MASTER OF SCIENCE BY RESEARCH

The Effect of Car Engine Oil on Arabidopsis thaliana and Cotswold Winter Vetch/Rye MSc by Research in Crop Improvement using Molecular Biotechnology

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The Effect of Car Engine Oil on *Arabidopsis thaliana* and Cotswold Winter Vetch/Rye

MSc by Research in Crop Improvement using Molecular Biotechnology

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Abstract

The aim of this project was to contribute to the knowledge and understanding of the behaviour and capabilities of oil degrading porous pavement systems (PPS) with particular reference to planted surfaces constructed of a variety of recycled materials.

Previous research has been performed on the effects of oil on *Panicum virgatum, Festuca arundinacea* and *Cajanus cajan* and has examined their oil biodegradation traits. Showing a decrease in their growth, they also tolerated the presence of oil and enhanced biodegradation. *Arabidopsis thaliana* and winter rye were subjected to growth in oil-contaminated growing medium in this project. Both plant species showed physical changes, their growth being impeded and alterations in the colour of their leaves on exposure to oil. Whole gene probing of mRNA of *Arabidopsis* also showed amplification in seven genes that provided tolerance to water and drought stresses.

Previous experiments at Coventry University showed that eukaryotic organisms gave good indications of oil degrading capabilities. This project indicated that there was greater diversity of these species in the planted PPS than in the non-planted PPS laboratory models. The granite and recycled material sub-bases also produced no significant leakage of oil in the effluent from the planted PPS. However, further work is required before one might conclude that the plants may have aided the degradation or retention process.

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1.0 Introduction

1.0 Introduction

The work in this project has been focused on the addition of oil to an environment to look at its effect on plants and on its movement through a porous pavement system. For this work it is important to consider the plants and the techniques of analysis, the system and also the oil. As the work has focused on plants, this has been the primary area for consideration of this report. Addition of plants to a model porous pavement system has also been briefly investigated in a preliminary experiment mainly aimed at evaluating a proposed model system design for use in future.

1.1 Sustainable Urban Drainage Systems (SUDS) and their relationship with plants

Sustainable urban drainage systems (SUDS) have been developed to manage the drainage of surface water, which with more conventional techniques could cause flooding following a storm. There are various types of drainage systems, such as: swale systems (SEPA, 2003), ponds and wetlands (SEPA, 2003), soakaways (CIRIA, 2003) and permeable paving (Puehmeier *et al.*, 2004). These SUDS reduce the impact of building developments by mimicking the natural processes of the infiltration of water into underground rocks and growing mediums (The Water Policy Team, 2001).

With increasing amounts of impervious surfaces in built-up areas, drainage of water needs to be efficient to prevent localised flooding but traditional measures often just move and concentrate the problem elsewhere resulting in severe flooding problems. Water quality issues are also factors that are becoming highly important. Once groundwater becomes polluted, it is extremely difficult to clean up (CIRIA, 2003). Conventional drainage systems do not take into account the pollution that may be present, so man-made drainage systems that filter and 'clean' storm-water are becoming more widely used.

Current research at Coventry University is analysing the use of recycled materials for the degradation of oil. The Porous Pavement Research Group has been funded, under the Landfill Tax Credit Scheme, by Waste Recycling Environmental Ltd. to assess degradation of oil by bacteria in porous pavements, which could be applied to drainage systems. The group are studying the use of recycled concrete and granite within porous pavement structures, in an attempt to discover the best materials for use in the constructions. Over many years crushed granite has shown that it functions effectively as a sub-base in a porous pavement system (Puehmeier *et al.*, 2004). In these types of system it is the complex microbial community that facilitates the bioremediation of engine oil. These are largely confined to the upper layers of the structure and, in particular, to the geotextile (Bond, 1999) (see section 2.14). Although not directly involved in the biodegradation of oil during the

long term slow accumulation from dripping cars, the sub-base does provide a sturdy base on which the pavement is built, it may influence the microbiology of the geotextile laid upon it and can form an enhanced surface upon which sorption and degradation can occur in the event of a major spill. Now, with the growing international interest in sustainability (e.g. Kyoto protocol, (Pearce, 2004)), recycled concrete is being studied as a potential sub-base material (Puehmeier *et al.*, 2004). However, the Coventry group has not previously worked with the planted porous pavement systems that form a sub-set of the PPS family (CIRIA, 2003; SEPA, 2003; Puehmeier *et al.*, 2004).

A combination of plants and porous pavement structures together could promote the use of recycled materials for the structure of pavements particularly if additional growing medium-borne bacterial organisms influenced by the presence of plants can be seen to enhance oil biodegradation. If plants can aid bioremediation through their interactions with microorganisms, then the amalgamation of planting crops within pavement constructions could promote further degradation of engine oil. There are various options of permeable surfacing available but for the growth of plants in this experiment, the *ex-situ* cast concrete system, Grassblock® (Figure 1), and the recycled polypropylene surfacing block INTEGRA 500 (Permavoid Ltd, Amsterdam, The Netherlands) (Figure 2) were used.

Figure 1 Grassblock®

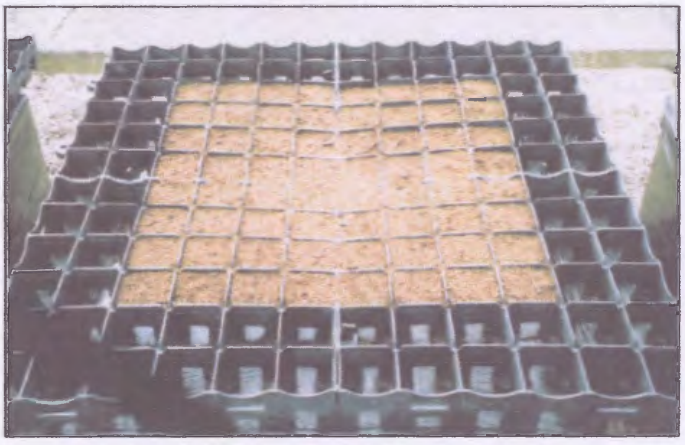


Figure 2 INTEGRA 500 porous paving block

Grassblock® provides a large surface area with voids that present space for the plants to grow. Intended uses for Grassblock® are to prevent erosion of land and to build car parks, driveways and access roads. If used for the construction of car parks and driveways, plants can be sown into the void spaces, and any oil that leaks from vehicles in these areas can be held in the system. Within the structures, it is proposed that bacteria will interact with the plant roots and the oil and promote degradation. Following the absorption and degradation of the oil in the growing

medium and sub-base, contaminated storm water would be able to continue migration through into underground watercourses and sewer systems (Newman *et al.*, 2002; Pratt *et al.*, 1999). The INTEGRA 500 polypropylene surfacing blocks provide a recycled alternative to the concrete Grassblock® blocks, supplying an option to the surface of the car park design. Whilst such systems have been studied over fairly long periods using a "black box" approach to pollutant retention (Brattebo and Booth, 2003) the long term aim of this research is to investigate if variation in plants and growing medium have an effect on the types of microorganisms present and thus, potentially, on the degradation process. As time was limited in this MSc project the effect on protozoa present within the structures was studied as part of an evaluation trial of a new design of outdoor model. As pointed out by Coupe *et al.* (2004) the protozoa would be expected to consume oil-degrading bacteria as their food source, and promote bacterial turnover and activity but much further work is required before any conclusions can be reached.

Growing medium has many organisms and sorption capacity, but without plants its drainage capability is reduced. Thus presence of plants maintains drainage of the growing medium and permits the designer of the PPS to make use of other properties of growing media, In addition, if plants are shown to be oil tolerant, they can have a good effect even if there is no real interaction with the growing medium.

The use of PPS in this work is aimed at a relatively small-scale but if a catastrophe was to occur on a larger scale, the PPS can be enhanced to resolve the problem. Even though aimed at the PPS uses, there are other agricultural situations where you need to grow things on oil contaminated ground, for example in oil production areas like Niger Delta. Niger Delta is an area consisting of oil-bearing communities, which suffers from being underdeveloped and from the effects of the environmental devastation caused by many decades of neglect and oil production by Shell (Abiama, 2004). As the main occupations of the area are subsistence farming and fishing, the need for restoration and revitalisation of the Niger Delta area is critical. By applying the PPS to this area of devastation, the systems could promote degradation of the oil in the clean up process to restore the area.

1.2 Growth of plants and the need for genetic analysis

At a very basic level, for human life to exist, it is essential that plants exist. Not only do they provide food, materials for shelter and clothing, and raw materials for industry, they also replenish the oxygen in the atmosphere for us to breathe (Friesen and Davis, 1998). Plants are considered to be crucial entities in our lives but if we wish to gain the most benefits from them, assessment of their responses to various environmental situations, whether this be resistance to pests, disease or pollution. To understand how they

grow and survive in the environment, particularly in the unusual environment of an oil-contaminated porous pavement system, great amounts of information can be gained through detailed analysis of plants to continue to improve their attributes. One way is to study the up- and down-regulation of genes within the genome that produces cellular components and proteins that are needed for plant growth. Further research must be performed to gain a better understanding of this work (Friesen and Davis, 1998).

During the past two decades, much work has been conducted on sequencing the genomes of various species of plants and animals, so a greater understanding is formed on genes sequences and their promoters. We may use plants for food sources, raw materials, and energy and in aesthetic application, but we still know rather little about them (Meyerowitz, 1999). Various plants species are currently being studied as each has a different genome and gene variation. One of the first plants to be sequenced was the thale cress, *Arabidopsis thaliana*. The *Arabidopsis* Genome Initiative (AGI) completed the sequence in 2000, identifying 25,498 genes in approximately 11,000 gene families (Bennetzen, 2001). Rice has also been sequenced and both plant species are providing tools for plant molecular biologists to use for the genetic analysis of the plant kingdom, investigating functions of potentially every plant gene (Pruitt *et al.*, 2003). In studies of the genome, many novel gene types have been discovered through the identification of mutants, providing foundations for subsequent research in plant

genetics, development and physiology. Many of the proteins characterised in *Arabidopsis* have been found in other plants and are now a source for cross-referencing genes in other kingdoms (Bennetzen, 2001). Finding new genes and integrating them into other plant genomes, through the use of genetic engineering, is providing advances in the understanding of plant biology. This allows the opportunity to identify gene function and to improve the tools and protocols that allow plant science and technology to develop.

In this work the growth of *Arabidopsis thaliana* and Cotswold winter vetch and winter rye plants in oil-contaminated growing medium was investigated. It is hoped that this will give new insights into the discovery of genes involved in plant survival in the environment. Previous work on environmental effects on plants has included the effect of sodium chloride on germination and the effects of calcium and potassium on *Acacia* seeds with salinity tolerance (Rehman *et al.*, 1996). The addition of knowledge on how the presence of oil in growing medium affects plant growth will add to this research and improve our understanding in this field.

1.3 *Arabidopsis thaliana*

Arabidopsis (Figure 3) may have been fully sequenced in the past decade, but first studies with this plant took place at the start of the Twentieth Century. As part of his Ph.D. in 1907, Friedrich

Laibach first began to study the plant as a cytogenetic model organism (Bressan *et al.*, 2001; Somerville and Koornneef, 2002; Pruitt *et al.*, 2003). Several dedicated scientists continued with the study and identification of mutants affecting the morphology and biochemistry of *Arabidopsis* until the 1940s (Pruitt *et al.*, 2003), with Erna Rheinholz being the most recognised for the work performed on mutational genetic experimentations. These studies in the 1940s resulted in the first report on the mutants in *Arabidopsis*, exposing the wide array of phenotypes that were controlled by single genes (Bressan *et al.*, 2001).

Figure 3 *Arabidopsis thaliana*

The research into *Arabidopsis* exploded into an intense subject in the 1980s. Intense plant molecular research was performed on its simple and small genome (Donahue *et al.*, 1997; Lin *et al.*, 1999; Sussman *et al.*, 2000; Somerville and Koornneef, 2002; Pruitt *et al.*, 2003). Consisting of 130-140Mb of highly repetitive regions of DNA, separated by introns approximately 4.6kb

apart (Marshall, 2001). *Arabidopsis* provides an excellent genetic model for plant biology research (Lin *et al.*, 1999; Somerville and Koornneef, 2002; Pruitt *et al.*, 2003). Not only is it easy to grow, easy to care for, self-fertilising and produces many seeds per plant, it also has a short life cycle of approximately 8 weeks, making it ideal to work with as results and subsequent progenies are quickly obtained (Somerville and Koornneef, 2002). *Arabidopsis* is, however, an extremely small plant. The typical height for a fully mature plant is around 300 mm, with a mean root length of about 45 mm and an average total exposed leaf area in the region of 580 mm^2. It takes approximately 50 days for the plant to complete its growth and flowering before it enters senescence (Boyes *et al.*, 2001). These authors pointed out that studies on particular components of *Arabidopsis* must be completed during the plant's growth stages so the optimum results can be gained, if specific observations are required.

As *Arabidopsis* grows, it develops, reproduces and responds to stresses and disease just as other plants of economic importance would. An extra-added bonus of *Arabidopsis* is that production of the plant is at an inexpensive cost and that many experiments can be performed on thousands of plants at a time (Friesen and Davis, 1998). A setback in an area of the *Arabidopsis* genome research is that it has taken some time to analyse the complex genomic organisation of the centromere sequence. The telomere area of the chromosomes within the genome also consisted of complex, highly

repetitive regions (Lin *et al.*, 1999; Sussman *et al.*, 2000). *Arabidopsis* may have been sequenced since the year 2000, but around only half of the plant's genes have been found to have specific functions – the remaining genes have functions that remain unknown (Ito *et al.*, 2002). This is where the direction of the study of *Arabidopsis* becomes much more interesting as research could benefit many scientists, farmers and breeders if gene function through breeding produces useful qualities towards their crops.

With the study of *Arabidopsis* in the context of a porous pavement system, it may be possible to determine if the plant interacts with the microorganisms that help with the degradation of engine oil that has leaked onto its surface.

Within the *Arabidopsis* genome, there are five chromosomes that possess all the genetic information of the plant (Figure 4). These five chromosomes contain 11,000 gene families. The number of families is equivalent to the hypothesised and known 11,000-15,000 gene families that exist in animals (Bennetzen, 2001).

Figure 4 The five chromosomes of *Arabidopsis thaliana* (Beis and Scheres, 2000)

In 1999, scientists studying *A. thaliana* had identified 7781 protein-coding genes. As 60% of the genes were found on chromosomes 2 and 4, it was hypothesised that there would be in the region of 25,000 proteins in the genome (Meyerowitz, 1999). This estimation was relatively accurate with 25,498 genes being uncovered within the gene families in 2000 (Bennetzen, 2001). Proteins encoded on the two chromosomes provide the functions for plant- and bacteria-specific processes, such as photosynthesis, metabolism, cell processes and intracellular transport (The *Arabidopsis* Genome Initiative, 2000). Knowing the functions of the proteins can enable comparisons to be made between plant and animal genes (Meyerowitz, 1999).

Together with the analysis of the *A. thaliana* genome for the genetic changes caused by the presence of oil contamination within growing medium, it is also important to discover the effects of the oil presence on model plants similar to those which might be included in a planting mixture. Winter vetch and winter rye were selected. Winter vetch and winter rye are commonly found in the type of rural environment where planted PPS may be installed. The effect of oil presence on these model species would give an indication on how other plant species might be affected at a physiological level. It would be important to investigate if the presence of the model species in a porous pavement system had an effect on the movement of oil from the pavement surface through the sub-base and out through drainage, and on the types of microorganisms within

the sub-base that aid degradation of the oil. However, as mentioned previously this could only be a preliminary study with the practical lessons learned from the design of the test rig probably of more value than the limited results obtained.

1.4 Winter vetch and winter rye

Winter vetch and winter rye are independently used as cover crops that are integral to organic cropping system, but may be found growing together in the wild. As a 'green manure', they are important in the maintenance of horticultural systems. As they are cultivated to preserve, accumulate and recycle organic matter and plant nutrients, they provide support for vegetable growing. Growth of vegetables is demanding on growing medium, causing the depletion in nutrients and the deterioration in growing medium structure (Rayns, 2003). Planting 'green manures' during the autumn and winter months allows time for these crops to renourish the growing medium for the essential nutrients required by the cash crops that grow throughout the remainder of the year.

As a member of the family Leguminosae, winter vetch (or *Vicia villosa*) is a perennial climbing legume (Figure 5). Its hermaphrodite flowers self-fertilise with the help from bees. Requiring moist growing medium, it is possible for winter vetch to grow in virtually all types of growing medium, ranging from light (sandy) to heavy (clay) growing media, at a wide range of pHs.

Figure 5 Winter vetch (Charters, 2003)

Ability to grow in semi-shade or no shade, this plant is extremely versatile. Although winter vetch is not a deep rooting legume species, it does have a symbiotic relationship with some growing medium bacteria. The bacteria fix atmospheric nitrogen by forming nodules on the roots of the legume. The fixed nitrogen is partly used by the legume itself but it is mainly used in protein synthesis by other plants that grow in close proximity (Plants for a Future, 2003[a]).

Winter rye (*Secale cereale* L.) (Figure 6) is a perennial cereal grain that is primarily grown in Continental Europe and North America (Allen, 2002). Used as a staple food, rye is more dependable in growth than other cereals such as wheat, as it is a hardier crop (hardy to minus 40°F) that is relatively frost and drought resistant (Schonbeck, 1988; Allen, 2002).

Figure 6 Winter rye (Seeds of Change, 2004)

Rye has a more extensive root system that holds growing medium in place during the winter and spring months when it is likely to be washed away by heavy rains. Possessing the ability to absorb and store nutrients such as nitrogen, rye provides a remarkable source of fibre and organic matter that helps to build a good growing medium structure and prevents leaching of nutrients (Schonbeck, 1988; Plants for a Future, 2003[b]; Hewitt's, 2004). A good growing medium structure may also provide the absorption and storage of oil where it can be maintained so bacteria have access to the substrate and aid its degradation. Rye also has other uses that can be of benefit to other crops once they are in season. Rye is able to suppress weeds (allelopathic) and pests, as well as preparing the growing medium for the legumes that are slow establishing (CTAHR, 2002). Having these qualities makes rye the ideal winter cover crop to provide protection not only against growing medium erosion but also for the balance of the growing medium when winter vetch is also present.

Winter vetch is a contrasting crop to winter rye. Binding the growing medium with its fibrous root system, rye stops the accidental removal of vetch, which is more taprooted. The growth of the rye stems in early spring gives vetch the support for its growth. Promoting good light penetration and air circulation for the vetch, dynamic growth occurs. Although quite contrasting in one sense, the crops combine together to stimulate weed suppression. The coverage of the vetch causes heavy shading of the ground, which instigates the competition for moisture with the rye and aids the allelopathic effect of the rye. Conclusively when the crops are turned under, the vetch helps the breakdown of the rye promoting the balance of carbon and nitrogen in the growing medium, maintaining the nutrient balance throughout the season (Schonbeck, 1988). Winter vetch and winter rye were chosen as models for this project as they are well understood and used at Warwick HRI as 'green manures' that are used to preserve horticultural systems by replenishing essential nutrients back into the growing medium. By including these plants to the outdoor rig experiment, it was possible to determine if vetch and rye could be used as models in a contaminated system.

1.5 Characterisation of other plant species

Other species that are also being characterised to widen the range of plants with well understood genomes. These include:

legumes such as *Medicago truncatula*; (Britt and May, 2003), *Antirrhinum majus* (Snapdragon), maize, petunia, tobacco, rice and moss (Pruitt *et al.*, 2003). Being able to attenuate expression levels of genes or to express foreign genes in these plants will have a huge impact on plant science and crop improvement (Britt and May, 2003). Changing genomic sequences to obtain specific proteins of interest would enhance the ability to test gene function within plants. A practical problem associated with this research effort appears to be that genes are being discovered quicker than their functions are being uncovered (Britt and May, 2003). Technologies are being developed to aid the analysis of gene expression and protein function, and concepts determined will be of benefit to plant science.

Eventually the rate at which the functions of the genes discovered should catch up and the analysis of gene function can be interlinked with other species. This process may be aided by the study of the effect of the presence of oil on the growth of plants in this report, a subject which is relatively untouched at present by the majority of research groups.

1.6 The effect of oil on plants

One objective of this research was to investigate if *Arabidopsis thaliana* and winter vetch and winter rye could be used to discover if plants could survive in oil contaminated environments by trying to demonstrate how the organism is affected physically and

genetically by the presence of engine oil. Plants take up minerals and metals from the growing medium and, in previous studies, have been seen to take up heavy metals that are in their environment (Ensley, 2000). However, if their surroundings have been contaminated by an oil spill, plants do not take up oil. Studies on spillage of oil onto vegetated land have demonstrated that biodegradation of oil occurred much quicker than on growing medium alone. Instead, the presence of the plants may aid the biodegradation of the contaminant. Their actively growing roots can stimulate a diverse population of growing medium microorganisms to enhance biodegradation by using the oil hydrocarbons as a food source (EPA, 2004). Three perennial species have been identified by Vavrek and Campbell (1999) that tolerate oil presence and enhance degradation. *Panicum virgatum, Festuca arundinacea* and *Cajanus cajan* have proven that they have the ability to survive in these contaminated conditions. When analysed, it was not possible to state whether these plants contributed little to the bioremediation of oil but a bioassay showed a reduction in growing medium toxicity.

As these species possess a tolerance to drought it was postulated that their water-use efficiency in the hydrophobic conditions of the growing medium might have provided the important trait that enabled them to be oil tolerant. In the same study it was shown by linear regression that the presence of petroleum hydrocarbons within the growing medium was positively related to the reduction in plant size, in particular, root length, weights and

area (Vavrek and Campbell, 1999). The bioassay was performed by growing lettuce (Iceburg) seeds in uncontaminated and oil-contaminated growing media as germination was shown to be reduced by 32.5% when grown in a contamination treatment. In the study it appeared that a few of the plant species increased remediation of the oil in contaminated growing media.

The plants do not take up oil but aid the biodegradation of the contaminant by interacting with the bacteria and fungi in the growing medium, enabling the microbes to use the oil as a source of nutrients. To possess a tolerance for drought and to promote the plant-microbial-remediation interactions could enhance bioremediation of oil without damaging any crops, if a spillage was to occur. When identified, these characteristics could be cloned from the plants and transformed into other species.

Although changes have been seen in the growth of plants, the exact traits that cause the alterations have not yet been clearly defined (Vavrek and Campbell, 1999). The three previous examples have shown that they possess a certain characteristic that enables them to tolerate oily conditions and to cause better remediation, compared to other species. Considering that the examples promoted greater bioremediation, it did not prove that they were totally comfortable with the conditions. Not only did they show changes in their metabolic and biochemical characteristics, they also showed a reduction in their normal plant growth performances (Vavrek and Campbell, 1999). The studies were designed for the

removal of oil and not for productivity of the crops, so the effect on the reduced crop growth was not important. In a PPS situation other characteristics such as aesthetics and traffic resistance would also be important.

Bioremediation of diffused or moderately contaminated soils is often employed by microbial degradation. An alternate approach to this is through the use of plants that promote contaminant remediation, namely phytoremediation (Díez Lázaro *et al.*, 2005). Phytoremediation is the use of green and vascular plants for the removal of the organic-contaminating compounds (including materials found in petroleum, nitrotoluenes and chlorocarbons) from the environment (Ensley, 2000; Gleba *et al.*, 1999), providing a low-cost method of soil remediation (Chaney *et al.*, 1997). At present, few plants have been studied for this purpose (Díez Lázaro *et al.*, 2005).

Kaimi *et al.* (2004) demonstrated that ryegrass lowered the dissipation threshold of the oil-contaminated soil it was grown in. The oil's residual rate in the soil was lowered by 55% with the development of ryegrass roots, compared to the contamination in root-free soil prior to growth.

The salt marsh plant, *Spartina alterniflora*, was shown to have significantly decreased biomass production, stem density and shoot height once transplanted into increasing levels of fuel-contaminated soil, in a dose-response experiment (Lin *et al.*, 2002). Results of

this experiment showed that the evapo-transpiration rates correlated with the total biomass response in the salt marsh.

Sagittaria lancifolia, a Louisiana freshwater marsh, was investigated for its effect on South Louisiana Crude oil in field conditions (Lindau *et al.*, 2003). With nitrogen (N) and phosphorous (P) also applied to some of the test plots, it was identified that a combination of the three variables promoted the greatest attenuation of the crude oil. A year following the application of oil to the plots, only trace hydrocarbons were detected in the marsh soil. This treatment demonstrated a relatively quick turnover of the oil in the study.

Gong *et al.* (2001) studied the germination and growth of four higher plant species (*Avena sativa* L. (oat); *Lepidium sativum* L. (cress); *Brassica rapa* Metzg. (turnip) and *Phaseolus vulgaris* L. (bush bean)) in mineral oil-contaminated soils. The results determined that shoot biomass provided more sensitive measurements than germination, as the seeds germinated within the contaminated soils but growth was affected by the nutrient status of the soils.

Phytoremediation of petroleum-contaminated soil was analysed using three tropical graminoid species, *Brachiaria brizantha*, *Cyperus aggregatus* and *Eleusine indica* (Merkl *et al.*, 2005). The study compared the specific root length (SRL), surface area, volume and average root diameter (ARD) of the three species involved. *B. brizantha* and *C. aggregatus* showed coarser roots in

the contaminated soils, compared to the control plants in oil-free soil. *B. brizantha* also had a larger specific root surface in the contaminated soil, with *C. aggregatus* showing significantly smaller SRL and surface area. The root structure of *E. indica* was not significantly affected by the presence of oil in the soil. This study, along with previous studies, showed that a greater specific root surface area gave greater degradation of petroleum hydrocarbons.

With these examples, it will be possible to identify areas where the oil-contaminated growing media will affect this project. The results obtained will give further information on the effect of the presence of oil on *A. thaliana*, winter vetch and winter rye.

Microbes in the growing medium and plant roots interact with each other in the rhizosphere, thus root characteristics may provide the interactions that promote remediation by the oil-degrading microorganisms. This makes the understanding of changes in the rhizosphere important. The rhizosphere is determined by the size, length, shape of the roots and the exudation pattern in which they grow in the growing medium. Basically the rhizosphere is the region of growing medium surrounding the root hairs of a plant, the area where the complex relationships between plants, growing medium microbes and the growing medium exist (Lennox, 2003) and is approximately 1 mm in width (Shigo, 1996). Within this environment, there is a constant mix of organisms that can influence the chemistry of the growing medium, including the pH and the nitrogen transformations (Lennox, 2003). The environment has a

major influence on the productivity and the health of the crops (Downie, 1998). Due to the close proximity of the plant roots and the growing medium, it is impossible to study the microbial populations independently from the plant. They are regarded as an integral part of the plant-microorganism-growing medium system (Dommergues and Krupa, 1978).

Nutrients absorbed by the roots are taken up as cations and anions, and the differential rates at which they are taken up causes the change in the pH in the rhizosphere. The most important ions involved in the pH change are nitrate (NO_3^-) and ammonium (NH_4^+). When there is a high concentration of NO_3^- in the growing medium, an efflux of hydroxyl (OH^-) ions, causing an increase of the rhizosphere pH. The pH of the rhizosphere is decreased when there is a high concentration of ammonium ions in the growing medium. This promotes an efflux of hydrogen (H^+) ions from the roots (Heckman and Strick, 1996). Any changes in the pH of the rhizosphere are localised to the growing medium area adjoining the root and not the bulk growing medium mass. This guarantees that other plants are not affected by a change in growing medium pH. As the roots are positioned within the contaminated source, they are in most contact with the oil. A characteristic that allows the roots to promote greater bacterial growth and enzyme production within the rhizosphere would increase the use of the oil as a food source for the bacteria. They would metabolise the oil supply and degrade it much more quickly. Examples of plant species that have shown an

increase in the production of oil degradation are: horseradish, tomato, water hyacinth and cotton (Vavrek and Campbell, 1999). Oxygen from the roots of these species was shown to diffuse towards the rhizosphere, providing a supply for the microbes. As the availability of oxygen increased the metabolism of the bacteria increased and thus, remediation was promoted. It was also shown in plants that were tolerant to flooding that an oxygen supply to the roots was maintained. This allowed aerobic respiration in parts of the plants that were below growing medium level (Vavrek and Campbell, 1999). As sub-surface aerobic microbes require oxygen, bioremediation of petroleum hydrocarbon-contaminated growing medium is increased if there is a supply of oxygen to them. In general this makes oxygen a limiting factor for the bioremediation of oil (Crocetti *et al.*, 1992).

It must also be considered that different plant species would promote interactions with different bacteria and microbes, once they had been identified. To be able to efficiently bioremediate an oil spill, the plant present must possess each trait available, and that would be highly unlikely. A collection of various plants with a variety of traits that have appropriate bioremediation characteristics would be after the best method of attempting to 'clean up' an oil spill and general oil leaks.

1.7 The effect of the presence of engine oil on *Arabidopsis thaliana*

At present, *Arabidopsis* has not been studied for its tolerance to oil-contaminated growing medium. *Arabidopsis* is a common weed, which is widespread in the UK. As mentioned previously it is quite likely, therefore, that the weed may be present at the site of a car park, designed on the porous pavement system, where an oil leakage is likely to occur and it is thus quite an appropriate model species. If *Arabidopsis* could be shown to possess a trait that either assists the bioremediation of oily substances or at least makes it tolerant of high oil loadings, the obtained information could then be utilised in other areas of pollution prevention and to plant breeding. The basic genome of *Arabidopsis* could be used to research this trait and thus investigate the effect upon other characteristics already present. The possession of such a favourable trait within the *Arabidopsis* genome could mean that the weed could exist at sites where oil leaks are more likely to occur, in anticipation of it happening, using the plant as a preventative measure. *Arabidopsis* genes could then be moved to appropriate species if they did not possess similar genes within their genomes. Theoretically, the application of genes into a variety of species would provide an excellent method as preventative measure against pollution. Whether this plant material would be permitted to be released into the wild would be reliant on how easily genes from transgenic crops

transfer from species to species and studies on this would progress over many years. A licence for the use of transgenic plants within porous pavement systems may be difficult to obtain as application as a car park would be seen as a low priority in relation to food crops transgenic against environmental conditions such as disease resistance, for example.

The study of how the presence of oil affects *Arabidopsis* attempts to discover the physical changes: for example plant colour, plant height, root length, and the genetic changes within the genome. The latter would be performed through the use of microarrays (see Section 1.12), analysing the mRNA to identify which, if any, genes are amplified by the presence of oil. *Arabidopsis* has quite a uniform growth pattern so the effect of the oil would be identified quite easily by a visible change.

In the case of *Amaranthus hybridus* L., previous work that has been performed has shown changes in mean height and leaf area, and more so in the levels of chlorophyll and protein obtained from the plant (Odjegba and Sadiq, 2002). With *Arabidopsis* being a well-studied plant, the effect of an oil contaminant would provide interesting reading for those who study its genes and growth.

1.8 The effect of the presence of engine oil on winter vetch and winter rye grass

The effect of engine oil on the legume winter vetch and the grass winter rye was assessed during this study.

The genome for the vetch and the rye are not fully sequenced; therefore analysis of their genomes in relation to the effect of oil on it has not been recorded. The way in which the oil affects the vetch and rye were compared to the affect on *Arabidopsis*. The physical change in appearance in the winter vetch and the winter rye were recorded, along with the statistical analysis of the change in mass between the wet and dry weights. It was possible to study *Arabidopsis* in greater detail, due to the completion of its sequenced genome and the availability of microarrays for analysis.

Prior to the analysis of the growth of both *Arabidopsis thaliana* and winter vetch and winter rye in an oil-contaminated growing medium, a short introduction to the growing medium will give a background to its components.

1.9 John Innes seedling compost

To ensure that the *A. thaliana* and winter vetch and winter rye seeds germinated successfully, they were planted in John Innes Seedling Compost. Prior to the specialised composts now available, horticulturalists and gardeners had to rely on 'good' growing

mediums or composts made from their own recipes to sow their seeds (John Innes Centre, 2001). These composts were based on trial and error, and as the growing media and composts were not sterilised or heat-treated, the seedlings were often attacked, damaged or destroyed by growing medium-borne diseases and insects (John Innes Centre, 2001).

William Lawrence and John Newell at the John Innes Horticultural Institute in the 1930s conquered the problems caused by the unreliable 'home-made' composts so that a compost could be created that gave consistently good, reliable results (John Innes Centre, 2001). Their main intentions were to promote reliable germination and better growth in their experimental material.

With the valuable knowledge of the requirements for optimum growth of plants, two standard composts were designed: a seed sowing compost and a potting compost. These standard composts were tried and tested in many seeding and potting situations, and they are still in popular use by gardeners and farmers today, many years after they were first designed. Although some nutrients in compost are combined with additional fertilisers, the four main components (loam, organic matter, sand and fertiliser) remain the same.

Loam forms the main body of compost. It provides the essential microelements and organic matter that for plants' basic nutrition. Growing medium gives the compost porosity, and improves aeration and the water-retaining capacity of the compost.

The growing medium eventually decomposes and develops humus, which is a fertile foundation material for planting (Geer, 2002). Sand provides drainage; preventing waterlogging that can damage the seeds before they even germinate. It also provides the extra stability required by larger plants to stand upright in the compost. The final component, fertiliser, provides the extra nutrients needed for plant growth. These include: nitrogen, phosphates, potash and trace elements. These elements can provide sufficient nutrients to support plant germination and growth for 1-2 months, before additional feed should be given (John Innes Centre, 2001). With these compost components and the correct environmental conditions, obtained usually quite well in a glasshouse (for example temperature and day-lighting hours), germination is typically successful in the majority of plant species.

Within this project, the germination and growth of *Arabidopsis thaliana* and winter vetch and winter rye seeds were assessed in oil-contaminated and non-contaminated growing medium in the glasshouse and the effect of used engine oil on established *Arabidopsis* and vetch and rye plants in planted porous pavement rigs, with the analysis of the effluent recovered from the rigs being examined also for the microbiological organisms degrading the added oil.

1.10.1 Ribonucleic acid (RNA)

RNA is a nucleic acid that is important in the transmission of information within cells. Its structure is formed from polymers of nucleotides (polynucleotides) and consists of the nitrogenous bases adenine (A), cytosine (C), guanine (G) and uracil (U), the pentose sugar ribose and a phosphate group. The phosphate groups link the pentose sugars together to form the sugar-phosphate backbone of the polynucleotide chain (Alberts *et al.*, 1998; Reed *et al.*, 2003). Alberts *et al.* (1998) point out that RNA is a single-stranded molecule that is "in the form of a gentle right-handed helix". They also point out that the helix is stabilised by base-sticking interactions that occur in the regions of the RNA that are self-complementary, leading to base pairing and the folding of the molecule into a 3D structure.

The levels of mRNA can give an impression of the levels of gene expression within a cell. This is particularly true for genes under transcriptional control. Some genes are controlled at the translational step and others activated by post-translational modifications. These will not be detected by changes in mRNA levels. RNA is the result of DNA-directed synthesis of protein in cells. It occurs from the disruption of the cells to release their contents, the removal of non-nucleic acid components (i.e. proteins) and the concentration of remaining nucleic acids. RNA is found in three forms in cells: ribosomal RNA (rRNA), transfer RNA (tRNA)

and messenger RNA (mRNA). Extracting RNA requires careful manipulation as the molecules degrade easily due to the action of any co-extracted, contaminating ribonucleases. The situation is complicated further by the fact that harsh treatment is required to dissociate RNA from the protein in the ribosomes (Reed *et al.*, 2003).

Transcription, the synthesis of mRNA that is complementary to the coding/template strand of DNA, occurs in the nucleus of a cell. The mRNA migrates from the nucleus to the cytoplasm where it provides a template for protein synthesis (translation) at the ribosome. The mRNA template is read as base sequences known as codons consisting of three nitrogenous bases, with each combination of the 3 bases corresponding to particular amino acids. tRNA brings the amino acids to the ribosomes where they are integrated together to form a polypeptide chain until a termination codon is reached, then a completed protein is released from the ribosome to the cytoplasm (Reed *et al.*, 2003).

The RNA from the *Arabidopsis thaliana* plants was extracted for further examination using microarrays, determining the effect of the oil presence on the plant.

1.10.2 Extraction of RNA and RNA amplification

In cases where RNA has been extracted from a source and little yield has been obtained, for whatever reason, it is necessary to

amplify the RNA to provide sufficient analyte for the analysis that is to take place. There are various methods of amplification, which have rather similar protocols to each other. One successful protocol was developed by Van Gelder *et al.* (1990). This method of RNA amplification was the basis for the MessageAmp™ II aRNA kit (patent pending). Used in this project, the methodology for this kit involves the reverse transcription of the RNA with the oligo(dT) primer that has a T7 promoter and results in the *in vitro* transcription of the resulting cDNA. With T7 RNA Polymerase, this makes up to 1000s of antisense RNA (aRNA) copies of each mRNA present in the sample (Ambion, Manual Version 0404, 2004).

The Van Gelder *et al.* (1990) protocol and MessageAmp™ II have one main difference: MessageAmp™ II uses ArrayScript™. ArrayScript™ is a reverse transcriptase that has been engineered to produce high yields of first strand cDNA. Without this enzyme, such yields would not be obtained with the wild type enzymes. ArrayScript™ enables the synthesis of almost full-length cDNA, which is then purified of unwanted RNA, primers, enzymes and salts from the double-stranded cDNA. These may cause the inhibition of a subsequent *in vitro* transcription (Ambion, 2004).

In vitro transcription reagents are also provided with the MessageAmp™ II kit. These reagents permit the synthesis of labelled and unlabelled RNA so that the samples can be used in microarray analysis. The purification of the aRNA discards the unincorporated NTPs, salts, enzymes and inorganic phosphate,

making the aRNA more stable and succeeding enzymatic manipulation easier (Ambion, 2004). Being able to work with RNA sample concentrations from 0.1-100ng, makes the MessageAmp™ II kit extremely attractive and the resulting aRNA samples can be used successfully for microarray analysis. If, following the conclusion of the amplification, there is insufficient RNA available for microarray analysis or other investigations, the RNA can be amplified for a second time with the MessageAmp™ II kit.

Determination of the concentration of RNA is available through the methods of formaldehyde agarose gel electrophoresis (Reed *et al.*, 2003) and through use of the NanoDrop® Spectrophotometer (NanoDrop®, 2004). A combination of both the methods provides both visual (agarose gel) and accurately quantitative (NanoDrop®) data required for further analysis.

1.11 Agarose gel electrophoresis

The most common method of obtaining the results of a DNA or RNA extraction or the products of PCR is to separate the samples by agarose gel electrophoresis. Obtained from seaweed, agarose is a natural linear polysaccharide that consists of galactose and 3,6 anhydrogalactose repeats (Reed *et al.*, 2003). Gels are formed by adding an electrophoresis buffer (e.g. Tris-Acetate-EDTA (TAE), Fisher Bioreagents) to the agarose at concentrations of 0.3-3.0% w/v (Reed *et al.*, 2003). During the preparation of gels hydrogen

bonds form with and between the agarose polymers, causing the formation of pores through which the movement of the proteins takes place during the separation (Reed *et al.*, 2003). The movement of DNA or RNA through the agarose is due to the attraction of their negative charge to the anode of the electrophoresis machine. Migration of the protein through the gel is determined by its individual charge density, with small linear fragments migrating faster than larger proteins through the separating matrix (Walker and Rapley, 1999). Bands separated by electrophoresis are visualised by adding a water-soluble anionic tracking dye (bromophenol blue) to the samples to see the migration (Reed *et al.*, 2003) and through the addition of ethidium bromide (EtBr) to the agarose gel (Brown, 1998). This enables the samples to be seen using UV light as the EtBr binds to the protein molecules by intercalation between the paired molecules (Reed *et al.*, 2003).

As RNA was analysed in this project it was necessary to add formamide and formaldehyde to the agarose gel. The addition of these chemicals prevents the anomalous mobility caused by the development of secondary structures formed by the RNA molecules, which would appear if the gel was not treated (Reed *et al.*, 2003).

1.12 Determining nucleic acid concentration with the NanoDrop ND-1000A UV-Vis Spectrophotometer

Over many years, separating samples by agarose gel electrophoresis has permitted the determination of DNA and RNA concentrations. By producing bands from the samples, approximate sizes and concentrations of the separated fragments can be verified by matching them to the similar size bands and the bands intensity of a control marker with known fragment sizes. The samples could then be analysed with a spectrophotometer to find that actual concentration in the sample.

New technology in the form of the NanoDrop® ND-1000A Spectrophotometer has made the measurement of DNA and RNA concentrations much easier. The NanoDrop® can produce many accurate measurements of nucleic acid samples, with an undiluted maximum concentration of 3700 ng μl^{-1}. As the NanoDrop® only requires 1 µl of the sample to measure, it does not need cuvettes nor capillaries like a normal spectrophotometer. This saves the cost of the cuvettes, the time it takes to clean material after use, and it also saves the majority of the sample from being used for measurement and reduces the risk of contamination. The system optics of the NanoDrop® measure the 1 µl sample across a controlled path length of 1 mm, which can be easily cleaned between samples. Many successive sample concentrations can also be measured with no carry-over. Plus, with each measurement

taking less than 10 s, many samples can be analysed quickly and efficiently, making the set up and running of an agarose gel, followed by normal spectrophotometer investigation, a long-winded process. The NanoDrop® scans the sample over a wavelength of 220-750 nm and calculates the A280, A260 and A230, providing the quantity of nucleic acid and the levels of impurities.

Determining the quantity of RNA accurately enables the decision to be made on whether the RNA is suitable for analysis with microarrays for investigation into the up- and down-regulation of genes that may have occurred in the experimental treatment.

1.13.1 Microarrays

The method of identifying specific genes from a genome has been developed in recent years. Previous analysis of genes involved a single gene per experiment, but now many genes can be analysed at once through the use of microarrays (DNA Microarray, 2002). This is a tool for analysing gene expression at the RNA level. The microarray consists of either a silicon chip or a slide that is usually made of glass but sometimes plastic or nylon membranes), that contains samples of many genes arranged in a regular pattern. The microarray takes advantage of the ability of a given mRNA molecule to bind specifically to, or hybridise to, the complementary DNA template from which it originated (NCBI, 2004). Thus, each DNA spot acts as a probe, enabling the determination of

levels of specific mRNA produced by the sample material (Sugnet, 1999). With many mRNA molecules able to hybridise with the DNA template at once, thousands of genes can be analysed per experiment. The basic protocol for a microarray involves the preparation of the DNA slide, the preparation of a hybridisation solution containing fluorescently-labelled cDNAs, the incubation of the DNA chip with the cDNA solution, detection of the fluorescently-bound cDNA to the DNA template, and the analysis of the data using various computational methods (NCBI, 2004).

Microarrays provide a powerful method of gene analysis as various levels of gene expression can be shown with the brightness of the fluorescence (detected by a laser) and mutated cDNA and mRNA sequences can easily be determined by the colour changes by the fluorescence (Figure 7).

Figure 7 Fluorescing microarray spots on a typical slide

Typical microarrays can carry several thousand spots per square centimetre, and up to 10,000 spots on a surface between 2-8 square centimetres (TAGC, 1999). Not all scientists use microarrays in the analysis of their genome research as the technology is relatively new, but many realise the benefits for performing many gene profiles in one attempt instead of many experiments. Microarrays are also quite expensive, costing several thousand pounds for a highly specific DNA template spots. Produced in-house, the cost can be reduced.

1.13.2 Manufacturing microarrays

There are many different types of microarrays but in general there are two construction methods that produce the best microarray slides (Sugnet, 1999) onto which the DNA molecules are chemically bonded. The fabrication methods are split into robotic spotting and *in-situ* synthesis (Stekel, 2003).

1.13.3 Robotic spotting

The process of spotting using a robot is the most common manufacturing method used by the majority of companies that produce microarray slides. The basic protocol involves the preparation of the DNA probes, the spotting of the DNA onto the microarray surface by a robot and then the post-spotting process of

the slide (Stekel, 2003). The type of spotted microarray then depends on the type of DNA probe and the way in which the probe is attached to the slide. The various probes are synthesised separately for microarrays; using polymerase chain reaction (PCR) for cDNA probes that have been amplified and purified from a clone library, or presynthesising DNA oligonucleotides with chemical synthesis (Sugnet, 1999; Stekel, 2003). Once the DNA probes have been prepared, they must be attached to the slide. For oligonucleotide probes, a primary aliphatic amine (NH_2) group is affixed to the 5' end of the DNA. This creates a covalent bond between the amine group and the chemical linkers for the probe on the slide (Stekel, 2003).

The attachment of the cDNA probes to the slides is by non-covalent bonding. Non-covalent bonding involves the electrostatic attraction between the phosphate backbone of the cDNA probe and the NH_2 groups attached to the glass slide surface. The cDNA probe is joined to the slide at many points as there are interactions at several locations on the phosphate backbone. It is difficult to attach oligonucleotides to the slide surface, as they are much shorter than cDNA and insufficient nucleotides bond with the surface (Stekel, 2003).

The probes, whatever their type, are made in microtitre well plates so that the robot can easily 'pick–up' probe samples from each of the wells with its grid series of pins. With the grid set-up of the pins, it is a practical and efficient method of producing many

identical microarray slides from the well plates as each probe will be in the same place, making the microarray slides easy to compare during analysis. The printing process is a relatively simple procedure. The pins are dropped in the wells by the robot to collect the DNA. The DNA is spotted onto different arrays, depending on the number of arrays required and the amount of samples that can be held by the reservoir of the pins. Following spotting, the pins are washed to remove any remaining solution and to prevent contamination. The pins are then dropped again into the next set of wells and the process continues, until the microarrays are complete (Stekel, 2003). To prevent the addition of surplus DNA to the arrays, the surfaces are 'fixed' so that they are ready for use in analysis. 100% ethanol is used to wash excess DNA from the slide, removing any opportunity for the DNA to bind to the fixed nucleotides. 'Fixing' the array surface also makes the slides more hydrophilic and helps the mixing of the target solution during hybridisation (Stekel, 2003). This method of microarray production is comparatively inexpensive and adaptable, used by many laboratories.

1.13.4 *In-situ* synthesis

This is also known as a deprotection method of producing microarrays. Unlike spotting, the oligonucleotides are not presynthesised. Instead, the oligonucleotides are built on top of

each other, base-by-base, onto the surface of the array. To build the bases onto each other, there is a covalent reaction between the 5' hydroxyl group of the sugar of the previous nucleotide attached and the phosphate group of the next nucleotide (Stekel, 2003).

To prevent the addition of more than one base in each synthesis, there is a protective group at the 5' end of the nucleotide. This protective group converts into the hydroxyl group through the presence of light or acid.

There are three technologies that involve deprotection treatment:

- Photodeprotection using masks
- Photodeprotection without masks
- Chemical deprotection with synthesis using ink-jet technology

When performing photodeprotection, light is the factor that converts the protective group into the hydroxyl group. Currently, only Affymetrix® process microarray slides using masks (Sugnet, 1999). The masks allow light to pass through to some areas of the slide, and not to other areas. This is termed photolithography. For each step of synthesis, a different mask must be made so different areas are subjected to light. This is an expensive process, but once the masks are made, identical arrays can be manufactured easily (Stekel, 2003).

Photodeprotection can also be performed without the use of masks. Directing the light from its source and bouncing the rays off the appropriate areas of microarray slides to produce the resulting arrays. The computer controls the direction of the light rays so the resulting nucleotide array data can be analysed from the computer files. Some similar protocols use an ink-jet like printer to 'fire' the oligonucleotide probes onto the microarrays (Sugnet, 1999). The computer controls the nozzles that fire the nucleotides onto the array so that analysis can be referred back to the sequences in a computer file (Stekel, 2003). As any oligonucleotide can be sprayed onto the microarrays, this technology is extremely flexible; however, problems may arise if many identical arrays are required. Once the printer runs out of probes, it must be refilled, causing a break in the manufacturing process.

These methods of attaching DNA to the microarray slides produce varying levels of success. The photodeprotection methods have a coupling efficiency of *ca.* 95%, meaning that the majority of the nucleotides are successfully adjoined to the slides. In the case of the chemical- or acid-mediated deprotection technology, this figure rises to *ca.* 98%. Both coupling efficiencies are dependent on the length of the nucleotide attached to the slides; the larger the oligonucleotide, the poorer the yield obtained (Stekel, 2003). The quality of the yield is reliant on capping, a process that is used by Affymetrix® in their microarray manufacture. If an oligonucleotide fails in its synthesis, the end of the sequence is capped to prevent

further synthesis. If the oligonucleotides continue to be created following the failure within synthesis, it is likely that bases would be missing from the sequences causing deletions in the data. Thus, it is better to cap and have shorter oligonucleotides than to allow full-length oligonucleotides to be synthesised that contain deletions (Stekel, 2003).

1.13.5 Spot quality

In order to obtain suitable data from microarrays, the spot quality must be taken into account. With each microarray, the quality of information attained from the spots is variable. If the spot is irregular, light from the array analysis may leak into overlapping feature, giving false readings (Stekel, 2003). Ensuring that the light is projected through the fluorescing spot by lining up the rays can be a time consuming procedure, but it does provide the best results. It has been shown that the ink-jet arrays produce the best spot quality as the nucleotides are 'printed' on the slides in a uniform pattern, without any possibility of overlapping.

1.14 Overall aim and specific objectives of the project

Throughout the duration of this project, the overall aim was to contribute to the knowledge and understanding of the behaviour and capabilities of oil degrading porous pavement systems (PPS) with particular reference to planted surfaces constructed on a variety of recycled materials. This led to a need to discover how model plants are affected by engine oil, to study the genes up-regulated in response to the presence of oil and to discover if the plants in a porous pavement system had an effect on the eukaryotic microbiology which indicates the degradation of engine oil.

Specific objectives were:

(a) To design and build a novel type of outdoor experimental planted porous pavement test model rig and use this rig to study a limited number of natural rainfall events to ascertain whether or not, and to what extent, oil was released from the porous pavement systems under study. These models would also be used to investigate changes in growth of *Arabidopsis thaliana*, winter vetch and winter rye, subsequent to used engine oil addition, and in microbial population, size and composition. It was intended to use microscopy and use of the Infra Red

Spectrometry in the form of the Horiba OCMA-310 Oil Content Analyser to make this assessment possible. The main intention was as a pre-cursor to further work to determine whether or not this outdoor rig design could be adopted successfully for further field trials.

(b) To ascertain the germination efficiency of the seeds, determining how many were required for substantial growth of plant material.

(c) To germinate the *Arabidopsis thaliana* and winter rye in increasing levels of oil-contaminated growing medium, prior to discovering the effect of the presence of oil on the growth of the species and statistically demonstrating the growth inhibition through statistical analysis.

(d) To use molecular techniques to extract mRNA from the *Arabidopsis thaliana* grown in oil-contaminated growing medium; to amplify and specifically label RNA with A-Cy3 and G-Cy5 fluorescent dyes (Stekel, 2003) and to identify genes upregulated by the presence of oil through the use of microarray analysis. To use a t-test within GeneSpring program to indicate gene upregulation shown by the microarrays.

2.0 Materials and Methods

2.0 Materials and methods

The plant species used in this project were:

- *Arabidopsis thaliana* ecotype Columbia 0, provided by Warwick HRI;
- *Arabidopsis thaliana* ecotype Columbia 4, provided by Coventry University;
- Transgenic *Arabidopsis thaliana* ecotype Columbia 4, (B8C). Transformed containing CaMV35S promoter and maize ABP1 cDNA (overexpressing ABP1 gene), provided by Dr. Colin Lazarus (Bristol University);
- Winter vetch (*Vicia villosa*) (Cotswold Seeds Ltd), provided by Warwick HRI.
- Winter rye (*Secale cereale* L.) (Cotswold Seeds Ltd), provided by Warwick HRI.

In the preliminary pot experiment of the growth in oil-contaminated growing medium, analysis of both winter vetch and winter rye was to occur. However, it was observed that the winter vetch did not grow whereas the rye did. It was decided to remove the vetch from the experiment as the rye provided enough plant material for analysis.

However, in the outdoor experiment at Warwick HRI, both the vetch and rye germinated and grew. This occurred over several months, giving time for the vetch to germinate and establish itself in

the growing medium. The growth of vetch was maintained in the experiment to preserve the original experimental plan.

A. thaliana and winter rye were tested for their ability to germinate and grow in oil-contaminated growing medium, and to discover their physical reaction (with winter vetch) following addition of used car engine oil, following their growth and establishment within a planted porous pavement system. Eukaryotic microorganism samples were also obtained from the effluent from the planted porous pavement systems and compared to those from non-planted porous pavement systems.

2.1 Sterilisation of *Arabidopsis thaliana* and winter rye seeds

To sterilise the seeds, 1.7 g dichloroisocyanate acid was pipetted into 100 ml of deionised water. To this, a few drops of Nonidet P40 were added and the solution was mixed thoroughly. The seeds were placed in microfuge tubes and sufficient solution was transferred to the tubes so all seeds were submerged under the solution. The contents of the microfuge tubes were mixed thoroughly on a whirlimixer for 6 min. The tubes were centrifuged at 10,000 *x g* for *ca.* 10 s to bring the seeds to the bottom of the tubes. The supernatant was discarded and sterile deionised water was used to wash the seeds. The tubes were placed on the whirlimixer briefly and centrifuged at 10,000 *x g* for 1 min and the excess water

discarded. The procedure was completed three times and the seeds were sterilised.

2.2 Germination efficiency assay of *Arabidopsis thaliana* and winter rye

To determine how many seeds were required to be sown on the growing medium to provide statistical data for the experiment, a germination assay was performed with each seed type on a general plant medium, Murashige and Skoog 30 (MS30) (Murashige and Skoog, 1962).

Solid MS30 medium (4.4 g/l MS macro and micro nutrients, 30 g/l sucrose, 8 g technical agar, pH 5.6-5.8) was autoclaved for sterilisation (15 min at +121°C) and was poured into 9 cm Petri dishes, prior to solidification at room temperature. Three replicates each of 30 acid-washed seeds of each *Arabidopsis* ecotype and the winter rye were placed on the growing medium and placed into +4°C for stratification. The plates were then transferred to the +24°C growing room with a 14 h photoperiod for 7 days to allow germination.

The number of germinated seeds provided information on the viability of the seeds and allowed the calculation of the number of seeds required for the experiment.

2.3 Growth of *Arabidopsis thaliana* and winter rye in oil-contaminated growth medium

A. thaliana and winter rye seeds were sown in plant pots containing increasing levels of oil-contaminated growing medium, John Innes Seed Compost. A previous attempt at this pot experiment identified that winter vetch did not germinate when sown in the pot with the rye. Thus only rye seeds were included in the contaminated growing medium, rather than the vetch/rye mixture.

Each of the 120 pots was filled with 220 g of the growing medium. The oil-treated samples were set up by adding a percentage volume (with a Gilson pipette) of the car engine oil (Castrol GTX) to the weight of the growing medium. A range from uncontaminated growing medium (0% oil) through 1%, 2.5%, 5%, 7.5% to 10% (v/w) oil-contamination for each of the four seed types were made, using an electric hand food whisk to mix the oil thoroughly into the growing medium. It was decided to mix the oil so that reproducible results from subsequent experiments could be established. A contamination range was also set up with the same oil concentrations but without any seed additions as a negative control.

The individual plant pots were arranged in the 24°C growing room in a randomised block design with four replicate blocks (Figure 8) on page 52.

Pot content	0.0%	1.0%	2.5%	5.0%	7.5%	10.0%

Block 1

Negative control	0	1	2.5	5	7.5	10
Rye	10	7.5	5	2.5	1	0
Columbia 0	1	0	7.5	10	2.5	5
Columbia 4	5	10	1	7.5	0	2.5
GM Columbia 4	7.5	2.5	10	0	5	1

Block 2

Columbia 4	7.5	1	10	2.5	5	0
Negative control	1	5	0	10	2.5	7.5
GM Columbia 4	10	0	7.5	5	1	2.5
Columbia 0	2.5	7.5	5	0	10	1
Rye	5	10	1	7.5	0	2.5

Block 3

GM Columbia 4	5	1	0	7.5	2.5	10
Columbia 0	0	5	10	2.5	1	7.5
Rye	7.5	0	2.5	5	10	1
Negative control	10	2.5	7.5	1	0	5
Columbia 4	1	10	5	0	7.5	2.5

Block 4

Columbia 0	10	2.5	1	7.5	5	0
GM Columbia 4	5	10	2.5	1	0	7.5
Columbia 4	1	0	7.5	5	2.5	10
Rye	2.5	1	0	10	7.5	5
Negative control	5	7.5	10	0	1	2.5

Figure 8 Randomised block design of the experiment

Within each block the pots with the four seed types and the negative control were randomised. Within each seed type the position of the oil concentrations were also randomised.

Throughout the experiment the pots were watered until they reached field capacity. The moisture content of the growing medium was measured each day using the Delta T HH2 Moisture Meter probe. Moisture content was also analysed at the end of the experiment using a gravimetric protocol (Black, 1965). Samples of

fresh weight from the negative controls were measured, before placing them in incubation at 105°C for 3 days and determining the volume of water by the loss of weight of the growing medium. The moisture meter data were recorded throughout the duration of the experiment and the appropriate statistical analyses used on the final reading, providing data for comparison with the samples from the gravimetric method.

2.4 Assessment of the affect of oil on the growth of winter rye

To analyse how the presence of oil added to the growing medium affected the growth of winter rye, the difference between the fresh and dry weight were analysed. To the growing medium 60 winter rye seeds (Cotswold Seeds Ltd.) were added to ensure germination of at least 20 of the seeds and were sterilised as mentioned previously.

The winter rye was harvested after 3 weeks' growth at 24°C (+/- 2°C) as the grass was outgrowing the plant pots. The stems of the grass were separated from their roots by harvesting above the level of the growing medium and the wet weight of the plant material was recorded immediately using a top-pan balance. The stems were placed in paper bags and were placed in an 80°C oven for 3 days to dry. The bags containing the plant material were re-weighed and the dry weight of the winter rye determined.

The measurements were used to statistically determine, using ANOVA and regression, if the presence of oil had an affect on the growth of the grass. The root morphology of the winter rye was also analysed once the excess growth medium was washed away using deionised water. Any signs of root damaged were noted, establishing if the concentration of oil was the cause of the effect.

2.5 RNA extraction from *Arabidopsis thaliana*

Arabidopsis thaliana ecotypes Columbia 0 and Columbia 4 seeds (HRI and Coventry University respectively) were sown in the growing medium. RNA was extracted from harvested plants so that it could be used in microarray analysis. Following the growth and harvest of *Arabidopsis thaliana*, plant material from each pot was flash frozen separately in liquid nitrogen to prevent RNA degradation.

The RNeasy Mini Protocol was used for the isolation of total RNA from plant cells and tissues. ß-Mercaptoethanol (ß-ME) was added to Buffer RLT (10 µl ß-ME was added to 1 ml RLT Buffer). Buffer RPE was also prepared by adding 4 volumes of 96-100% ethanol to provide a working solution.

100 mg of the flash-frozen plant material was weighed out and ground with a pestle and mortar in the presence of liquid nitrogen. The powder was transferred to a liquid-nitrogen-cooled 2 ml microcentrifuge tube. The liquid nitrogen was allowed to evaporate

but the tissue was not allowed to thaw out. To the ground tissue, 450 µl of Buffer ß-ME-RLT was added and the mixture was vortexed vigorously. The lysate was pipetted directly onto a QIAshredder spin column and the column was centrifuged at 13,000 *x g* for 2 min. The supernatant from the flow-through was transferred to a new microcentrifuge tube without disturbing the pelleted cell-debris. A 0.5 volume of 96-100% ethanol was added to the cleared lysate and the sample was mixed immediately by pipetting. The sample *ca.* 650 µl was applied to an RNeasy mini column (to bind the RNA to the membrane as the sample progressed through the wash stages of the protocol) and the tube was centrifuged for 15 s at 10,000 *x g* and the flow-through discarded. The column was washed with 700 µl Buffer RW1. The flow-through and collection tube were discarded. The RNeasy column was transferred into a new 2 ml collection tube and 500 µl Buffer RPE was pipetted onto the column. The tube was centrifuged for 15 s at 10,000 *x g* to wash the column and the flow-through was discarded. An additional 500 µl Buffer RPE was added to the RNeasy column and the tube was centrifuged at 10,000 *x g* for 2 min to dry the silica-gel membrane. A fresh 2 ml collection tube was placed on the RNAeasy column and re-centrifuged for 1 min at 13,000 *x g* to ensure that no ethanol was carried over during elution. To elute the RNA, the RNeasy column was transferred to a new 1.5 ml collection tube and 30 µl RNase-free water was added. The tube was left to stand at room temperature for 1 min and

centrifuged at 10,000 *x g* for 1 min. The concentration of RNA in samples was determined using the NanoDrop® Spectrophotometer.

2.6 RNA purification using QIAquick PCR purification kit protocol

To clean the RNA, 5 volumes of Buffer PB were added to 1 volume of the RNA sample and mixed. The sample was applied to a QIAquick spin column (like the RNeasy column, RNA bound to the membrane to prevent it being washed away during the cleaning process of the protocol) and centrifuged at 13,000 *x g* for 1 min. The flow-through was discarded and the QIAquick columns were returned to the collection tubes. The samples were washed with 0.75 ml Buffer PE and the columns re-centrifuged for an additional minute to discard any residual ethanol. The columns were placed in a clean 1.5 ml microcentrifuge tube and 30 µl elution buffer was added to the centre of the QIAquick membrane to elute the RNA. The columns were left to stand for a minute at room temperature and then centrifuged at 13,000 *x g* for 1 min.

2.7 RNA gel electrophoresis

To assess whether the RNA extraction had been successful, the samples were analysed on a 1.3% formaldehyde agarose gel. The gel tray was pre-prepared by soaking it in EDTA and further

cleaned with RNase Away so ribonucleases were not present to denature the RNA.

Into an RNase free 250 ml flask, 0.78 g Agarose (1%) and 52.5 ml DEPC treated water were added and the agarose melted using a microwave. The flask was transferred to a water bath at 55°C and held for 30 min to ensure that the agarose was not too hot when the formaldehyde was added. To avoid exposure to toxic fumes, 1 ml of formaldehyde and 6 ml of 10x MOPS were added to the agarose in a fume cupboard. The flask contents were mixed gently and poured into the gel tray. The gel was left to set in the fume cupboard for 1 h. The RNA samples were prepared for loading by mixing 5 µl RNA with 2 µl 10x MOPS, 3.5 µl formaldehyde, 10 µl deionised formamide and 1 µl of ethidium bromide (10 ng ml^{-1}). The gel was run at 50 V for 3 h and the bands viewed using UV illumination.

2.8 Ethanol precipitation of RNA extraction

RNA extracted from both the control and treated *A. thaliana* samples was concentrated by ethanol precipitation. To each of the samples, 1/10 volume of 3M NaCl and 2 volumes of absolute ethanol were added and mixed thoroughly. The samples were held overnight at –20°C and centrifuged at 13,000 *x g* for 10 min at 4°C. The supernatant was discarded and the pellet was covered with 500 µl of 70% ethanol and re-centrifuged at 13,000 *x g* for 7 min at 4°C.

The supernatant was discarded carefully and the pellet was left to air dry. The pellet was resuspended in RNase-free water and the concentration was adjusted to 10 µg $µl^{-1}$ with 10 µl of RNase free water. To check the quality of the RNA, 1 µl of each sample was diluted with 9 µl of RNase free water and analysed using the Nanodrop® system and by formaldehyde agarose gel electrophoresis. Although the quality of the RNA was good, there was insufficient quantity of RNA for the microarray so the samples were pooled together to give adequate RNA to perform the protocol.

2.9.1 RNA amplification for microarray analysis (MessageAmp™ II aRNA)

Prior to carrying out the protocol, the aRNA wash buffer was prepared by adding 24 ml 100% ethanol. For amplification 10 µl of total RNA was mixed with 1 µl T7 Oligo(dT) primer in sterile non-stick, RNase-free 0.5 ml microfuge tube. Nuclease-free water (1 µl) was added to bring the total volume to 12 µl. The samples were vortexed briefly to mix, centrifuged to bring the contents of the tube to the bottom and incubated at 70°C for 10 min.

Following incubation, the RNA samples were placed on ice. In a second nuclease-free tube, the reverse transcriptase master mix was prepared at room temperature. To this 2 µl of 10x First Strand Buffer; 1 µl of ribonuclease inhibitor; 4 µl of dNTP mix and 1 µl of reverse transcriptase were added per sample. The master mix was

vortexed, centrifuged for *ca*. 5 s to bring the mix to the bottom of the tube and placed on ice. To each of the RNA samples, 8 µl of the reverse transcription master mix was added, mixed well and placed at 42°C for 2 h. After incubation each sample was centrifuged briefly for *ca*. 5 s to collect the reaction at the bottom of the tube and the samples placed on ice.

2.9.2 Second strand cDNA synthesis

In a microfuge tube 20 µl of cDNA sample from the previous step 2.9.1 was mixed with 63 µl of nuclease-free water, 10 µl of 10x Second Strand Buffer, 4 µl of dNTP mix, 2 µl of DNA polymerase and 1 µl of RNase H and mixed gently. Each sample was incubated at 16°C for 2 h before purification of the cDNA.

2.9.3 cDNA purification

Nuclease-free water was preheated to 50°C for *ca*. 10 minutes. The cDNA filter cartridge for each cDNA sample was equilibrated by adding 50 µl of cDNA binding buffer to the filter in the cDNA filter cartridges and incubated at room temperature for 5 min. cDNA binding buffer (250 µl) was added to each sample and mixed thoroughly by repeated pipetting. The mixtures were applied to the cDNA filter cartridge and centrifuged at 10,000 *x g* for 1 min. The flow-through was discarded and washed with 500 µl cDNA wash

buffer and centrifuged for 1 min at 10,000 *x g*. The flow-through was discarded and the cDNA filter cartridge was re-centrifuged for an additional minute to remove any remaining traces of ethanol. The cDNA filter cartridges were then transferred to cDNA elution tubes (1.5 ml). Nuclease-free water preheated to 50°C (10 µl) was pipetted to the centre of the cDNA filter cartridge. The cartridges were left at room temperature for 2 minutes and then centrifuged for *ca*. 1.5 min at 10,000 *x g*. A second elution using 10 µl nuclease-free water was used to ensure that the double stranded cDNA had eluated (*ca*. 16 µl). The volume of the cDNA samples were adjusted to 16 µl and placed on ice.

2.9.4 *In vitro* transcription to synthesise aRNA.

To reduce pipetting error and contamination the 40 µl transcription reaction components were combined in a master mix at room temperature. This mix included per sample 16 µl of double-stranded cDNA, 4 µl of T7 ATP Soln (75 mM), 4 µl of T7 CTP Soln (75 mM), 4 µl of T7 GTP Soln (75 mM), 4 µl of T7 UTP Soln (75 mM), 4 µl of T7 10x reaction buffer and 4 µl of enzyme mix. The mixture was pipetted gently and the tube centrifuged briefly (*ca*. 5 s) to collect the reaction mix. The sample was incubated for 14 h at 37°C. For removal of the template cDNA from the aRNA, each sample was treated with 2 µl DNase I and incubated at 37°C for 30

min. aRNA was purified by the addition of 60 µl elution solution to each tube, making the total volume 100 µl.

2.9.5 aRNA purification

To each sample, 350 µl aRNA binding buffer was added and mixed, then 250 µl of 100% ethanol was added. Immediately the mixture was applied to the centre of the aRNA filter cartridges and centrifuged for 1 min at 10,000 *x g*. The flow-through was discarded and the aRNA filter cartridge was replaced back into the aRNA collection tubes. The filter cartridge was washed with 650 µl aRNA wash buffer by centrifugation at 10,000 *x g* for 1 min. The flow-through was discarded and the aRNA filter cartridge was centrifuged for an addition minute to remove traces of ethanol. The filter cartridge was transferred to a fresh aRNA collection tubes. To elute the aRNA from the filter cartridges, 50 µl of preheated nuclease-free water was added to the centre of the aRNA filter cartridge and incubated at 50°C. The cartridge was left at room temperature for 2 min and centrifuged at 10,000 *x g* for *ca.* 1.5 minutes. The elution was repeated using an additional 50 µl of nuclease-free water, ensuring the aRNA had transferred to the 100 µl elution solution in the bottom of the aRNA collection tube.

2.9.6 Concentration of the purified aRNA

Samples were precipitated using 5M NH_4OAc and ethanol. 1/10 volume of 5M NH_4OAc (*ca.* 10 µl) and 2.5 volumes of 100% ethanol were added, the samples were mixed well and incubated at -20°C for 30 min. The samples were centrifuged at 13,000 *x g* for 15 min at 4°C and the supernatant was removed carefully and discarded. The pellet was washed with 500 µl of 70% ethanol, centrifuged and the ethanol was discarded. Remaining traces of ethanol was removed by centrifugation and aspiration using a fine tipped pipette. The pellets were air dried and resuspended in 30 µl nuclease-free water.

2.9.7 aRNA storage

The samples were stored in aliquots of 5-20 µl at -80°C prior to labelling for microarray studies to minimise repeated freeze-thawing and damage to the sample.

2.10.1 Second round amplification of aRNA using MessageAmp™ II aRNA

To generate additional aRNA from the first procedure using the MessageAmp™ II aRNA kit, the reagents were used in a similar fashion in a second round of amplification.

2.10.2 Synthesis of first stand cDNA (Second round)

From the purified aRNA, 2 µg of the sample was pipetted into sterile RNase-free microfuge tube and 2 µl of Second Round Primers were added, with the volume made up to 12 µl using nuclease-free water. The protocol was followed as previously until the completion of the 2 h incubation at 42°C. Following incubation, the sample was re-centrifuged before adding 1µl RNase H to the reaction. This sample was mixed and incubated for 30 min at 37°C.

2.10.3 Synthesis of the second strand (Second round)

To the sample, 5 µl T7 Oligo(dT) Primer was added and the sample was incubated at 70°C for 10 min. To this, 58 µl of nuclease-free water, 10 µl of 10x Second Strand Buffer, 4 µl of dNTP mix and 2 µl of DNA polymerase were added. The sample was vortexed and centrifuged briefly to bring the reagents to the bottom of the tube, and incubated at 16°C for 2 h. cDNA purification and aRNA synthesis were completed as previously described, concluding with the storage of the aRNA at -80°C.

2.11 Labelling of cDNA for microarray analysis

Once completion of the amplification of RNA had occurred, it was labelled to promote the fluorescence of the microarray. From

the amplified RNA, 10 µl was dispensed into a microcentrifuge tube together with 3 µl of random hexamers. The RNA was denatured at 65°C for 10 min and annealed with the hexamers during the 10 min incubation at room temperature. The annealing process was stopped by placing the tube on ice for 2 min. A mix of 5.0 µl of 5x RT reaction buffer, 2.0 µl of dNTPs, 2.0 µl of Cy3-dCTP or Cy5-dCTP (1mM), 2.0 µl of 0.1M DTT and 1.0 µl of Superscript II (200 U, Invitrogen) was added to the tube and mixed gently before bringing the contents to the bottom of the tube. The mix was incubated at 42°C for 2 h to allow the incorporation of the Cy3 and Cy5 dyes with the RNA. The reaction was terminated with 6.25 µl of 1M NaOH prior to a second incubation at 65°C for 10 min. Neutralisation of the reaction was made by adding 6.25 µl of 1M HCl and 200 µl of TE buffer. Any unincorporated dNTPs, fluorescent dyes and random hexamers were removed using the Qiagen PCR Purification kit. The resulting cDNA was eluted in 30 µl of nuclease-free water that had been pre-heated to 70°C. The concentration of the cDNA was determined using NanoDrop® analysis and equal volumes of Cy3 and Cy5 was combined together in one tube. The excess nuclease-free water was evaporated until the cDNA was almost dry. The labelled cDNA was eluted in 60 µl of a formamide-based hybridisation buffer (25% formamide, 5x SSC, 0.1% SDS, 0.5 mg ml^{-1} yeast tRNA, RNase-free water) and stored at –20°C in preparation for use.

2.12 Hybridisation of the microarray slides

To prepare the microarray slides for analysis they were hybridised so the cDNA would bond with the spots. The prehybridisation buffer (5x SSC, 0.1% SDS, 1% BSA, RNase-free water) was warmed to 42°C for *ca.* 45 min. The slides were immersed in prehybridisation buffer for an hour and were washed five times with Milli-Q water. A final wash with isopropanol, followed by the removal of the excess moisture by centrifugation at 500 *x g* for 2 min. 60 µl of the labelled cDNA was pipetted onto the microarray slide and a cover slip was lowered gently onto the slide. The slide was placed in a hybridisation chamber containing Milli-Q water to prevent the slide drying out. The chamber was transferred to a 42°C incubator overnight.

Wash buffers were prepared to remove the hybridisation buffer from the microarray slides. Wash buffer 1 (40 ml 2x SSC, 4 ml 0.1% SDS), wash buffer 2 (20 ml 1x SSC) and wash buffer 3 (2ml 0.1x SSC) were made to a volume of 400 ml each and were preheated to 30°C. The microarray slide cover slips were removed and the slides were washed with 200 ml of wash buffer 1 for 5 min at room temperature. The same procedure followed with wash buffers two and three, prior to the slides drying in centrifugation at 500 *x g* for 2 min. The slides were analysed using the Affymetrix ArrayScanner 428™ before the genes were obtained using GeneSpring (Silicon Genetics). Following fluorescing of the spots onto a grid image, the

spots were lined up with the CATMA grid on the computer screen. The line-up of the grid with the image was performed by eye. The spots were trimmed to avoid including some of the background, which can cause inference. This would have affected the flagging of the data, as complete spots would not have been obtained. This is where more microarray replicates of the data are required so that any non-0 value flags can be checked against other slides, this will prevent the possibility of any false positives/negatives in the data.

2.13 Analysis of plant-growing medium porous pavements

A series of plant-growing medium porous pavements with a Grassblock® surface were used to study the movement of oil and water within the systems over the course of this study. The work fitted in with the other research in the programme and formed part of an overall strategy to study the performance of these systems. The design of these systems was novel and was decided upon after consideration of a series of alternative designs based on metal-framed glass and plastic and systems constructed from traditional building materials such as breezeblocks. All of these systems were rejected on the grounds of practicality and cost and the finally chosen design made use of the product which was marketed by a company funding other work within the research group, the Permavoid box.

Permavoid boxes were originally intended as load bearing, void-forming units for use as a sub-base replacement in porous pavements. However, their strength, light weight and the ease with which they can be joined together lends themselves for use in construction when a light yet strong support system is required. The design required was a robust system in which the drainage could be segregated so as to avoid edge effects. This was necessary because in real scale pavements liner surface/aggregate bed volume ratios are much smaller than in a model system and it was felt that in this scale of model it was necessary to take this into account.

2.14 Construction of the porous pavement rigs with planted surface blocks at Warwick HRI, Wellesbourne

The rigs were constructed using 4 polypropylene Permavoid boxes (708 mm x 354 mm x 150 mm) joined at the ends using nylon cable ties and were arranged to form open-top rigs, as illustrated in Figure 9, on page 68.

Figure 9 Permavoid boxes joined to form an open-top rig

The base of the rig structure consisted of two halves of a Permavoid box, providing support for the sub-base. The rig was lined with a colourless horticultural grade low-density polyethylene (LDPE) liner (0.5 mm) (Figure 10), with holes punched, using a garden fork, in the base to allow drainage. The patterns of the drainage holes were such that two separate pathways of effluent were established. One pattern of drainage holes was in the centre of the LDPE to collect the sample effluent and the other pattern followed the perimeter of the LDPE liner to allow rejection of the effluent that had passed through the system, largely in contact with the outer edges of the sub-base and the LDPE.

Figure 10 LDPE lining the Permavoid rig structure

The sub-base (50 mm crushed granite (Figure 11a) or nominally 50 mm recycled aggregate (Figure 11b) obtained from Coventry University stores and Bardon Recycling respectively) was added to the LDPE liner, filling up to 100mm from the top of the rig structure.

Figure 11a Addition of the granite sub-base material to the rig

Figure 11b Addition of the recycled aggregate sub-base material to the rig

In fact this recycled aggregate was as supplied from the specification given to the contractor and consisted of a roughly 1:1 mix of crushed concrete and crushed clay bricks. Whilst 50 mm was specified the size range that was supplied included particles as small as 5 mm. This was a lesson in itself as it indicated that the specifications in the recycling industry are somewhat more varied than in the virgin aggregate industry.

A layer of non-woven, heat bonded polypropylene/polyethylene geotextile (Terram 1000®) prevented the gravel layer mixing with the sub-base (Figure 12).

Figure 12 Separating the gravel layer from the sub-base

The gravel layer filled the remainder of the rig structure (Figure 13), before being covered by another a layer of geotextile (Figure 14).

Figure 13 The gravel layer

Figure 14 An additional layer of geotextile

Four Grassblocks (Grass Concrete Limited) were placed on top of six of the rigs (Figure 15) and four polyethylene INTEGRA 500 surfacing blocks (Permavoid Limited) were placed on top of the remaining three rigs (Figure 16) and the voids filled with a 50:50 mixture of John Innes No. 1 and gravel (Figure 17).

Figure 15 Permavoid rig with a Grassblock® surface

Figure 16 Permavoid rig with a INTEGRA 500 surface

Figure 17 Completion of the rig with growing medium filling the surfacing blocks' voids

Figure 18 shows an illustrated cross-section diagram following the completion of the construction of the rig structure.

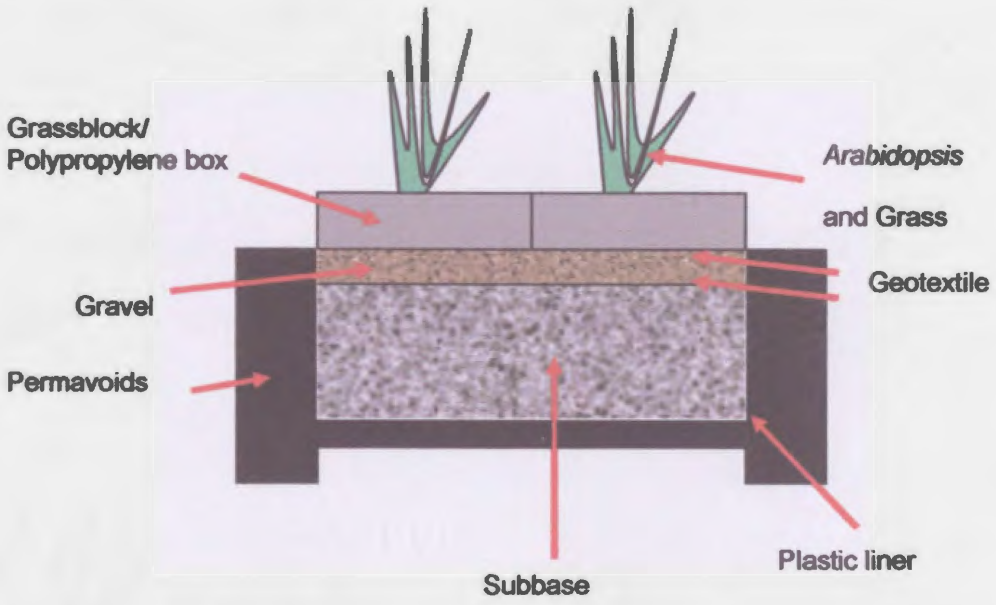


Figure 18 A cross-sectional diagram of a rig

2.14.2 Addition of used engine oil to the plant-growing medium porous pavement rigs

Winter vetch and winter rye seeds (Cotswold Seeds Ltd.) were sown in the outer Grassblock® voids and *Arabidopsis thaliana* (ecotype Columbia 0) (Warwick HRI, Wellesbourne) was planted in the centre void.

Once the seeds had germinated and established themselves within the growing medium, 100 ml of used engine oil (Freelander diesel engine, Landrover UK) was applied to the growing medium in the four large square voids and the five central voids subsequent to the collection of a base line, oil-free sample. 100 ml of used engine oil was applied to the rigs as this has been shown to give measurable oil concentrations. The volume of oil was applied all at

once whereas it would have been better to apply the oil to the growing medium over the duration of the experiment. However, the *Arabidopsis* grew slowly and experimental time ran out. Reflecting on this, perhaps only the vetch and rye should have been planted in the surfacing blocks, ensuring plant growth in each void.

At the time of construction, rigs containing (a) no plants but with oil addition, and (b) no oil addition but with plants, should have been constructed to provide a negative control and a positive control respectively. The original aim of the construction of the rigs was to compare the sub-base materials on their oil degradation but during microscopy and oil content analysis of the effluent samples, the microbiological and oil retention properties were influenced by the presence of the growing medium, thus these results were not expected.

One-litre samples were collected following rainfall events that occurred naturally with the weather in the region. A Pyrex® dish was situated in the centre of the base of the rig, so only effluent from the centre of the rig's sub-base region was obtained. The effluent was analysed for oil content using the Horiba OCMA-300 Oil Content Analyser, an instrument that is based on solvent extraction into a proprietary, totally halogenated, solvent and IR spectrometry. Microscopic eukaryotic species present in the samples were studied by light microscopy at 100x and 200x magnifications following the sample preparation step outlined on the next page:

50 ml of the effluent from each rainfall was centrifuged at 1500 *x g* for 5 min and 45 ml of the supernatant was discarded. From the remaining 5 ml of effluent, 100 µl was pipetted onto a microscope slide and analysed, recording microorganism numbers as they were observed. Microorganisms were identified by comparing those observed on the microscope slide with the photographs in 'Free-Living Freshwater Protozoa: a Colour Guide' (Patterson and Hedley, 1992) and by crosschecking with Dr S. Coupe for confirmation that the species identification was accurate. Microorganism species were recorded for each rainfall sample and compared between the sub-base types.

3.0 Results

3.0 Results

This section provides the results of the experiments from this project. The germination of *Arabidopsis* and the winter rye were assessed, determining the growth inhibition effects of the presence of oil using statistical analysis. Moisture content of the negative controls was analysed using both the moisture meter and the gravimetric methods and these were compared to determine the most suitable method of analysis. Effluent samples obtained from the outdoor rigs were analysed for microbiological data and oil content within the effluent.

3.1 Germination of *Arabidopsis thaliana* and winter rye

Table 1 shows the seed germination following stratification and 1 week incubation at +24°C (+/- 2°C).

Table 1 Results of the germination efficiency assay on *Arabidopsis thaliana* and winter rye

Seed type	Mean germination (% ± se)
A. thaliana Columbia 0	79 ± 2.9
A. thaliana Columbia 4	53 ± 3.3
A. thaliana GM Columbia 4	12 ± 2.2
Winter rye	52 ± 2.9

From the results it can be seen that the Col-0 ecotype had a reasonably high germination percentage, with approximately half of the Col-4 seeds germinating, and the transgenic Col-4 showing very low germination.

3.2 Growth of *Arabidopsis thaliana* in oil-contaminated growing medium

The growth of the three *Arabidopsis thaliana* ecotypes in the oil-contaminated growing medium was studied. It was shown that growth occurred in all plant pots that were not treated with oil (0%); less growth occurred in pots containing 1% oil contamination and little, if any, in pots containing 2.5% or greater oil contamination.

Analysis of variance (ANOVA) determined that oil had a significant effect on the growth of Col-0, with the fresh and dry weight changes giving p-values of 0.02 and 0.007 respectively

(Appendix I). Regression analysis of Col-0 was not significant for both fresh ($p = 0.227$) and dry weight ($p = 0.19$) (Appendices II and III). Figures 19a and 19b give an indication of the change of weight between the treatments.

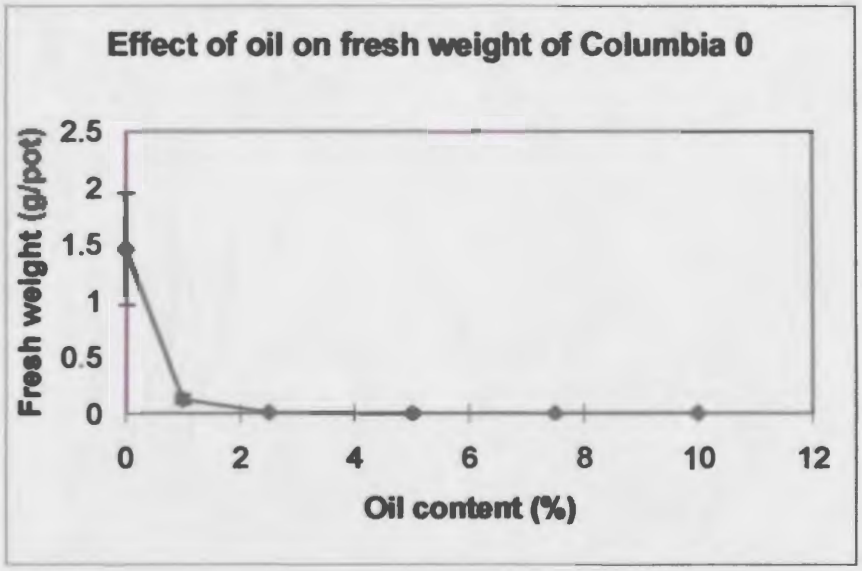


Figure 19a Change in weight of fresh plant material from Col 0

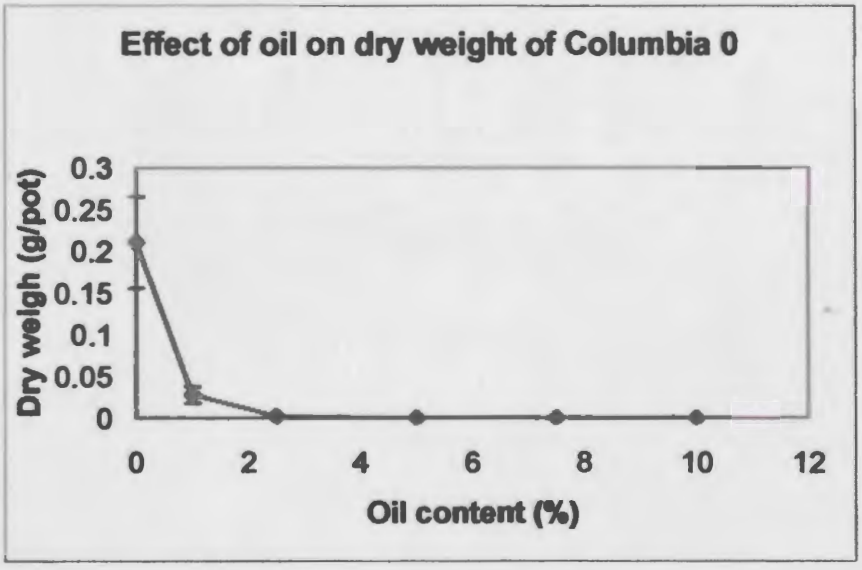


Figure 19b Change in weight of dry plant material from Col-0

The ANOVA of the Col-4 ecotype showed that oil had a significant effect on the dry weight change with a p-value of 0.04, but an insignificant effect on the fresh weight (p = 0.21) (Appendix IV). Regression analysis of the fresh and dry weight determined that there was no significance in the plant material weight as the oil concentrations increased in the growing medium (Appendices V and VI). Figures 20a and 20b show the decrease in plant tissue weight as the oil concentrations increased.

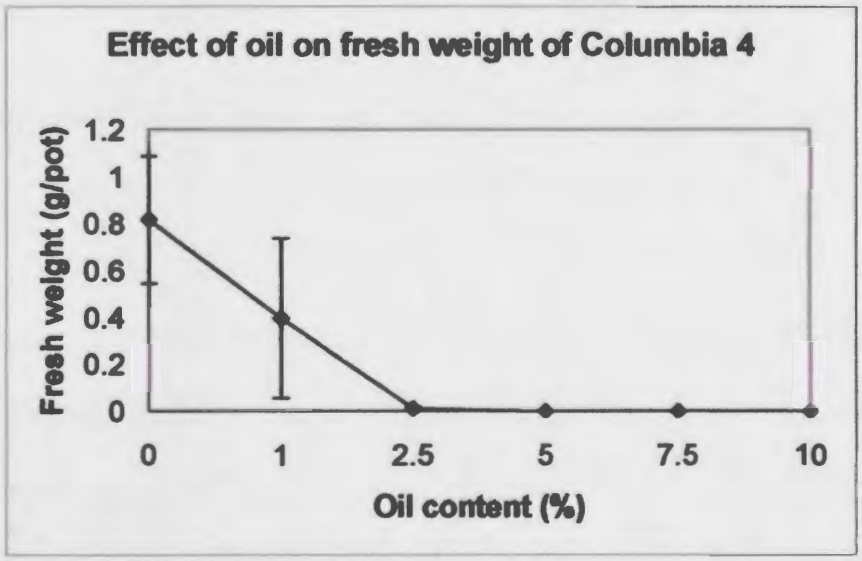


Figure 20a Change in weight of fresh plant material from Col-4

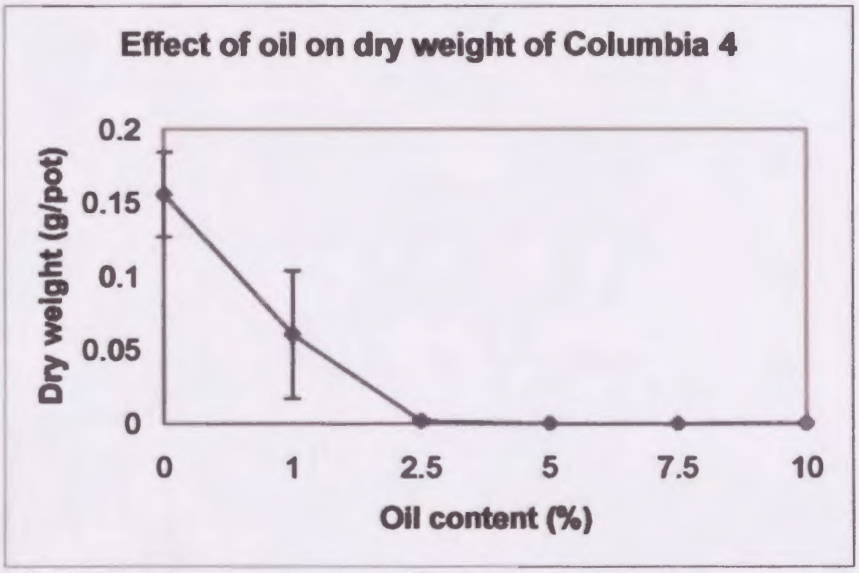


Figure 20b Change in weight of the dry plant material from Col-4

The ANOVA of the transgenic Col-4 ecotype also showed that oil had a significant effect on growth. Change in the fresh and dry weight had p-values of 0.046 and 0.012 respectively (Appendix VII). Regression analysis of the fresh and dry weight determined that there was no significance in the plant material weight (Appendices VIII and IX). Figures 21a and 21b show the decrease in plant material weight as the oil concentrations in the pots increased.

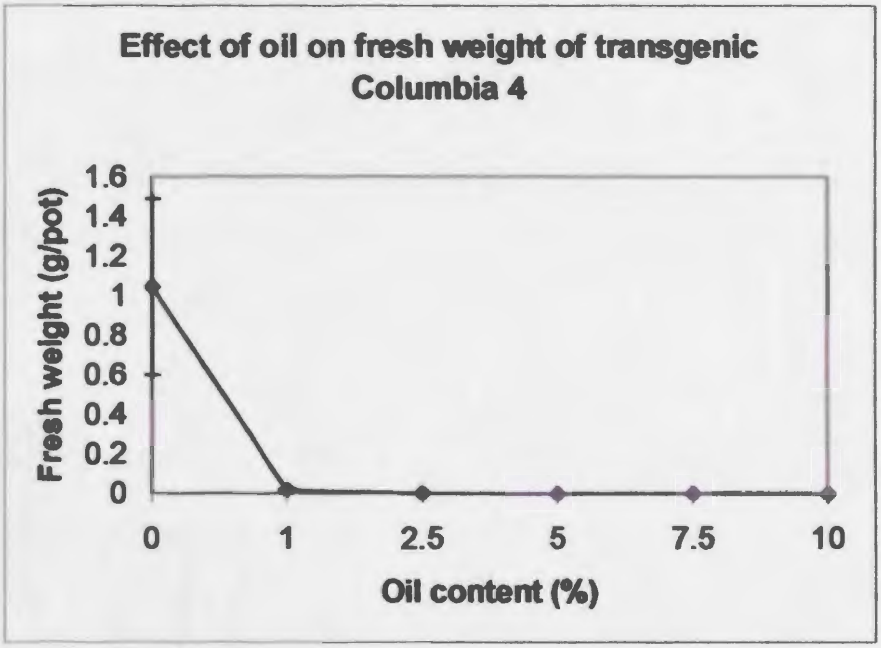


Figure 21a Change in weight of the fresh plant material from the transgenic Col-4 ecotype

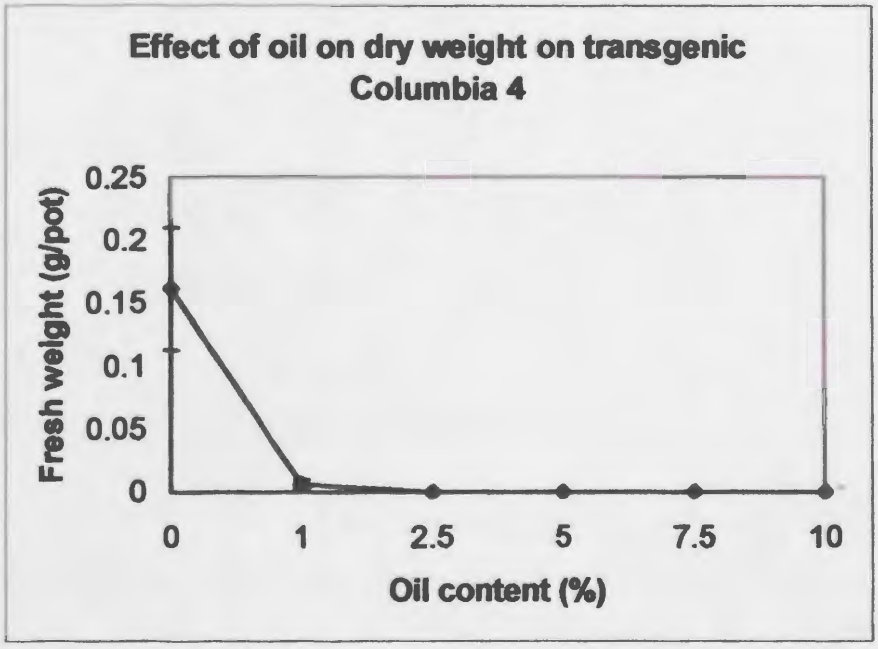


Figure 21b Change in weight of the dry plant material from the transgenic Col-4 ecotype

3.3 Comparison of the *Arabidopsis thaliana* ecotypes, subsequent to their growth in oil-contaminated growing medium

The ecotypes were compared to each other following their growth, using an ANOVA of two factors with replication (Appendix X). The ANOVA established that the oil had a significant effect on the decrease in growth as the concentration of oil increased. However, there was no significant difference between the ecotypes' growth at each oil concentration level. This was true for both the fresh and dry weight, with p-values of $4.6E^{-05}$ and $1.89E^{-07}$ for the significant decreases in growth, and p-values of 0.718 and 0.618 of insignificant different between the ecotypes' growth, respectively.

3.4 Leaf area of *Arabidopsis thaliana* ecotypes

Each *Arabidopsis* plant grown in the oil-contaminated growing medium was measured by the WinDIAS Image Analysis System (Delta-T Devices) to obtain leaf area. Total leaf area was determined for each pot and ANOVA was performed for the Col-0 and Col-4 ecotypes. P-values resulting from ANOVA showed that oil had a significant effect on the growth of Col-0 plants ($p = 0.006$) (Appendix XV) but not on Col-4 plants ($p = 0.248$) (Appendix XVI), as there was more growth in the plant pots containing 1% oil contamination than the pots containing no oil (0%) (Appendix XVIII). As growth only occurred in 0% and 1% oil-contaminated pots of the

transgenic Col-4, a paired t-test was applied to the data (Appendix XIX). This analysis found that there was no significant effect of the oil on growth ($p = 0.088$).

3.5 Weight loss on drying of winter rye following growth in oil-contaminated growing medium

Following growth of the winter rye, the sheaths of grass were harvested above growing medium level and the fresh weight from the different treatments were recorded. The rye was dried at 80°C for 3 days and the dry weight were also recorded. Analysis of variance confirmed the fresh and dry plant material had p-values of 0.0002 and 0.0003 that were obtained respectively (Appendix XI), stating that oil had significantly affected the growth of the rye. This was also proved using regression analysis on the data, which produced p-values of 0.002 for the fresh material (Appendix XII) and 0.001 for the dry material (Appendix XIII), and can be seen in Figures 22a and 22b.

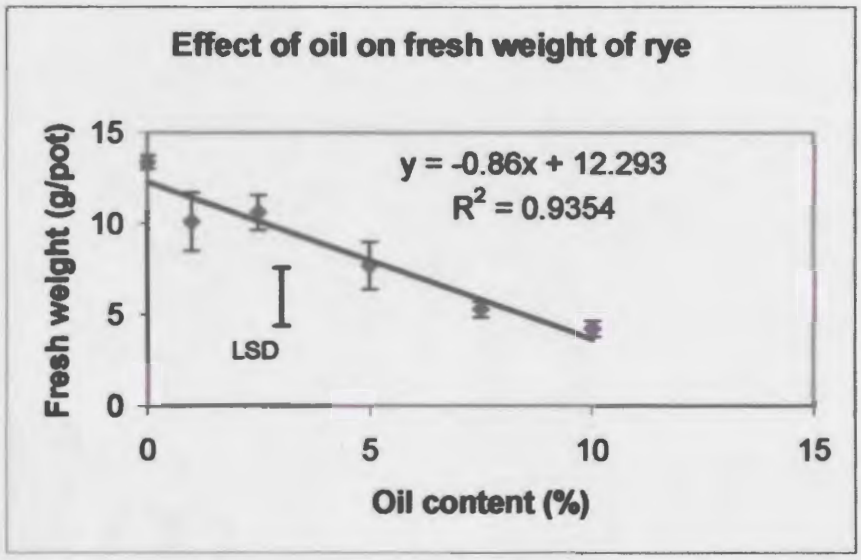


Figure 22a The effect of oil on the fresh weight of winter rye

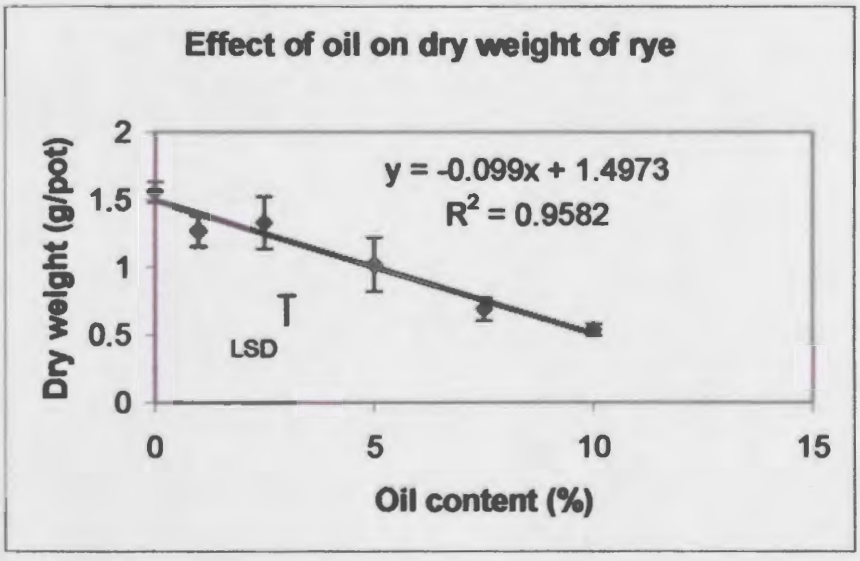


Figure 22b The effect of oil on the dry weight of winter rye.

The difference in moisture content of the rye was also measured.

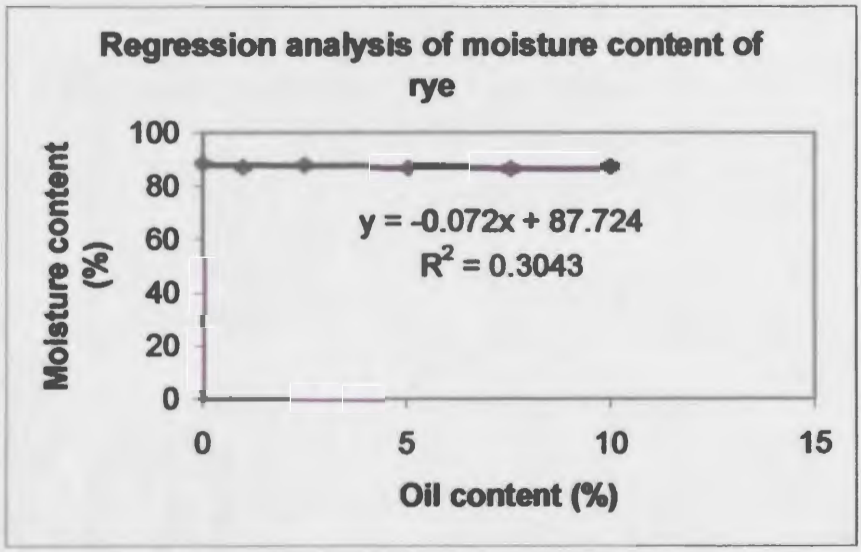


Figure 23 Regression analysis of moisture content from rye

Using regression analysis (Figure 23), the data determined that the oil did not affect the moisture content of the rye ($p = 0.257$), (Appendix XIV).

Following the removal of excess growing medium from the roots, it was possible to observe that there was little, if any, damage. The roots possessed a creamy-white colour and had an exudation pattern within the growing medium of each pot.

3.6 Moisture change in the oil-contaminated growing medium

Subsequent to the completion of growth, the moisture content of the negative controls was measured using the Delta-T HH2 moisture meter and the gravimetric method.

The ANOVA of the moisture meter data determined that the oil had no significant effect on the moisture content of the growing medium (p = 0.466) and the regression analysis determined a similar result (p = 0.402), shown in Figure 24 (Appendix XX).

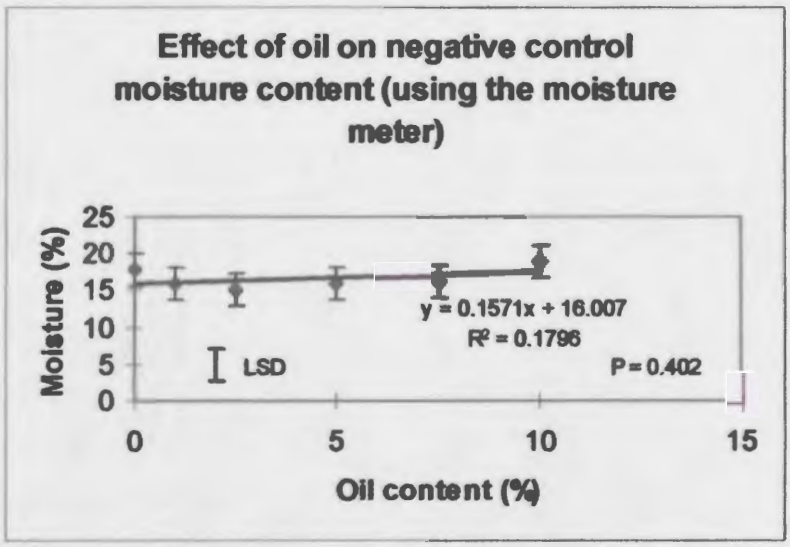


Figure 24 Regression analysis of final moisture content of the negative controls

The ANOVA of the gravimetric data determined that the presence of oil had no significant effect on the moisture content of the growing medium (p = 0.5) (Appendix XXI). Figure 25 shows the moisture content of the growing medium, following regression analysis of the data (Appendix XXII and XXIII).

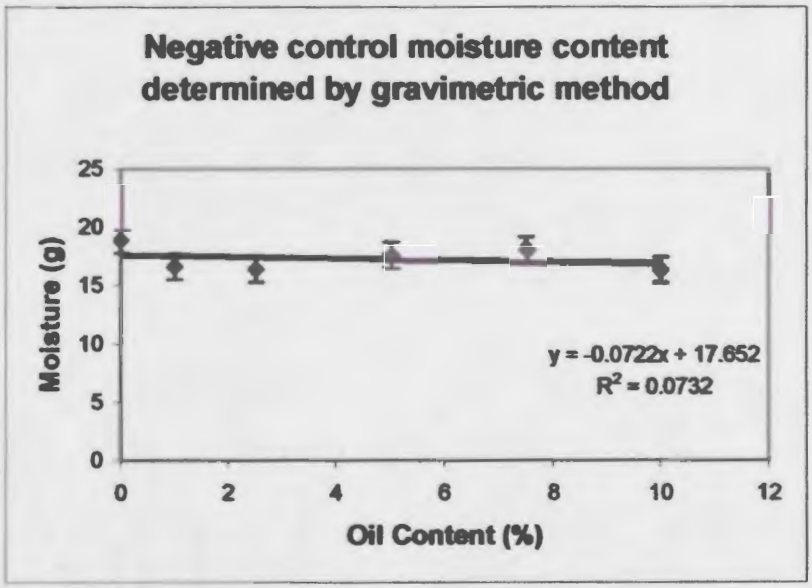


Figure 25 Regression analysis of the final moisture content data using the gravimetric method

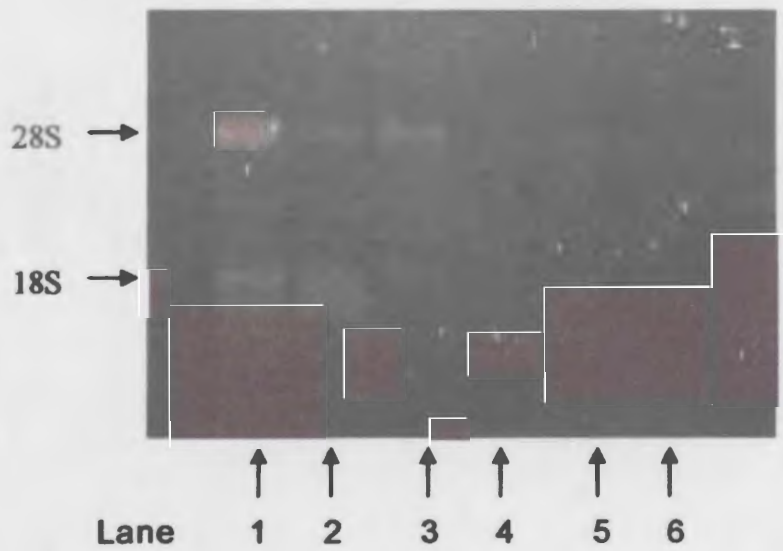
Comparing the data obtained from the moisture meter and the gravimetric method, a paired t-test determined that there was no significant difference ($p = 0.411$) between the methods of moisture determination (Appendix XXIV).

This data concludes that oil does not have a significant effect on the moisture content of the growing medium but significantly affects the growth of the plants within the growing medium.

3.7 RNA extraction from *Arabidopsis thaliana*

RNA was extracted from the harvested *Arabidopsis* and was analysed by formaldehyde agarose gel electrophoresis and using the NanoDrop® spectrophotometer. The agarose gel (Figure 26)

showed two bands for each of the samples, the bands being 28S and 18S. This showed that the RNA was intact and not denatured. However, extraction was poor and levels were low, thus the separate extractions were pooled together to provide greater concentrations of RNA.



Lane	*A. thaliana* line and oil conc. (%)
1	Col 4 in 0% oil contamination
2	GM Col 4 in 0% oil contamination
3	Col 4 in 1% oil contamination
4	GM Col 4 in 1% oil contamination
5	Col 4 in 2.5% oil contamination
6	GM Col4 in 2.5% oil contamination

Figure 26 Formaldehyde agarose gel electrophoresis of *Arabidopsis* RNA

The NanoDrop® was used to measure the concentration of RNA in the samples and the results can be seen in Table 2. The

greater the concentration of oil in the peat the less the concentration of RNA extracted.

Table 2 *Arabidopsis* RNA concentrations recorded using the NanoDrop® after formaldehyde agarose gel electrophoresis

	After Extraction		1st Amplification and Precipitation		2nd Amplification and Precipitation	
Sample	Volume µl	Total µg	Volume µl	Total µg	Volume µl	Total µg
Uncontaminated	191	24.67	10	16.6	100	223.2
GM Uncontaminated	191	24.02	10	16.8	100	88.6
1%	299	19.44	10	10.4	100	2.5
GM 1%	399	5.04	10	3.4	100	4.0
2.5%	141	5.44	10	6.3	100	167.1
GM 2.5%	91	6.56	10	4.4	100	7.1

The concentrations of extracted, intact RNA of Col-0 and non-transgenic Col-4 ecotypes were sufficient to use in microarray analysis provided amplification was used. After the first amplification, there was sufficient RNA in the uncontaminated and the non-transgenic 1% oil contaminated samples to use in microarray analysis (minimum 5 µg required). Following the second amplification, sufficient RNA was obtained from both the non-transgenic and transgenic samples of uncontaminated and 2.5% oil contamination. These samples were used in microarray analysis.

3.8 Microarray analysis of *Arabidopsis thaliana* cDNA

Following hybridisation to the microarray slides with the *Arabidopsis* RNA, the spots were lined up with CATMA grids and intensity images were created. This provided an intensity signal for each gene and its identity. The images were imported into GeneSpring (Silicon Genetics) for statistical analysis. The data was normalised using the Standard Two Colour scenario (Lowess) and log and linear interpretations were set for the data. Standard deviation filtered out flags that had a value greater than 0 and the spots that did not alter in expression. Through t-test analysis at 10%, data showed that 1055 genes had been amplified up to 2-fold, with their p-values ranging from 7.15E-05 to 0.998. The best-amplified genes had p-values between 7.15E-05 and 0.0994. The 20 genes that were most upregulated can be seen in Table 3 on page 93.

Table 3 Best amplified genes from the CATMA microarray

CATMA5a31570	hypothetical protein similar to unknown protein (gb|AAD26867.1)
CATMA1a09620	expressed protein
CATMA4a34735	homeobox protein (ATH1)
CATMA5a45900	transport inhibitor response protein, putative E3 ubiquitin ligase SCF complex F-box subunit
CATMA3a17940	C2 domain-containing protein
CATMA2a20200	SEC14 cytosolic factor, putative / phosphoglyceride transfer protein
CATMA4a31120	prolidase-related contains similarity to prolidase
CATMA1a58840	UbiE/COQ5 methyltransferase family protein
CATMA4a37470	aconitate hydratase, cytoplasmic / citrate hydro-lyase / aconitase (ACO)
CATMA3a14500	exonuclease family protein contains exonuclease domain
CATMA2a29390	tRNA synthetase class I (C) family protein
CATMA4a30580	casein kinase, putative similar to casein kinase I, delta isoform
CATMA2a24170	expressed protein
CATMA5a19910	expressed protein
CATMA4a37600	L-ascorbate peroxidase, putative similar to ascorbate peroxidase
CATMA1a62500	sugar transporter family protein
CATMA3a06150	expressed protein
CATMA1a54520	expressed protein
CATMA5a46470	CCAAT-box binding transcription factor Hap5a, putative
CATMA1a12910	eukaryotic translation initiation factor 5A-1

From the remainder of the 1055 slightly upregulated genes, seven were identified as being responsive to dehydration stress. A list of these seven genes can be seen in Table 4.

Table 4 Upregulated genes related to water stress in *Arabidopsis thaliana*

CATMA1a03260	Dehydration-responsive protein-related, similar to early-responsive to dehydration stress ERD3 protein
CATMA2a38550	Dehydration-responsive family protein similar to early-responsive to dehydration stress ERD3 protein
CATMA3a23300	Dehydration-responsive protein-related, similar to early-responsive to dehydration stress ERD3 protein
CATMA4a16170	Senescence/dehydration-associated protein-related similar to senescence-associated protein 12
CATMA4a37620	Senescence/dehydration-associated protein-related similar to senescence-associated protein 12
CATMA5a12890	Universal stress protein (USP) family protein similar to ER6 protein
CATMA5a45190	Drought-responsive family protein similar to drought-induced mRNA, Di19

3.9 Oil content analysis of the effluent from the Grassblock® rigs from Warwick HRI, Wellesbourne

Effluent was collected from the samples following natural rainfall events, as described earlier in Section 2.14.2. The samples were analysed for the oil content using the Horiba Oil Content Analyser OCMA-300. The control samples were collected (25 June

2004) before used oil was applied to the surface. Each of these samples gave effluent readings below detection. The rainfall samples following the application of the oil, taken on 28 June 2004, 30 June 2004 and 2 July 2004 also gave readings of 0 mg/l of oil present.

On 5 July 2004 the granite and recycled concrete-sub-based rigs with the concrete Grassblock® surfaces gave readings below detection. However, the rigs containing the recycled concrete and the INTEGRA 500 recycled polypropylene surface gave readings between 0.6-1.0 mg/l of oil. This needs to be considered in the context of the 5 mg/l limit put on a class 1 oil interceptor system (Environmental Alliance, 2003).

3.10 Microbiological analysis of the Grassblock® rigs from Warwick HRI, Wellesbourne

The rainfall samples obtained from the rigs at Warwick HRI were analysed to determine if various species of protozoa and metazoa were present. The control samples obtained on the 25th June 2004 contained the species listed in Table 5, on page 96.

Table 5 List of eukaryotic microorganisms found in base line samples (prior to oil addition) from the Grassblock® rigs

Rig Subbase	Eukaryotic Microorganisms Present
Granite	
	Amoeba spp
	Euplotes
	Actinophrys
	Lacrymaria
	Heteromita globosa
	Nematode
	Stylonychia
Recycled Concrete	
	Actinophrys
	Heteromita globosa
	Sacpingoeca
Recycled Concrete and Polypropylene Box	
	Amoeba spp
	Lacrymaria
	Leptopharynx
	Colpoda spp
	Nematode

Following the addition of used oil to the rigs, natural rainfall events occurred prior to taking the effluent samples that had drained through the sub-base. In the effluent samples (28th June 2004) the same microorganisms that were in the control samples recorded were also present in these samples, and several examples of these are shown in Figures 27-31. Additional genera of *Rotifer* (Figure 27) and *Pompholyxophrys* (Figure 28) were found in the concrete

rigs, *Amoeba radiosa* was found in the granite rigs, and *Rotifers* and *Gastrotrich* (Figure 29) were found in the Recycled Materials (RM) rigs.

Figure 27 *Rotifer* (Bruderhof Communities, 2005)

Figure 28 *Pompholyxophrys* (Microscope, 2004)

Figure 29 *Gastrotrich* (McCord and Davison, 2005)

Two days later (30th June 2004) a heavy rainfall occurred and further microbiological species were identified in the effluent samples. Within the granite samples, *Euglypha rotunda*, floating amoeba and *Vannella* were found along with fungal spores and cysts of nuclearia. *Euglypha rotunda, Arcella* and a second species of *Euplotes* (different from that named in the control sample list) were present in the recycled concrete rigs. From the RM rigs included additional species of *Tetrahymena* (Figure 30), *Colpidium* and *Arcella* (Figure 31) were detected along with more fungal spores.

Figure 30 *Tetrahymena* (Microbial Culture Collection, 2004)

Figure 31 *Arcella* (de Oliveira, 2004)

Two days later the next effluent samples were collected. No additional species were found in the samples from the granite rigs,

in comparison to the previous rainfall events. The effluent from the rigs with the concrete sub-bases produced samples containing *Colpidium* and either *Bodo saltans* or *Monosiga* (defining characteristics for the definite species identification were not seen). The RM rig effluent samples contained the species as previously mentioned, with the addition of *Gastrotrich* being identified. Another heavy rainfall occurred on 4th July 2004, effluent samples were collected on the 5th July 2004. An additional species of *Pompholyxophrys* was found with previously mentioned protozoa in the granite rigs. In the effluent from the concrete rigs, *Cyclidium* was discovered with many structures that looked like cysts of nuclearia. Final samples from the RM rigs showed no additional species that had not been identified previously.

3.11 Final sampling, three months after used engine oil application

Final effluent samples were taken from the rigs three months after the previous analysis. Effluent samples showed relatively little change from the analysis on the 5th July 2004. More fungal spores were present in the samples but there were no other microorganisms observable.

3.12 Experiences with new model design

The new model design proved to be relatively easy to construct and in practice it was possible to use the arrangement of the rigs to good effect to make economic use of these relatively expensive products. An initial problem was the fact that the half-Permavoid boxes used as the base to allow drainage bowed downwards under the weight of the aggregate. At first this was considered to be a potential immediate failure point but in practice this was not the case as the "creep" was not continuous.

Whilst the Permavoid boxes are themselves expensive the whole construction was cheap because it could be done without the labour of tradesmen.

The differential drainage system used to overcome edge effects seemed to work to a degree but even at the time of construction it seemed that preferential pathways to the sampling drain could be created. When the half-Permavoid support bowed downwards even greater fears of non-segregation of the two effluents were engendered.

The major problem associated with this system came when consideration had to be given to dismantling and disposal of the contaminated components. Disposal of these systems after heavy oil loading would seem to be very difficult without either risking polluting the ground around the area or having to destroy the Permavoid boxes. This is neither economically or environmentally

sound and in retrospect is perhaps something, which could have been considered in the design phase.

Following a great deal of thought and debate within the research group a new approach to these outdoor model systems has been taken. This is addressed briefly in the discussion section.

4.0 Discussion

4.0 Discussion

This section discusses the work that has taken place in this project, in retrospect of the experiments and the data analysis that discovered if oil had an effect on the growth of *Arabidopsis thaliana* and winter rye, and if planted porous pavement structures provided support in the removal of oil that had been applied to the pervious surface. The structure of this discussion will bring together relevant subjects that have been previously mentioned in the thesis.

As the world becomes more environmentally aware of potential disasters, ways and means of attempting to prevent these catastrophes are becoming important not only in a financial sense but also as a practical sense. In recent decades, technologies and machinery have evolved that are more ecologically friendly and cause less pollution and use less resources. There is, however, an ever-present possibility that a problem could arise, causing an environmental catastrophes at a variety of scales, such as the severe flooding problems mentioned in Section 1.1. Most environmental attention seems to be concerned with major incidents such as tanker spill but the oil pollution issue is an excellent example of the fact that the cumulative effects of numerous small-scale incidents can bring about greater harm than large-scale events. For an incident involving engine oil on a small scale (i.e. a loss of a full sump on a car park), typical methods of

decontaminating the location would include the use of detergents and other cleaning materials that could also harm the environment if used in large quantities and allowed to enter a drainage system. A more insidious problem is the fact that on a typical car park, cars leak around 200 mg of oil onto each square metre every week (Bond, 1999. unpublished Ph.D. thesis). This corresponds to around 120 g per year for each car park bay. This low level of contamination could accumulate if not treated. Car parks drained with traditional positive drainage will inevitably contribute to oil pollution unless an efficient oil interceptor is used.

In systems considered in this work, as with much of the work within the group where the research was carried out, technologies to facilitate the removal of oil are combined with devices designed to retain infiltrate stormwater and thus control flooding. Many factors influence the control of oil in car parks using pervious surfaces. These include drainage system design, acceptable methods of addition of inorganic fertiliser, addition of degrading microorganisms or, in the case of planted systems the selection of appropriate plants. Ultimately a combination of all these may result from this work.

This project attempted to take initial steps towards understanding the effects of the presence of engine oil on plants as model systems to reflect the plants growing on planted parking

surfaces, assessing whether they could be sufficiently oil resistant to play a role in the clean up of polluted planting media and making very preliminary inroads into understanding how they may do this. Previous work on this subject is not profuse in comparison to the amount of work that has been performed on heavy metals and some other chemical contaminants in relation to their effect on plants (as mentioned in Section 1.2) so the information obtained from this work will form a basis for further study.

Prior to the commencement of the project, it was predicted the addition of engine oil to the growing medium in which the plants grew might cause a water availability problem to the plants. This was a reasonable assumption as this may have been the result of the oil binding the growing material particles together and allowing the water to drain through the pot, or that the oil's physical properties repelled the water from the growing medium, is uncertain. Further work on the combination of oil and the growing medium would shed light on this question.

A limitation to the growth of plants in oil-contaminated growing medium would be the distribution of the oil within the growing medium. If the oil and growing medium were not mixed, it would not be certain that the desired oil concentration would be available to the plant. By mixing the oil and medium together, it ensures that each pot has been treated similarly and replicate pots should

produce similar data and significant data analysis. The growing medium and oil was thoroughly mixed, without leaving residues on the mixing equipment so the use of an electric hand whisk provided a relatively clean method. After mixing, the distribution of oil may change within the plant pot, as the oil may be mobile within the growing medium. For the application of oil to be as authentic as leaks on a car park bay from an engine, it may be better to drop the volume of oil required (as mentioned on page 104 at the start of this discussion) onto the growing medium surface. Leaks from an engine would not ensure that the oil was thoroughly mixed into the growing medium, as in the experiment in this project, so this would need to be investigated further if the experiment was to be performed again. This would simulate oil drips onto a planted porous pavement surface and give a more realistic view of how it may occur in an actual car park. Both approaches have their advantages and disadvantages, but as this was the first set of data on *A. thaliana*, it was decided to mix the oil so that readily reproducible experiments could be established.

One of the main objectives of this project was to discover the effect the engine oil had on germination and growth of *Arabidopsis thaliana* and winter rye used in this project.

As the *Arabidopsis thaliana* lines grew in the oil-contaminated growing medium, it could be visualised that the presence of the contaminant inhibited growth and the effect on plant size (and

growth) increased as the percentage of the contamination increased. The pots with plants but no oil (0%) produced healthy-looking plants, with green leaves and a developed flower head after three weeks of growth. The majority of the plants growing in the 1% oil-contaminated growing medium showed stunted growth compared with those at 0% contamination. Leaf area of the plants showed a significant decrease at a probability level of $p < 0.05$, compared with those unaffected by the oil (0%). The plants at 1% oil contamination were not as green as their controls. In pots containing greater concentrations of oil contamination there was little, if any, growth. Growth that did occur did not produce sufficient plant material that could allow significant data analysis. This may be due to the direct effect of the oil on the plants or some indirect effect such as a reduction in the amount of water reaching the plant roots.

Work on *Panicum virgatum, Festuca arundinacea* and *Cajanus cajan* indicated that it was possible for a plant to tolerate oil within the soil, even if growth was affected (Vavrek and Campbell, 2003). With growth of plant material below 2.5% oil contamination in this project, it can be said that *A. thaliana* has limited tolerance to oil-contaminated growing medium, making growth above this oil contamination level almost impossible. With ANOVA and regression analysis on *Arabidopsis*, it was proved that the presence of oil had a negative significant effect on the growth as oil concentrations increased. Any growth that did occur may be the result of limited

localised regions of the growing medium that had not been mixed as well as the rest of the growing medium in the pots. For another method of analysing this problem, it may be worth considering reproducing the experiment in laboratory-prepared growing medium that had oil added to the medium ingredients and by growing the plants in sterile propagating pots with ventilated lids. Within the sterile environment these pots maintain, the plants would receive all the nutrients required for growth, as well as the contamination of the oil treatment, without external influences. Although the plants would not be produced in the way they have been grown in this experiment. The medium would provide both the required nutrients and the oil contaminant for growth. This would be suitable as a controlled laboratory experiment but would not imitate the experiment that has been performed in this thesis. The media prepared for these pots are usually sterile and the absence of bacteria in the medium would not promote remediation of the oil, as would happen in the growing medium. Data obtained from an experiment such as this would provide a base line for the growth of the plant in increasing levels of oil concentration but not on actual treatment as was in this project.

Previously published material on the subject of the effect of the presence of oil-contaminated soils showed that the main effect of the contamination was on growth and size of the plant material. In comparison the results of this project, it can be said that the presence of oil in the growing media had an effect on the growth of

A. thaliana and winter rye, in a similar fashion to other species. Like the salt marsh plant *Spartina alterniflora*, *A. thaliana* and winter rye's biomass and shoot heights decreased significantly as the level of oil contamination increased. Thus the experiment performed for this study can be referred to as a dose-response experiment as increases in the treatment dose had greater effect on the plants.

The work published by Gong *et al.* (2001) also showed that the biomass of a plant provides more sensitive measurements rather than the germination of the seeds. This is also true to this project as the seeds germinated on the surface of the growing media, despite the volume of oil within the media. Measuring the biomass gave an indication of the amount of growth of the plants and a more accurate view of the effect of the treatments.

When comparing the results of this project to the work published by Merkl et *al.* (2005), more work needs to be performed on the effect of the presence of oil in contaminated growing media on the roots of the plants. At a glance, the roots of both the *A. thaliana* and winter rye plants seemed to have grown as in non-contaminated growing media. They possessed a cream colour and had grown in an exudation pattern throughout the pots. For an accurate analysis of the growth of the roots, the roots would have to be studied using microscopy for measurements and length, and then compared to roots obtained from control plants. This data would then add further knowledge to that of how the presence of oil affects plant growth.

Further analysis using ICP technology would also allow the detection of the heavy metals from oil within plant tissue and digestions for thin layer chromatography (TLC) would determine how chlorophyll and other plant cell structures are affected by the presence of oil. This was not performed during this project, as there was insufficient time. This further analysis would provide more answers on the questions posed by the effect of an oil contaminant on plants.

Several methods of mixing the oil into the growing medium so that the oil was thoroughly mixed and that would have had the most contact with the plant roots. Mixing by hand, a food blender and and electric hand whisk were assessed. The electric hand whisk provided thorough mixing whereas when mixed using a food blender or by hand, the oil may have remained localised within the growing medium and may not have been present in the vicinity of the plants' roots were. The method of mixing the oil into the growing medium using a blender then shows to be ineffective, as data obtained would not necessarily show that the plants were affected by oil. The oil within the growing medium could also migrate to the base of the plant pot, away from the roots, or the oil could dissolve in the water used to irrigate the plant pots. This would dilute the oil concentration within the pot. This concern for homogeneous mixing is perhaps an unfortunate artefact of the research situation, brought about by a need to carry out a robust experiment but not really

reflecting reality. In a situation where the plants existed in a planted porous parking surface, the growing medium found in the voids of the surfacing blocks would not have been thoroughly mixed with the engine oil as in this experiment. The oil concentrations of a parking bay would be localised in the top surface of the growing medium, as this would be where the oil dripped to from the car engine. An additional application to make the experiment more realistic in a sense of the plants existing in a planted car-parking bay would be to apply used car engine oil to the growing medium as this would simulate the oil that drips from a car engine. Virgin engine oil has advantages such as being easier and more pleasant (and indeed safer) to work with and provides a basis of what can happen, but with the heavy metals and toxins that exist in used oil plants may tolerate this environment with a different approach, research that can be performed at a later date.

With this insight, seeds from a transgenic *Arabidopsis thaliana* Columbia 4 ecotype were planted alongside non-transgenic *Arabidopsis thaliana* Columbia 4 ecotype. Previous unpublished work has suggested that the transgenic Columbia 4 ecotype is possibly more tolerant to drought stress, which would make the line more likely to endure a water deficiency. Comparing the effect of the oil on the putative drought-tolerant transgenic and the non-transgenic *Arabidopsis thaliana*, it was possible to see that there was little difference in the growth between the two ecotypes, with

experimentally-measurable growth of plant material in 0% and 1% oil-contaminated growing medium and little growth in higher concentrations for both strains. Growth of *Arabidopsis* also occurred in 2.5% and 10% oil-contaminated medium but there was insufficient material to analyse statistically. With plant material that was obtained, microarray analysis was performed on its RNA to determine if the presence of oil had caused stress tolerant genes to be amplified.

Arabidopsis thaliana is a model experimental plant. The availability of a fully sequenced genome and molecular biotechnological tools make it possible to study the plant thoroughly in research. Furthermore, as work in this project had indicated that oil affected plant growth, it enabled additional work to be carried out compared to the rye.

To investigate further how oil affects *A. thaliana* growth and development microarrays, Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis and SDS-PAGE analysis (Reed *et al.*, 2003) of proteins are some of the ways that could be employed to identify genes that promoted tolerance to oil. Using this knowledge, similar genes could be targeted in other plant species identifying if they also possess tolerance to oil contaminant. One effect from the presence of oil in the growing medium could be a reduction in the available water and so the moisture of the growing medium can be measured and the effect on growth, as in this project.

A mixture of winter vetch and winter rye was planted in a previous experiment at increasing concentrations of oil contaminations but at harvest, only rye had grown, with no evidence of vetch growth. Consideration was given to the reason for this and it was found that vetch seed germination does not require cold stratification to commence (USDA, NRCS. 2004) but a moist growing medium is needed for growth (Plants for a Future, 2003[a]). The growing medium may have been too dry for the vetch to germinate and grow. One other possibility of the vetch not growing could be that the rye had not established substantial root development itself in the growing medium to support the growth of the legume, as previously mentioned in Section 1.4 (Schonbeck, 1988). Schonbeck (1988) states in the 'New Alchemy Quarterly' (No. 33), that in agricultural settings rye grows quicker than vetch, providing support with an extensive rooting system that aids the prevention of the heaving of short-rooted vetch. However, this does not explain the lack of initial growth and definite answers to why the vetch did not grow would need to be investigated. After considering the results of the first experiment and having decided that a new, better-designed experiment was required, a decision was made that the growth of rye in oil-contaminated growing medium would provide sufficient plant material for statistical analysis thus the vetch was removed from this work.

Growth of rye occurred in all pots from 0% oil contamination to 10%. Through the range of contamination levels, it was possible to

see a decrease in growth between the pots, as oil concentration increased. Rye seeds germinated at levels of contamination above 5% oil, producing weedy, purple/brown-coloured stems. This indicates that the rye was more tolerant of the oil than the *Arabidopsis*, as it is to many other environmental situations, such as acidic, neutral and alkaline soils; sandy, loamy and clay soils and although it requires a moist environment, it can also tolerate drought conditions (Plants for a Future, 2003[b]). By determining the fresh and dry weight of the rye following its growth, it could be seen with regression analysis that the increasing concentrations of oil in the growing medium resulted in less growth of the rye, in quality and weight of the plant material, and this data was significant at a probability level of $p < 0.05$.

Assuming that the presence of oil in growing medium had an effect on the growth of the plants, this may have happened indirectly as a result of the interactions of the oil with the water within the growing medium, as mentioned earlier in the discussion. As oil is hydrophobic, it repels water molecules away from it. This could therefore repel the water away from the plant roots, thus preventing them from receiving the water they need to survive. The experiment discussed in this thesis was set up in a temperature-controlled growth room where the environment had a consistent temperature of 24°C, thus preventing the pots from drying out too much. As the moisture levels in the controls showed no difference to the oil-treated plants it is unlikely that the presence of oil had an effect on

the moisture content of the growing medium, even though the oil had an effect on the growth of *Arabidopsis* and the rye. The use of the moisture meter probe presents problems. The probe of the meter measured approximately 10 cm. The probe was placed at the same depth in each pot so that the moisture of the localised growing medium around the roots was measured. By placing the probe slightly deeper into the growing medium an increase in the moisture percentage could be obtained. As the water drained through the pots and remained in the saucers, the base of the plant pots was constantly in the water and therefore maintaining a saturated environment. To enable reproducible results each time, each application of the probe into the growing medium was placed at a depth as similar as possible, so the readings were measured as accurately as possible. The final moisture content readings were also analysed by measuring the fresh weight of the growing medium and by drying the samples in an 105°C over three days (gravimetric method, see Section 2.3). This method, in conjunction with the moisture meter data, indicated that the presence of oil did not affect the moisture content of the pot, and regression analysis and ANOVA statistically proved this (see Sections 3.2 to 3.6). It may be possible to obtain enhanced data if better regulating moisture meter probes that measure the same areas of the plant pots were available. These conditions would standardise the readings and experimental design.

From analysis of the data for the moisture content of growing medium, it was seen that although there was a range of oil contamination from no oil (0%) to 10% (v/w), there was no difference in the moisture content of the growing medium. This was confirmed by regression analysis of the data from the moisture readings. By measuring the moisture content of the centre of the plant pots, the growing medium that was not directly in contact with the roots was analysed and not the actual effect of the water deficiency on the plant. If the plant's roots become covered in oil, it could cause the plant to die so measuring the whole growing medium environment of the pot was not the best method of analysis. For a plant to grow, direct water availability at the root/soil interface is a far more important factor in plant growth than water content of the bulk of the growing medium. It was not possible to measure the available water during the experiment as the Delta-T HH2 Moisture Meter only measured the moisture percentage of the growing medium.

Once sufficient RNA had been obtained from *Arabidopsis* extractions, amplified, purified and hybridised onto the microarray slides, it was possible to acquire data on the genes that had been amplified by the presence of oil within the growing medium, by detection of the fluorescently-bound cDNA to the template, and the analysis of the data using various computational methods (NCBI, 2004). Discovering the cDNA obtained from the *Arabidopsis* in this project did not have a high enough concentration for the microarray

analysis, samples with the same treatment were pooled together to provide sufficient material. This therefore means that the data was not genuinely replicated, thus the data generated by the microarray spots would not show amplification from genuine samples and a combination of samples may present false results. Much plant material from subsequent growth in oil-contaminated medium would be required for this analysis to be genuine.

Analysis of the microarrays was performed twice with the pooled material. The data obtained for this thesis was based on the first microarray created. The microarray was hybridised with the non-transgenic *Arabidopsis* Columbia 4 ecotype, combining the control (0% oil contamination) and the treated plants in 2.5% oil contamination. Not only did these samples provided the most raw materials after harvesting and flash-freezing and provided the greatest amounts of cDNA after extraction, they also gave examples of plant material that was treated and non-treated, thus showing any change in gene amplification between the two samples. The second microarray failed to produce any results and was abandoned when the cDNA labelling was unsuccessful. Following the labelling of the cDNA, the dyes did not produce a colour. This may have been a result of exposure to light, which would bleach the dyes, or the dyes may simply not have incorporated with the cDNA. At the time of the first microarray hybridisation it was possible to perform one colour-swap microarray, but having two microarray slides with one set of

colour-swap data on it does not provide sufficient reliable evidence for full t-test analysis. Obtaining a greater number of replicates is possible by substituting the cover slip of the microarray when it is ready for hybridisation for another microarray slide. This produces two slides with each procedure of the protocol, saving much labour and time. Placing the two slides together does increase the risk of smudging and damaging the microarray spots; therefore so extra care is required if performing this process. This will be carried out in future work, however, there was insufficient time within the current project.

The microarrays of the control cDNA and the cDNA obtained from 2.5% oil contamination were hybridised, however some spots seemed to be missing on some parts of the slide. As such a number of genes may be missed, some genes from within each grid should be present. One reason for the missing spots could be that they had not hybridised in this section. More replicates of the hybridised cDNA would support this.

Spots that were present were analysed using the GeneSpring computer program. The resulting microarray highlighted a total of 1055 genes that had been amplified (at a maximum of 2-fold) with p-values between 0.998 and $7.15E^{-05}$ in the t-test at $p = < 0.1\%$. Out of these 1055 genes, few were related to water stress or drought tolerance. For these genes, raw data was analysed at a 25% t-test and 7 genes were amplified that were responsive to water stress and drought tolerance. These genes included two drought-

responsive protein-related genes, two drought-responsive family proteins, two senescence/dehydration-associated proteins and one universal stress protein. These genes may not have been completely amplified if the spots had not been fully hybridised with the labelled cDNA or if the genes on the microarray had been partially missing (Stekel, 2003). The genes that had amplified within the 10% of the t-test contained a variety of proteins that performed various functions. By accessing The *Arabidopsis* Information Resource (TAIR) website (http://www.arabidopsis.org) it was possible to obtain information on the proteins that were amplified by searching for their identification number. These genes include transport inhibitor response proteins, homeobox proteins, tRNA synthetase, CCAAT-box binding transcription factor and many others. Several genes that were amplified were found to be 'expressed proteins', without a definite function. This illustrates that genes are being discovered before their function is known. There are many project groups globally that are studying various functions of genes so these 'unidentified' genes will soon possess an identity. By performing further microarrays with the cDNA and the CATMA microarray slides, it will be possible to gain a greater knowledge of the genes that are amplified by the plants as they endure the presence of oil in their growing conditions.

In future work, RNA obtained from the transgenic *Arabidopsis* plants will also be assessed to see if any differences in gene

expression are found by the presence of the oil in the growing medium in comparison to the non-transgenic *Arabidopsis*.

During analysis of the protozoa obtained from the Grassblock® rigs at Warwick HRI, it was revealed that before oil was applied to the rigs, there was a higher biodiversity of microbiological species present than is typically found in non-planted rigs treated with oil that were based at Coventry University (see Coupe 2004). From microscopy analysis, many large organisms were identifiable and common between the different types of rigs. This latter case is important since it indicates the high pH caused by concrete in the recycled aggregate was not causing changes in the eukaryotic microbiology of the systems. Many different species of microorganisms were present within the samples, indicating that the samples had been obtained from soil and sub-base combinations that had an already well-established eukaryotic microbial population. The species present in the control samples included bacteria, fungi, protist groups (including flagellates, ciliates heliozoa, gymnamoeba and testate amoeba) and metazoa (nematodes, rotifers and gastrotrich). Following the addition of oil to the Grassblock® rigs, a variety of additional species were present in the effluent samples besides those found in the samples before oil was applied. The majority of the species from the control samples were present within the effluent samples obtained. This was a limitation of the experiment as there were no

controls to refer samples back to. *Stylonychia* and *Sacpingoeca* were observed in the baseline effluent samples from the granite and recycled concrete Grassblock® rigs respectively, but were not seen in subsequent samples. Other species of microorganism identified in the baseline samples were present in subsequent samples. Whether the presence of oil caused the inhibition of these two species of microorganism would require further work. In analysis of the effluent samples, many large diverse organisms in a wide diversity were observed in subsequent samples following the rainfalls and this indicates that the rigs maintain a healthy range of organisms, despite the addition of used engine oil. For the two species absent, it is possible they were not detected in the samples. Determining if the organisms had been obtained only from effluent samples of the sub-base would be difficult. Growing media have naturally rich and diverse microorganism habitats so samples that were analysed would have had an influence from the microorganisms that had migrated from the growing medium into the sub-base. From the results acquired from the effluent, it may be stated the addition of the engine oil may have increased the variety of species of microorganisms in the porous pavement system, this may be due to the combination of the organisms from the soil and the sub-base that are naturally present and an insufficient sampling period/effort prior to adding the oil to allow the true baseline diversity to be established. This is an important lesson, which will need to be taken into account when planning further work. The

numbers of testate amoeba and gymnamoeba were low in the effluent samples. The project described was a first attempt at identifying the eukaryotic microorganisms obtained from a porous pavement system and the author's first experience of eukaryotic microbiology. Thus further studies of model pervious pavement systems in both planted and non-planted forms would increase the knowledge of the microbiology of the system and would certainly be necessary in future work. Further work on the planted porous pavement system in comparison to non-planted rigs may make it easier to identify definite microbiological variations and would show if the combination of plants with the pavement system promotes better degradation of engine oil than if no plants were incorporated. As plants photosynthesise, the use of carbon dioxide/oxygen relations in planted model systems may be difficult and Coupe's indicator organism approach may be one of few options available to identify when a PPS is established as an oil biodegrading one. The high background protozoan populations in non-oiled control samples would, however, indicate a need for much more research before this is relied upon. Perhaps a quantitative approach in which protozoan/metazoan density or biomass estimates may be a better approach than simply looking for a change in diversity

In terms of the practical application of the new test rig, there were certain considerations, which could affect any future design. In fact following the background learnt from this study it has been decided not to adopt the design used for future work in this research

group. Both the difficulties in differential drainage and the disposal problems have been overcome by adopting a new design based on a 0.5 m^3 plastic stillage constructed as an injection moulding from high density polyethylene (HDPE). This will allow internal baffles to be welded to the inside of the "box" to direct water flows appropriately.

The stillage system has provision for being picked up using the forks of a forklift truck. This will allow the entire contents to be lifted such that they can be tipped easily into a skip, allowing the expensive stillage box to be retained intact for cleaning and re-use. The system also has another very practical advantage. It can be picked up, intact and moved around. This will allow temporary access to the hard standing for maintenance purposes in long-term experiments and the possibility of rotating the position of models to overcome non-randomisation effects.

Overall this project has shown statistically that oil has an effect on the growth of winter rye and *Arabidopsis thaliana* but not on the available moisture to the plants whilst they are in the pots. The change in plant tissue mass of rye and both plant tissue mass and leaf area in *Arabidopsis thaliana* determined effect on growth. Both methods of moisture determination proved that there was little difference between the different oil concentrations, thus proving that water cannot have played a part in the reduction in growth of the plants.

The microarray analysis showed that genes are amplified when plants are under stress, with specific genes amplified in relation to the stress. No conclusions can be drawn from the analyses, as further replicates are required so statistical analyses can prove the likelihood of the data is significant.

The rig structures at Warwick HRI did not leak any oil in their effluent. The rigs would need to be dismantled to discover if the oil had migrated to the sub-base of had become trapped in the growing medium surface or the geotextile. Microorganisms in the effluent gave a strong indication that the planted surface had aided the maturation of the rigs, due to the broad variety of species present.

Taking into account all of the data obtained from the experiments, it can be seen that further analysis on each aspect of the project could lead to strong conclusive results on the effect of oil on different plant species, the effect oil of the genome of *Arabidopsis thaliana*, on the microorganisms that exist in planted PPS and on the oil retention capabilities of planted PPS.

5.0 Conclusion and Further Work

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Following the completion of this project, it can be stated that the presence of oil did have an effect on the physical properties and the genome of the non-transgenic *Arabidopsis thaliana*, by amplifying many genes in the genome, including various dehydration resistant genes. To clarify that these genes provide the plant with some protection against the contaminant, further microarrays must be performed.

To determine the effect of the presence of oil on the *A. thaliana* that has been transgenic to resist drought, microarray analysis will also be performed, allowing comparisons with the non-transgenic plant. This information will provide data for a planned publication on the effect of the presence of engine oil on both the transgenic and the non-transgenic *Arabidopsis*.

Further studies on the planted porous pavement systems will also provide the conclusion of whether plants do assist the degradation or the retention of oil within these systems. Additional analysis of the pavement systems will increase the data of the microbiology within the systems, determining if the addition of soil/peat to the surface of the pavement supplies supplementary eukaryotic organisms that aid the degradation of oil.

Further analysis on both the planted porous pavements and the microarrays is mentioned in the discussion and requires the techniques used in this research. The results will provide the

foundation of the knowledge on contaminants that have not been assessed before, providing valuable information, not only for scientific and environmental researchers but also for those in the automotive and engineering industry.

6.0 References

6.0 References

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7.0 Appendix

Appendix I Analysis of variance of Columbia 0 growth

Fresh weight
Anova: Two-Factor Without Replication

SUMMARY	Count	Sum	Average	Variance
1	3	1.9013	0.633766667	0.886240663
2	3	0.6499	0.216633333	0.050445613
3	3	2.8493	0.949766667	2.378393243
4	3	0.9253	0.308433333	0.282350763
0	4	5.8214	1.45535	0.99204483
1	4	0.4959	0.123975	0.007444743
2.5	4	0.0085	0.002125	1.80625E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	1.002688623	3	0.334229541	1.004781442	0.45293976	4.757055194
Columns	5.199026285	2	2.599513143	7.81481659	0.021345498	5.143249382
Error	1.995834282	6	0.332639047			
Total	8.19754919	11				

Dry weight
Anova: Two-Factor Without Replication

SUMMARY	Count	Sum	Average	Variance
1	3	0.2372	0.079066667	0.013581143
2	3	0.1449	0.0483	0.00227251
3	3	0.3987	0.1329	0.03835321
4	3	0.1759	0.058633333	0.010138703
0	4	0.843	0.21075	0.01192059
1	4	0.1115	0.027875	0.000402776
2.5	4	0.0022	0.00055	0.00000121

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	0.012781209	3	0.004260403	1.056624944	0.434123875	4.757055194
Columns	0.104498615	2	0.052249308	12.95837997	0.006643506	5.143249382
Error	0.024192518	6	0.004032086			
Total	0.141472343	11				

Appendix II Regression of Columbia 0 growth (fresh mass)

SUMMARY OUTPUT (Fresh weight)

Regression Statistics	
Multiple R	0.590901313
R Square	0.349164362
Adjusted R Square	0.186455453
Standard Error	0.527984055
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.598218984	0.598218984	2.145944947	0.216808869
Residual	4	1.115068651	0.278767163		
Total	5	1.713287635			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.649514643	0.339837098	1.911252912	0.128560175	-0.294026358	1.593055644	-0.294026358	1.593055644
X Variable 1	-0.088817802	0.060630442	-1.464904416	0.216808869	-0.257155245	0.07951964	-0.257155245	0.07951964

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	0.649514643	0.805835357
2	0.560696841	-0.436721841
3	0.427470137	-0.418970137
4	0.205425632	-0.205425632
5	-0.016618874	0.016618874
6	0.238663379-	0.238663379

Appendix III Regression of Columbia 0 growth (dry mass)

SUMMARY OUTPUT (dry weight)	
Regression Statistics	
Multiple R	0.615420153
R Square	0.378741964
Adjusted R Square	0.223427455
Standard Error	0.07428679
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.013457196	0.013457196	2.43854851	0.193412489
Residual	4	0.022074109	0.005518527		
Total	5	0.035531305			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.097863214	0.047814715	2.046717513	0.110099973	-0.034891992	0.23061842	-0.034891992	0.23061842
X Variable 1	-0.013321319	0.008530638	-1.561585255	0.193412489	-0.037006216	0.010363579	-0.037006216	0.010363579

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	0.097863214	0.112886786
2	0.084541896	-0.056666896
3	0.064559918	-0.062359918
4	0.031256621	-0.031256621
5	-0.002046676	0.002046676
6	-0.035349973	0.035349973

Appendix IV Analysis of variance of Columbia 4 growth

Anova: Two-Factor Without Replication (fresh)

SUMMARY	Count	Sum	Average	Variance
1	3	0.3764	0.125467	0.030768
2	3	1.7843	0.594767	0.540463
3	3	1.2906	0.4302	0.514536
4	3	1.4471	0.482367	0.521929
0	4	3.2662	0.81655	0.295097
1	4	1.5869	0.396725	0.46469
2.5	4	0.0453	0.011325	0.000219

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	0.36219	3	0.12073	0.377709	0.77281	4.757055
Columns	1.297565	2	0.648782	2.029742	0.212191	5.143249
Error	1.917827	6	0.319638			
Total	3.577582	11				

Anova: Two-Factor Without Replication (dry)

SUMMARY	Count	Sum	Average	Variance
1	3	0.1179	0.0393	0.002986
2	3	0.3008	0.100267	0.00903
3	3	0.2175	0.0725	0.01538
4	3	0.2358	0.0786	0.010119
0	4	0.6227	0.155675	0.003291
1	4	0.242	0.0605	0.007544
2.5	4	0.0073	0.001825	9.48E-06

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	0.005731	3	0.00191	0.42766	0.740601	4.757055
Columns	0.048228	2	0.024114	5.398318	0.045581	5.143249
Error	0.026802	6	0.004467			
Total	0.08076	11				

Appendix V Regression of Columbia 4 growth (fresh mass)

SUMMARY OUTPUT (Fresh weight)

Regression Statistics	
Multiple R	0.739522
R Square	0.546893
Adjusted R Square	0.433616
Standard Error	0.255041
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.314037	0.314037	4.827929	0.092937
Residual	4	0.260183	0.065046		
Total	5	0.57422			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.483003	0.164157	2.942323	0.042291	0.027229	0.938777	0.027229	0.938777
X Variable 1	-0.06435	0.029287	-2.19725	0.092937	-0.14567	0.016963	-0.14567	0.016963

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	0.483003	0.333547
2	0.418652	-0.02193
3	0.322124	-0.3108
4	0.161245	-0.16124
5	0.000365	-0.00037
6	-0.16051	0.160789

Appendix VI Regression of Columbia 4 growth (dry mass)

SUMMARY OUTPUT (Dry weight)

Regression Statistics	
Multiple R	0.713892
R Square	0.509642
Adjusted R Square	0.387053
Standard Error	0.049468
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.010173	0.010173	4.157309	0.111076
Residual	4	0.009788	0.002447		
Total	5	0.019961			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.086561	0.031842	2.718639	0.053061	-0.00184	0.174963	-0.00184	0.174963
X Variable 1	-0.01158	0.005681	-2.03895	0.111076	-0.02735	0.004189	-0.02735	0.004189

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	0.086561	0.069114
2	0.074979	-0.01448
3	0.057605	-0.05578
4	0.028649	-0.02865
5	-0.00031	0.000307
6	-0.02926	0.029488

Appendix VII Analysis of variance of transgenic Col 4 growth

Anova: Two-Factor Without Replication (fresh)

SUMMARY	Count	Sum	Average	Variance
1	3	2.2654	0.755133	1.701182
2	3	0.909	0.303	0.242496
3	3	0.1388	0.046267	0.003929
4	3	0.9217	0.307233	0.283177
0	4	4.1717	1.042925	0.794868
1	4	0.0632	0.0158	0.000299
2.5	4	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	0.781173	3	0.260391	0.973832	0.464646	4.757055
Columns	2.857237	2	1.428619	5.342863	0.046496	5.143249
Error	1.604329	6	0.267388			
Total	5.24274	11				

Anova: Two-Factor Without Replication (dry)

SUMMARY	Count	Sum	Average	Variance
1	3	0.2539	0.084633	0.020785
2	3	0.1447	0.048233	0.005057
3	3	0.047	0.015667	0.000434
4	3	0.2269	0.075633	0.017161
0	4	0.6472	0.1618	0.009412
1	4	0.0253	0.006325	4.2E-05
2.5	4	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	0.008677	3	0.002892	0.881604	0.501757	4.757055
Columns	0.067189	2	0.033594	10.2395	0.011635	5.143249
Error	0.019685	6	0.003281			
Total	0.095551	11				

Appendix VIII Regression of transgenic Col 4 (fresh mass)

SUMMARY OUTPUT (Fresh weight)

Regression Statistics	
Multiple R	0.553075
R Square	0.305892
Adjusted R Square	0.132365
Standard Error	0.395436
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.275647	0.275647	1.762794	0.254978
Residual	4	0.625478	0.15637		
Total	5	0.901126			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.437712	0.254522	1.719737	0.160599	-0.26896	1.144381	-0.26896	1.144381
X Variable 1	-0.06029	0.045409	-1.3277	0.254978	-0.18637	0.065787	-0.18637	0.065787

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	0.437712	0.605213
2	0.377422	-0.36162
3	0.286986	-0.28699
4	0.136261	-0.13626
5	-0.01446	0.014465
6	-0.16519	0.16519

Appendix IX Regression of transgenic Col 4 (dry mass)

SUMMARY OUTPUT (Dry weight)

Regression Statistics	
Multiple R	0.565503
R Square	0.319794
Adjusted R Square	0.149742
Standard Error	0.060477
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.006878	0.006878	1.880569	0.242168
Residual	4	0.01463	0.003658		
Total	5	0.021508			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.06929	0.038926	1.780041	0.149677	-0.03879	0.177367	-0.03879	0.177367
X Variable 1	-0.00952	0.006945	-1.37134	0.242168	-0.02881	0.009758	-0.02881	0.009758

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	0.06929	0.09251
2	0.059767	-0.05344
3	0.045481	-0.04548
4	0.021672	-0.02167
5	-0.00214	0.002138
6	-0.02595	0.025947

Appendix X Analysis of variance analysing the difference in growth between the lines

Anova: Two-Factor With Replication

SUMMARY	Col 0	Col 4	GM4	Total
0				
Count	4	4	4	12
Sum	5.8214	3.2662	4.1717	13.2593
Average	1.45535	0.81655	1.042925	1.104942
Variance	0.992045	0.295097	0.794868	0.644113
1				
Count	4	4	4	12
Sum	0.4959	1.5869	0.0632	2.146
Average	0.123975	0.396725	0.0158	0.178833
Variance	0.007445	0.46469	0.000299	0.15687
2.5				
Count	4	4	4	12
Sum	0.0085	0.0453	0	0.0538
Average	0.002125	0.011325	0	0.004483
Variance	1.81E-05	0.000219	0	9.09E-05
Total				
Count	12	12	12	
Sum	6.3258	4.8984	4.2349	
Average	0.52715	0.4082	0.352908	
Variance	0.745232	0.325235	0.476613	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	8.396332		24.198166	14.78991	4.6E-05	3.354131
Columns	0.190266		20.095133	0.335148	0.718165	3.354131
Interaction	0.957496		40.239374	0.843301	0.51004	2.727766
Within	7.664043		270.283853			
Total	17.20814		35			

Appendix XI Analysis of variance of winter rye growth

Fresh mass

SUMMARY	Count	Sum	Average	Variance
A	6	52.28	8.713333	17.12407
B	6	49.61	8.268333	14.10326
C	6	52.87	8.811667	7.815177
D	6	50.78	8.463333	22.45703
0	4	53.54	13.385	0.543633
1	4	40.48	10.12	10.3762
2.5	4	42.53	10.6325	3.706492
5	4	30.82	7.705	6.8335
7.5	4	21.14	5.285	0.739633
10	4	17.03	4.2575	0.692092

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	1.08715	3	0.362383	0.080425	0.969648	3.287383
Columns	239.9101	5	47.98203	10.64887	0.000164	2.901295
Error	67.5875	15	4.505833			
Total	308.5848	23				

Dry mass

SUMMARY	Count	Sum	Average	Variance
A	6	6.74	1.123333	0.253347
B	6	7.14	1.19	0.22676
C	6	6.45	1.075	0.11399
D	6	5.29	0.881667	0.220937
0	4	6.24	1.56	0.025267
1	4	5.08	1.27	0.053467
2.5	4	5.31	1.3275	0.149358
5	4	4.09	1.0225	0.160425
7.5	4	2.76	0.69	0.029
10	4	2.14	0.535	0.0073

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	0.316283	3	0.105428	1.650461	0.220078	3.287383
Columns	3.117	5	0.6234	9.759262	0.000264	2.901295
Error	0.958167	15	0.063878			
Total	4.39145	23				

Appendix XII Regression of winter rye growth (fresh mass)

SUMMARY OUTPUT (fresh mass)

Regression Statistics	
Multiple R	0.967169
R Square	0.935417
Adjusted R Square	0.919271
Standard Error	0.983861
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	56.0806	56.0806	57.9355	0.001599
Residual	4	3.871933	0.967983		
Total	5	59.95253			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	12.29314	0.633263	19.41239	4.15E-05	10.53492	14.05137	10.53492	14.05137
X Variable 1	-0.85996	0.112981	-7.61154	0.001599	-1.17364	-0.54627	-1.17364	-0.54627

Appendix XIII Regression of winter rye growth (dry mass)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.978883
R Square	0.958212
Adjusted R Square	0.947765
Standard Error	0.090008
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.743078	0.743078	91.72148	0.000664
Residual	4	0.032406	0.008101		
Total	5	0.775483			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	1.497286	0.057934	25.8448	1.33E-05	1.336436	1.658136	1.336436	1.658136
X Variable 1	-0.09899	0.010336	-9.57713	0.000664	-0.12769	-0.07029	-0.12769	-0.07029

Appendix XIV Regression analysis of moisture content from rye

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.551594
R Square	0.304255
Adjusted R Square	0.130319
Standard Error	0.473921
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.39288	0.39288	1.749237	0.256522
Residual	4	0.898403	0.224601		
Total	5	1.291283			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	87.72357	0.305039	287.5813	8.77E-10	86.87665	88.5705	86.87665	88.5705
X Variable 1	-0.07198	0.054422	-1.32259	0.256522	-0.22308	0.079122	-0.22308	0.079122

Appendix XV Analysis of variance of leaf area in Col 0

Anova: Two-Factor Without Replication (Col 0)

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	2	5.024	2.512	0.403202
Row 2	2	2.534	1.267	0.144722
Row 3	2	3.459	1.7295	3.950861
Row 4	2	1.648	0.824	1.116018
Column 1	4	9.203	2.30075	0.74976
Column 2	4	3.462	0.8655	0.788839

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	3.12088	3	1.040293	2.087661	0.280472	9.276619
Columns	4.119885	1	4.119885	8.267785	0.063769	10.12796
Error	1.494917	3	0.498306			
Total	8.735683	7				

Appendix XVI Analysis of variance of leaf area of Col 4

Anova: Two-Factor Without Replication (Col 4)

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	2	1.211	0.6055	0.002965
Row 2	2	3.014	1.507	0.060552
Row 3	2	1.217	0.6085	0.612725
Row 4	2	1.538	0.769	0.761378
Column 1	4	4.525	1.13125	0.114653
Column 2	4	2.455	0.61375	0.555541

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1.108575	3	0.369525	1.22901	0.434709	9.276619
Columns	0.535613	1	0.535613	1.781403	0.274231	10.12796
Error	0.902006	3	0.300669			
Total	2.546194	7				

Appendix XVII Analysis of variance of leaf area of transgenic Col 4

Anova: Two-Factor Without Replication (GM Col 4)

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	2	1.875	0.9375	1.443301
Row 2	2	1.304	0.652	0.36125
Row 3	2	0.822	0.411	0.11045
Row 4	2	1.804	0.902	1.627208
Column 1	4	5.314	1.3285	0.321794
Column 2	4	0.491	0.12275	0.009993

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.360817	3	0.120272	0.568626	0.672839	9.276619
Columns	2.907666	1	2.907666	13.74691	0.034096	10.12796
Error	0.634542	3	0.211514			
Total	3.903026	7				

Appendix XVIII Analysis of variance comparing lines Col 0 and Col 4

Anova: Two-Factor Without Replication
(Col 0)

SUMMARY	Count	Sum	Average	Variance
1	3	47.64	15.88	499.9477
2	3	20.3	6.766667	44.87463
3	3	57.5	19.16667	877.7324
4	3	26.93	8.976667	235.6006
0	4	135.26	33.815	295.9974
1	4	16.62	4.155	7.639033
2.5	4	0.49	0.1225	0.060025

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	302.9934	3	100.9978	0.996532	0.456025	4.757055
Columns	2708.215	2	1354.107	13.3608	0.006165	5.143249
Error	608.0958	6	101.3493			
Total	3619.304	11				

Anova: Two-Factor Without Replication (Col 4)

SUMMARY	Count	Sum	Average	Variance
1	3	10.07	3.356667	19.57263
2	3	53.03	17.67667	529.6726
3	3	37.45	12.48333	415.5001
4	3	48.71	16.23667	595.428
0	4	98.05	24.5125	338.8036
1	4	49.45	12.3625	438.93
2.5	4	1.76	0.44	0.393133

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	373.0385	3	124.3462	0.380391	0.771055	4.757055
Columns	1159.005	2	579.5025	1.772774	0.248343	5.143249
Error	1961.342	6	326.8903			
Total	3493.385	11				

Appendix XIX Paired t-test for two treatments in transgenic Columbia 4

t-Test: Paired Two Sample for Means (GM Col 4)

	0	*1*
Mean	26.7175	0.6475
Variance	426.9288	0.356492
Observations	4	4
Pearson Correlation	-0.37571	
Hypothesized Mean Difference	0	
df	3	
t Stat	2.495464	
P(T<=t) one-tail	0.044029	
t Critical one-tail	2.353363	
P(T<=t) two-tail	0.088058	
t Critical two-tail	3.182449	

Appendix XX Analysis of variance on moisture meter data from the negative controls

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	6	93.1	15.51667	7.185667
Row 2	6	99.5	16.58333	5.117667
Row 3	6	94.4	15.73333	12.63467
Row 4	6	113.5	18.91667	9.125667
Column 1	4	71.2	17.8	1.766667
Column 2	4	63.9	15.975	4.6425
Column 3	4	60.5	15.125	4.4025
Column 4	4	63.6	15.9	40.43333
Column 5	4	65.2	16.3	5.06
Column 6	4	76.1	19.025	1.095833

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	43.56792	3	14.52264	1.693476	0.21106	3.287383
Columns	41.68375	5	8.33675	0.972143	0.465736	2.901295
Error	128.6346	15	8.575639			
Total	213.8862	23				

Appendix XXI Analysis of variance using gravimetric data

Anova: Two-Factor Without Replication (fresh)

SUMMARY	Count	Sum	Average	Variance
1	6	175.2389	29.20648	10.22792
2	6	221.593	6.931671	83.7579
3	6	216.9262	36.15437	27.40636
4	6	230.9774	38.49623	1.523797
0	4	139.4865	34.87163	73.29479
1	4	135.9473	33.98675	36.04269
2.5	4	122.5862	30.64655	21.41997
5	4	143.2064	35.8016	5.608499
7.5	4	134.0562	33.51405	17.09516
10	4	169.4502	42.36255	217.0323

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	304.1811	3	101.3937	1.883943	0.175671	3.287383
Columns	307.2806	5	61.45611	1.141884	0.381319	2.901295
Error	807.2992	15	53.81995			
Total	1418.761	23				

Anova: Two-Factor Without Replication (dry)

SUMMARY	Count	Sum	Average	Variance
1	6	85.2058	14.20097	3.375097
2	6	91.2013	15.20022	1.088246
3	6	114.912	19.152	12.23583
4	6	124.8176	20.80293	1.732336
0	4	75.5255	18.88138	36.07162
1	4	66.5452	16.6363	20.08828
2.5	4	65.5605	16.39013	13.92621
5	4	70.6243	17.65608	2.957678
7.5	4	72.4048	18.101	26.348217
10	4	65.4764	16.369	13.548408

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	178.2447	3	59.41491	12.62777	0.000221	3.287383
Columns	21.58105	5	4.316210	0.917347	0.49621	2.901295
Error	70.5765	15	4.7051			
Total	270.4023	23				

Appendix XXII Regression of fresh growing medium mass using the gravimetric method

SUMMARY OUTPUT fresh

Regression Statistics	
Multiple R	0.648084
R Square	0.420013
Adjusted R Square	0.275017
Standard Error	3.337464
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	32.26548	32.26548	2.896709	0.163976
Residual	4	44.55466	11.13867		
Total	5	76.82014			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	32.37061	2.14816	15.069	0.000113	26.40635	38.33487	26.40635	38.33487
X Variable 1	0.652287	0.383254	1.701972	0.163976	-0.4118	1.716373	-0.4118	1.716373

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	32.37061	2.501016
2	33.0229	0.963854
3	34.00133	-3.35478
4	35.63205	0.169554
5	37.26276	-3.74871
6	38.89348	3.469067

Appendix XXIII Regression of dry growing medium mass using the gravimetric method

SUMMARY OUTPUT dry

Regression Statistics	
Multiple R	0.270565
R Square	0.073205
Adjusted R Square	-0.15849
Standard Error	1.118068
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.394961	0.394961	0.31595	0.604057
Residual	4	5.000302	1.250075		
Total	5	5.395263			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	17.65176	0.719645	24.52844	1.64E-05	15.6537	19.64982	15.6537	19.64982
X Variable 1	-0.07217	0.128392	-0.56209	0.604057	-0.42864	0.284306	-0.42864	0.284306

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	17.65176	1.229616
2	17.57959	-0.94329
3	17.47134	-1.08121
4	17.29092	0.365158
5	17.1105	0.990704
6	16.93007	-0.56097

Appendix XXIV Comparing the moisture content methods (Paired t-test)

t-Test: Paired Two Sample for Means

	moisture meter (%)	*gravimetric water mass*
Mean	16.6875	17.85815833
Variance	9.299402174	48.79560312
Observations	24	24
Pearson Correlation	0.261986632	
Hypothesized Mean Difference	0	
df	23	
t Stat	-0.837133675	
P(T<=t) one-tail	0.2055659	
t Critical one-tail	1.713870006	
P(T<=t) two-tail	0.411131801	
t Critical two-tail	2.068654794	