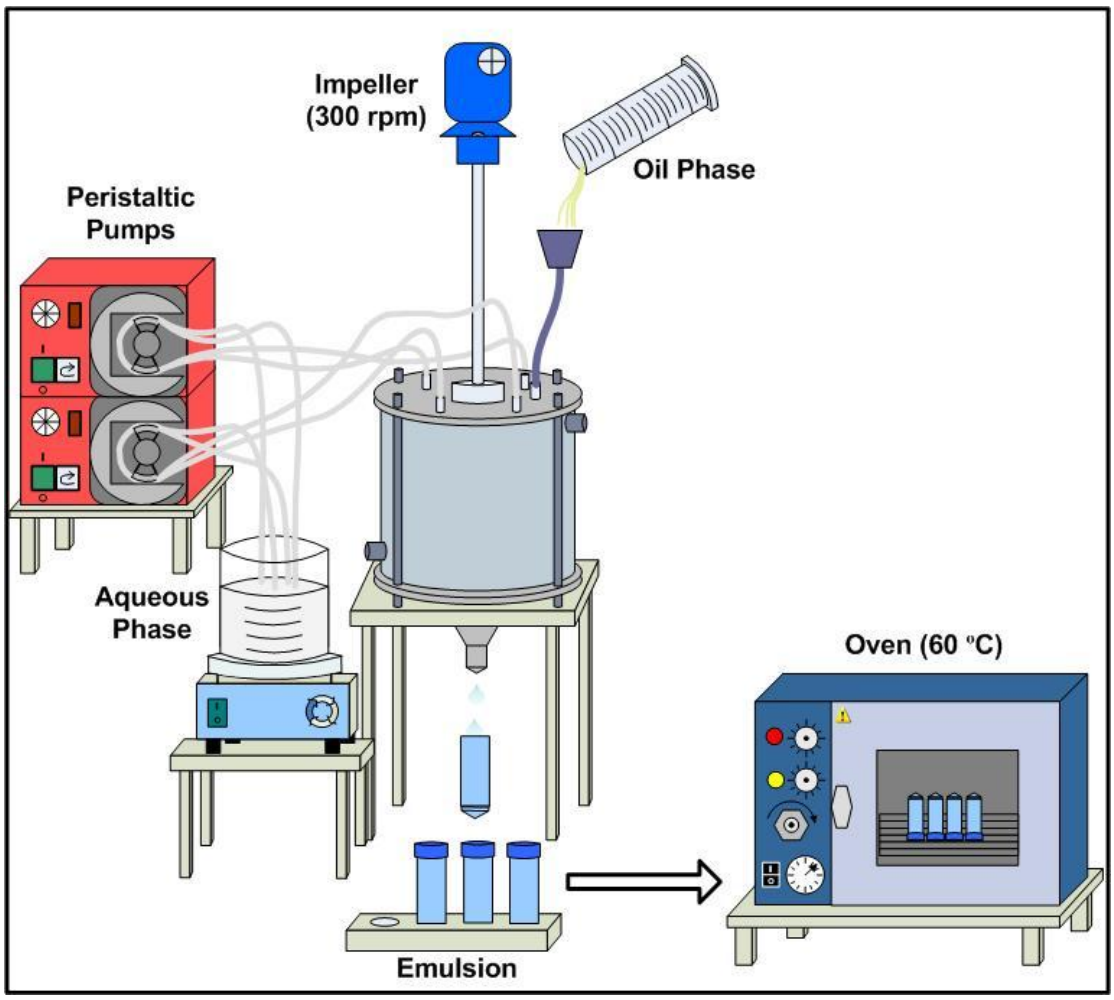


Figure 13: Schematic diagram of the apparatus used for PHP preparation (Calkan, 2007)

The polymer is then cut into 4 mm discs, then dried in a conventional oven at 60^{O}C.

Sulphonation

The discs were soaked in concentrated sulphuric acid for 2 hours, then microwaved in a conventional 1 kW kitchen microwave oven at full power for 30 seconds x 5 in a fume cupboard, opening the door between each session to allow fumes to escape and to cool and the discs were turned over to help obtaining even sulphonation. The sulphonated discs were washed with deionised water for 30 minutes twice, to remove any excess surfactant and initiators followed by 60 minutes using 2.5N ammonium hydroxide to neutralise any acid left. The pH was then finally adjusted to 5-7 by adding acetic acid and washing to remove any excess nitrogen. The discs were then dried in a fume cupboard and cut into small cubes. The resulting product (Figure 14) was then ready for use.

Figure 14: Finished PHP product

2.3 Water absorption capacity of polymer

The sulphonated polymer is hydrophilic and absorbs water. Its absorption capacity was determined by weighing a dry polymer disc, soaking in deionised water until absorption equilibrium was reached – ie when the weight stopped increasing. This was achieved in 5 minutes. After 5 minutes, the excess water was removed from the surface with tissue and the discs reweighed.

The water absorbency was then calculated using the equation:

$$\text{Water absorbency} = \frac{W_W - W_D}{W_D}$$

Where W_W = wet weight & W_D = dry weight

Water absorption results

Dry weight (W_D) = 0.38 (average of 3)

Wet weight (W_W) = 5.93 (average of 3)

$$\therefore \text{Water absorbency} = \frac{5.93 - 0.38}{0.38} = 14.6$$

Some variation was observed between different batches of polymer although the conditions were the same. Water absorbency ranged from 13.2 – 16.5. The age of the

ingredients seemed to have an effect – with older ingredients, polymerisation did not take place as readily and was sometimes incomplete with some unpolymerised liquid remaining in the tubes and on these occasions the water absorbency was less.

Chapter 3: Development of polyHIPE as a slow release fertiliser

The use of chemical fertiliser, in particular nitrogen, has a major impact on crop yield. But it is highly expensive, uses non-renewable resources, its production contributes massively to global warming and a large proportion gets washed away by run off, leaching, volatilisation or denitrification and is never of any benefit to the plants. It has been estimated that 50-70% of applied fertiliser can be lost to the environment and never provides any benefit to the plants (Abraham and Pillai, 1996). One method of reducing this waste and pollution is the development of slow release fertiliser. These fertilisers reduce the rate of loss from the soil and therefore increase the efficiency of utilisation, sustaining the supply of nutrients to the crop for a longer period, reducing the frequency of application and reducing environmental pollution (Liu *et al*, 2007). PHP may have potential to be used as a slow release fertiliser.

The method of manufacture of the polymer was modified in order to produce a product with ammonium sulphate as an integral part of the polymer. Ammonium sulphate is a key ingredient of many fertilisers. Experiments were conducted using peas and soybeans to investigate the effect of using polymer produced by this method on plant growth rate. Ammonium sulphate is produced in the polymer and must dissolve and diffuse out through the small pores in the polymer thereby prolonging the time scale over which the fertiliser is released, so the plant will benefit over a much longer period and less fertiliser will be lost through leaching, run off, volatilisation or denitrification. Vermiculite was used in the initial experiments which is an inert substance used as a growth medium in place of soil. It contains no nutrients so all inputs can be controlled. The nutrients were added in the water in the form of nitrogen free (Hoaglands) nutrient solution (appendix 1, p159), which contains all essential nutrients for plant growth except nitrogen.

Summary of experiments using PHP with slow release fertiliser:

- Soybeans grown in vermiculite using PHP neutralised with NH_4OH
- Peas grown in vermiculite using PHP neutralised with NH_4OH
- Peas grown in vermiculite using PHP neutralised with NH_4OH & KOH
- Observation of root growth in vermiculite in Petri dishes
- Peas grown in soil using PHP neutralised with NH_4OH & KOH

3.1 Soybeans grown in vermiculite with & without polyHIPE neutralised with ammonium hydroxide

3.1.1 Materials and Methods

The polymer was manufactured, sulphonated and neutralised with ammonium hydroxide as normal, but then instead of washing the excess nitrogen out using acetic acid, sulphuric acid was added instead which formed ammonium sulphate in the polymer. Sulphuric acid was added drop wise while stirring the solution containing the sulphonated PHP particles to lower the pH to 5 – 7. The liquid was then drained and the polymer discs were dried in a fume cupboard for 48 hours.

Vermiculite was used as a growth medium, supplied by Sinclair Horticulture Ltd, Gainsborough, Lincolnshire. Soybeans (*Glycine* max, variety *Pan*) from South Africa were used in this experiment. *Bradyrhizobium japonicum* in a peat carrier was obtained from Soygro Biofertiliser, South Africa and Hoagland's nitrogen free nutrient solution was used to water the plants. Chemicals required for Hoagland's solution were obtained from Sigma-Aldrich, Dorset, UK.

Nitrogen Analysis

The nitrogen content of the PHP was measured using a Carlo Erba 1108 Elemental Analyser. The N content of PHP prepared by this method (neutralising with H_2SO_4) was compared with PHP prepared by the normal method of neutralising with acetic acid and washing the excess nitrogen out.

Examination of polymer with root penetration under SEM.

Biological samples must first be fixed before they can be examined under SEM. Pieces of polymer with roots growing through them were washed by immersing in Phosphate Buffer Solution (PBS) for 10 minutes, followed by immersion in 2% glutaraldehyde (EM grade) in PBS for 24 hours. Samples were then washed again by immersing in PBS for 10 minutes. They were then dehydrated by immersing in increasing concentrations of ethanol - 10%, 25%, 50%, and 75%, for 10 minutes at each concentration. Samples can then be stored in 100% ethanol at 4^0C until examination under SEM. Samples were then critical-point dried with liquid CO_2, then mounted on metal stubs using double sided adhesive tape. The surface of the samples to be examined was broken rather than cut to avoid damage to the structure during cutting. The samples were then gold coated using a Polaron e1500 Sputter Coater and then examined using a Cambridge s240 scanning electron microscope (Figure 15).

Figure 15: Cambridge s240 scanning electron microscope

Planting of soybeans

Plants were grown in a greenhouse in 13cm diameter pots. PHP is normally added to soil at the rate of 0.5% by weight (Akay and Burke, 2010) and was therefore added to the vermiculite at the equivalent weight. These pots hold 500g of soil, therefore 2.5g of polymer was added to each pot. Polymer was only added to the vermiculite below the

level of the seed. One soybean seed was placed on top of 0.5g (a small spatula) of *Bradyrhizobium japonicum* in the centre of each pot and covered with a further 25 mm of vermiculite. One seed was planted in each pot and each treatment was carried out in triplicate. Plants were grown for 6 weeks, watering twice a week with Hoagland's nitrogen free nutrient solution. After 6 weeks, the plants were harvested and the length, fresh weight and dry weight of the shoots and fresh weight and dry weight of the roots were recorded. Since dry weights give the most representative result, only dry weights and not fresh weights are recorded here. Results were analysed by one way ANOVA at 95% confidence interval (ie a significant result if $p < 0.05$) using Minitab statistical software.

3.1.2 Results & Discussion

Elemental analysis

Nitrogen content of polymer prepared by neutralising excess ammonium hydroxide with concentrated sulphuric acid was compared with neutralising with acetic acid and washing the excess nitrogen out of the polymer (Table 1).

	Nitrogen %
Acetic acid/washing	6.89
Sulphuric acid	11.94

Table 1: Nitrogen % of polymer

Nitrogen content has increased by 73.3% by neutralising with sulphuric acid compared with acetic acid/washing. With sulphuric acid, the nitrogen has been locked in the polymer in the form of ammonium sulphate, a key ingredient of many nitrogen fertilisers. When the polymer comes in contact with soil moisture, it will dissolve and diffuse slowly through the polymer over a much longer timescale than when fertiliser is applied directly to the soil, so the plant will benefit over a much longer period.

SEM of polymer

Before sulphonation, the surface of the pore has a smooth appearance, but after sulphonation it is much more granular due to the deposition of ammonium sulphate on the surface (Figure 16 a & b).

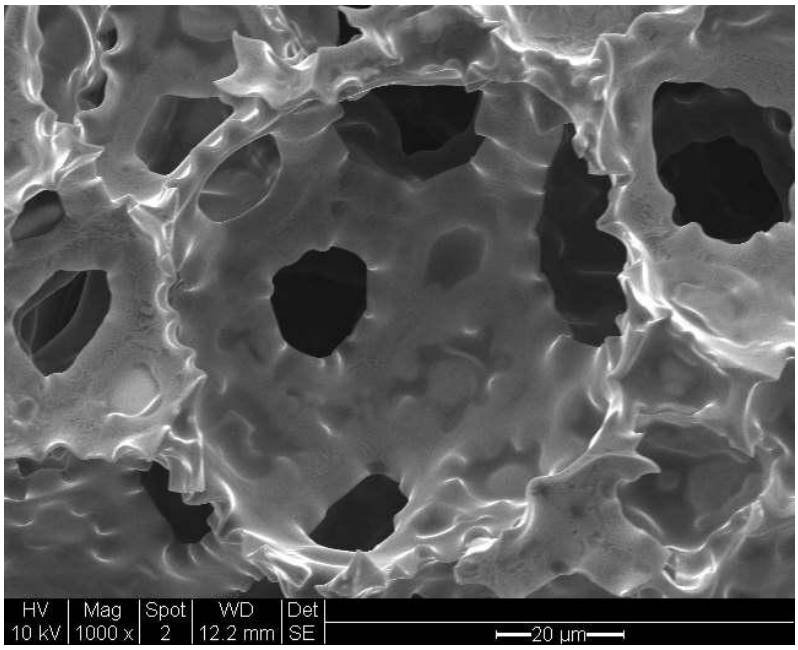


Figure 16(a): Before sulphonation x1000 (scale bar 20µm)

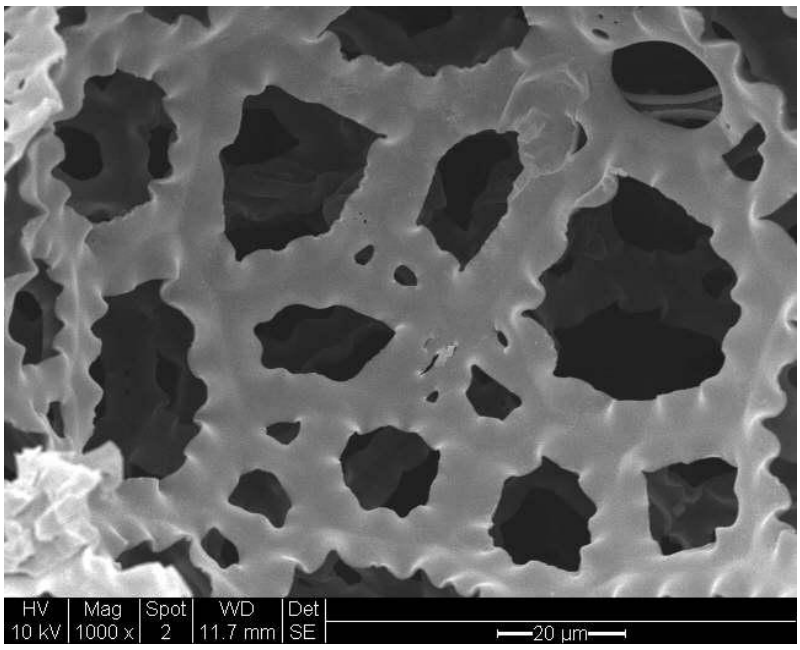


Figure 16(b): After sulphonation and neutralisation x1000(scale bar 20µm)

Growth of plants

After 2 weeks growth, plants plus polymer were visually larger than the control plants. After 3 weeks growth there was an obvious difference both in size and colour of the shoots plus polymer compared to the control plants (Figure 17). The darker colour of the plants plus polymer is an indication that they have received more nutrients, in particular nitrogen.

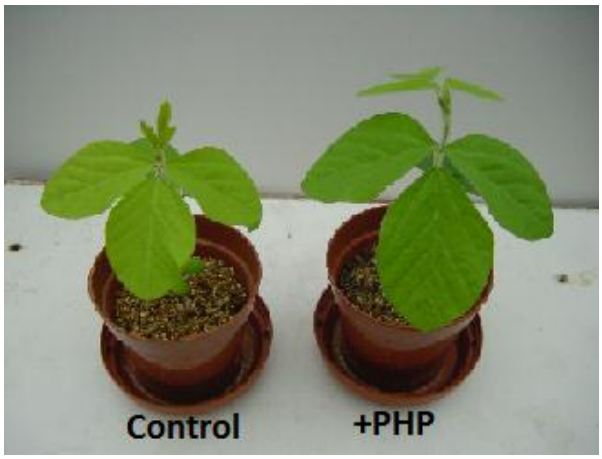


Figure 17: Soybean shoots after 3 weeks growth

Figure 18: Root nodules on soybean root

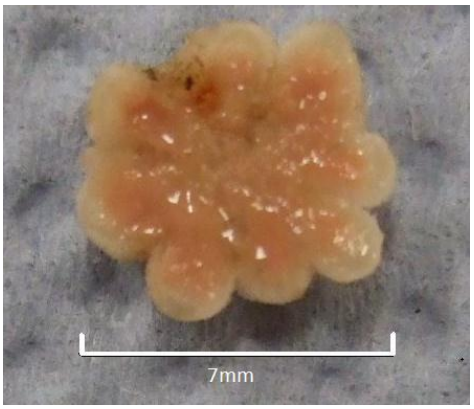


Figure 19: x section of soybean root nodule

Most root nodules on dicotyledonous plants like soybeans and peas are crown nodules which are located at the base of the tap or main root and on the lateral roots close to the tap root (Figure 18). A cross section of a nodule shows the pink colour of a healthy, active nodule (Figure 19).

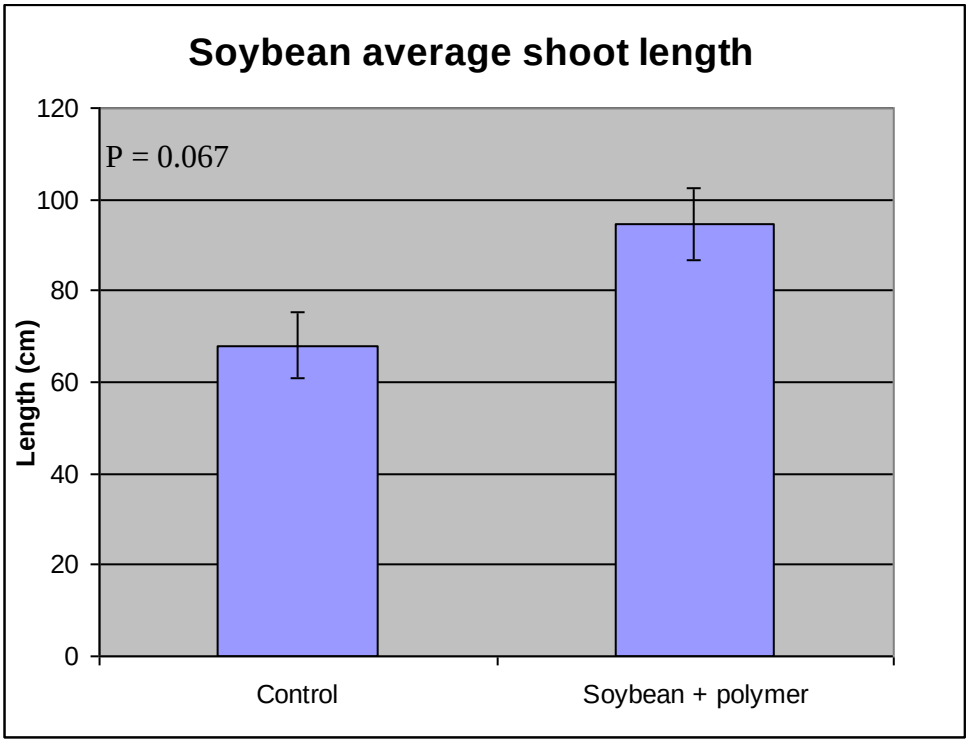


Figure 20: Soybean average shoot length

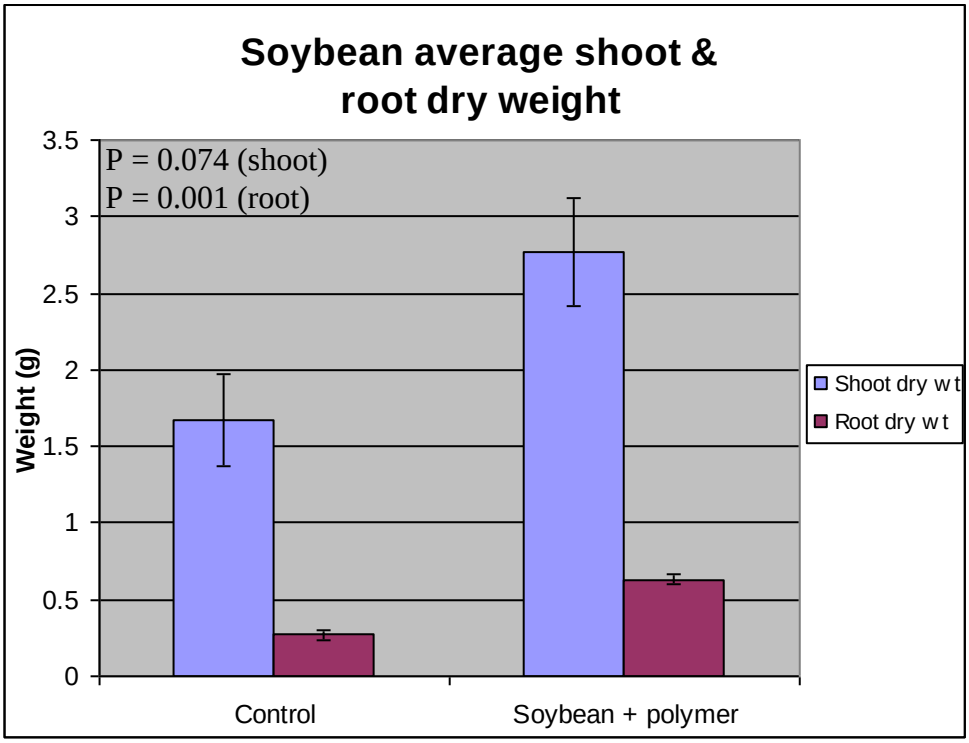


Figure 21: Soybean average shoot & root dry weight

The results showed a significant increase in average shoot length, shoot weight and root weight of soybeans compared to control plants with no polymer added, although further analysis showed that not all data is significantly different although the averages for

plants plus polymer were higher (appendix 2, p163). P values (95%CI) were 0.067 and 0.074 for shoot length and shoot dry weight. Only root weights showed a significant weight increase with more detailed analysis with a P value of 0.001. A P value < 0.05 indicates a significant difference at 95% CI. Shoots of plants with polymer added were on average 39.2% longer than those with no polymer (Figure 20)**.** Shoot dry weights for plants with polymer increased 65.9% and root dry weights increased by 133% (Figure 21).

3.2 Peas grown in vermiculite with & without polyHIPE

The same experiment was repeated using peas (*Pisum sativum L*, variety *Phoenix*) from Sudwestaat, Rheinfeld, Rastatt, Germany. The bacteria used were *Rhizobium leguminosarum* in peat obtained from Becker Underwood Ltd, Saskatchewan, Canada. The experiment was set up the same as for soybeans (p40). Again, the addition of polymer produced a positive result compared to control plants with no polymer.

3.2.1 Results & Discussion

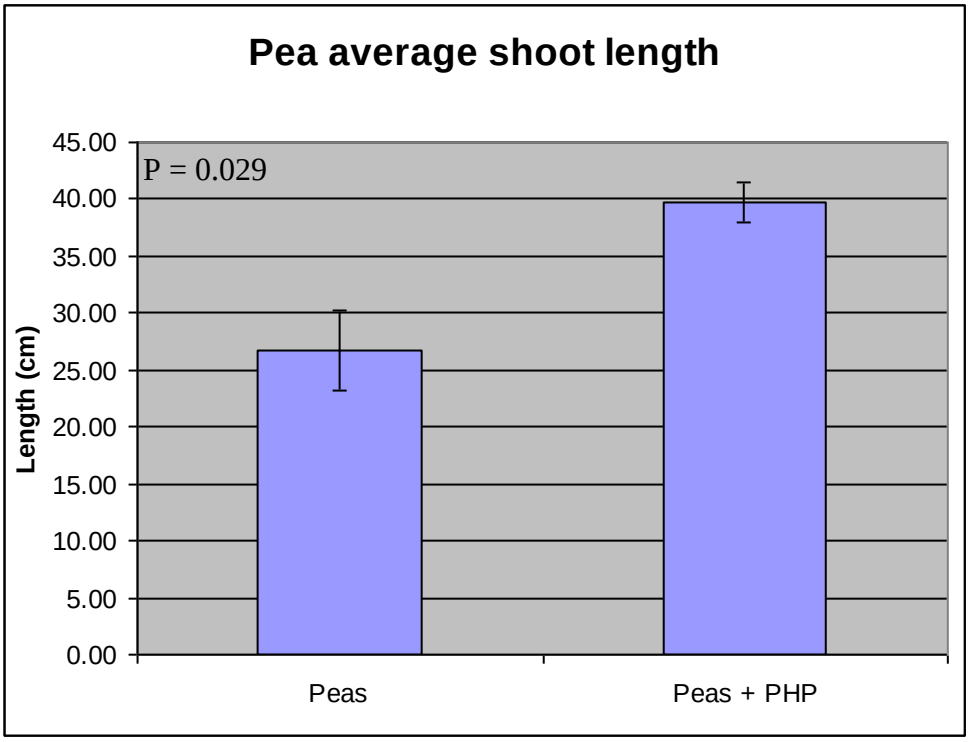


Figure 22: Pea average shoot length

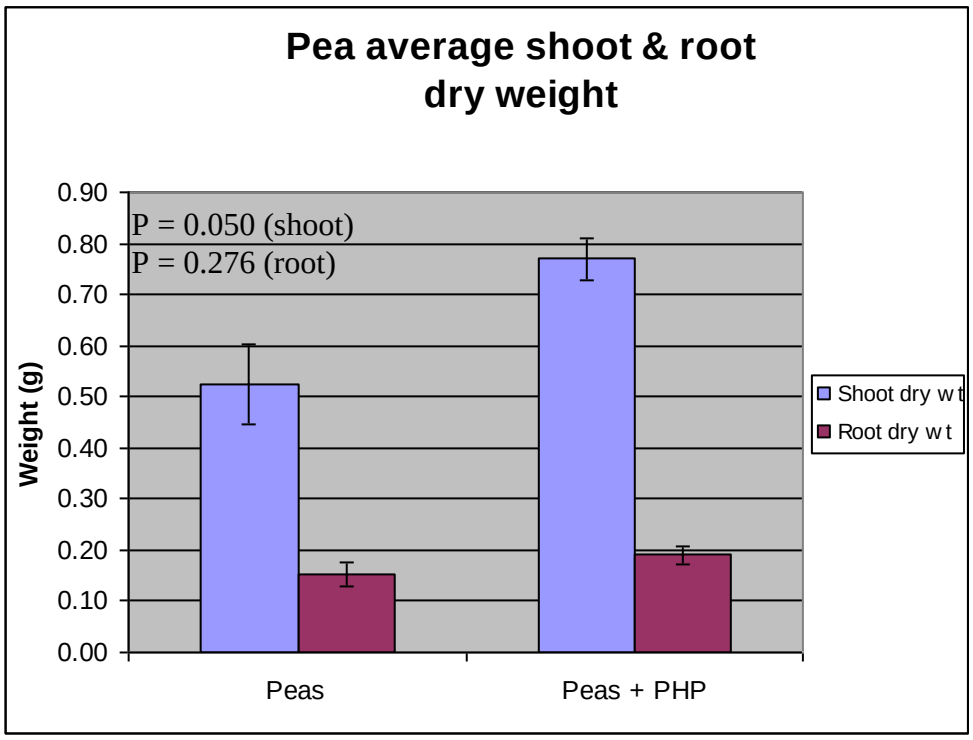


Figure 23: Pea average shoot & root dry weight

After 6 weeks, shoot length was 48.7% greater for plants with polymer compared to those without (Figure 22). Shoot dry weights for plants with polymer added increased by 48.1% and root dry weight increased by 26.7% (Figure 23). Statistical analysis showed that shoot length and dry weight were significantly higher for plants plus polymer than for those without polymer (P = 0.029 and 0.05 respectively). Root weights were not significantly higher with a P value of 0.276 (Appendix 2, p164).

Results for both beans and peas indicate that polymer produced by this method produces a significant increase in plant growth and therefore has potential to act as a fertiliser over an extended period unlike conventional fertiliser which is highly soluble and is washed away very quickly. Further experiments were conducted to verify that the enhanced growth was due to nitrogen contained in the polymer.

3.3 Peas grown in vermiculite with & without PHP neutralised with ammonium hydroxide and potassium hydroxide

In order to confirm that the increase in yield was the result of extra nitrogen in the polymer, the experiment was repeated but using potassium hydroxide instead of ammonium hydroxide to neutralise the excess sulphuric acid. There was therefore no nitrogen present in the production of the polymer. Nitrogen is the primary constituent

of fertiliser which produces the biggest yield response so if it is nitrogen in the polymer that is producing the positive result, then its absence should reduce or eliminate the effect. Peas were used in this experiment, variety Early Onward which is an earlier maturing variety than Phoenix and was used in all subsequent experiments with peas. Three treatments were used; (1) control with no polymer added; (2) polymer neutralised with ammonium hydroxide; (3) polymer neutralised with potassium hydroxide, with three replicates of each treatment. Vermiculite was again used as the growth medium and the experiment was conducted in a Weiss Gallenkamp growth cabinet (Figure 24) for eight weeks. Conditions in the cabinet were the same for all experiments: 16 hours light, 1 hour dawn and dusk, 6 hours dark, 24^0C, and 70% relative humidity.

Figure 24: Weiss Gallenkamp growth cabinet

It was also repeated in the greenhouse but very high temperatures resulted in many plants dying so only growth cabinet results are recorded here.

3.3.1 Results & Discussion

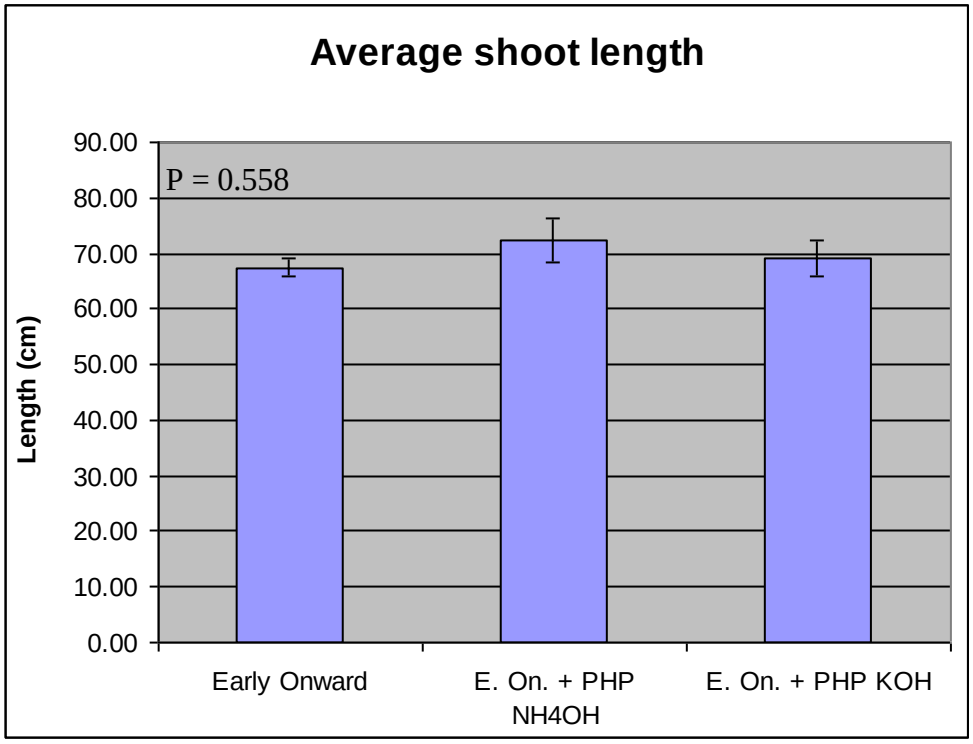


Figure 25: Peas (Early Onward) average shoot length (vermiculite)

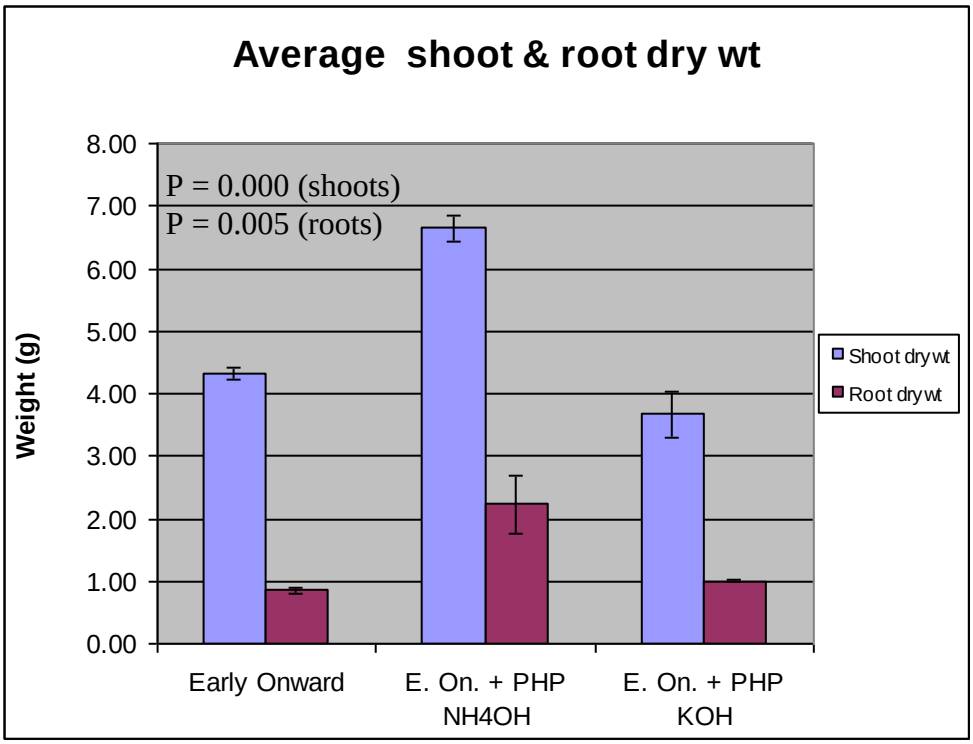


Figure 26: Peas (Early Onward) average shoot & root dry weight (vermiculite)

Unlike the previous experiment, there was no significant difference between shoot lengths of different treatments (Figure 25), possibly due to the fact that this was conducted in a growth cabinet with constant, high light and temperature levels whereas

the previous experiment was in a greenhouse where light and temperature levels were highly variable. But plants grown with polymer neutralised with ammonium hydroxide again produced a significant weight gain of both shoots and roots (Figure 26). Shoot dry weight was 53.3% higher than control plants and root dry weight was 162.3% higher. Plants with polymer neutralised with potassium hydroxide produced a slight weight decrease of shoots compared to the control plants and roots produced a small increase. Shoot dry weights were 15.2% lower and root dry weights were 18.8% higher (Figure 26, Appendix 2, p165).

This result confirms it is the nitrogen in the polymer that is producing the positive effect because when no nitrogen is present, there was no weight increase compared to the control plants.

3.4 Observation of root growth in vermiculite

In order to carry out these experiments in a controlled environment where all inputs are known, vermiculite was used as the growth medium and all essential nutrients except nitrogen were added in the water. Vermiculite is a nutrient free, neutral pH, chemically and biologically stable growth substrate, but it has a very light, open structure so the behaviour of roots growing in it may be different from their behaviour growing in soil which is much more dense and compacted. In order to investigate the behaviour of roots growing in vermiculite, pea seeds were germinated on top of vermiculite in vertically held Petri dishes with the top and bottom halves taped together with Parafilm and a section cut off the top edge.

3.4.1 Results & Discussion

As roots grew down through the vermiculite, they were observed to avoid penetrating the polymer but go round it (Figure 27).

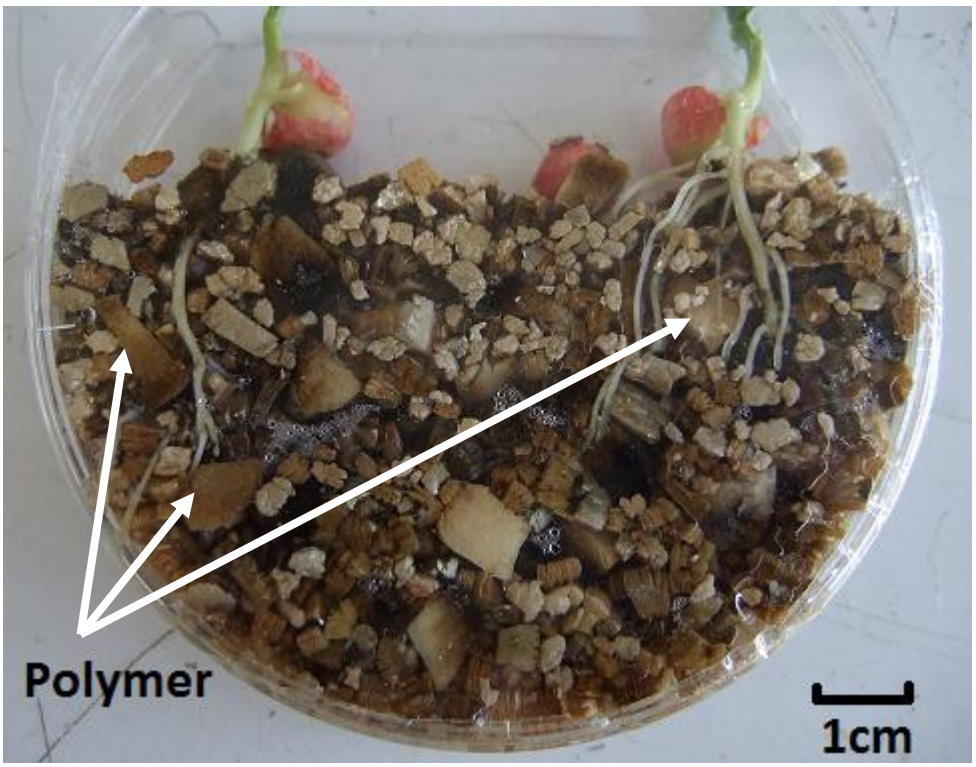


Figure 27: Observation of pea roots growing in vermiculite

The loose structure of the vermiculite with large spaces between the particles makes this the easiest route for the roots to take. It was not possible to repeat the experiment using soil because the roots would not be visible in situ in the Petri dish. However, when plants were removed from pot experiments, the roots were observed to have penetrated the polymer extensively. There are two possible reasons why roots should behave in this manner – polymer is less dense than soil so roots will be able to penetrate it easier than soil. And secondly, the polymer, being hydrophilic, attracts water and roots are naturally attracted to water by the phenomenon known as hydrotropism (Graham *et al*, 2006).

3.5 Peas grown in soil with & without PHP neutralised with ammonium hydroxide and potassium hydroxide

Roots are known to be attracted to and grow through the polymer when grown in soil (Burke, 2007) but, since this has not been observed when using vermiculite, the previous experiment using PHP neutralised with ammonium hydroxide and potassium hydroxide was repeated using soil to investigate if a similar result was observed. The soil mixture used was 75% John Innes No.3 and 25% horticultural sand. The experiment was conducted in a growth cabinet. The experiment was again set up as p40.

3.5.1 Results & Discussion

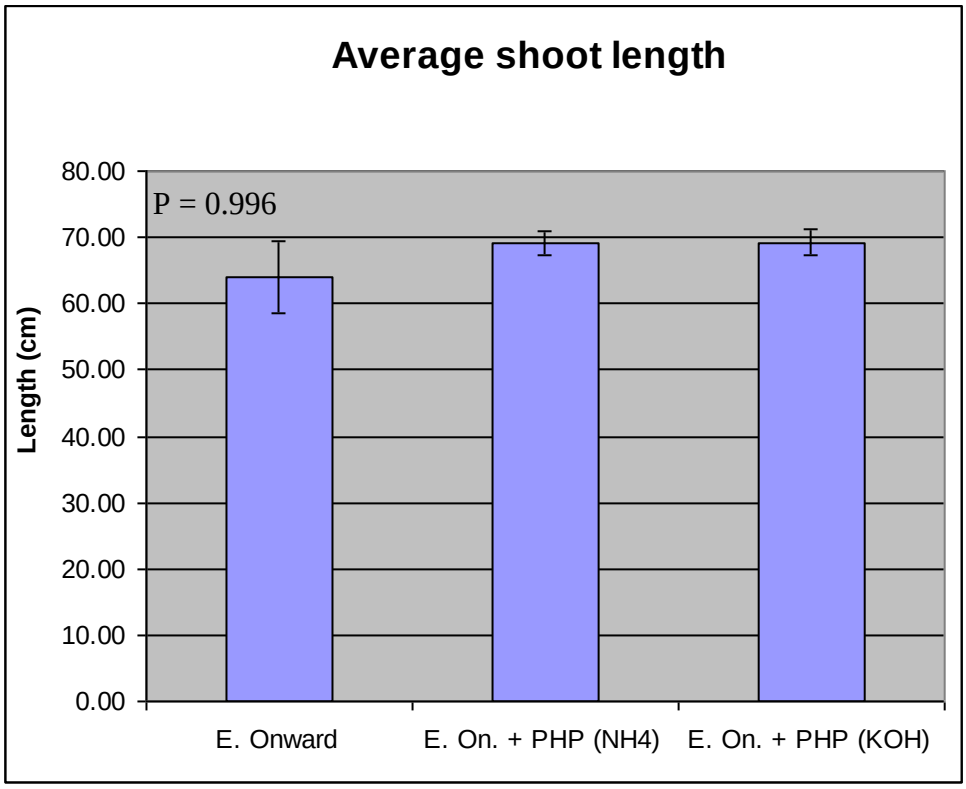


Figure 28: Peas (Early Onward) average shoot length (soil)

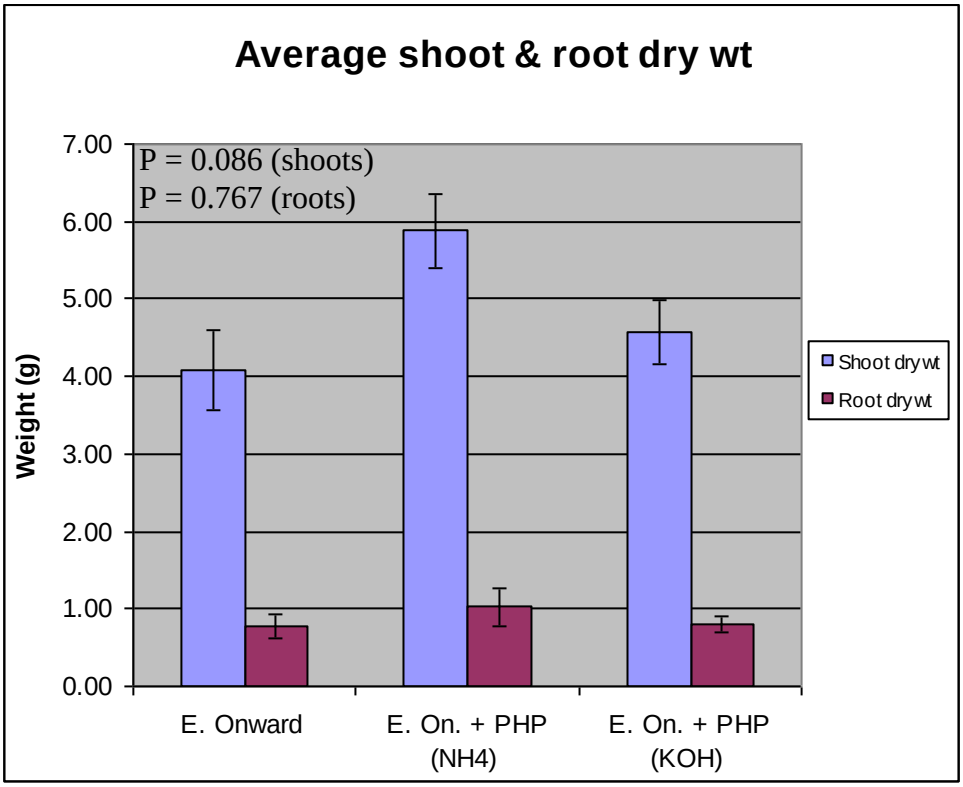


Figure 29: Peas (Early Onward) average shoot & root dry weight (soil)

As with the previous result using vermiculite, no significant difference was observed in the length of shoots (Figure 28), but there were differences between both shoot and root

weights for plants grown with polymer neutralised with ammonium hydroxide but not with potassium hydroxide (Figure 29). Shoot dry weights for plants with PHP neutralised with ammonium hydroxide were 43.8% higher than control plants and root dry weights were 30.8% higher. Dry weights for plants with PHP neutralised with potassium hydroxide were not significantly different from control plants. Further statistical analysis showed that the result for both shoots and roots neutralised with ammonium hydroxide were not significant (P = 0.086 and 0.767 respectively) due to the large variation between replicates (appendix 2, p166). Roots of all plants were observed to penetrate the polymer and become intimately associated with it (Figures 30 & 31), unlike plants grown in vermiculite.

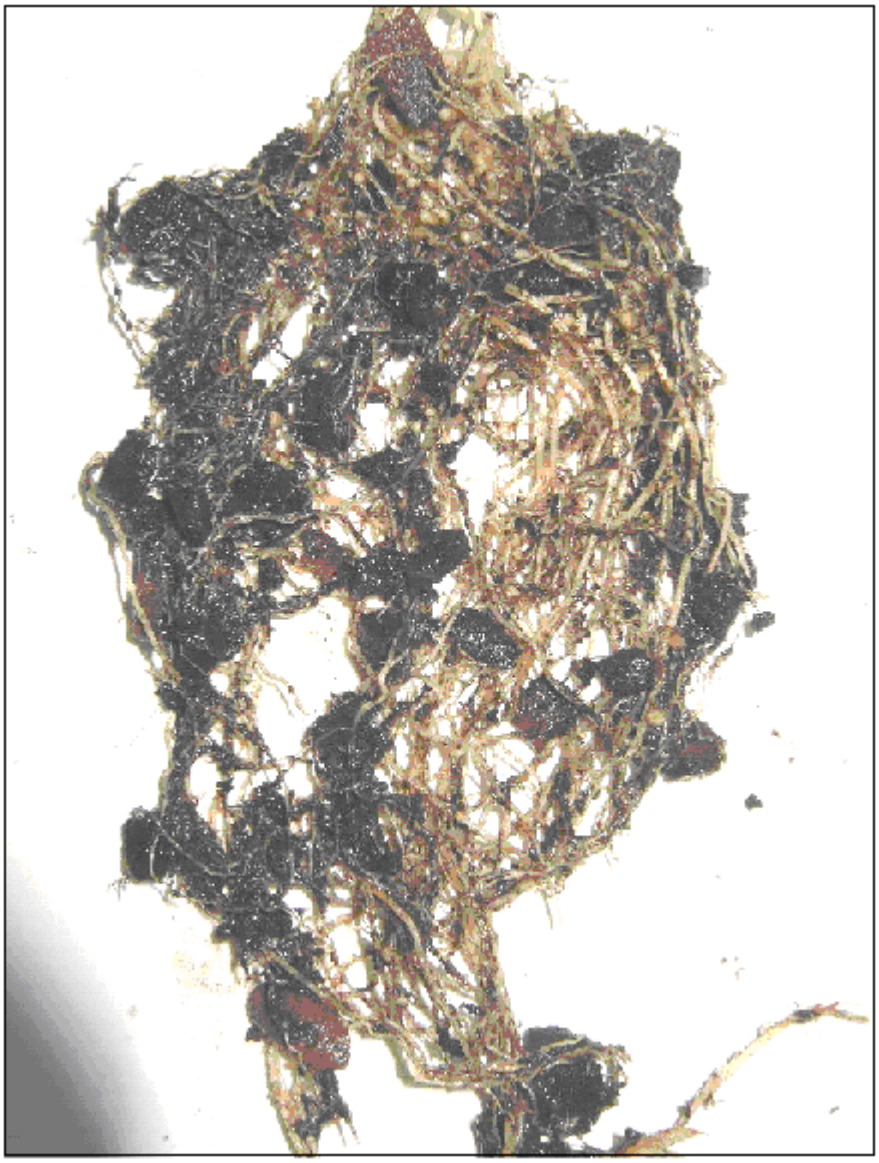


Figure 30: Pea roots showing root nodules and root penetration of polymer

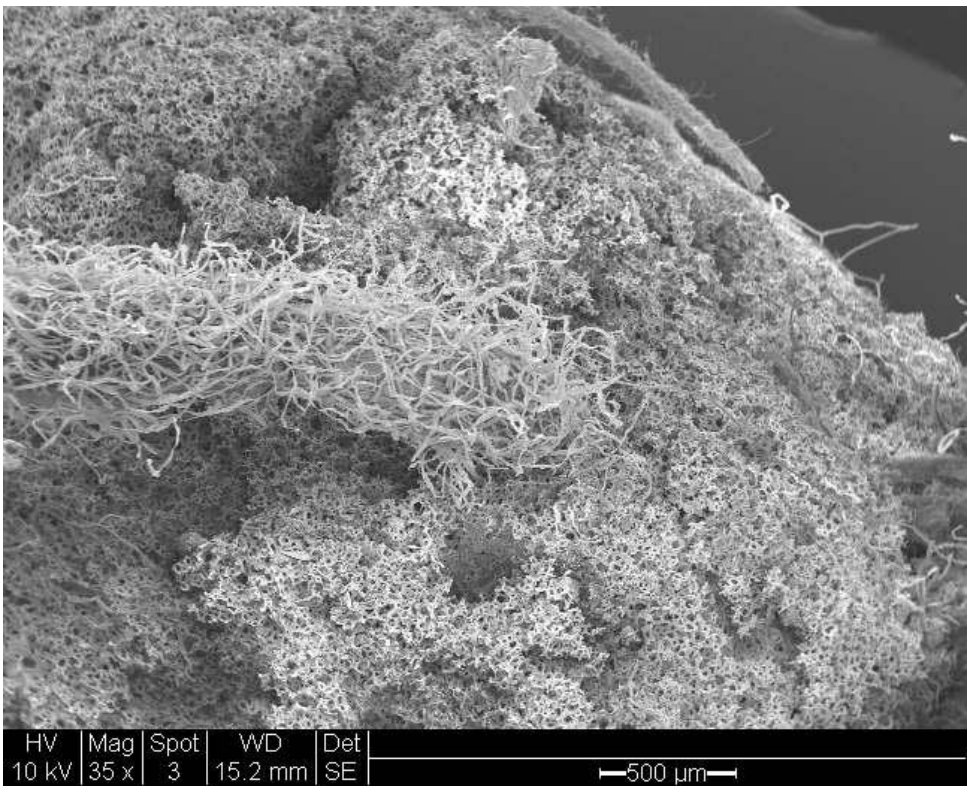


Figure 31: SEM of pea root growing through polymer 35X magnification

As the root penetration of the polymer was only observed when plants were grown in soil and not vermiculite, only soil was used in all further experiments.

Root nodules were concentrated around the top of the lateral roots (Figure 32), although not as many were concentrated on the tap root as they are on the soybeans.

Figure 32: Pea root showing root nodules

Pea nodules have a more elongated structure compared with the soybean nodules which are more round. Pea nodules contain a meristem and can continue to grow over long periods producing elongated structures. Soybean nodules cease to divide and grow by expansion rather than division producing round nodules.

Conventional methods of fertiliser application involves the use of highly soluble products which dissolve very quickly when they come in contact with moisture and a large proportion gets leached out of the soil and is never of any benefit to the plants, as well as causing environmental problems by polluting water systems. For plants growing in soil, the roots are widely dispersed through the soil, so a lot of water and fertiliser is nowhere near any plant roots. By using PHP, the roots are attracted to the polymer and become intimately associated with it, so it brings the roots into close proximity with the fertiliser in the polymer. The fertiliser is released slowly, close to the plant roots and therefore allowing a much greater proportion of the fertiliser to be utilised by the plant so there will be much less wasted making it more economic as well as causing less pollution.

Chapter 4: Development of polyHIPE as a soil additive to enhance crop yield in semi-dry environments

A vast and increasing expanse of land area is covered by semi dry land that can at best only produce crop yields substantially reduced compared to adequately watered land. Experiments were conducted in order to follow on form previous work (Burke, 2007) to investigate the effect of PHP on plant growth in reduced watering conditions.

Summary of drought experiments:

- Observation of root attraction to polymer
- Soybean in semi dry environment
- Jatropha in semi dry environment
- Polyurethane as an alternative to PHP

4.1 Observation of Root Attraction to Water

Sulphonated polymer is hydrophilic and attracts water which can then act as a reservoir to supply the plant in drier conditions. Plant roots are naturally attracted to a water source by the phenomenon known as hydrotropism. This experiment used whole polymer discs watered via Pasteur pipettes to demonstrate the attraction to the polymer by plant roots.

4.1.1 Materials and Methods

Two polymer discs were placed in a 10cm pot of soil (75% John Innes No3, 25% horticultural sand) one below the other, one inch from the surface and one inch between them. A Pasteur pipette was stuck into each disc for watering them. Fifteen grass seeds were placed in the centre of the pot and covered with soil mixture (Figure 33).

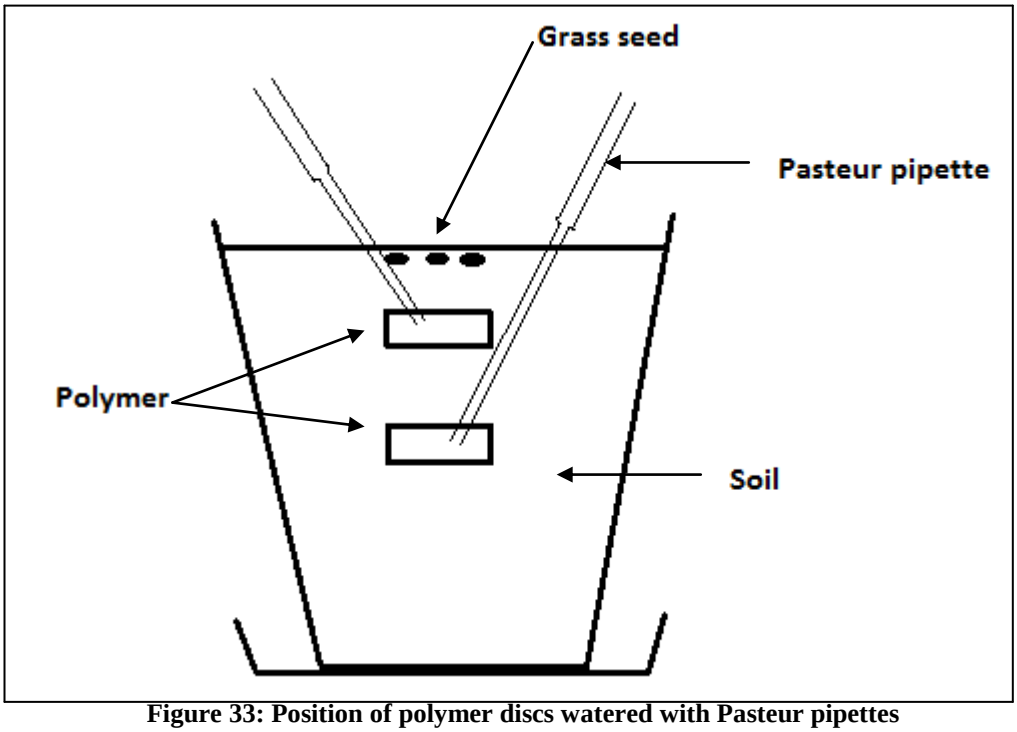


Figure 33: Position of polymer discs watered with Pasteur pipettes

A control was also used, watered with pipettes but with no polymer. There were 4 replicates of each treatment. Pots were watered normally until grass germinated, then watering was via the pipettes, 3ml/pipette every 3 days. Plants were harvested after 3 weeks and the roots examined to observe the interaction with the polymer.

4.1.2 Results & Discussion

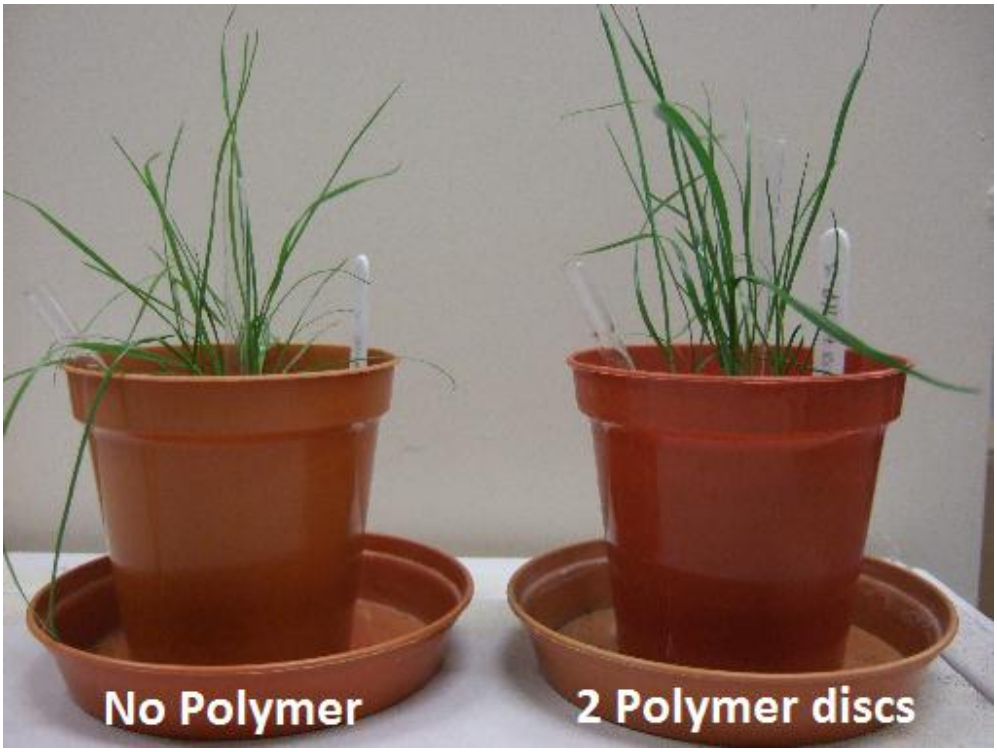


Figure 34: Grass growth after 3 weeks

After 3 weeks growth, control plants looked wilted compared to the plants with polymer which remained fresh and healthy (Figure 34), indicating that the control plants were suffering some water stress.

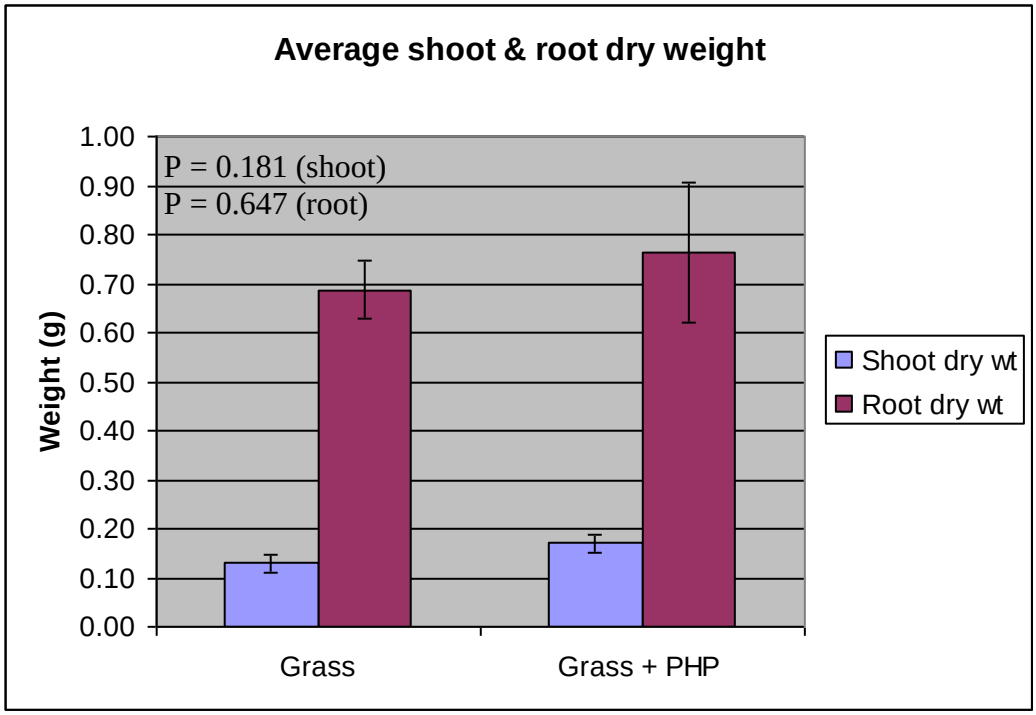


Figure 35: Average shoot & root weight after 3 weeks growth

Average shoot dry weight of plants with polymer was 30.8% heavier than control plants after 3 weeks growth and root dry weight of plants with polymer was 10.1% heavier than control plants but none of the results were significant (Figure 35, appendix 2, p167).

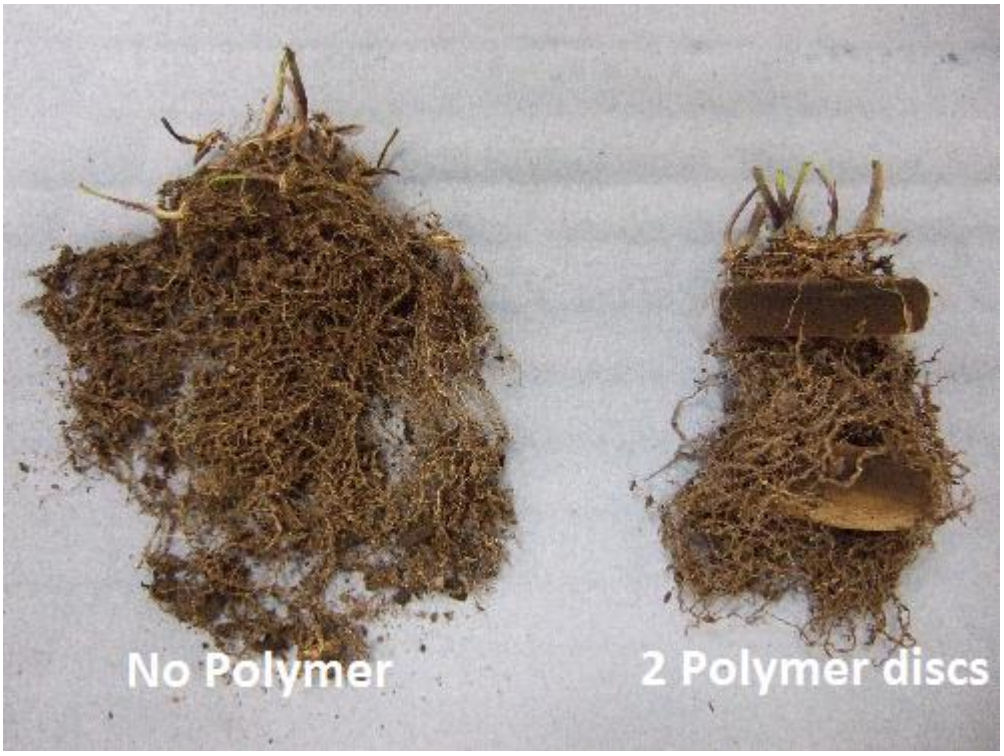


Figure 36: Roots after 3 weeks growth

From the picture of roots (Figure 36), it looks like the control plants have a larger root volume, but it looks exaggerated because the control plant roots are nearly 2 dimensional, lying flat on the surface whereas the roots with the polymer is 3 dimensional, held up by the polymer.

Water and any nutrients it contains is normally fairly evenly distributed in soil and in light, sandy soils it drains away easily, but in the presence of the hydrophilic polymer more water is retained in the soil which would otherwise be drained away which therefore allows plants to survive longer in semi dry environments. Plant roots are naturally attracted to water, by the phenomenon known as hydrotropism, so the roots are then attracted to the polymer and become intimately associated with it.

4.2 Growth of Soybeans in semi dry environments

The soybean plant (*Glycine max*) is an annual plant native to South East Asia. It is a leguminous plant, a member of the pea family and the beans are a source of high quality protein. It grows 40-100cm tall and produces yellow, spherical beans the size of peas in pods containing 3 or 4 seeds. It has been used as a food source for more than 5000 years.

The experiment was to investigate the effect of the addition of PHP on the growth of soybeans with normal and reduced watering. In order to simulate less fertile conditions, the soil was washed to reduce the nutrient level. The washing was monitored by measuring the conductivity with a conductivity meter. As more solutes were washed out of the soil the conductivity of the water increases. The nitrogen, phosphorus & potassium content of the soil were measured, in triplicate, before and after washing.

4.2.1 Elemental Analysis

Nitrogen analysis

Nitrogen content was analysed using a Leco FP428 nitrogen analyser (Figure 37).

Figure 37: Leco FP428 nitrogen analyser

Air dried samples were ground with a pestle and mortar and 0.25g samples were weighed to 4 decimal places, wrapped in silver foil and placed in the machine which purges atmospheric gases, then is dropped into a hot furnace (850^0C) and flushed with pure oxygen to achieve a rapid combustion. The combustion products, mainly $C0_2$, H_2O, NO_x and N_2 are passed through a thermoelectric cooler to remove some of the water. The gas mixture is then analysed by passing through hot copper which removes oxygen and changes NO_x to N_2. The sample then passes through a desiccant which removes CO_2 and water, leaving only nitrogen which is measured in a thermal conductivity cell.

Phosphorus analysis

Phosphorus content was analysed by a colorimetric method using ammonium molybdate and measuring the absorbance of the blue coloured phospho-molybdate

complex using a spectrophotometer. Phosphorus was extracted from the soil and the concentration determined by the addition of ammonium molybdate and ascorbic acid to form a blue phospho–molybdate complex. The intensity of the blue colour is proportional to the concentration of phosphate in the sample. The concentration was determined by measuring the absorbance using a U.V. spectrometer and comparing the result with a standard graph prepared using solutions of known phosphorus concentration.

Potassium analysis

Potassium content was analysed using a PFP7 flame photometer (Figure 38).

Figure 38: PFP7 Flame photometer

The test solution is sucked into the machine via a capillary tube and passed through an atomiser producing a fine mist which is drawn into the flame. The light produced by the combustion of the elements is then analysed by a photoelectric cell. The readout produced is an arbitrary figure which is converted to a potassium concentration by producing a standard curve by measuring the readouts of solutions of known potassium concentration. At higher concentrations, the graph flattens off producing a curve rather than a straight line and therefore a less accurate result, so if the potassium concentration is higher, a more accurate result is obtained by diluting the sample so that a reading can be obtained from the lower end of the graph where there is a direct relationship between the reading and potassium concentration so a more accurate result is obtained.

4.2.2 Materials & Methods

4.2.2.1 Soil preparation

The growth medium used was made up of 75% Wilkinsons soil from Wilkinsons Hardware Stores Ltd, Nottingham, S80 3YY and 25% horticultural sand. The soil mixture was washed to remove some of the nutrients producing a product with lower fertility so that the effect the polymer was having on plant growth would be evident earlier. The soil was washed by adding 10 litres of water to 2.5kg of dry soil. Washing continued for 48 hours, stirring occasionally, changing the water after 24 hours. The washing was monitored by measuring the conductivity of the water over the washing period. The conductivity of tap water was 315µs/cm. After 24 hours, with occasional stirring, the conductivity increased to 775µm/cm (average of 3 samples). The water was drained and a further 10 litres of water added. After a further 24 hours, the conductivity averaged 506µm/cm. The conductivity due to soil electrolytes was therefore reduced from 460µS/cm to 191µS/cm. Soil was then dried in trays and sterilised in an autoclave in 500g aliquots in autoclavable plastic bags.

4.2.2.2 Elemental Analysis

Nitrogen analysis

Air dried samples were ground with a pestle and mortar and 0.25g samples were weighed to 4 decimal places, wrapped in silver foil and placed in the analyser.

Phosphorus analysis

Soil was air dried and ground with a pestle and mortar. 5.00g $\pm$ 0.01g was weighed into a 250ml plastic bottle, 100ml 0.5M sodium hydrogen carbonate solution was added to each bottle, stoppered and shaken on an orbital shaker at 275 rpm at room temperature for 30 minutes. The samples were then filtered and 2ml samples were pipetted into test tubes along with phosphorus calibration solutions and a blank solution. 8ml of colour reagent were added to each tube, mixed and allowed to stand for 60 minutes. Tubes were then placed in a water bath at 90^0C for 10 minutes, cooled to 20^0C, mixed thoroughly, then the absorbances were measured at 712nm using a uv spectrophotometer. A standard curve was constructed by measuring the absorbance of solutions of known phosphorus concentration, then the phosphorus concentration of the samples can be read from the graph.

Potassium analysis

Air dried samples were ground with a pestle and mortar. 10g of each sample was weighed into a 250ml plastic bottle, 50ml 1M ammonium nitrate was added to each bottle and they were shaken for 30 minutes at 275 rpm at room temperature. Samples were then filtered, then analysed in the flame photometer. The system was flushed with deionised water between each sample. A standard graph was produced using samples of known potassium concentration from which the potassium concentration of the samples was determined.

4.2.2.3 Planting of soybeans

Soybeans (*Glycine* max, variety *Pan*) from South Africa were used in these experiments. Plants were grown in the washed soil / sand mixture in a greenhouse from 13/7/09 – 23/10/09 in 13cm diameter pots. PHP was added to the soil at the rate of 0.5% by weight (Akay and Burke 2010). These pots hold 500g of soil, therefore 2.5g of PHP was added to each pot. Polymer was only added to the soil below the level of the seed. Four treatments were used in this experiment: (1) soybeans, normal watering; (2) soybeans, reduced watering; (3) soybeans + PHP, normal watering and (4) soybeans + PHP, reduced watering. Normal watered plants were watered twice a week with 100ml water. Reduced watered plants were watered twice a week with 50ml water. Each treatment was replicated 6 times. Temperature ranged from a minimum of 18^0C to a maximum of 37^0C. Plants were harvested after 15 weeks and the length, fresh weight and dry weight of the shoots and the fresh weight and dry weight of the roots were recorded. Shoot length and shoot and root dry weights are recorded here.

4.2.3 Results & Discussion

4.2.3.1 Elemental Analysis

Nitrogen analysis

	Before washing	After washing	% reduction
Nitrogen	0.22%	0.14%	36.4%

Table 2: Nitrogen analysis of soil

Washing has reduced the nitrogen content of the soil by 36.4% (Table 2).

Phosphorus analysis

Standard graph

[P] standard solutions (µg/ml)	Absorbance (712nm)
0	0
5	0.248
10	0.425
15	0.561
20	0.642

Table 3: Phosphorus standard graph

From the absorbencies of the known phosphorus concentrations (Table 3), the phosphorus standard graph was constructed (Figure 39), from which the phosphorus concentration of the samples was determined.

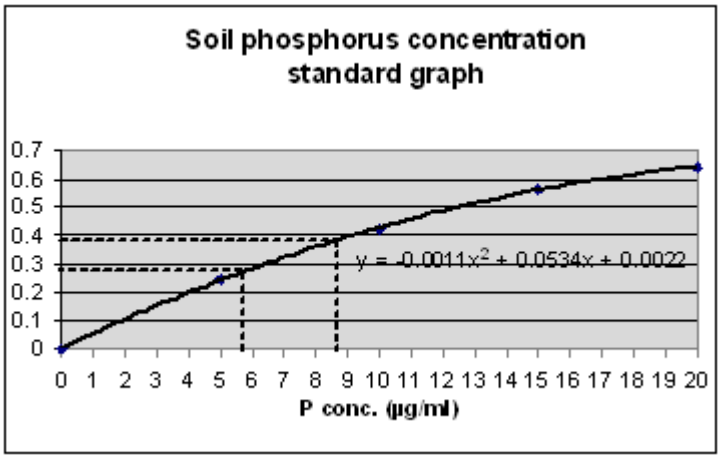


Figure 39: Phoshorus standard graph

	Before washing	After washing	% reduction
Absorbance (av.of 3)	0.382	0.278	
[Phosphorus]	8.66µg/ml	5.87µg/ml	32.2

Table 4: Phosphorus analysis of soil

Washing has reduced the phosphorus content of the soil by 32.2% (Table 4).

Potassium analysis

Standard graph

[K] standard solutions (µg/ml)	Readout
0	0
5	36
10	67
15	113
20	127
25	150

Table 5: Potassium standard graph

From the absorbencies of the known potassium concentrations (Table 5), the potassium standard graph was constructed (Figure 40), from which the potassium concentration of the samples was determined.

Samples were diluted 10X to bring them within the range of the standard graph.

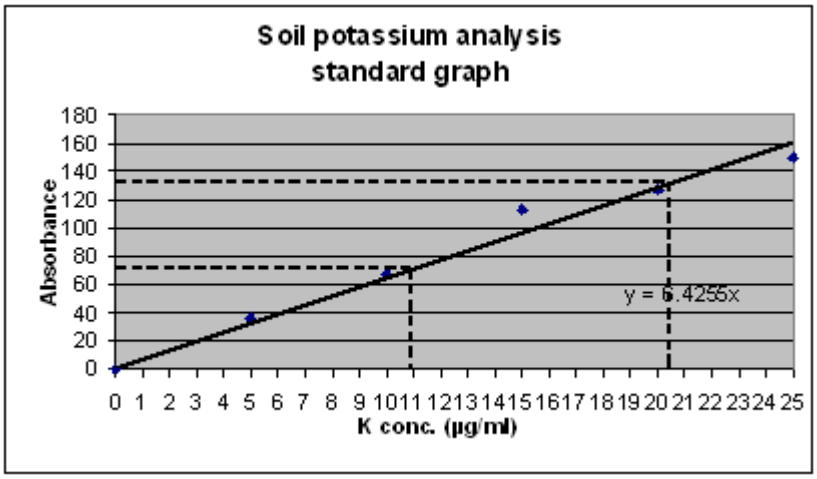


Figure 40: Potassium standard graph

	Before washing	After washing	% reduction
Absorbance (av.of 3)	130.4	69.7	
[Potassium]	203.0µg/ml	108.5µg/ml	46.5

Table 6: Potassium analysis of soil

Washing has reduced the potassium content of the soil by 46.5% (Table 6).

The reduced nutrient levels of the washed soil was then more representative of less fertile soil conditions, so any effect the polymer was having would be evident sooner.

4.2.3.2 Effect of polymer on soybean growth

Results show that the addition of polymer significantly increased the length and weight of both shoots and roots compared to the control plants with no polymer after 15 weeks of growth, both of normal watered and reduced watered plants.

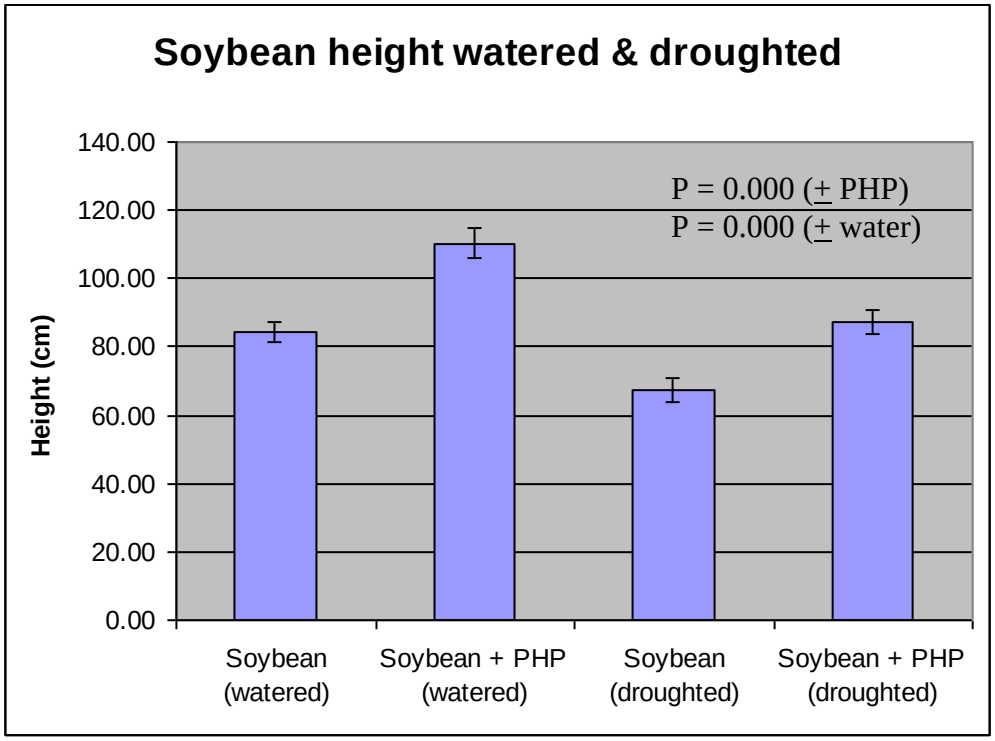


Figure 41: Average soybean height watered & droughted

The addition of PHP has had a significant effect on the shoot length both of normally watered plants and droughted plants (Figure 41). Watered plants with PHP were 30.8% longer than control plants with no polymer. Although droughted plants were 20.2% shorter than the normally watered control plants, the addition of PHP increased the length of droughted plants by 29.7% compared to the droughted plants with no PHP, making them similar in length to the normally watered control plants with no polymer.

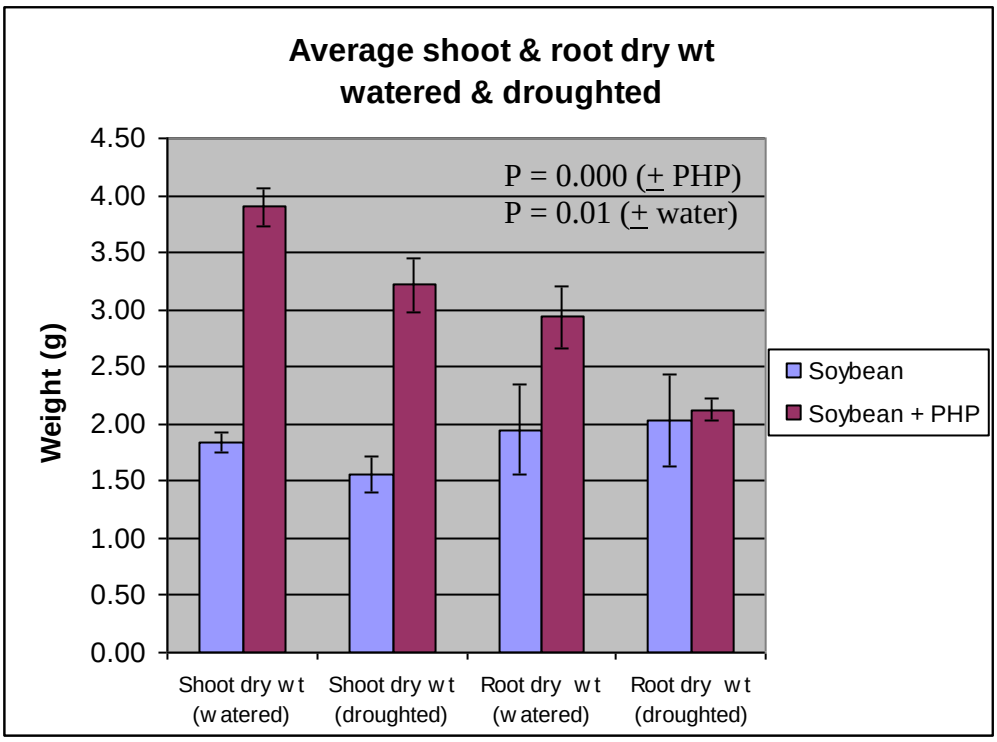


Figure 42: Average soybean shoot & root dry weight watered & droughted

Watered shoot dry weight with PHP increased by 111.9% compared to control with no PHP and droughted shoot dry weight with PHP increased by 106.4% compared to control with no PHP (Figure 42). Watered root dry weight with PHP increased by 50.8% compared to control with no PHP and droughted root dry weight with PHP increased by 4.4% compared to control with no PHP. This result is consistent with previous results using grass grown under drought conditions with the addition of PHP (Burke, 2007). Shoot dry weight of droughted plants plus PHP was increased by 106.4% although the root dry weight was only increased by 4.4%. This would suggest that the roots are more efficient in the presence of polymer than they are without it. It is not a normal plant response to drought which is for plants to increase the root size in drought conditions to increase the area of ground from which they can absorb water. Results were analysed by 2 way ANOVA and showed a significant effect of both addition of water and of PHP, but there was no significant interaction between the 2 treatments (P = 0.247), (appendix 2, p171). SEM pictures of the PHP (Figure 43) with root penetration showed the intimate association of the roots with the polymer therefore bringing the roots into close proximity with any available water and nutrients in the polymer.

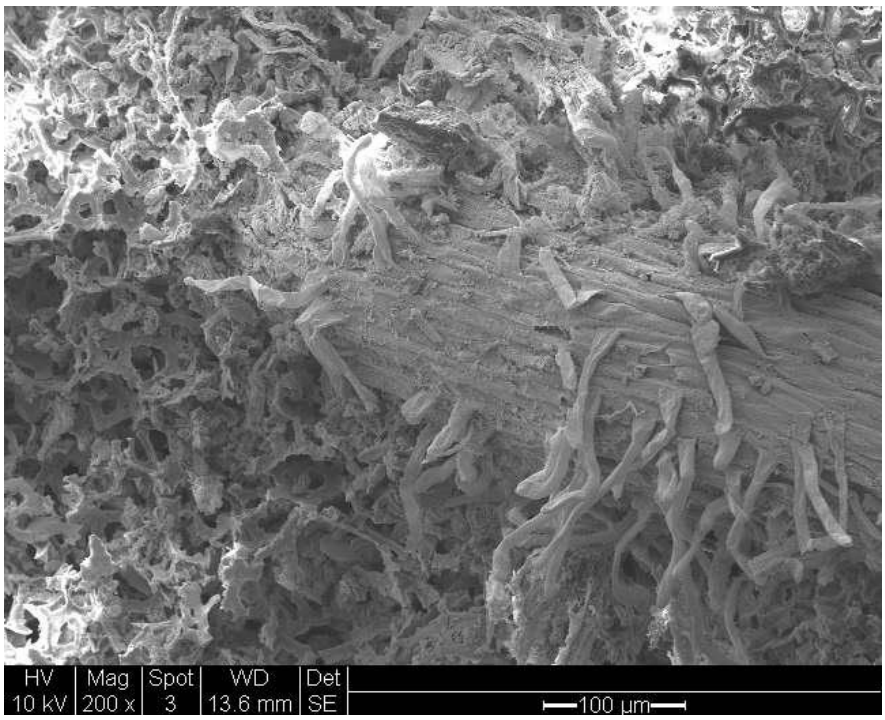


Figure 43: SEM of root penetration of the PHP

The results indicate that, although a positive effect has been obtained for droughted plants, the polymer also produces a positive effect when water is not limiting so it is also of benefit in non drought conditions. This can be attributed to the nitrogen content of the polymer which is still present even when neutralised with acetic acid and washing out the excess, some nitrogen still remains which will have a positive effect on plant growth (p 42).

4.3 Jatropha

Diminishing supplies of fossil fuels and the accumulation of greenhouse gasses are dictating that alternative sources of fuel must be developed. One of the most important energy sources for the future could be biomass from which biofuel can be produced and used as a substitute for fossil fuels. Biofuel is a renewable energy source which has several benefits over fossil fuels including greater energy security, reduced environmental impact by a reduction in greenhouse gas emissions, reduced dependence on imports of fossil fuels and protection against the volatile price of fossil fuels (Achten *et al*, 2010). Biodiesel is carbon neutral – there is an equal balance between the amount of CO_2 emissions and the amount of CO_2 absorbed by the growing plants that produce the fuel.

Jatropha has emerged as a potentially suitable plant for cultivation for biofuel production. Jatropha curcas L. is a perennial, deciduous, stem succulent plant which produces seeds with up to 35% oil which can easily be converted to biodiesel (Maes *et al*, 2009[1]; Maes *et al*, 2009[2]). The processed oil can, after minor modifications, be used directly in diesel engines or it can be blended with conventional diesel (Parawira, 2010). A major problem with growing biomass as a source of biofuel is the conflict between growing plants for food and for fuel. Jatropha plants can potentially overcome this problem by being able to grow on marginal semi arid land which is unsuitable for food production and is at present unproductive. There are vast areas of such land in many countries – for example 40 million hectares in India (Kaushik *et al*, 2007). The promotion of such enterprises can help in the development of these countries and create employment in otherwise very underdeveloped areas.

Figure 44: Jatropha bush in Zimbabwe (www.reuk.co.uk/Jatropha-for-Biodiesel-Figures.htm, 2010)

Jatropha plants can survive on semi arid tropical land for up to 50 years reaching a height of 5 metres (Figure 44), producing a crop of seed for more than 30 years and therefore turn barren wasteland into productive ground without interfering with food production (Fairless, 2007). Erosion is becoming a serious problem in some countries where bare land is exposed to the elements, in particular wind and water. Yearly, more than 10 million hectares of valuable crop land are degraded and lost because of wind and water erosion of soil (Pimentel and Pimentel, 2006). Jatropha helps to reduce erosion by providing ground cover on otherwise barren land and therefore help to protect it (Figure 45).

Figure 45: Jatropha cultivation transforms barren land in India into lush productive land (Fairless, 2007)

Once the plants have been established and the ground has been stabilised, conventional food crops can then be grown in strips between the jatropha plants (Makkar & Becker, 2009).

Jatropha originated in Central America has been cultivated in Asia and Africa since the 16th century when it was used for medicinal purposes. Its name is derived from Greek 'jatros' meaning doctor and 'trophe' meaning food/nutrition (Fairless, 2007; Krishnan & Paramathma, 2009). The seeds are poisonous to humans and most animals and the plant is non palatable to browsing animals. It can therefore be used as a hedging plant used for shelter belts and stock control, both to fence animals in and to protect growing crops from browsing animals and soil erosion. It is drought tolerant but can grow over a wide range of rainfall regimes – from 200 – 1500 mm per annum (Openshaw, 2000). It normally produces one crop of seeds per year, but with irrigation up to 3 crops can be produced. Seed yield is very variable from 0.4 – 12 tonnes/hectare/year after 5 years, the yield depending mainly on the amount of rainfall, but also the nutrient level. After the oil is removed from the seed, the residue can be used as a fertiliser (Openshaw, 2000; Fujimaki, 2010).

The plant is not affected by many serious pests or diseases when grown on a small scale but several African and Asian countries are now planting large scale plantations which may result in serious consequences if a virulent disease gets established in these areas. Very little work has been carried out on genetic improvement and agronomy of the native plants to produce higher yielding varieties and in some countries large scale plantations have been established but have resulted in very low yields of seeds, making them non viable (Divakara, 2010). There also needs to be adequate regulations in place to ensure that these plantations do not compete directly for ground that is suitable for

food production and therefore reduce food availability in areas where it is already scarce (Parawira, 2010).

Although it is claimed that jatropha can grow and produce a good yield of seeds on marginal land, experience has shown that in some places this is not happening. In several instances, for example, some plantations in Mozambique jatropha has been planted on arable land with good nutrient levels and irrigation and is therefore competing directly with land for food production and have still not produced the yield levels required to make the crop viable (www.jatropha.de). So perhaps large scale planting before sufficient research has taken place to establish the best strains has been a bit premature.

Nanotechnology is becoming a multi million pound industry with applications in many fields including catalysts, semiconductors, drug carriers, cosmetics and microelectronics (Nel *et al*, 2006). As a result, engineered nanoparticles (ENPs) are being released into the environment. ENPs are defined as materials with at least one dimension less than 100 nanometers (Nel *et al*, 2006). Some ENPs are taken up by plants which could have toxic effects on the plants although some have been reported to have a beneficial effect (Ma *et al*, 2010). Tomato plants have been shown to increase germination and growth rate by the addition of ENPs which penetrated the seed and promoted water uptake (Ma *et al*, 2010). However, the main concern is that ENPs will have a toxic and growth inhibitory effect on plants. Reduced germination, growth rate and root elongation in the presence of ENPs have all been observed (Ma *et al*, 2010). Some ENPs are also taken up by the plants and accumulate in the leaves. Iron oxide nanoparticles have been shown to accumulate in both the roots and leaves of pumpkin plants (Zhu *et al*, 2008). Samples of the jatropha root and stem were examined with SEM to observe if the addition of PHP had any physical effect on the structure. They were also analyzed by EDX to observe any differences in the composition of the roots before and after passing through the PHP. EDX (Energy Dispersive X-ray Analysis) is a facility attached to an electron microscope which identifies the elemental composition of a sample. Samples must first be carbon coated rather than gold coated when analyzed by EDX. Samples of the stems, leaves and roots were also chemically analyzed to observe any differences with the addition of PHP.

The experiment was to investigate the effect of the addition of PHP on jatropha plants watered normally and with reduced watering.

4.3.1 Materials & methods

Jatropha seeds were obtained from Kurunegala, Colombo, Sri Lanka. Plants were grown in a growth room in 13cm diameter pots. Growth conditions were 28^0C day and 22^0C night temperature, 16 hours day light and 8 hours dark. The growth medium was 75% Wilkinson's compost, 25% horticultural sand. The germination of the jatropha seeds was very poor (approximately 65%) therefore they were planted in small (10cm) pots and allowed to grow for 1 month to ensure enough seeds germinated before transplanting into 13cm pots with polymer. There were 4 treatments: (1) normal watering (200ml twice/week) with PHP; (2) normal watering without PHP; (3) reduced watering (100ml twice/week) with PHP; (4) reduced watering without PHP. There were 5 replicates of each treatment, with one seed per pot. 0.5% w/w of PHP was added to pots containing polymer. Pots held 500g of soil mixture therefore 2.5g of PHP was added to each pot. Liquid fertiliser was applied every 8 weeks. Miracle-gro All Purpose concentrated liquid plant food was used and applied at the recommended dosing rate of 11ml concentrate in 1 litre of water. Each pot was given 50ml diluted solution every 8 weeks. The height of each plant was measured and a picture taken every month from 3 months until harvest at 8 months. Shoots were harvested and weighed after 8 months and the fresh and dry weights recorded. Shoot length and dry weights are recorded here. Roots were very brittle and broke into small pieces when harvested so collection for weighing was not possible. Pictures of root and stem samples were taken with SEM, analysed by EDX and chemically analysed.

Elemental Analysis

Two samples each of leaves, stem and root were analysed by NRM laboratories, Bracknell, Berkshire for nitrogen, sulphur, phosphorus, potassium, calcium, magnesium, manganese, copper, zinc, iron and boron content.

Nitrogen and sulphur

Samples were dried, ground and passed over a 0.5mm screen. Analysis was carried out using a Carlo Erba NCS 2500 analyser. Samples were totally combusted in an oxygen

rich environment, then the oxides of the gases were separated using a chromatographic column and measured by thermal conductivity.

Phosphorus, potassium, calcium, magnesium, manganese, copper, zinc, iron and boron analysis

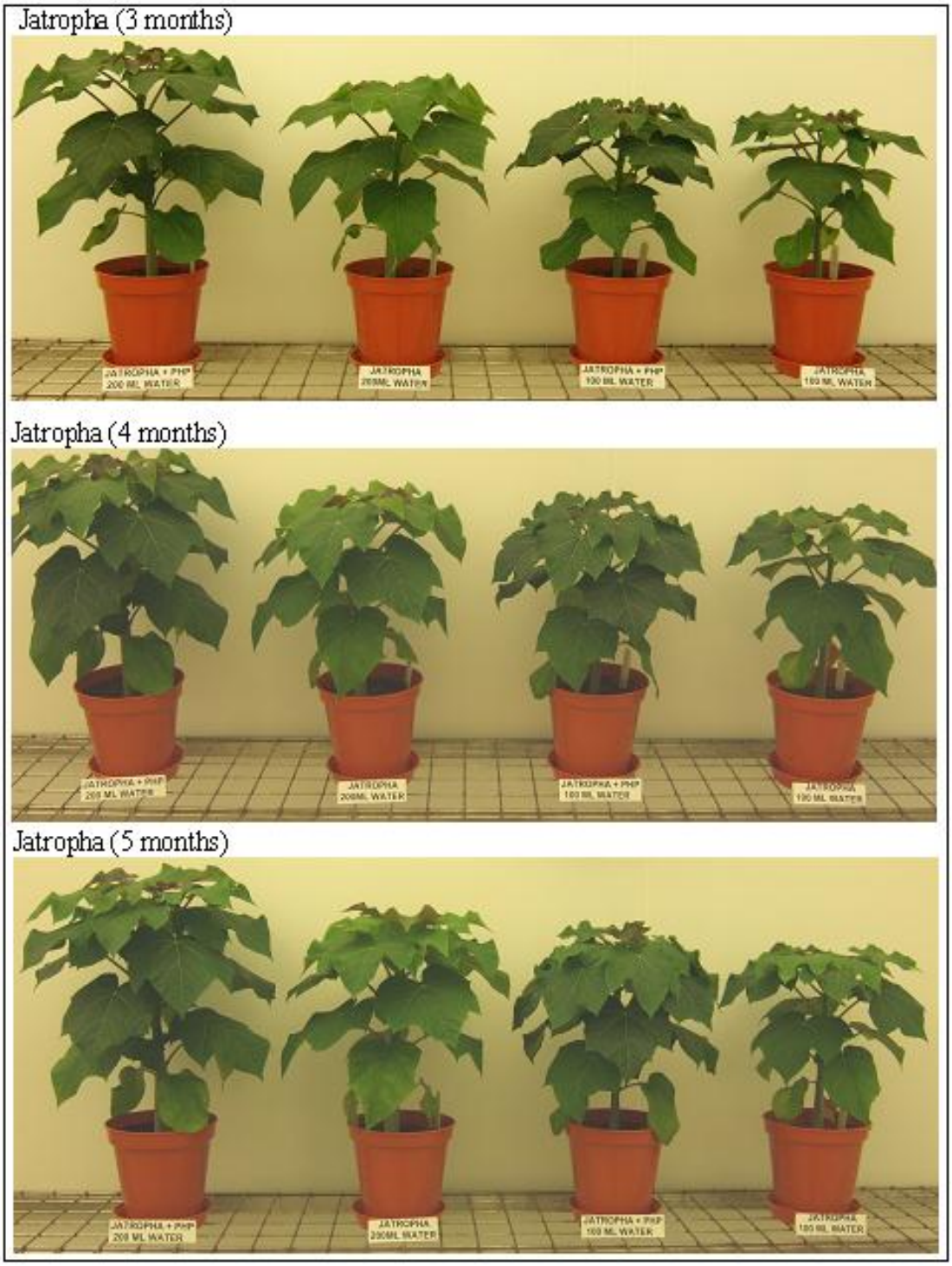
Samples were dried, ground and passed over a 1mm screen. The samples were ashed at 550^0C and the residue was dissolved in hydrochloric acid. The concentration of each element in the solution was determined by Inductively Coupled Plasma-Optical Emission Spectroscopy using a Perkin Elmer - Optima 5300 DV.

4.3.2 Results & Discussion

Jatropha plants produce 1 - 2 cm dark oval seeds containing up to 35% oil (Figure 46).

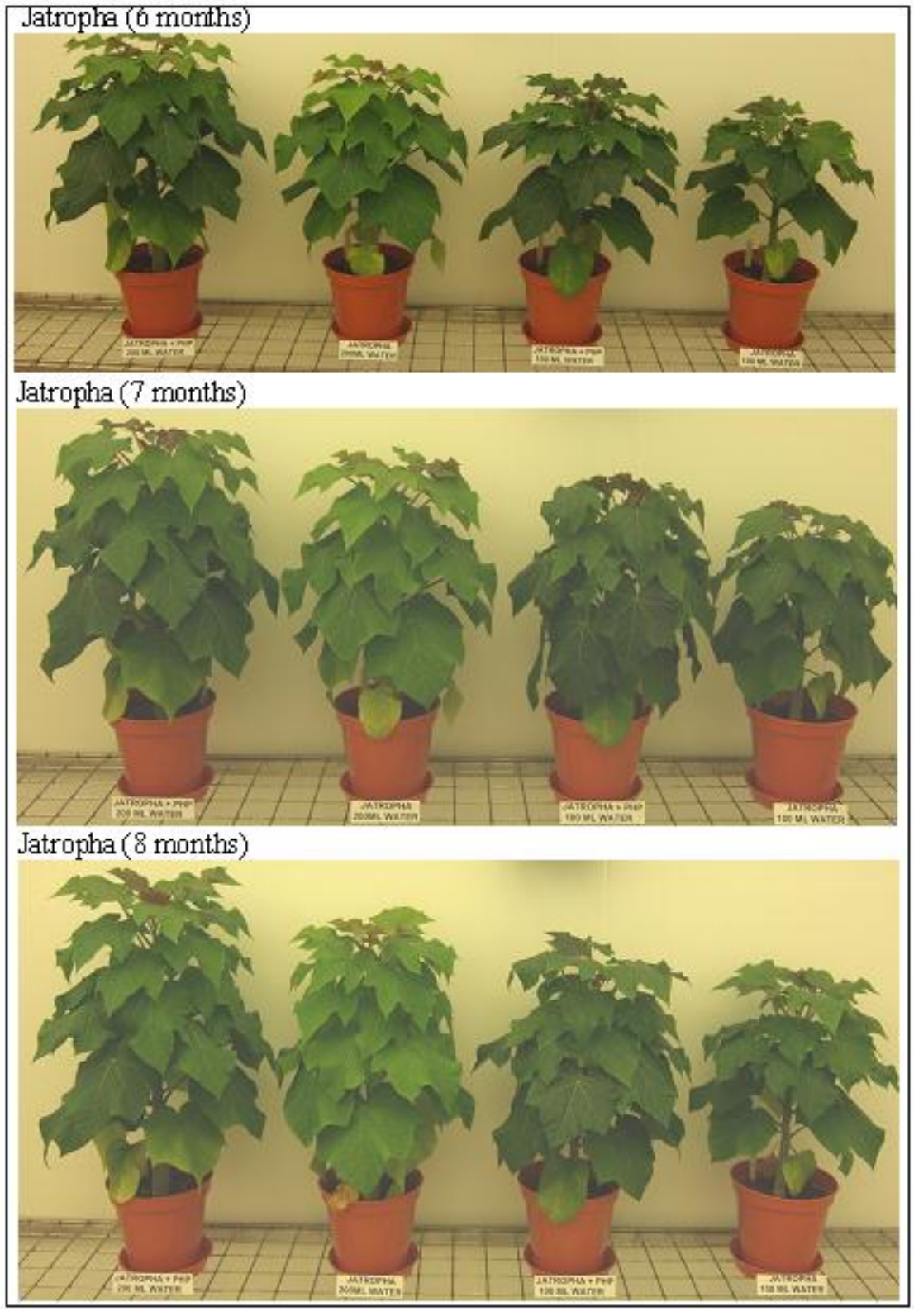
Figure 46: Jatropha seeds (Jatropha curcas)

A picture was taken every month from 3 – 8 months (Figure 47 a & b).



Jatropha + PHP 200ml water	Jatropha 200ml water	Jatropha + PHP 100ml water	Jatropha 100ml water

Figure 47(a): Jatropha growth (3 – 5 months)



Jatropha + PHP 200ml water	Jatropha 200ml water	Jatropha + PHP 100ml water	Jatropha 100ml water

Figure 47(b): Jatropha growth (6 - 8 months)

Normally watered plants plus PHP were obviously visibly larger after 3 months and the difference became larger as the months progressed. Droughted plants plus PHP were also larger than plants without PHP but the difference was not so obvious visibly.

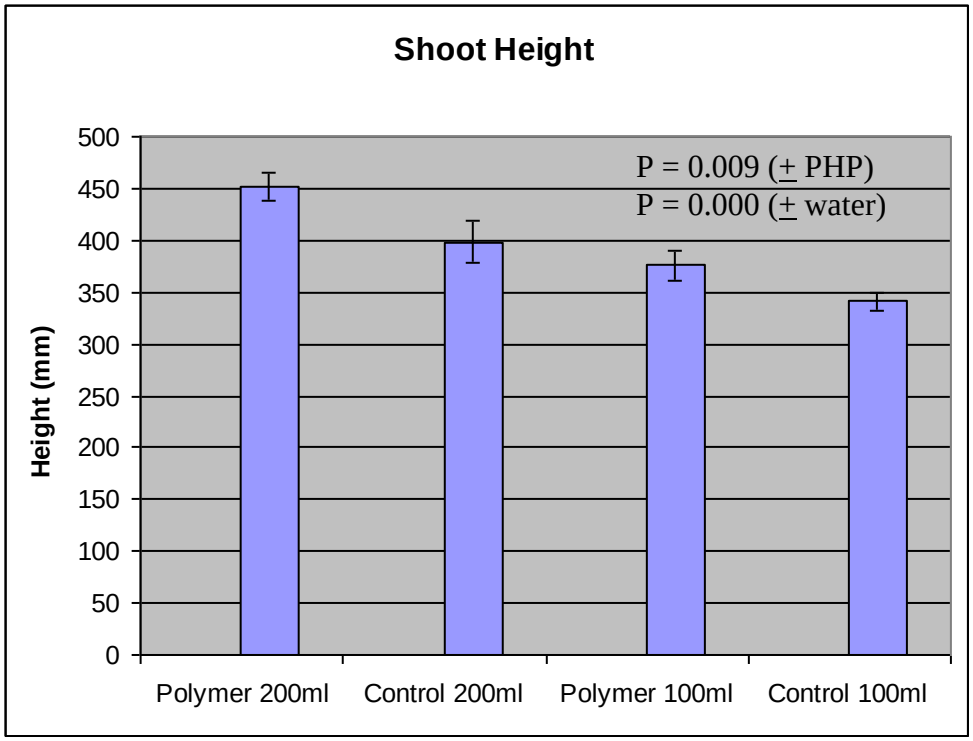


Figure 48: Jatropha shoot height

Normal watered plants with PHP were 13.5% taller than those with no PHP and droughted plants with PHP were 10.2% taller than those with no PHP (Figure 48).

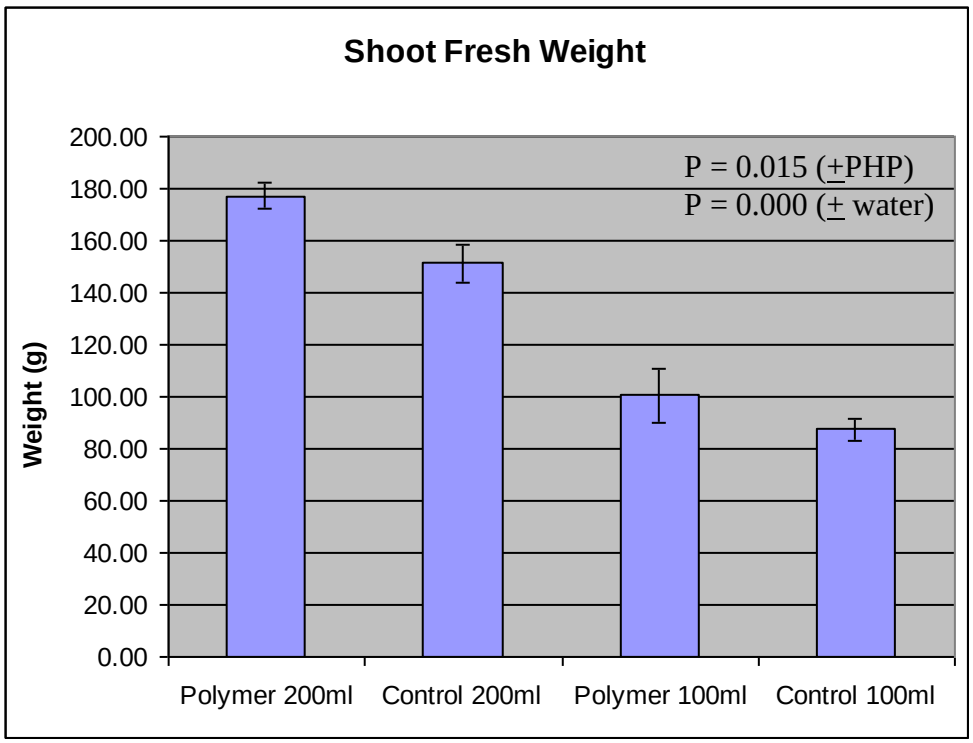


Figure 49: **Jatropha shoot weight**

The fresh weight of plants with normal watering (200ml) was 73.3% heavier than plants with reduced watering (100ml). Normal watered plants with PHP were 17.1% heavier

than those with no PHP and droughted plants with PHP were 15.4% heavier than those with no PHP (Figure 49). The results demonstrate that water is a major factor in limiting plant growth.

For both normal watered and reduced watered plants, the addition of PHP has also significantly increased the dry weights compared to the plants with no PHP.

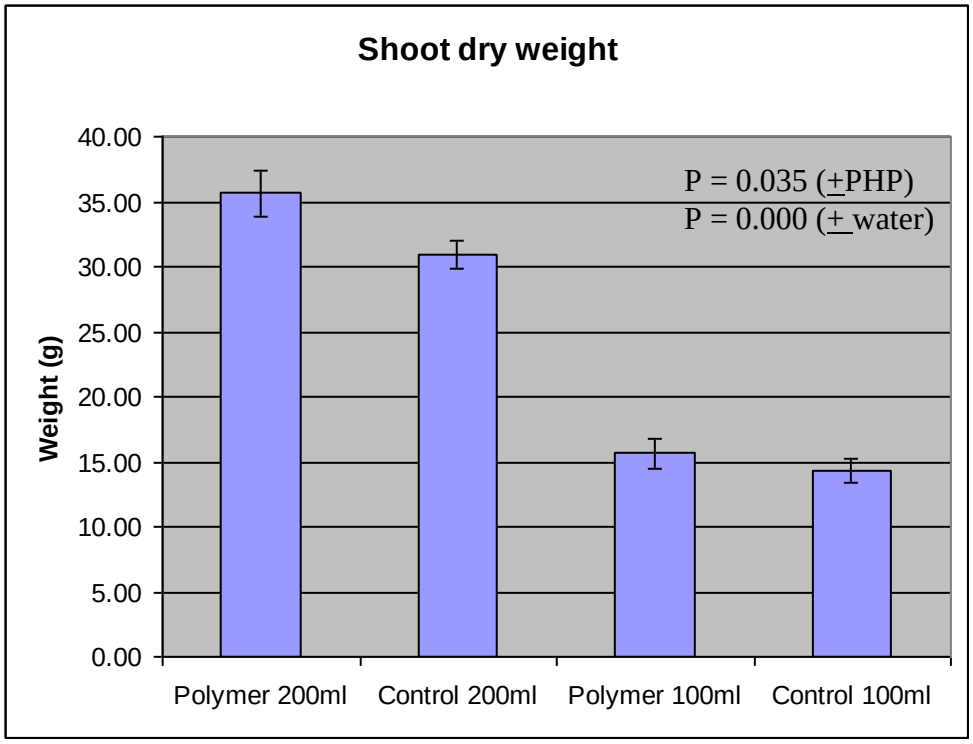


Figure 50: Jatropha shoot dry weight

The dry weight of plants with normal watering (200ml) was 114.9% heavier than plants with reduced watering (100ml). Normal watered plants with PHP were 15.3% heavier than those with no PHP and droughted plants with PHP were 8.8% heavier than those with no PHP (Figure 50). The smaller percentage increase of the dry weight compared to the fresh weight of droughted plants would suggest that in drought conditions the plants store extra water. They are known as stem succulent plants – ie they can store water in the stems. Analysis by 2 way ANOVA showed that water had the most significant effect (P = 000), PHP also had a significant effect, (P = 0.035) but there was not a significant interaction (P = 0.203) (appendix 2, p172).

The positive increase in weight of plants with PHP is much smaller for jatropha compared to soybeans because jatropha is a perennial plant with a much slower growth rate, taking 5 years to reach maturity whereas soybean is an annual plant which grows and produces seed in one season.

SEM pictures of roots

SEM pictures of samples were compared before and after passing through the polymer to observe if any physical changes were apparent (Figures 51 & 52).

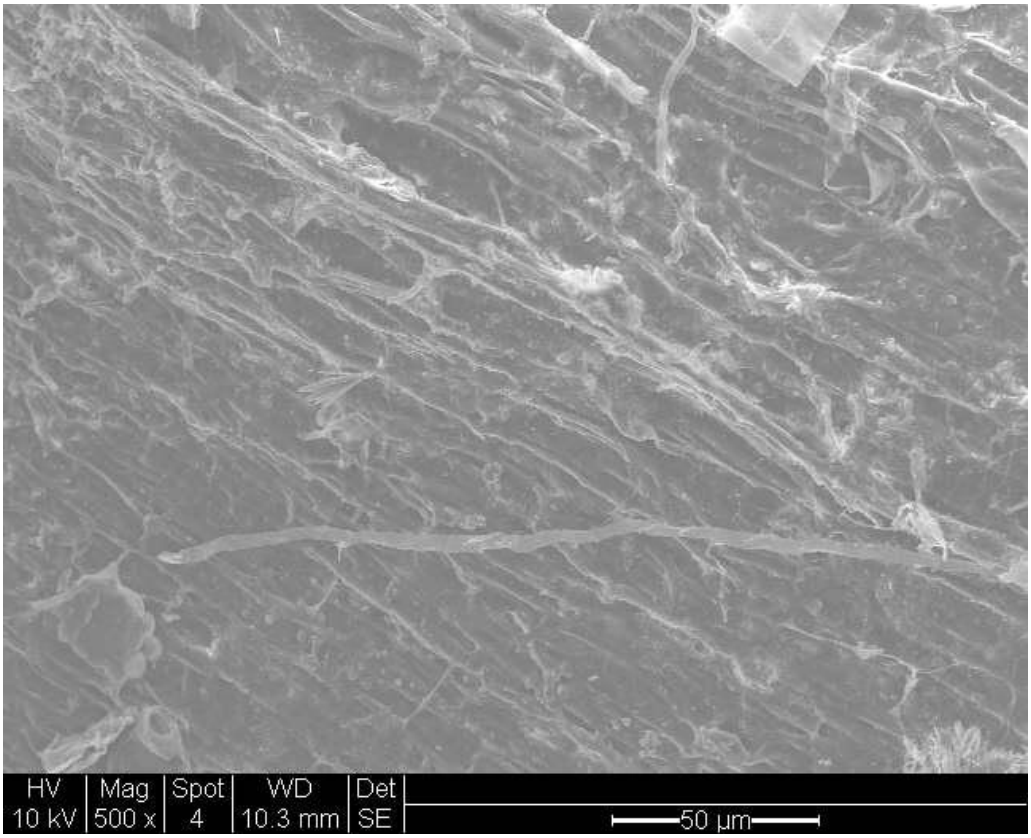


Figure 51: SEM of root before passing through polymer

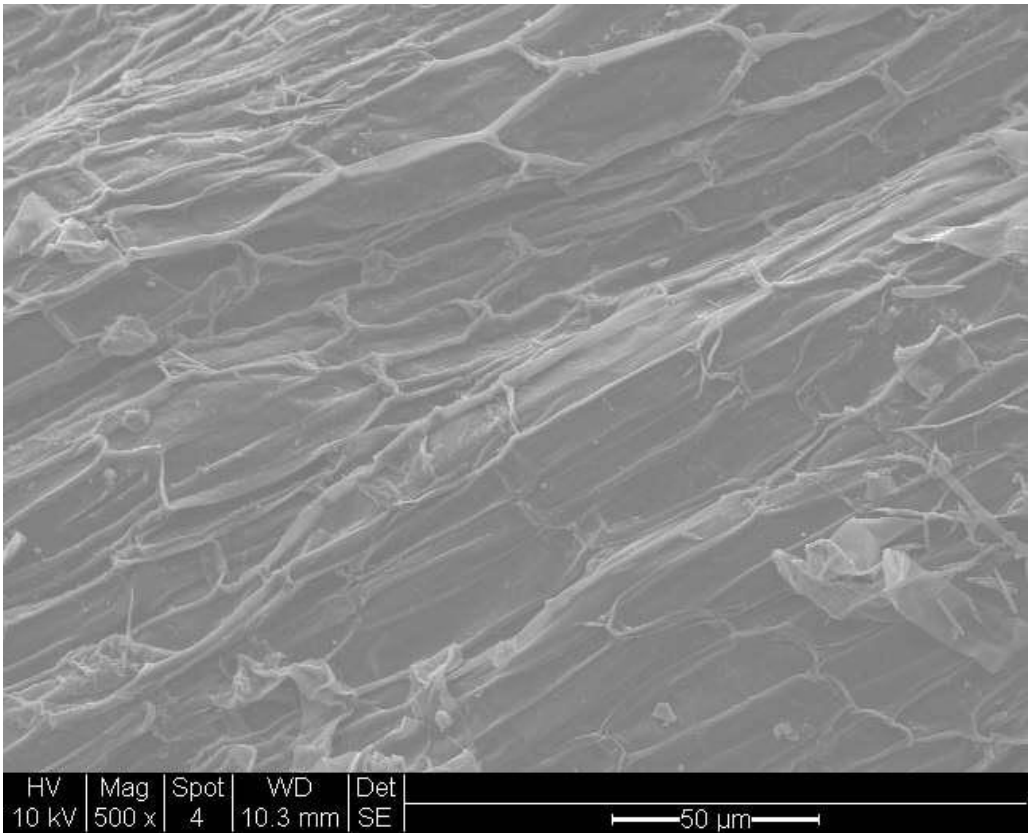


Figure 52: SEM of root after passing through polymer

There did not appear to be any physical difference between the roots before and after the polymer other than after the polymer the cells seemed to be cleaner probably by the physical action of pushing through the spongy material of the polymer.

SEM of longitudinal section of stem

SEM pictures were taken of longitudinal sections of stem to observe if addition of PHP had any physical effect on the structure of the stem (Figure 53 a, b & c).

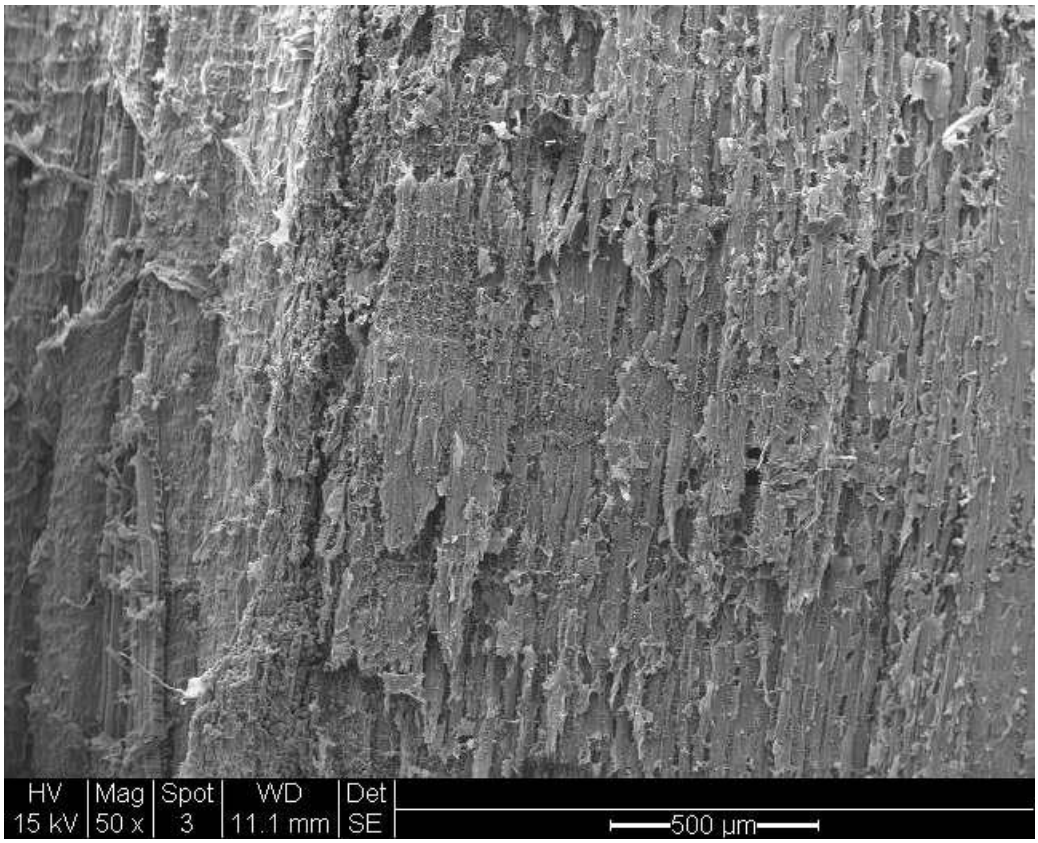


Figure 53(a): Longitudinal section of stem 50X magnification soybean root nodule

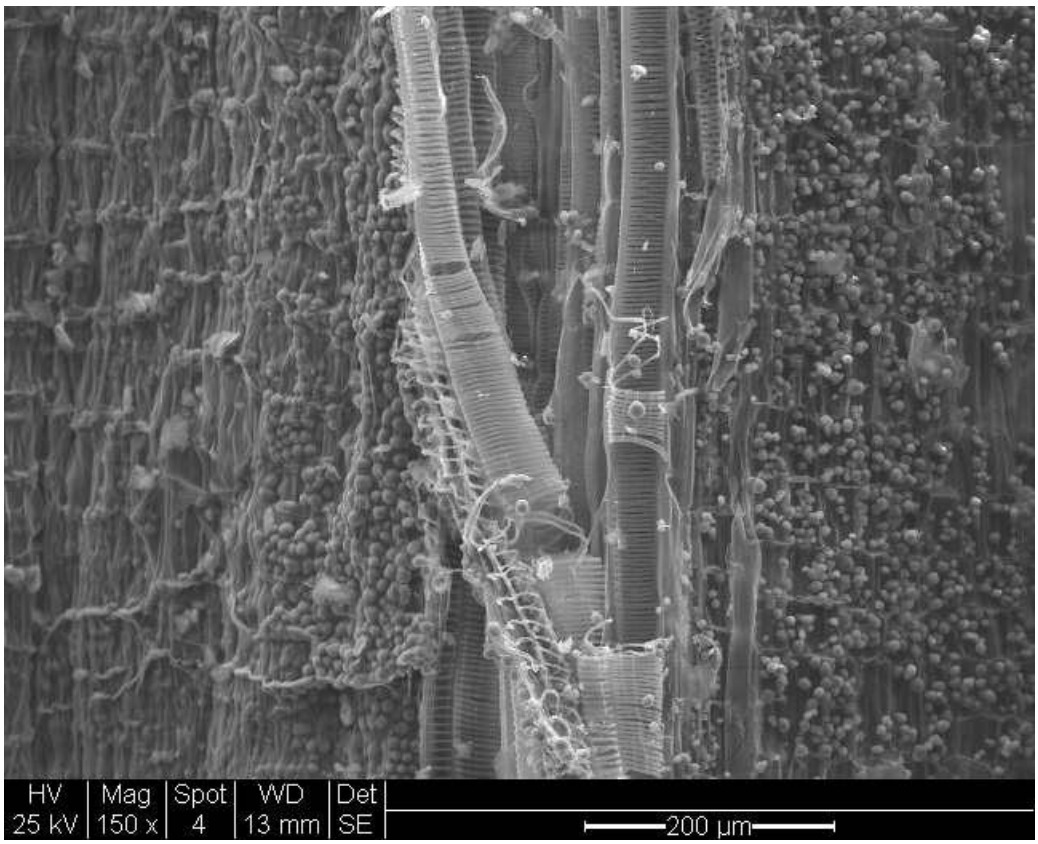


Figure 53(b): Longitudinal section of stem showing xylem vessels 150X magnification

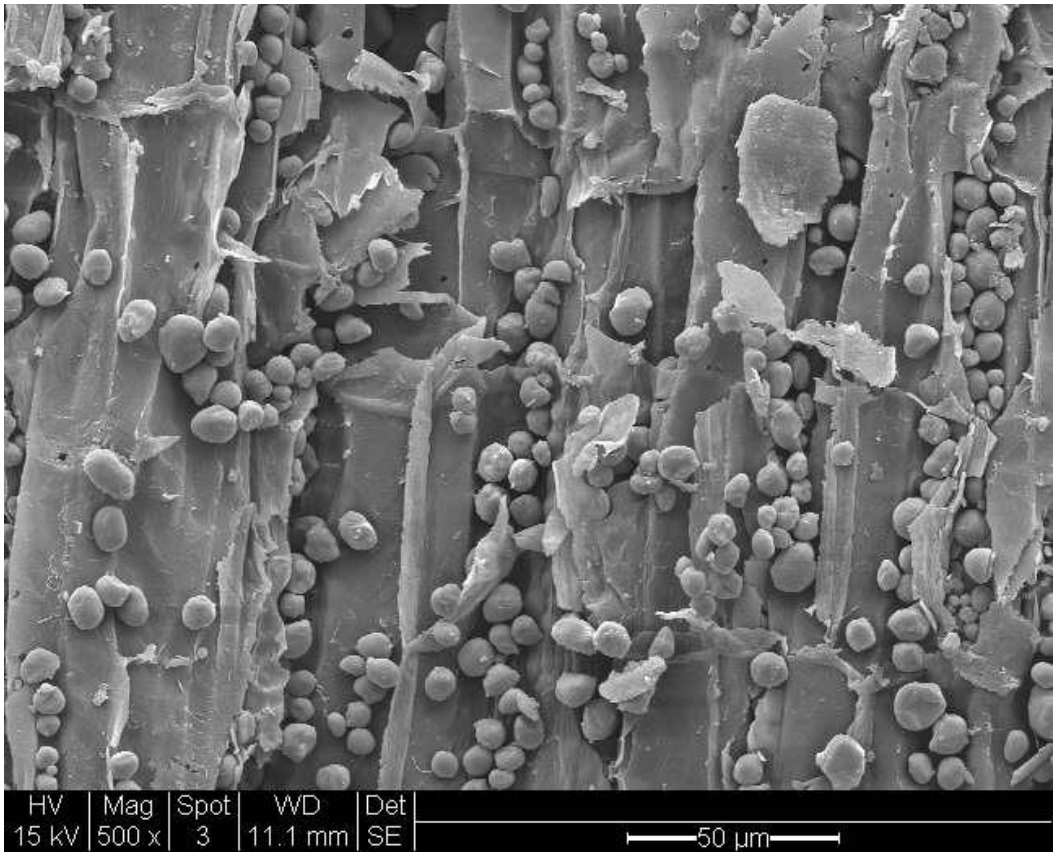


Figure 53 (c): Longitudinal section of stem 500X magnification

No difference was observed between pictures with or without polymer so only one set of pictures is included. All stems appeared to be full of small globules. These have been identified as latex globules as the stems are a source of latex which has several medicinal uses including a remedy for external skin diseases, a blood coagulant and an anti cancer drug (Thomas *et al*, 2008). A clear liquid was observed to ooze from the wound left on the stem after the leaves were removed which would be the latex.

Chemical analysis of roots, stems & leaves

Root, stem and leaf samples were chemically analysed to see if addition of PHP produced any chemical differences to the plants (Figure 54a, b & c).

Chemical analysis of jatropha root

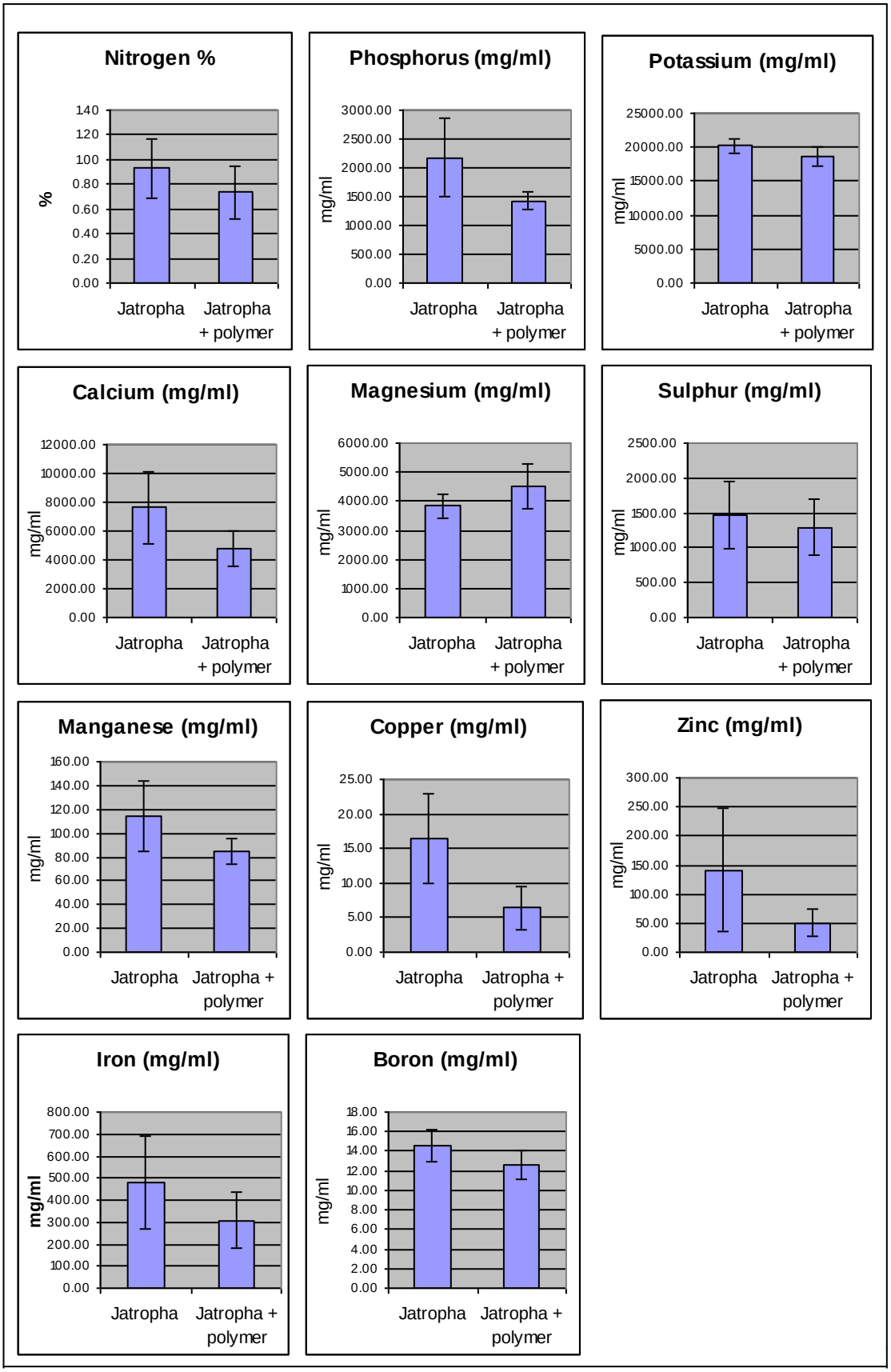


Figure 54(a): Root chemical analysis

Chemical analysis of jatropha stem

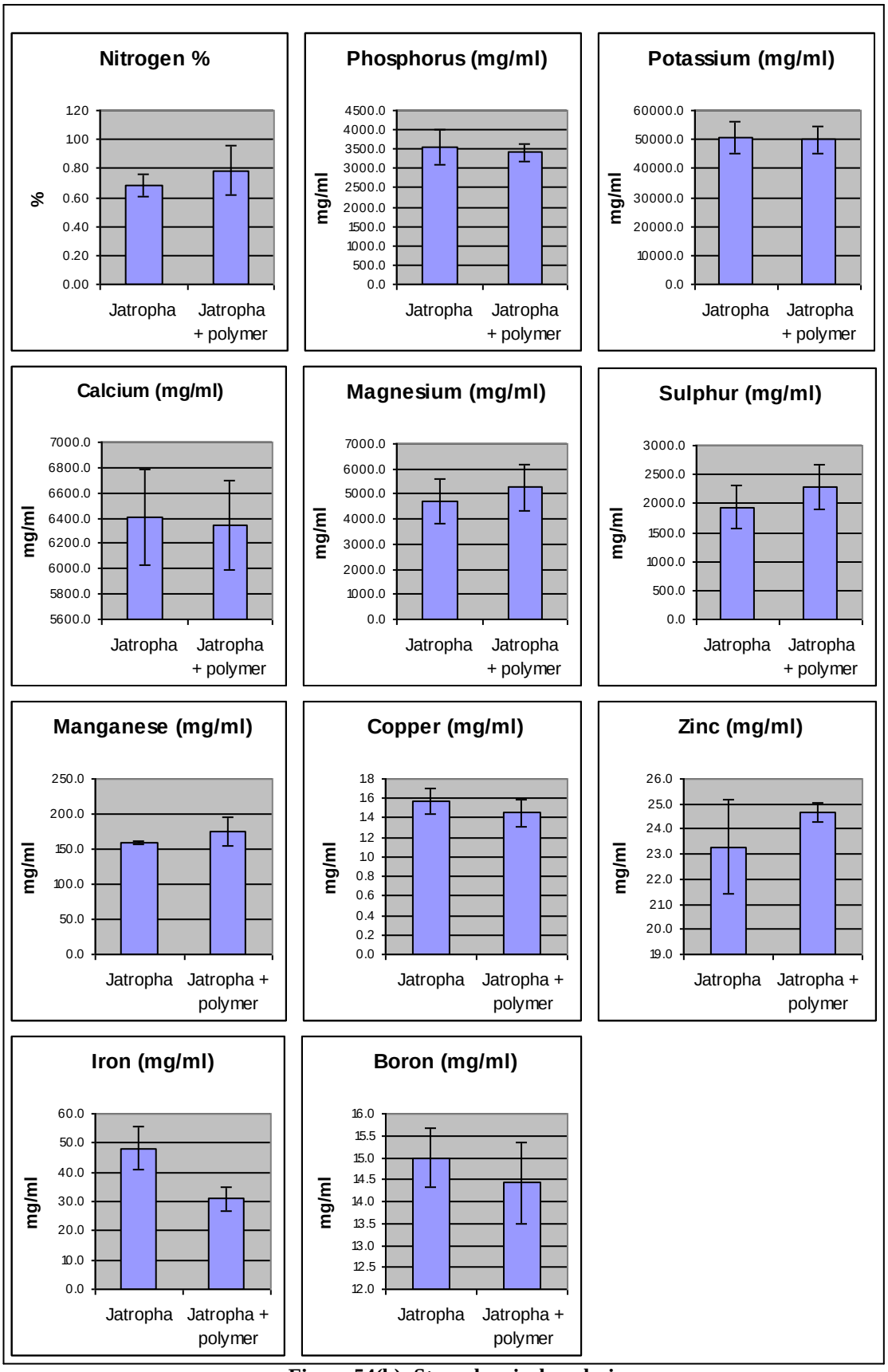


Figure 54(b): Stem chemical analysis

Chemical analysis of jatropha leaf

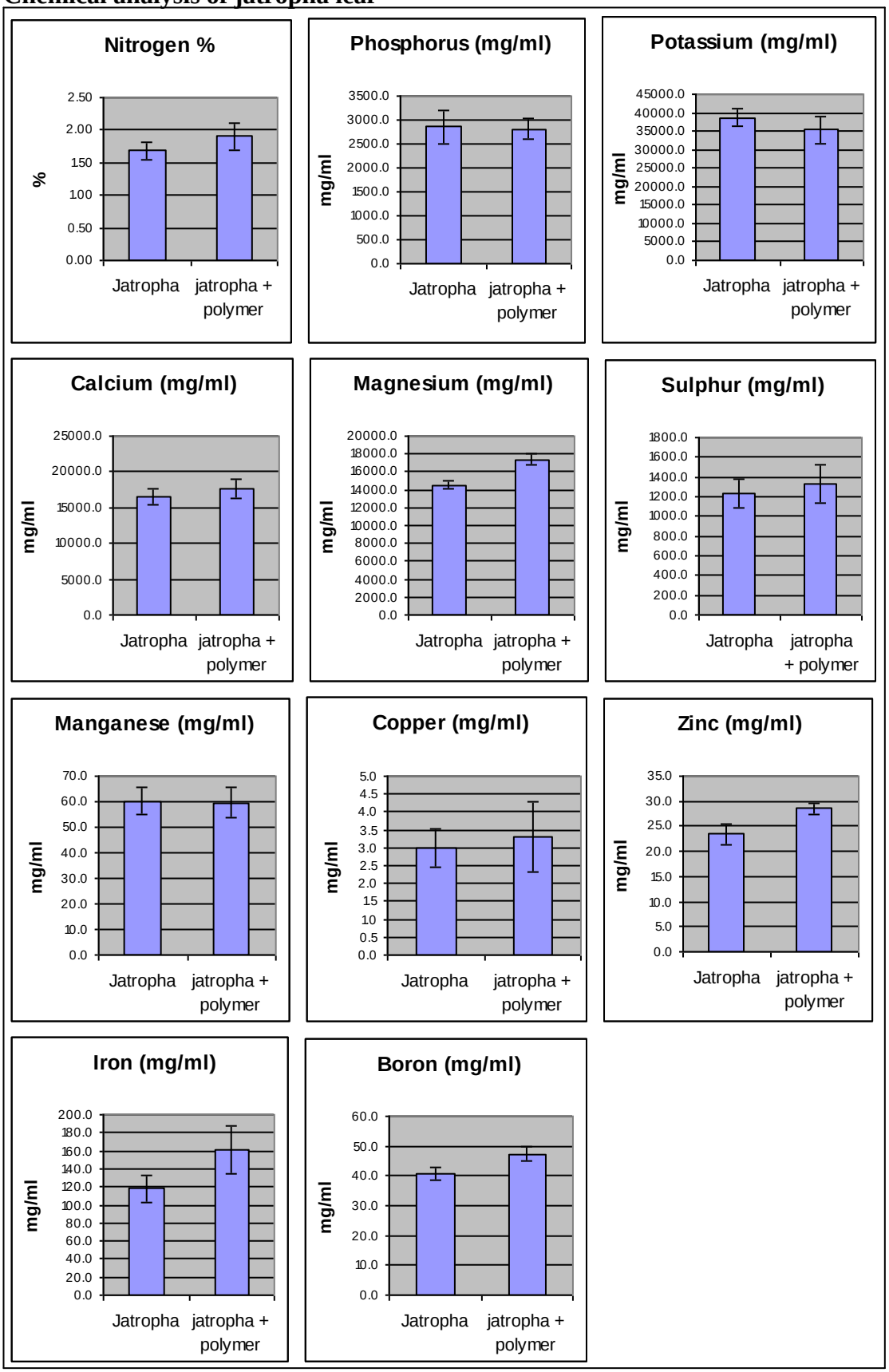


Figure 54(c): Leaf chemical analysis

Chemical analysis of leaves, stems and roots showed that there was no significant difference between the chemical composition of plants with or without the addition of

PHP, with the exception of iron content of stems and magnesium & boron content of leaves which showed a slight variation although it was small. Several other elements showed a big variation between replicates of the same treatment. The element which might have been expected to show the biggest variation was sulphur which may have been taken up by the plant as it grew through the polymer, but there was no evidence of this as there was no significant difference between the sulphur content of leaves, stems or roots of plants with or without PHP added.

Analysis by EDAX also showed very little difference between the chemical composition of the roots of plants with or without PHP added and certainly no difference between the levels of sulphur (pink) (Figure 55).

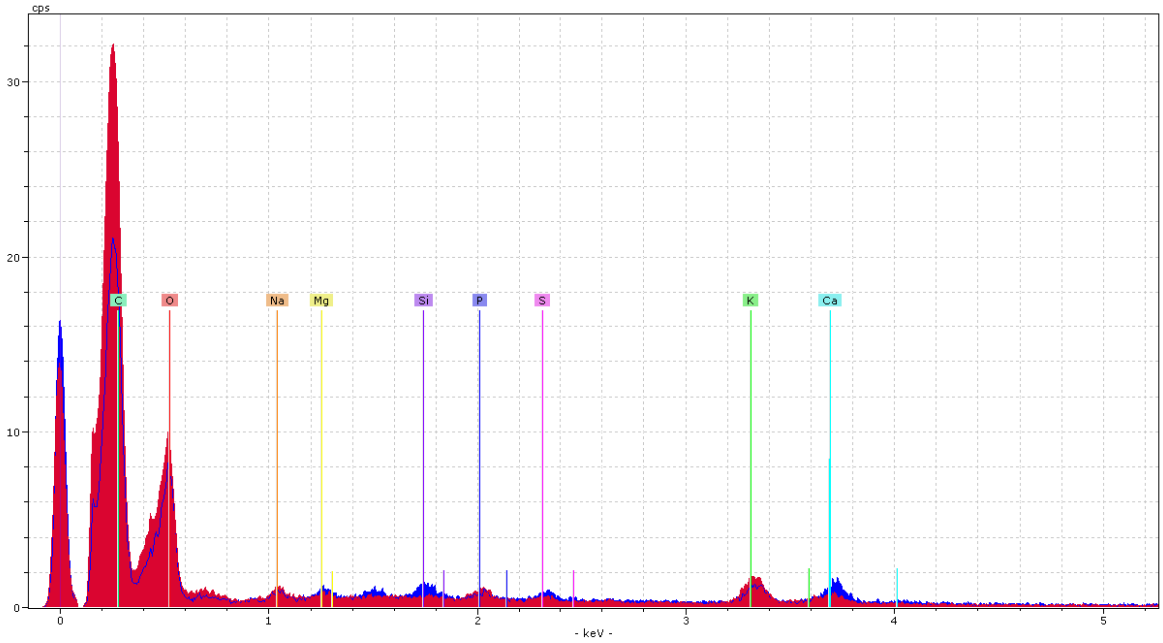


Figure 55: EDX analysis of root surface ± PHP

Root surface - no PHP (red)
Root surface - plus PHP (blue)

It can therefore be concluded that ENPs have not been taken up by the plant as a result of the roots growing through the polymer.

After 8 months in the soil the PHP did not appear to have been eroded – it still looked the same as initially and still had its spongy texture. Some of this PHP was re-potted, mixed with the same soil mixture as previously and grass was planted and left for a further 12 months so it was in the soil for 20 months in total. Samples of the polymer

were then examined with SEM to observe if the extended time in the soil had produced any detrimental effect on the polymer. The polymer appeared to be very stable and, even after 20 months did not show any signs of degrading and was still spongy although slightly more brittle than originally (Figure 56 a & b).

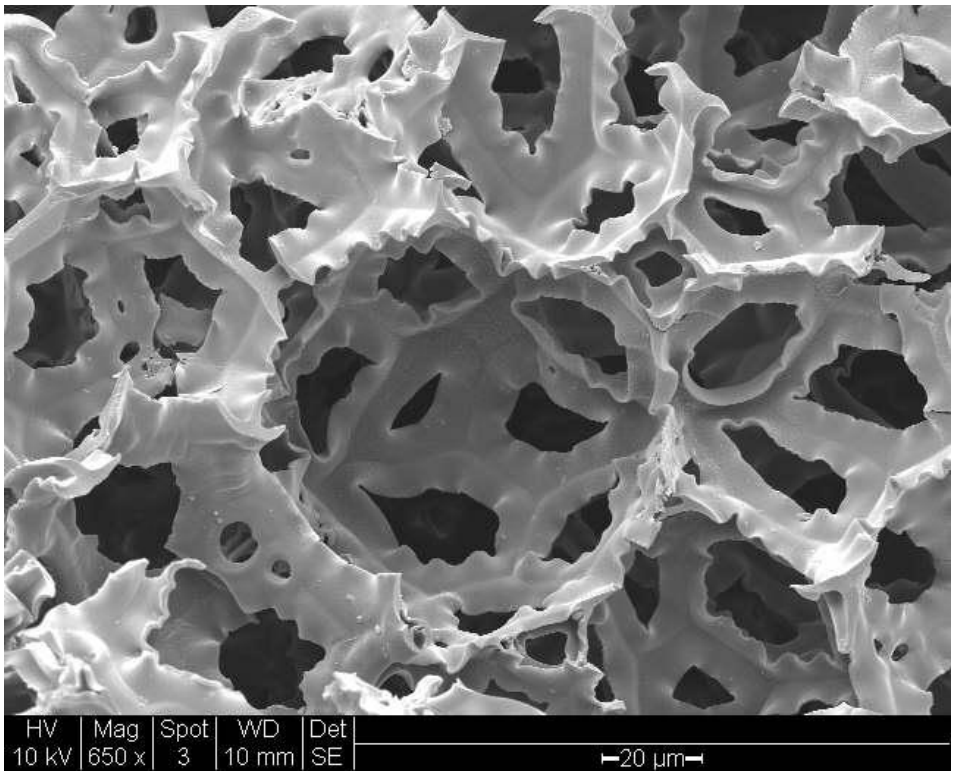


Figure 56(a): PHP removed from soil after 20 months (mag 650X)

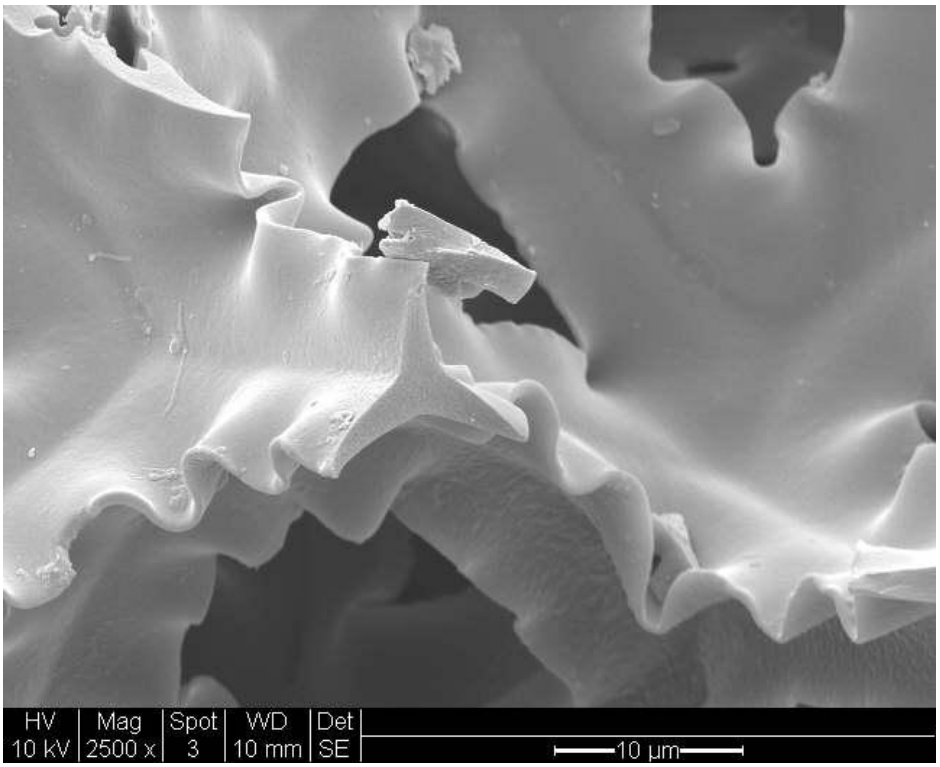


Figure 56 (b): PHP removed from soil after 20 months (mag 2500X)

One of the attractions of jatropha as a fuel plant is that it can grow on semi dry areas where other plants do not grow. This experiment has shown that it does grow in reduced water conditions, but it grows better when water is not a limiting factor. Farmers, being mainly driven by economics, would therefore be inclined to grow it on more fertile ground where water is not a limiting factor if it was going to prove more profitable than food crops. So there would need to be appropriate incentives or legislation to ensure that the crop is not grown on ground that would otherwise grow food crops, especially in areas where food production must be increased to satisfy the needs of a growing population.

4.4 Use of Polyurethane sponge as an alternative soil additive

A major factor to consider in determining whether PHP could be a viable product is one of economics. The purpose of this experiment was to determine if a cheaper alternative could produce the same effect as PHP and polyurethane is cheap and readily available. An ordinary household sponge was used for this purpose and grass was used in this experiment. The effect of polyurethane (PU) sponge on grass growth was compared with grass plus PHP and control plants with no additive.

In most experiments, the addition of PHP has a significant positive effect, but occasionally, the weight gain was small or negligible. In order to investigate if the age of the polymer had any influence on how effective it is, the polymer used in this experiment was manufactured, then stored for 4 months before planting. The CHN content was measured when manufactured, then again after 6 months, at the time when the experiment was completed to ascertain if the nitrogen content of the PHP was reduced over time when in storage.

4.4.1 Materials and Methods

A polyurethane (PU) sponge from Wilkinsons store was cut into 5mm cubes the same as PHP cubes. Grass was grown in 10cm pots using 75% John Innes No 3 and 25% horticultural sand. 250g soil mixture was added per pot and PHP was added at the rate of 0.5%w/w = 1.25g/pot. The density of the sponge was much lower than the PHP (PHP density = 0.1g/cm^3, sponge density = 0.02g/cm^3), so adding 1.25g was too much volume of sponge per pot, therefore the volume of 1.25g PHP was measured and the

same volume of sponge was used in each pot (25ml). The grass was Johnsons Lawn Seed, Inkberrow, Worcestershire. There were three treatments: (1) grass; (2) grass plus PHP; (3) grass plus PU sponge. There were three replicates of each treatment. 0.5g grass seed was added to each pot and covered with a light covering of soil/sand mixture. The experiment was conducted in a growth room with 16 hours daylight, 8 hours dark, 28^0C day temperature and 22^0C night temperature. Plants were watered twice weekly with 100ml tap water. The shoots were harvested after 3, 6 and 9 week intervals and the roots were harvested at 9 weeks.

The CHN content of the PHP used in this experiment was measured using a Carlo Erba 1108 Elemental Analyser immediately after manufacture and again after storage for 6 months.

4.4.2 Results & Discussion

CHN content

	N%	C%	H%
New PHP	6.99	41.13	5.24
6 months old PHP	8.03	39.27	4.65

Table 7: CHN content of PHP

The CHN results (Table 7) showed that the nitrogen content has not decreased during the 6 month storage time. It has increased slightly from 6.99% to 8.03%. The reason for occasional instances where the polymer does not produce a significant positive effect can therefore not be explained by the nitrogen content being reduced with age. The slight increase in nitrogen content could possibly depend on what position the sample came from on a polymer disc. Samples from the outside edge may have a slightly higher nitrogen content than samples from the centre because it would take longer for the ammonium hydroxide solution to soak in to the centre of the disc. Another possible explanation for the PHP occasionally having no effect may be the age of the ingredients. As the ingredients get older, polymerisation becomes more erratic and sometimes is not complete or can sometimes not polymerise at all and the ingredients separate into 2 phases.

SEM pictures

SEM pictures of PHP and PU were taken at the same magnification showing the much less dense structure of the PU compared to the PHP (Figure 57 a & b).

Density of PHP = 0.1g/cm^3; Density of PU = 0.02g/cm^3

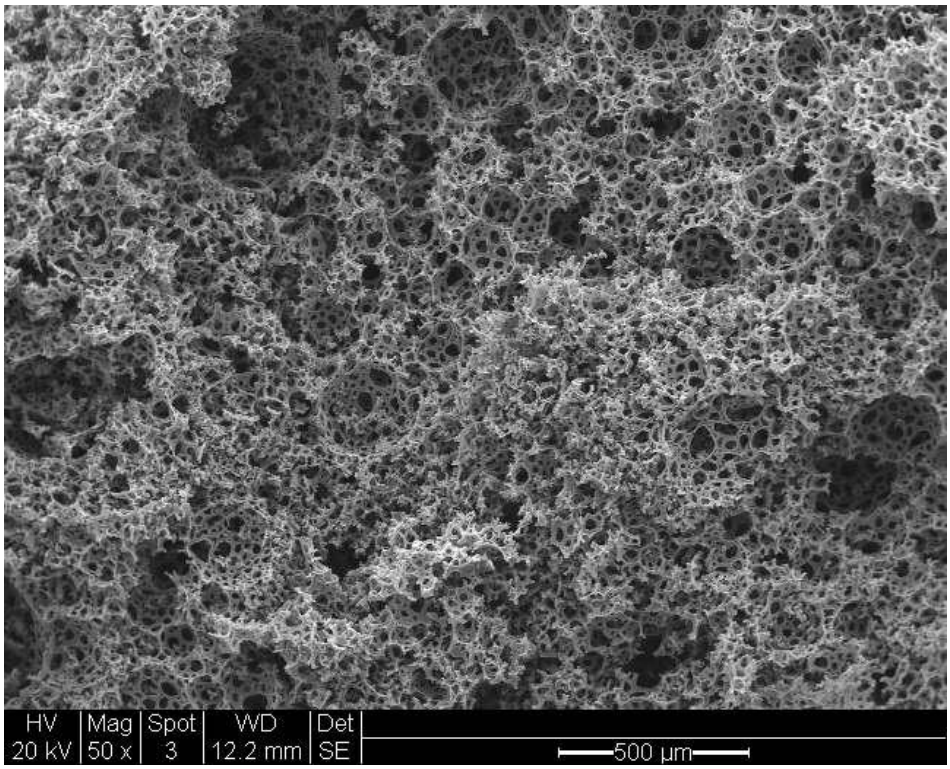


Figure 57(a): SEM of PolyHIPE (50x magnification)

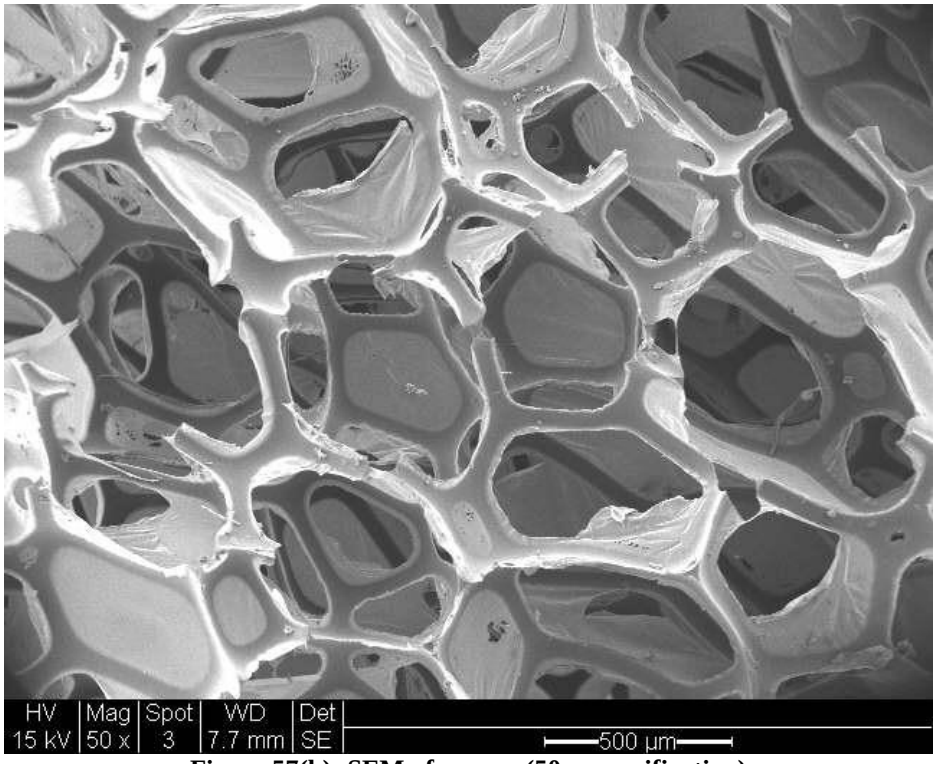


Figure 57(b): SEM of sponge (50x magnification)

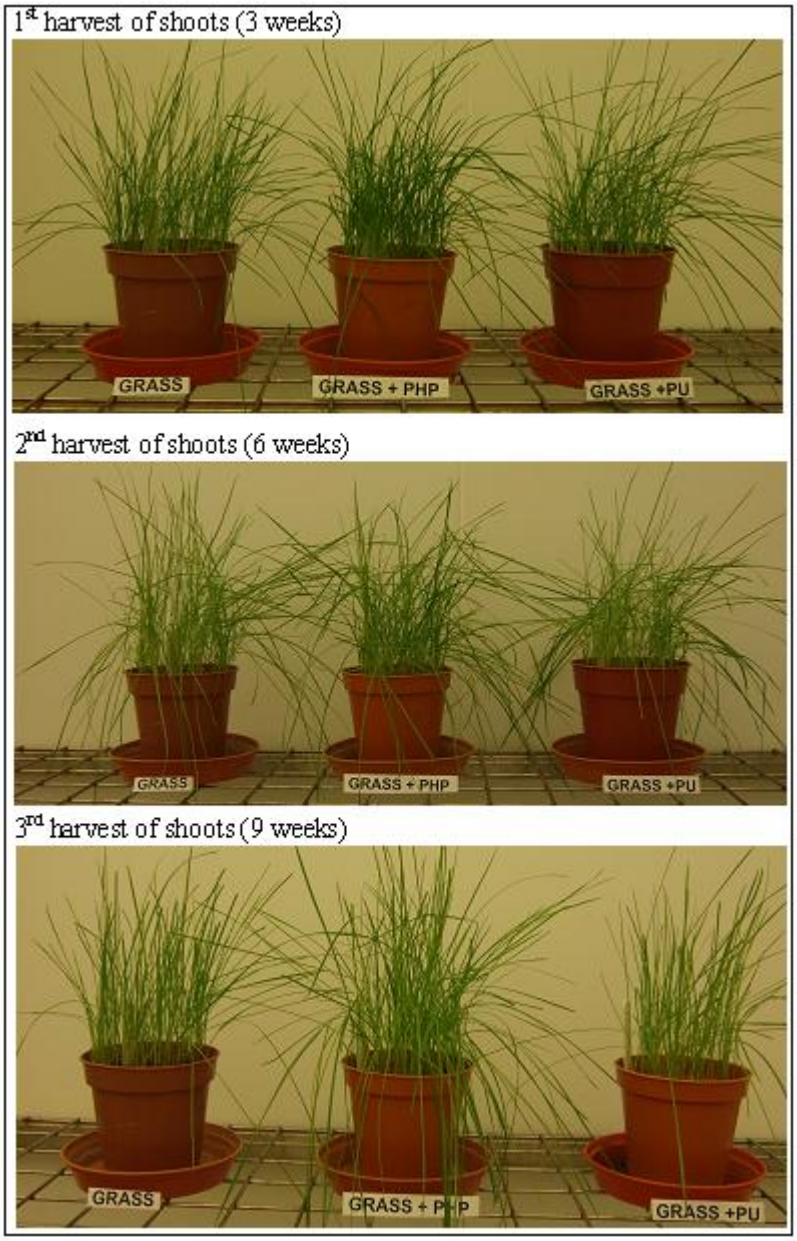


Figure 58: Grass + PHP & PU in growth room

No obvious visible difference was apparent after the 1st two harvests, but by the 3rd harvest, the plants plus PHP were obviously heavier than control plants and plants plus PU were lighter (Figure 58).

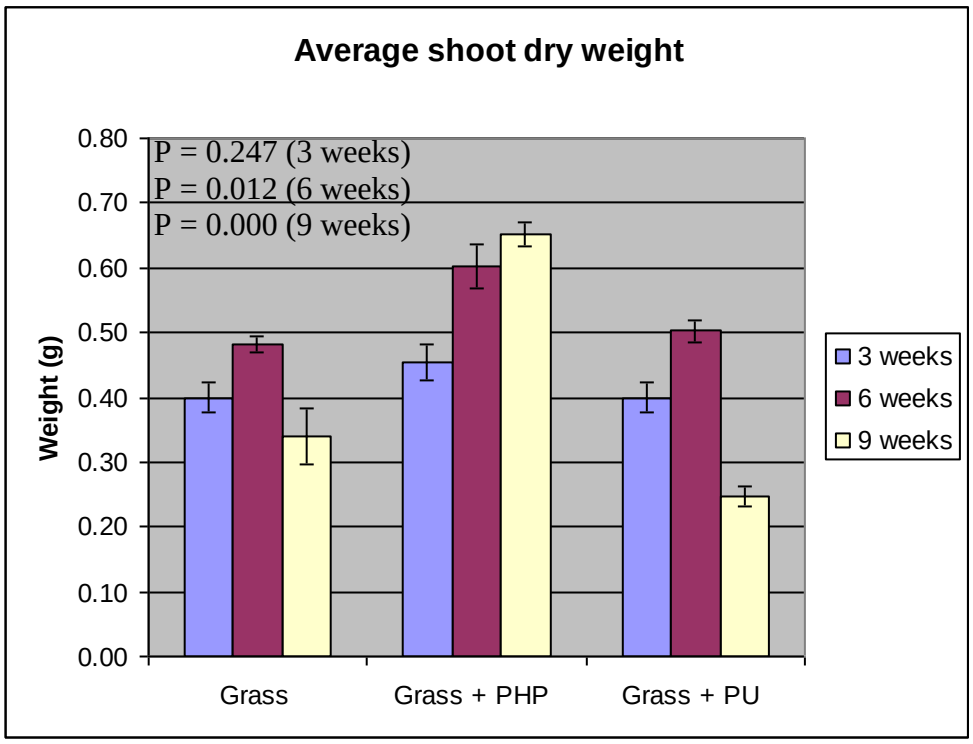


Figure 59: Grass + PHP & PU – average shoot dry weight

After three weeks, there was no significant difference between any of the shoot dry weights (Figure 59; Appendix 2, p169). But by 6 weeks, the shoot dry weights of plants plus PHP were 25% heavier than the control plants (P = 0.012), but the plants + PU were still not significantly heavier than the control plants. After 9 weeks, the plants plus PHP had continued to out grow the control plants and were now 91.2% heavier than the control plants (P = 0.000). The plants plus PU had continued to perform poorly and were now 26.5% lighter than the control plants.

Roots of plants with both PHP and PU had been attracted to, and grown through the polymers (Figures 60 & 61).

Figure 60: Roots of grass + PHP & PU after 9 weeks growth

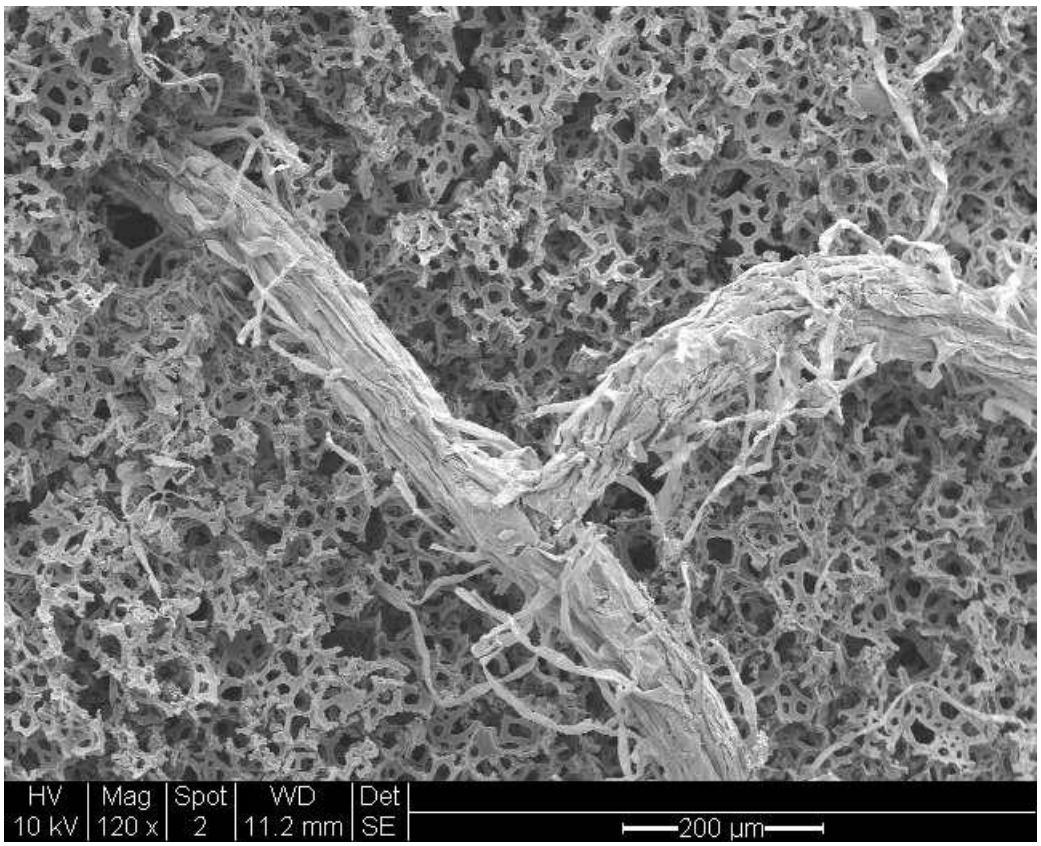


Figure 61(a): SEM of grass roots growing through PHP

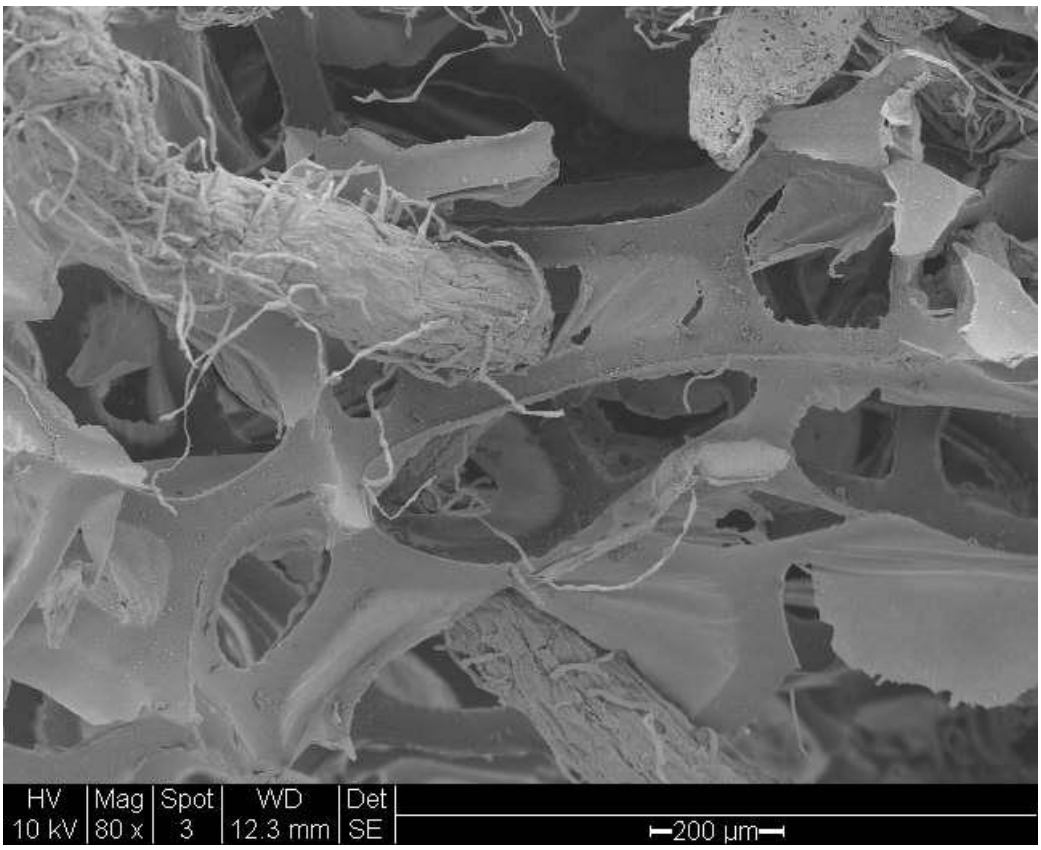


Figure 61(b): SEM of grass roots growing through PU

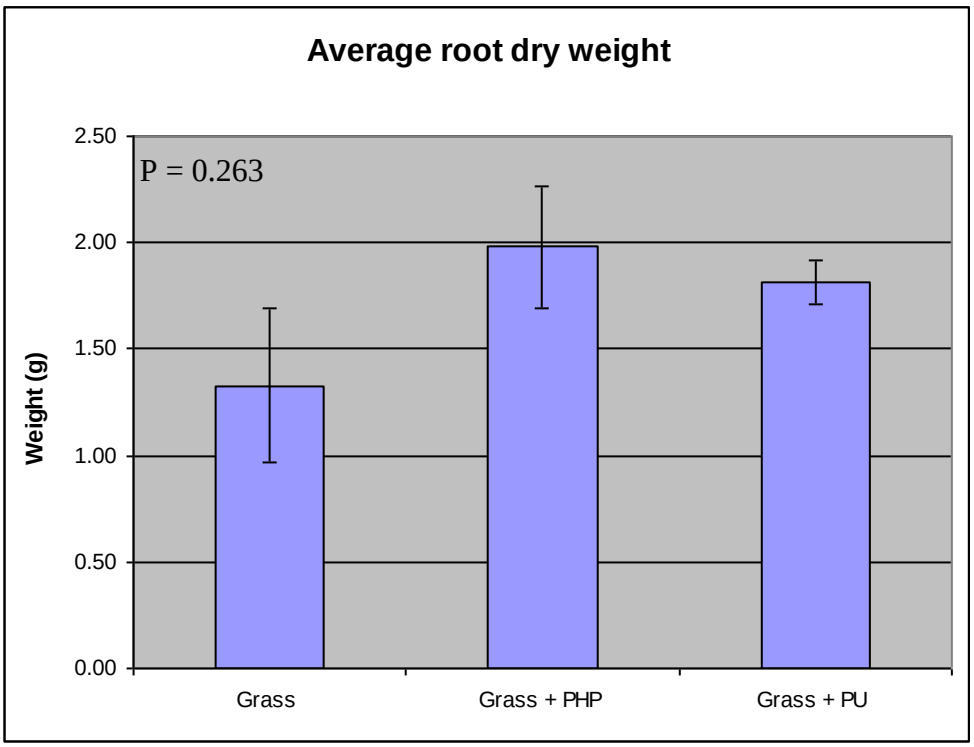


Figure 62: Grass + PHP & PU – average root dry weight

Dry weight of roots plus PHP was 48.9% heavier than control plant roots, but the difference was not significant due to the big variation in weights of replicate plants (Figure 62, Appendix 2, p169). Plants plus PU were 36.1% heavier than control plants but this also was not significant.

PHP has produced a significant increase in shoot weight as it usually does, but polyurethane produced no effect. This would add further credence to the suggestion that it is the residual nitrogen within the PHP that produces the positive effect. A similar effect was observed in the previous experiments when potassium hydroxide was used to neutralise the polymer instead of ammonium hydroxide (p52). When potassium hydroxide was used and therefore no nitrogen was present, the polymer did not produce a positive effect. Plant roots were however still attracted to the polyurethane and grew through it.

Although root weights of plants with PHP were heavier than control plants, the difference was not significant because of the big variation between replicates which was to a large extent due to the difficulty in removing the polymer and sponge without removing roots as well, so it does not give an accurate representation of the root weights.

PU sponge has not had a positive effect on plant growth but it may still have potential as a reservoir for bacteria or water in semi dry environments, although the larger pore sizes would mean water and any nutrients it contained would be released faster than from the PHP with its smaller pore sizes and therefore would not be as effective as a slow release fertiliser over longer periods.

4.5 Modification of PU sponge to make it more hydrophilic

PU sponge is hydrophobic but for water retention it needs to be hydrophilic so some experiments were carried out to attempt to make it more hydrophilic. Experiments were conducted to try to coat the surface of the PU sponge with PHP in order to make it more hydrophilic. Several methods were tried to do this:

1) Make poly High Internal Phase Emulsion (polyHIPE) then insert it into the PU sponge;

2) Add polar solvents (toluene and chlorobenzene) to the PU sponge;

3) Add ethyl hexylacrylate to the oil phase in the preparation of polyHIPE;

4) Add vinyl pyridine to the oil phase in the preparation of polyHIPE.

1) Make polyHIPE then insert into PU sponge.

The emulsion is viscous so a syringe was used to get it into the PU. 1g of $5mm^3$ PU sponge cubes was put in a 100ml glass syringe. An excess of a solution of 40g styrene, 20g DVB and 1g lauroyl peroxide, was taken into the syringe (30g). This was left overnight at room temperature then removed by compressing the syringe. A 90% aqueous phase volume emulsion was made as normal. This was heated to 60^0C for 30 minutes, then 30g was sucked into the syringe after fully compressing to squeeze all the air out of the sponge. The emulsion was ejected from the syringe, then re sucked in to ensure that the emulsion fully occupied the interior of the sponge. The PU cubes were then removed from the syringe and incubated at 60^0C overnight in a sealed container for polymerisation to take place.

Polymerisation did not take place. The liquid was poured off and kept to see if it separated into oil and water phases which it did not. Only the aqueous phase remained so the oil phase must have been absorbed into the sponge. The sponge pieces had expanded and turned from white to yellow but remained spongy (Figure 63).

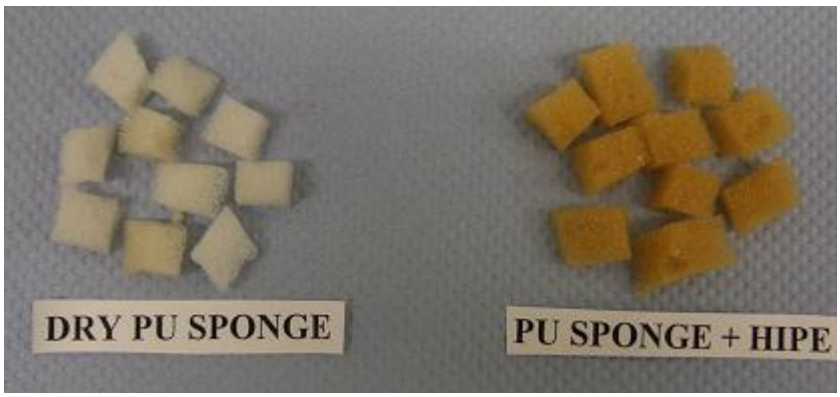


Figure 63: Sponge modification with HIPE

Sponge pieces were washed in a soxhlet with isopropanol for 3 hours followed by 3 hours with distilled water, then dried on the bench.

Samples were examined by SEM which showed that no polymerisation had taken place but ridges had formed on the surface of the sponge compared to normal sponge where the surface is smooth (Figure 64 a, b, c & d).

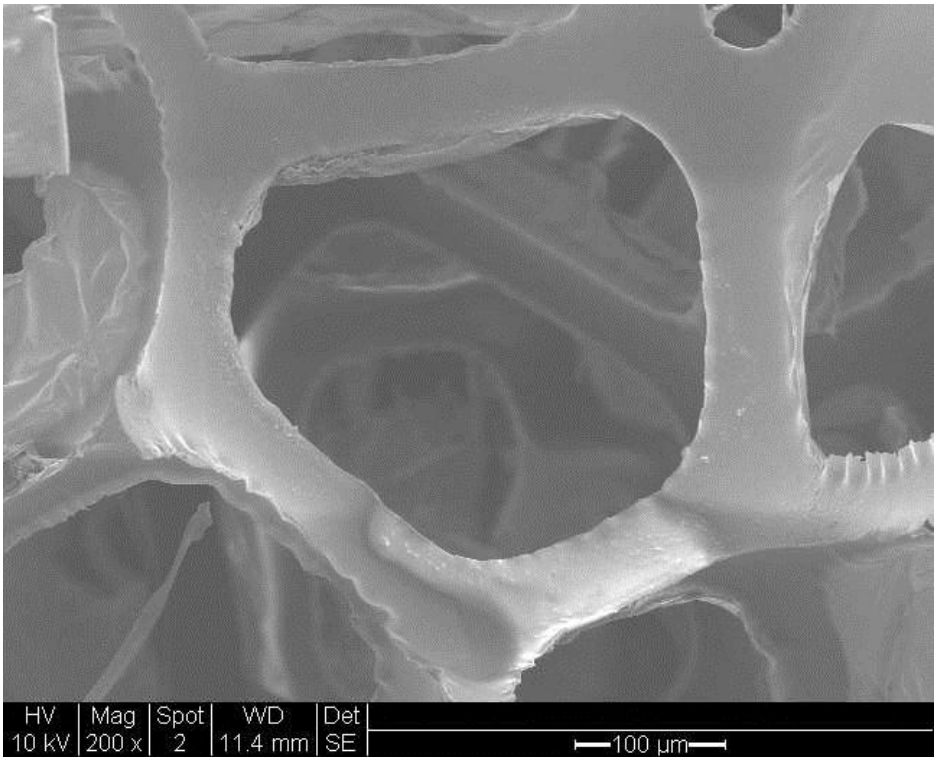


Figure 64(a): Normal sponge (200X magnification)

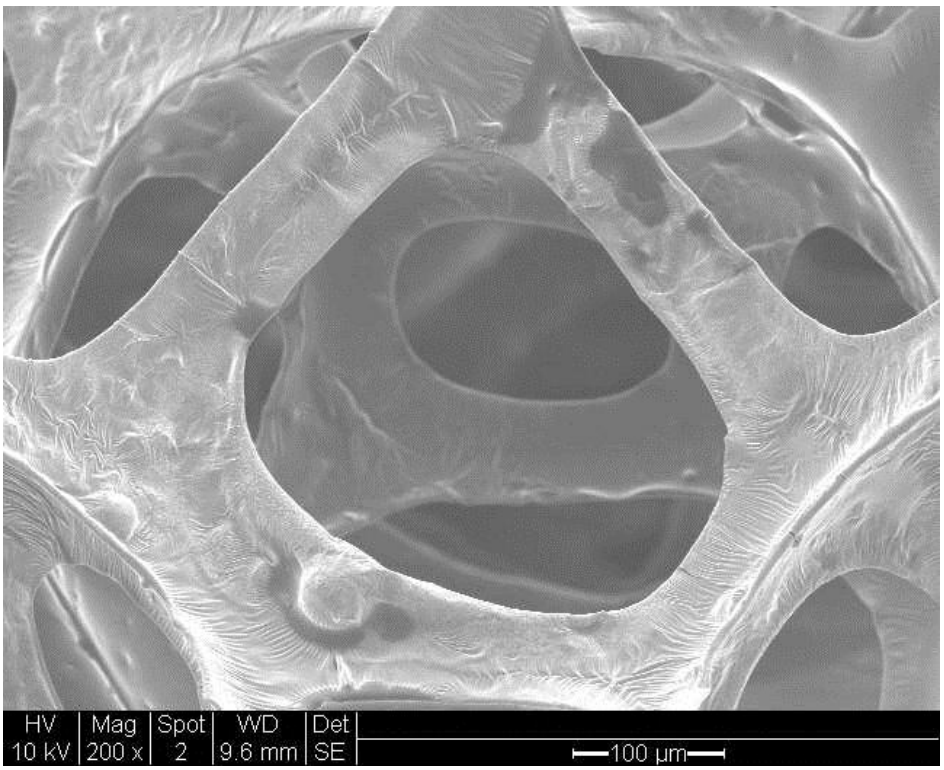


Figure 64(b): Sponge + HIPE (200X magnification)

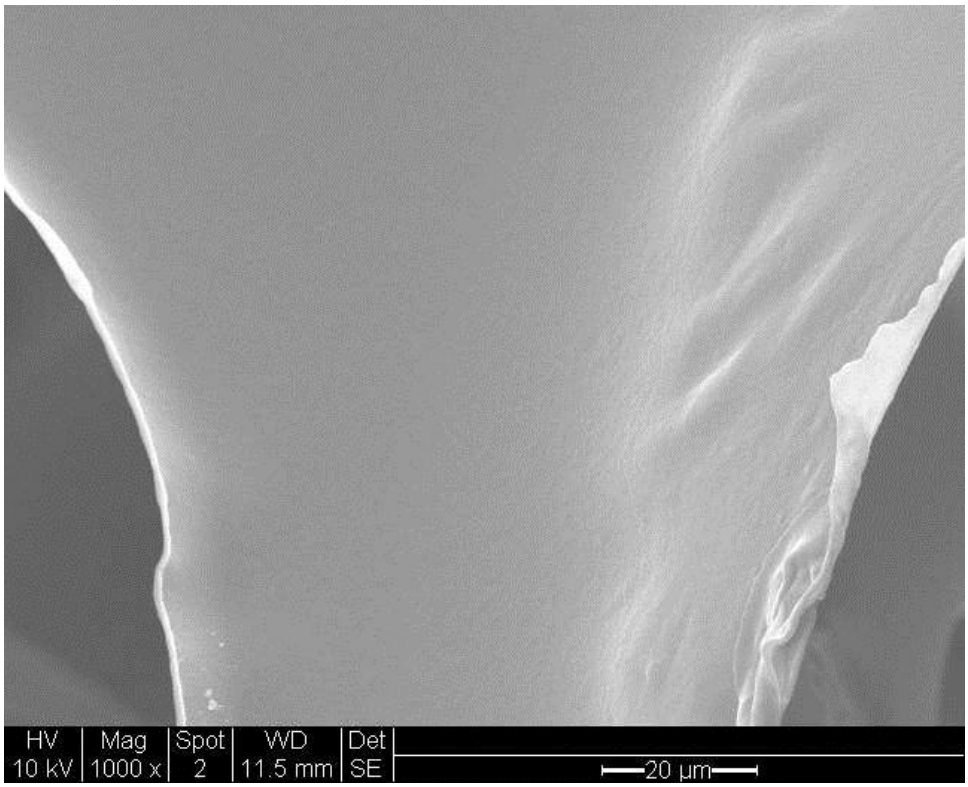


Figure 64(c): Normal sponge (1000X magnification)

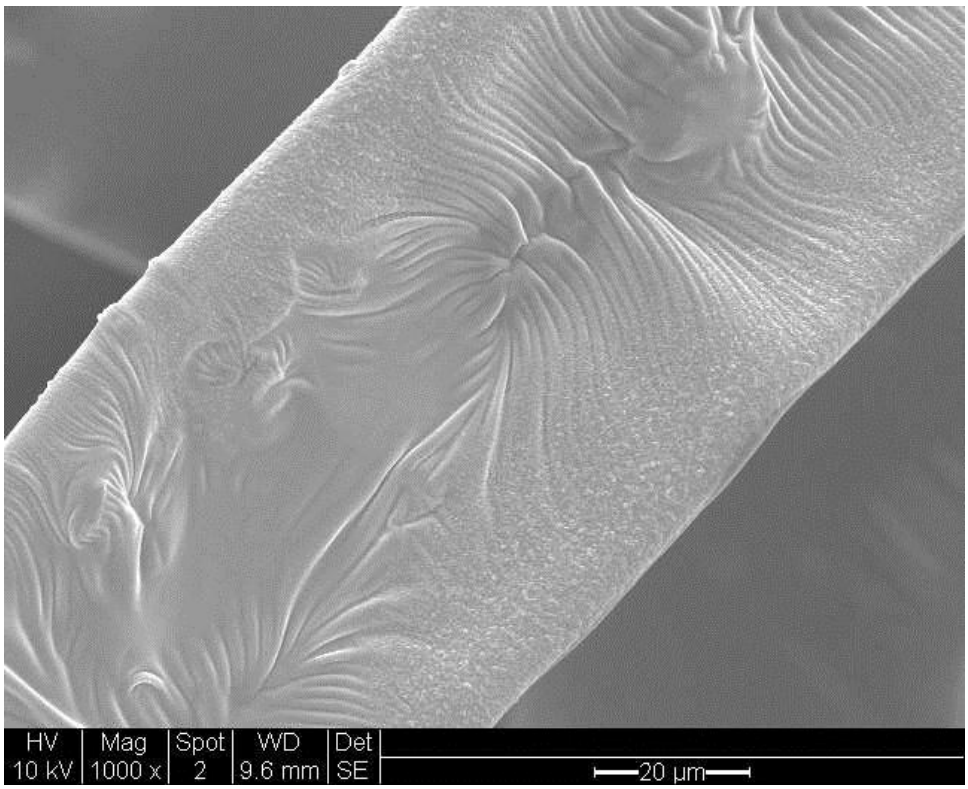


Figure 64(d): Sponge + HIPE (1000X magnification)

Sulphonation

It was not possible to sulphonate the sponge pieces because they dissolved in 98% sulphuric acid. They were soaked in 10% sulphuric acid for 2 hours, but then they melted when heated both in a microwave and in an oven at 95^0C.

2) Add polar solvents (toluene and chlorobenzene) to the PU sponge

Polar solvents make the PU swell and they also act as porogens which increase the surface area, but it produces a mechanically weaker polymer. Excess toluene was added to 0.3g of 5mm sponge cubes in a glass syringe and left for 24 hours. The cubes were observed to have expanded slightly after 24 hours. Excess toluene was then removed from the syringe and the weight of the wet sponge was determined (1.35g). A solution containing 10g of styrene, 10g of DVB and 1g of lauroyl peroxide was made up and sucked into the syringe after squeezing all the air out of the sponge pieces. After ensuring all the sponge pieces were wet with the new solution, some liquid was removed until the weight of sponge plus liquid was 3 - 4 times the weight of the original sponge (4.5g). Sponge pieces were then sealed in a glass bottle and incubated overnight in an oven at 60^0C. After 24 hours some polymerisation had taken place but some unpolymerised liquid remained and the sponge pieces had expanded slightly and gone solid.

Sulphonation

After soaking in 98% sulphuric acid for two hours, the sponge pieces were microwaved for 2 minutes after which they had turned black but remained solid.

Heating overnight in an oven at 95^0C also turned the sponge pieces black but they remained solid.

The same experiment was repeated but soaking in chlorobenzene instead of toluene initially. As with toluene, there was not much expansion after soaking for 24 hours in chlorobenzene, but more after polymerisation had taken place (Figure 65). After polymerisation, the sponge pieces went solid but were very fragile and when attempts were made to sulphonate them, they disintegrated.

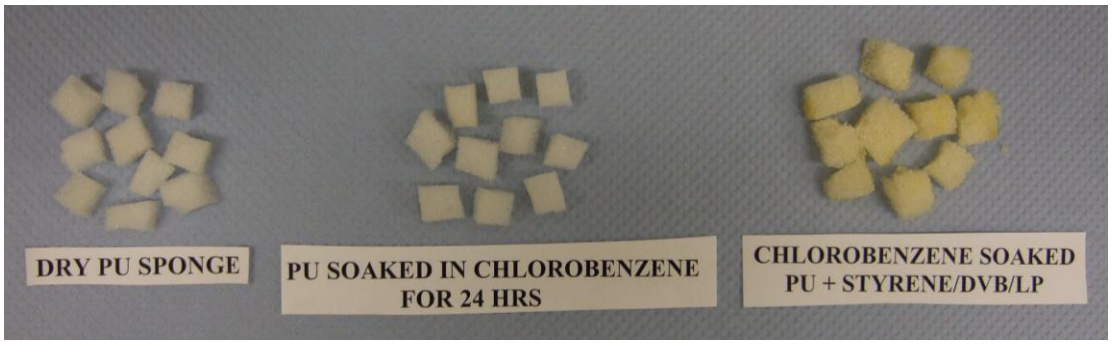


Figure 65: PU + chlorobenzene

3) Add ethyl hexylacrylate to the oil phase

A more elastic polymer can be obtained by adding ethyl hexylacrylate to the oil phase. An oil phase was made up consisting of 15.6% styrene, 62.4% 2-ethyl hexylacrylate, 8% divinyl benzene and 14% Span 80. 0.3g of lauroyl peroxide was added as an initiator. The emulsion was added to the PU sponge using a syringe and allowed to polymerise for 2 hours before removing excess liquid from the syringe, so that it became more viscous and would therefore be more likely to adhere to the PU sponge pieces. The emulsion with sponge pieces was then incubated overnight at 60^0C. The emulsion again failed to polymerise.

4) Add vinyl pyridine to the oil phase

Vinyl pyridine can be used as an additive to the polymer which makes it hydrophilic without the need to sulphonate it. An oil phase was made up consisting of 68% styrene, 8% 2-vinyl pyridine, 10% divinyl benzene and 14% Span 80. Normal aqueous phase was used. Polymer was made with 80% aqueous phase and 20% oil phase using a dosing time of 5 minutes and a mixing time of 25 minutes. The emulsion was absorbed

onto the sponge by compression in a syringe, then incubated at 40^0C for 6 hours then 60^0C for a further 6 hours. The emulsion again failed to polymerise.

None of these methods were successful in producing a hydrophilic PU sponge, but the sponge may still have potential use as a reservoir for bacteria, or nutrients, although the larger pore sizes would mean water and any nutrient it contained would be released faster than from the PHP with its smaller pore sizes.

Chapter 5: Development of polyHIPE as a biofertiliser

Soil is a living environment that contains a vast array of microorganisms. Most of them are harmless, but a few of these can have a big impact on plant productivity, either pathogenic organisms that have a detrimental effect, or beneficial organisms that stimulate plant productivity by supplying limited nutrients to the plant (Van der Heijden *et al*, 2008). The relatively small numbers of beneficial organisms normally present in most soils mean they do not have a significant effect on plant production, but if the number of these organisms could be increased, their effect could then become more significant. PHP may have potential to act as a reservoir for these beneficial organisms, offering a protective environment for them to grow without competition from all the other soil organisms. If the numbers of beneficial organisms can be increased, then they have potential to make a significant contribution to the nutrient requirements of the crop. The two most important categories of beneficial organisms are nitrogen fixing bacteria and mycorrhizal fungi.

Several experiments were conducted to investigate the potential of PHP impregnated with bacteria or fungi to act as a biofertiliser using peas, clover and grass.

Summary of biofertiliser experiments:

- Investigation into the effect of PHP and *Rhizobium leguminosarum* on pea growth
- Investigation of the effect of adding PHP to bacterial broth at different stages of the growth curve of *Rhizobium trifoli*
- Investigation into the effect of PHP and *Rhizobium trifoli* on clover growth
- Investigation into the use of free living bacteria and fungi as a biofertiliser

5.1 Investigation into the effect of PHP and *Rhizobium leguminosarum* on pea growth

Peas were grown in vermiculite with and without polymer soaked with *Rhizobium leguminosarum* to investigate the effect on plant biomass.

5.1.1 Materials & Methods

Preparation of nutrient solution for growing bacteria

1 litre of nitrogen free nutrient solution was made from 200ml Hoaglands nutrient solution (Appendix 1, p159), 0.2g sodium carbonate, 800ml deionised water, 10g mannitol, and 1g yeast. Agar plates were made by adding 3.75g agar to 250ml of the resulting solution. 60ml aliquots were put in 250ml flasks and sterilised in an autoclave at 121^0C for 20 minutes. *R. leguminosarum* were isolated from the bacteria in peat from Becker Underwood Ltd, Saskatchewan, Canada. They were streaked for single colonies on nitrogen free nutrient agar plates, grown for 4 days, then a single colony was replated on another plate and grown for a further 4 days to ensure a pure culture was obtained. A starter culture was produced by inoculating a single colony from the agar plate into 60ml sterile nitrogen free nutrient solution and grown for 24 hours in a shaker incubator at 26^0C and 160 rpm. A growth curve was produced as p107. A sample was Gram stained and examined under a light microscope. 100µl from the starter culture were then inoculated into the 60ml aliquots of nutrient solution and incubated at 26^0C and 160rpm for 72 hours. The cultures were then added to 2.5g sterilised polymer in sterile plastic beakers and left for a further 72 hours. Polymer used in these experiments was produced by washing excess nitrogen out rather than neutralising it with sulphuric acid.

Gram staining procedure

A droplet of the culture was spread on a glass microscope slide and dried by passing smear side up through a low bunsen flame. The cells were then fixed by passing once slowly face down through the bunsen flame. This sticks the cells to the glass so that they are not washed off when flooded with stain. The slide was then immersed in crystal violet for two minutes then rinsed with water and the excess liquid was drained off, followed by immersion in Lugol's iodine for one minute. Absolute alcohol was then dripped onto the slide until the blue dye is only just removed from the smear, then washed again with water. The slide was then counter stained with neutral red for two minutes, then washed with water and blotted dry. The slide can then be examined under a microscope. Gram positive cells are stained blue and Gram negative cells are stained red.

Planting of peas

Peas (variety Phoenix) were grown in vermiculite in a greenhouse from 19/5/08 – 14/7/08 in 13cm diameter pots. There were 3 treatments: (1) peas; (2) peas plus PHP; (3) peas plus PHP soaked with *R. leguminosarum*. Six replicates of each treatment were planted. One seed was planted in each pot and covered with 25mm of vermiculite. The polymer was mixed with vermiculite below the level of the seed. Plants were watered with Hoaglands nutrient solution. Plants were grown for 8 weeks, but very high temperatures in the greenhouse resulted in all plants dying. Temperature ranged from a minimum of 8^0C to a maximum of 39^0C. However, the difference in growth rate in the earlier stages produced weight differences that were still worth recording. The shoot length and shoot & root dry weights are recorded here.

5.1.2 Results & Discussion

White coloured single colonies on an agar plate were obtained with a characteristic glistening texture (Figure 66).

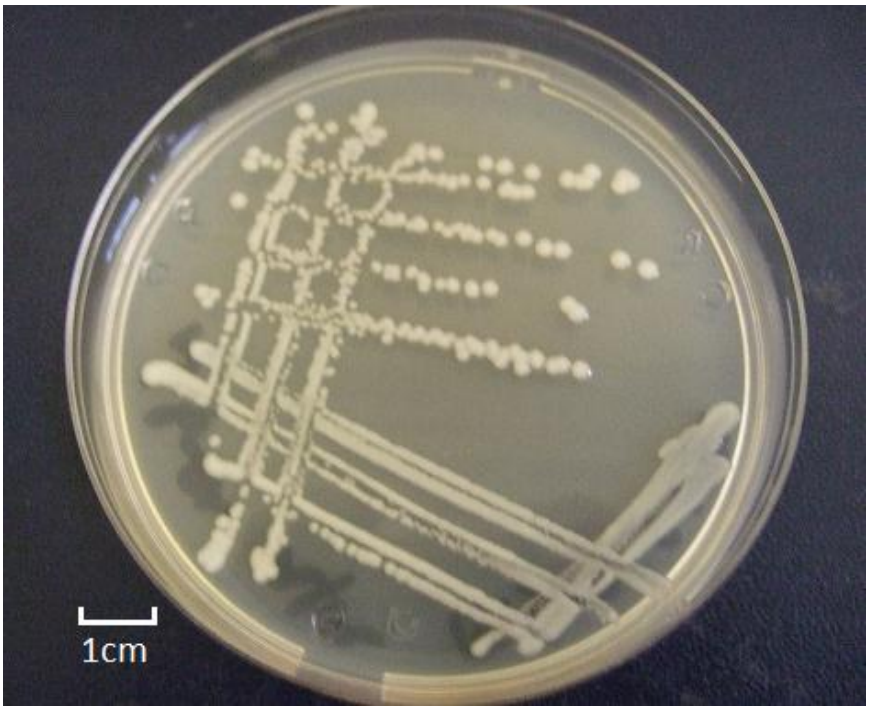


Figure 66: *Rhizobium leguminosarum* on nitrogen free agar plate

Examination of a Gram stained sample under a light microscope showed the bacteria to be Gram negative rods (Figure 67).

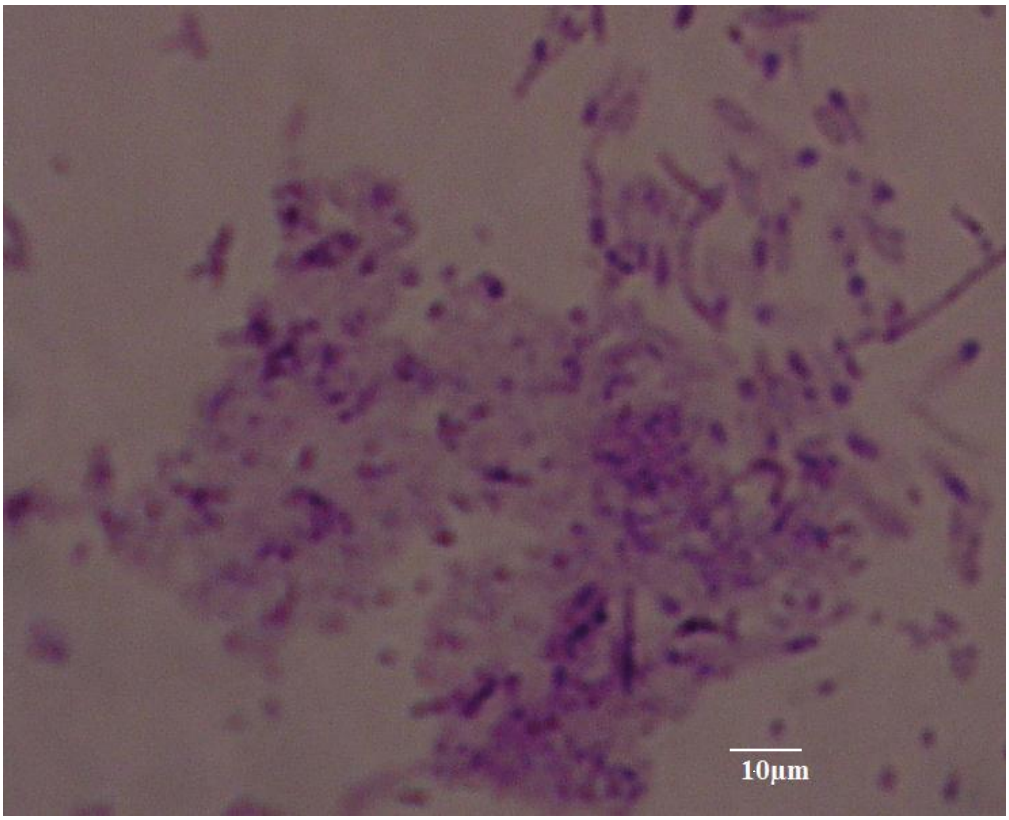


Figure 67: Gram stain of *Rhizobium leguminosarum*

***Rhizobium leguminosarum* growth curve**

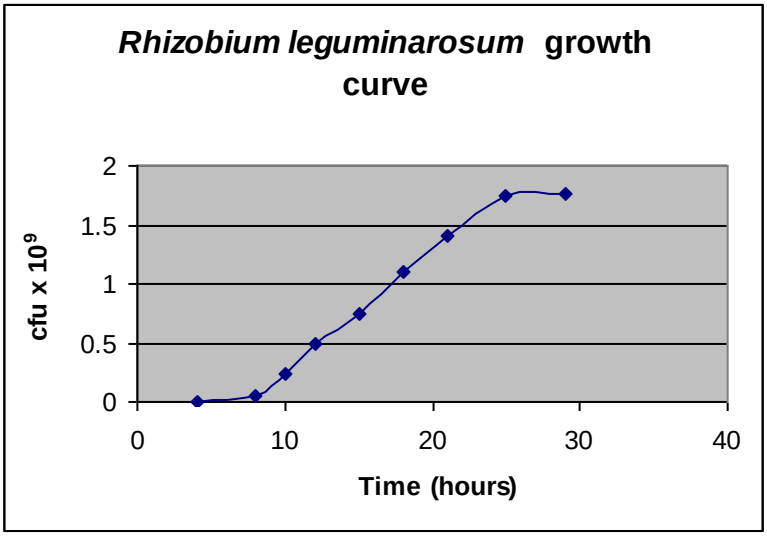


Figure 68: *Rhizobium leguminosarum* growth curve

Rhizobium leguminosarum are slow growing bacteria. The lag phase lasted approximately 8 hours, then the log phase started and continued until 25 hours when the stationery phase started (Figure 68).

Harvest of plants

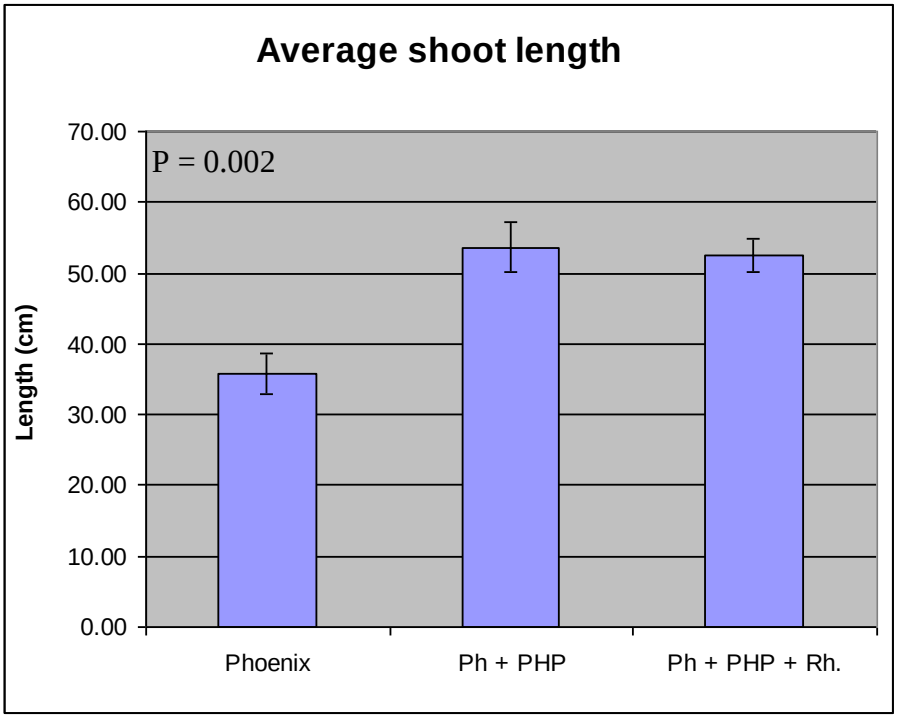


Figure 69: Pea average shoot length

No significant difference was observed between plants with polymer plus or minus bacteria, but all shoots of plants with polymer were significantly longer and heavier than the control plants (Figure 69; Appendix 2, p174). Plants plus polymer were 49.9% longer than control plants and plants with polymer plus bacteria were 46.6% longer than control plants.

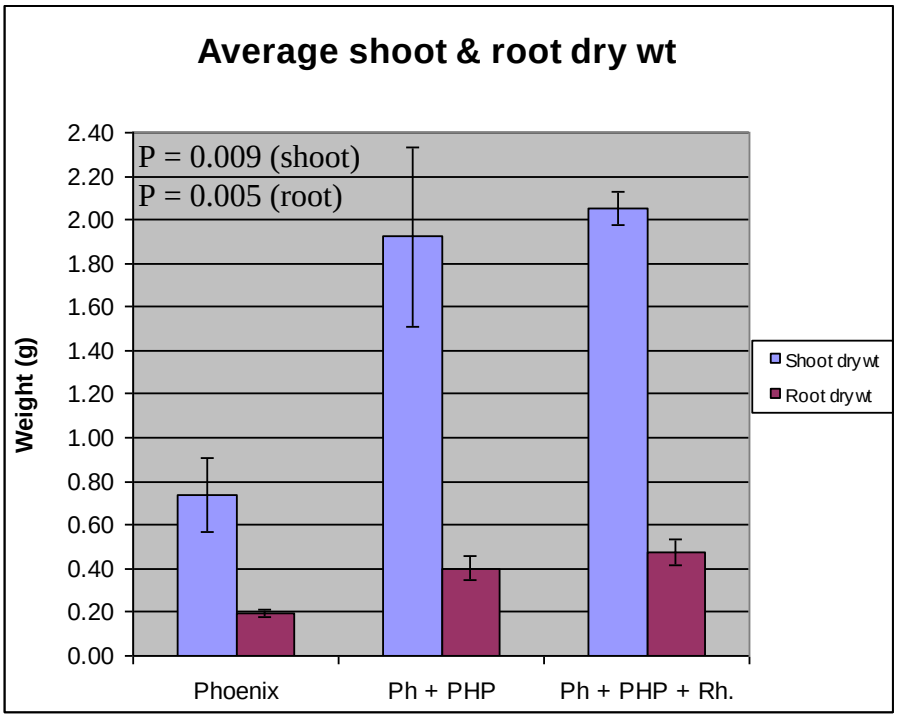


Figure 70: Pea average shoot & root dry weight

Shoot dry weights of plants with polymer were 159.4% heavier than control plants and shoot dry weights of plants with polymer plus bacteria were178.4% heavier. Root dry weights of plants with polymer were 100% heavier than control plants and root dry weights of plants with bacteria were 140% heavier than control plants (Figure 70).

The effect of polymer on its own has produced a yield response similar to that obtained in earlier experiments, but addition of bacteria to the polymer has not had a significant additional effect. As the experiment was conducted using vermiculite as the growth medium, there was not any intimate association of the polymer with the plant roots, so any effect the growing bacteria were having on nitrogen availability would not be evident so early. The high temperature during the growing period would be above the optimum for the bacteria to grow so their growth would be restricted if not completely stopped. Root nodules were observed on the pea roots but the plants would not be growing long enough for the nodules to develop and start fixing nitrogen and have a positive effect on plant growth. High temperatures would mean the plants would be growing fast which would enhance the difference between the control plants and those with PHP, until they suffered temperature stress and died because the temperature became too high.

5.2 Investigation of the effect of adding PHP at different stages of the growth curve of *Rhizobium trifoli*

An experiment was conducted to investigate how to get the maximum number of bacteria into the polymer in the shortest time, by producing a growth curve for R trifoli, then adding polymer to growing cultures of bacteria at different stages of the growth curve. Samples were taken from each culture and examined with SEM to ascertain the optimum time to add polymer to the culture to obtain maximum number of bacteria in the polymer in the shortest necessary time.

5.2.1 R. trifoli growth curve

Bacterial growth curve

All bacteria produce a similar type of growth pattern when grown in a fixed volume of liquid, or batch culture. The curve can be divided into 4 distinct phases: lag phase, exponential or log phase, stationery phase and death phase (Figure 71).

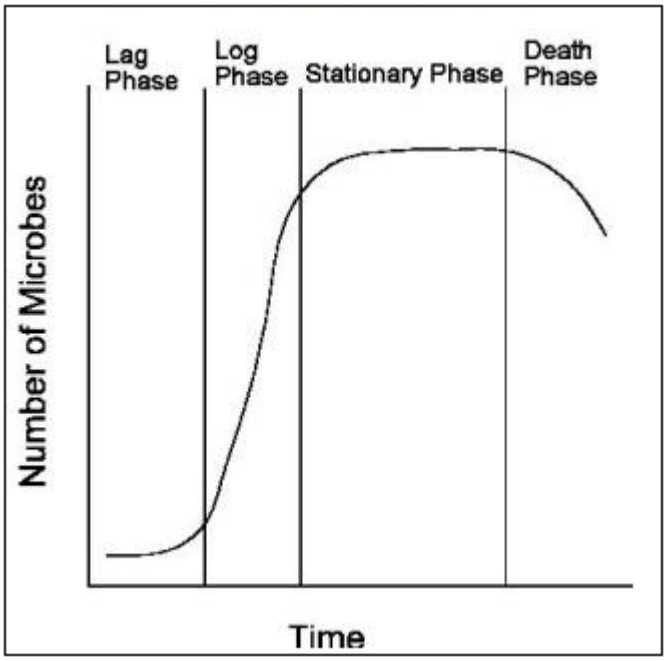


Figure 71: Bacterial growth curve

Lag phase

Immediately after inoculation of cells into a fresh medium, the number of cells remains constant. During this time the cells are adapting to their new environment and synthesising the necessary enzymes required for cell division to take place. The length of the lag phase depends on several factors including the size of the inoculum, the type of nutrients available in the growth medium and time required to synthesise the necessary enzymes required to utilise the available nutrients.

Exponential phase

Once the cells have adapted to the system, they begin to divide regularly by division into two (binary fission), thereby producing an exponential increase in cell numbers. One cell divides to produce two cells in the first generation, then four cells in the second generation, then 8 cells in the third etc.

Stationery phase

In an enclosed system, or batch culture, the exponential growth cannot continue indefinitely. The rate of growth slows down because of the exhaustion of nutrients in the medium and the accumulation of toxic end products. The rate of cell division slows down and the rate of cell death increases so there is no further net increase in cell numbers.

Death phase

As nutrients become totally exhausted and toxic by-products accumulate, the rate of cell death overtakes cell division and the net number of cells declines.

The same basic pattern of growth is observed for most bacteria grown in batch culture, with the major difference being the time scale. The generation time is the time interval required for the number of cells to double and can vary from a few minutes for *Escherichia coli* to several hours for *Rhizobium trifoli.*

5.2.2 Materials and Methods

A sample of *R. trifoli* was taken from storage in glycerol at -80^{0}C and streaked for single colonies on a nitrogen free agar plate. A starter culture was produced by inoculating a single colony from the nitrogen free agar plate into 60ml sterile nitrogen free nutrient solution in a 250ml conical flask and grown for 24 hours in a shaker incubator at 26^{0}C and 160 rpm. A growth curve was then initiated by inoculating 100µl from the starter culture into 60ml fresh sterile nitrogen free nutrient solution in 250ml flasks in triplicate. Samples were removed periodically over a period of 57 hours. For each sample, a series of dilutions were made up – 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}. Each dilution was mixed thoroughly with a vortex mixer before sampling and a fresh pipette tip was used for each successive dilution. 20µl of each dilution was spotted in duplicate on a nitrogen free agar plate. Three dilutions were spotted on each plate, therefore making 6 spots per plate (Figure 72). After allowing the spots to dry, the plates were turned upside down and incubated at 26^{0}C for 3 days. A sample was kept in long term storage by adding 0.85ml bacterial broth to a sterile Eppendorf tube with 0.15ml sterile glycerol, mixing on a vortex mixer and storing at -80^{0}C.

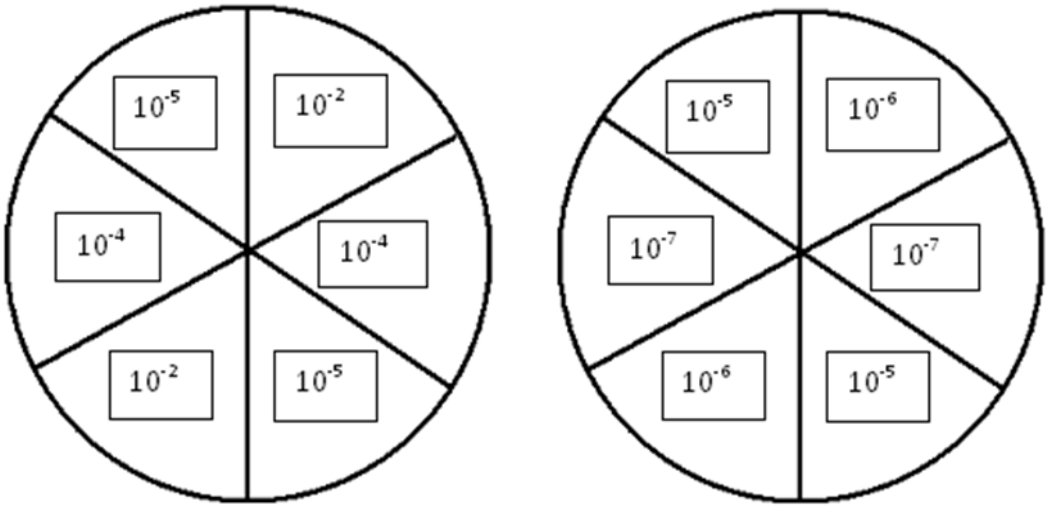


Figure 72: Diagram showing dilutions spotted on each plate

Somewhere within the range of dilutions there will be a countable number of colony forming units (cfu) from which the concentration of cells in the original culture can be calculated. The number of cfu was an average of six – two duplicates on each plate and replicated three times. The concentration of cells/ml in the original sample = number of cfu x dilution factor x 50 (because 20 µl added/plate and the required figure is /ml). The turbidity of each sample was also measured at 540nm using a spectrophotometer. After the first 3 samples, when the turbidity of the culture indicated cells were growing rapidly, then only the higher dilutions were plated. The colonies were counted using the spot with the lowest dilution that it is possible to count. A growth curve plotting the cell number against time can then be constructed.

5.2.3 Results & Discussion

Plates were incubated for 3 days then the colonies were counted using the 10^{-6} spots (Figure 73).

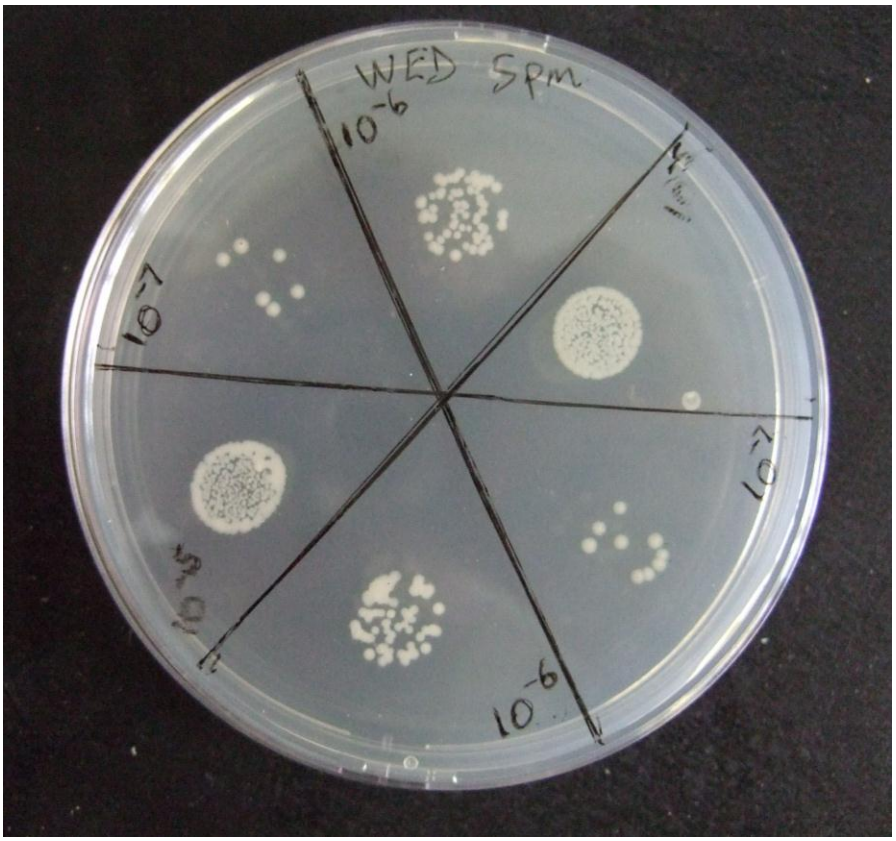


Figure 73: Nitrogen free plate showing growth of serial dilutions of *R. trifoli* after 3 days.

A growth curve could then be constructed (Figure 74).

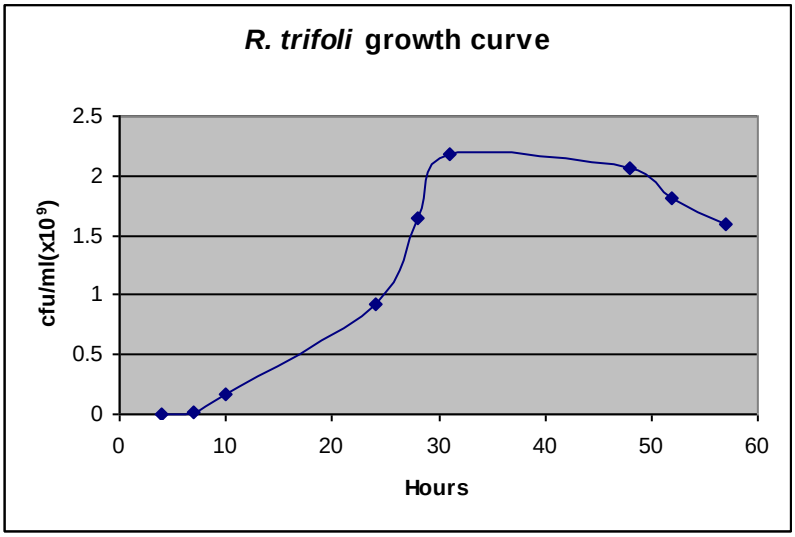


Figure 74: *Rhizobium trifolium* growth curve

The growth curve indicates that the lag phase lasts for approximately 7 hours, then the bacteria start growing exponentially until 30 hours when the stationery phase starts. This continues for 15 hours after which numbers start to decline in the death phase.

Investigation of the effect of adding PHP to bacterial broth at different stages of the growth curve

The results from the growth curve were then used to investigate the timing of adding PHP to the growing culture to maximise the number of bacteria in the PHP while minimising the time the culture was left growing. A starter was again produced and 100µl was added to another three 250ml flask containing 60ml sterile nitrogen free nutrient solution which was again incubated at 26^0C and 160 rpm on a shaker incubator. Flasks were incubated for 16 hours, until the log phase had started and the bacteria would be growing rapidly. 1.25g of PHP was then added to one flask, then incubation continued for a further 5 hours when 1.25g PHP was added to the second flask. This would be approximately half way up the log phase. After a further 5 hours, 1.25g PHP was added to the third flask. This would be approaching the end of the log phase. Flasks were incubated for a further 1 hour, then a sample was removed from each flask, fixed with gluteraldehyde, stored in 100% ethanol, then critical point dried, then examined under SEM (Figure 75 a, b & c).

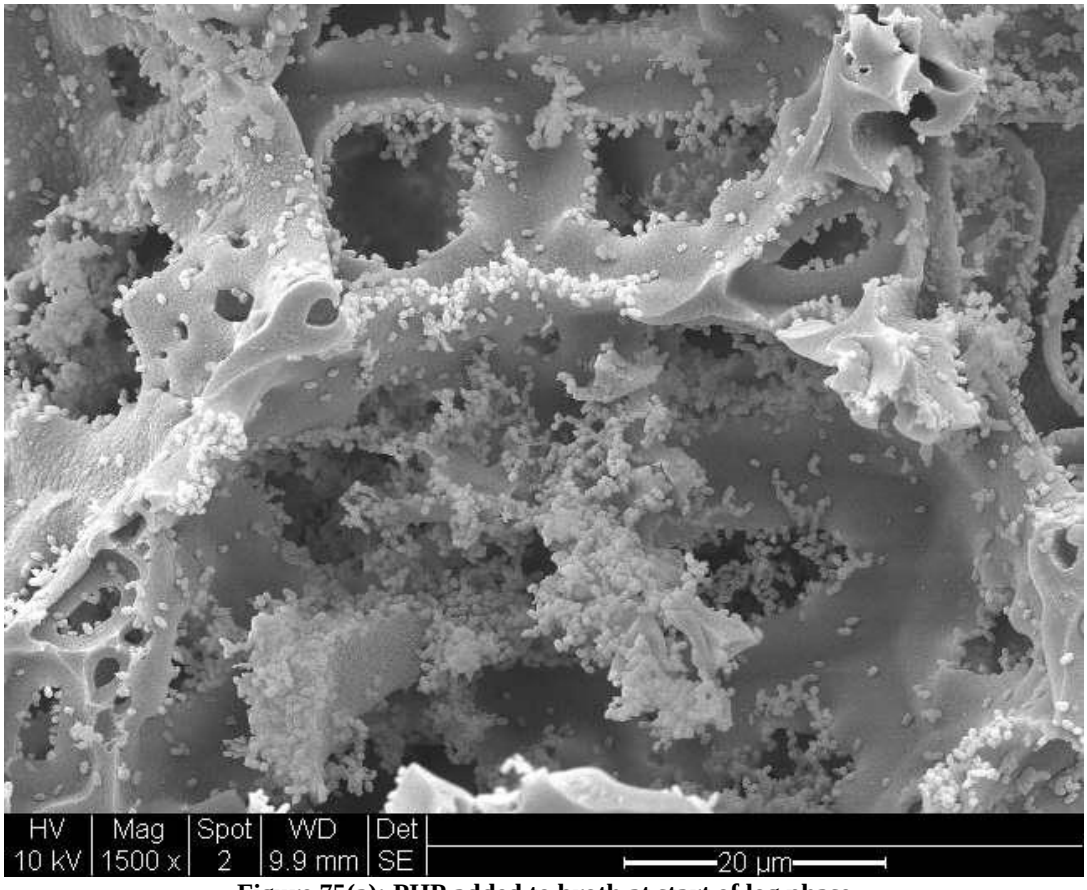


Figure 75(a): PHP added to broth at start of log phase

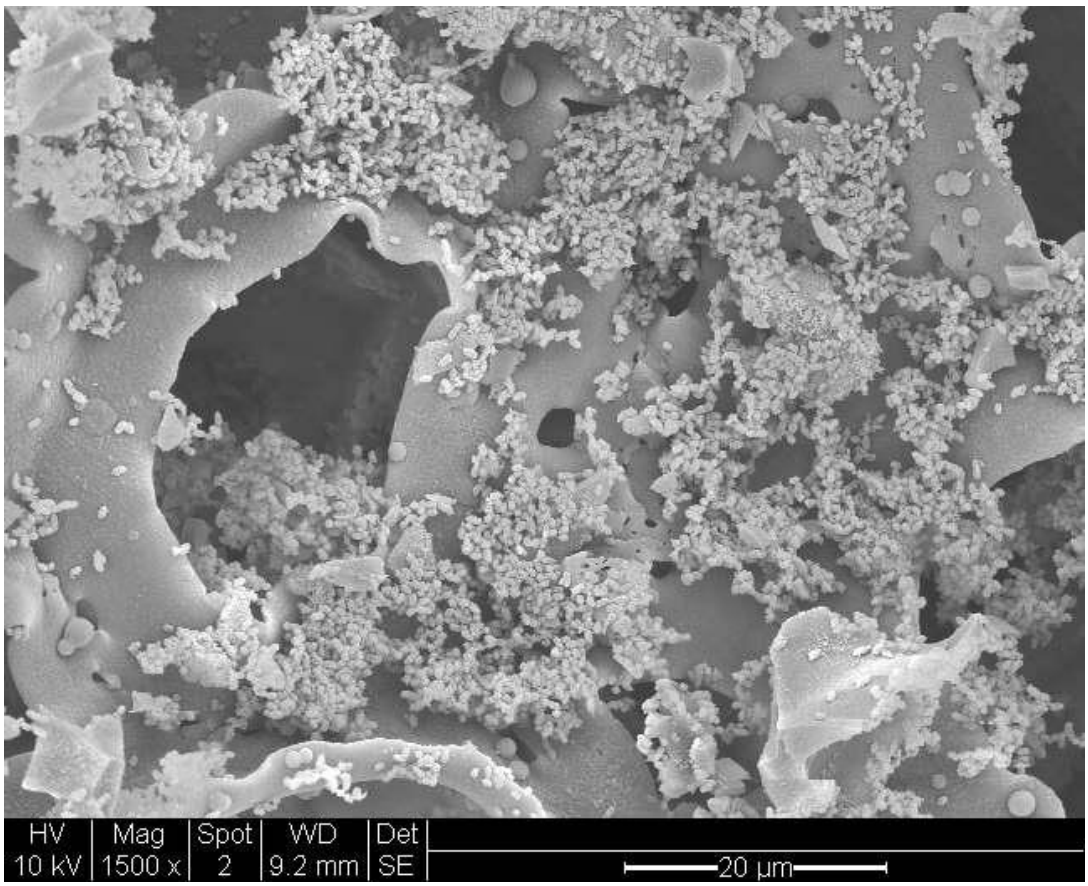


Figure 75(b): PHP added to broth approx. half way through log phase

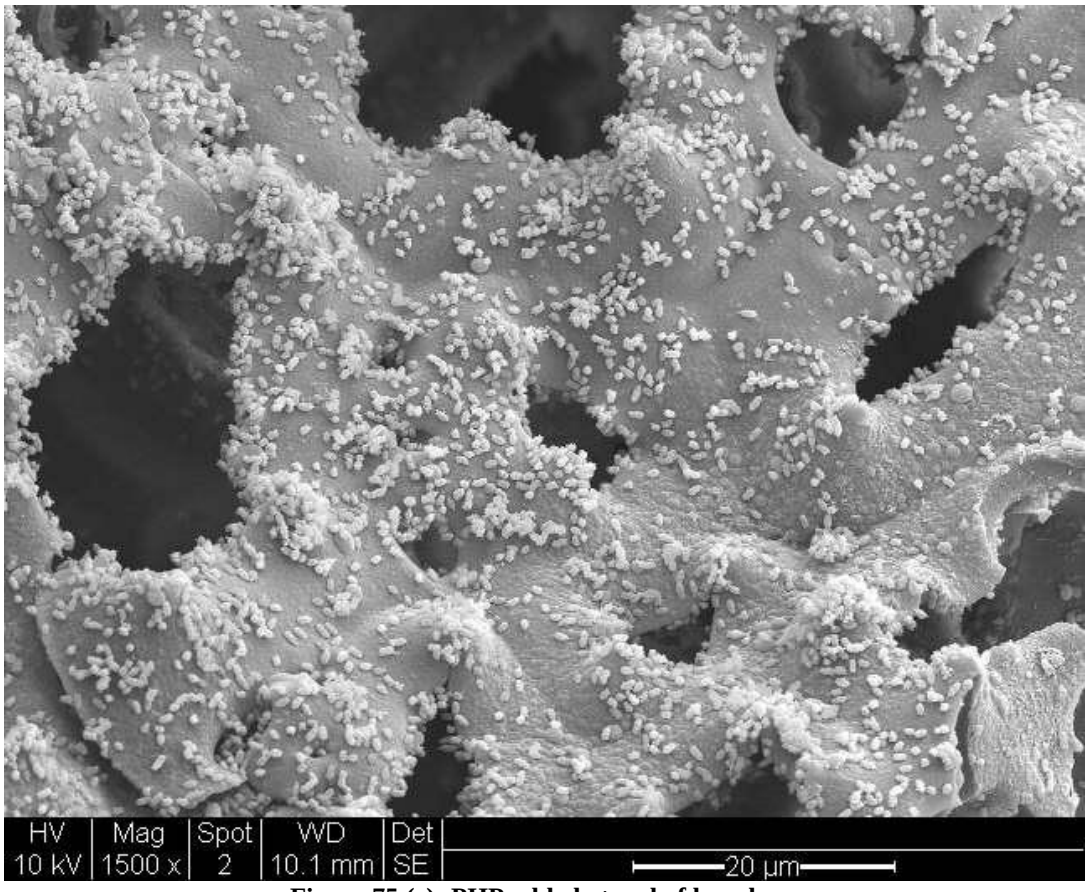


Figure 75 (c): PHP added at end of log phase

When PHP was added early in the log phase, a few bacteria have entered the polymer and have been growing and dividing in the polymer because clumps of bacteria were evident within the polymer. But there was significant damage to the polymer caused by the shaking action of the incubator over a longer period. The abrasive action of the pieces of polymer rubbing against each other in the shaker also caused significant erosion of the edges of the polymer.

When polymer was added in the middle of the log phase, more bacteria would be in the broth, so more would be initially attracted into the polymer and they have continued to divide for the remainder of the log phase again producing clumps of bacteria within the polymer.

When the polymer was added at the end of the log phase, they would be nearly stopped growing so there were no clumps of bacteria evident in the polymer – only single bacteria.

Although bacteria were evident in the polymer in all experiments, they were not in all areas of the polymer – there were large areas with no bacteria present. There tended to be, but not always, more bacteria nearer the outer surface of the polymer.

In order to produce the optimum result of most bacteria in the polymer in the shortest time without significantly damaging the polymer, adding the PHP half way through the log phase appeared to be the best procedure so in future experiments with bacteria this was the protocol used.

5.3 Investigation into the effect of PHP and *Rhizobium trifoli* on clover growth

Experiments were conducted with clover to examine the effect the addition of polymer and *Rhizobium trifoli* had on plant growth. *R. trifoli* is the nitrogen fixing bacteria that specifically associates with clover, forming nodules on the roots.

Five experiments were conducted using clover:

- Clover with and without PHP soaked with *R. trifoli* in growth cabinet.
- Clover with and without PHP soaked with *R. trifoli* in greenhouse.
- Clover with and without PHP soaked with *R. trifoli* in greenhouse using autoclaved soil.
- Clover with and without PHP soaked with *R. trifoli* in greenhouse using washed, autoclaved soil.
- Clover with *R. trifoli* and PHP containing the additional components of silica (Bendzil 10) and hydroxyapatite in growth cabinet.

5.3.1 Clover with & without polymer soaked with Rhizobium trifoli

5.3.1.1 Materials and Methods

Clover was grown in soil in 10cm pots in a growth cabinet. Four treatments were used: (1) clover; (2) clover plus PHP; (3) clover plus *R. trifoli* broth; (4) clover plus PHP soaked with *R. trifoli*. There were 4 replicates of each treatment. *R. trifoli* was obtained from DSMZ Sales, Inhoffenstrabe 7 B, 38124 Braunschweig, Germany. The soil mixture used was 75% John Innes No.3 and 25% horticultural sand. The pots hold 250g of soil mixture and polymer was added at 0.5% wt therefore 1.25g of polymer was added to each pot. 100µl of *R. trifoli* culture from a starter culture was added to 60ml sterile aliquots of nitrogen free nutrient solution in 250ml flasks and shaken at 26^0C and 160rpm on a shaker incubator. After 24 hours 1.25g sterile PHP was added to each flask and incubated for a further 5 hours. The excess bacterial broth was removed from the flasks and measured (30ml), so 30ml had been absorbed into the polymer. Therefore 30ml was the volume added to pots containing only bacterial broth. Wild white clover seed was obtained from Victoriana Nursery, Challock, Ashford, Kent. 0.1g clover seed was added to each pot and covered with a light covering of soil mixture. Plants were watered twice weekly.

Shoots were harvested and weighed at 3, 6 and 9 week intervals and the roots were also washed and weighed after 9 weeks. Dry weights are recorded here. Pictures were taken at 3 week intervals before harvesting.

5.3.1.2 Results & Discussion

Control plants and those with polymer plus bacteria were visibly smaller than the other treatments after 3 weeks growth, but after 6 and 9 weeks, these plants have made up to the extent that there is no obvious visible difference between any of the plants (Figure 76).

Figure 76: Clover + PHP & *R. trifoli* in growth cabinet

After 9 weeks growth in favourable growing conditions of the growth cabinet, all plants had grown extensive roots with all the pots tightly packed with roots to the extent that the pots were limiting root growth and making it impossible to clean the soil off the roots without losing a significant amount of roots, so root weights could not be recorded (Figure 77). Roots were however observed to have extensively penetrated the polymer pieces (Figure 78).

Figure 77: Clover showing dense root structure after 9 weeks

Figure 78: Clover roots showing penetration of polymer by roots after 9 weeks

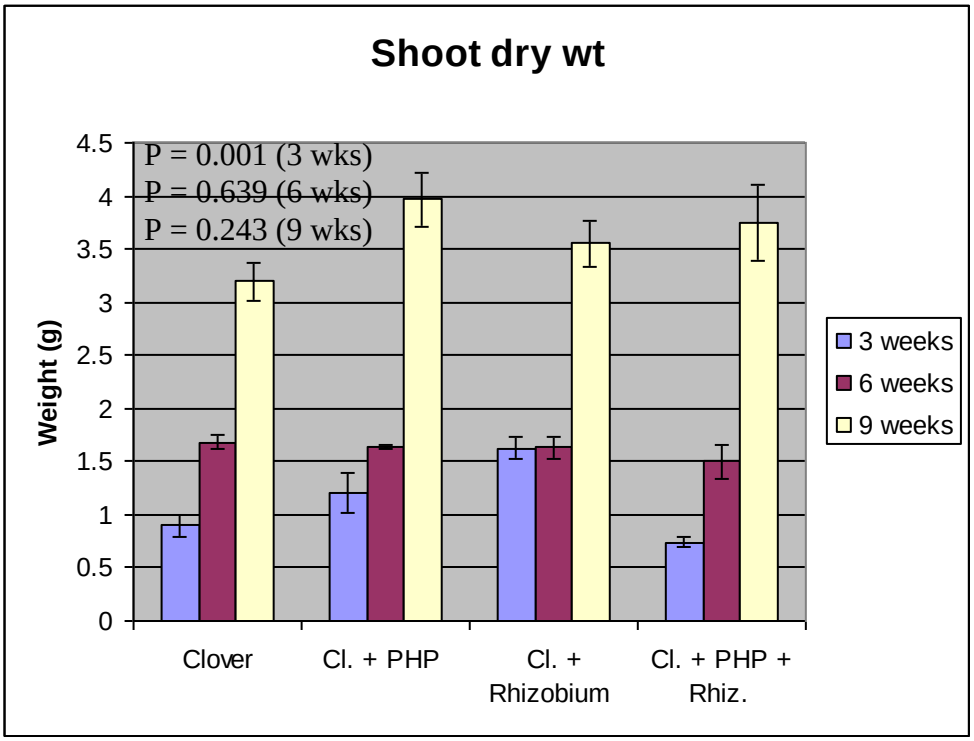


Figure 79: Dry weights of clover + PHP & *R. trifoli* in growth cabinet

After 3 weeks growth, dry weights of plants with polymer have increased by 33.3% and plants with bacterial broth have increased by 81.1% compared to the control. But plants with polymer soaked in bacteria have decreased in weight by 17.8% compared to the control (Figure 79; Appendix 2, p 175).

By 6 weeks, there was no significant difference between the dry weights of any plants, but by 9 weeks, all treated plants were significantly heavier than control plants. Plants plus PHP, plus bacteria and plus PHP with bacteria were 24.4%, 11.3% and 17.5%

heavier than control plants respectively. These three treatments were not significantly different from each other, but they are all significantly heavier than the control plants.

Even when polymer is manufactured by neutralising with acetic acid and washing the excess nitrogen out there is still some residual nitrogen left in the polymer which could account for the positive weight gain normally observed with the addition of polymer.

When bacteria were added in the polymer, they would be competing with the plants for the available nitrogen in the polymer initially while they grow and divide and develop nodules on the plant roots. The nitrogen available for the plants would therefore be reduced, resulting in a lower weight at the first harvest. As nodules become developed and start to fix nitrogen from the air, this would subsequently become available to the plants, so these plants would then grow faster and they have made up the lost ground compared to the other treatments, so that by the 2nd harvest there was no difference between the weights of any plants. Time and space constraints meant many experiments were terminated after 9 weeks, but if this had been continued for another 3 weeks, perhaps the faster weight gain observed in plants with PHP plus bacteria compared to the other treatments would have been continued and they would have ended up heavier than the other treatments.

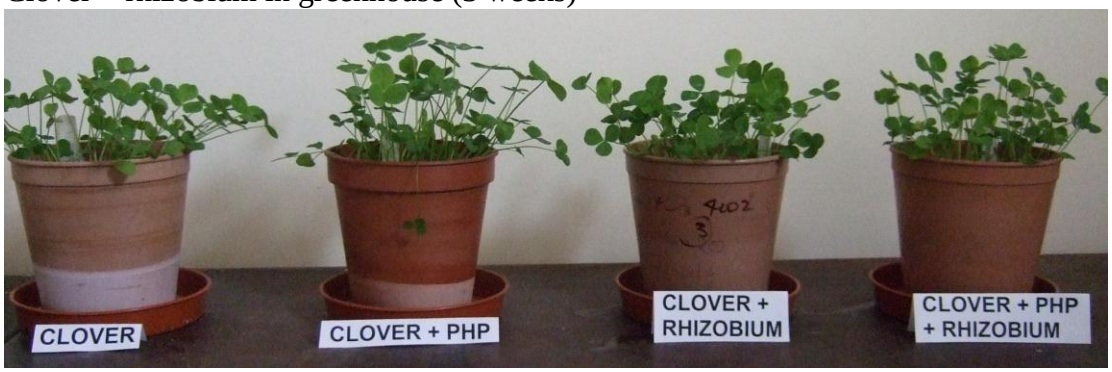
5.3.2 Clover with & without polymer soaked with *Rhizobium trifoli* in greenhouse

The same experiment was repeated in the greenhouse from 1/4/10 – 8/6/10, setting up the same as previously (p113). Temperature ranged from a minimum of 5^{0}C to a maximum of 36^{0}C.

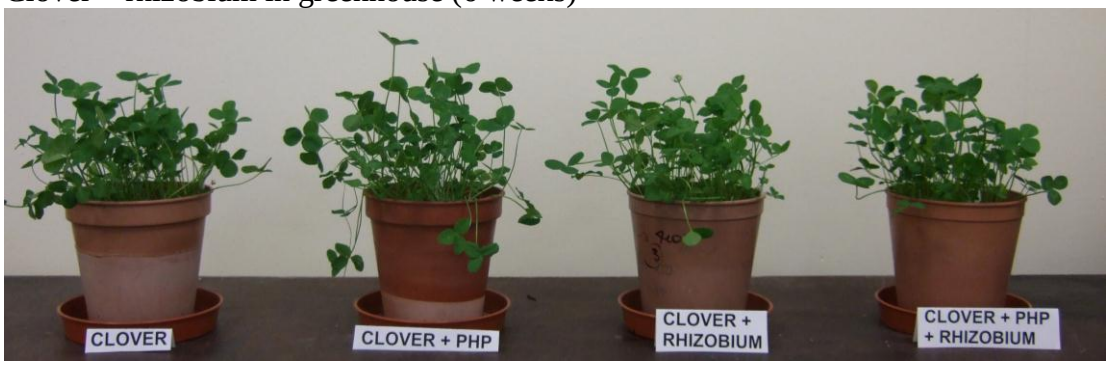
5.3.2.1 Results & Discussion

In individual pots, the growth is much less uniform than in the pots from the growth cabinet because of the much more variable growing conditions and lower temperatures in the greenhouse compared to the growth cabinet where the constant favourable growth conditions produce much more uniform plants. Unlike the growth cabinet plants, there was no obvious visible difference between any of these plants (Figure 80).

Clover + rhizobium in greenhouse (3 weeks)



Clover + rhizobium in greenhouse (6 weeks)



Clover + rhizobium in greenhouse (9 weeks)

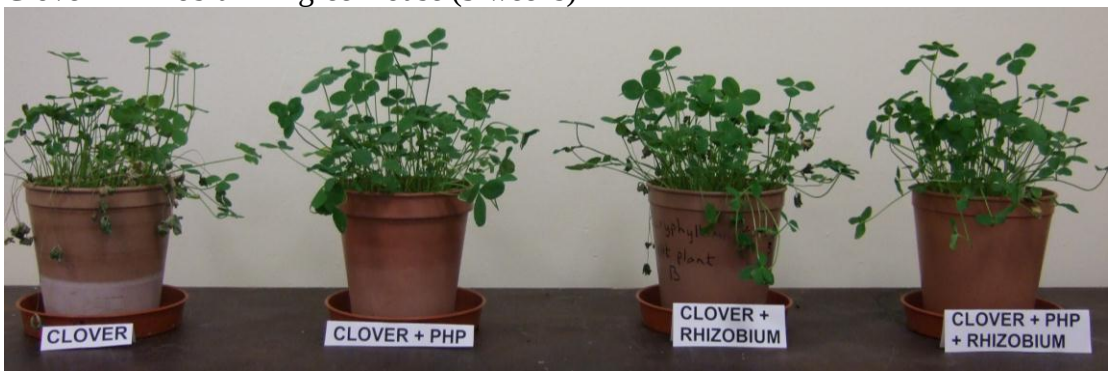


Figure 80: Clover + PHP & *R. trifoli* in greenhouse

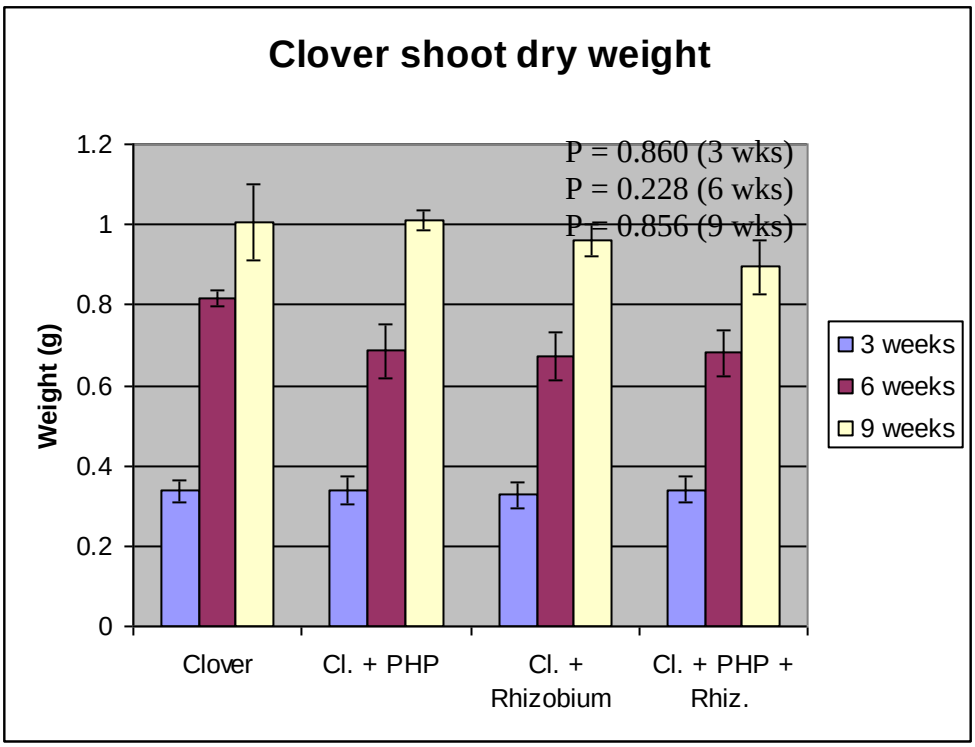


Figure 81: Clover + PHP & *R. trifoli* in greenhouse – shoot dry weights

The less favourable conditions of the greenhouse compared to the growth cabinet are reflected in the much lower weights of the plants which were only approximately one quarter of the weight of the plants from the cabinet. None of the treatments produced a positive effect compared to the control plants (Figure 81; Appendix 2, p176). After 3 weeks growth, there was no significant difference between any of the plants. By 6 weeks, the control plants were heavier than all the treated plants, but there was no significant difference between any of the different treatments. Control plants were 15.8%, 18.3% and 17.1% heavier than plants plus PHP, plants plus bacteria and plants plus PHP with bacteria respectively. Further statistical analysis showed that this was not significant (P = 0.228) because of the wide variation in the replicates. After 9 weeks, there was no difference between any of the treatments. Examination of the roots indicated that nodules were present on all plants including the control plants (Figure 82), and there was no obvious difference in the number of nodules between any plants, but there were too many very small nodules to allow them to be counted accurately.

Figure 82: Roots of control plants showing root nodules

The bacteria are present naturally in the soil which must be present in sufficient numbers to enable the plant to form enough nodules without the addition of any extra bacteria, so the extra bacteria added have no positive effect on plant growth, either as a broth or in the polymer. The lower growth rate of the plants compared to the previous experiment in the cabinet will also mean nutrients available in the soil will not be depleted so rapidly so any effect the bacteria or polymer have on plant growth will not be evident so early. If the plants had been left a further three weeks then perhaps some significant differences in weights would have been observed.

The addition of polymer has, unusually, not produced a positive effect compared to the control although root penetration of the polymer has been observed (Figure 83). In order to investigate if the age of the polymer had any influence on its effectiveness, the experiment was repeated using freshly made polymer in the greenhouse in June but high temperatures when plants were germinating resulted in many plants dying so the experiment was terminated. Another experiment was conducted using 6 month old polymer which showed that age did not appear to have an influence on the effectiveness of the polymer (p88).

Figure 83: Clover roots showing root nodules and root penetration of PHP

5.3.3 Clover with & without polymer soaked with Rhizobium trifoli in greenhouse using autoclaved soil

The same experiment was then repeated again but the soil was sterilised before planting to remove the resident bacteria in the soil so that any positive effect would come from bacteria added as broth or in the polymer. The experiment ran from 2/7/10 till 10/9/10 and the minimum temperature was 10^0C and the maximum was 31^0C. 250g aliquots of soil/sand mixture were autoclaved at 121^0C for 20 minutes in autoclavable plastic bags then the experiment was set up again as previously (p113), again with 4 replicates.

5.3.3.1 Results & discussion

After 3 weeks growth, plants with PHP plus bacteria appear to have a higher germination percentage with more plants growing than the other treatments (Figure 84).

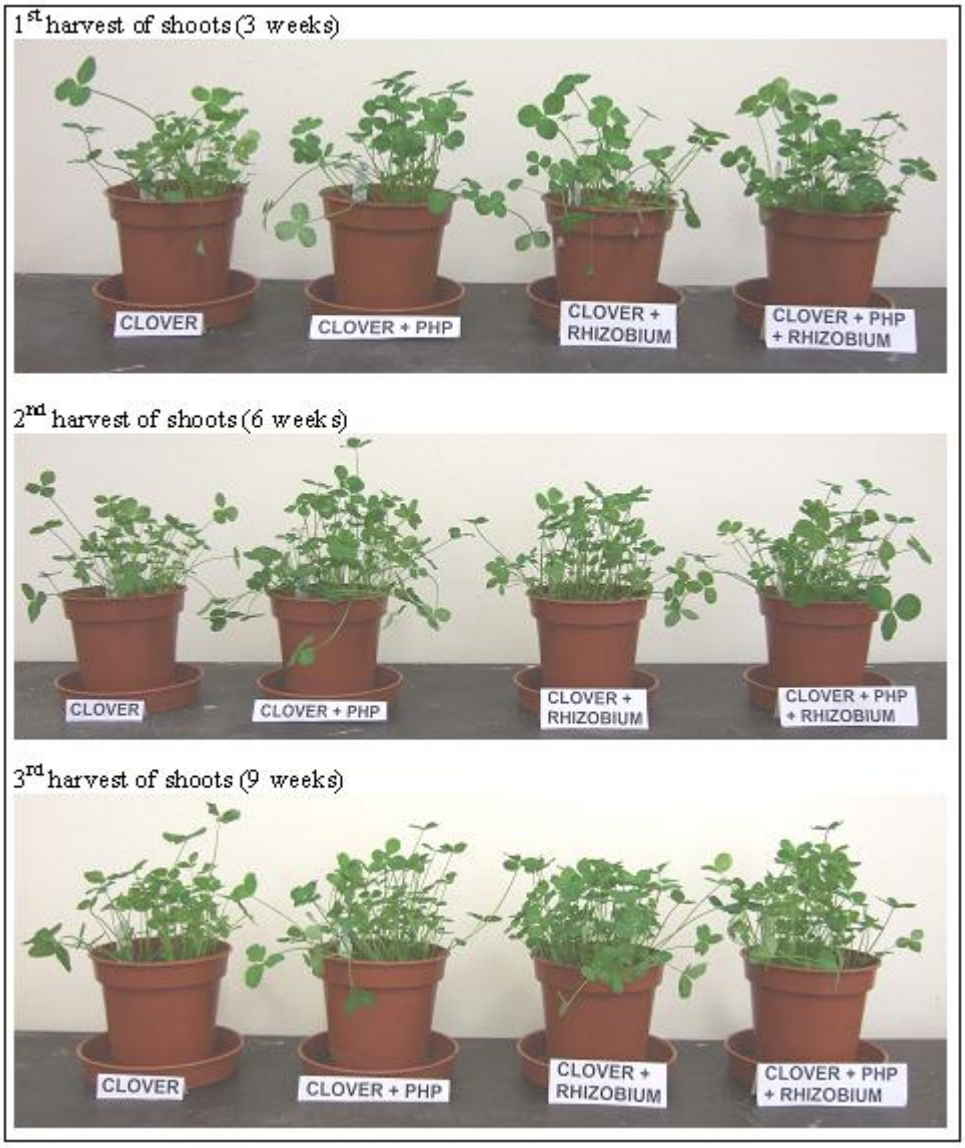


Figure 84: Clover + PHP & *R. trifoli* in sterilised soil in greenhouse

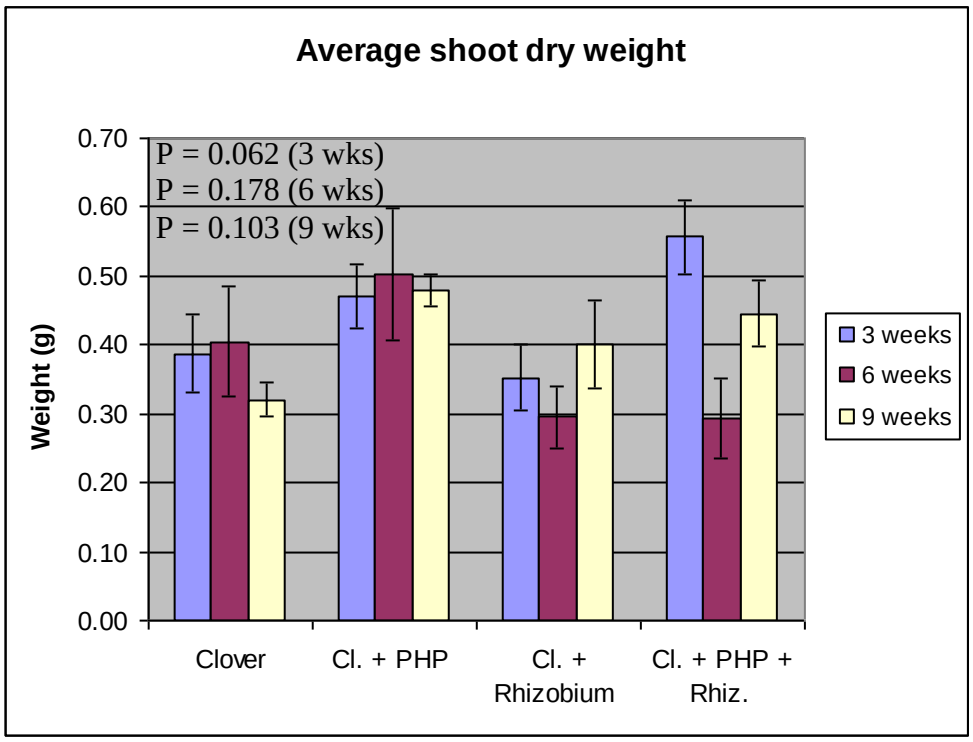


Figure 85: Clover + PHP & *R. trifoli* in sterilised soil in greenhouse – shoot dry weights

After 3 weeks, plants with polymer, with bacteria and with polymer plus bacteria were 20.5%, -10.3% and 43.6% heavier than control plants respectively (Figure 85; Appendix 2, p177). Plants plus polymer soaked with bacteria were heavier than the other treatments although it was not significant. This is not a typical result but, by looking at the picture, it is evident that more seeds have germinated in this pot than the others which would explain the heavier weight at the first harvest. It also demonstrates the importance of seed rate – more plants have grown initially, producing a heavier average shoot weight, but in subsequent harvests, the other treatments with fewer seeds germinated have grown faster and ended up gaining the lost ground so that the shoot weights were as heavy as these plants in subsequent harvests. So more seeds growing initially does not mean the plants will eventually produce the highest yield.

After 6 weeks, plants with polymer were 21.9% heavier than the control plants although this was not significant. Plants with bacteria and plants with polymer plus bacteria were 26.8% and 29.3% lighter than control plant respectively. This was not a significant difference although these two treatments were both lighter than the plants with polymer alone.

After 9 weeks, all treated plants were heavier than control plants. Plants with polymer, with bacteria and with polymer plus bacteria were 50%, 25% and 40.6% heavier than

control plants. Further statistical analysis showed that these were not significant differences (P = 0.103) (Appendix 2, p177).

Examination of the roots showed that the control plants and those with only polymer added and no bacteria, still had a few root nodules present. Plants with bacterial broth added had produced more root nodules, but plants with bacteria added in the polymer did not appear to have produced any more nodules (Figure 86 a & b).

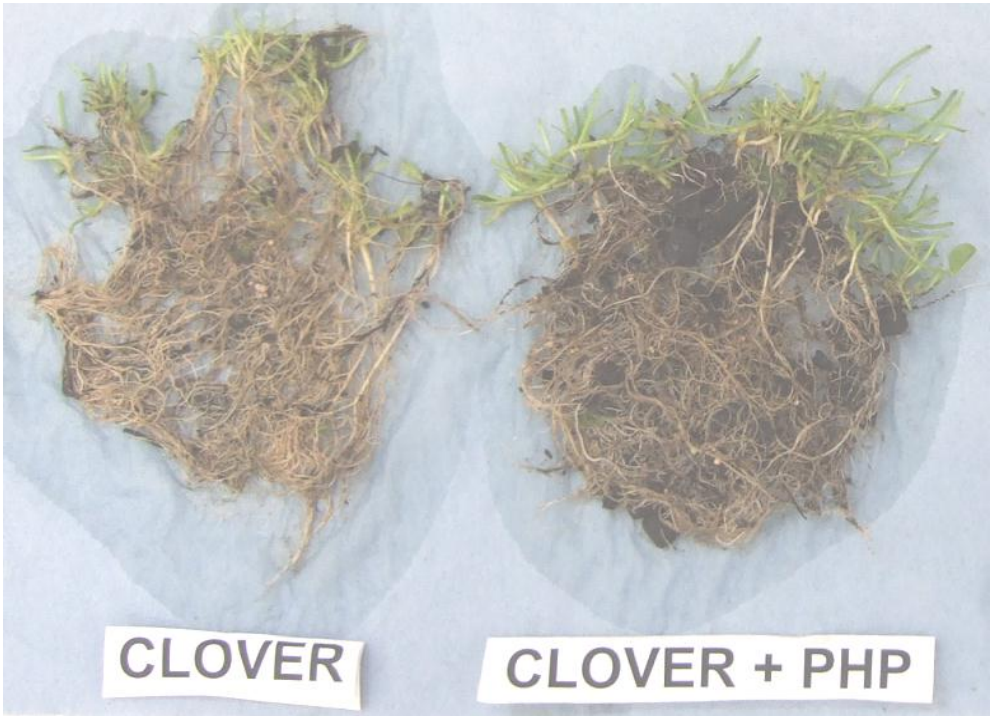


Figure 86(a): Close up of roots

Figure 86(b): Close up of roots

There must therefore have been a few bacteria still present in all pots which could be on the surface of the clover seed or the standard autoclave procedure of 20 minutes at 121^0C may not be sufficient to completely sterilise the bigger bulk of the soil samples. Addition of *R. trifoli* to the polymer has not had a positive effect on the growth of clover as the result was not significantly different to polymer alone.

5.3.4 Clover with & without polymer soaked with Rhizobium trifoli in greenhouse using washed, autoclaved soil

The experiment was repeated again using washed autoclaved soil. This would lower the nutrient status of the soil, as well as remove any soil borne bacteria, so any effect the addition of bacteria or polymer should become apparent earlier. The soil was washed and the washing was monitored by following the conductivity so that the nutrient concentration left in the soil would be approximately the same as previously (p62). The conductivity of tap water was 300μs/cm. After 6 hours the conductivity increased to 1115μs/cm, then the water was changed and left for a further 24 hours when the conductivity was 470μs/cm. Soil was then dried in trays and sterilised in an autoclave in 500g aliquots in autoclavable plastic bags.

The experiment with washed, autoclaved soil was again set up as previously (p113) again with 4 replicates, from 14/7/10 – 15/9/10. Temperature ranged from 10^0C minimum to 31^0C maximum.

5.3.4.1 Results & Discussion

High temperatures at germination produced very uneven germination and continued high temperatures meant plants never recovered and the results produced were very erratic (Figure 87).

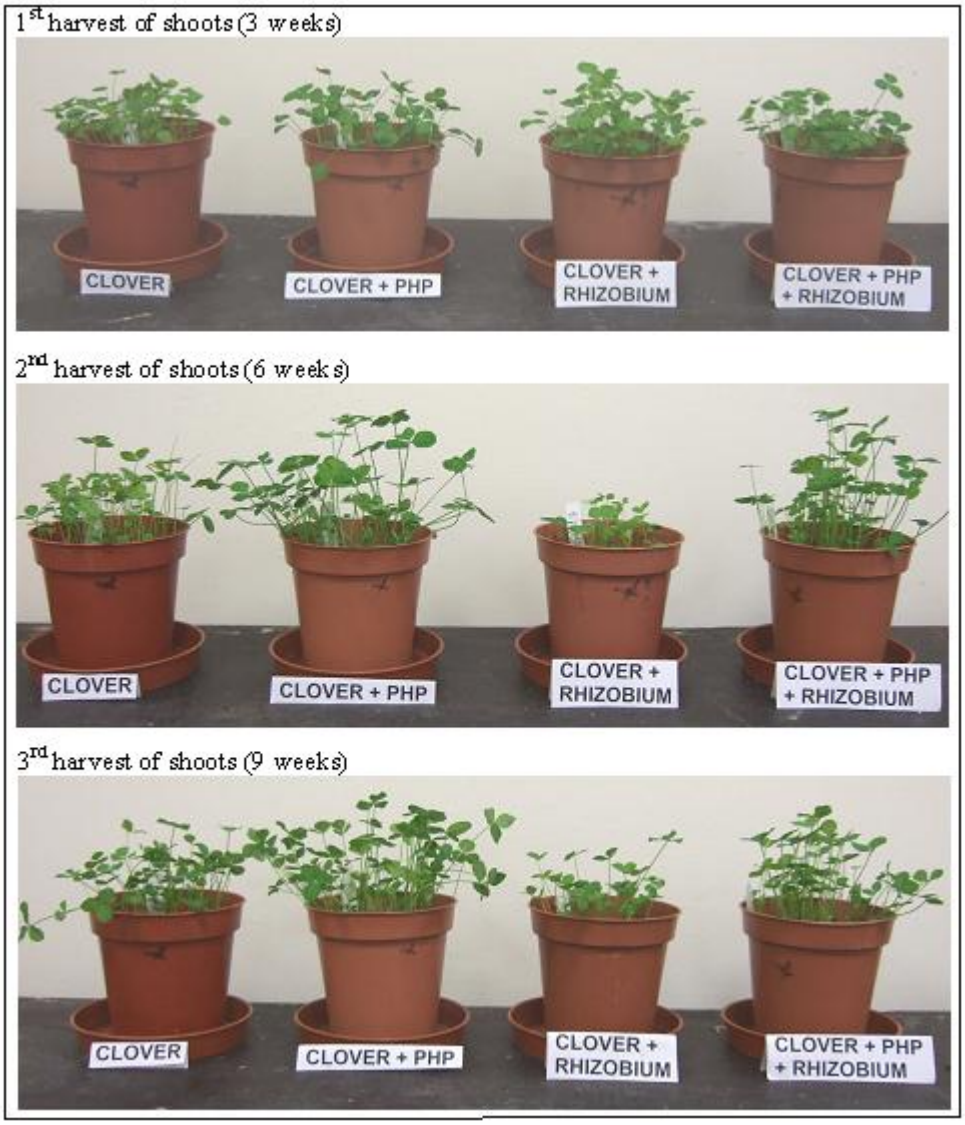


Figure 87: Clover + PHP & *R. trifoli* in sterilised, washed soil in greenhouse

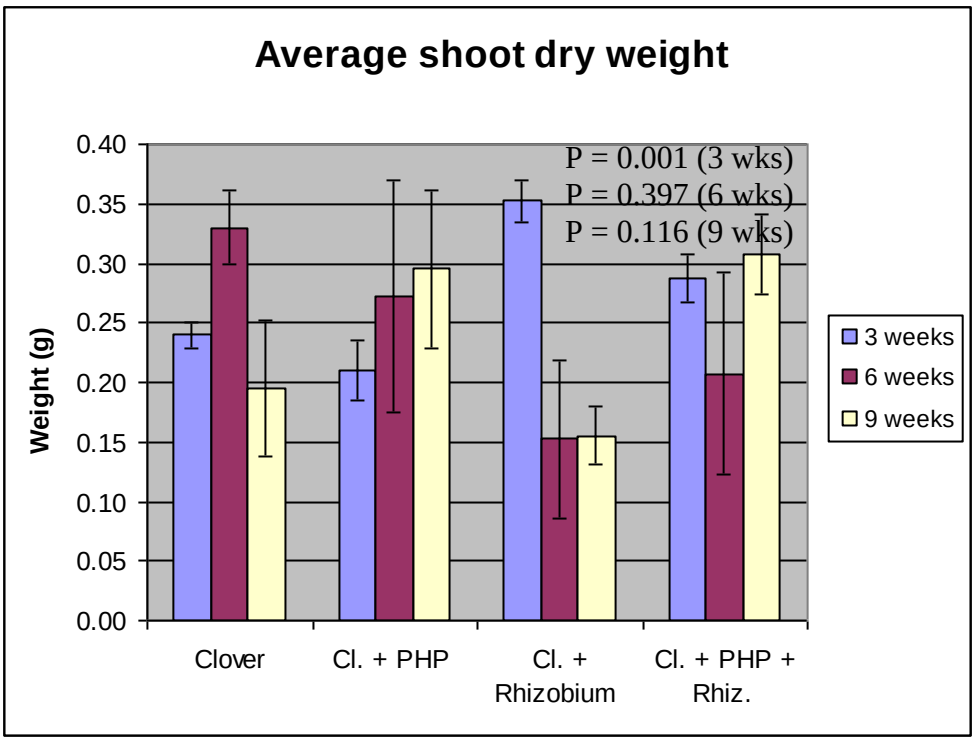


Figure 88: Clover + PHP & *R. trifoli* in sterilised, washed soil in greenhouse – shoot dry weights

Plants with bacterial broth added unusually produced a positive result with plants heavier 45.8% than control plants after 3 weeks (Figure 88; Appendix 2, p178). But this result was not sustained in subsequent harvests when these plants became very stunted and unhealthy looking. Large variation in the replicates of all other treatments meant there was no significant difference between any of the other results. The lateness of the season meant it was not possible to repeat the experiment.

5.3.5 Clover + rhizobium and PHP with silica (Bindzil 10) and hydroxyapatite

The original experiment with clover (p113) was repeated using PHP with the additional 2 ingredients in its manufacture of Bindzil 10 and hydroxylapatite to see if they had an effect on clover growth.

PHP + Bindzil 10

Sulphonated polymer was prepared as previously, then soaked in Bindzil 10 overnight then allowed to dry at room temperature. Bindzil is a colloidal silica solution that binds to the surface of the PHP and increases the surface area of the polymer.

PHP + hydroxyapatite

Hydroxyapatite is a calcium phosphate ($Ca_{10}(PO_4)_6OH_2$)

Sulphonated polymer was prepared as previously. Hydroxyapatite solution was prepared by dissolving 5g hydoxyapatite in 200ml distilled water containing 30 ml phosphoric acid, then made up to 1 litre (Bokhari, 2003). The polymer was then soaked in the hydroxyapatite solution overnight, dried, then soaked in 1M potassium hydroxide, dried, then washed to remove excess potassium hydroxide.

There were 8 treatments in total: (1) clover; (2) clover + PHP; (3) clover + rhizobium; (4) clover + PHP & rhizobium; (5) clover + PHP with Bindzil 10; (6) clover + PHP with Bindzil 10 & rhizobium; (7) clover + PHP with hydroxyapatite; (8) clover + PHP with hydroxyapatite & rhizobium. There were 4 replicates of each treatment. Pots were planted as previously (p113). Shoots were harvested and weighed at 3 week intervals for 4 harvests and the roots were harvest at the 4th harvest. Roots were tightly packed in the pots and it was difficult to remove the soil without losing roots as well so it was not possible to obtain root weights.

5.3.5.1 Results & discussion

SEM pictures showed that for both polymer samples with additional ingredients, some material had been deposited on the surface of the polymer (Figure 89 a, b & c).

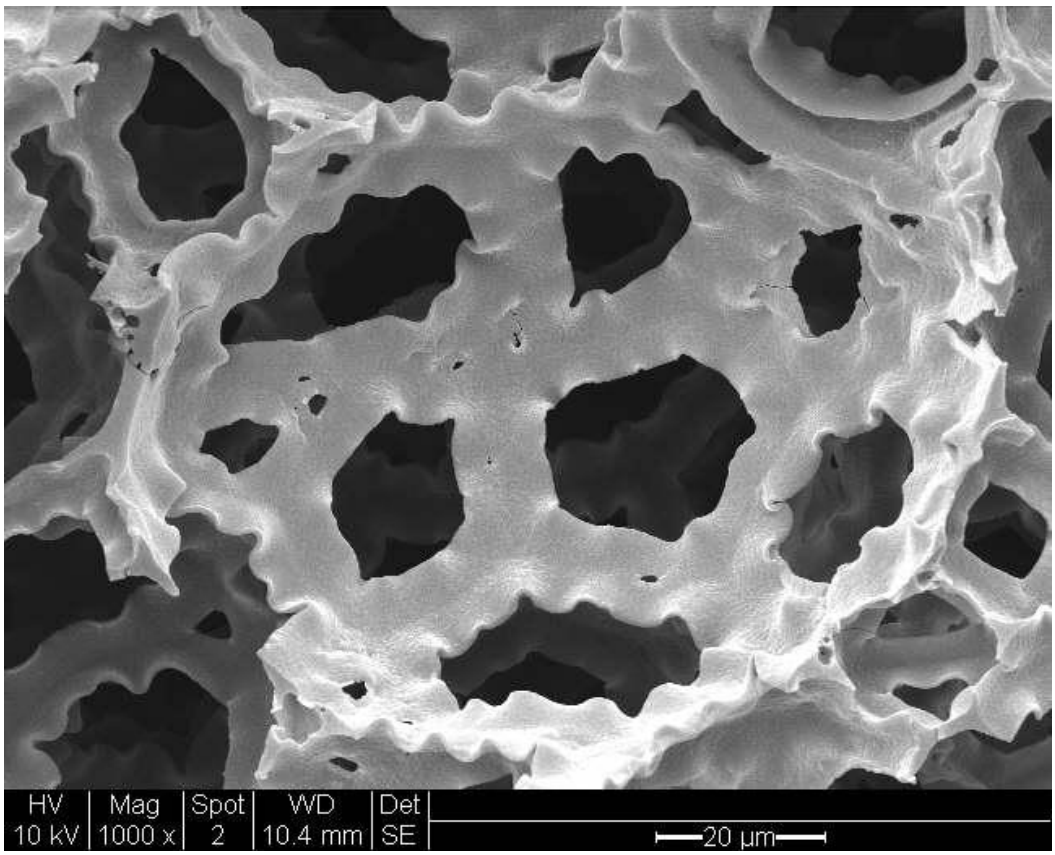


Figure 89(a): Sulphonated polymer

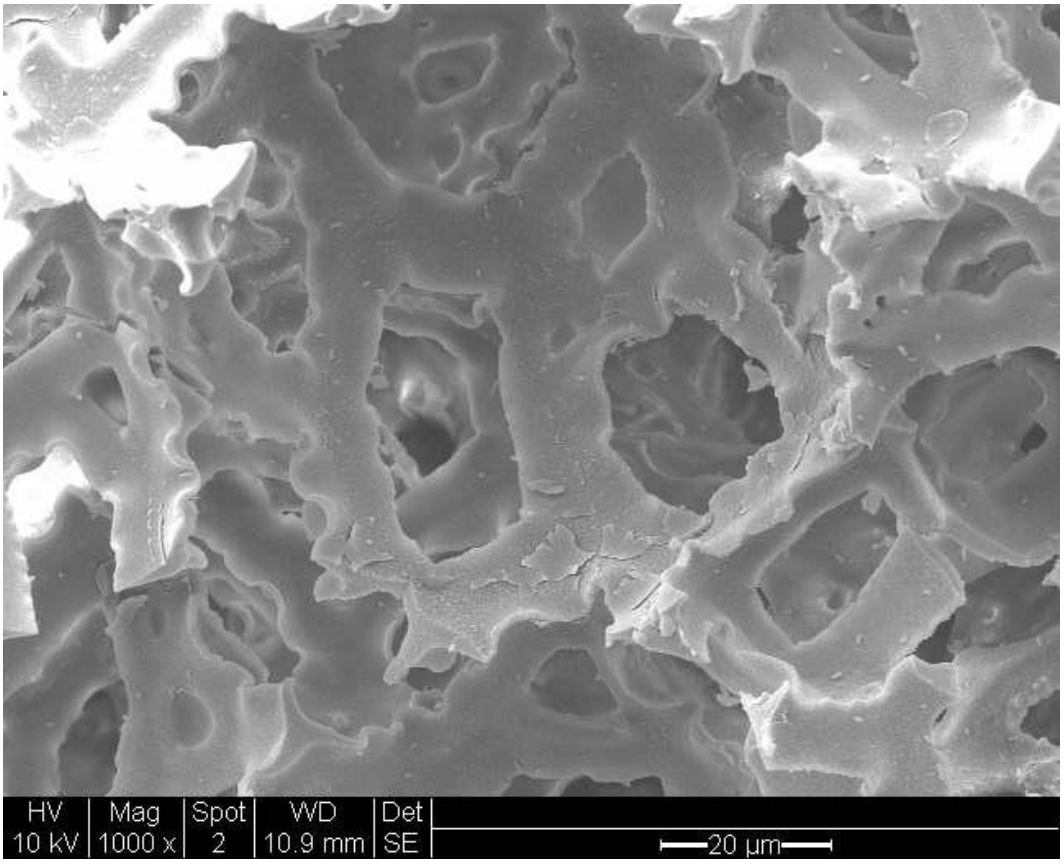


Figure 89(b): Sulphonated polymer + Bindzil 10

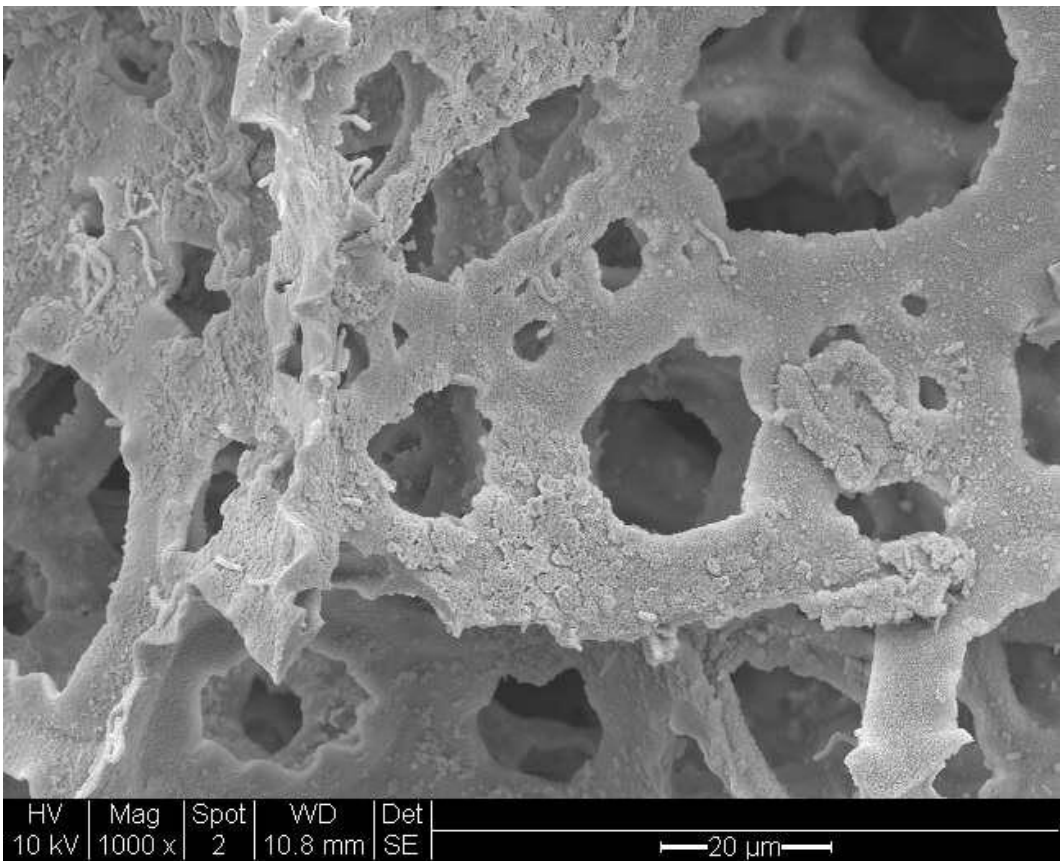


Figure 89(c): Sulphonated polymer + hydroxyapatite

Harvest of plants

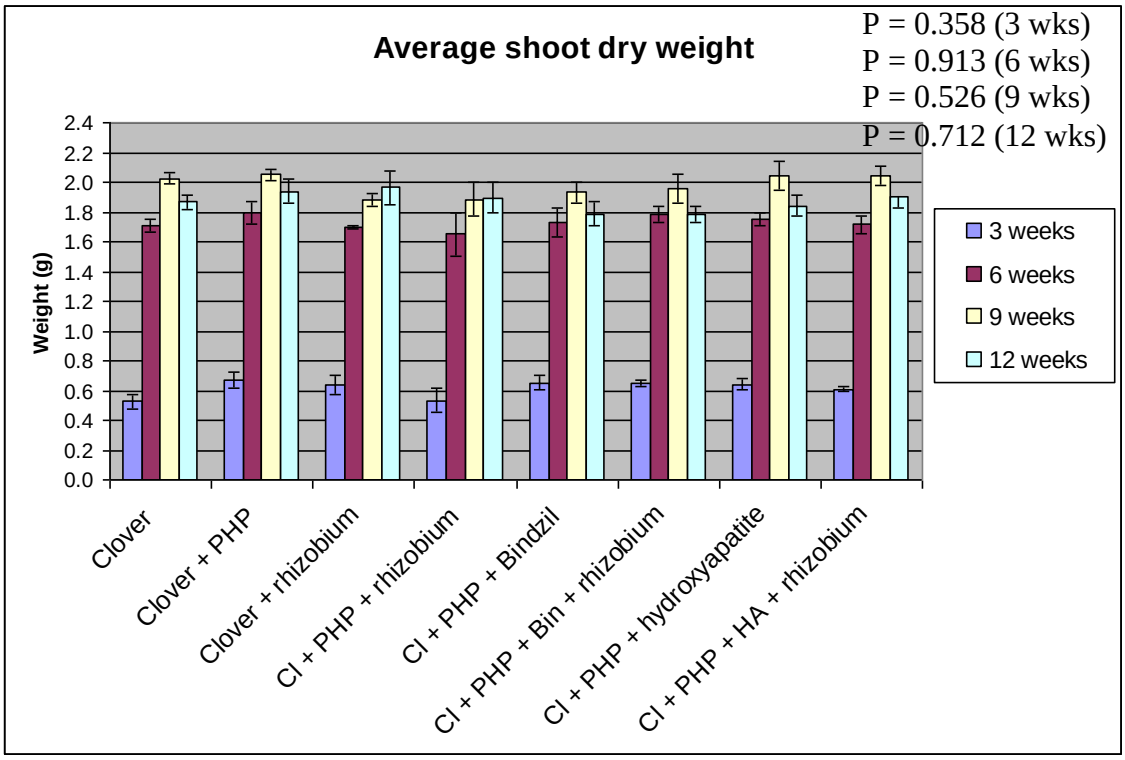


Figure 90: Clover + *R. trifoli* and PHP with Bindzil 10 & hydroxyapatite

	Cl. + PHP	Cl. + rh.	Cl. + PHP + rh.	Cl. + PHP + Bin.	Cl + PHP + Bin + rh.	Cl. + PHP + HA	Cl. + PHP + HA + rh.
3 weeks	26.4	20.7	0.0	22.6	22.6	20.7	15.1
6 weeks	4.7	(1.2)	(3.5)	1.2	4.1	2.3	0.0
9 weeks	1.5	(6.9)	(6.9)	(4.4)	(3.0)	1.0	1.0
12 weeks	4.3	5.4	1.6	(3.8)	(4.3)	(1.1)	2.1

Table 8: % increase (decrease) of shoot dry weight compared to controls

After 3 weeks, all treatments except PHP with *R. trifoli* have produced an increase in shoot dry weight but it was not significant (Figure 90; Table 8; Appendix 2, p180). But in subsequent harvests the positive effect has been reduced or eliminated with most plants showing no significant increase or a decrease in weight compared to the control plants. Only plants with PHP alone consistently showed an increase although it was not significant. Neither Bindzil 10 nor hydroxyapatite has produced a positive effect on plant growth with or without bacteria so their inclusion in the polymer was not continued in any further experiments.

The first 4 treatments in this experiment is a repeat of the first experiment with clover. The same effect was observed in both experiments for the PHP plus *R. trifoli* after 3 weeks growth. The bacteria in the polymer reduce the effectiveness of the polymer compared to plants with polymer alone, probably because the bacteria are competing with the plants for the nitrogen available in the polymer initially while they were growing and forming nodules, therefore reducing the amount of available nitrogen to the plants. As time progresses and the bacteria in the nodules begin to fix nitrogen from the air, this will become available to the plants so their growth rate would then increase compared to other treatments. By the 4th harvest, although they were not heavier than other treatments, the plants plus PHP with bacteria were not significantly different from the other treatments although they were significantly lighter at the 1st harvest. The plants were left for an extra harvest compared to most experiments (12 weeks instead of 9), and the optimum conditions in the growth cabinet meant the roots were tightly packed in the pots to the extent that this may have limited further growth of all plants.

Results for all clover experiments were very erratic and no decisive conclusions can be drawn from them due, to a large extent because of high temperatures in the greenhouse. For most, although not all experiments, plants with PHP plus bacteria initially grow slower but during the course of the experiment, they make up the lost ground and end as heavy as other treatments, suggesting that the bacteria compete with the plant for available nitrogen initially, but once established they then start to fix nitrogen from the air which ultimately becomes available to the plant, therefore enhancing growth rate of the plants in the later stages of the experiment.

5.4 The use of free living bacteria and fungi as a biofertiliser

Free living nitrogen fixing bacteria do not form symbiotic relationships with any specific plants and do not form root nodules, so they can potentially benefit any plant and therefore makes their potential as a biofertiliser much greater than bacteria like rhizobium that form root nodules on specific plants.

The potential of *Azospirillum brasilense* and *mycorhiza spp.* to act as a biofertiliser to enhance the growth of grass was investigated.

Two experiments were conducted using *A. Brasilense* and *mycorrhiza spp*:

- Grass + *A. brasilense* & *mycorrhiza spp* in greenhouse

- Grass + *A. brasilense* & *mycorrhiza spp* in growth cabinet

5.4.1 Grass + Azospirillum brasilense & mycorrhiza spp in greenhouse

Azospirillum are Gram negative free living nitrogen fixing bacteria. They can use various C and N metabolism pathways which makes them well adapted to the competitive environment of the rhizosphere. Ammonium, nitrate, nitrite, amino acids and molecular nitrogen can all serve as nitrogen sources. In unfavourable conditions, such as nutrient limitation or desiccation, they produce cysts which develop an outer coat of polysaccharides and accumulate poly-β-hydroxybutyrate granules which act as a carbon and energy source in stress and starvation conditions. They exhibit positive chemotaxis towards root exudates secreted by the plant and can colonise the interior of plant roots (Steenhoudt and Vanderleyden, 2000). Wheat yield has been increased by up to 30% by inoculation with *Azospirillum brasilence* (Hayat *et al*, 2010).

Mycorrhiza spp can enhance plant growth by increasing the surface area of the roots and enhance water and nutrient absorption from the surrounding soil and therefore increase crop yield.

5.4.1.1 Materials & Methods

There were 7 treatments in total: (1) grass; (2) grass + PHP; (3) grass + *A. brasilense*; (4) grass + *Mycorrhiza spp*; (5) grass + PHP with *A. brasilense*; (6) grass + PHP with *Mycorrhiza spp*; (7) grass + PHP with *A. brasilense* & *Mycorrhiza spp.* There were 4 replicates of each treatment.

Preparation of mycorrhiza spp.

The *mycorrhiza spp*. were from the commercially available product 'Rootgrow' from Plantworks Ltd, 1-19 Innovation Buildings Kent Science Park, Sittingbourne, Kent. The product contained both arbuscular mycorrhizal and ectomycorrhizal fungi. Some 'Rootgrow' was added to the centre of a Potato Dextrose Agar (PDA) plate and the fungus allowed to grow out almost to the edge. An agar block was then cut from growing edge on the plate with a sterile scalpel and put on a fresh plate to obtain a pure fungal sample. The fungus grew over the plate after 2 weeks. As nutrients became depleted in the agar, it started to produce spores. After 6 weeks the spores were

harvested from the plate by flooding with sterile PBS. The plate was gently agitated, then the liquid spore suspension was collected and stored in Eppendorf tubes with glycerol at -80^{0}C.

The spores were counted using a haemocytometer. The number of cells in the central 1mm square containing 25 small squares was counted and from the volume contained in this area, the number of cells/ ml can be calculated.

Volume = 1mm x 1mm x 0.1mm (depth) = 0.1mm^3

= 1/10,000 ml

$\therefore$ no. of cells/ml = no. counted x 10,000

0.2ml spore suspension was added to 15ml sterile PD broth, then the solution was added to 1.25g sterile PHP in 6 Petri dishes, followed by incubation at 26^{0}C for 3 weeks. Four were for PHP + *mycorrhiza spp*. and the other 2 were for PHP + both *mycorrhiza spp*. & *A. brasilense.* The spore suspension and PD broth mixture was also added to another 4 Petri dishes containing 20g sterile soil for adding only *mycorrhiza spp*. to the soil. The surplus spore suspension was placed in Eppendorf tubes with 0.85ml/tube plus 0.15ml sterile glycerol and stored at -80^{0}C.

Azospirillum brasilense growth curve

A growth curve was produced as previously (p107) to determine the time taken to reach the end of the exponential phase and therefore obtain the maximum number of bacteria in the shortest time.

Preparation of bacteria

The growth medium was nitrogen free nutrient solution (Appendix 1, p159). Nine 60ml aliquots were sterilised in 250ml flasks. A starter solution was grown in one flask by inoculating with *A. brasilense* from stock stored in 15% glycerol at -80^{0}C. This was grown for 24 hours in an orbital shaker at 26^{0}C and 160rpm, then the other 8 flasks were inoculated with 500µl from the starter solution. Six 1.25g aliquots of PHP were sterilised in universal bottles in an autoclave and added to the growing bacterial broth after 24 hours. Four were for PHP + *A. brasilense* and two for PHP + both *A. brasilence* and *Mycorrhiza spp*. The remaining two flasks were for adding bacterial broth direct to the pots. Flasks were incubated for another 8 hours after the addition of PHP before planting.

Preparation for planting

PHP containing either *A. brasilense* or *mycorrhiza spp*. or both was mixed in the soil then put in 10cm pots. For pots with broth added, this was added on the surface before planting the seeds. 0.5g grass seed was added to each pot, then covered with a light covering of soil mixture. The experiment ran from 8/7/10 – 30/9/10 and the minimum temperature was 10^0C and the maximum was 31^0C. Plants were watered twice weekly and the shoots were harvested at 3 week intervals. The experiment was terminated after 12 weeks at the fourth harvest when then roots were also washed and weighed.

5.4.1.2 Results & Discussion

A growth curve was produced as previously (p107), (Figure 91).

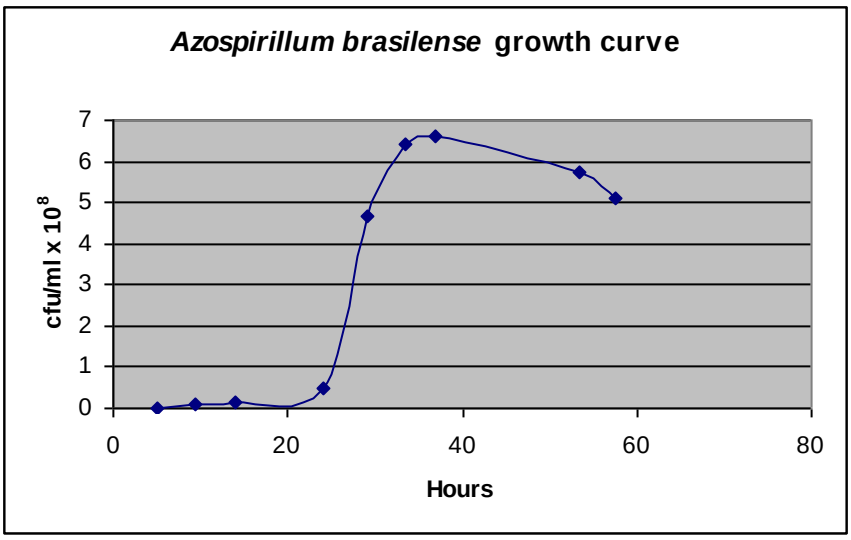


Figure 91: *Azospirillum brasilense* growth curve

A. brasilense is a slow growing bacteria the same as *Rhizobium*. The lag phase lasted approximately 20 hours, followed by a log phase of 12 hours. PHP was added after 24 hours when the cultures were at the beginning of the log phase and they were left for a further 8 hours when they would be approaching the end of the log phase.

Spore count

Average no. of spores in 25 squares on haemocytometer = 25.76

$\therefore$ no. of spores/ml = 257,600/ml

SEM of spores

The fungus has proliferated through the PHP and produced spores inside it (Figure 92).

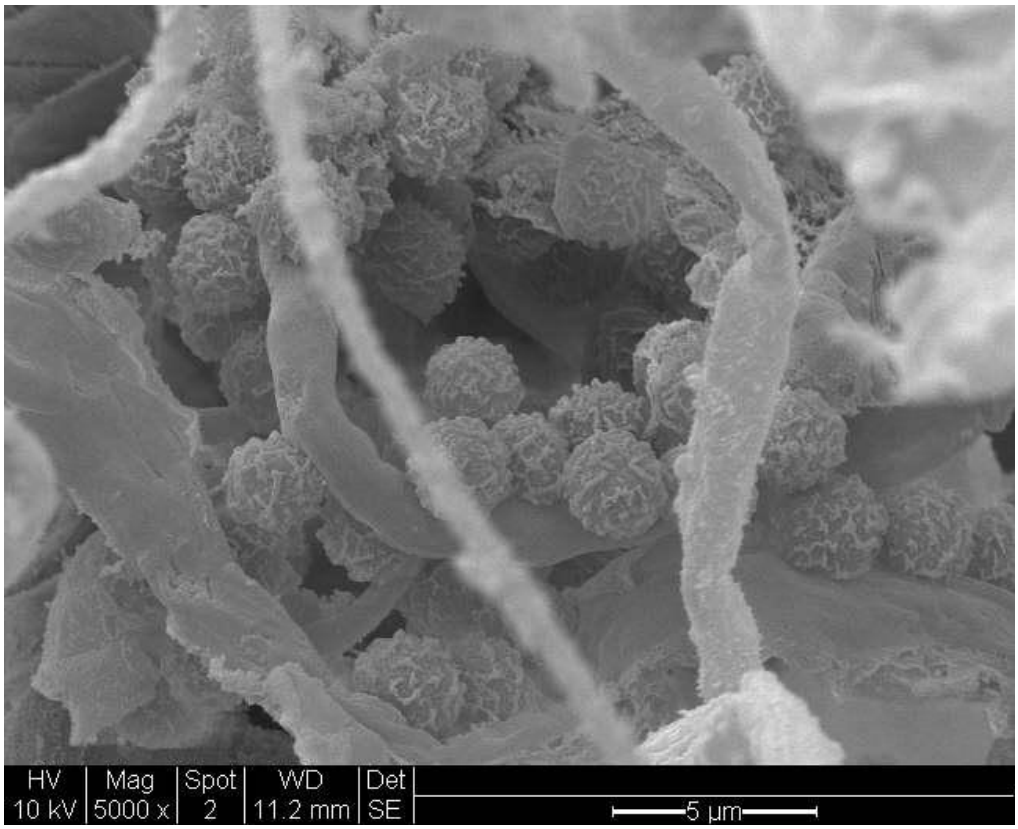


Figure 92: Fungal spores in PHP

The size of the spores was approximately 2 – 3 µm which suggested the fungus that had been isolated was not mycorrhiza, because mycorrhiza spores are mostly much larger (50 – 100+ µm). When the fungus from the commercial product was grown on the PDA plate, there was one obviously dominant species which was assumed to be mycorrhiza, but this was an incorrect assumption. DNA analysis was carried out by Geneius Laboratories Ltd, INEX Business Centre, Newcastle University on the isolated spores to identify the species, which was identified as a zygomycete (*Rhizopus oryzae*). The experiment was however completed using this fungus as the plants were already planted.

A picture was taken of the growing plants every 3 weeks and the shoots were harvested and fresh and dry weights recorded. Dry weights are recorded here. No obvious visible difference could be observed at the first 2 harvests, but by the 3rd harvest, plants with PHP in them were visibly larger than the others. By the 4th harvest, plants with PHP plus *Azospirillum* were obviously the largest, followed by PHP plus both *Azospirillum* and fungus, and PHP alone. All other plants were slightly larger than the control plants (Figure 93).

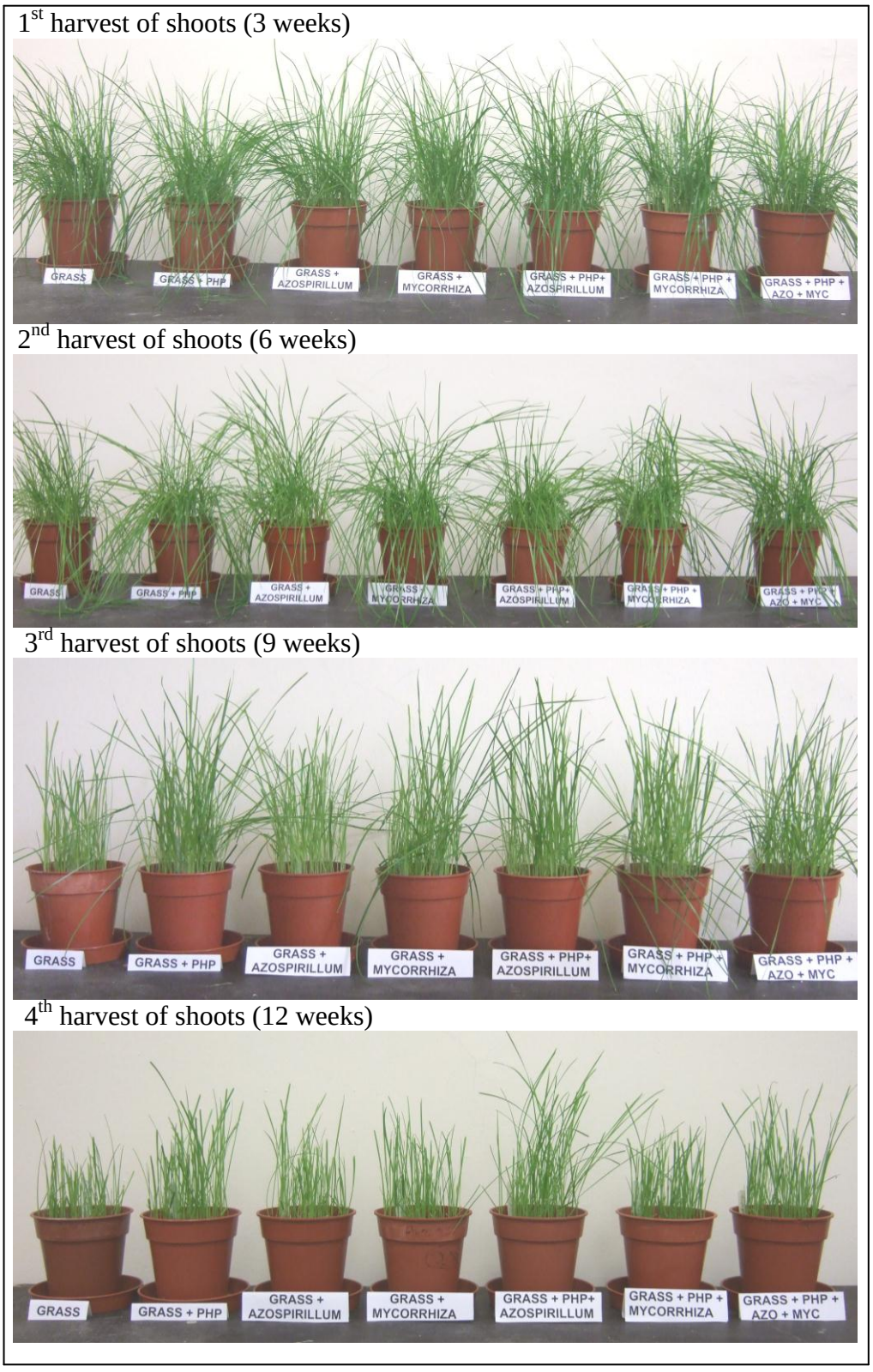


Figure 93: Grass + A*zospirillum brasilense* & fungi in greenhouse

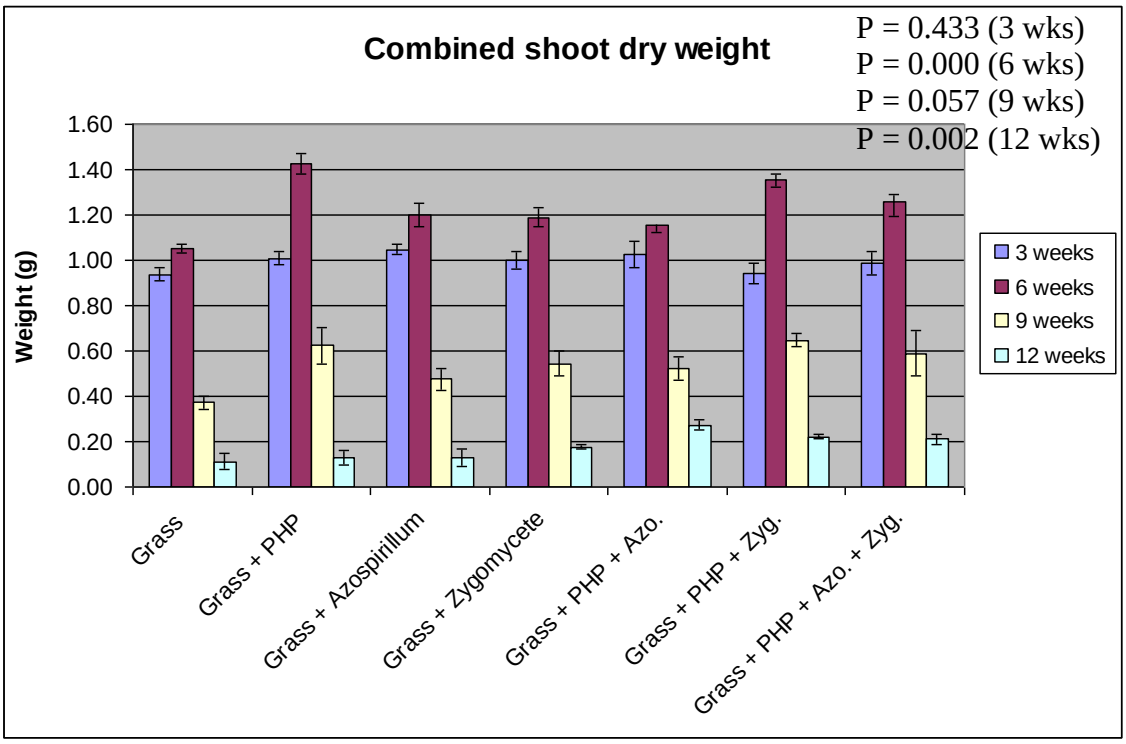


Figure 94: Grass + *Azospirillum brasilense* & fungi in greenhouse - shoot dry weights

Percentage increase of dry weights compared to control plants was calculated.

	Grass + PHP	Grass + azo	Grass + zyg	Grass + PHP + azo	Grass + PHP + zyg	Grass + PHP + azo + zyg
3 wks	7.4	11.7	6.4	9.6	0.0	5.3
6 wks	36.2	14.3	13.3	9.5	28.6	20.0
9 wks	70.3	29.7	45.9	40.5	75.7	59.4
12 wks	18.2	18.2	63.6	145.4	100.0	90.9

Table 9: % increase of shoot dry weight compared to controls

First harvest of shoots (3 weeks)

After 3 weeks growth, all treatments except PHP + fungus have produced a positive increase in dry weight of shoots (Figure 94, Table 9). Plants with *A. brasilense* either as a broth or in the polymer have produced the biggest weight increase at 11.7% and 9.6% respectively. Plants with PHP alone have increased by 7.4%. Plants with fungus alone and plants with PHP containing *A. brasilense* and fungus have not significantly increased the dry weight. Further statistical analysis showed no results were significant (Appendix 2, p182).

Second harvest of shoots (6 weeks)

After 6 weeks, all treatments have produced increased shoot dry weights compared to the 3 week harvest and the variation between different treatments is becoming larger. Plants with PHP alone have produce the greatest weight increase at 36.2%, but all plants with PHP added have produced the most significant increase in dry weight, except for those with PHP containing *A. brasilense* which has increased by the lowest amount at 9.5%.

Third harvest of shoots (9 weeks)

After 9 weeks, the dry weights of the harvested shoots were reduced to less than half those of the 6 week harvest but the differences between different treatments continued to increase. Again, the biggest increases were in the plants containing PHP, except for those with PHP + *A. brasilense* which again did not produce such a large increase in dry weight compared to the control as the other plants containing PHP, but none of the results were statistically significant.

Fourth harvest of shoots (12 weeks)

After 12 weeks, the dry weights obtained have again been reduced compared to the first harvest, with some plants only yielding approximately one tenth of the yield obtained in the first harvest. This could be attributed to the available nutrients in the soil becoming depleted, but a major factor would probably be the lateness of the season when all plant growth is slowing down because of reduced daylight hours and reduced temperature. The final harvest was on 30/9/10. Although the weights were much lower, the comparison between the different treatments has now changed compared to the previous harvests. The plants plus PHP alone have now increased by the lowest percentage, at 18.2% compared to the control plants, the same as plants plus *A. brasilense* broth. Plants plus PHP with *A. brasilense* which were previously one of the lowest weights were now the highest, having increased by 145.4% compared to the control plants ($P = 0.002$, Appendix 2, p182). Plants plus PHP with fungus and with both *A. brasilense* and fungus have both increased by much greater percentage at 100.0% and 90.9% respectively. Plants plus fungus alone have continued to increase and were now 63.6% heavier than control plants. Although the fungus has been shown not to be *mycorrhiza* it has still produced a positive effect compared to the control plants.

Roots were tightly packed in the pots and it was difficult to wash all the soil out without also removing some roots as well, so accurate measurement of root weights was not possible. There was extensive penetration of the polymer by the roots (Figure 95).

Figure 95: Close up of roots + PHP

The results for plants plus PHP only would suggest that the polymer is acting as a slow release fertiliser with the increase in dry weight not being so great after the first harvest but increasing in the second and third harvests. But by the fourth harvest, the effect of the polymer has been very much reduced suggesting that the nitrogen reserves in the polymer were becoming depleted and therefore the plant growth rate has been reduced. Plants plus *A. brasilense* broth had a positive effect for the first three harvests, but by the fourth harvest, the effect was becoming reduced, possibly due the competition from other naturally occurring bacteria in the soil competing for limited nutrients. However, plants with PHP and *A. brasilense* have produced a different effect with only a modest increase compared to the control plants for the first two harvests, increasing to 40.5% by the third harvest and 145.4% by the fourth harvest. When nitrogen is available in the soil or in the polymer, nitrogen fixing bacteria use this rather than the more energy intensive alternative of fixing nitrogen from the air. The bacteria added in the polymer will be competing with the plants for nutrients including nitrogen and therefore initially the plant yield was reduced. As the available nitrogen is used up, the bacteria then start

to fix nitrogen from the air. The life of the bacteria is relatively short – only a few days. So when they die and decompose the nitrogen they assimilate from the air becomes available to the plants and produces increased yields observed in the third and fourth harvests. Time and space constraints have meant that most experiments have been allowed to continue for nine weeks, but by leaving this experiment to continue for a further three weeks, the effect of the bacteria has become much more pronounced. Any available nutrients in the soil will have become depleted so the nitrogen fixing activities of the bacteria then gave a much more positive effect.

5.4.2 Grass + Azospirillum brasilense & mycorrhiza spp in cabinet

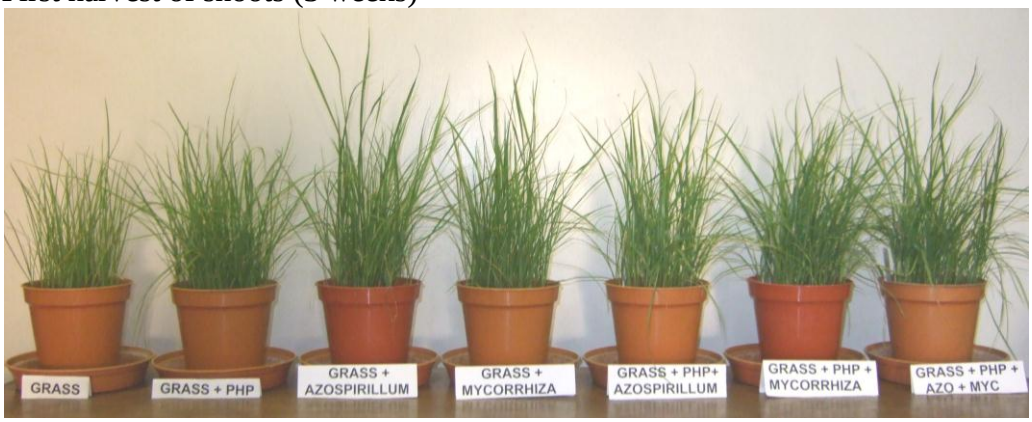
The lateness of the season did not allow the previous experiment to be repeated in the greenhouse but it was repeated in the growth cabinet. Growth conditions were 16 hours light, 1 hour dawn and dusk, 6 hours dark, 20^0C, and 70% relative humidity.

The same procedure was followed as the previous experiment (p134), except that 0.2ml fungal spore suspension was added to 1.25g sterile PHP, then immediately mixed with soil for planting rather allowing it to grow in the polymer for three weeks. The plants were planted before in became known that that the fungus was not mycorrhiza.

5.4.2.1 Results & Discussion

Favourable conditions in the growth cabinet have meant the plants have grown faster than in the greenhouse, but differences between treatments were not as obvious as the greenhouse experiment (Figure 96). After the 4th harvest, plants plus only bacteria or fungus were visibly smaller than the others. All the others including the control plants were not visibly different (Figure 96), although there were differences when they were weighed (Figure 97, Table 10).

First harvest of shoots (3 weeks)



Second harvest of shoots (6 weeks)

Third harvest of shoots (9 weeks)

Fourth harvest of shoots (12 weeks)

Figure 96: Grass + *Azospirillum brasilense* & fungi in cabinet

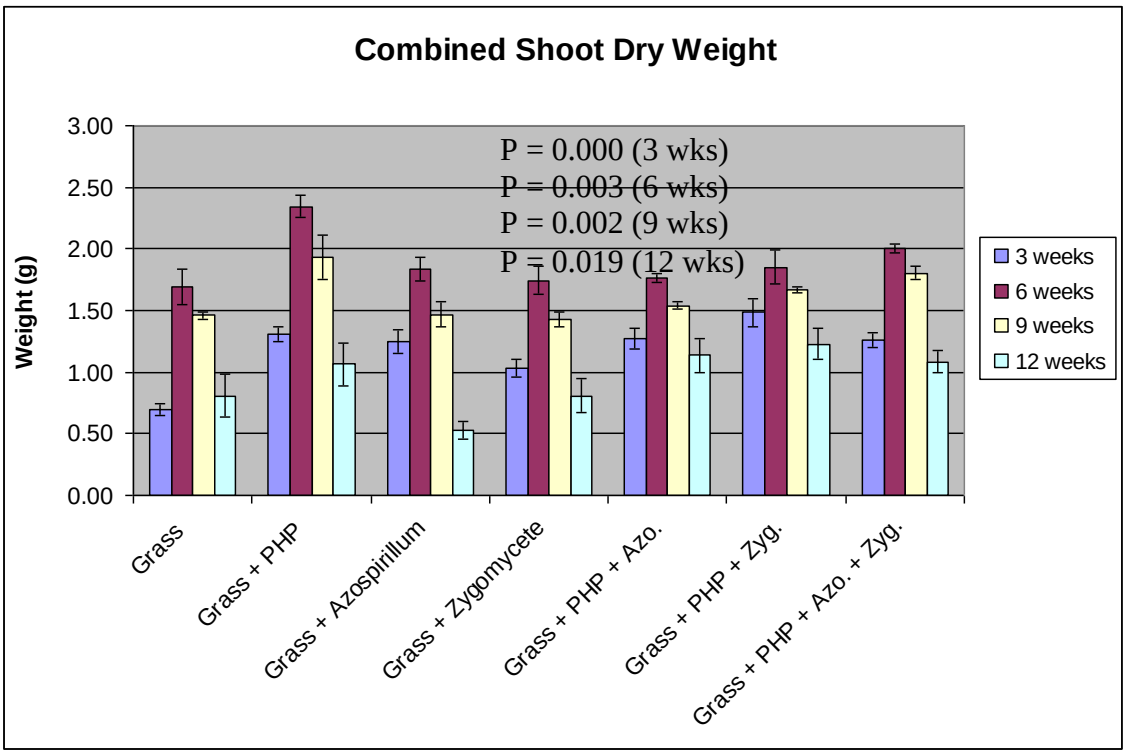


Figure 97: Grass + *Azospirillum brasilense* & fungi in cabinet - shoot dry weights

	Grass + PHP	Grass + azo	Grass + zyg	Grass + PHP + azo	Grass + PHP + zyg	Grass + PHP + azo + zyg
3 wks	87.1	78.6	48.6	81.4	111.4	80.0
6 wks	39.0	8.9	3.6	4.7	9.5	18.9
9 wks	32.9	0.7	(2.0)	5.5	14.4	23.3
12 wks	30.9	(34.6)	0.0	40.7	51.8	34.6

Table 10: % increase (decrease) of shoot dry weight compared to controls

First harvest of shoots (3 weeks)

The biggest increases were observed in all plants with PHP. Plants with PHP plus fungus were up to 111.4% increase in dry weight compared to the control plants. Plants plus *A. brasilense* or fungus with no PHP did not produce such a big increase but they were however still significantly higher than the control plants (P = 0.000, Appendix 2, p184).

Second harvest of shoots (6 weeks)

The dry weight increases observed after 6 weeks were greatly reduced compared to the control plants, but this is partly due to the control plants producing a much higher dry weight at the second harvest. The dry weight of the control plants at the second harvest was 141.4% higher than the first harvest. Plants plus PHP alone have produced the

biggest increase at 39.0% higher than the control plants. Plants plus PHP reached a maximum dry weight of 2.35g compared to 1.43g for the equivalent plants in the greenhouse showing that the ideal conditions in the cabinet can produce a much higher yield compared to the sub optimal conditions in the greenhouse. The biggest reductions were again observed in plants with PHP plus either bacteria or fungus or both. The bacteria or fungi would be competing with the plants for available nutrients so the plant yield would be less. Plants with PHP alone were the only ones significantly different from the others (P = 0.003) (Appendix 2, p184).

Third harvest of shoots (9 weeks)

After 9 weeks, the percentage increase of plants plus PHP was beginning to decline compared to the control plants. Plants with bacterial broth or fungus are not now significantly different from the control plants (Appendix 2, p184). Plants with PHP plus *A. brasilense*, fungus or both were now showing a slight increase in dry weight compared to the control plants. As nutrient availability in the soil declines, the bacteria would be fixing nitrogen from the air which would gradually become available to the plants as the older bacteria die and decompose releasing nutrients which can then be utilised by the plants.

Fourth harvest of shoots (12 weeks)

The percentage increase for plants plus PHP continued to decline and was now not significantly higher than the control plants. Plants plus *A. brasilense* broth appear to have had a detrimental effect with plants now significantly lighter than control plants and fungus alone has not now produced any effect. Plants plus PHP with *A. brasilense*, fungus or both, were now producing a positive effect with all treatments producing a dry weight heavier than the control plants although they were not significantly different from each other.

Roots were very tightly packed in the pots the same as plants grown in the greenhouse and were very difficult to clean without losing some of the root material so accurate weighing of root material was not possible.

The grass has grown faster in the more favourable conditions in the cabinet compared to the greenhouse because of higher temperature and constant light in the cabinet. The seasonal decline in weights observed in the greenhouse in the later stages of the

greenhouse experiment has been removed because of the constant conditions in the cabinet so the dry weights observed are much higher and remain higher than they did in the greenhouse. The decline in weights in the later harvests can now be totally attributable to nutrient availability because the seasonal factor has been removed. Big increases in dry weights of all treatments compared to the controls were observed after 3 weeks growth. The faster growth rate has meant this has happened sooner than the plants in the greenhouse where this was not observed until the second harvest. By the second harvest, plants with only PHP have produced the biggest percentage increase (39.0%) compared to the control. Other weights have decreased compared to the first harvest. The bacteria and fungi would be competing with the plants for available nutrients and therefore reduce the availability of nutrients to the plants. In the third and fourth harvests the dry weights of plants with PHP alone continued to decline as the available nitrogen in the polymer becomes depleted. The opposite was observed for plants with polymer plus bacteria and/or fungi - the dry weights have now started to increase again so that by the fourth harvest they are similar to plants plus PHP alone. As the nitrogen is becoming depleted by this stage, the bacteria would be fixing nitrogen from the air which would subsequently become available to the plants and therefore increase the growth rate. The growth of all plants might be limited by the fourth harvest because all plants had become pot bound – the roots were very tightly packed in the pots to the extent that it was probably limiting growth. The tightly packed roots would not allow this to happen as freely as if the roots had enough space to grow uninhibited. Plants with only fungi and no polymer had no effect on plant growth and plants with only *A. brasilense* had a negative effect – by the fourth harvest their dry weights were less than the control plants. These organisms did not have the protection of the polymer so would have to compete with other organisms in the soil and would therefore not be so prolific and not contribute as much nitrogen that would become available to the plants.

Chapter 6: Conclusions & future work

6.1 Conclusions

PHP has proved to be a versatile material which can be used in a wide variety of applications. It has been shown to have potential to increase plant biomass by several mechanisms, including a slow release fertiliser, water retention, and a reservoir for beneficial bacteria.

Alternative methods of fertiliser production are required because the present method used to make most fertiliser is very energy intensive as well as environmentally damaging so it is not sustainable as a long term production method. Using modified PHP to incorporate a fertiliser component as an integral part of its composition offers a more environmentally friendly product which also brings the fertiliser into close proximity to the plant roots and therefore reduces waste as well as pollution caused by run off and leaching of some of the fertiliser applied using conventional methods. The production method does have the disadvantage that it produces large quantities of concentrated sulphuric acid as a waste product. Perhaps this could be reduced by recycling the acid, finding alternative uses for it, or finding other sulphonating agents that are less toxic.

Increasing areas of semi dry land which are suffering from reduced crop yield because of water shortage may benefit from application of PHP to act as a water reservoir and sustain the plant for longer in dry conditions. Other additives which are water absorbent and store water in a similar manner are available which can store much larger quantities of water than PHP but they are relatively short lived and must be frequently replaced. PHP has the advantage that it is very stable and survives in the soil intact for several years and therefore does not need replacing like other alternatives, therefore making it a more economic alternative.

Perhaps the application with the most potential is the use of PHP as a biofertiliser. If beneficial bacteria can be added in the PHP and they become established in the soil, then they will be self-sustaining and will not need regular replacement unlike applying PHP containing fertiliser which will eventually become depleted and will have to be replaced. Other forms of inoculations of beneficial bacteria must be re-inoculated

regularly because they cannot survive in the soil against all the competition from other soil bacteria, but they will be able to survive much longer in the protected environment of the polymer.

Although the amount of polymer added to a plant pot is small – 1.25g in a 10cm pot, if the amount per hectare is calculated by extrapolation, then the weight and volume required becomes relatively large (2.65tons/hectare, Appendix 3, p185). The use of PHP cannot therefore offer a viable alternative to fertiliser usage in intensive agriculture in developed countries due to difficulties of application on a large scale typical of modern farming and the volume required would make in uneconomic, but it may have applications in developing countries where agriculture is on a much smaller scale and in many places, no alternative source of fertiliser is available, although the cost would probably make it prohibitive for most even if the raw materials are cheaper. It may have applications in more intensive production systems such as horticulture, or recreational areas like golf courses and bowling greens, or in forestry where the polymer would be placed beside each tree seedling rather than spread over the whole area, thereby reducing the volume required.

A major factor in determining if the use of PHP in agriculture, horticulture, amenity or forestry is a viable product is one of economics. Producing polymer in small amounts for lab scale experiments is relatively expensive, but mass production will reduce the costs associated with its manufacture and continued escalation of the price of artificial fertilisers will mean any alternatives, such as PHP will become more attractive.

6.2 Future work

- Is enhanced biomass production eventually converted into increased crop yield? Time and space constraints meant plants could not be grown for long enough to produce a crop, so plants need to be grown for a longer period to see if the effect is still produced over a longer timescale and the increased biomass is converted into bigger crop yield.

- Can positive results observed with the addition of PHP in pot experiments be achieved in field conditions? Pot experiments in the controlled environment of growth cabinet and greenhouse have shown the addition of PHP can have a

positive effect but field experiments will be required to investigate if a positive result can still be achieved in the more varied sub-optimal environment that prevails in field conditions.

- The large amount of polymer required to add in field conditions would make it non-viable both in cost and practicalities of application. Can application of reduced amounts still produce a positive effect? Or can a positive effect be obtained by mixing polymer with another additive, eg biochar?

- Investigation of cyst production by *Azospirillum brasilense*. A major limitation on the commercial production of biofertiliser may be the shelf life of the polymer containing live bacteria. *A. brasilense* produces cysts which can withstand more adverse conditions, so by creating appropriate conditions to stimulate the bacteria to produce cysts, then impregnating the PHP with them, then a product with a longer shelf life could be produced (Lamm & Neyra, 1981; Sadasivan & Neyra, 1985; Sadasivan & Neyra, 1987).

- Can inoculation with bacteria increase the availability of other essential nutrients? Phosphorus is an essential nutrient for plant growth which can be available in the soil in either organic or inorganic forms. Before insoluble phosphates can be utilized by plants they must be converted to soluble forms. Several bacteria, including strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are capable of solubilising insoluble phosphates which then become available for utilization by plants. This is frequently achieved by the production of organic acids, especially gluconic acid. By inoculating the soil with phosphate solublising bacteria, it may be possible to enhance the phosphate availability to plants where soluble phosphate availability in the soil is low (Hayat *et al*, 2010). Or possibly by adding P as part of the polymer – for example hydroxyapatite.

- Can inoculants with mixed bacteria produce a synergistic effect and enhance yields further? Simultaneous inoculation with two different nitrogen fixing bacteria, for example *Azospirillum* and R*hizobium*, can produce an enhanced yield increase compared to the effect each bacteria has separately (Graham & Vance, 2000).

- Spore production of mycorrhizae. Further work needs to be done on isolation of mycorrhiza spores and investigating if it is possible impregnate PHP with them and if the infected polymer will have a positive effect on plant growth.

References

Abraham, J. and V. N. R. Pillai (1996). "Membrane encapsulated controlled-release urea fertilisers based on acrylamide copolymers." Journal of Applied Polymer Science **60**: 2347-2351.

Achten, W. M. J., W. H. Maes, *et al*. (2010). "Jatropha: From global hype to local opportunity." Journal of Arid Environments **74**: 164-165.

Akay, G. (1995). "Flow induced phase inversion in powder structuring by polymers, ." Chapter 20 in Polymer Powder Technology M. Narkis and N. Rozenzweig Editors(Wiley: New York): 542-587.

Akay, G. (1998). "Flow induced phase inversion in the intensive processing of concentrated emulsions." Chemical Engineering Science **53**(2): 203-223.

Akay, G. and D. R. Burke (2010). "Synthetic symbiosis system as soil additives to deliver active ingredients through plant roots for enhanced plant and crop yield." Patent Publicaton Number WO/2010/040996.

Akay, G., M. Dogru, *et al*. (2005[1]). Biomass processing in biofuel applications. Chapter 4 in:biofuels for fuel cells, Editor: A. Moreno, London, IWA publicashing**:** 71-75.

Akay, G. and S. Fleming (2011). Engineered Ecosystem Development for Agro-Process Intensification. Ecosystems and Sustainable Development VIII. Y. Villacampa and C. A. Brebbia, WIT Press, Southampton**:** 485-495.

Akay, G., Z. Z. Noor, *et al*. (2005[2]). "Process intensification in Water-in-Crude Oil Emulsions Separation by Simultaneous Application of Electric Field and Novel Demulsifier Absorbers based on PolyHIPE Polymers." Microreactor Technology and Process Intensification. Y. Yang and J. M. Holladay. Washington DC, American Chemical Society **914**: 378-392.

Akay, G. and R. J. Wakeman (1994). "Mechanisms of permeate flux decay, solute rejection and concentration polarisation in crossflow filtration of a double chain ionic surfactant dispersion
" Journal of Membrane Science **88**(2-3): 177-195.

Aldhous, P. (2008). "Genes for Greens." New Scientist **197**(2637): 28-31.

Andrews, M., S. Hodge, *et al*. (2010). "Positive plant microbial interactions." Annals of Applied Bioogy **157**: 317-320.

Anon (2006). "World planting of biotech crops is increasing." Chemical & Engineering News **84**(4): 29.

Atkinson, C. J., J. D. Fitzgerald, *et al*. (2010). "Potential mechanisms for achieving agricultural benefits from biochar application to temperate soils: a review." Plant & Soil **337**: 1-18.

Bai, W., H. Zhang, *et al*. (2010). "Effects of super-absorbent polymers on the physical and chemical properties of soil following different wetting and drying cycles." Soil Use and Management **26**(3): 253-260.

Barby, D. and Z. Haq (1982). "European Patent No. 0060138."

Bartels, D. and R. Sunkar (2005). "Drought and Salt Tolerance in Plants." Critical Reviews in Plant Science **24**: 23-58.

Bashan, Y. (1998). "Inoculants for plant growth-promoting bacteria for use in agriculture." Biotechnology Advances **16**: 729-770.

Bashan, Y. and H. Levanony (1988). "Adsorption of the rhizosphere bacterium Azospirillum brasilense cd to soil, sand and peat particles." Journal of general microbiology **134**: 1811-1820.

Bhalla, P. L. (2006). "Genetic engineering of wheat - current challenges and opportunnities." Trends in biotechnology **24**(7): 305-311.

Bhattacharjee, R. B., A. Singh, *et al*. (2008). "Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: prospects and challenges." Applied Microbiology and Biotechnology **80**: 199-209.

Bhumgara, Z. G. (1995). "A Study of the Development of PolyHIPE Foam Materials for use in Separation Processes." PhD Thesis, University of Exeter.

Blanke, A., S. Rozelle, *et al*. (2007). "Water saving technology and saving water in China." Agricultural Water Management **87**: 139-150.

Bockman, O. (1997). "Fertilisers & biological N fixation as sources of plant nutrients: Perspectives for future agriculture." Plant & Soil **194**: 11-14.

Bokhari, M. A. (2003). "Bone tissue engineering using novel microcellular polymers." PhD Thesis, Chemical Engineering and Advanced Materials, Newcastle University.

Bolan, N. S. (1991). "A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants." Plant & Soil **134**(2): 189-207.

Burke, D. (2007). "Agroprocess Intensification: Development of Novel Materials for Soil Enhancement to Maximise Crop Yield." PhD Thesis, Chemical Engineering and Advanced Materials, Newcastle University.

Calkan, O. F. (2007). "Intensified, Integrated Gasification System Development." PhD Thesis, Chemical Engineering and Advanced Materials, Newcastle University: pp.158.

Calkan, O. F., M. Dogru, *et al*. (2005). "Nano-porous electrically conductive polymers: fabrication and applications." in 7th world Congress of Chemical Engineering, Glasgow, Scotland.

Cameron, N. R. (2005). "High internal phase emulsion templating as a route to well defined porous polymers." Polymer **46**(5): 1439-1449.

Cerdeira, A. L., D. L. P. Gazziero, *et al*. (2011). "Agricultural Impacts of Glyphosate-Resistant Soybean Cultivation in South America." Journal of Agricultural and Food Chemistry **59**(11): 5799-5807.

Charpentier, M. and G. Oldroyd (2010). "How close are we to nitrogen-fixing cereals?" Current Opinion in Plant Biology **13**: 556-564.

Choudhury, A. T. M. A. and I. R. Kennedy (2004). "Prospects and potentials for system of biological nitrogen fixation in sustainable rice production." Biology and fertility of soils **39**(4): 219-227.

Cominelli, E. and C. Tonelli (2010). "Transgenic crops coping with water scarcity." New Biotechnology **27**(5): 473-477.

Curtis, T. P., W. T. Sloan, *et al*. (2002). "Estimating prokaryotic diversity and its limits." Proceeds of the Natural Academy of Sciences **99**: 10494-10499.

De Fraiture, C., X. Cai, *et al*. (2003). "Addressing the Unanswered Questions in Global Water Policy: A Methodology Framework." Irrigation & Drainage **52**: 21-30.

Deng, X.-P., L. Shan, *et al*. (2005). "Water-saving approaches for improving wheat production." Journal of the Science of Food and Agriculture **85**: 1379-1388.

Divakara, B. N., H. D. Upadhyaya, *et al*. (2010). "Biology and genetic improvement of Jatropha curcas: A review." Applied Energy **87**: 732-742.

Dixon, R. O. D. and C. T. Wheeler (1986). "Nitrogen fixation in plants." Blackie, USA.

Dobbeleare, S., A. Croonenborghs, *et al*. (2001). "Responses of agronomically important crops to inoculation with *Azospirillum*." Australian Journal of Plant Physiology **28**: 871-879.

Donahue, R. L., R. W. Miller, *et al*. (1983). Soils: an introduction to soils and plant growth, Prentice-Hall.

Epstein, P. (2000). "Is global warming harmful to health?" Scientific American **283**(2): 50.

Fairless, D. (2007). "Biofuel: the little shrub that could - maybe." Nature **449**: 652-655.

Fernandes, P. I., T. G. Rohr, *et al*. (2009). "Polymers as carriers of rhizobial inoculant formulations." Pesquisa Agropecuaria Brasileira **44**(9): 1184-1190.

Flowers, T. (1999). "Salinisation and horticultural production." Scientia Horticulturae **78**: 1-4.

Follett, R. F. and J. A. Delgado (2002). "Nitrogen Fate and Transport in Agricultural Systems." Journal of Soil and Water Conservation **57**(6): 402-408.

Fred, E. B., I. L. Baldwin, *et al*. (1932). "Root nodule bacteria and leguminous plants." University of Winconsin.

Fujimaki, H. and N. Kikuchi (2010). "Drought and salinity tolerances of young Jatropha." International Agrophysics **24**: 121-127.

Garg, N. (2007). "Symbiotic nitrogen fixation in legume nodules: process and signalling. A review." Agronomy for Sustainable Development **27**: 59-68.

Gilland, B. (2002). "World population and food supply. Can food production keep pace with population growth in the next half century." Food Policy **27**: 47-63.

Glover, J. D. (2005). "The necessity and possibility of perennial grain production systems." Renewable Agriculture and Food Systems **20**(1): 1-4.

Glover, J. D., C. M. Cox, *et al*. (2007). "Future Farming: A Return to Roots?" Scientific American **297**(2): 82-89.

Glover, J. D., J. P. Reganold, *et al*. (2010). "Increased Food and Ecosystem Security via Perennial Grains." Science **328**: 1638-1639.

Godfray, H. C. J., J. R. Beddington, *et al*. (2010). "Food security: The Challenge of Feeding 9 Million people." Science **327**: 812-818.

Goff, S. and J. Salmeron (2004). "Back to the future of cereals." Scientific American **291**(2): 42-49.

Govindarajan, M., J. Balandreau, *et al*. (2008). "Effects of Inoculation of *Burkeholderia vietnamensis* and Related Endophytic Diazotrophic Bacteria on Grain Yield of Rice." Microbial Ecology **55**(1): 21-37.

Graham, L. E., J. M. Graham, *et al*. (2006). Plant biology (2nd Edition), Pearson Prentice Hall.

Graham, P. H. and C. P. Vance (2000). "Nitrogen fixation in perspective: an overview of research and extension needs." Field Crops Research **65**: 93-106.

Hainey, P., I. M. Huxham, *et al*. (1991). "Synthesis and Ultrastructural Studies of Styrene-Divinylbenzene PolyHIPE Polymers." Macromolecules **24**: 117.

Hayat, R., S. Ali, *et al*. (2010). "Soil beneficial bacteria and their role in plant growth promotion: a review." Annals of Microbiology **60**: 579-598.

Horner-Devine, M. C., M. A. Leibold, *et al*. (2003). "Bacterial diversity patterns along a gradient of primary productivity." Ecology Letters **6**: 613-622.

Huttermann, A., L. J. B. Orikiriza, *et al*. (2009). "Application of Superabsorbant Polymers for Improving the Ecologial Chemistry of Degraded or Polluted Lands." Clean - Soil, Air, Water **37**(7): 517-526.

Jatropha (2010). "Jatropha for biodiesel figures." Retrieved 7/6/2011, from www.reuk.co.uk/Jatropha-for-Biodiesel-Figures.htm.

Jatropha (2011). "The jatropha system." Retrieved 7/6/2011, from www.jatropha.de.

Jeffries, P. (1987). "Use of Mycorrhizae in Agriculture." Critical Reviews in Biotechnology **5**(4): 319-357.

Jhurry, D. (1997). "Agricultural Polymers." AMAS Food & Agriculture Research Council, Mauritius: 109-113.

Kar, G., H. Verma, *et al*. (2006). "Effects of winter crop and supplemental irrigation on crop yield, water use efficiency and profitability in rainfed rice based cropping system of eastern India." Agricultural Water Management **79**: 280-292.

Kaushik, N., K. Kumar, *et al*. (2007). "Potential of Jatropha curcas for biofuels." Journal of Biobased Materials and Bioenergy **1**(3): 301-314.

Krishnan, P. R. and M. Paramathma (2009). "Potentials and Jatrohpa species wealth of India." Current Science **97**(7): 1000-1004.

Ladha, J. K. and P. M. Reddy (2003). "Nitrogen fixation in rice systems: state of knowledge and future prospects." Plant & Soil **252**: 151-167.

Lamm, R. B. and C. A. Neyra (1981). "Characterisation and cyst production of azospirilla isolated from selected grasses growing in New Jersey and New York." Canadian Journal of Microbiology **27**: 1320-1325.

Leake, J. R., D. Johnson, *et al*. (2004). "Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning." Canadian Journal of Botany **82**: 1016-1045.

Lehmann, J., J. P. da Silva Jr, *et al*. (2003). "Nutrient availability and leaching in archeological Anthrosol and a Ferralsol of the Central Amazon basin: fertiliser, manure and charcoal amendments." Plant and Soil **249**: 343-357.

Lehmann, J. and S. Joseph (2009). "Biochar for Environmental Management." Earthscan, London, UK.

Lenton, T. (2006). "Climate change to the end of the millenium." Climate Change **76**(1-2): 7-29.

Levitt, J. (1972). "Responses of plants to environmental stress." Academic press: New York.

Li, Y. (2006). "Water saving irrigation in China." Irrigation & Drainage **55**: 327-336.

Liang, R. and M. Liu (2006). "Prepartion and Properties of a Double-Coated Slow-Release and Water-Retention Urea Fertiliser." Journal of Agricultural Food Chemistry **54**: 1392-1398.

Liu, M., R. Liang, *et al*. (2007). "Preparation of superabsorbent slow release nitrogen fertiliser by inverse suspension polymerisation." Polymer International **56**: 729-737.

Lugtenberg, B. and F. Kamilova (2009). "Plant Growth Promoting Rhizobacteria." Annual Review of Microbiology **63**: 541-556.

Ma, X., J. Geiser-Lee, *et al*. (2010). "Interactions between engineered nanoparticles (ENPs) and plants: Phytotoxicity, uptake and accumulation." Science of the Total Environment **408**: 3053-3061.

Maes, W. H., W. M. J. Achten, *et al*. (2009[1]). "Plant - water relationships and growth strategies of Jatropha curcas L. seedlings under different levels of drought stress." Journal of Arid Environments **73**: 877-884.

Maes, W. H., A. Trabucco, *et al*. (2009[2]). "Climatic growing conditions Of Jatropha curcas L." Biomass and Bioenergy **33**: 1481-1485.

Mahajan, S. and N. Tuteja (2005). "Cold, Salinity and Drought stresses: an overview." Archives of Biochemistry and Biophysics **444**(2): 139-158.

Makkar, H. P. S. and K. Becker (2009). "Jatropha curcas, a promising crop for the generation of biodiesel and value-added coproducts." European Journal of Lipid Science and Technology **111**: 773-787.

Makoi, J. H. J. R. and P. A. Ndakidemi (2009). "The agronomic potential of vesicular-arbuscular mycorrhiza (VAM) in cereals-legume mixtures in Africa." African Journal of Microbiology Research **3**(11): 664-675.

McMichael, A. J., J. W. Powles, *et al*. (2007). "Food, livestock production, energy, climate change and health." The Lancet **370**(9594): 1253-1263.

Mia, M. A. B. and Z. H. Shamsuddin (2010). "Rhizobium as a crop enhancer and biofertiliser for increased cereal production." African Journal of Biotechnology **9**(37): 6001-6009.

Miransari, M. (2011). "Arbuscular mycorrhizal fungi and nitrogen uptake." Archives of Microbiology **193**(2): 77-81.

Mittelman, G., O. Mouchtar, *et al*. (2007). "Large-Scale Solar Thermal Desalination Plants: A review." Heat Transfer Engineering **28**(11): 924-930.

Mooney, B. P. (2009). "The second green revolution? Production of plant-based biodegradable plastics." Biochemical Journal **418**: 219-232.

Morse, S., R. M. Bennett, *et al*. (2007). "Inequality and GM crops: a case study of Bt cotton in India." AgBioforum **10**(1): 44-50.

Ndlovu, T. M. (2009). "Bioprocess Intensification of Antibiotic Production using Functionalised PolyHIPE Polymers." PhD Thesis, Chemical Engineering and Advanced Materials, Newcastle University.

Nel, A., T. Xia, *et al*. (2006). "Toxic potential of materials at the nanolevel." Science **311**: 622-627.

Normatov, J. and M. S. Silverstein (2008). "Highly porous elastomer-silsesquioxane nanocomposites synthesised within high internal phase emulsions." Journal of polymer science part a - polymer chemistry **46**(7): 2357-2366.

Openshaw, K. (2000). "A review of Jatropha curcas: an oil plant of unfullfilled promise." Biomass and Bioenergy **19**: 1-15.

Orts, W. J., R. E. Sojka, *et al*. (1999). "Preventing Soil Erosion with Polymer Additives." Polymer News **24**: 406-413.

Paranychianakis, N. and K. Chartzoulakis (2005). "Irrigation of Mediterranean crops with saline water: from physiology to management practices." Agriculture, Ecosystems & Environment **106**: 171-187.

Parawira, W. (2010). "Biodiesel production from Jatropha curcas: A review." Scientific Research and Essays **5**(14): 1796-1808.

Parry, M. A. J. and M. J. Hawkesford (2010). "Food security: increasing yield and improving resource use efficiency." Proceedings of the Nutrition Society **69**: 592-600.

Paton, C. (2010). "Sahara forest project." Retrieved 7/6/2011, from www.saharaforestproject.com.

Peterson, R. L., H. B. Massicotte, *et al*. (2004). "Mycorrhizas: Anatomy and Cell Biology." CABI Publishing.

Pimentel, D. and M. Pimentel (2006). "Global environmental resources versus world population growth." Ecological Economics **59**: 195-198.

Postgate, J. (1998). "Nitrogen Fixation." Cambridge University Press.

Rai, P. (2007). "Wastewater management through biomass of Azzola pinnata: An ecosustainable approach." AMBIO **36**(5): 426-428.

Raun, W. R. and G. V. Johnson (1999). "Improving nitrogen use efficiency for cereal production." Agronomy Journal **91**(3): 357-363.

Renner, R. (2007). "Rethinking Biochar." Environmental Science & Technology **41**(17): 5932-5933.

Rondon, M. A., J. Lehmann, *et al*. (2007). "Biological nitrogen fixation by common beans (*Phaseolus vulgaris L.*) increases with bio-char additions." Biology and fertility of soils **43**: 699-708.

Rosling, A. (2009). "Fungi provide nutrition for trees." Sustainability Journal Formas **July 2009**(2).

Sadasivan, L. and C. A. Neyra (1985). "Flocculation in Azospirillum brasilense and Azospirillum lipoferum: exopolysaccharides and cyst formation." Journal of Bacteriology **163**: 716-723.

Sadasivan, L. and C. A. Neyra (1987). "Cyst production and brown pigment formation in aging cultures of Azospirillum brasilense." Journal of Bacteriology **169**: 1670-1677.

Saikia, S. P. and V. Jain (2007). "Biological nitrogen fixation with non-legumes: An achievable target or a dogma?" Current Science **92**(3): 317-322.

Saito, M. and T. Marumoto (2002). "Inoculation with arbuscular mycorrhizal fungi: the status quo in Japan and the future propects." Plant & Soil **244**: 273-279.

Seawatergreenhouse (2010). "A new approach - restorative agriculture." Retrieved 7/6/2011, from www.seawatergreenhouse.com.

Sepaskhah, A. R. and Z. Mahdi-Hossainabadi (2008). "Effect of polyacrylamide on the erodibility factor of a loam soil." Biosystems Engineering **99**(4): 598-603.

Shantharam, S. and A. K. Mattoo (1997). "Enhancing biological N fixation: An appraisal of current & alternative technologies for N input into plants." Plant & Soil **194**: 205-216.

Sharma, M. and B. Sood (2011). "A banana or a syringe: journey to edible vaccines." World Journal of Microbiology & Biotechnology **27**(3): 471-477.

Smith, R. S. (1992). "Legume inoculant formulation and application." Canadian Journal of Microbiology **38**(6): 485-492.

Smith, S. E. and D. J. Read (1997). "Mycorrhizal symbiosis." 2nd ed Academic Press, London, UK.

Soffe, R. J., Ed. (2003). The Agricultural Notebook (20th edition), Blackwell.

Sojka, R. E., D. L. Bjorneberg, *et al*. (2007). "Polyacrylamide in Agriculture and Environmental Land Management." Advances in Agronomy **92**: 75-162.

Steenhoudt, O. and J. Vanderleyden (2000). "Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects." FEMS Microbiology Reviews **24**: 487-506.

Stern, N. (2007). "The Economics of Climate Change: The Stern Review." Cambridge University Press: 1-24.

Swaminathan, M. S. (2007). "Can science and technology feed the world in 2025." Field Crops Research **104**: 3-9.

Tang, G., J. Qin, *et al*. (2009). "Golden Rice is an effective source of vitamin A." American Journal of Clinical Nutrition **89**(6): 1776-1783.

Thomas, D. and N. Middleton (1993). "Salinisation: new perspective on a major desertification issue." Journal of Arid Environments **24**: 95-105.

Thomas, R., N. K. Sah, *et al*. (2008). "Therapeutic biology of Jatropha curcas: a mini review." Current Pharmaceutical Biotechnology **9**(4): 315-324.

Thorburn, P. J., F. J. Cook, *et al*. (2008). "Improving trickle irrigation: Better matching trickle systems design to soils." Acta Horticulturae **792**: 669-677.

Van der Heijden, M. G. A., R. D. Bardgett, *et al*. (2008). "The unseen majority: microbes as drivers of plant diversity and productivity in terrestrial ecosystems." Ecology Letters **11**: 296-310.

Verma, S. C., J. K. Ladha, *et al*. (2001). "Evaluation of plant growth promoting and colonisation ability of endophytic diazotrophs from deep water rice." Journal of Biotechnology **91**: 127-141.

Vessey, J. K. (2003). "Plant growth promoting rhizobacteria as biofertilisers." Plant & Soil **255**: 571-586.

Wakeman, R. J., Z. G. Bhumgara, *et al*. (1998). "Ion exchange modules formed from polyhipe foam precursors." Chemical Engineering Journal **70**(2): 133-141.

Wang, H., C. Liu, *et al*. (2002). "Water-saving agriculture in China: An overview." Advances in Agronomy **75**: 135-171.

Wang, W., B. Vinocur, *et al*. (2003). "Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance." Planta **218**: 1-14.

Warnock, D. D., J. Lehmann, *et al*. (2007). "Mycorrhizal responses to biochar in soil - concepts and mechanisms." Plant & Soil **300**: 9-20.

Welch, R. M. and R. D. Graham (1999). "A new paradigm for world agriculture: meeting human needs - Productive, sustainable, nutritious." Field Crops Research **60**(1-2): 1-10.

Williams, J. M., A. J. Gray, *et al*. (1990). "Emulsion stability and rigid foams from styrene or divinylbenzene water-in-oil emulsions." Langmuir **6**(2): 437-444.

Yanni, Y. G., R. Y. Rizk, *et al*. (1997). "Natural endophytic association between Rhizobium leguminosarum bv. trifolii in rice roots and assessment of its potential to promote rice growth." Plant and Soil **194**: 99-114.

Yuksel, I. (2008). "Global warming and renewable energy sources for sustainable development in Turkey." Renewable energy **33**: 802-812.

Zhu, H., J. Han, *et al*. (2008). "Uptake, translocation and accumulation of manufactured iron oxide nanoparticles by pumpkin plants." Journal of Environmental Monitoring **10**: 713-717.

Zwingmann, N., B. Singh, *et al*. (2009). "Zeolite from alkali modified kaolin increases NH_4^+ retention by sandy soil: Column experiments." Applied Clay Science **46**: 7-12.

Appendix 1: Materials & Methods protocols

Hoaglands solution (nitrogen free nutrient solution for watering plants)

The solution is used as a liquid fertiliser added to water for watering plants where no nitrogen is required in the fertiliser.

Hoaglands solution is made from 6 stock solutions:

Macro ingredients in 500ml deionised water:

(1) 1M $MgSO_4.7H_20$ – 123.24g/500ml

(2) 1M KH_2PO_4 - 68.045g/500ml

(3) 0.1M FeDTA – 16.384g/500ml

(4) 1M $CaCl_2.2H_20$ – 73.51g/500ml

(5) 50mM KCl – 1.85g/500ml

Micro ingredients in 500ml deionised water (6):

0.715g H_2BO_3 (boric acid)

0.223g $MnSO_4.4H_20$

0.288g $ZnSO_4$

0.062g $CuSO_4.2H_2O$

0.0063g $Na_2MoO_4.2H_2O$ (sodium molybdate)

!0 litres of full strength nitrogen free solution are then prepared from the stock solutions using deionised water and the following volumes:

(1) 10ml 1M $MgSO_4$

(2) 30ml 1M KH_2PO_4

(3) 5ml 0.1M FeDTA

(4) 10ml 1M $CaCl_2$

(5) 10ml 50mM KCl

(6) 20ml micro nutrients

Adjust pH to 6.8 with 1M NaOH if necessary

Nitrogen free nutrient solution for growing bacteria

The nutrient solution contains all the essential nutrients for bacterial growth except nitrogen.

To prepare 1 litre: 200ml Hoaglands solution
0.2g sodium carbonate
800ml deionised water
10g mannitol
1g yeast
Autoclaved before use

To prepare nitrogen free agar plates
250ml (enough for 10 plates):
50ml Hoaglands solution
0.05ml sodium carbonate
200ml deionised water
2.5g mannitol
0.25g yeast
3.75g agar
Autoclave and pour plates

Potato Dextrose Agar (growth medium for isolating fungi)

200g diced potato boiled slowly in 800ml deionised water.
Filter using a vacuum filter
Add 10g dextrose
Adjust pH to 6 if necessary
Make up to 1 litre with deionised water
Add 15g agar
Sterilise in autoclave at 121^0C for 20 minutes

Phosphorus analysis

Preparation of 0.5M sodium hydrogen carbonate solution, pH 8.5.

Weigh 210g $\pm$ 0.5g sodium hydrogen carbonate into a 2 litre beaker

Add 1200ml deionised water and stir to dissolve

Transfer to a 5 litre volumetric flask and make up to 4 litres deionised water

Adjust the pH to 8.5 by adding 5M sodium hydroxide dropwise while stirring

Make final volume up to 5 litres and store in 5 litre bottles

Check the pH of the reagent on the day of use

Preparation of sulphomolybdic solution

Add 800ml deionised water to a 2 litre beaker

Carefully add 560ml 98% sulphuric acid in small aliquots while stirring.

Allow to cool

Add 98.16g $\pm$ 0.01g ammonium molybdate to the sulphuric acid solution and stir to dissolve.

Make up to 2 litres in a volumetric flask

Store in an amber glass bottle

Preparation of colour reagent

Add 720ml deionised water to a 2 litre beaker

Add 1g ascorbic acid and stir to dissolve

Add 0.05g sodium thiosulphate and stir to dissolve

Add 15ml sulphomolybdic acid

Add 65ml 5M sulphuric acid

Store in a 1 litre amber glass bottle

Sorensens Phoshate Buffer (pH 7.3) 0.1M (for washing samples before fixing for SEM)

Solution A: potassium di-hydrogen orthophosphate (mw136.09)

1.36g KH_2PO_4 in 100ml distilled water

Solution B: di-sodium hydrogen orthophosphate (mw 177.99)

8.895g $Na_2HPO_4.2H_2O$ in 500ml distilled water

To make up, mix together: 28.5ml solution A with 71.5ml solution B (store at 4^0C)

Glutaraldehyde (for fixing samples for SEM)

2ml 25%Glutaraldehyde (EM grade) plus 23ml phosphate buffer

(Store frozen in labelled plastic tubes)

Appendix 2: Results data

	Soybean plants in vermiculite harvested after 6 weeks growth				
12/10/2007	Shoot length (cm)	Shoot wt (g)	Shoot dry wt (g)	Root wt (g)	Root dry wt (g)
Soybean 1	54	8.4	1.1	3.3	0.2
Soybean 2	72	14.4	2.1	5.7	0.3
Soybean 3	78	12.9	1.8	3.9	0.3
mean	68.00	11.90	1.67	4.30	0.27
Soybean + PHP 1	109	21.1	3.4	10.8	0.7
Soybean + PHP 2	93	13.1	2.2	10.1	0.6
Soybean + PHP 3	82	17.9	2.7	9.2	0.6
mean	94.67	17.37	2.77	10.03	0.63

Analysis by one way ANOVA

Treatment versus height P = 0.067

```
                          Individual 95% CIs For Mean Based on Pooled StDev
           N   Mean  StDev  ------+---------+---------+---------+---
Soybean    3  68.00  12.49  (---------*---------)
Soy + PHP  3  94.67  13.58               (---------*---------)
                           ------+---------+---------+---------+---
                                60        80       100       120
```

Treatment versus shoot dry weight P = 0.074

```
                            Individual 95% CIs For Mean Based on Pooled StDev
           N    Mean   StDev    +---------+---------+---------+---------
Soybean    3  1.6667  0.5132    (----------*----------)
Soy + PHP  3  2.7667  0.6028                (----------*----------)
                              +---------+---------+---------+---------
                            0.80      1.60      2.40      3.20
```

Treatment versus root dry weight P = 0.001

```
                            Individual 95% CIs For Mean Based on Pooled StDev
           N     Mean    StDev  --------+---------+---------+---------+-
Soybean    3  0.26667  0.05774  (-----*-----)
Soy + PHP  3  0.63333  0.05774                          (-----*-----)
                              --------+---------+---------+---------+-
                                    0.30      0.45      0.60      0.75
```

Peas grown in vermiculite with & without the addition of polyHIPE					
07/05/2008	Pea plants harvested after 6 weeks			Variety Phoenix	
	Shoot length (cm)	Shoot wt (g)	Shoot dry wt (g)	Root wt (g)	Root dry wt (g)
Phoenix 1	33	5.12	0.68	3.06	0.19
Phoenix 2	21	3.53	0.45	2.57	0.16
Phoenix 3	26	3.00	0.44	1.83	0.11
Mean	26.67	3.88	0.52	2.49	0.15
Phoenix + PHP 1	39	4.90	0.71	2.72	0.16
Phoenix + PHP 2	37	6.14	0.75	2.68	0.19
Phoenix + PHP 3	43	7.32	0.85	3.55	0.22
Mean	39.67	6.12	0.77	2.98	0.19

Analysis by one way ANOVA

Treatment versus shoot length P = 0.029

```
                          Individual 95% CIs For Mean Based on Pooled StDev
               N    Mean  StDev  ------+---------+---------+---------+---
Phoenix        3  26.667  6.028  (--------*---------)
Phoenix + PHP  3  39.667  3.055                   (---------*--------)
                                 ------+---------+---------+---------+---
                                    24.0      32.0      40.0      48.0
```

Treatment versus shoot dry weight P = 0.05

```
                            Individual 95% CIs For Mean Based on Pooled StDev
               N    Mean   StDev  -------+---------+---------+---------+--
Phoenix        3  0.5233  0.1358  (-----------*-----------)
Phoenix + PHP  3  0.7700  0.0721                   (----------*-----------)
                                  -------+---------+---------+---------+--
                                       0.45      0.60      0.75      0.90
```

Treatment versus root dry weight P = 0.276

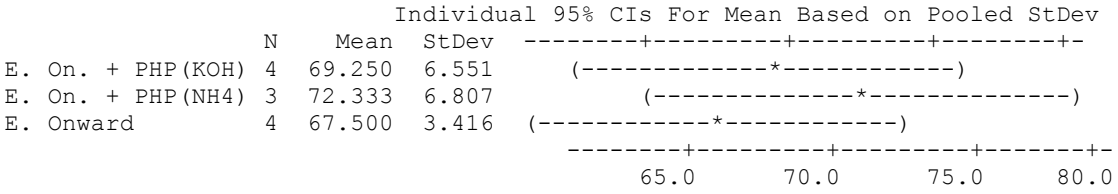
```
                            Individual 95% CIs For Mean Based on Pooled StDev
               N     Mean    StDev  ------+---------+---------+---------+--
Phoenix        3  0.15333  0.04041  (-------------*--------------)
Phoenix + PHP  3  0.19000  0.03000           (--------------*-------------)
                                  ------+---------+---------+---------+---
                                      0.120     0.160     0.200     0.240
```

28/08/2008	Peas (Early Onward) grown in vermiculite + PHP neutralised with NH_4OH & KOH				
	Shoot length (cm)	Shoot weight (g)	Shoot dry wt (g)	Root weight (g)	Root dry wt (g)
E. Onward 1	69	21.19	4.14	8.36	0.81
E. Onward 2	67	24.49	4.36	8.98	0.84
E. Onward 3	63	23.62	4.25	8.16	0.80
E. Onward 4	71	27.08	4.58	10.64	0.95
Mean	67.50	24.10	4.33	9.04	0.85
E. On. + PHP (NH_4) 1	67	31.40	6.33	15.69	1.37
E. On. + PHP (NH_4) 2	70	33.04	6.57	16.55	2.30
E. On. + PHP (NH_4) 3	80	33.77	7.03	22.90	3.01
Mean	72.33	32.74	6.64	18.38	2.23
E. On. + PHP (KOH) 1	77	23.40	4.35	12.33	1.01
E. On. + PHP (KOH) 2	70	17.58	3.11	11.54	1.02
E. On. + PHP (KOH) 3	69	24.23	4.24	10.65	1.01
E. On. + PHP (KOH) 4	61	17.17	2.99	11.40	0.98
Mean	69.25	20.60	3.67	11.48	1.01

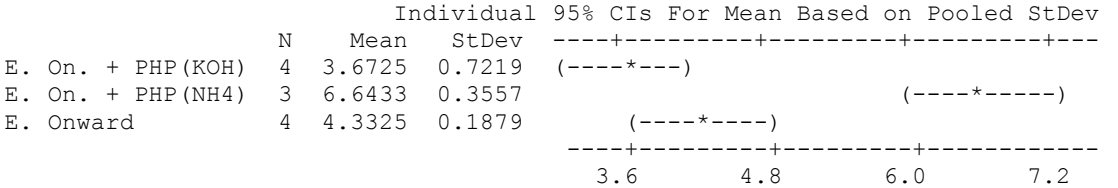
(E. On. = Early Onward-pea variety)

Analysis by one way ANOVA

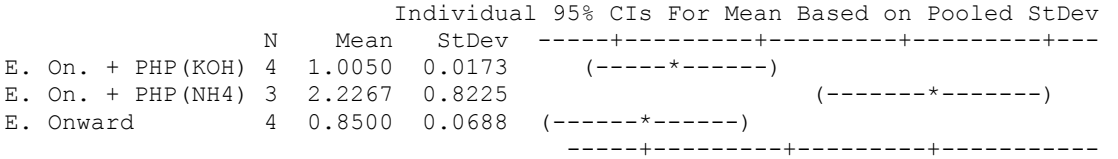
Treatment versus height P = 0.558



Treatment versus shoot dry weight P = 0.000



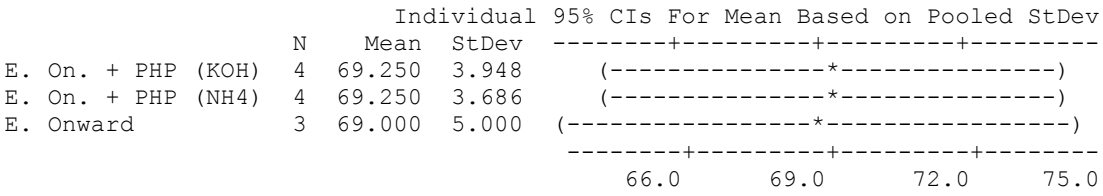
Treatment versus root dry weight P = 0.005



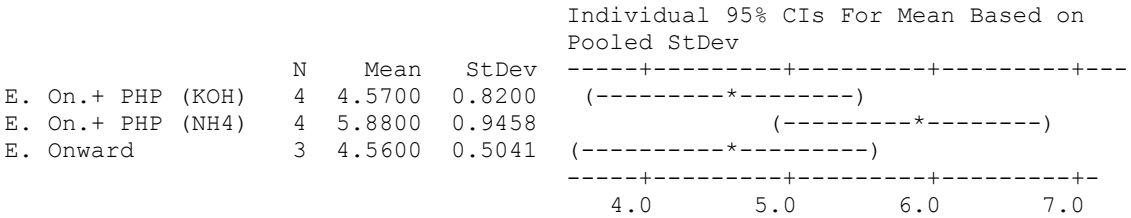
3/9/2008	Peas (Early Onward) grown in soil + PHP neutralised with NH_4OH & KOH				
	Shoot length (cm)	Shoot weight (g)	Shoot dry wt (g)	Root weight (g)	Root dry wt (g)
E. Onward 1	69	21.81	5.00	9.97	1.24
E. Onward 2	64	15.92	4.01	4.54	0.46
E. Onward 3 *	49	11.91	2.68	8.89	0.75
E. Onward 4	74	18.34	4.67	6.29	0.65
Mean	64	17.00	4.09	7.42	0.78
E. On. + PHP (NH_4) 1	69	26.09	6.31	14.30	1.68
E. On. + PHP (NH_4) 2	74	26.48	6.65	9.89	0.79
E. On. + PHP (NH_4) 3	69	24.65	6.05	7.55	0.52
E. On. + PHP (NH_4) 4	65	21.27	4.51	11.01	1.10
Mean	69.25	24.62	5.88	10.69	1.02
E. On. + PHP (KOH) 1	75	20.59	5.01	9.68	1.00
E. On. + PHP (KOH) 2	68	15.01	3.69	5.16	0.54
E. On. + PHP (KOH) 3	68	23.77	5.48	10.98	0.74
E. On. + PHP (KOH) 4	66	18.50	4.10	11.25	0.92
Mean	69.25	19.47	4.57	9.27	0.80

* Discarded – very poor growth of shoot. Analysis by one way ANOVA

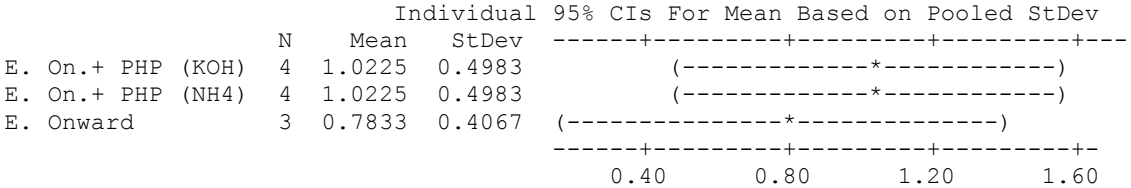
Treatment versus shoot length P= 0.996



Treatment versus shoot dry weight P = 0.086



Treatment versus root dry weight P = 0.767



	Grass + 2 PHP discs with pipettes			
	Shoot wt (g)	Shoot dry wt (g)	Root wt (g)	Root dry wt (g)
Grass	0.73	0.12	1.97	0.72
Grass	0.74	0.09	1.55	0.51
Grass	0.75	0.13	2.56	0.75
Grass	1.08	0.18	2.66	0.77
Mean	0.83	0.13	2.19	0.69
Grass + PHP + pipettes 1	0.56	0.12	0.92	0.48
Grass + PHP + pipettes 2	0.91	0.17	2.18	1.16
Grass + PHP + pipettes 3	0.91	0.18	1.61	0.74
Grass + PHP + pipettes 4	1.15	0.21	1.67	0.67
Mean	0.88	0.17	1.60	0.76

Analysis by one way ANOVA

Treatment versus shoot dry weight P = 0.181

```
                          Individual 95% CIs For Mean Based on Pooled StDev
               N     Mean    StDev  ------+---------+---------+---------+---
Grass          4  0.13000  0.03742  (------------*------------)
Grass+PHP+pip  4  0.17000  0.03742             (------------*------------)
                                    ------+---------+---------+---------+---
```

Treatment versus root dry weight P = 0.647

```
                          Individual 95% CIs For Mean Based on Pooled StDev
               N    Mean   StDev  ----+---------+---------+---------+-----
Grass          4  0.6875  0.1201  (----------------*----------------)
Grass+PHP+pip  4  0.7625  0.2869       (----------------*---------------)
                                   ----+---------+---------+---------+-----
                                     0.48      0.64      0.80      0.96
```

	Grass + PHP & PU in growth room								
	3 weeks		6 weeks		9 weeks				
	Shoot wt (g)	Shoot Dry wt (g)	Shoot wt (g)	Shoot Dry wt (g)	Shoot wt (g)	Shoot Dry wt (g)	Root wt (g)	Root dry wt (g)
Grass 1	3.23	0.34	6.06	0.46	2.56	0.33	10.29	2.29
Grass 2	4.18	0.41	6.70	0.51	3.48	0.46	8.20	1.27
Grass 3	4.07	0.40	6.18	0.50	2.59	0.31	9.39	1.21
Grass 4	4.87	0.45	5.69	0.46	2.03	0.26	5.82	0.54
Mean	4.09	0.40	6.16	0.48	2.67	0.34	8.43	1.33
Grass + PHP 1	3.56	0.38	6.68	0.53	6.23	0.70	10.43	1.19
Grass + PHP 2	4.73	0.48	8.01	0.57	5.63	0.64	14.84	2.17
Grass + PHP 3	5.41	0.51	8.07	0.69	5.80	0.66	14.74	2.03
Grass + PHP 4	4.61	0.45	7.28	0.62	5.80	0.61	13.39	2.53
Mean	4.58	0.46	7.51	0.60	5.87	0.65	13.35	1.98
Grass + PU 1	3.38	0.35	6.58	0.54	2.28	0.21	12.20	1.82
Grass + PU 2	3.49	0.38	5.89	0.48	2.70	0.24	9.45	1.61
Grass + PU 3	4.22	0.46	5.95	0.47	2.24	0.26	11.19	1.72
Grass + PU 4	3.93	0.41	6.49	0.52	2.54	0.28	11.78	2.10
Mean	3.76	0.40	6.23	0.50	2.44	0.25	11.16	1.81

Grass + PHP & PU in growth room

Analysis by one way ANOVA

Treatment versus shoot dry weight (3 weeks) P = 0.247

```
                                Individual 95% CIs For Mean Based on Pooled StDev
             N     Mean    StDev   -+---------+---------+---------+--------
Grass        4  0.40000  0.04546   (----------*----------)
Grass + PHP  4  0.45500  0.05568              (----------*----------)
Grass + PU   4  0.40000  0.04690   (----------*----------)
                                    -+---------+---------+---------+--------
                                   0.350     0.400     0.450     0.500
```

Treatment versus shoot dry weight (6 weeks) P = 0.012

```
                                Individual 95% CIs For Mean Based on Pooled StDev
             N     Mean    StDev  --------+---------+---------+---------+-
Grass        4  0.48250  0.02630  (-------*--------)
Grass + PHP  4  0.60250  0.06898                    (-------*--------)
Grass + PU   4  0.50250  0.03304     (--------*--------)
                                   --------+---------+---------+---------+-
                                         0.480     0.540     0.600     0.660
```

Treatment versus shoot dry weight (9 weeks) P = 0.000

```
                                Individual 95% CIs For Mean Based on Pooled StDev
             N     Mean    StDev  --------+---------+---------+---------+-
Grass        4  0.34000  0.08524        (----*---)
Grass + PHP  4  0.65250  0.03775                             (----*---)
Grass + PU   4  0.24750  0.02986  (----*---)
                                   --------+---------+---------+---------+-
                                         0.30      0.45      0.60      0.75
```

Treatment versus root dry weight P = 0.263

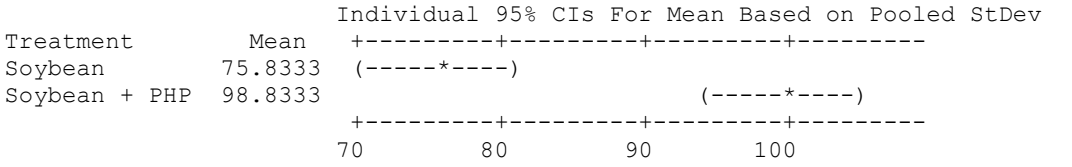
```
                               Individual 95% CIs For Mean Based on Pooled StDev
             N    Mean   StDev  ------+---------+---------+---------+---
Grass        4  1.3275  0.7220  (------------*-----------)
Grass + PHP  4  1.9800  0.5672              (------------*-----------)
Grass + PU   4  1.8125  0.2100           (-----------*------------)
                                  ------+---------+---------+---------+---
                                     1.00      1.50      2.00      2.50
```

Soybeans in greenhouse + PHP, watered & droughted					
09/10/2009					
	Height (cm)	Shoot wt	Root wt	Shoot dry wt	Root dry wt
Soybean 1	82	7.45	18.66	1.72	2.67
Soybean 2	81	7.76	14.90	1.86	1.30
Soybean 3	83	8.69	23.99	2.06	2.36
Soybean 4	91	9.18	26.77	2.09	3.33
Soybean 5	94	6.93	13.84	1.67	1.14
Soybean 6	75	7.01	14.06	1.61	0.91
mean	84.33	7.84	18.70	1.84	1.95
Soybean + PHP 1	106	12.97	30.99	3.46	2.53
Soybean + PHP 2	102	14.10	31.54	3.70	3.36
Soybean + PHP 3	99	14.23	43.65	3.93	3.91
Soybean + PHP 4	112	16.80	27.84	3.93	2.31
Soybean + PHP 5	130	13.21	41.90	4.65	3.22
Soybean + PHP 6	113	15.32	34.46	3.75	2.28
mean	110.33	14.44	35.06	3.90	2.94
Droughted (50ml)					
Soybean 1	71	7.72	29.82	2.00	3.66
Soybean 2	60	6.99	29.89	1.77	2.67
Soybean 3	67	4.81	13.48	1.14	1.11
Soybean 4	62	5.10	17.61	1.34	1.51
Soybean 5	61	5.01	13.16	1.14	1.23
Soybean 6	83	7.88	27.13	1.94	2.03
mean	67.33	6.25	21.85	1.56	2.04
Soybean + PHP 1	79	11.18	25.53	3.81	2.19
Soybean + PHP 2	81	8.14	21.18	2.17	2.15
Soybean + PHP 3	90	11.39	29.17	3.30	2.29
Soybean + PHP 4	100	12.04	27.26	3.65	2.39
Soybean + PHP 5	93	10.56	32.25	3.27	1.73
Soybean + PHP 6	81	11.10	30.99	3.11	2.01
mean	87.33	10.74	27.73	3.22	2.13

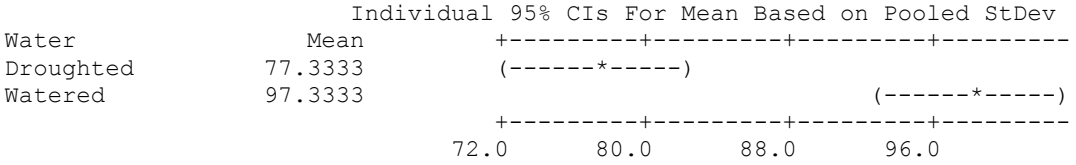
Soybeans in greenhouse + PHP, watered & droughted (cont.)

Analysis by two way ANOVA

Height versus treatment, water



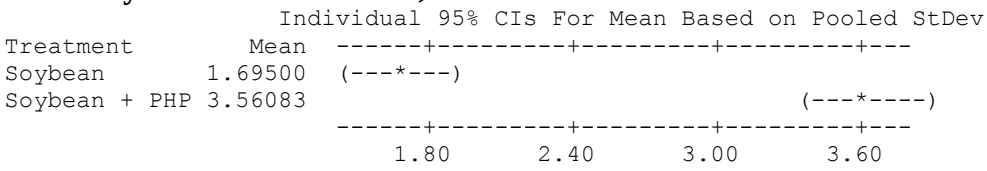
P = 0.000



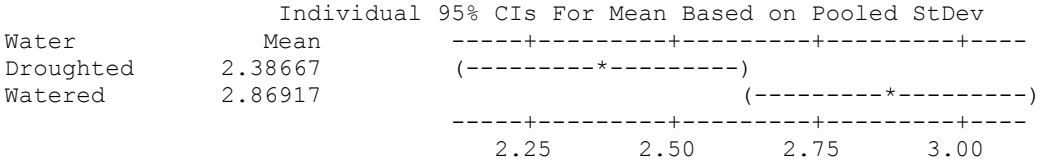
P = 0.000

Interaction P = 0.419

Shoot dry wt versus treatment, water



P = 0.000



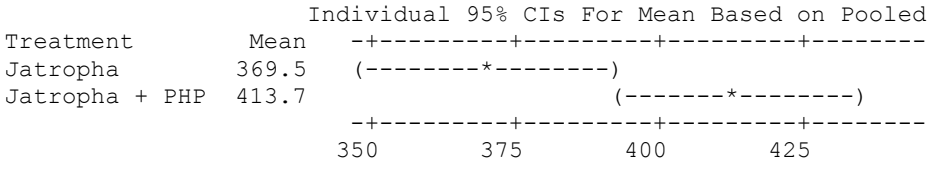
P = 0.01

Interaction P = 0.247

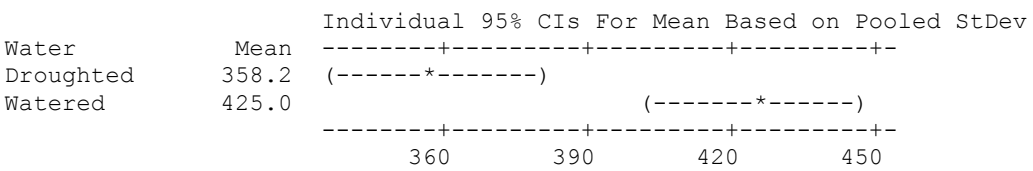
Jatropha			
8 months	Height (mm)	Shoots fresh wt	Shoot dry wt
Jatropha100a	329.00	87.73	14.53
Jatropha100b	395.00	104.39	16.10
Jatropha100c	340.00	89.07	16.58
Jatropha100d	320.00	77.78	12.93
Jatropha100e	320.00	77.63	11.75
Mean	340.80	87.32	14.38
Jatropha + PHP100a	368.00	99.28	18.24
Jatropha + PHP100b	431.00	116.64	16.35
Jatropha + PHP100c	409.00	116.14	17.67
Jatropha + PHP100d	350.00	94.26	14.25
Jatropha + PHP100e	320.00	77.31	11.72
Mean	375.60	100.73	15.65
Jatropha200a	409.00	112.18	27.61
Jatropha200b	420.00	171.17	33.26
Jatropha200c	432.00	149.57	33.43
Jatropha200d	360.00	159.13	30.09
Jatropha200e	370.00	164.76	30.15
Mean	398.20	151.36	30.91
Jatropha + PHP200a	431.00	163.98	32.48
Jatropha + PHP200b	484.00	192.12	38.90
Jatropha + PHP200c	454.00	177.38	41.09
Jatropha + PHP200d	440.00	173.75	32.30
Jatropha + PHP200e	450.00	178.72	33.40
Mean	451.80	177.19	35.63

Analysis by 2 way ANOVA

Height versus treatment, water



P =0.009

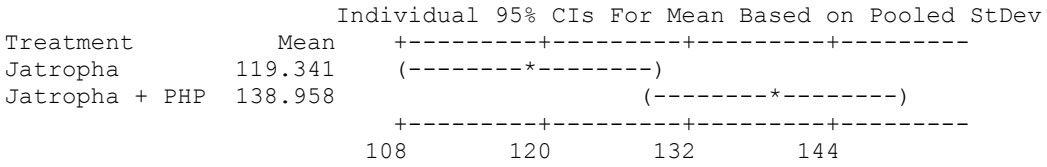


P = 0.000

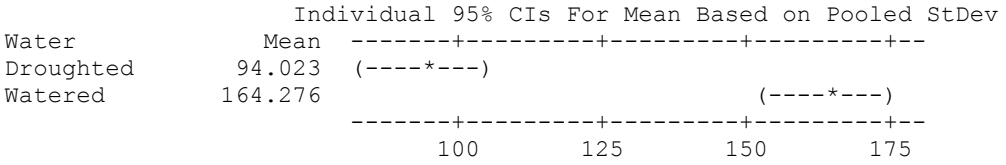
Interaction P = 0.534

Jatropha (cont.)

Shoot fresh weight versus treatment, water



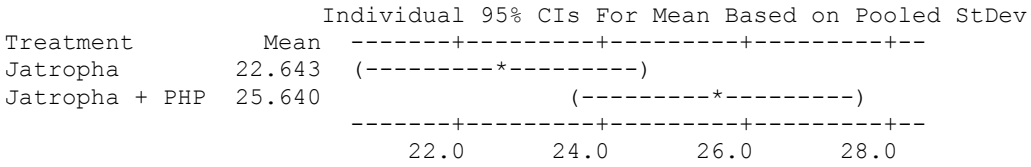
P = 0.015



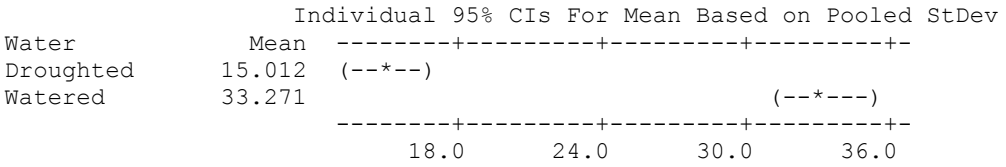
P = 0.000

Interaction P = 0.401

Shoot dry weight versus treatment, water



P = 0.035



P = 0.000

Interaction P = 0.203

18/07/2008		**Peas + PHP soaked with rhizobium**			8 weeks
	Shoot length (cm)	Shoot weight (g)	Shoot dry wt (g)	Root weight (g)	Root dry wt (g)
All plants nearly dead due to high temperatures					
Phoenix 1	42	3.97	1.12	2.96	0.23
Phoenix 2	40	2.42	0.64	3.15	0.22
Phoenix 3	33	4.23	1.16	3.41	0.22
Phoenix 4	38	2.15	0.46	1.84	0.14
Phoenix 5	26	0.88	0.32	2.68	0.18
Mean	35.80	2.73	0.74	2.81	0.20
Ph + PHP 1	45	5.04	1.59	4.47	0.32
Ph + PHP 2	48	4.58	1.33	3.77	0.32
Ph + PHP 3	69	12.93	3.91	8.02	0.66
Ph + PHP 4	57	5.35	1.68	3.69	0.39
Ph + PHP 5	54	6.18	1.86	4.57	0.38
Ph + PHP 6	49	5.88	1.16	3.78	0.34
Mean	53.67	6.66	1.92	4.72	0.40
Ph + PHP + Rh. 1	42	4.09	2.02	2.65	0.25
Ph + PHP + Rh. 2	56	6.37	1.94	6.22	0.55
Ph + PHP + Rh. 3	55	7.98	2.26	5.59	0.48
Ph + PHP + Rh. 4	55	6.19	1.91	3.33	0.39
Ph + PHP + Rh. 5	57	7.64	2.32	5.83	0.52
Ph + PHP + Rh. 6	50	5.77	1.88	6.64	0.66
Mean	52.50	6.34	2.06	5.04	0.48

Analysis by one way ANOVA

Treatment versus height P = 0.002

```
                                Individual 95% CIs For Mean Based on Pooled StDev
                N    Mean  StDev  ----+---------+---------+---------+-----
Ph + PHP        6  53.667  8.664                          (-------*-------)
Ph + PHP + Rh.  6  52.500  5.683                         (-------*------)
Phoenix         5  35.800  6.419  (--------*-------)
                               ----+---------+---------+---------+-----
                                32.0      40.0      48.0      56.0
```

Treatment versus shoot dry weight P = 0.009

```
                                Individual 95% CIs For Mean Based on Pooled StDev
                N    Mean   StDev  --------+---------+---------+---------+-
Ph + PHP        6  1.9217  1.0056                   (-------*--------)
Ph + PHP + Rh.  6  2.0550  0.1889                     (-------*-------)
Phoenix         5  0.7400  0.3826  (--------*-------)
                               --------+---------+---------+---------+-
                                     0.70      1.40      2.10      2.80
```

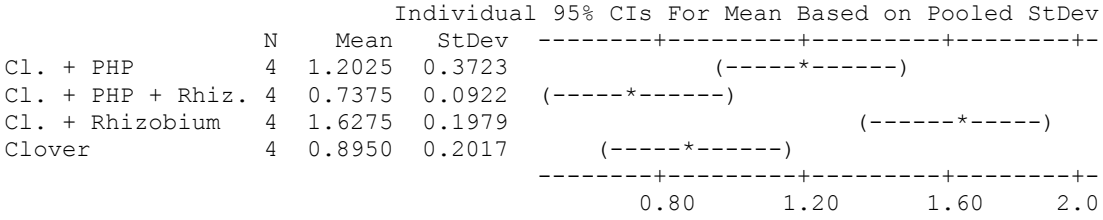
Treatment versus root dry weight P = 0.005

```
                                Individual 95% CIs For Mean Based on Pooled StDev
                N    Mean   StDev  ----+---------+---------+---------+-----
Ph + PHP        6  0.4017  0.1300                (------*------)
Ph + PHP + Rh.  6  0.4750  0.1412                     (------*-----)
Phoenix         5  0.1980  0.0377  (------*-------)
                               ----+---------+---------+---------+-----
                                  0.15      0.30      0.45      0.60
```

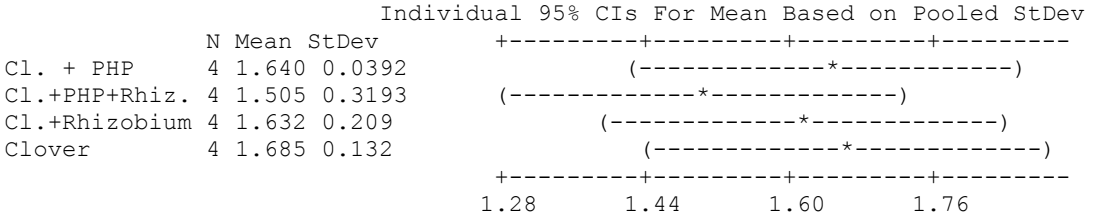
	Clover + rhizobium in growth cabinet (harvest of shoots)		
	3 weeks	6 weeks	9 weeks
	Shoot dry wt (g)	Shoot dry wt (g)	Shoot dry wt (g)
Clover 1	0.74	1.79	3.04
Clover 2	0.80	1.77	3.47
Clover 3	1.19	1.50	3.49
Clover 4	0.85	1.68	2.77
Mean	0.90	1.69	3.19
Cl. + PHP 1	1.26	1.63	3.74
Cl. + PHP 2	1.53	1.68	3.42
Cl. + PHP 3	1.35	1.66	4.09
Cl. + PHP 4	0.67	1.59	4.62
Mean	1.20	1.64	3.97
Cl. + Rhizobium 1	1.40	1.36	3.32
Cl. + Rhizobium 2	1.54	1.66	3.06
Cl. + Rhizobium 3	1.85	1.87	3.88
Cl. + Rhizobium 4	1.72	1.64	3.95
Mean	1.63	1.63	3.55
Cl. + PHP + Rhiz. 1	0.69	1.47	3.96
Cl. + PHP + Rhiz. 2	0.77	1.13	4.45
Cl. + PHP + Rhiz. 3	0.85	1.91	3.84
Cl. + PHP + Rhix. 4	0.64	1.51	2.75
Mean	0.74	1.51	3.75

Analysis by one way ANOVA

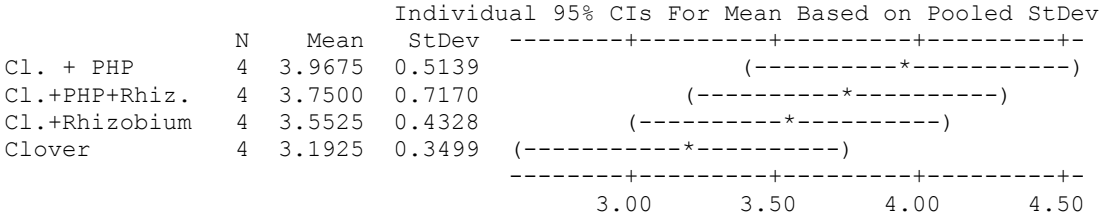
Treatment versus dry weight (3 weeks) P = 0.001



Treatment versus dry weight (6 weeks) P = 0.639



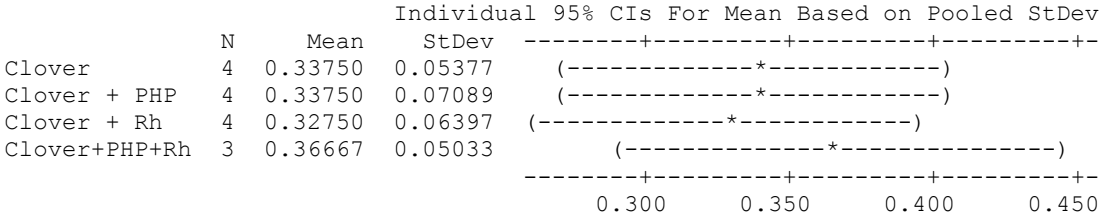
Treatment versus dry weight (9 weeks) P = 0.243



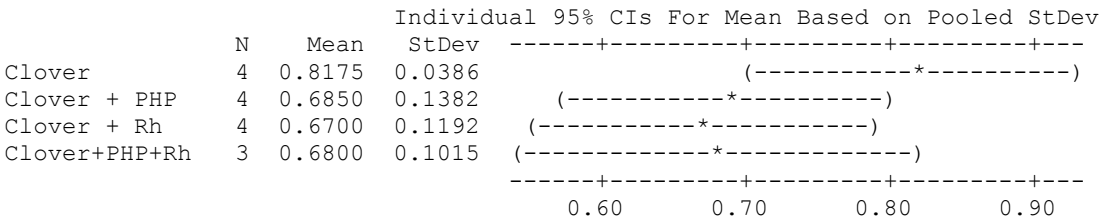
	Clover + PHP & rhizobium in greenhouse			
	3 weeks	6 weeks	9 weeks	9 weeks
	Shoot dry wt (g)	Shoot dry wt (g)	Shoot dry wt (g)	Root dry wt (g)
Clover 1	0.37	0.86	1.12	1.39
Clover 2	0.32	0.79	0.80	1.05
Clover 3	0.27	0.84	0.90	1.08
Clover 4	0.39	0.78	1.21	0.95
Mean	0.34	0.82	1.01	1.12
Clover + PHP 1	0.39	0.86	0.96	2.17
Clover + PHP 2	0.33	0.56	0.98	1.74
Clover + PHP 3	0.24	0.59	1.07	1.46
Clover + PHP 4	0.39	0.73	1.04	1.41
Mean	0.34	0.69	1.01	1.70
Clover + Rhizobium 1	0.25	0.55	0.95	1.35
Clover + Rhizobium 2	0.38	0.68	0.91	1.97
Clover + Rhizobium 3	0.38	0.83	1.08	1.65
Clover + Rhizobium 4	0.30	0.62	0.91	2.02
Mean	0.33	0.67	0.96	1.75
Clover + PHP + Rh 1*	0.26	0.19	0.73	0.97
Clover + PHP + Rh 2	0.42	0.70	0.88	2.45
Clover + PHP + Rh 3	0.36	0.57	0.91	1.41
Clover + PHP + Rh 4	0.32	0.77	1.06	2.62
Mean	0.34	0.68	0.90	1.86

* Discarded because very poor growth. Analysis by one way ANOVA

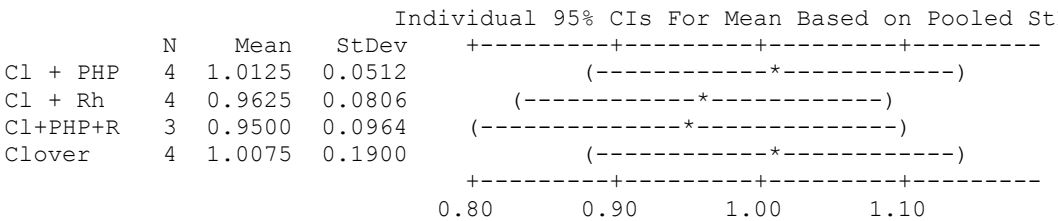
Treatment versus dry weight (3 weeks) P = 0.86



Treatment versus dry weight (6 weeks) P = 0.228

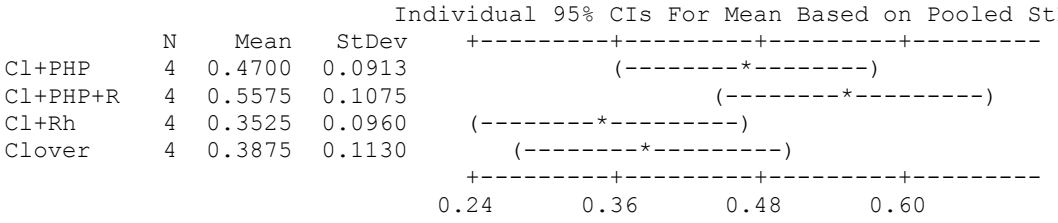


Treatment versus dry weight (9 weeks) P = 0.856

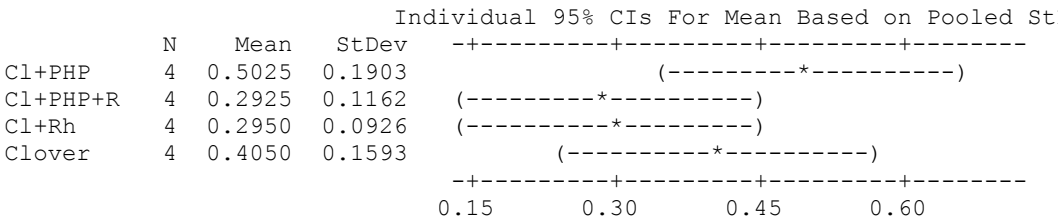


	Clover in greenhouse (autoclaved soil)			
	3 weeks	6 weeks	9 weeks	
	Shoot dry wt (g)	Shoot dry wt (g)	Shoot dry wt (g)	Root dry wt (g)
Clover 1	0.24	0.18	0.25	0.58
Clover 2	0.36	0.49	0.36	0.36
Clover 3	0.46	0.41	0.32	0.53
Clover 4	0.49	0.54	0.35	0.59
Mean	0.39	0.41	0.32	0.52
Clover + PHP 1	0.42	0.31	0.42	0.44
Clover + PHP 2	0.57	0.69	0.53	1.27
Clover + PHP 3	0.37	0.37	0.49	1.12
Clover + PHP 4	0.52	0.64	0.48	0.77
Mean	0.47	0.50	0.48	0.90
Clover + Rhizobium 1	0.26	0.24	0.34	0.96
Clover + Rhizobium 2	0.47	0.43	0.59	1.04
Clover + Rhizobium 3	0.39	0.28	0.35	0.96
Clover + Rhizobium 4	0.29	0.23	0.32	0.56
Mean	0.35	0.30	0.40	0.88
Clover + PHP + Rh 1	0.59	0.45	0.58	0.96
Clover + PHP + Rh 2	0.69	0.20	0.44	0.75
Clover + PHP + Rh 3	0.44	0.21	0.37	0.95
Clover + PHP + Rh 4	0.51	0.31	0.39	0.54
Mean	0.56	0.29	0.45	0.80

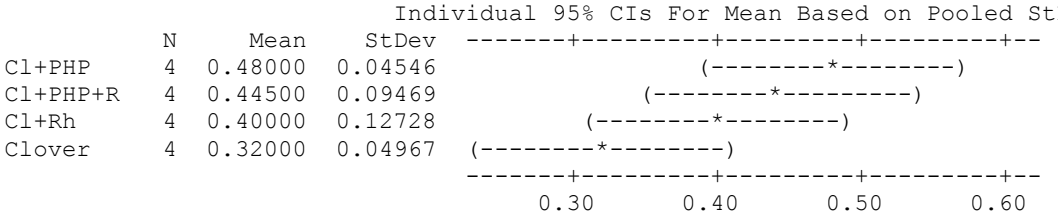
Treatment versus dry weight (3 weeks) P = 0.062



Treatment versus dry weight (6 weeks) P = 0.178

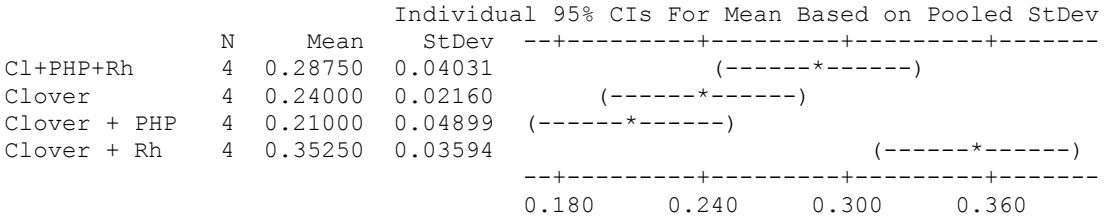


Treatment versus dry weight (9 weeks) P = 0.103

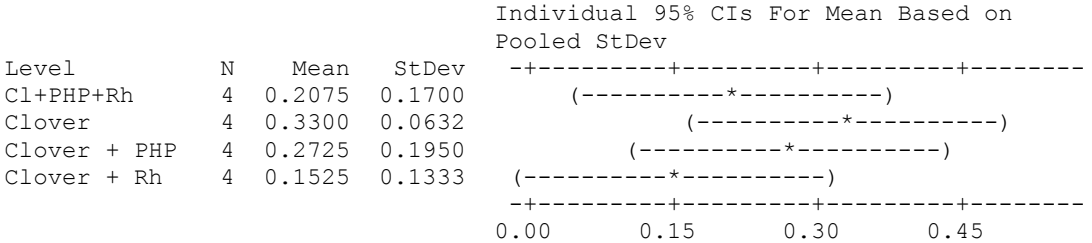


	Clover in greenhouse (autoclaved, washed soil)			
	3 weeks	6 weeks	9 weeks	9 weeks
	Shoot dry wt (g)	Shoot dry wt (g)	Shoot dry wt (g)	Root dry wt (g)
Clover 1	0.27	0.25	0.26	0.91
Clover 2	0.22	0.39	0.32	0.95
Clover 3	0.23	0.31	0.11	0.88
Clover 4	0.24	0.37	0.09	0.66
Mean	0.24	0.33	0.20	0.85
Clover + PHP 1	0.19	0.26	0.44	0.91
Clover + PHP 2	0.15	0.11	0.16	0.37
Clover + PHP 3	0.25	0.17	0.21	0.95
Clover + PHP 4	0.25	0.55	0.37	1.16
Mean	0.21	0.27	0.30	0.85
Clover + Rhizobium 1	0.30	0.35	0.22	0.86
Clover + Rhizobium 2	0.36	0.09	0.10	0.54
Clover + Rhizobium 3	0.38	0.11	0.15	1.03
Clover + Rhizobium 4	0.37	0.06	0.15	0.46
Mean	0.35	0.15	0.16	0.72
Clover + PHP + Rh 1	0.29	0.45	0.39	0.95
Clover + PHP + Rh 2	0.23	0.20	0.33	0.61
Clover + PHP + Rh 3	0.32	0.10	0.27	0.74
Clover + PHP + Rh 4	0.31	0.08	0.24	0.61
Mean	0.29	0.21	0.31	0.73

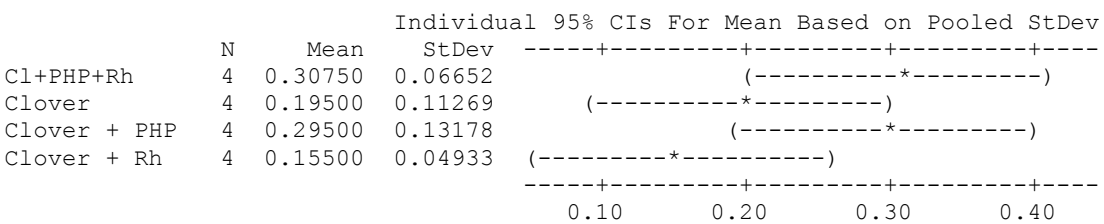
Treatment versus dry weight (3 weeks) P = 0.001



Treatment versus dry weight (6 weeks) P = 0.397



Treatment versus dry weight (9 weeks) P = 0.116

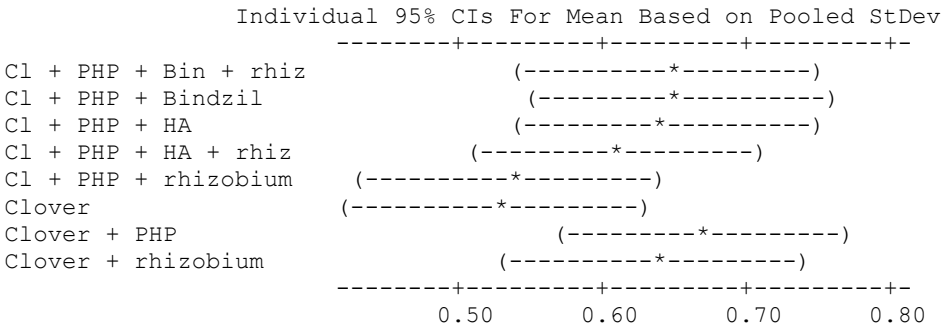


	Clover + Rhiz. with Hydroxy Apatite & Bindzil in cabinet				
	3 weeks	6 weeks	9 weeks	12 weeks	12 weeks
	Shoot dry wt (g)	Shoot dry wt (g)	Shoot dry wt (g)	Shoot dry wt (g)	Root dry wt (g)
Clover 1	0.39	1.82	2.10	1.74	8.11
Clover 2	0.52	1.69	2.08	1.84	6.92
Clover 3	0.62	1.62	1.98	1.98	12.88
Clover 4	0.58	1.71	1.94	1.90	6.71
Mean	0.53	1.71	2.03	1.87	8.66
Clover + PHP 1	0.58	1.63	2.14	1.74	8.41
Clover + PHP 2	0.59	2.00	1.97	1.92	11.32
Clover + PHP 3	0.82	1.79	2.07	2.12	6.79
Clover + PHP 4	0.69	1.75	2.02	1.98	6.77
Mean	0.67	1.79	2.05	1.94	8.32
Clover + rhizobium 1	0.49	1.70	1.78	2.19	12.40
Clover + rhizobium 2	0.57	1.73	2.00	1.88	15.51
Clover + rhizobium 3	0.79	1.67	1.85	1.68	11.10
Clover + rhizobium 4	0.70	1.68	1.90	2.10	13.78
Mean	0.64	1.70	1.88	1.96	13.20
Cl + PHP + rhizobium 1	0.60	1.77	2.17	1.64	7.50
Cl + PHP + rhizobium 2	0.50	1.76	1.88	2.13	13.17
Cl + PHP + rhizobium 3	0.70	1.86	1.86	1.90	13.27
Cl + PHP + rhizobium 4	0.34	1.21	1.63	1.91	7.01
Mean	0.54	1.65	1.89	1.90	10.24
Cl + PHP + Bindzil 1	0.68	1.73	2.11	1.82	9.52
Cl + PHP + Bindzil 2	0.74	1.79	1.95	2.01	11.12
Cl + PHP + Bindzil 3	0.51	1.94	1.75	1.66	8.84
Cl + PHP + Bindzil 4	0.68	1.46	1.91	1.66	13.64
Mean	0.65	1.73	1.93	1.79	10.78
Cl + PHP + Bin + rhiz1	0.64	1.79	2.00	1.82	16.04
Cl + PHP + Bin + rhiz2	0.70	1.64	1.75	1.90	10.44
Cl + PHP + Bin + rhiz3	0.61	1.82	2.19	1.79	10.69
Cl + PHP + Bin + rhiz4	0.64	1.89	1.89	1.63	5.25
Mean	0.65	1.79	1.96	1.79	10.61
Cl + PHP + HA1	0.56	1.75	1.81	2.04	16.50
Cl + PHP + HA2	0.63	1.85	1.95	1.76	4.87
Cl + PHP + HA3	0.74	1.75	2.14	1.77	11.18
Cl + PHP + HA4	0.64	1.64	2.27	1.80	11.14
Mean	0.64	1.75	2.04	1.84	10.92
Cl + PHP + HA + rhiz1	0.57	1.64	2.11	1.97	9.44
Cl + PHP + HA + rhiz2	0.60	1.65	1.91	2.07	11.37
Cl + PHP + HA + rhiz3	0.64	1.67	2.20	1.87	14.08
Cl + PHP + HA + rhiz4	0.63	1.90	1.95	1.70	7.66
Mean	0.61	1.72	2.04	1.90	10.64

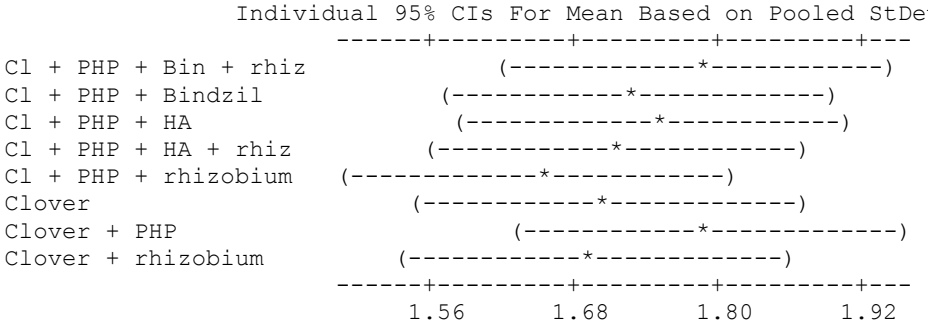
Clover + Rhizobium with Hydroxy Apatite & Bindzil in cabinet

Analysis by one way ANOVA

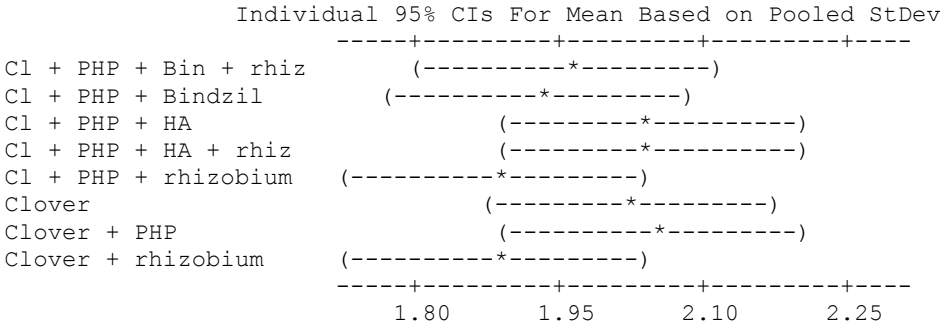
Treatments versus dry weight (3 weeks) P = 0.358



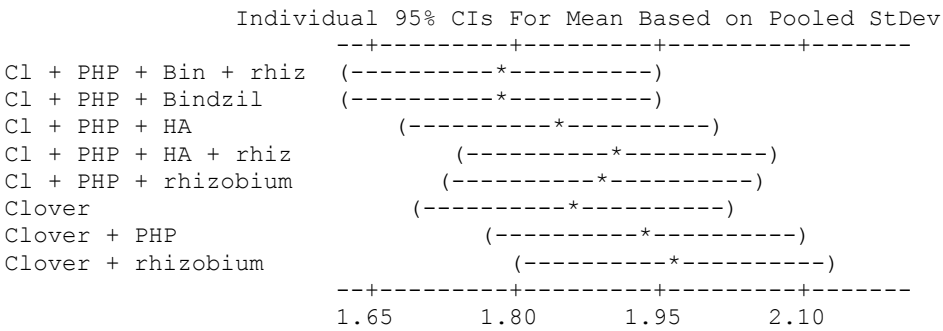
Treatment versus dry weight (6 weeks) P = 0.913



Treatment versus dry weight (9 weeks) P = 0.526



Treatment versus dry weight (12 weeks) P = 0.712

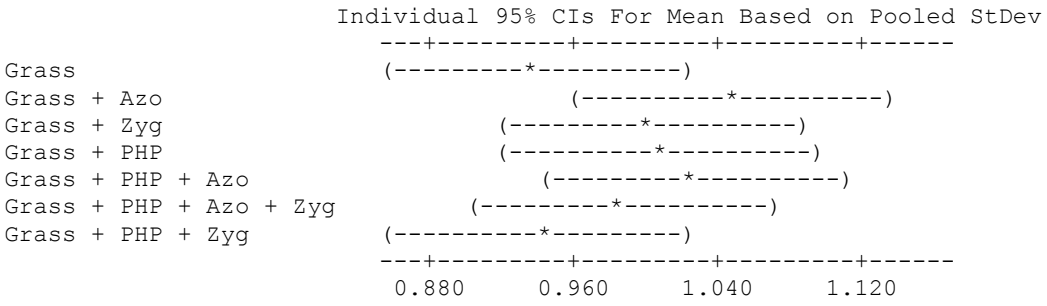


	Grass+*Azospirillum* & *Zygomycete* in greenhouse				
	3 weeks	6 weeks	9 weeks	12 weeks	12 weeks
	Shoot dry wt (g)	Shoot dry wt (g)	Shoot dry wt (g)	Shoot dry wt (g)	Root dry wt (g)
Grass 1	1.01	1.04	0.44	0.21	8.74
Grass 2	0.96	1.08	0.33	0.07	17.39
Grass 3	0.90	1.00	0.32	0.08	10.40
Grass 4	0.88	1.08	0.40	0.09	25.12
Mean	0.94	1.05	0.37	0.11	15.41
Grass + PHP 1	0.97	1.38	0.40	0.07	20.43
Grass + PHP 2	0.95	1.52	0.65	0.09	15.70
Grass + PHP 3	1.07	1.33	0.78	0.16	24.53
Grass + PHP 4	1.04	1.48	0.67	0.20	22.61
Mean	1.01	1.43	0.63	0.13	20.82
Grass + *Azospirillum* 1	1.07	1.23	0.51	0.09	23.63
Grass + *Azospirillum* 2	1.04	1.06	0.58	0.06	18.47
Grass + *Azospirillum* 3	1.09	1.30	0.45	0.24	16.41
Grass + *Azospirillum* 4	0.99	1.21	0.36	0.13	32.17
Mean	1.05	1.20	0.48	0.13	22.67
Grass + *Zygomycete*1	1.09	1.29	0.47	0.15	31.58
Grass + *Zygomycete* 2	0.92	1.08	0.53	0.18	34.80
Grass + *Zygomycete* 3	1.04	1.20	0.47	0.17	21.03
Grass + *Zygomycete* 4	0.96	1.19	0.70	0.20	29.54
Mean	1.00	1.19	0.54	0.18	29.24
Grass + PHP + *Azo*. 1	1.05	1.17	0.47	0.22	30.90
Grass + PHP + *Azo*. 2	1.10	1.22	0.66	0.28	40.34
Grass + PHP + *Azo*. 3	1.10	1.12	0.53	0.26	17.12
Grass + PHP + *Azo*. 4	0.86	1.10	0.43	0.33	17.95
Mean	1.03	1.15	0.52	0.27	26.58
Grass + PHP + *Zyg*. 1	0.95	1.44	0.71	0.25	19.34
Grass + PHP + *Zyg*. 2	0.98	1.28	0.63	0.22	12.22
Grass + PHP + *Zyg*. 3	1.02	1.35	0.67	0.23	14.91
Grass + PHP + *Zyg*. 4	0.81	1.34	0.58	0.19	16.95
Mean	0.94	1.35	0.65	0.22	15.86
Grass + PHP + *A* + Z 1	0.87	1.29	0.53	0.18	10.71
Grass + PHP + *A* + Z 2	1.12	1.42	0.44	0.19	22.28
Grass + PHP + *A* + Z 3	1.01	1.14	0.51	0.20	19.46
Grass + PHP + *A* + Z 4	0.95	1.17	0.88	0.28	18.74
Mean	0.99	1.26	0.59	0.21	17.80

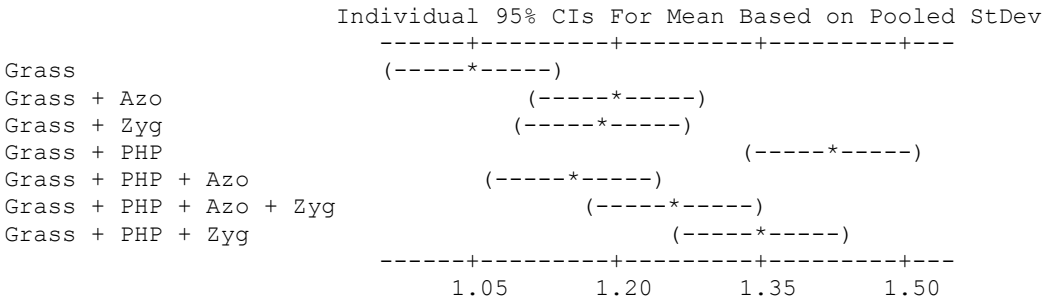
Grass+Azospirillum & Mycorrhiza in greenhouse (cont.)

Analysis by one way ANOVA

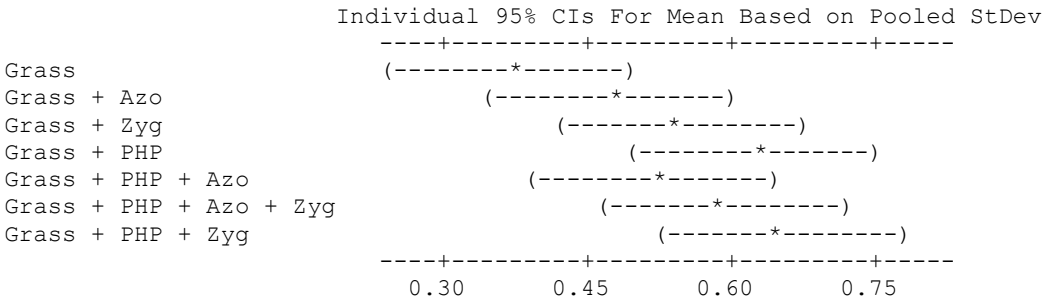
Treatment versus dry weight (3 weeks) P = 0.433



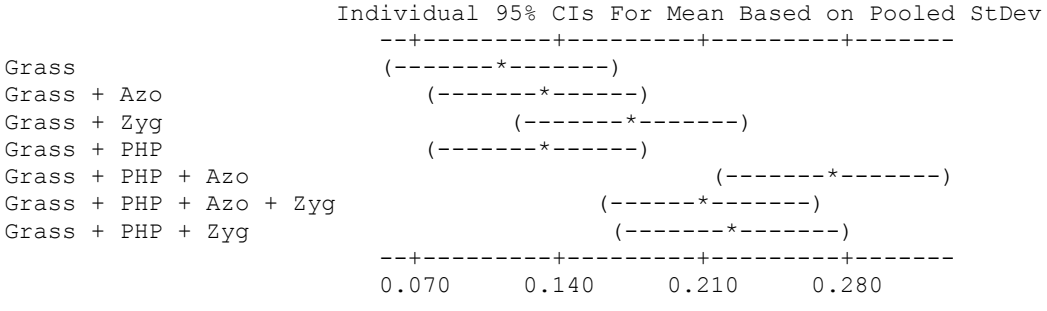
Treatment versus dry weight (6 weeks) P = 0.000



Treatment versus dry weight (9 weeks) P = 0.057



Treatment versus dry weight (12 weeks) P = 0.002

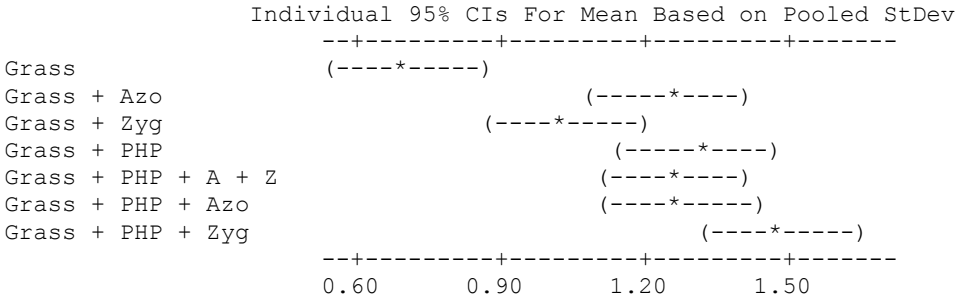


	Grass+*Azospirillum* & *Mycorrhiza* in cabinet				
	3 weeks	6 weeks	9 weeks	12 weeks	12 weeks
	Shoot dry wt	Shoot dry wt	Shoot dry wt	Shoot dry wt (g)	Root dry wt (g)
Grass 1	0.72	1.84	1.50	0.62	30.52
Grass 2	0.73	1.46	1.41	0.92	28.01
Grass 3	0.77	2.03	1.41	0.45	67.96
Grass 4	0.57	1.43	1.53	1.24	47.80
Mean	0.70	1.69	1.46	0.81	43.57
Grass + PHP 1	1.46	2.60	1.62	0.83	48.00
Grass + PHP 2	1.22	2.31	1.71	0.71	79.24
Grass + PHP 3	1.34	2.23	2.00	1.40	71.46
Grass + PHP 4	1.21	2.24	2.42	1.31	78.17
Mean	1.31	2.35	1.94	1.06	69.22
Grass + *Azospirillum* 1	1.34	2.02	1.64	0.45	90.41
Grass + *Azospirillum* 2	1.40	1.96	1.64	0.59	87.42
Grass + *Azospirillum* 3	0.96	1.78	1.35	0.69	84.63
Grass + *Azospirillum* 4	1.29	1.59	1.24	0.37	37.53
Mean	1.25	1.84	1.47	0.53	75.00
Grass + *Zygomycete* 1	0.94	1.93	1.50	1.03	80.15
Grass + *Zygomycete* 2	0.88	1.62	1.55	1.02	75.77
Grass + *Zygomycete* 3	1.15	1.94	1.35	0.74	81.54
Grass + *Zygomycete* 4	1.17	1.49	1.30	0.45	86.99
Mean	1.04	1.75	1.43	0.81	81.11
Grass + PHP + *Azo*. 1	1.44	1.80	1.48	0.97	62.75
Grass + PHP + *Azo*. 2	1.33	1.70	1.58	1.32	72.72
Grass + PHP + *Azo*. 3	1.03	1.71	1.51	1.41	62.90
Grass + PHP + *Azo*. 4	1.28	1.86	1.59	0.84	77.99
Mean	1.27	1.77	1.54	1.14	69.09
Grass + PHP + *Zyg*. 1	1.36	1.95	1.63	1.49	70.62
Grass + PHP + *Zyg*. 2	1.25	2.19	1.63	0.98	63.15
Grass + PHP + *Zyg*. 3	1.59	1.60	1.69	1.42	75.28
Grass + PHP + *Zyg*. 4	1.73	1.66	1.72	1.03	75.76
Mean	1.48	1.85	1.67	1.23	71.20
Grass + PHP + *A* + Z 1	1.37	2.06	1.95	1.09	83.27
Grass + PHP + *A* + Z 2	1.37	2.02	1.80	0.90	64.64
Grass + PHP + *A* + Z 3	1.17	1.91	1.73	1.34	72.83
Grass + PHP + *A* + Z 4	1.13	2.03	1.73	1.01	49.04
Mean	1.26	2.01	1.80	1.09	67.45

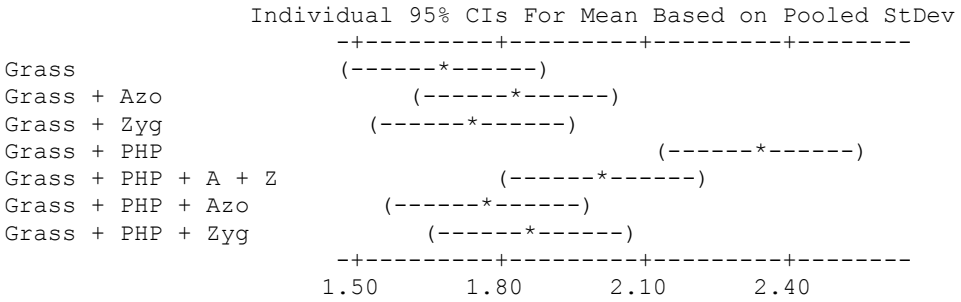
Grass+Azospirillum & Mycorrhiza in cabinet (cont.)

Analysis by one way ANOVA

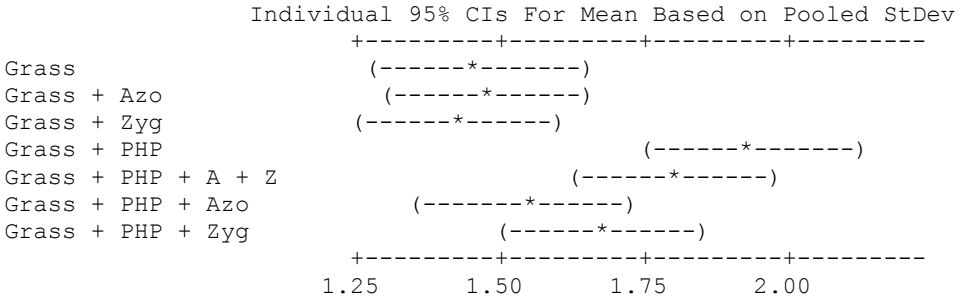
Treatment versus dry weight (3 weeks) P = 0.000



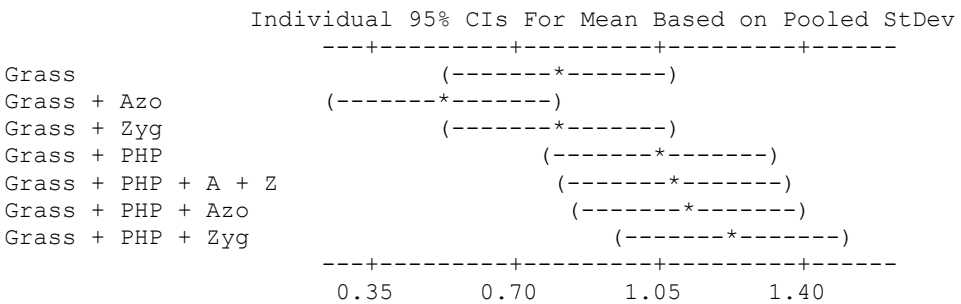
Treatment versus dry weight (6 weeks) P = 0.003



Treatment versus dry weight (9 weeks) P = 0.002

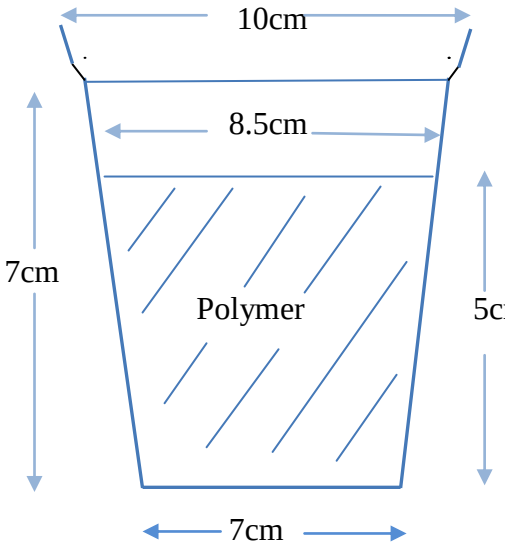


Treatment versus dry weight (12 weeks) P = 0.019



Appendix 3: polymer requirement/hectare

Weight of polymer required for 1 hectare if 1.25g added to a 10cm diameter pot



Soil depth = 7cm
Soil with polymer = 5cm

Volume of truncated cone = $(\pi \times h \div 3) \times (R^2 + r^2 + (R \times r))$

h = height of soil containing polymer

R = Radius at top of soil containing polymer (4.25cm)

r = Radius of base (3.5cm)

Volume of soil with polymer in 1 pot = $(3.14 \times 5 \div 3) \times (4.25^2 + 3.5^2 + (4.25 \times 3.5))$

$= 5.23 \times 45.19$

$= 236cm^3$

$1cm^3 = 10^{-6} m^3 \therefore 236cm^3 = 0.000236cm^3 = 2.36 \times 10^{-4}m^3$

Volume of $1m^2$ 5cm deep = $100 \times 100 \times 5 = 50000cm^3 = 0.05m^3$

$\therefore$ No. of pots in $1m^2$ 5cm deep = $\frac{0.05}{0.000236}$ = 212 pots

$1.25g/pot = 1.25 \times 212 = 265g/m^2$

$10{,}000m^2$ = 1 hectare

$\therefore$ Weight of polymer / hectare = $265 \times 10{,}000 = 2650000g = 2650kg/hectare$

Density

Density of PHP = $0.1g/cm^3$

$1cm^3 = 10^{-6} m^3$

$\therefore$ Density = $100{,}000g/m^3$

$= 100kg/m^3$

Density of conventional fertiliser = approximately $900kg/m^3$