Evaluation of the Release and uptake of the fungicide tebuconazole from a novel microencapsulated formulation

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A cryo SEM image of a typical yeast cell (Saccharomyces cerevisiae) showing the cell wall and exposed internal contents within the cell membrane. Reproduced with permission, (Duckham et al., 2003).

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LIST OF ABBREVIATIONS AND ACRONYMS

~	Approximately
°	Degrees
°C	Degrees Celsius
AI	Active Ingredient
ANOVA	Analysis of variance
a.u.	Arbitrary units
BCPC	British Crop Protection Council
cm	Centimetre
d	Day
DCM	Dichloromethane
DEFRA	Department of Environment Food and Rural Affairs
DMI	Demethylation Inhibiting
EBI	Ergosterol Biosynthesis Inhibitor
FID	Flame Ionisation Detector
e.g.	e*xempli gratia*
et al.	*et alli*
g	Grams
g	Gravity
GC	Gas Chromatography
h	Hours
ha	Hectare
HGCA	Home Grown Cereals Authority
HPLC	High Performance Liquid Chromatography
i.e.	*id est*
kg	Kilogram
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
Log	Logarithm
m	Metre
MeOH	Methanol
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
MS	Mass Spectroscopy
ng	nanogram
P	Probability
pers. com.	Personal Communication
QoI	Quinone Outside Inhibitors
s	Seconds
SDW	Sterile Distilled Water
SEM	Scanning Electron Micrograph
SIM	Selected Ion Monitoring (Mass Spectroscopy)
SCF	Stem Concentration Factor
SPE	Solid Phase Extraction
TCSF	Transpiration Stream Concentration Factor
µg	microgram

µL	Micro Litre
µm	micrometre
v	Volume
w	Weight

ABSTRACT

Triazole fungicides, such as tebuconazole, have been pivotal in the control of both foliar and seed/soil-borne diseases of many crops, including cereals, since their introduction in the 1970s. Although routinely applied as a foliar spray, tebuconazole is also used as a seed treatment. The systemic nature of the fungicide may confer protection of plants from disease during the early stages of development. However, application rates are limited to between 1 and 7 g active ingredient (AI) per 100 kg of seed due to seed application causing reduced germination and stunting during early plant development.
A novel technique whereby fungicides, including tebuconazole, are encapsulated using yeast cells as pre-formed microcapsules may provide a solution to the problem of phytotoxicity and allow seed treatments to be applied at higher rates, resulting in a reduction in total fungicide application required during the growing season. Several batches of microencapsulated tebuconazole were produced using yeast from different sources. These where tested for their potential for uptake, retention and subsequent delivery of the fungicide. The release of tebuconazole from the different yeast batches into water was assessed over a time course using liquid-liquid extraction (LLE) and GC-MS. Results showed no differences in the release characteristics of tebuconazole observed between batches. Further to this the age of the batches tested was found to have no effect on release of tebuconazole into water, suggesting shelf-life would not be a problem with this technology. Repeated washing of a sample of microencapsulated tebuconazole with water suggested that the novel formulation could provide the gradual delivery of tebuconazole.

An investigation of the phytotoxic effect of tebuconazole, applied as seed treatments at higher than recommended rates (40 g AI per 100 kg seed), showed the microencapsulated formulation had a significantly reduced impact on germination and early plant development, when compared to two commercial formulations of the fungicide, Mystique and Raxil. Further analysis, of the amount of tebuconazole accumulated by plants grown from treated seed, using solid phase extraction (SPE) and GC-MS), showed there to be initially less fungicide in plants treated with the microencapsulated formulation. As plant development continued, the amount of tebuconazole recovered from plants treated with both the microencapsulated and conventional formulations became similar. Variation in the results, between replicate samples and also between growth stages led to a series of experiments investigating the possible effect of the growth environment and seed sowing density on tebuconazole uptake. However, no significant effects of these factors were observed.

A comparison of the uptake of seed-applied microencapsulated and conventional formulations of tebuconazole between 2 and 19 weeks after sowing, showed there to be no significant differences between formulations used in the concentration of the fungicide accumulated in plants. However, a pronounced increase in plant accumulation of the fungicide was consistently observed between 17 and 19 weeks. It was suggested that this might be associated with roots becoming densely packed within the compost, encouraging fungicide uptake from the compost in the localised dressing zone around the seed. It was shown that tebuconazole became readily bound to the loam-based compost used during the research. Experiments conducted in 1m columns, containing tebuconazole applied to specific compost layers within the columns, confirmed the ability of wheat roots to recover the fungicide from the compost when they made contact with the specific layers.

CHAPTER 1: GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

"In agriculture, the conditions and rules have changed in an unprecedented way over recent years. Not only have the rules changed, but alternative techniques are increasingly challenging the chemical approach to disease control. Chemical crop protection has a vital role in securing healthy food supplies for a growing global population. We have to make the point again and again: without chemically protecting our agricultural commodities there would not be enough food to eat."

(From the Welcome Address of Jost Harr, Chairman of the 10th IUPAC Congress on Chemistry of Crop Protection, 2002 (Harr, 2002).

Increases in world population have made it vital that the production of food is as efficient as possible (Hazell and Wood, 2008). For most of recorded history increases in global population have averaged below 0.2% per annum (Hewitt, 1998). However, the advent of improved medical techniques and increased birth rates allied with an increased ability of food growers to produce a more varied array of crops at consistent yields and quality, has led to a rapid population increase since the early 19th century (Hewitt, 1998; Hazell and Wood, 2008). The majority of this initial increase was in what the United Nations, now class as developed countries; Europe, the former Soviet Union, N. America, Australia, New Zealand and Japan (Hewitt, 1998). The current world population is estimated to be at 6.7 billion and is predicted to increase, if present growth rates continue, to between 9.5 and 10.5 billion by the year 2050 (Anon, 2009). However, by the year 2100 only a small proportion of the total global population will be living in these regions with the majority living in the developing world (Hewitt, 1998;

IDB, 2008). This increase in population will demand a linked increase in the quantity of food required. However, despite the development and improvement of every aspect of food production, leading to huge increases in the amount of food produced over the past 50 years, there is still food insecurity (Strange and Scott, 2005). Food is not uniformly distributed throughout the world and not all that is produced is consumed, meaning that there is a surplus of food in some places and a chronic shortage in others (Hewitt, 1998; Strange and Scott, 2005). This imbalance impacts the developing world hardest of all, where the majority of the world's undernourished population exist. Over 800 million people suffer from malnutrition (Delmer, 2005; Strange and Scott, 2005), many of these people live in developing counties, where losses due to plant disease are substantially higher (Strange and Scott, 2005). These losses also often occur in the poorest of the developing countries, which are unable to afford expensive crop protection chemicals and which can least afford the loss. A distribution map of the countries with the highest percentages of undernourished people is given in Figure 1.1 (Waller *et al.*, 2002; IDB, 2008). As the most fertile and irrigable lands come under greater pressure to meet food production requirements, lower yielding land must increasingly be bought into use. This in turn means that economically sound production of crops relies more than ever on fertiliser application but also on the reduction of losses due to disease (Hazell and Wood, 2008).

Figure 1.1 A distribution map showing the percentage of the population that are undernourished in the countries of the world 2002-2004 (FAO, 2008).

1.2 LOSSES AND IMPACT ON YIELD ASSOCIATED WITH PLANT DISEASE

It is estimated that between 31 and 42% of all crops produced worldwide are lost as a result of disease, insects and weeds. Of this total, 14.1% are lost to plant disease equating to approximately $220 billion worth of crops pre-harvest (Ghini *et al.*, 2008). These losses impact greatly on global food production and represent a significant threat to global food security (Strange and Scott, 2005). Predictions of the world's ability to produce enough food to sustain the predicted population increases vary from those that foresee no problem to those that predict a major shortfall (Hewitt, 1998). Estimations show that only a proportion of the land available for cultivation is actually employed in food production; being approximately 25% in S. America, 20% in Africa and slightly more than 45% in Asia. Overall, it is estimated that 23% of the global total available arable land would need to be used for arable crop production by 2050 to feed predicted population levels (Weber, 1994; Hazell and Wood, 2008). However, the ecological impact of using these lands would be likely to be severe and it is more likely that food

requirements will be met through increases in yield and the use of technology or methods to restrict losses (Hewitt, 1998; Hazell and Wood, 2008). Of the total necessary additional increases in production, only up to 26% can be achieved by expanding cultivated areas and 14% through the intensification of production process. This means that up to 60% of the required increase must be attained by increasing the yield of the crops grown (Strange and Scott, 2005). Public investments in modern scientific research for agriculture have led to dramatic yield breakthroughs in the 20th century. The story of English wheat, for example, is typical. It took almost 1000 years for yields to increase from 0.5 to 2 t ha^{-1}. However, with the advent of modern plant breeding, improved agronomy and development of modern fertilisers and agrochemicals during the last 40 to 50 years yields have further increased to an average 7.9 t ha^{-1} (HGCA, 2010). Much research and investment has gone into efforts to increase yields using several strategies. However, this huge goal may only be attained by the use of better fertiliser and seed stock and through the reduction of pre and post harvest losses due to pathogens (Jørgensen and Olesen, 2002). It is therefore paramount that crop protection measures available are as effective as possible and that the correct measures are integrated and employed where and when they are needed.

1.3 CROP PROTECTION

Pathogens have challenged farmers since the first crop plants were domesticated during the transition to agriculture that occurred globally starting 12,000 years ago (Balter, 2007; Stukenbrock and McDonald, 2008). Control of diseases in plants is defined as "keeping disease severity below the level at which it may become economically significant" (Roberts and Boothroyd, 1972). Control measures may be split into several sub groups: regulation, cultural, physical, chemical and biological (Jones and Clifford, 1978; Heitefuss, 1989; Hewitt, 1998). Broadly, plant disease control follows one of two epidemiological

principles; either to reduce the amount of disease causing inoculum present or to slow its rate of increase thus reducing the likelihood that inoculum may reach sufficient levels to cause disease (van der Plank, 1968). Every control measure affects the pathogen either directly or indirectly by targeting susceptible hosts or altering the hosts environment (Roberts and Boothroyd, 1972); There are seven principle methods of achieving this control.

I. **Exclusion** – cultural measures such as quarantining foreign plant material, certification for pathogen free seed stocks and the practice of barrier cropping to prevent the dispersal of a pathogen (Heitefuss, 1989; Mathews, 2002).

II. **Eradication** – killing the pathogen, e.g. through physical means, during the survival phase of its life cycle, including approaches such as soil treatments, thorough tillage, crop rotation and eliminating alternative hosts/sources of inoculums (Martin and Woodcock, 1983; Katan *et al.*, 1987; Heitefuss, 1989)

III. **Therapy** – Therapeutic application of agents such as fungicides, antibiotics and bio-control agents to eradicate established disease (Narayanasamy, 2001).

IV. **Vertical Resistance** – Breeding crops resistant to infection by a specific race of pathogen (Parlevliet and Zadoks, 1977; Ma and Michailides, 2005).

V. **Horizontal Resistance** – Breeding crops resistant to all races of a pathogen (Ma and Michailides, 2005; Wyand and Brown, 2005).

VI. **Protection** – Application of chemical agents to prevent infection process of pathogen (Roberts and Boothroyd, 1972; Narayanasamy, 2001).

VII. **Avoidance** – Planning of sowing date to reduce exposure of crop to disease causing inoculum (Roberts and Boothroyd, 1972; Hewitt, 1998; Narayanasamy, 2001; Ma and Michailides, 2005).

The use of cultural and physical practices to control the prevalence of pathogens varies in their effectiveness to reduce diseases (Narayanasamy, 2001). Indeed, used individually they are ineffective as a control strategy, the use of an integrated approach, employing a

combination of several different forms of control is almost always the most efficient and cost effective method to control disease (Hewitt, 1998). Though there is extensive literature available on the interactions, advantages and disadvantages of the different options for disease control, the scope of this project is primarily concerned with the use of chemicals to protect crops from disease.

1.3.1 Chemical control measures for crop protection

The use of chemicals as plant protection agents must be carefully thought out. The relevant advantages and disadvantages of chemical use must be considered and if used in combination with other control methods they may provide excellent levels of pest and pathogen control. A chemical pesticide may be defined as a substance which kills harmful organisms which are free living at some stage in their life cycle. They may be classified by name referring to the type of pest that they control for example; insecticides, acaricides, nematicides, rodenticides, fungicides and herbicides. Some of the chemicals used can fall into several of the categories but all pesticides need to meet certain criteria to be successful (Mogul *et al.*, 1996). The use of chemicals may be further sub-divided into three main modes; Eradicative, Protective and Curative (Jones and Clifford, 1978; Hewitt, 1998; Strange and Scott, 2005). These distinctions are important as they will dictate the manner by which the chemical is employed:

Eradicative methods encompass the use of chemicals outside the plant to destroy potentially disease causing pathogens before they actually cause disease, for example a seed treatment. They may also be used to destroy a pathogen that is in the colonisation stage of its life cycle, for example, the use of fungicides to control mycelial growth of powdery mildew.

Protective methods employ chemicals that are applied to the surface of the plant to be protected. Agents in this group are applied prophylactically and provide protection against attack and invasion by plant pathogens such as fungi. The pest or pathogen that this type of treatment protects against generally penetrates the plant epidermis in some way in the initial stages of infection (Hewitt, 1998); a good example of this is the penetration peg of an appressoria produced by an attacking fungal pathogen such as powdery mildew (Heitefuss, 1989). As such the attacking pest or pathogen is rapidly killed as a result of being in contact with the chemical agent.

Curative methods use chemical agents that may help prevent further damage or loss of yield in plants that have already become infected by a pathogen. As such these agents must be able to penetrate the plant epidermis to become effective from the inside out or systemic. Many of the most effective fungicides could be classed within this group. They may be used to target a single pathogen during critical periods of infection, for example an outbreak of a disease epidemic.

With some agents, of which fungicides are an example, the definitions of eradicative, curative and protective may become blurred. In some instances the chemical agent may perform well at controlling disease once it is established but may also be employed as a pre-emptive treatment to help prevent the disease becoming a problem in the first place (Heitefuss, 1989; Narayanasamy, 2001). Chemical agents that possess this ability are highly sought after for obvious reasons. For a chemical to be employed as an agent for the control of disease, be it in a role as an eradicative, protective or curative role, it must meet some basic requirements. A general summary of these ideal targets may be seen in Table 1.1

Table 1.1 A Summary of ideal targets for chemical plant protection agents (Narayanasamy, 2001).

Attribute	Type of Product Improvement
Safety	• Safe to user and consumer • Environmentally acceptable
Performance	• Broad disease control spectrum • Control period and reliability increase • Ability to overcome resistance • Reduced levels of phytotoxicity
Use	• Compatible with other products • Easy to use formulation
Cost	• Low cost (compared to commercial formulations: - Low required dose + number of treatments - Cheap cost of purchase and application

1.4 FUNGICIDES AS CHEMICAL CONTROL AGENTS

"Fungicides are agents of natural or synthetic origin which can act to protect plants against invasion by fungi and/or to eradicate fungal infection" (Hewitt, 1998). Since their discovery fungicides have been used and developed continually alongside other agrochemicals as agents to increase yield, and quality of produce. Pressures such as increasing world population and the need to produce better quality produce for lower costs have pushed fungicide usage and development from the start (Hazell and Wood, 2008). Some of the first and oldest recorded uses of fungicidal chemicals employed elemental sulphur and copper compounds (Ogawa *et al.*, 1977). The wild predecessors to present day food crops would have been susceptible to many types of plant disease long before humans began to cultivate them for food production (Hollomon and Wheeler, 2002). Powdery mildew certainly affected plants grown by the Romans and Greeks who used sulphur to control the disease (Martin and Woodcock, 1983; Hollomon and Wheeler, 2002). Formulations such as lime-sulphur, first formulated in

the early 19th century, improved the potential array of agents available for the treatment of powdery mildew (Martin and Woodcock, 1983). However, it was not until the discovery of the first organic fungicides in 1934 that the speed of development of chemical agents for the treatment or prevention of plant disease increased (Hollomon and Wheeler, 2002).

1.4.1 Modern fungicide formulations

The discovery of the systemic antibiotic agent griseofulvin, although never widely employed in plant protection strategies, was an important breakthrough as it illustrated the potential for systemic fungicides that could be used to treat plant diseases (Hollomon and Wheeler, 2002). This stimulated the subsequent development of several systemic compounds. During the 1970s and 80s the triazole (sterol de-methylation inhibitors or DMIs) were introduced and have played an important role in the treatment and control of major plant diseases. Triazoles belong to the azole family of fungicides and contain five number rings incorporating three nitrogen atoms. Some triazoles have been employed effectively as clinical fungicides for medical applications, for example, fluconazole, which has been used successfully in the treatment cryptococcosis and coccidioidomycosis and can be administered orally and intravenously (Al-Mohsen and Hughes, 1998). Triazoles are active against ergosterol biosynthesis (Ergosterol biosynthesis inhibitors or EBIs), this class of fungicide inhibits the demethylation of sterols in fungi. Ergosterols are the predominant type of sterol in most fungal species and are responsible for the maintenance of membrane structure and function (Iris and Loeffler, 1993; Turner *et al.*, 2000). Specifically, DMI fungicides bind with the active site of 14α-demethylase, which catalyses the C-14 demethylation of 2,4-methylene dihydrolanosterol, inhibiting the action of the haem prosthetic group within the cytochrome P-450 complex within the enzyme. The presence of the fungicide in this position blocks

the binding of an activated oxygen molecule which is required for the hydroxylation of the 14α-methyl group on the developing sterol molecule. This inhibition results in an accumulation of C-14 methyl sterols and a subsequent depletion of ergosterol, disrupting membrane function and activation chitin and β-1,3 glucan synthesis ultimately preventing fungal growth (Kang *et al.*, 2001; Griffiths *et al.*, 2003). Their systemic nature, broad range of action against several important pathogens, such as rust, smut, bunt and mildew, of food producing crops and longevity of effective use have made triazoles pivotal in integrated control strategies against plant diseases (Cremlyn, 1991; Morton and Staub, 2008). In 2005 this class of fungicide accounted for over 20% of total fungicide sales worldwide (Morton and Staub, 2008).

1.4.2 Tebuconazole usage

First reported in 1986, tebuconazole (a triazole fungicide) was introduced by Bayer CropScience in South Africa in 1988. Tebuconazole is a systemic fungicide with protective, curative and eradicative modes of action and is rapidly adsorbed into vegetative parts of the plant and translocated principally acropetally. Active against a wide range of pathogens in many crops including cereals, tebuconazole is used to treat *Tilletia* spp., *Ustilago* spp., *Urocystis* spp., *Septoria nodorum* (seed borne), rusts (*Puccinia* spp.) and powdery mildew (*Blumeria graminis*) to mention a few (Tomlin, 2009). Tebuconazole has been used consistently in the UK over the past 16 years with total use increasing over the past six years - Table 1.2.

Table 1.2 A summary of tebuconazole usage in the UK between 2004 and 2008 (Pesticide Usage Statistics, 2010)

Year	Total Area Treated (ha)	Total Weight Applied (kg)
2008	2,384,270	190,727
2006	1,952,362	153,186
2004	1,668,704	126,430
2002	1,603,103	118,175

In 2006, 97.3% of the total area of arable crops planted was treated with fungicides. In total, 79.61 tonnes of raw (i.e. unformulated) tebuconazole was applied, to fields in the U.K. This equated to 3.3% of the total fungicides applied to arable crops in the UK ranking the 6th most used Active Ingredient (AI) (Garthwaite *et al.*, 2010).

1.5 APPLICATION OF FUNGICIDES

Application of fungicides for the control of plant pathogens can be undertaken using various means. Principally there are three main forms of application - either as seed treatments, foliar sprays or as a combination of these (Hewitt, 1998; A.A.B., 2010). Often pesticides are applied to crops via broadcast (spraying) methods, Table 1.3 lists some of the common formulation types currently used.

Table 1.2 Common agrochemical formulation types (adapted from Lainsbury, 2009)

Formulation Type	Description
EC	Emulsifiable Concentrate
ES	Emulsion for seed treatment
EW	Oil in water emulsion
LS	Solution for seed treatment
FS	Flowable Concentrate (for seed treatment)
WP	Wettable Powder

The scope of this research is primarily aimed at the use of fungicide formulations as seed dressings. Such applications have been primarily employed as a method of protecting seedlings from diseases caused by seed and soil-borne pathogens (Taylor and Harman, 1990; McGee, 1995). However, more recently, this form of fungicide application is routinely employed to apply systemic materials (Hewitt, 1998). Seed treatment with agrochemicals has been an effective way to control many pests and diseases in plants for several years. Crops including

cereals, cotton, oilseed rape, potatoes and sugar beet, benefit from the use of seed treatments (Hewitt, 1998).

Many of the substances or formulations employed as seed treatments today are systemic in nature and include a wide range of active ingredients including fungicides such as tebuconazole, fluquinconazole and triticonazole. Such substances provide excellent broad spectrum activity against a wide range of pathogens and are especially effective during the early stages of growth (Brandl and Biddle, 2001). The use of fungicides, such as triazoles, as seed treatments can also provide convenient and economically beneficial application options. This sector of the fungicide market has showed considerable growth globally from €560 million in 1994 to €780 million between 1994 and 1999 (Hicks, 2000). Systemic treatments also confer benefits in terms of their toxicological and ecotoxicological impact when compared to other commercial methods of application, such as spraying (Asrar *et al.*, 2004). There are many advantages to the use of seed treatments over other methods of application available, the main example being the ability to target application of treatments in a specific environment. The inherent inaccuracy of the use of sprayers for the application of plant protection chemicals, due to factors such as spray drift and problems with adhesion of materials to plant surfaces means that, often, a higher application rate is required to achieve an adequate level of protection (A.A.B., 2010). Although the advent of systemic materials has reduced required application rates, factors such as the environmental and economic impact of the use of these chemicals are still of concern (Hewitt, 1998; Hicks, 2000). The total amount of active ingredient applied to a crop, and subsequently the environment, may be greatly reduced by the use of systemic products as seed treatments. Further advances in the formulation of systemic fungicides that may give a slow release of the chemical into the environment, possibly conferring an improved and prolonged level of protection against disease, have

further increased the potential for the use of seed dressings (Bahadir, 1990; Asrar *et al.*, 2004).

One limitation of this type of treatment, even when using fungicides that are systemic in nature, is that the level of protection offered to the plant is typically only significant in the early stages of plant growth. The effectiveness of the treatment declines due to environmental degradation of the active ingredient (AI), low uptake efficiency and short half lives of the AI in the plant tissue itself. These chemicals may also be subjected to leaching, evaporation and degradation by photolysis, hydrolysis and microbial actions, all of which decrease the amount of available AI that reaches the desired target (Mogul *et al.*, 1996). Allied with this, it has proved difficult to maintain significant levels of AI in the plant as it matures, especially in later growth stages as the plants biomass increases (Ashley *et al.*, 2003). Aside from the issue of maintaining a reasonable concentration of AI in the plant as it grows there is also the problem of phytotoxicity causing stunting and emergence problems in young plants. Many systemic treatments, especially seed treatments, may have a phytotoxic effect on plants, examples include triarimol, tridemorph and azepine-A2151. All have been found to cause phytotoxic effects when applied at necessary dose rates to attain adequate disease control (Maude *et al.*, 1984). This is an issue in itself but it also limits the amount of AI that may be applied directly onto the seed potentially decreasing the potential for disease control. Therefore AIs used for seed treatments are preferably required to cause low levels of phytotoxicity and provide an adequate level of protection from pests and/or disease (Brandl and Biddle, 2001).

1.6 TEBUCONAZOLE AS A SEED TREATMENT

A commercially available formulation of tebuconazole, used as a seed treatment formulation exists in the form of a flowable suspension called Raxil produced by Bayer CropScience. Registered for use in over 100

countries on over 90 crop types, Raxil is one of the main formulations used in the prevention of seed and soil borne diseases in cereals such as *Tilletia* spp., *Ustilago* spp., *Septoria* spp. (BayerCropScience, 2010). Tebuconazole is nominally applied as a seed hygiene treatment at a rate of between 1 and 7.5 g AI per 100 kg seed (BayerCropScience, 2010). Although no longer employed as a single AI product Raxil still exists as a formulation incorporating tebuconazole in mixtures with other AIs. Many chemicals used in disease control can be phytotoxic to plants as well as the disease causing organisms the agent is applied to treat. Tebuconazole, like several other triazoles, can have a phytotoxic effect on plants. This problem is especially serious for seeds that have a higher water content which may be easily penetrated by applied fungicides causing injury to the seed endosperm (McGee, 1995). Excessive application of tebuconazole can cause damage to seeds and reduce germination. This effect is caused by the inhibition of gibberellin synthesis by the blocking of P450 dependant mono-oxygenases which inhibits the oxidation of kaurene to kaureonic acid (Rademacher, 2000). Gibberellin hormones play a vital role throughout plant development, and are vital for processes such as seed germination, stem elongation flower induction, anther development and seed and pericarp growth (Hedden and Kamiya, 1997). Triazole fungicides can also inhibit demethyl-sterol synthesis in plants. The recommended rate of application for tebuconazole as a seed treatment is between 1 and 7.5 g of AI/100 kg of wheat seed, applied at this rate tebuconazole is effective against many early season diseases affecting food crops such as powdery mildew (Asrar *et al.*, 2004; Tomlin, 2009). However application of the fungicide in this manner requires a further foliar application of the treatment later in the growing season to maintain adequate levels of protection against plant diseases (Mogul *et al.*, 1996)

1.7 CONVENTIONAL VERSUS NOVEL FORMULATIONS OF AGROCHEMICALS AS SEED TREATMENTS

The science of pesticide formulation is a broad and varied field covering aspects not only of development, production and storage of the formulations but also the interaction of the pesticide with the environment, including effects on plants, insects, animals, soil, air and water (Walker *et al.*, 2001; Markus and Linder, 2006). The choice of formulation is influenced by several factors. The physical and chemical properties of the pesticide must be considered. The crop to be treated and biological/environmental properties of the pesticide (crop selectivity, transport through soil/ground water and LD_{50} for mammalian and non-mammalian species) are also important. Finally the economic cost of the above is also an important consideration (Markus and Linder, 2006).

Often applications are performed at very high and potentially toxic rates to attain a high enough initial concentration of the chemical to be effective against the target disease (Kydonieus, 1980). This type of application usually results in a rapid decrease in available concentrations of the fungicide and subsequent repeats of the treatment later in the growing season to maintain disease control (Markus and Linder, 2006). Novel formulations must therefore be designed to meet several simultaneous demands of efficacy and suitability to mode of application (e.g. seed dressing) whilst minimising environmental impact (Markus and Linder, 2006).

Depending on the method by which a chemical control agent, such as tebuconazole, is employed, up to 90% of the total applied may be lost before the AI reaches its target objective and the desired effect is produced (Mogul *et al.*, 1996). Attaining the desired biological response at the right concentration and at the correct time is more complicated.

To combat these difficulties agents may need to be applied regularly and non-specifically. This increases the cost of the treatment and also results in other undesirable effects such as increased selective pressure on pathogens. This may lead to potential problems with fungicide efficacy and may also produce adverse effects on the plant, for example stunting (Mogul *et al.*, 1996). The emergence of technology whereby the release rate of these chemicals can be controlled and sustained has the potential to alleviate many of the inherent problems and inadequacies (Mogul *et al.*, 1996).

One of the ways by which the ideal situation of reduced phytotoxicity and prolonged disease protection may be obtained is by controlling the rate at which the AI is made available to the plant. The principal advantage of controlled release formulations of these chemicals is that much less chemical may need to be applied overall to attain the same effect (Kydonieus, 1980). Allied to this is the potential for the extension of the half-life of some of the more volatile compounds used. The AI is loaded into a carrier which then allows the molecule to be released in a controlled and sustained manner. Conversely the AI could be applied at higher rates without risking adverse phytoxicological (plant damage) or environmental effects whilst, at the same time, conferring longer term protection with a higher level of AI available for longer periods of time (Markus, 1996; Markus and Linder, 2006). By encapsulating the AI its application to a soil environment, as a seed dressing may result in the release of the AI by diffusion after hydration, in a sustained manner. The effects of leaching, environmental degradation and evaporation should also be reduced. The use of such formulations should therefore mean that each application would be far more efficient per unit weight whilst also providing longer protection per unit applied. Saturation of the ecological environment could also be reduced, meaning less risk of leaching of harmful pesticides into waterways and drinking water supplies. If these formulations could be shown to reduce reliably the

leaching affect observed with some of these treatments, these chemicals could in turn be used, reliably, near irrigation canals/other water ways and also near crops that might be sensitive to the treatment (Mogul *et al.*, 1996)

Apart from the advantages listed above there also exists the possibility of combining incompatible pesticides allowing for a more comprehensive strategy to be employed against potential pathogens and pests (Asrar *et al.*, 2004). The use of synthetic chemicals, such as fungicides, to increase yields by reducing the impact of plant pathogens, is considered vital to produce adequate food stocks (Gonzalez-Rodriguez *et al.*, 2008). The use of fungicides specifically, is pivotal in the control of both pre and post harvest pathogens that can have significantly detrimental effects on production (Clarke *et al.*, 1997). However, many of the chemicals used for these applications are not only toxic but also persistent in nature. Food is the main source of exposure of the general population to pesticides and accounts for more than 90% of total exposure (Gonzalez-Rodriguez *et al.*, 2008). Pesticide residues in food and crops are as a direct result of the application of these chemicals to crops growing in the field but also from crop residues in the soil environment of fields where food crops are grown (Businelli *et al.*, 1992). As a result, there is a growing desire to reduce the amount of pesticide use in agriculture (Freidberg, 2003). Fungicide application may also result in exposure of the general population to toxic compounds. Controlled release formulations of agrochemicals that allow less total chemical to be applied to the environment may help provide the answer to this problem.

1.8 MICROENCAPSULATION OF AGROCHEMICALS AS CONTROLLED RELEASE FORMULATIONS

1.8.1 Theory of microencapsulation

The microencapsulation of materials has evolved from examples in nature, where numerous examples exist ranging from macro to nano scale. The simplest examples of these are a bird egg or plant seed on the macroscopic scale and a cell and its contents on the microscopic scale (Bishop *et al.*, 1998; Hemsley and Griffiths, 2000). The development of microencapsulation began with the preparation of microcapsules containing dyes which were incorporated into paper for copying purposes and replaced carbon paper (Schleicher and Green, 1956). The pharmaceutical industry has long used microencapsulation for the preparation of capsules containing active ingredients, though as time passed, a variety of new technologies have been developed in this field (Ghosh, 2006). The approach of microencapsulating active materials has subsequently been utilised in a variety of applications in industries including agricultural products, food, cosmetics and textiles (Ghosh, 2006). Microencapsulation may be described as a process of enclosing micron sized particles, of solids, droplets of liquid or gases in an inert shell, which in turn isolates and protects the material from the external environment (Benita, 1996). The process also provides the possibility of combining the properties of different types of, otherwise incompatible, material (e.g. inorganic and organic chemicals). This possibility has implications for the agrochemical industry and specifically the formulation of fungicide materials for use as seed treatments, where often, the most effective treatments available involve a combination of different active ingredients (Taylor and Harman, 1990; Benita, 1996; Brandl and Biddle, 2001; Ghosh, 2006).

The resultant products of the microencapsulation process are termed microcapsules which, nominally, are of around a micron in size and have a spherical or irregular shape. The core or intrinsic part of the microcapsule, contains the active ingredient (e.g. a fungicide), while the

shell or extrinsic part, protects the core area from the external environment, this is summarised in Figure 1.2 (Benita, 1996; Gouin, 2004; Ghosh, 2006).

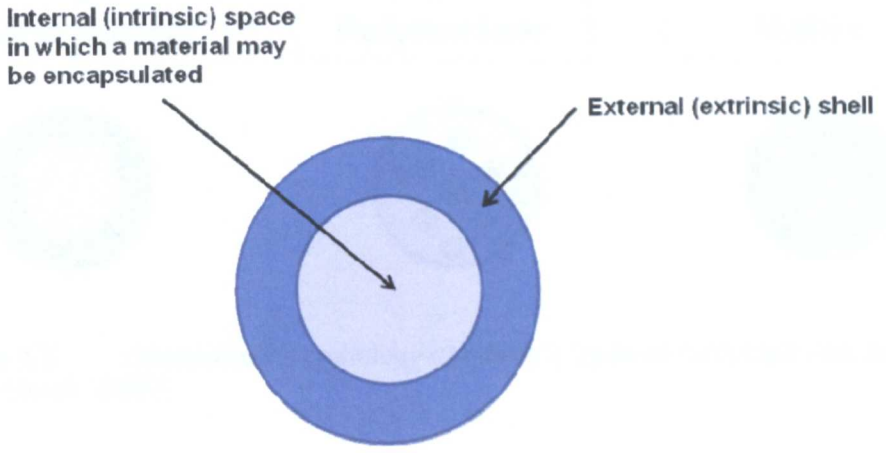

Figure 1.2 Generalised schematic of a microcapsule

For successful encapsulation there must be a sufficient degree of compatibility of the material to be loaded with in the core of the microcapsule. Pre-treatment of the core material is often required to facilitate increased encapsulation efficiencies and the size of the core material may also play a role in subsequent levels of diffusion and permeability especially in formulations where controlled release is desirable (Benita, 1996; Asrar *et al.*, 2004; Ghosh, 2006). The release of an encapsulated material is also governed by the thickness of the shell wall and this is an important criterion for choosing an appropriate microcapsule for industrial applications (Ghosh, 2006). The morphology of microcapsules may differ significantly and are dependent on the core material and are classified as three main types summarised in Figure 1.3. Mononuclear (core-shell) microcapsules contain the shell around the core, poly-nuclear contain multiple core structure contained within an external shell. In matrix encapsulation the core material is distributed homogenously throughout the shell material.

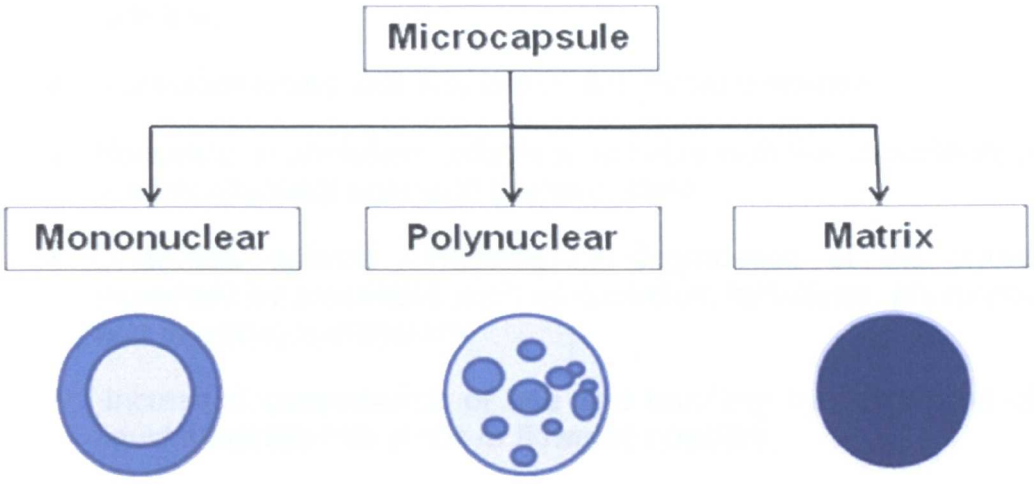

Figure 1.3 Generalised morphology of different types of microcapsules. Adapted from (Ghosh, 2006).

1.8.2 Advantages of microencapsulation

Generally speaking, the use of microencapsulation to deliver active materials such as agrochemicals to the environment has many potential advantages, these are summarised below (Ghosh, 2006).

- Protection of unstable and/or sensitive materials from their environment.
- Better processability (improving solubility, dispersibility and flowability.
- Enhancement of half-life by prevention of degarding reactions.
- Controlled, sustained and/or timed release
- Safe and convenient handling of toxic materials.
- Controlled and targeted delivery of materials.

When the material employed is a chemical pesticide, e.g. a fungicide, the use of microencapsulation may confer several further, more specific advantages (Kydonieus, 1980; Markus and Linder, 2006), these are summarised below;

- An extension in the duration of activity of the active ingredient for an equal rate of application and a decreased cost associated with this.
- Controlled timing and rate of active ingredient release.
- Reduction in phototoxic effects associated with the application of several chemical agents to plants or seed.
- Protection against environmental degradation of the active ingredient by processes such as oxidation, hydrolysis, photolysis and microbial metabolism.
- Increased convenience of use and handling by conversion of liquid materials into solids or flowable powders.

Many formulations of microencapsulated pesticide are manufactured for their ability to release the active material in a controlled manner. However, some formulations of fungicides, such as foliar sprays, are required to release the encapsulated active ingredients as quickly as possible to facilitate better uptake by target pathogens before factors such as wash off induced by rainfall or watering cause a reduction the amount of chemical available (Benita, 1996). The potential advantages of applying microencapsulated formulations of fungicide treatments are clear. However, there are some disadvantages associated with this type of formulation. The cost of producing, processing and testing controlled release formulations may be higher when compared to conventional forms of the fungicide (Kydonieus, 1980). There may be an environmental impact caused by the use of non-biodegradable or harmful materials as microcapsules. Similarly, additives and solvents used to increase the efficiency of the loading process during microencapsulation may also be hazardous, not only to the environment, but also to operators using the product and the plant to which the product is being applied (Tewes *et al.*, 2006). This problem may, however, be overcome by the use of biodegradable microcapsules such as yeast.

1.8.3 Yeast cells as preformed microcapsules

The potential for the use of microorganisms, such as yeast, to be used as preformed natural and biodegradable microcapsules for the controlled delivery of active materials has first recognised in the 1970s. Early work on yeast cells (*Saccharomyces cerevisiae*) showed that, when pre-treated with a plasmolyser, the cells were observed to be able to retain water soluble flavour compounds (Serozym Laboratories, 1973). Other research carried out in 1970s by the company Swift and Co., used specially pre-prepared yeast containing high lipid concentration (>40%) to encapsulate lipophillic materials (Swift and Company, 1977). Subsequently, there have been several examples of synthetic polymeric delivery vehicles investigated for the delivery of pharmaceutical materials (Langer and Peppas, 2003; Langer and Tirrell, 2004). Increasing amounts of research into the use of microorganisms for this application, especially for the application of molecules such as bioactive proteins and vaccines have been carried out over the past 15 years. The use of yeast cells for this purpose could include the production and subsequent release of a desirable active, such as a vaccine, *in-situ* within the environment of application, such as the human gut (Blanquet *et al.*, 2004).

Yeast are unicellular fungi, averaging approximately 5 micrometers (for *Saccharomyces* spp.) in diameter, confined by a rigid cell wall which protects the internal membrane and organelles from the external environment. The cell wall is composed of complex and highly linked glucan, mannan and chitin and is approximately 100-200 nm thick comprising 15-25% of the dry mass of the cell. In live yeast cells, the cell wall surrounds the much thinner (<10 micrometers) plasma membrane which consist of a typical bi-layer unit membrane comprising (of) phospholipids, sterols and neutral lipids, mainly by triacyl glycerols and sterol esters (Nelson *et al.*, 2006). The structure of a typical yeast cell can be seen in Figure 1.4. A freeze fracture cryo scanning electron

micrograph (SEM) image is seen in Figure 1.5. The image shows the exposed internal contents of a yeast cell. It is these contents which are removed to allow the microencapsulation of materials to take place within the cell wall of the yeast cell. The cell membrane provides the main barrier to the uploading of active ingredients into the yeast's structure and inactive yeast is therefore more effective at carrying AIs (Nelson *et al.*, 2006).

Figure 1.4 Diagrammatic representation of structure of a typical yeast cell (adapted from Duckham *et al.*, 2003)

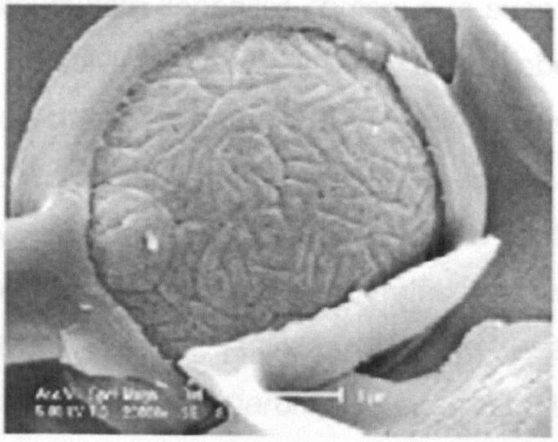

Figure 1.5 A cryo SEM image of a typical yeast cell (*Saccharomyces cerevisiae*) showing the cell wall and exposed internal contents within the cell membrane. Reproduced with permission, (Duckham *et al.*, 2003)

1.8.4 Microencapsulation in yeast cells

Yeast cells are one of the most important classes of micro-organism with applications in the brewing, ethanol production, baking and recombinant protein and biopharmaceutical industries (Nelson *et al.*, 2006). The encapsulation of chemicals in microorganisms was established as a patented technology in the late 1980s by AD2 Ltd. (AD2, 1987). The technology became commercialised in subsequent years initially for the delivery of flavour volatiles but in more recent times, for other AIs (Duckham *et al.*, 2003). The process involves the cytoplasmic contents of the cell being removed and the material to be encapsulated taking its place (Nelson *et al.*, 2006). Yeast used could be the industrial bi-product of other primary industrial processes and, as such, the yeast would be available in large quantities and at low, cost-effective prices. The size and physical make (lipid content) up of the yeast microcapsules could also be controlled easily *in vitro* by altering the nutrient balance in the fermentation medium (Nelson *et al.*, 2006). The use of specific yeast strains grown on high nitrogen media enabled the accumulation of high concentrations, upwards of 40%, of lipid material. These strains (*Torulopsis lipofera* and *Endomyces vernalis*)

were able to accumulate lipophillic compounds such as dyes, vitamins and drugs by dissolving the AIs in the lipid globules found in the cytoplasm (Swift and Company, 1977). This technology progressed further when AD2 Ltd. proved it was possible to encapsulate lipophillic substances in yeast cells with less than 5% lipid content (Nelson *et al.*, 2006). This allowed the use of yeast strains that were already being employed in other industries such as brewing, bio-ethanol production and baking, including the *Saccharomyces* species (Nelson *et al.*, 2006).

The encapsulation of lipid soluble compounds has been found to be possible in either living or dead yeast cells (Bishop *et al.*, 1998) The technique for encapsulation of essential oils and other chemical was confirmed by the presence of droplets found within the 4-5 micron yeast capsule by Bishop *et al.* (1998). Yeasts from several different processes have been tested for this application. Bi-product yeast from bio-ethanol production is non-viable due to its prior treatment, other yeasts used in the past have been still living when used (Duckham *et al.*, 2003). However, the specifics of the release mechanism are not fully understood (Duckham *et al.*, 2003). It is hoped that by microencapsulating AIs such as fungicides the yeast can offer some form of protection from environmental effects and that a more prolonged and controlled release can be achieved (Salvage, 2004).

A company called Micap PLC, formerly based in Runcorn, England developed and patented a novel microencapsulation technology (Pannel, 1987). Micap, the registered trade name for the technology, used yeast cells to enclose active ingredients. The encapsulation process (using strains of the yeast *Saccharomyces cerevisiae* available as bi-products from the baking and brewing industries) has been described (Bishop *et al.*, 1998; Nelson *et al.*, 2006) and is summarised in Figure 1.6 The process involves the mixing of the active to be

encapsulated with yeast and water in the presence of a loading solvent. The mixtures are then separated by centrifugation and spray dried prior to use.

Encapsulation levels generally attained 20 to 40 % (w/w) but can sometimes reach 80 % (Nelson and Crothers, 2003). The technology has been used successfully in the food industry where it has been used to encapsulate essential oils and synthetic flavours and protect them from evaporation. These molecules can then be released from the capsules on contact with the moist tongue surface without the yeast cell being disrupted (Nelson and Crothers, 2003). The release mechanism is not by cell fracture but by diffusion, and the yeast cells are able to withstand high pressures and shear forces (Nelson and Crothers, 2003). The structural integrity of the capsules is vital to protect the active molecules during the application process.

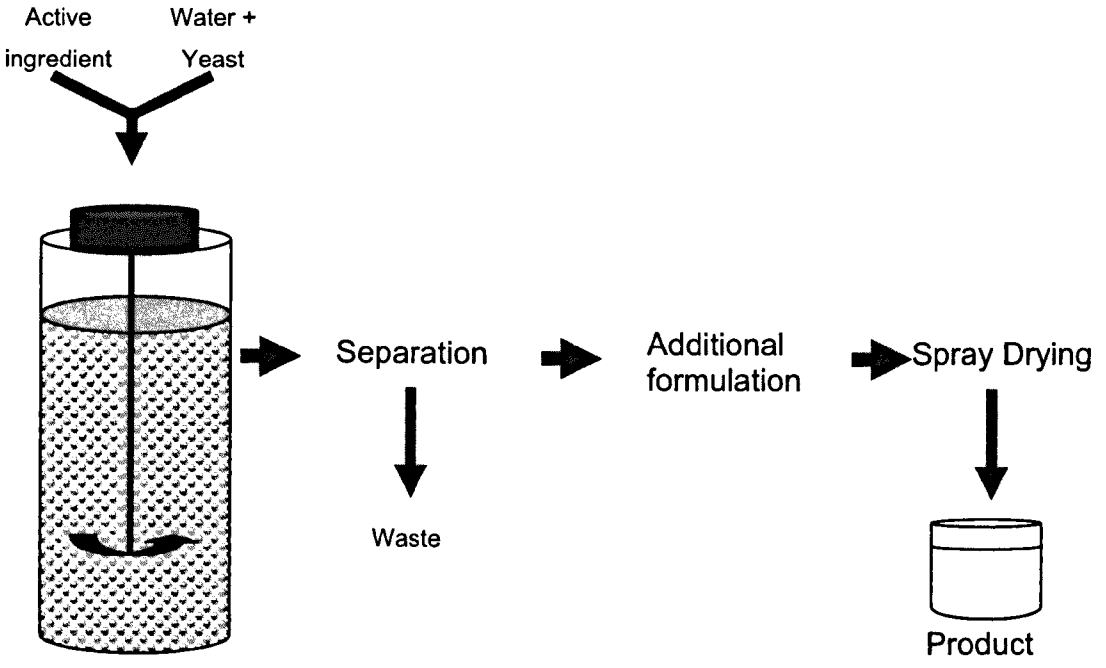

Figure 1.6 Process steps in the Micap yeast-based encapsulation process (Adapted from Pannel, 1987)

Yeast cells can be used to encapsulate a wide range of commercially important active ingredients, including fungicides (Nelson and Crothers, 2003). The yeast cells are indeed an ideal vehicle for the delivery of fungicides as they have some natural bio-adhesive effects (Nelson and Crothers, 2003), which would allow them to stick to the surface of the plant tissue. In addition, in suspension, agglomerates of yeast cells disperse very quickly to one or two cells, which are approximately five microns in diameter (Nelson and Crothers, 2003). These characteristics suggest the feasibility of good delivery as the yeast cells which are small enough not to clog the equipment used by growers during a spraying process. Unfortunately Micap PLC ceased trading in 2007, within the period of the research undertaken for this project.

1.9 DETECTION AND EXTRACTION OF FUNGICIDE RESIDUES

1.9.1 Detection

Fungicides are used widely in agriculture to control a variety of diseases and belong to various chemical classes including triazoles, benzimidazoles and carbamates (Sanchez-Brunete *et al.*, 2002). There are many, varied, published methods for the extraction, detection and quantification of fungicide residues in plants, vegetables, fruits, soil, water, and even honey bees (Hiemstra *et al.*, 1995; Torres, 1997; Wu *et al.*, 2001; Rial-Otero *et al.*, 2004; Khummueng *et al.*, 2006; Charlton and Jones, 2007). Conventional methods for the detection and quantification of fungicide residues usually consist of the following steps: sampling and sample preparation, liquid-liquid partition and cleanup followed by determinative procedures (Sherma, 1975). There are several detection techniques available for the detection of fungicide residues. These can include the use of analytical techniques such as reverse phase high performance liquid chromatography (RP-HPLC) with column switching and diode array detection (DAD) (Michel *et al.*, 2006). Techniques using LC-MS/MS have also been reported as a

method of for assessing systemicity of fungicides in wheat plants without the use of radio-labelled compounds and/or efficacy tests (Klittich *et al.*, 2008). There has also been some research into the use of molecular methods for the detection of fungicide residues (Danks *et al.*, 2001). However, the pre-eminent technique employed in the detection and quantification of fungicide residues, and pesticide residues in general, is Gas Chromatography Mass Spectrometry (GC-MS) (Scott and Gutnikov, 2000).

The introduction of gas chromatography in the 1960s revolutionised residue detection methodology (Fong *et al.*, 1999; Scott and Gutnikov, 2000). Gas chromatography has very low detection limits and it is for this reason that it has evolved as the method of choice for pesticide residue analysis (Seiber *et al.*, 1999). The principle of GC is to focus on the unique structural features of each analyte and allow detection of these even if other chemicals are present at higher concentrations (Seiber *et al.*, 1999). This is achieved by separating the compounds so that they may be presented to the detector (e.g. mass spectrometer) for identification. Samples, dissolved in a small volume of solvent (e.g. methanol or dichloromethane) are injected into the GC through a plastic membrane seal into a heated glass injector column where they become vaporised. The subsequent vapour is then forced down a column by a constant flow of analytical grade helium (or nitrogen). A thin liquid phase coating the inside of the column absorbs each chemical element of the injected samples to different degrees allowing the different components to travel down the capillary column at different rates. The column is installed in an oven maintained at a sufficient temperature to prevent the sample from condensing; the manner in which the temperature of the oven changes during an analytical run can be adjusted to optimise the separation of the samples.

The column terminates within a component of the MS known as the source, which is under a constant vacuum and high temperature, samples are transferred to this section of the GC-MS via heated transfer line again to prevent samples from condensing. Molecules of the compounds that have exited the column are bombarded with a constant stream of electrons, generated by a filament similar to that found in a light bulb, turning the molecules into ions. The ionised sample components exit the source under the influence of an electrical field forming an ion beam which is then focussed by a series of charged plates known as lenses and onto the mass analyser by the electrical field generated by four, charged rods known as a quadrupole. On entering the mass analyser a complex series of varying electrical fields consisting of radio frequencies and maintained static electrical voltage allow ions of a certain mass, dictated by the mass spectroscopy scan program, to pass. These ions enter the detector where they are accelerated onto a conversion dynode forming electrons, which continue onto a phosphor. Electrons striking the phosphor emit light which is converted into a measurable electrical signal by a photomultiplier and analysed (Fisons GC 8000 operating manual, 2005). Using GC-MS both qualitative and quantitative analysis may be performed on many fungicide residues including the analysis of multiple residues (Sanchez-Brunete *et al.*, 2002).

1.9.2 Extraction

Despite the selectivity and sensitivity of analytical techniques, such as GC-MS, there is often a requirement for the pre-concentration of trace amounts of compounds due to their low concentrations encountered. This is especially true in the analysis of water samples (Rial Otero *et al.*, 2003). Furthermore, high levels of other components often accompany analyte of interest and may make subsequent detection more difficult. In the past, liquid to liquid extraction (LLE), usually involving organic solvents has been a staple means of removing

analytes of interest from sample materials (Camel, 2003; Rial Otero *et al.*, 2003). More recently solid phase extraction (SPE) has been employed to selectively remove and also concentrate the analyte of interest from samples whilst simultaneously removing other interfering compounds and improving detection limits (Liao *et al.*, 1991; Rial Otero *et al.*, 2003). The first experimental applications of SPE started over 50 years ago. However, the technique only became widely used as an alternative to LLE for the sample preparation in the mid 1970s (Lika, 2000). SPE has been shown to be an applicable tool for the extraction and purification of analytes in many different applications (Kakalíková *et al.*, 1996; Bernal and Del Nozal, 1997; Bernal *et al.*, 1997; Herrero *et al.*, 2006).

1.9.2.1 Solid Phase Extraction (SPE)

The principle of SPE is similar to that of LLE involving the partition of solutes between two phases. However, instead of two immiscible liquid phases, such as water and dichloromethane, SPE involves the partitioning between a liquid (sample matrix) and a solid (sorbent) phase (Camel, 2003). The basic approach involves passing a liquid sample through a column containing an adsorbent which retains the analyte which are then subsequently recovered or eluted from the sorbent bed using an appropriate solvent.

An SPE method comprises a series four successive steps; the sorbent bed is first conditioned using an appropriate solvent followed by a further volume consisting of the same solvent as that of the sample. This step of the method is vital as it serves to wet and to remove air from the sorbent bed, activate (solvate) functional groups of sorbent bed and to remove any impurities remaining after production of the cartridge (Lika, 2000). During extraction the cartridge must not be allowed to dry out between samples as this may lead to poor retention

of analytes and subsequent lower than optimum recoveries. Similarly, if the cartridge is to be used repeatedly then appropriate care must be taken to re-condition the sorbent bed between samples (Rial Otero *et al.*, 2003).

The sample is then passed through the SPE cartridge in an appropriate volume of solvent, (ranging from 1-1000 mL depending on application) this may be achieved using gravity, pumping, vacuum aspiration or by an automated system. This step concentrates and retains the analyte on the sorbent bed (Camel, 2003). Trace elements are usually adsorbed onto the solid phases of the cartridge through Van der Vaals forces or hydrophobic interactions, especially if the sorbent bed is highly non-polar or reverse phase (Lundgren and Schilt, 1977). This second step is commonly followed by a third step, where by the sorbent bed is 'washed' by passing a further volume of solvent with a low elution strength through the cartridge. This eliminates any matrix components that may be retained within the sorbent bed and may also remove excess water left in the cartridge which may have an adverse effect on subsequent extract concentration and/or hinder analysis (Camel, 2003).

The fourth step consists of the elution of the analyte from the sorbent bed using a suitable volume of solvent to ensure a dilute final concentration of analyte to solvent, an appropriate flow rate of elution solvent through the sorbent must also be used to ensure efficient elution. For the elution phase of the method to be effective the analyte must have a high solubility in the solvent selected to ensure efficient partioning from the sorbent bed into the solvent. The elution solvent must also be selected to elute the target analyte only (Camel, 2003).

1.9.2.2 Advantages of SPE

The use of LLE to extract concentrations of analyte from samples are often time consuming and labour intensive, especially if high numbers of replicate samples are to be analysed. The use of SPE is beneficial for a number of reasons; LLE uses comparably high volumes of solvents, SPE reduces total volumes required and the associated expense of disposal (Majors, 1986). SPE also allows the simultaneous extraction, concentration and removal of interfering compounds into a final sample volume smaller than that of the initial sample. Further to this, SPE offers a high degree of selectivity compared to other extraction options, such as LLE, by only pre-concentrating trace elements of interest and avoiding elution and subsequent detection problems associated with major ions (Castillo *et al.*, 2001). This facet of SPE may also allow a further degree of speciation for compounds, whereby analytes that may hinder subsequent detection may be removed individually thus further increasing potential limits of detection for the analyte of interest (González-Toledo *et al.*, 2001).

1.10 UPTAKE AND FATE OF FUNGICIDES IN PLANTS

1.10.1 Uptake pathways

For the most part, fungicides are synthetic man-made chemicals and, as such, would not exist in the environment naturally. It is therefore reasonable to think of fungicides applied, even intentionally, to the environment as a form of pollutant in so far as without human intervention, these compounds would not be present naturally. The uptake of organic chemical compounds, such as fungicides, into plants is a complex combination of numerous factors (Trapp, 1995). The topic of the accumulation of pesticides in food crops, their persistence and fate in the environment and methods for the determination of these residues has been the topic of a huge amount of research (Gonzalez-Rodriguez *et al.*, 2008).

There are at least three principle pathways by which chemicals may transit from the environment into plants.

- Root uptake into conduction channels and subsequent translocation by the transpiration stream, as a result of desorption of chemicals from the soil via water or air in the soil.
- Uptake of chemicals from compounds in the surrounding air, through the plant epidermis or via natural openings such as stomata.
- External contamination of shoots by soil or dust resulting in uptake by retention on and subsequent penetration through the plant cuticle. A summary of these pathways is depicted in Figure 1.7. In most cases the uptake of a chemical into a plant is facilitated by a combination of these pathways (Topp *et al.*, 1986).

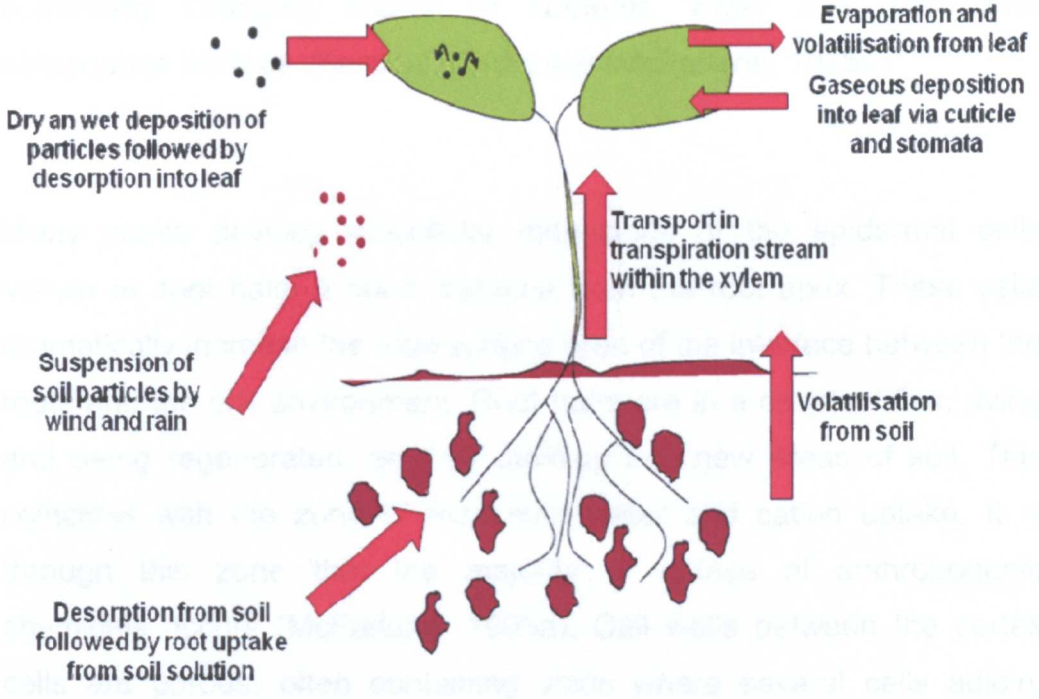

Figure 1.7 A summary of the principle pathways for the uptake of organic chemicals by plants. (Adapted from Collins *et al., 2006)*

The scope of this research is primarily concerned with the uptake of fungicides applied to the soil as seed treatments and not via other means. Uptake of fungicides from the soil occurs via the root system of the plant and is predominately a passive process. Uptake occurs via the vapour or water phases of the soil by the process of diffusion. There are, however, some exceptions to this, most notably some hormone like chemicals such as the phenoxy acid class of herbicide for which there has been shown some evidence of active uptake (Bromilow and Chamberlain, 1995). Roots provide anchorage, permit storage of energy rich molecules and undergo a physical as well as a chemical interaction with the soil. As with the visible, aerial parts of plants, the root architecture of each species of plant may differ greatly (McFarlane, 1995a). The manner in which the roots develop is driven by a variety of factors such as genetically controlled branching characteristics, radial growth, development of secondary cell walls and layering of suberin. Root development is also driven by other factors such as soil characteristics, available nutrients, water, pathogens, meteorology and solar radiation (Amir and Sinclair, 1991). Root growth provides a continually changing source of nutrients, water and also other compounds such as chemicals in the soil (McFarlane, 1995a).

Many plants develop unicellular extensions of the epidermal cells known as root hairs a short distance from the root apex. These cells dramatically increase the total surface area of the interface between the roots and the soil environment. Root hairs are in a constant flux, dying and being regenerated, as they develop into new areas of soil. This coincides with the zone of maximum water and cation uptake. It is through this zone that the majority of uptake of anthropogenic chemicals occurs (McFarlane, 1995a). Cell walls between the cortex cells are porous, often containing voids where several cells adjoin. Water containing dissolved solutes moves freely from the soil solution to the interior of the roots via the capillary spaces between these cells.

This is known as apoplastic movement. This movement, summarised in Figure 1.8, is stopped before entry into the plants vascular system at the endodermis where a band exists of waxy material formed around anticlinal walls of these specialised cells called the casparian strip. At this point dissolved solutes carried into the plant via the roots must pass through at least one cell membrane to move into a plant's vascular system. This is classed as symplastic movement. The pace of this movement has been shown to be affected only by the rate at which water moves through the plant driven by evaporative loss of water from the leaves i.e. transpiration (McFarlane, 1995a). Experiments involving the uptake of non-ionised chemicals from a hydroponic solution into plant roots have shown that the process consists of two components. Firstly equilibration, where chemical concentrations in the aqueous phase of the plant root and the surrounding solution become balanced. Secondly, sorption of the chemical onto lipophillic root solids found in the cell membranes of and cell walls of cells in the roots (Paterson *et al.*, 1991). Once in the roots the chemical may then disperse to other areas of the plant via the plants vascular system.

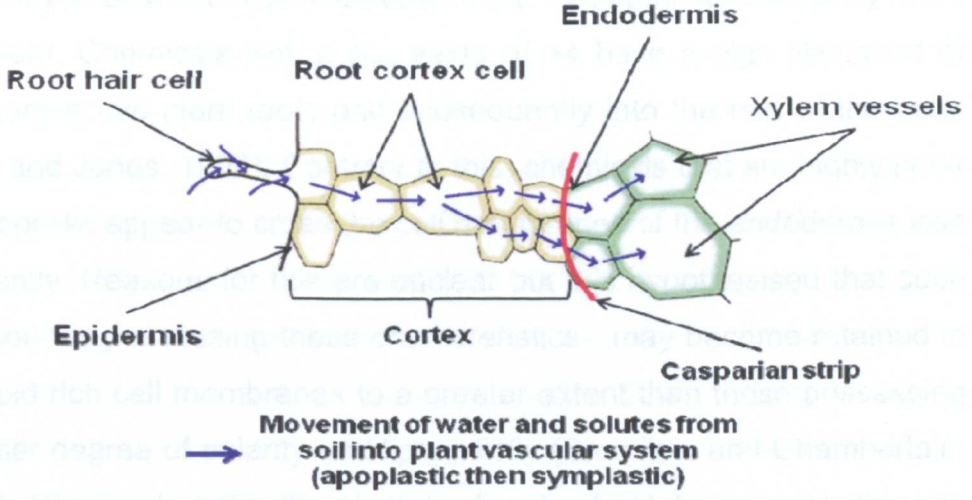

Figure 1.8 Wheat root cross section showing the route of water and dissolved solutes from the soil into the plant. (Adapted from McFarlane, 1995a)

1.10.2 Factors affecting uptake

There are several factors affecting the degree to which a chemical is available for uptake from the soil. Initially, uptake is governed by the physiochemical properties of the chemical in question. This is highlighted, in the first instance, where chemicals are taken into the roots and travel, via the apoplastic pathway to the endodermis. Here, they are then required to cross a cell membrane to enter into other parts of the plant. The implication of this is that for a compound, such as a seed applied fungicide, to be successfully taken in by the plant to provide protection from pathogens it must be lipophylic to a high enough degree to achieve this transition across the membrane. For this reason, lipophilicity is one of the principle factors governing the uptake of chemicals into plants from the environment (Bromilow and Chamberlain, 1995). The degree of lipophilicity may be measured by assessing a chemicals 1-octanol/water partion co-efficient or K_{ow}, previous experiments have shown there to be a linear relationship between K_{ow} and the accumulation of non-ionised chemicals in roots (Briggs *et al.*, 1983). Chemicals that have higher K_{ow} have a higher tendency to bind with lipid elements in the roots and subsequently enter the plant. Chemicals with a K_{ow} value of >4 have a high likelihood of partitioning into plant roots and subsequently into the rest of the plant (Wild and Jones, 1992). Contrary to this, chemicals that are highly polar or lipophillic appear to cross the cell membranes of the endodermis less efficiently. Reasons for this are unclear but it is hypothesised that such compounds possessing these characteristics may become retained in the lipid rich cell membranes to a greater extent than those possessing a lesser degree of polarity and lipophilicity (Bromilow and Chamberlain, 1995). Chemicals enter the plant as dissolved solutes in water through the roots the level of water solubility of a given chemical is also important. Ultimately, it is a combination of water solubility and lipid solubility which determine the degree to which a chemical is able to enter the plant and move within it (McFarlane, 1995b).

1.10.3 Movement of solubilised chemicals within the plant

Water and dissolved solutes are transported in the transpiration stream upwards within the plant from the roots to other areas, this is termed acropetal transport (Collins *et al.*, 2006). This is driven by the rate of transpiration. A relationship for predicting concentrations of chemicals in the transpiration stream from the concentration of the chemical in the soil solution and the K_{ow} of the chemical was derived by Briggs *et al.* (1983). The research undertaken investigated the uptake of a limited number of non-ionised chemicals into barley plants. The findings of the work showed that the transpiration stream concentration factor (TSCF), could be predicted by dividing the concentration of the chemical in the xylem sap by its concentration in the external solution. This was found to be at a maximum for chemicals with a log K_{ow} of around 1.8 (Briggs *et al.*, 1983). Different work by other authors have found potentially large differences in results especially for compounds at the higher and lower end of the log K_{ow} scale (i.e. >4 or <1) (Hsu *et al.*, 1990; Burken and Schnoor, 1998). This suggests that the model is not perfect and differing local concentrations of lipids and other solutes in the plant may change the way in which a chemical moves through the plant.

Once in the transpiration stream solubilised chemicals are transported throughout the plant and may become concentrated in plant shoots as well as other areas. This accumulation may take place as a result of equilibration of the chemical between the aqueous phase of the plant shoot aqueous phase and the xylem. This can be affected by lipid concentrations in the shoot and the relationship, or stem concentration factor (SCF) has been shown to be linear when compared to the log K_{ow} of the compound (Barak *et al.*, 1983; Briggs *et al.*, 1983). The effect of lipid concentrations in the stem, on uptake, will increase as a chemical moves further up the stem. As a result of this, highly lipophillic compounds (log K_{ow} >4) are unlikely to perform well as systemic products as they would have to be applied at significantly higher

concentrations to permit an even distribution throughout the plant (Uchida, 1980; McCrady *et al.*, 1987).

1.11 GENERAL AIMS AND OBJECTIVES

The aim of the research was to assess the release and uptake of tebuconazole from a novel formulation of the fungicide. Yeast cells were used as microcapsules to allow the production of a microencapsulated form of the fungicide. The main premise behind the production of this formulation was that the yeast cells would be able to provide protection of the fungicide from degradation but also confer a more controlled and sustained release of the AI into the environment. It was hoped to be able to demonstrate improved levels of crop safety by reducing the effect of phytotoxicity associated with tebuconazole when applied as a seed dressing. It was also hoped that the new formulation could be shown to provide tebuconazole available for plant uptake for a longer period then existing formulations of the fungicide. The main research objectives were as follows:

- To assess the release characteristics of tebuconazole from the yeast microcapsules into water.
- To select and optimise a suitable method for the detection and quantification of tebuconazole released from microcapsules into water.
- To assess the release and uptake into plants of tebuconazole from the yeast microcapsules when applied as a seed dressing and compare this to fungicide release and uptake from a commercially available formulation.
- To select and optimise an easily repeatable and accurate method for the extraction and quantification of tebuconazole from treated plant samples.

CHAPTER 2 – *IN VITRO* MICROENCAPSULATED TEBUCONAZOLE RELEASE INTO WATER

2.1 INTRODUCTION

The use of fungicides to provide effective control of plant disease remains vital. Yield reductions are estimated to be 20% in the major food and cash crops worldwide (Oerke *et al.*, 1994; Oerke and Dehne, 2004). The use of single target site fungicides, including triazoles, has steadily increased since their introduction in the 1960s and this class of fungicide tended to replace older multi site actives (Gulliano *et al.*, 2000). However, these older formulations still play an important role in fungicide mixtures, especially when employed in anti-resistance strategies. Despite the launch of QoI fungicides (strobilurins) in the late 1990s and early 2000s, serious resistance problems with these compounds, has ensured that triazoles still have an important role to play in current crop protection strategies. The imminent launch of succinate dehydrogenase inhibitors (SDHIs) fungicides will, however, still permit a market for triazoles as the new formulations will be sold as a mixture of the two active ingredients.

Triazole fungicides, such as tebuconazole (Figure 2.1), provide excellent control of a wide range of diseases, particularly in early crop growth stages (Asrar *et al.*, 2004). However, increasing attention is being directed to reducing the cost and amount of pesticide used in the protection of crops. As much as 90% of applied conventional agrochemicals never reach their target objective in sufficient time and concentration to provide the desired effect (Mogul *et al.*, 1996). The resultant over application of agrochemicals is undesirable in terms of both cost and environmental impact and may be reduced by the use of novel formulations of fungicide. One such approach is that of microencapsulation – where the active ingredient is released over a

prolonged period of time. Various microencapsulation processes have been described for the encapsulation of pesticides (Markus, 1996), one example being the use of yeast cells as bio-capsules.

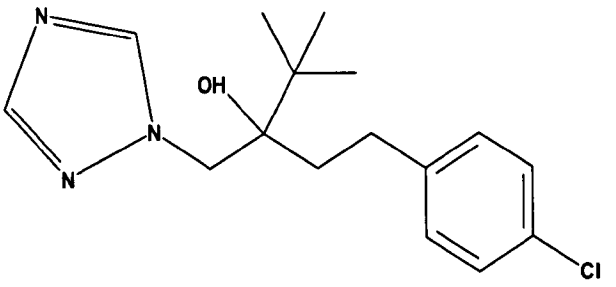

Figure 2.1 The chemical structure of the triazole fungicide tebuconazole (P.P.D.B., 2010).

In an agricultural sense the use of microencapsulation, giving a more prolonged and controlled release, may have other benefits. The use of fungicides, such as tebuconazole, as seed treatments has the ability to control a wide range of plant diseases especially in early growth stages (Asrar *et al.*, 2004). The effectiveness of these seed treatments can be nullified over time by influences of environment, poor uptake efficiency and potential short half life of the AI in plant tissue. It is also difficult to maintain an effective concentration of active within the plant due to increasing plant size and mass (Asrar *et al.*, 2004). Many of the chemical formulations used as actives, including seed treatments, are also phytotoxic to plants and, as such, must only be used in limited concentrations, higher rates of use are also potentially damaging to the environment, even in the case of systemic products (Asrar *et al.*, 2004). The use of microencapsulation could therefore prove beneficial as a more controlled release could provide a solution to many of these issues as well as providing other benefits such as prolonged disease protection (Salvage, 2004).

Several batches of microencapsulated tebuconazole were produced using a method patented and developed by Micap PLC. Yeast obtained from different sources was evaluated by Micap and unpublished research suggested that waste yeast from the process of bio-ethanol production may be the most efficient type to use due to improved levels of release of the active observed from this type of yeast and also due to the low cost of the material (Duckham and Wheeler, 2005a). This type of yeast was used in the preparation of a new microencapsulated tebuconazole sample for the purposes of this research. The formulations being tested could be used as a spray or seed dressings and the release of tebuconazole from the yeast microcapsules is dependent on their hydration. The interaction between the yeast microcapsules and leaf or seed surface and the surrounding environment will dictate the amount of tebuconazole available for uptake by the plant. As release of tebuconazole from the yeast microcapsules is dependent on the presence of water, this will be the medium linking the microcapsules to the surrounding environment. An understanding of the release profile in water may help in the further understanding the behaviour of microencapsulated tebuconazole formulations in actual application.

Several experiments were undertaken to assess the release characteristics of tebuconazole from the yeast microcapsules into water. Reliable and accurate extraction, detection and quantification methods were first developed to allow this release to be assessed. Yeasts from different sources were formulated to contain tebuconazole. Several batches were initially assessed including formulations of different ages to investigate if the type and storage of yeast had any effect on the release of the fungicide. A formulation of microencapsulated tebuconazole was also tested for its potential to release tebuconazole over a sustained period.

2.2 SUMMARY OF AIMS AND OBJECTIVES

- Develop a reliable extraction, detection and quantification method for tebuconazole in water.
- Test and explain the release kinetics of tebuconazole from Micap formulations in water.
- Examine the release properties of several batches of microencapsulated tebuconazole from different yeast sources.
- Examine the potential effect of formulation age on the release of tebuconazole.

2.3 MATERIALS AND METHODS

2.3.1 Production of microencapsulated tebuconazole samples

A 5 g aliquot of technical grade tebuconazole (manufactured by Bayer BayerCropScience, supplied by Micap PLC., Runcorn, England) was weighed and added to a 10 mL volume of acetophenone. The solution was mixed using a heated (40°C) magnetic stirrer and further 1 g aliquots of tebuconazole added until the solution was saturated. A yeast (washed Aventine *Saccharomyces cerevisiae*) slurry was produced by the mixing of yeast and sterile distilled water (SDW) at the ratio of 5:3 water:yeast. The slurry was mixed using an overhead stirrer and paddle in a heated water bath (45°C) for 30 min. The tebuconazole solution was added to the yeast slurry at a ratio of 1:2 solution:slurry and left to mix for a minimum period of 12 h. Yeast cells were recovered by centrifugation at 3400 g for 20 min at room temperature. The resultant pellet was washed by re-suspension in SDW, and adjusted to contain a maximum of 20% solids prior to spray drying. A Büchi B-290 laboratory scale mini spray drier (Postfach, Switzerland) was used to form a dry powdered sample of microencapsulated tebuconazole.

Finished samples were assessed in triplicate for their tebuconazole content by solvent extraction. A 50 mg (±0.05g) sample of the yeast was weighed into a 2 mL Eppendorf tube and hydrated with 350 µL of SDW. A vortex mixer was used to agitate the sample and, after 30 min standing time, 1.4 mL methanol (MeOH) added. The sample was mixed again and then sonicated (sonicleaner 644i sonic bath, Ultrasonics Ltd. Kettering, England) for 30 min. The yeast was removed from the sample by centrifugation (22,660 g for 5 min) and the supernatant filtered using a nitrile filter and syringe. The pellet was re-suspended in a fresh 1.4 mL volume of MeOH and the supernatant recovered in the same manner. The replicate samples were then analysed using Gas Chromatography linked to a flame ionisation detector (GC-FID) method

developed by Micap PLC for the purpose. An aliquot of 1 µL was injected using a split injector (250°C) onto a HP-5 column (Agilent Technologies, Stockport, England) with dimensions of 30 m length x 0.32 mm internal diameter x 0.25 µm film thickness. The column was installed in an Agilent 6890N gas chromatograph fitted with an autosampler and split/splitless inlet and FID (Agilent Technologies, Stockport, England). The software package GC ChemStation was used to program the equipment. An initial start temperature of 50°C (hold time 2 min) increasing at a rate of 10°C min^{-1} to a maximum of 300°C (hold time 2 min) was used. The carrier gas used was Helium at a flow rate of 1 mL min^{-1}. The detector (FID) was set at a temperature of 300°C with gas flows set at 30 mL min^{-1} H_2, 400 mL min^{-1} Air and 45 mL min^{-1} N_2. The results were processed to give the percentage active loaded into the yeast and also the efficiency of the extraction. Results of the GC-FID analysis were quantified by comparison with standards of know concentration allowing an estimation of the amount of encapsulated fungicide to be made.

2.3.2 Aqueous *in vitro* release testing of Micap formulation

The release kinetics of the tebuconazole from the dry yeast cells into water was assessed. Three batches of microencapsulated tebuconazole (Table 2.1) produced from different yeast sources were initially tested. Tebuconazole is frequently applied as a spray at a water volume of 200 L ha^{-1} containing 1 L of a commercial tebuconazole formulation such as Folicur which is manufactured to contain 250g L^{-1} tebuconazole. As such, the fungicide active ingredient is applied at a rate of 1.25 g L^{-1}. The Micap formulations to be assessed were prepared to be equivalent to this application rate.

Table 2.1 Sample details of microencapsulated tebuconazole formulations tested in preliminary release experiments

Batch Number	Tebuconazole encapsulated (%)	Mass equivalent to 1.25 g L^{-1} (g)	Yeast source
4292	9.6	0.65	Baker's yeast (autolysed)
3122	19.0	0.33	Baker's yeast (non-autolysed)
4282	21.7	0.29	Bi-product Bio-ethanol production

A preliminary experiment was carried out to test the different batches of microencapsulated tebuconazole for their ability to release tebuconazole into an aqueous solution. Three batches of microencapsulated tebuconazole were weighed (Table 2.1) and mixed with 50 mL of SDW in 50 mL plastic Sterilin universal tubes and mixed using a vortex mixer. Each replicate sample was analysed over a time course – 5, 15, 30, 60, 240, 480 and 1440 min respectively. At each sampling point the samples were centrifuged for 5 min at 3400 g and a 5 mL aliquot removed and set aside for extraction. A 5 mL volume of fresh SDW was added to each sample which were then re-suspended using a vortex mixer and set aside until the next sampling point. During the course of the same experiment the pH of the solutions was also monitored. The pH was recorded at the start of the time course and at each sampling point thereafter.

2.3.3 Extraction and quantification of tebuconazole

Samples were analysed by GC-MS and needed extraction prior to analysis by using an LLE method with dichloromethane (DCM). Quantification of sample fungicide concentrations was performed by comparison with known quantities of tebuconazole prepared as standards. The external standard series was run as part of the sequence of samples being analysed by GC-MS. The peak area for the

external standard, observed on the GC-MS chromatogram, was recorded and compared to the peak area obtained for tebuconazole in the sample, allowing the amount of tebuconazole in the sample to be calculated.

Initial results obtained allowed for the quantification method to be refined by the addition of an internal standard. An internal standard consists of a compound that is structurally and chemically similar, but is not identical to the compound of interest in the samples (Skoog *et al.*, 2007). The internal standard was added to each sample during the extraction process. As such, any loses of analyte during the extraction technique will be paralleled by losses of the internal standard compound. The internal standard used was chosen to provide a GC-MS signal that was similar to the analyte signal, but, sufficiently different so that the two signals were readily distinguishable from each other.

Once analysed the ratio of the analyte GC-MS signal can be compared to the internal standard signal, which is of known concentration. This data can be compared to the GC-MS signal retrieved from a standard containing known concentrations of both the internal standard and the analyte, thus allowing quantification of the analyte of interest in the samples. The ratio of the internal standard is compared to the analyte in the standard is compared to the ratio of internal standard and analyte in the sample allowing the concentration of the analyte of interest in the sample to be calculated. The numerical data required to do this is taken by analysing the peak area seen on a chromatogram obtained from the sample and the standard. The equation used to calculate the amount of tebuconazole recovered from samples in µg/sample is as follows:

$$\frac{Peak\ Area\ Tebuconazole\ (sample)}{Peak\ Area\ Tebuconazole\ (standard)} \times \frac{Peak\ Area\ Flusilazole\ (standard)}{Peak\ Area\ Flusilazole\ (sample)}$$

The internal standard chosen was flusilazole, another triazole fungicide, as can be seen from Figure 2.3 and Table 2.1, flusilazole is relatively similar in structure and properties to tebuconazole allowing co-analysis of the two compounds to be made.

Table 2.2 Chemical properties of tebuconazole and flusilazole

Name / Property		Tebuconazole	Flusilazole
Chemical Group		Triazole	Triazole
CAS No.		107534-96-3	85509-19-9
Chemical Formula		$C_{16}H_{22}ClN_3O$	$C_{16}H_{15}F_2N_3Si$
Molecular Mass (g mol^{-1})		307.82	315.39
Melting point (°C)		105	53.2
Boiling point (°C)		Decomposes before boiling	Decomposes before boiling
Solubility in water at 20°C (µg mL^{-1})		36	41.9
Solubility in DCM (mg mL^{-1})		High (200)	High (250)
Octanol-water partition coefficient (K_{ow}) at pH 7, 20°C	P	5.01×10^3	7.41×10^3
	Log P	3.7	3.87

Notes on table: A solubility of <50 µg mL^{-1} in water is classed as low. A 'high' solubility in DCM (dichloromethane) is classed as any solubility limit ≥200 g L^{-1}. A Kow (Robertson and Pope) of over 10 dictates a high degree of hydrophobicity and a high soil sediment adsorption co-efficient. **(Adapted from: The Pesticide Properties Database PPDB, 2010).**

Knowing the chemical properties of tebuconazole and flusilazole, detailed in Table 2.1, it was possible to predict how the tebuconazole and internal standard would act in solution and therefore plan an

extraction technique. A solvent liquid-liquid extraction method was used for the extraction of the fungicide from the water phase of the solution. Tebuconazole and flusilazole are relatively hydrophobic with a Log P of 3.7 (see Table 2.2) the presence of an organic, non-polar, solvent such as di-chloromethane (DCM) would cause the fungicide to preferentially dissolve into the non-polar DCM.

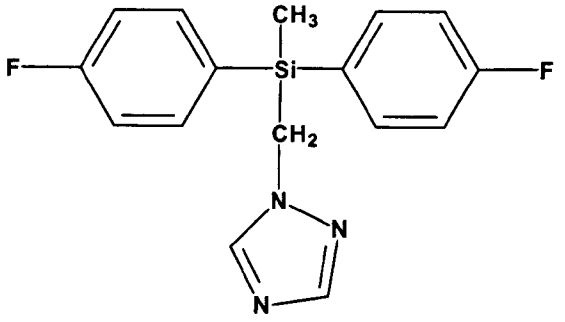

Figure 2.3 The chemical structure of the fungicide flusilazole (adapted from PPDB, 2010).

A 5 mL volume of DCM was measured into glass test tubes. Aliquots (5 mL) from the aqueous samples were then added to the DCM and thoroughly mixed using a vortex mixer. A volume equivalent to 100 µg of PESTANAL Flusilazole Analytical Standard (Riedel-de-Haën, Seezle, Germany) dissolved in methanol was added at this point to act as an internal standard. As DCM is immiscible with water the samples formed two distinct phases allowing the transfer of approximately 2 mL of this phase into GC-MS vials using a Pasteur pipette.

2.3.4 GC-MS method for the detection of tebuconazole released into water

An aliquot (1 µl) was injected using a split injector onto a ZB1 column (Phenomenex, Macclesfield, England). The column was 30 m in length with an internal diameter of 0.25 mm and a 0.25 µm thick dimethylpolysiloxane non polar stationary phase. The column was also

fitted with a 1 m long retention gap to prolong its useful life. The column was installed in a Fisons (Fisons Scientific Instruments, Manchester, England) GC 8000 series GC fitted with a Fisons AS800 auto sampler and linked to a Fisons MD 800 mass spectrometer. The software package MassLynx v3.2 (Micromass, Manchester, UK) was used to program the equipment and also to analyse the results. The oven program was set up to start at 140°C and increase in temperature at a rate of 12.5°C min $^{-1}$ to a maximum temperature of 280°C. The temperature program held this temperature for 4 min before cooling. A helium gas column head pressure of 22.5 p.s.i. and an injector temperature of 250°C were used. The mass spectrometer was operated in full scan and electron impact positive (EI^+) mode with a source temperature of 200°C.

2.3.5 Analysis of tebuconazole release into water from microencapsulated formulations between 2 – 30 min

Following the first time course investigation, a second experiment was performed to analyse tebuconazole release between 2 and 30 min (5 replicates per time point). The same method detailed earlier in this section was used for the extraction, detection and quantification of tebuconazole from the water phase of the samples. As all three batches of yeast tested in the initial experiment behaved in the same manner, only one batch (#4282) of the Micap formulation was tested in this instance. In this method individual samples were used for each data point rather than re-suspending the yeast pellet after each sampling point. A further experiment examined a time course with sampling points at 30, 60, 120 and 240 min. Although the experiment could have been taken further in terms of the time course, the results seen in the preliminary experiments performed up to this point in the research pointed towards there being very little change after 240 min point. Similar to the experiment examining the earlier stages of the time course this experiment followed the same protocol and methods as the

first two but had more sampling points and more replicates at each point. Again only a single batch of yeast (#4282) was tested.

2.3.6 Assessment of the effect of microencapsulated formulation age/storage on tebuconazole release into water.

A new formulation (batch 8129 containing 11.1% tebuconazole w/w) of microencapsulated tebuconazole was prepared following the same procedure as described previously in section 2.3.1. Samples of the new batch of microencapsulated tebuconazole where accurately weighed out to be equivalent to the other batches being tested. Batch 8129 was tested against three older formulations to ascertain if there were any differences in release between them. Samples were taken over a time course with sampling points at 2, 6, 10, 15, 25, 30, 60, 120 and 240 min. The sampling and extraction method followed the same protocol as described earlier in this chapter.

2.3.7 Assessment of the potential for sustained release of tebuconazole from microencapsulated formulations into water.

Five weighed samples of microencapsulated tebuconazole batch (#4282), containing an equivalent of 2 mg total of the fungicide, were placed into individual (five) centrifuge tubes. To these 50 mL of SDW was added and then the sample was mixed for a few seconds using a vortex mixer. The samples were then left for 30 min then centrifuged for 3 min at 3400 g. A 5 mL aliquot was then taken from each sample and partioned with 5 mL of DCM in preparation for analysis using GC-MS. After being centrifuged each sample was re-suspended in a fresh 50 mL volume of sterile water using a vortex mixer. The samples were set aside for a 30 min period after which the process was repeated. Each replicate sample of yeast was washed a total of 30 times and the amount of tebuconazole determined. Due to the number of washes

needed the experiment was undertaken over a 48 h period. When left overnight samples were left dry in sealed tubes at 4°C. Freezing was considered as an option, although this was dismissed as the samples of the yeast may have become damaged by the freezing process subsequently, affecting the results

2.4 RESULTS AND DISCUSSION

2.4.1 Confirmation of detection of tebuconazole and flusilazole using GC-MS

The validity of the GC-MS method was tested by analysing standards of tebuconazole and flusilazole at a concentration of 20 µg mL^{-1}. Standards were prepared using PESTANAL Tebuconazole/Flusilazole Analytical Standard at a concentration of 1 mg mL^{-1} in methanol and diluted as required. This allowed a standard chromatogram and spectra of tebuconazole and flusilazole to be recorded.

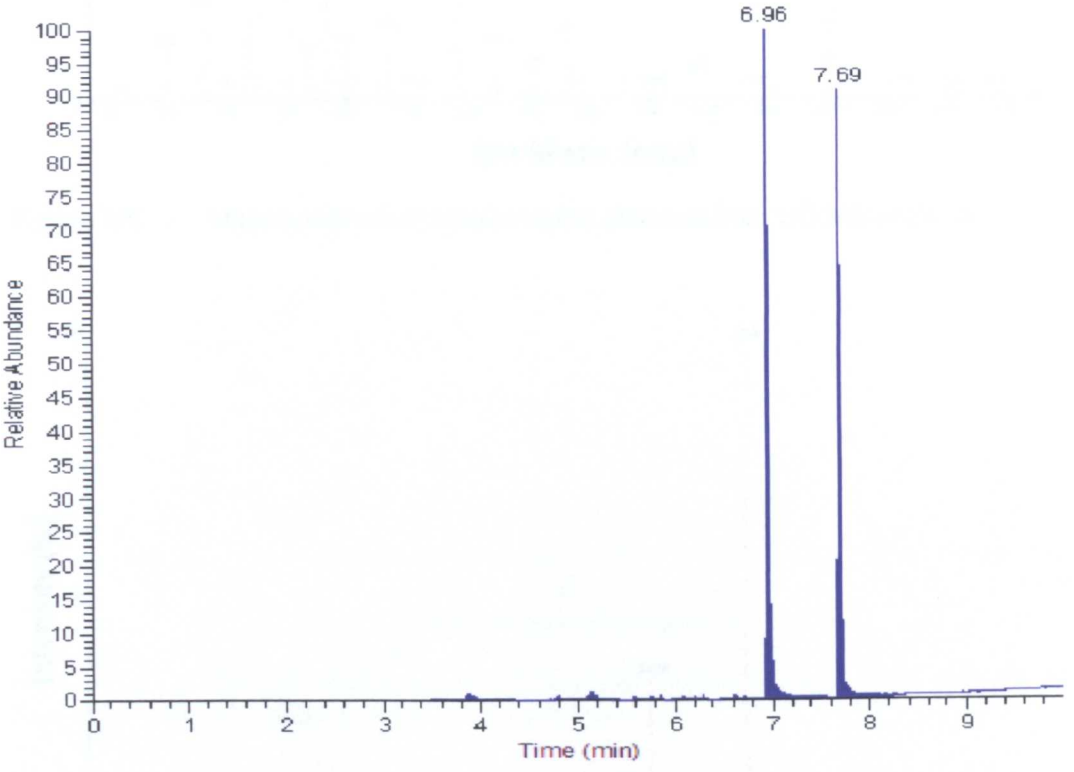

Figure 2.4 Chromatographic analysis of a standard containing the fungicides flusilazole (6.96 min) and tebuconazole (7.69) min at a concentration of 20 µg mL^{-1}.

The results of initial GC-MS runs to detect tebuconazole are shown in Figure 2.4. The mass spectra of the peaks were analysed and were shown to be that of tebuconazole (Figure 2.5) and flusilazole, (Figure

2.6). A standard curve was determined by analysis of a series of standard vials consisting of varying concentrations of tebuconazole and the same concentration of flusilazole.

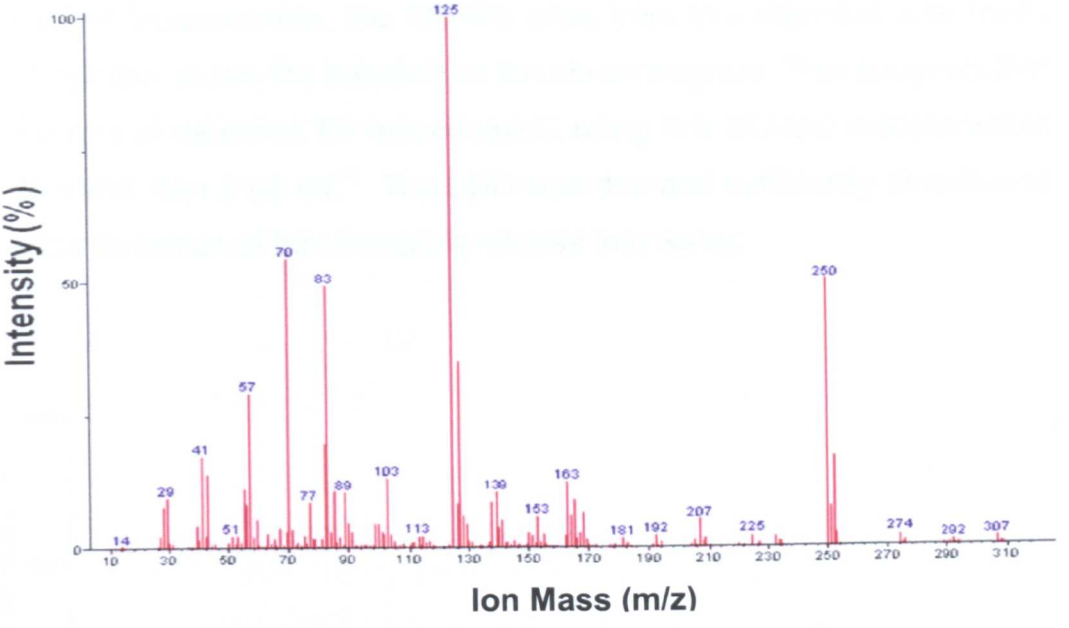

Figure 2.5 Mass spectrum of tebuconazole obtained from GC-MS analysis.

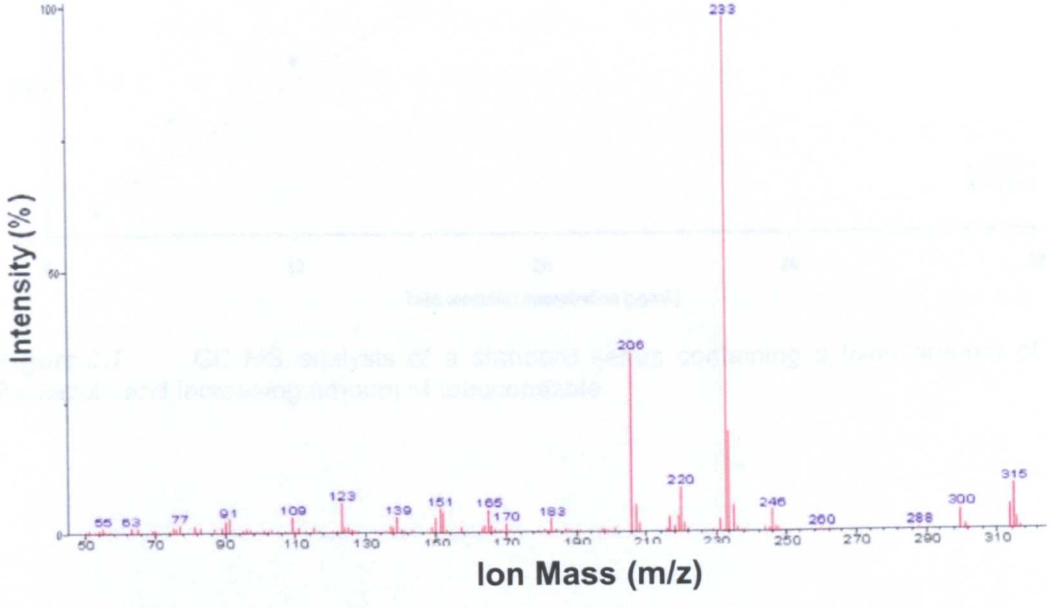

Figure 2.6 Mass spectrum of flusilazole obtained from GC-MS analysis.

Figure 2.7 displays a calibration curve obtained form analysing a standard series containing known concentrations of tebuconazole. As can be seen from the figure the ratio of tebuconazole to flusilazole increased in a linear fashion over the the standard series. The minimum concentration of tebuconazole detected in this standard series was 2 µg mL^{-1} of tebuconazole, the GC-MS peak from this standard was easily detectable above the baseline of the chromatogram. This suggests that the limit of detection for tebuconazole using this GC-MS method would be lower than 2 µg mL^{-1}. The LOD was deemed sufficiently sensitive to allow detection of tebuconazole release into water.

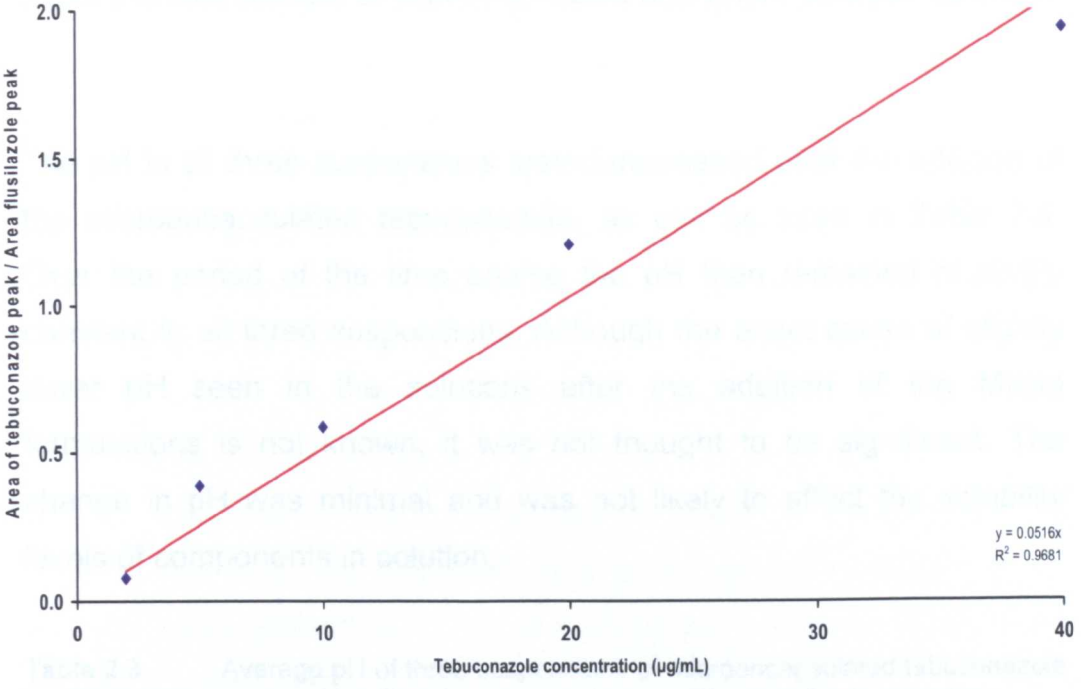

Figure 2.7 GC-MS analysis of a standard series containing a fixed amount of flusilazole and increasing amount of tebuconazole

2.4.2 Assessment of release of tebuconazole from microencapsulated formulations into water

2.4.2.1 pH testing

Changes in the pH can affect the level of solubility of components within a solution. Different batches of the Micap formulation were suspended in water and the pH of the suspensions recorded. Three batches of microencapsulated tebuconazole in yeast were tested in triplicate. As the different batches provided by Micap each contained differing percentages of tebuconazole, the samples where adjusted to make the total amount of active ingredient equivalent between batches.

The pH in all three suspensions tested decreased after the addition of the microencapsulated tebuconazole, as can be seen in Table 2.4. Over the period of the time course the pH then remained relatively constant in all three suspensions. Although the exact cause of slightly lower pH seen in the solutions after the addition of the Micap formulations is not known, it was not thought to be significant. The change in pH was minimal and was not likely to affect the solubility levels of components in solution.

Table 2.3 Average pH of three suspensions of microencapsulated tebuconazole recorded over a time course. Results are an average of three replicate samples

Time (min)	0	10	60	240	480	1440
Yeast Batch	pH					
4292	6.40	5.90	6.10	6.20	6.10	6.10
standard deviation	0.57	0.10	0.00	0.57	0.00	0.00
3122	6.20	5.20	5.20	5.40	5.50	5.40
standard deviation	0.10	0.57	0.10	0.00	0.00	0.00
4282	5.80	4.80	5.10	5.20	5.30	5.30
standard deviation	0.20	1.52	0.10	1.00	0.00	0.00

2.4.2.2 Assessment of tebuconazole release into water over a time course.

The ability of three formulations of microencapsulated tebuconazole (4282, 21.7% AI; 3122, 19.0% AI and 4292, 9.6% AI) to release tebuconazole into water was tested and the different batches compared to each other. Formulations were weighed to be equivalent to a commercial application rate, mixed with water then sampled over a time course. The amount of tebuconazole released was quantified by GC-MS and the use of an external standard series as detailed earlier.

Results obtained from this initial experiment (Figure 2.8) showed that in the early stages of the time course the solution had an average tebuconazole concentration of 436, 335 and 281 μg mL^{-1} (batches 4282, 3122 and 4292 respectively). All three batches being tested followed the same trend; the concentration of tebuconazole in solution decreased quickly over the early part of the time course. There was no discernable difference in the concentration of tebuconazole released from the different formulations all three batches behaving in a similar way. After a period of 240 min the concentration of tebuconazole in solution appeared to reach a constant state. After 1440 min or 24 h the level of the fungicide observed in solution remained stable at an average concentration of 41, 49 and 46 μg mL^{-1} (batches 4282, 3122 and 4292 respectively).

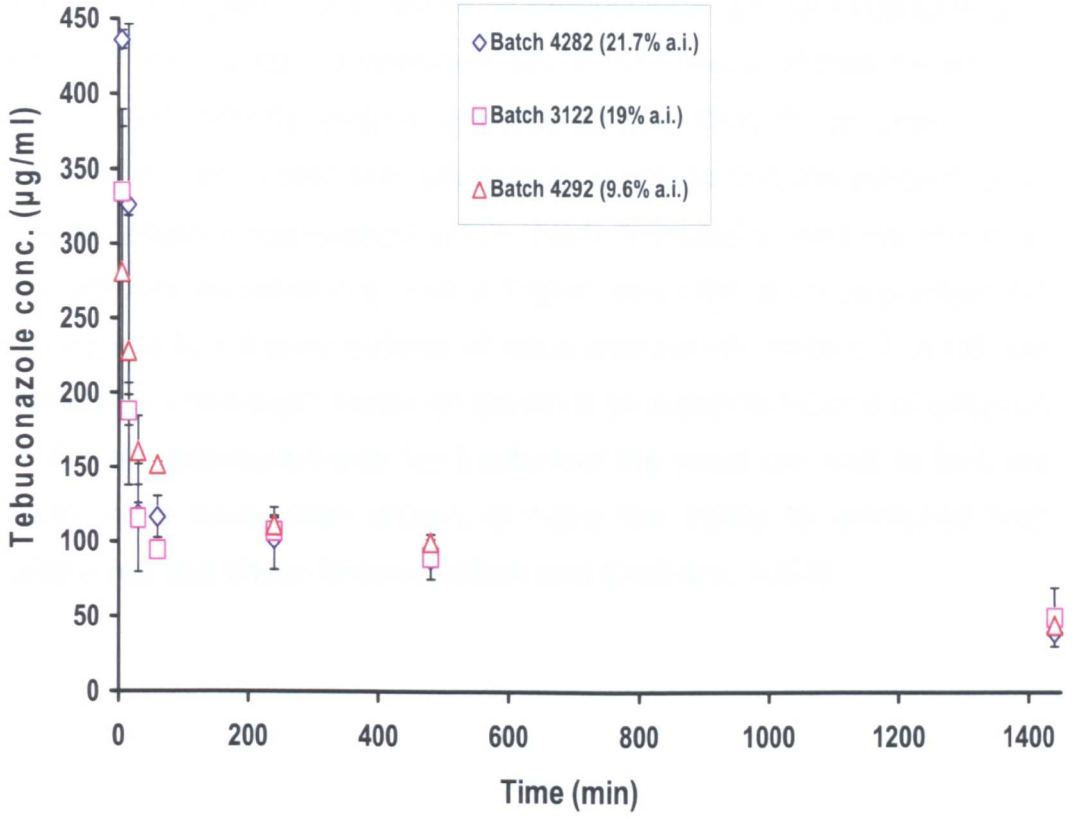

Figure 2.8 Mean concentration of tebuconazole released into water from three different microencapsulated tebuconazole formulations over a time course (Each data point represents an average value from 3 replicate samples. Error bars show standard deviation).

It was suggested that the high concentrations of tebuconazole released from yeast microcapsules seen in Figure 2.8 could be attributed to the age of the formulation. Testing the potential shelf life of the Micap formulations was also of interest. A new batch of microencapsulated tebuconazole was also assessed. Batch 8129 (11.1% active) was tested alongside three older batches (4282, 3122 and 4292), each having been produced a minimum of 2 years before testing, to ascertain any potential differences the age of the formulations might have on their performance. The data seen in Figure 2.9 clearly illustrates that there was no detectable differences between tebuconazole release from the new formulation and the other three older formulations. The new formulation, batch 8129, followed the same trend as the other formulations. This showed that the age of the batch being tested had no effect on tebuconazole release characteristics into

solution. The trend observed by all formulations was identical to results seen in the previous experiment. An initial release of tebuconazole at high concentrations early in the time course then fell to lower levels over 10-20 min. It was also possible to conclude that the percentage of tebuconazole encapsulated within each formulation had no effect on the amount released (i.e. that a higher percentage encapsulation did not equate to a higher release of tebuconazole into water). The release mechanism has been shown in previous research to be one of diffusion and is not associated with the fracture of the yeast cell wall. In fact, the yeast cells have been shown to have the ability to withstand high pressures and shear forces (Nelson and Crothers, 2003).

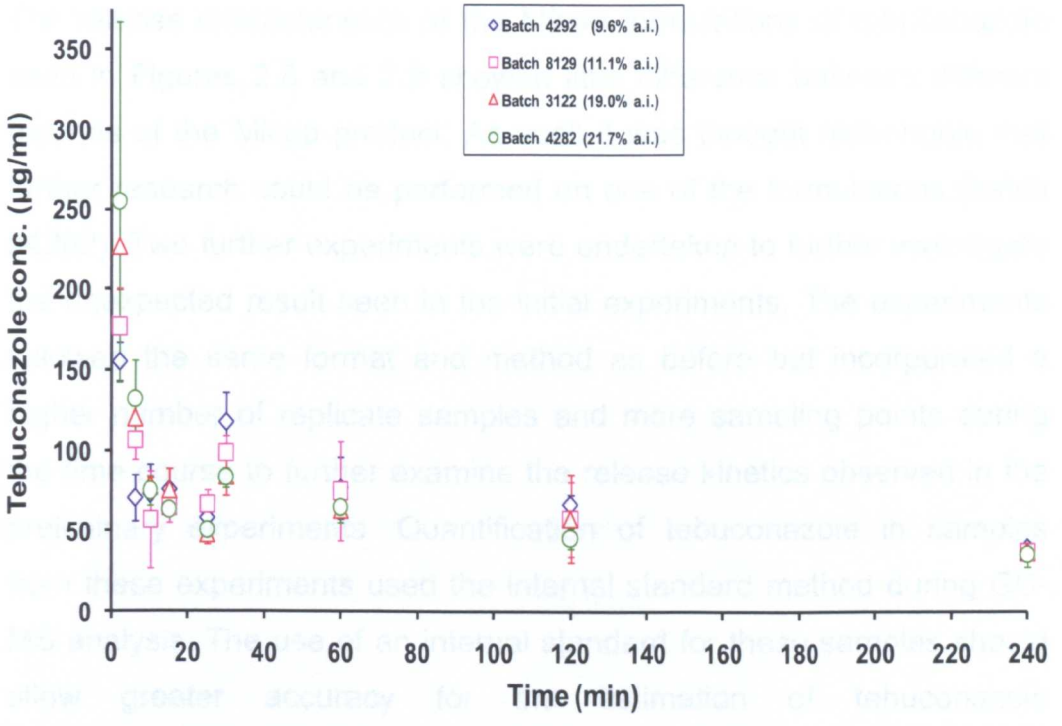

Figure 2.9 Mean concentration of tebuconazole released into water from a new batch of microencapsulated tebuconazole (8129) compared against 3 older samples over a time course. Each data point represents an average value of 3 replicate samples. Error bars show standard deviation.

The results seen in Figures 2.8 and 2.9 confirmed that all four formulations tested were able to release tebuconazole into an aqueous solution. The timing of release was near instantaneous after the addition of the SDW. The profile of the release of tebuconazole into water was, however, unexpected as all four formulations tested released tebuconazole into the surrounding aqueous environment at concentrations that appeared to be many times higher than the published solubility rate for tebuconazole in water of 36 μg mL^{-1} (P.P.D.B., 2010). For this reason the experiment was further repeated twice to verify the authenticity of the findings. The analyses of these experiments gave identical results and, as such, have not been given.

2.4.3 Short time course analysis of tebuconazole release into water from microencapsulated formulations.

The release characteristics of the Micap formulations of tebuconazole seen in Figures 2.8 and 2.9 showed little difference between different batches of the Micap product. As such it was thought reasonable that further research could be performed on one of the formulations (batch #4282). Two further experiments were undertaken to further investigate the unexpected result seen in the initial experiments. The experiments followed the same format and method as before but incorporated a higher number of replicate samples and more sampling points during the time course to further examine the release kinetics observed in the preliminary experiments. Quantification of tebuconazole in samples from these experiments used the internal standard method during GC-MS analysis. The use of an internal standard for these samples should allow greater accuracy for the estimation of tebuconazole concentrations released into water.

As can be seen from Figure 2.10 the results obtained (aimed at analysing the early stages of tebuconazole release in more detail) were

concurrent with those seen in the preliminary experiment shown in Figure 2.8. Average concentrations of 502 to 370 µg mL^{-1} were observed after suspension for between 2 and 6 min. The upper values recorded equate to nearly 14 times more tebuconazole in the solution than the published solubility limit for tebuconazole in water of 36 µg mL^{-1} (P.P.D.B., 2010). Finding the same levels of tebuconazole in solution using the internal standard method of sample analysis confirmed that the results were authentic and not merely a sampling or calibration artefact. Observation of the second half of the time course showed that the average concentration of tebuconazole recovered from the solution steadily decreases and stabilises, as seen in Figures 2.8 and 2.9.

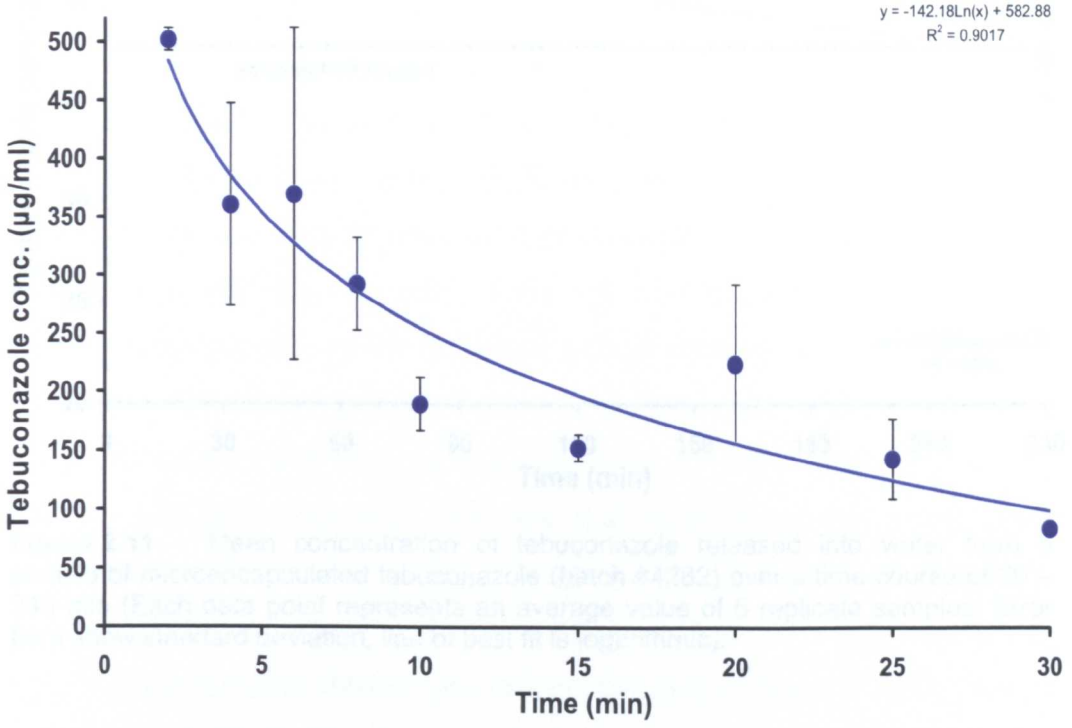

Figure 2.10 Mean concentration of tebuconazole released into water from a sample of microencapsulated tebuconazole (batch #4282) over a time course between 2 – 30 min after suspension. (Each data point represents an average value of 5 replicate samples. Error bars show standard deviation, line of best fit is logarithmic).

The internal standard system was also used to determine the concentration of tebuconazole in water over a longer time course. The

change in the concentration of the fungicide in solution over the time course, shown in Figure 2.11, followed the same trend as seen previously. At the 30 min time point, the concentration of tebuconazole recovered from the solution was higher than the published solubility limit. Over the course of the experiment, the concentration of tebuconazole decreased and after 4 h, had reached a level of around 36 µg mL^{-1}.

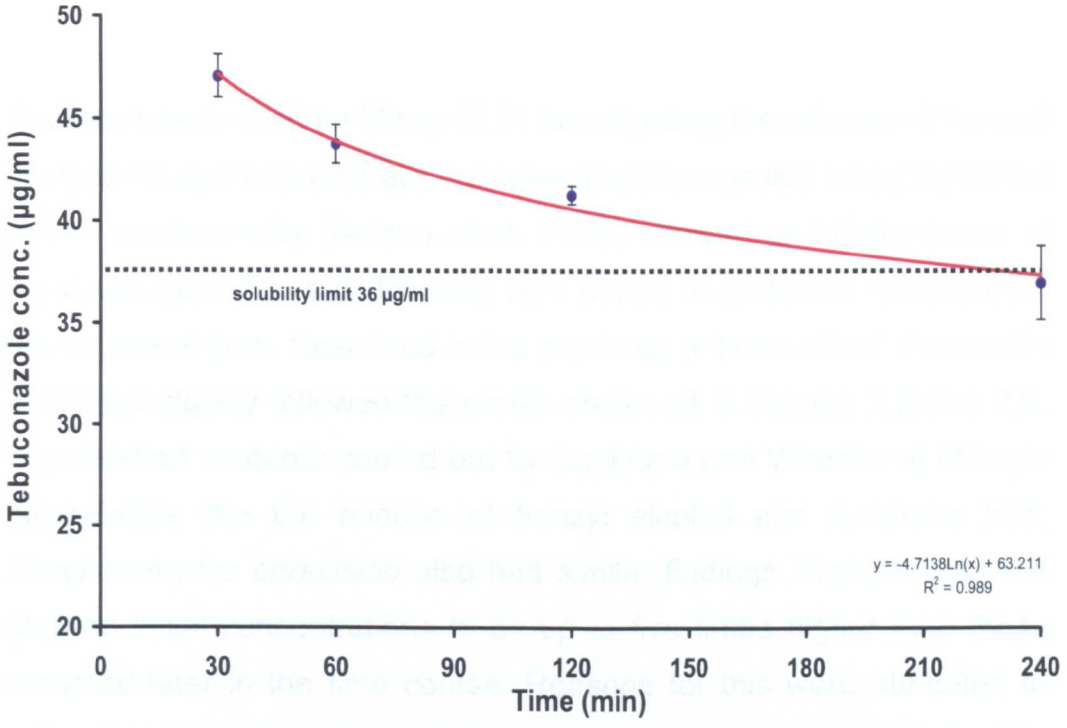

Figure 2.11 Mean concentration of tebuconazole released into water from a sample of microencapsulated tebuconazole (batch #4282) over a time course of 30 – 240 min (Each data point represents an average value of 5 replicate samples. Error bars show standard deviation, line of best fit is logarithmic).

The occurrence of the 'super-saturated' solutions observed in the analysis of release of tebuconazole from microencapsulated formulations may be attributed to several causes. The process by which the Micap formulations are manufactured may have led to excess un-encapsulated tebuconazole being held on the surface of the yeast cells. This tebuconazole may have been released into solution when the

formulation was hydrated causing a phase separated suspension of tebuconazole to form within the solution itself. The chemical properties of tebuconazole, as detailed earlier in this chapter (Table 2.2), show that the molecule has a high K_{ow} value and is highly hydrophobic. As such the tebuconazole may have formed micelle like structures or droplets within the solution. These pockets of highly concentrated tebuconazole could have ended up in aliquots of the supernatant taken from the early stages of the time course and led to the high concentrations of tebuconazole observed in solution.

Research performed by Micap PLC. investigating the release of the anti cholesterol agent fenofibrate from yeast microcapsules when delivered to the duodenum by (Nelson *et al.*, 2006) showed an initial release of the active from the yeast followed by a period of sustained release over the course of time. Described in the article as a 'burst effect' the results published closely followed the profile observed in Figures 2.8 and 2.9. Unpublished research carried out by Duckham and Wheeler at Micap's laboratories into the release of benzyl alcohol and limonene from *Saccharomyces cerevisiae* also had similar findings. Results obtained showed initial concentrations to be up to five times higher than those recorded later in the time course. Reasons for this were attributed to non-encapsulated material held on the surface of the yeast cells (Duckham and Wheeler, 2005). Similar research carried out into the release of limonene from yeast cells found that the process was driven by diffusion but also dependant on the porosity of the cell wall. This in turn was driven by the degree to which the yeast cells had become hydrated (Normand *et al.*, 2005; Dardelle *et al.*, 2007).

Research into the release of agrochemicals from other types of controlled release formulation into water have generally found the process to be that of diffussion following Fick's first law. The law

essentially states that the diffussion will be from a region of high concentration to a region of low concentration (Markus and Linder, 2006). However, release of chemicals from such formulations is not often easily explained by simple models and is affected by factors individual to each formulation (Fernandez-Perez *et al.*, 1998; Pérez-Martínez *et al.*, 2001; Singh *et al.*, 2009a). Nevertheless, as can be seen in Figure 2.11, after an incubation period of 240 min the concentration of tebuconazole in solution equilibrated, to around the published solubility limit for tebuconazole in water. Again, exact reasons for this are unclear; however, the highly hydrophobic nature of the tebuconazole may cause the excess tebuconazole in solution to re-encapsulate by a physical partition effect, from the solution back into the yeast cells themselves, paralleling the encapsulation phenomenon. Research carried out by Nelson and Crothers (Nelson and Crothers, 2003) pointed out the role of swelling and disruption of the yeast cell wall caused when spray dried formulations were hydrated. It was argued that this disruption might provide a route by which encapsulated materials could re-enter the yeast cells. Overall, the results show that, when hydrated, the Micap formulations release an initial burst of tebuconazole. Where the microencapsulated formulations were required to be used with water (e.g. a spray application) a minimum stabilisation period of 4 h could be used to allow the concentration of tebuconazole to reach a steady state. Where the formulations were employed as a dry seed treatment the initial burst effect was unlikely to adversely affect the performance of the formulations as the tebuconazole will be released in the proximity of the seed. Although soil holds an inherent level of moisture, this will not be close to the total volume of available water tested in these experiments. When treated seeds are watered, there will be an increase in the water content of the soil around the seed causing the formulations to release tebuconazole into the environment. The tebuconazole would be released into the immediate environment of the seed and then reach a state of

equilibrium as the immediate area around the seed would become saturated with the fungicide.

2.4.4 Assessment of the potential for sustained release of tebuconazole from microencapsulated formulations into water.

An investigation was undertaken to quantify the release of active ingredient from a sample of microencapsulated tebuconazole when repeatedly washed to simulate release in the environment. A weighed sample of Micap (batch #4282 equivalent to a total of 2 mg tebuconazole was repeatedly washed with volumes of SDW and the resulting samples extracted into DCM and analysed by GC-MS as described in section 3.7.

The results (Figure 2.12) show a similar profile to those seen previously. Samples taken early in the experiment, i.e. after only one or two washes, showed high levels of tebuconazole in solution. Over the course of the experiment the concentration of tebuconazole, recovered from the solution after each wash sequence, gradually became lower and lower. After 30 washes the average concentration of tebuconazole recovered from the sample was 28.4 µg mL^{-1}. This is slightly lower than the published solubility limit of tebuconazole in water of 36 µg mL^{-1}. Thus, the Micap formulation displayed good potential for providing a sustained and prolonged release of active ingredient. The formulation of the Micap yeast tested in this instance was still delivering concentrations of tebuconazole over 28 µg mL^{-1} after 30, repeated washes, totalling 1.5 L. The total of the tebuconazole recovered from the solution was 1695 µg over 30 sequential washes. As a total of 2 mg of tebuconazole was used in this experiment, the total tebuconazole recovered from the yeast was 84.7% of the total available. This indicates that micro-encapsulated tebuconazole in yeast cells provided

the potential for gradual sustained release of active ingredient over a considerable time window – the active molecule is not released immediately on contact with water. However, the formulation still allows release of tebuconazole showing that, once encapsulated the fungicide does not remain permanently bound within the yeast cells. Research carried out into the sustained release of benzyl alcohol and limonene encapsulated in yeast when repeatedly washed found similar results (Duckham and Wheeler, 2005a).

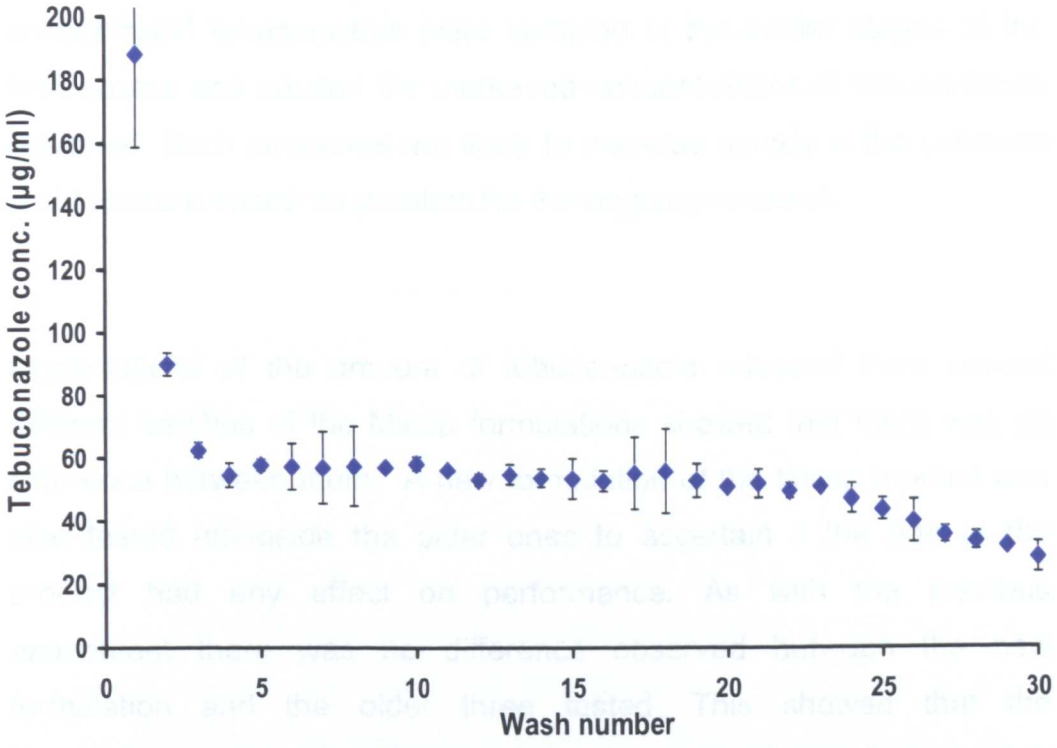

Figure 2.12 Mean amount of tebuconazole released into water from a sample of microencapsulated tebuconazole repeatedly washed in SDW Each data point represents an average value of 5 replicate samples. Error bars show standard deviation.

2.5 CONCLUSIONS

The principal aim of the research described in this chapter was to understand the release kinetics of microencapsulated tebuconazole produced by Micap into an aqueous solution. The initial burst release effect in the preliminary experiments was unexpected. This may be due to the formation of micelle like structures of hydrophobic tebuconazole soon after the formulation has been hydrated. The initially high concentrations of tebuconazole released from the yeast microcapsules observed after only a short period of time in solution equated to a large proportion of the total tebuconazole applied. These areas of highly concentrated tebuconazole were sampled in the earlier stages of the time course and caused the increased concentrations of tebuconazole observed. Such structures are likely to disperse quickly in the solutions and therefore posed no problem for the ongoing research.

Observations of the amount of tebuconazole released from several different batches of the Micap formulations showed that there was no difference between them. A new formulation of the Micap product was also tested alongside the older ones to ascertain if the age of the product had any effect on performance. As with the previous experiment there was no difference observed between the new formulation and the older three tested. This showed that the formulations were not affected by age in terms of their behaviour in solution. Most commercially available fungicides formulations have a minimum shelf life of two years (Robertson and Pope, 2006). As batches 4292 and 4282 were a minimum of two years old at the point of testing it is thought reasonable that the age of the formulations can be discounted as a factor for results obtained.

As the formulations behaved in a similar manner in solution any of the formulations available could be used in the ongoing research. One

formulation (batch 4282), a bi-product of bio-ethanol production and thus potentially cheap to obtain, was selected for further testing to examine, in further detail, the burst release effect seen in the early experiments. The results from these experiments were consistent with those seen before. The same formulation was tested for its ability to release tebuconazole into solution over a sustained and prolonged period. The batch tested performed well releasing nearly the entire amount of tebuconazole held within it over a large number of washes. Ultimately, the release of any encapsulated product from a microcapsule into water will be subject to several driving forces such the lipid and water solubility limits of the compound and the rate at which the microcapsules become hydrated sufficiently to allow diffusion of the AI out of the microcapsules to occur. It would appear that in this instance, release of tebuconazole from the yeast microcapsules into water was achievable. To provide an improvement over existing formulations the novel formulation would be required to release a high percentage of the total microencapsulated fungicide they contained. It is also proposed that the encapsulated formulations would need to sustain the release of active molecules over a prolonged period; this would also appear to be feasible. The release profiles observed also suggest that there would be good potential for plant uptake of tebuconazole from the microencapsulated formulation via the water phase of the soil and the plant root system. The manner in which tebuconazole was released from the yeast microcapsules, whereby a large proportion of the tebuconazole contained within the yeast was released into solution, should be beneficial for the subsequent availability of the fungicide for plant uptake when applied as a seed treatment.

CHAPTER 3: DETECTION AND ANALYSIS OF UPTAKE OF SEED APPLIED TEBUCONAZOLE FORMULATIONS

3.1 INTRODUCTION

In the U.K. over 90% of wheat seed sown is routinely treated with a fungicide for seed hygiene reasons as an insurance measure against a wide range of seed borne diseases. The cost of this application (ranging from £40-150/tonne of seed) is relatively small in comparison to foliar applied alternatives. This is primarily due to the fact this method is more targeted in its application and as such requires less AI to attain the same effect (Clark *et al.*, 2004). Where systemic products are used as seed treatments a further potential benefit of this type of application is the protection of young plants from early season foliar diseases alongside protection from seed borne pathogens (Brandl and Biddle, 2001). This method of employing systemic fungicides is not, however, without its drawbacks. Many systemic fungicide formulations, tebuconazole included, are known to cause problems with seed germination and development if applied at excessive concentrations. This effect is termed phytotoxicity and in practice means that, often, fungicide application rates are limited in an effort to reduce this effect. It is therefore difficult to apply systemic fungicides at sufficient dose rates to maintain effective levels of fungicide in plants as they develop to protect them against non-seed borne diseases (Bahadir, 1990; Fullerton *et al.*, 1995; Asrar *et al.*, 2004; Karabulut *et al.*, 2006).

The problem of phytotoxicity may be increased if application of the seed treatment is not performed correctly (Ashley *et al.*, 2003). In addition, effects of other factors such as environmental degradation, poor uptake efficiency, short half life of AIs in the environment and plant tissue and reduction in dose as plant biomass increases further decrease the effectiveness of seed applied fungicides whilst also increasing the need

for higher application rates (Mogul *et al.*, 1996). Maintenance of adequate levels of chemical in plant tissue can be easily attained by a further application of fungicides as a foliar spray at a later stage during the growing season. However, increased concerns for the amount of agrochemicals applied to the environment from several viewpoints such as effects on local wildlife (Charlton and Jones, 2007), fungicide residues in food (Gonzalez-Rodriguez *et al.*, 2008) and water ways (Bermúdez-Couso *et al.*, 2007) make less targeted application methods such as spraying less desirable. Allied to this, there is an increased cost associated with a further application of fungicide making a single, seed application more desirable (Mogul *et al.*, 1996).

Tebuconazole is a widely used systemic fungicide often applied as a seed treatment to several types of crop (Asrar *et al.*, 2004). A commercially available formulation of tebuconazole called Raxil is produced by Bayer CropScience in the form of a flowable suspension. Although no longer marketed as a single AI product (it is now formulated as a combined formulation with other AIs such as prothioconazole and triazoxide) for the purposes of this research an experimental sample of the tebuconazole only formulation was obtained. The recommended application rate for tebuconazole is between 1 and 7.5 g AI per 100 kg of seed (Tomlin, 2003). Applications of the fungicide at this rate do, however, require a subsequent foliar application in order to provide adequate levels of protection against disease later in the growing season. Application at greater dose rates to achieve adequate protection, reducing the need for repeated application, causes associated effects of phytotoxicity and is therefore not possible. A tebuconazole formulation that allowed higher dose rates to be applied whilst alleviating the negative effects of phytotoxicity could provide a solution to this problem whilst also reducing overall cost and risk to the environment (Markus, 1996).

It was hypothesised that the microencapsulated formulation of tebuconazole produced using the method developed by Micap may be able to provide the optimum combination of the higher application rates combined with reduced levels of phytotoxicity. Further to this, when applied at higher rates it was also suggested that an adequate level of disease protection could be maintained for longer periods reducing the need for further applications. Previous research carried out at the University of Nottingham had shown microencapsulated formulations applied as a seed dressing to be more effective than commercial alternatives at controlling foliar diseases such as powdery mildew. The results of the trials carried out showed that lower application rates of the Micap product were equally effective at controlling the pathogen when compared to dose rates up to four times higher (Rossall, pers. com.). Micap in partnership with the agrochemical company Nufarm, were also interested in producing a microencapsulated formulation to be comparable to another commercial seed treatment formulation, Jockey, which is a combination of fluquinconazole and prochloraz produced by BASF, may be applied at higher rates than tebuconazole at up to 75 g AI per 100 kg of seed (BASF, 2010). This aim was never realised due to the fact that Micap PLC ceased to exist as a trading company in 2007.

In an effort to verify if the improved efficacy results could be attributed to increased availability and uptake of tebuconazole from the microencapsulated formulations a series of experiments were undertaken. A reliable method for the extraction, detection and quantification of the fungicide was first optimised. There are several methods in the literature for the recovery and analysis of specific organic chemicals from plant material (Yunis *et al.*, 1991; Rial Otero *et al.*, 2003). Previous studies involving the extraction and quantification of organic chemical residues in plants have involved several extraction and purification steps to remove the majority of potentially interfering

compounds, which may be present at higher concentrations than the compound of interest itself and thus interfere with subsequent detection (Rial Otero *et al.*, 2003). A method commonly employed uses a liquid-liquid extraction as a prior isolation step using solvents such as methanol and DCM followed by further concentration and purification using solid-phase extraction (SPE) (Bernal and Del Nozal, 1997). The amount of analyte in resulting aliquots of solvent are then determined using by further analysis using a technique such as GC-MS.

A trial to assess whether the microencapsulated formulations could be applied at higher rates without having a phytotoxic effect on emerging plants was also performed. Following this a detailed assessment of the level of fungicide uptake, distribution of the fungicide within plants and potential effect of different growth condition on uptake was carried out. These results were compared to those from plants treated with commercially available formulations of tebuconazole, Raxil and Mystique. Plant uptake and distribution of organic chemicals such as fungicides occurs via a number of pathways. Fungicides applied as a seed treatments are taken in from the soil via the plants root system. This process has been shown to be a passive, diffusive one, whereby the chemical is taken up via the water phases of the soil (Collins *et al.*, 2006). The water and solutes taken in by the roots are then transported acropetally to the rest of the plant mainly via the apoplastic pathway and transpiration stream (Trapp, 1995). For a compound such as tebuconazole to enter a plant via the root system it must first cross at least one cell membrane. Due to the high lipid content of cell membranes it is a combination of a compound's solubility in water and lipid solubility that will determine its mobility within the plant (McFarlane, 1995b). The polarity of the chemical may also affect its ability to cross hydrophobic lipid membranes. Molecules with a log K_{ow} of between 2.15 and 3.62 have been demonstrated to possess a high potential for root uptake and subsequent *in-planta* translocation (Duarte-Davidson

and Jones, 1996). As tebuconazole has a log K_{ow} of 3.7, it should demonstrate good uptake and translocation ability.

3.2 AIMS AND OBJECTIVES

This section of the research aimed to investigate the following:

- Develop and refine a reliable method for the efficient extraction, detection and quantification of tebuconazole from plant samples.
- Assess the potential phytotoxic effect on emerging plants of different formulations of tebuconazole when applied at a higher than recommended rate.
- Assess the ability of the microencapsulated formulation of the fungicide to release tebuconazole available for plant uptake when applied as a seed dressing, the rate of this uptake and how the amounts of resulting tebuconazole in plant material compare to those of plants grown from seeds treated with commercial formulations of the fungicide.
- Examine distribution and amount of accumulation of the fungicide within individual plant/leaf samples.
- Investigate potential effects of environmental factors on the rate of tebuconazole uptake.

3.3 MATERIALS AND METHODS

3.3.1 GC-MS method development

Previous research has shown average tebuconazole residues recovered in green forage to be as low as 17 mg/kg of plant tissue (FAO, 1994). It was also unknown how formulations of tebuconazole (both commercial and microencapsulated), applied as a seed dressing would behave and the amounts of fungicide that would accumulate in plant tissue. The existing GC-MS method (Chapter 2 – section 2.3.4), although adequate for the detection of tebuconazole released into water, would need to be improved to detect the lower concentrations expected from treated plant samples. An improvement in chromatographic quality - peak height and shape - and hence a lower limit of detection could be achieved by optimising the temperatures the GC-MS was operating under. Different combinations of a) start temperature and b) temperature ramp (rate of temperature change) were assessed to improve the GC-MS method. Initially, the sensitivity of the method was improved by changing the injection system from a split injection, where a proportion of a sample is prevented from entering onto the GC-MS column to a splitless injection whereby the entire sample is analysed. Additionally the mass spectrometer was programmed to analyse samples in selected ion monitoring mode (SIM), whereby only ions of interest, as found during initial GC-MS analysis (detailed previously in Chapter 2 – Figures 2.5 and 2.6), were detected.

An experimental planning software package, Design Expert 7 (Stat-Ease Inc, Minneapolis, USA) was used to design a two factorial experiment including replicate analysis points, based around the temperature conditions (start temperature, 140°C, temperature ramp, 12.5°C) used in the original GC-MS method (Chapter 2). A start

temperature range of between 100 and 180°C and a temperature ramp of between 5 and 20 °C min^{-1} were assessed. A standard of analytical grade tebuconazole and flusilazole (Riedel-de-Haën, Seezle, Germany) dissolved in HPLC grade methanol (Fisher Scientific, Loughborough, U.K.) at a concentration of 2 µg mL^{-1} of each fungicide was used for the optimisation; An aliquot (1 µl) of the standard solution was injected in splitless mode using a Fisons (Fisons Scientific, Manchester, England) AS8000 autosampler onto a ZB1, 30 m x 0.25 mm ID x 0.25 µm dimethylpolysiloxane non polar stationary phase column (supplied by Phenomenex, Macclesfield, England). The column was installed in a GC 8000 series gas chromatograph linked to a Fisons MD 800 set to operate in SIM mode (m/z 125, 250, 233 and 315) and electron impact positive (EI^+) mode with a source temperature of 200°C. The peak areas of the results were obtained using MassLynx v3.2. This data was then further analysed using Design Expert 7 to gauge the improvement in chromatography which was assessed using the following criteria; increase in peak height, increase in peak area, decreased area:height ratio (a marker of peak resolution) and decreased retention time. The amount of tebuconazole was then quantified based on a comparison of the peak areas obtained from chromatograms.

After the initial assessment a second experiment was performed centred around the temperature combination that had shown the greatest improvement in chromatography. A response surface quadratic design was used to assess the effect of an initial oven temperature of between 100 and 125°C and a rate of oven temperature increase of between 15 and 22.5 °C min^{-1}. All other experimental conditions were the same as used in the initial experiment.

3.3.2 Finalised GC-MS method

The following GC-MS method was used for the analysis of tebuconazole amounts in all plant samples and subsequent research. An aliquot (1 µl) of each sample was injected (injector temperature 280°C) in HPLC grade methanol by an AS 3000 autosampler (Thermo Fisher Scientific, Loughborough, England) using a splitless injection onto a ZB1-Inferno column (30 m x 0.25 mm I.D. x 0.25 µm film thickness dimethylpolysiloxane non polar stationary phase - Phenomenex, Macclesfield, England), selected for its high maximum operating temperature of 460°C and fitted with a 1 m long retention gap to prolong its useful life. The column was installed in a Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, Loughborough, England) set to start the analysis at 125°C and increase to 325°C (maintained for 2 min before cooling) at a rate of 24°C min^{-1}. The column was linked to a Thermo DSQ II mass spectrometer (Thermo Fisher Scientific, Loughborough, England) operated in selected ion monitoring (SIM) mode (m/z 125, 250, 233 and 315) and electron impact positive (EI^+) mode with a source temperature of 200°C. The carrier gas was helium used at a column head pressure of 22.5 p.s.i. The software package Xcalibur v.2 (Thermo Fisher Scientific, Loughborough, England) was used to program the equipment and also to analyse the results.

3.3.3 Validation and optimisation of method for extraction and purification of tebuconazole using methanol extraction and SPE based systems.

Solvent extraction followed by further cleanup and concentration of the fungicide, performed using solid phase extraction (SPE), was selected as the method for recovery and analysis of tebuconazole from treated plant samples. Methanol was selected for this purpose due to its miscibility with water (high water content of plant material) and the high solubility of tebuconazole (and flusilazole) in this solvent. The validity and accuracy of a method for the recovery and purification of

tebuconazole from solution, using SPE, was tested and refined using known concentrations of fungicides dissolved in methanol. The method was also required to reliably extract flusilazole, to be used as an internal standard, from samples. The similar chemical properties of tebuconazole and flusilazole suggested that they should be eluted from the SPE cartridge at the same time. Strata-X^{TM} 33 µm reversed phase polymeric sorbent SPE cartridges with a 200 mg absorbent limit, a 6 mL capacity and a pore size of 88 Å (Phenomenex, Macclesfield, England) were selected for this purpose. These were used connected to a Büchner flask under vacuum to draw the solvent volume through the cartridge.

A series of 5 individual, randomly selected, cartridges were tested repeatedly for their ability to retain both tebuconazole and flusilazole. A 4 mL volume of HPLC grade methanol (Fisher Scientific, Loughborough, U.K.) followed by 10 mL SDW was used to condition and activate each cartridge prior to use. Replicate (5) samples of 5 mL 50:50 methanol/SDW (v/v) containing 1 µg each of tebuconazole and flusilazole were passed through each cartridge. Each sample volume was followed by a wash step consisting of 2.5 mL of 50:50 SDW: methanol. The fungicides were then recovered from the cartridge using 4 mL of HPLC grade methanol which was collected and the analysed by GC-MS as detailed previously in this chapter. The method is summarised in Figure 3.1.

The SPE extraction method was improved by assessing the optimum volume of methanol required to elute fungicides from the sorbent bed of SPE cartridges. Replicate 5 mL volumes of 50:50 methanol/water v/v containing a total of 1 µg each, tebuconazole and flusilazole, were passed through randomly selected cartridges. After the wash step,

detailed above, 4 mL of HPLC grade methanol, was passed through the cartridge and the elutant collected in five 400 µL fractions. Each fraction was then analysed for its tebuconazole and flusilazole content using the GC-MS method previously detailed in this chapter.

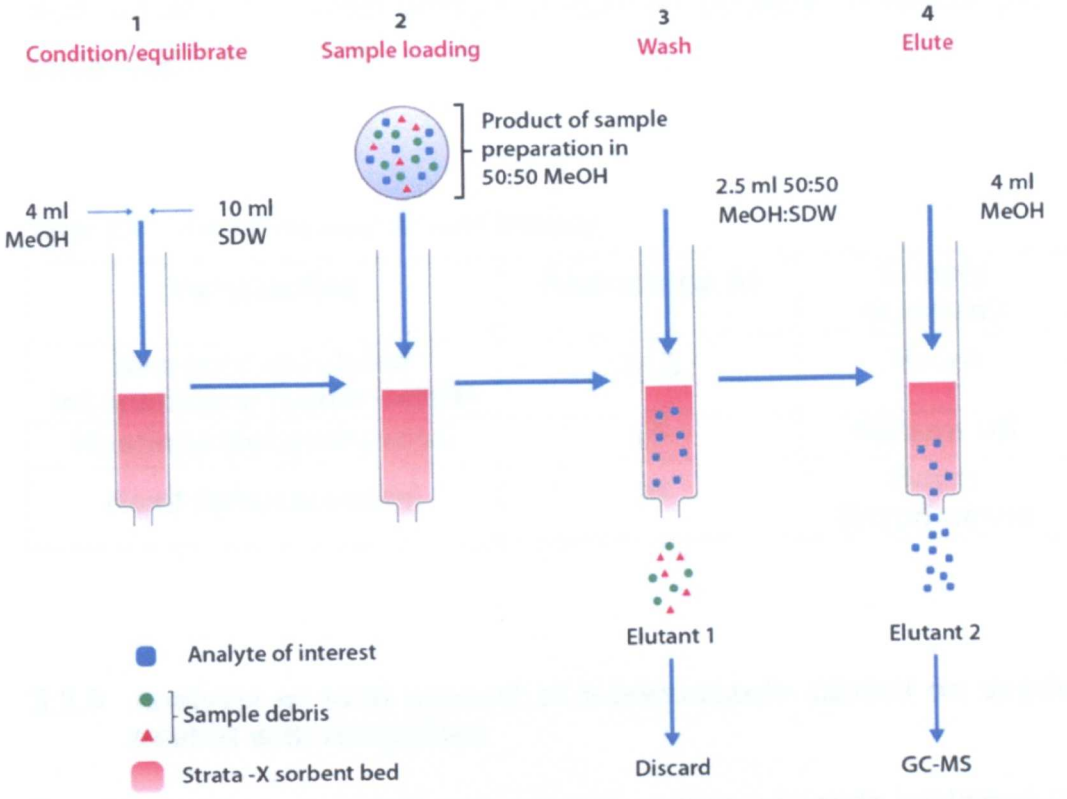

Figure 3.1 Summary of SPE extraction method used to recover and concentrate fungicides from aqueous solutions prior to quantification of fungicide amount using GC-MS analysis (adapted from Camel, 2003)

3.3.4 Preparation of fungicide treated seed stocks

Wheat seeds (*Triticum aestivum L cv. Claire*) were treated with different formulations of microencapsulated tebuconazole and two commercial ones (Table 3.1). Amounts of each formulation, containing different total amounts of the fungicide, were adjusted to be equivalent to each other and applied at the required dose rate expressed as grams of active ingredient per 100 kg of seed. Calculated amounts of each formulation were added to 100 g seed in glass powder jars. The jar was then

sealed and vigorously shaken continuously for a minimum period of 5 min until all seeds were coated. Where a liquid formulation had been applied seeds were placed on foil and dried for 12 h in a laminar flow cabinet Samples of the same seed were also professionally treated using a Hede 11 (spinning disk) seed coating drum by a third party (Eurofins-Agrisearch based in Melbourne, U.K.). Treated seed samples were stored out of direct sunlight at room temperature, in sealed glass containers.

Table 3.1 Fungicides used for seed dressing

Name/active	Percentage AI	Supply company
Microencapsulated tebuconazole (batch #4564)	16.3	Micap
Mystique (tebuconazole)	25	Nufarm UK
Raxil (tebuconazole)	25	Bayer CropScience

3.3.5 Analysis of total amount of tebuconazole carried on seeds treated with fungicides

The thousand grain weight and average number of seeds contained in a 100 g mass of wheat seed (variety Claire) was calculated using a Contador seed counter (Pfeuffer. Kitzengen, Germany). It was then possible to estimate the amount of tebuconazole per seed at a given application rate. This was also confirmed in the laboratory, seeds treated with three formulations of tebuconazole (Table 3.1) applied at a rate of 40 g active ingredient (AI) per 100 kg of seed, using the seed coating method and those treated commercially were tested. A series of 5, randomly selected seeds from all three treatments were hydrated with 5 mL SDW over 24 h. An aliquot (200µl) of flusilazole at a concentration of 20 µg mL^{-1} was added, to act as an internal standard. Samples were then partitioned with 5 mL DCM, thoroughly mixed using a vortex mixer and incubated for 48 h. The high K_{ow} or Log P of 3.7 for

tebuconazole and 3.87 for flusilazole combined with a solubility limit of 200 and 250 g L^{-1} for tebuconazole and flusilazole in DCM (PPDB, 2010) would see the compounds preferentially dissolve into the DCM from the water phase of the samples. Samples were then transferred into 2 mL GC-MS vials using a glass Pasteur pipette and analysed using the GC-MS method set out previously in this chapter.

3.3.6 General plant growth method

All plants grown for the purposes of this project have been grown using the same method unless otherwise stated. The research was carried out solely on wheat plants grown from seeds (variety Claire) provided by Plant and Crop Sciences the University of Nottingham. John Innes No. 3 (consisting of 7 parts loam to 3 parts peat and 2 parts sand) compost was used as the growth medium. Tebuconazole was applied to the seed at a rate of 40 g AI per 100 kg of seed unless otherwise stated using the seed coating method detailed previously in this chapter. Plants were grown in 13 cm plastic pots at a sowing density of ≤8 seeds per pot in controlled conditions with a day temperature of 18°C, a night temperature of 12°C and a day length of 12 h. The plants were watered once, daily unless otherwise specified or required. Where plants were grown in growth rooms or natural light (where 12 h day length could not be achieved), light was provided by sodium bulbs with a radiance measured at 69 µmol m^2 s^{-1}

3.3.7 Initial analysis of fungicide uptake into plants from seeds treated with a microencapsulated tebuconazole

The release and subsequent uptake of tebuconazole into plants from seeds treated with microencapsulated formulations of the fungicide was tested. The experimental planning program, Design Expert 7 (Stat-Ease Inc, Minneapolis, USA) was used to plan a two factorial experimental

design including replicate analysis points. Factors investigated were; fungicide dose rate (between 5 and 40 g AI/100 kg seed) applied as a seed dressing and plant development (between growth stages (GS) 12 and 16 (Zadocks growth scale). Seed stocks were prepared using microencapsulated tebuconazole (Micap batch #4564) applied at the different application rates using the seed treatment method detailed previously in this chapter. Plants were grown in until they had reached the relevant growth stage (Zadocks Growth Scale) under standard conditions (Section 3.3.6) in John Innes No. 3 compost. Harvested samples were prepared for GC-MS analysis using the following, finalised, extraction method.

3.3.7.1 Finalised solvent/SPE method for extraction of tebuconazole from treated plant samples

Harvested samples were frozen and stored at -20 °C until required. Stored samples were thawed and HPLC grade methanol (Fisher Scientific, Loughborough, U.K.) added to the samples at a ratio of 2:1 mL solvent: g plant tissue to extract the fungicide. An aliquot (200 µL) of analytical grade flusilazole dissolved in methanol, equivalent to 2 µg mL^{-1}, to act as an internal standard during GC-MS analysis was added to samples which were then homogenised using a Polytron homogeniser for a minimum time of 20 s until plant material had been completely pulped. Samples were then diluted by 50% v/v with SDW and the solid content removed by filtration using grade 1, 11 µm cellulose filter papers (Whatman, Maidstone, England). Tebuconazole and flusilazole were then concentrated from the sample using the SPE cleanup technique detailed earlier in this chapter (Figure 3.1). Optimisation of this method highlighted that, when 4 mL of methanol was passed through the cartridge, tebuconazole and flusilazole were eluted from the sorbent bed in the second 400 µL fraction of the solvent. Thus the first 400 µL of solvent was discarded and the second 400 µL collected and analysed using the GC-MS method detailed

earlier. The remaining volume of methanol was also discarded but remained part of the final method to re-condition the cartridge for re-use.

3.3.8 Observations of effect of fungicide seed treatment on emergence and development of young plants.

The effect of phytotoxicity, causing delayed emergence and plant stunting during early growth, is one of the main problems associated with the application of tebuconazole as a seed dressing. Use of the microencapsulated formulations was hoped to combat this effect by releasing the active ingredient over a sustained period. Samples of untreated wheat seed (variety Claire) were treated with three formulations of tebuconazole (microencapsulated tebuconazole batch #4564 and two commercial formulations, Raxil and Mystique) at an equivalent rate of 40 g AI/100 kg of seed. A total of 90 pots per treatment were sown at a sowing density of 8 seeds per pot and a third of the population (30 pots) harvested after 10, 15 and 20 days after sowing. A population (90 pots - 8 seeds per pot) of untreated controls were also grown and harvested at each time point. Harvested plants were frozen and then analysed for percentage emergence (germination) and development in terms of height, (measured from tip of longest leaf to point of emergence from seed coat) and weight. The results from each population were then compared against each other and statistically analysed using the ANOVA function in Microsoft Excel. Five replicate samples taken from each population were also analysed for their tebuconazole content using the SPE extraction and GC-MS method detailed earlier.

3.3.9 Assessment of tebuconazole distribution within individual plants

To gain a more comprehensive understanding of the uptake and distribution of tebuconazole in young plants grown form seeds treated with different formulations of the fungicide, individual plant samples were analysed in more detail. Several populations of plants were grown from seeds treated with microencapsulated tebuconazole (batch #4564), Mystique and Raxil, applied at a dose rate of 40 g AI/100 kg seed. Plants were grown under the same conditions stated in the general plant growth method (detailed previously in this chapter) until they had reached growth 12, 14 and 16 (Zadoks growth scale) and then harvested. Replicate samples were then split into pairs of leaves based on leaf age, resulting in plants grown to GS 12 being analysed as one pair of leaves, plants grown to GS 14 being analysed as two, pairs of leaves and GS 16 as 3 pairs of leaves. Leaves were analysed by solvent extraction, SPE and GC-MS, the methods of which have been stated earlier in this chapter (Sections 3.3.7.1 and 3.3.2).

3.3.10 Assessment of tebuconazole distribution within individual leaves

In a further investigation of the previous experiment the distribution of tebuconazole within individual leaves was also investigated. Plants were grown, as before, from seed stocks treated with microencapsulated tebuconazole (batch #4564, Mystique and Raxil at an equivalent applications rate of 40 g AI/100kg seed. Individual leaves were split into longitudinal sections; each leaf was cut into sections consisting of the leaf tip, middle and base. Leaf samples were also divided into lateral sections consisting of the leaf margin and mid section. Several samples were combined to allow for easier detection using the GC-MS method. Prior to analysis all samples were weighed to allow an accurate comparison to be made between samples after solvent/SPE extraction and GC-MS determination.

3.3.11 Effect of growth environment on fungicide uptake

3.3.11.1 Temperature

The effect of the environment in which the plants were grown on the rate of tebuconazole uptake was investigated. To allow the environment to be more closely controlled, a series of growth rooms were utilised for these experiments. Populations of plants grown from seed stock treated with microencapsulated tebuconazole (batch #4564), Mystique and Raxil at an equivalent application rate of 40 g AI/100kg seed in average (day and night) temperatures of 6, 14, 18 and 21°C. The plants were grown till they had reached GS 14, harvested, weighed and frozen at -20°C. Tebuconazole content of replicate samples was then analysed by solvent/SPE extraction (section 3.3.7.1) and GC-MS (section 3.3.2) analysis as before.

3.3.11.2 Rate of transpiration

The effect of increased transpiration rates on the rate of tebuconazole uptake into emerging plants from treated seeds was assessed. Two identical populations of plants were grown from seeds treated with microencapsulated tebuconazole, Mystique and Raxil as before. The rate of transpiration was artificially increased in one population using a pedestal fan to achieve an increased air flow whilst the other was grown in normal conditions without increased air airflow. Plants were grown to GS 14 and were exposed to the increased air flow from emergence to harvesting. The amount of tebuconazole in harvested plants were analysed in the same manner as before using solvent/SPE extraction and GC-MS analysis.

3.3.11.3 Seed sowing density

A series of 13 cm pots were sown with seed stocks treated with Micap tebuconazole, Mystique and Raxil at an equivalent application rate of 40 g AI/100kg seed. Seed sowing densities of 1, 3, 6, 10, 15 and 21 seeds per pot were used. Several pots were planted at each sowing density to allow for a minimum of 5 replicate plants to be harvested from each treatment. The plants were grown to GS 12 in a glass house under the conditions detailed in the general plant growth method set out earlier, harvested and prepared for GC-MS analysis as before.

3.4 RESULTS AND DISCUSSION

3.4.1 GC-MS method development

Analysis of a range of GC-MS temperature parameters based around those used in the initial stages of the research (Chapter 2) showed that there was a significant ($P = <0.05$) impact of the combination of start temperature and temperature ramp on chromatographic quality. The results of the initial parameters tested, given in Table 3.2, showed that a combination of a lower start temperature and higher temperature ramp returned increased peak areas and heights when compared to other combinations. This was also the case for peak area, where a lower value showed a better peak resolution. Conversely, the retention time was shortened by the combination of a lower start temperature and ramp. This, however, was unimportant as the aim of the experiment was to improve chromatographic quality and the GC-MS used was able to perform injections automatically using the fitted autosampler.

The results also showed that there was potential for further improvements to be made in the method leading to a further experiment based on the initial findings. The results of this second testing, displayed in Table 3.3, showed that, of the new parameters, a higher temperature ramp gave the larger peak height and ratio of peak area:height. The results of these experiments lead to the selection of a start temperature of 125°C and a temperature ramp of 24°C min^{-1} for optimum chromatographic quality, the full details of the finalised method are detailed in the materials and method section of this chapter (section 3.3.1). The optimised GC-MS parameters allowed the detection of tebuconazole amounts as low as 0.05 mg which was a significant improvement on the previous method and also sufficient for the purposes of the ongoing research.

Table 3.2 Results of chromatographic analysis of a standard containing tebuconazole obtained as part of the preliminary GC-MS method optimisation experiment.

Temperature		Results			
Start Temp. (°C)	Temp. Ramp (°C min $^{-1}$)	Peak (a.u.)		Retention Time (min)	Peak ratio
		Height	Area		Area:Height
100	5	521	5060	25.1	9.71
100	5	486	5370	25.2	10.98
180	5	240	5030	10.0	20.96
180	5	250	5260	10	21.04
140	12.5	1305	5310	9.9	4.07
140	12.5	1220	4970	9.9	4.07
140	12.5	1210	5000	9.9	4.13
140	12.5	1241	5060	9.9	4.17
100	20	2135	5360	9.5	2.51
100	20	1960	5690	9.5	2.90
180	20	536	4590	5.6	8.56
180	20	525	4372	5.6	8.32

Table 3.3 Results of chromatographic analysis of a standard containing tebuconazole obtained as part of the from secondary GC-MS method optimisation experiment.

Temperature		Results			
Start Temp. (°C)	Temp. Ramp (°C min $^{-1}$)	Peak (a.u.)		Retention Time (min)	Peak ratio
		Height	Area		Area:Height
75	15	777	3330	13.29	4.29
75	15	680	2925	13.30	4.30
75	18	1130	3510	11.68	3.11
75	25	1170	3930	9.41	3.36
75	25	1140	3060	9.42	2.68
88	20	1050	3500	10.19	3.33
100	15	490	2150	11.6	4.39
100	15	618	2770	11.6	4.48
100	25	1160	3020	8.28	2.66
108	18	1010	3260	9.84	3.23
113	22.5	1050	3630	8.3	3.46
125	15	552	2475	9.9	4.48
125	15	770	3080	9.97	4.00
125	20	1000	3040	8.33	3.04
125	25	1060	2850	7.34	2.69
125	25	1170	3270	7.35	2.79

3.4.2 Solvent extraction and SPE method validation

The ability and efficiency of the SPE cartridges to repeatedly remove and return 1 µg aliquots of tebuconazole and flusilazole from a solution was tested. Randomly selected cartridges were tested with 5 replicate solutions containing the fungicides and the elutants analysed using GC-MS. The results, given in Figure 3.2, showed that there were no significant (P = >0.05) differences between cartridges when used repeatedly. The percentage recovery for each fungicide, 90% (±6.5) for tebuconazole and 90% (±5.8) for flusilazole, was deemed acceptable for the forthcoming analysis of plant material.

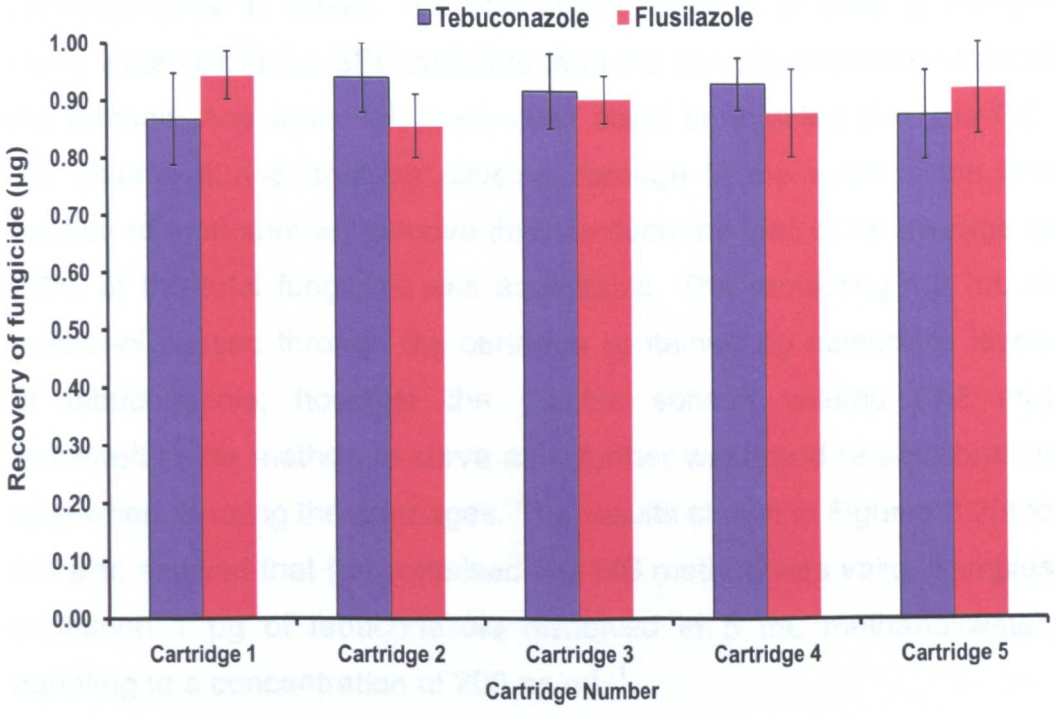

Figure 3.2 Mean recovery of 1 µg aliquots of fungicides tebuconazole and flusilazole from SPE cartridges. Results represent an average value of 5 replicate samples per cartridge. Error bars show standard deviation.

The elutant phase of the SPE extraction method was also tested by GC-MS analysis as a series of fractions. The aim of the analysis was to ascertain the volume of methanol needed to elute the tebuconazole and flusilazole from the SPE cartridges and thus optimise the extraction method. The cartridges were loaded with 1 µg each of tebuconazole and flusilazole. A 2 mL volume of methanol was passed through the SPE cartridge and collected in 400 µl volumes. These volumes were analysed using the GC-MS method set out earlier in this chapter. As can be seen from the results, in Figure 3.3, an average of 89.6% of tebuconazole and 89.0% flusilazole was recovered in the second 400 µl fraction of methanol analysed. On average, 2.4% tebuconazole and 2.6% flusilazole of total fungicides applied to the SPE cartridge were recovered in the first fraction of methanol analysed. The SPE method, detailed earlier, dictates that the first 400 µl of methanol eluted from the cartridge goes to waste. The first elution fraction is likely to contain some water left in the SPE cartridge from the sample and wash steps of the method. Any water left in samples could be injected onto the GC-MS column during analysis causing damage to the system; the first fraction of methanol will remove this. As such the loss of an average of 2.5% of the total fungicide was acceptable. The remaining 1.2 mL of methanol passed through the cartridge contained no detectable levels of tebuconazole; however the surplus solvent volume (3.2 mL) remained in the method to serve as a further wash and re-equilibration step when re-using the cartridges. The results shown in Figures 3.2 and 3.3 also showed that the optimised GC-MS method was valid. Samples contained 1 µg of tebuconazole dissolved in 5 mL methanol/water, equating to a concentration of 200 ng mL^{-1}.

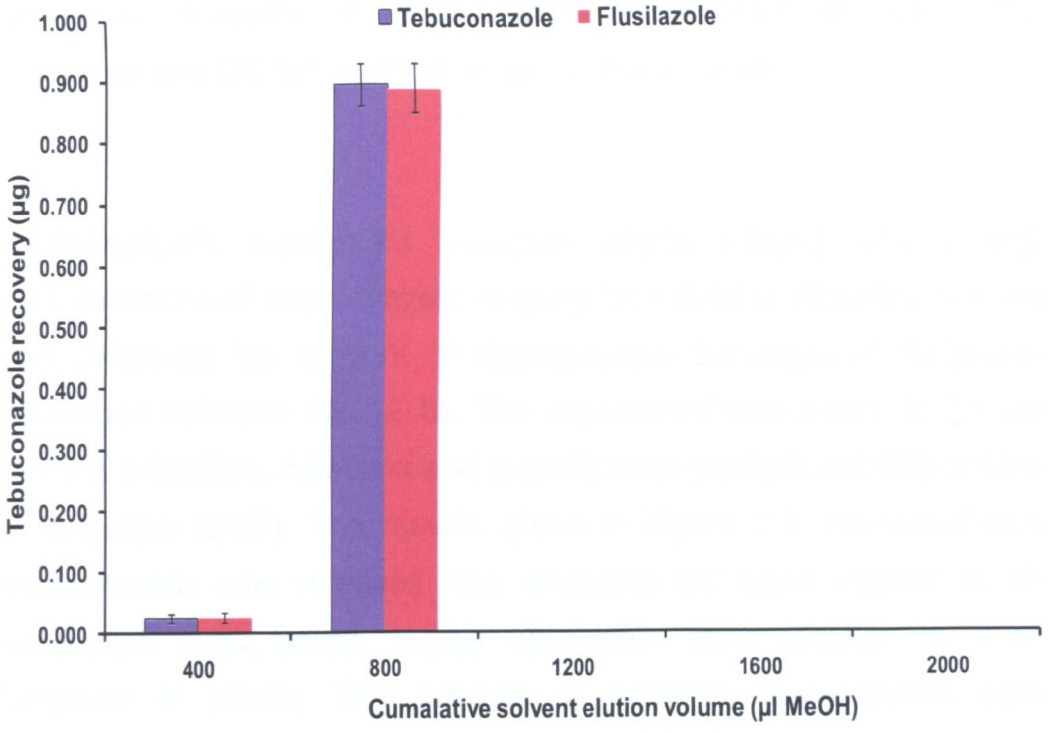

Figure 3.3 Analysis of the amount of tebuconazole returned from fractions of methanol recovered during the elution phase of the SPE extraction method. Each result is the average of 5 replicate samples. Error bars show standard deviation.

3.4.3 Preliminary assessment of release and plant uptake of tebuconazole from microencapsulated formulations of the fungicide

The microencapsulated tebuconazole formulation produced by Micap was tested as a seed dressing and for its basic behaviour as a controlled release formulation. The Micap formulation was to be compared to two commercially available formulations of tebuconazole which were Raxil and Mystique. Although Mystique is not applied as a seed dressing commercially it was selected for use as another tebuconazole only formulation for comparison. Seed stocks were initially treated with 40, 80 and 160 g AI/100kg of seed grown to growth stage (Briggs *et al.*) 12 (Zadocks), harvested and prepared for GC-MS analysis. The results (data not presented) showed that the plants treated with 40 g a.i per 100kg of tebuconazole accumulated easily

detectable amounts of tebuconazole when analysed using the extraction and GC-MS method set up for the purpose.

A subsequent experiment analysed seeds treated with varying concentrations of tebuconazole ranging from 5-40 g AI/100kg of seed and assessed the amount of tebuconazole accumulated as plants developed between GS 12-16. The experiment also aimed to further test the extraction, detection and quantification method and find its limit of detection (LOD). The results, given in Figure 3.4, confirmed that tebuconazole was released and available for plant uptake at all application rates tested. Higher application rates resulted in more fungicide in plants. The amount of fungicide accumulated also increased as the plant developed between GS 12 and 16 suggesting a ready supply of tebuconazole and confirmed the results of the sustained release experiment (Chapter 2) carried out earlier in the research. The results also served to validate the solvent extraction and SPE cleanup method. Using the finalised GC-MS method it was possible to detect tebuconazole amounts as low as 50 ng.

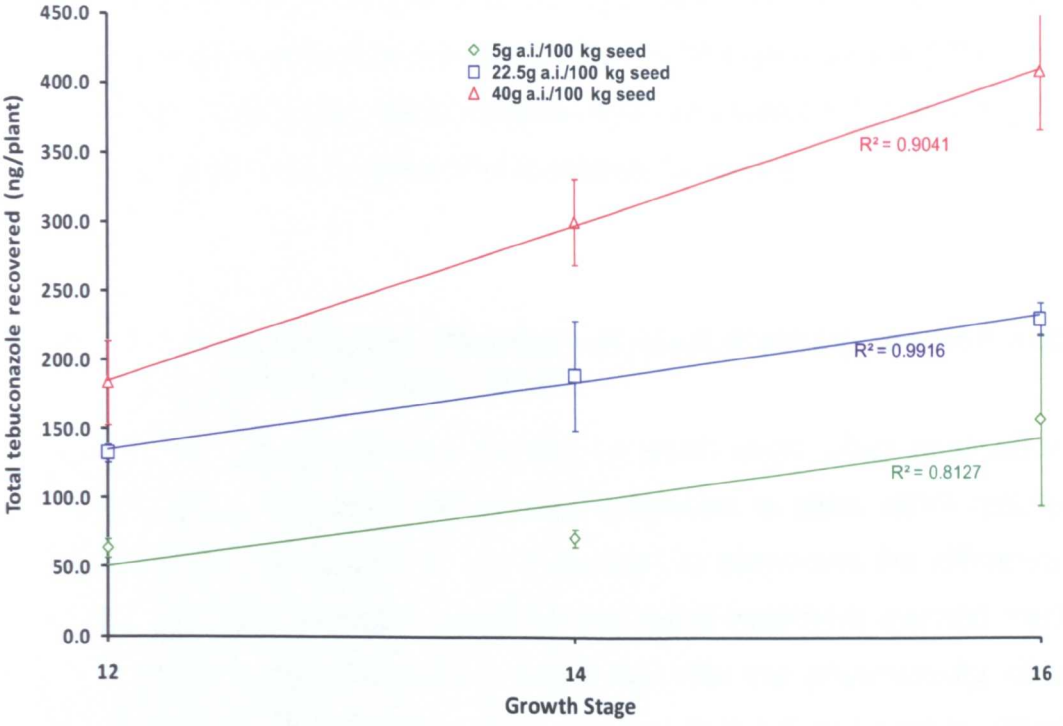

Figure 3.4 Mean tebuconazole amounts recovered from plants grown from seed treated with microencapsulated tebuconazole (batch #4564) at increasing application rates as the plant developed. Data points represent an average of three replicate samples. Error bars how the standard deviation of the means Lines of best fit are linear.

3.4.4 Application of microencapsulated tebuconazole compared with two commercial formulations of the fungicide and assessment of uptake and effect on developing plants

Fungicides that are used as seed treatments, such as tebuconazole, may have a delaying or stunting effect on plant emergence and early development. One of the possible advantages of the encapsulated tebuconazole product was that it would release the AI in a controlled manner. This would permit for a higher rate of fungicide to be applied without causing the negative effects of phytotoxicity. Previous results (Figure 3.3.5) showed that there were detectable tebuconazole concentrations in plants grown from seeds treated with the Micap product at an application rate of 5 g AI per 100 kg of seed. However, the purposes of the research were to test the ability of the encapsulated formulation of tebuconazole to release the fungicide over a prolonged period and provide protection later into the growth period of treated

plants. A dose rate of 40 g AI per 100 kg of seed was also thought to be an appropriate application rate for further testing as it was significantly higher than application rates currently used of between 1 and 7.5 g AI per 100 g seed and compared more closely to Jockey.

3.4.4.1 Assessment of efficiency of seed dressing method and of amount of AI per seed

The amount of tebuconazole carried by each seed, after application using the seed dressing method was assessed to allow other results obtained to be interpreted. It was important to determine the efficiency and identify any potential variations the seed treatment method may have caused. Other research carried out into the phytotoxicity of a formulation of tebuconazole (Raxil) applied to hard red spring wheat seed found the amount of fungicide on individual seed could vary between 25 and 400% of the recommended application rate as a result of the application method (Ashley *et al.*, 2003). The known amount of individual seed in 100 g (Table 3.4) and the amount of tebuconazole applied (at a rate of 40 g AI per 100 kg seed) were used to estimate the expected total per seed at 19.3 µg (assuming no variation between seed size and no losses of fungicide applied as a result of the seed dressing process).

Table 3.4 Average grain weights for wheat seed (variety *Claire)*

Sample weight Replicate	1000 Grain Weight (g)	Total seeds in 100 g sample
1	48.7	2132
2	47.9	2093
3	47.2	2084
4	48.1	2118
5	47.9	2103
Mean	**48.0**	**2104**

This estimate was further tested in the laboratory where tebuconazole was extracted into DCM from replicate seeds treated at the same rate and the amount of fungicide assessed using GC-MS. The results, given in Figure 3.5, showed there to be no significant (P = >0.05) differences, when analysed using a two way ANOVA, between the amount of fungicide recovered from seeds treated with the three treatments. The amount of tebuconazole recovered from the seeds was (18.3, 18.9 and 17.8 µg/seed for Micap, Mystique and Raxil respectively) on average slightly lower than the estimated amount of 19.3 µg. During the seed coating method it was noted that the fungicide was evenly coated on seeds and that there was negligible residue left on the interior surfaces of the jars used. Samples of each fungicide were also applied using a commercial seed coating drum by a third party (Eurofins, Agrisearch) and the amount of tebuconazole assessed in the same manner. The results obtained showed identical results and as such have not been included here.

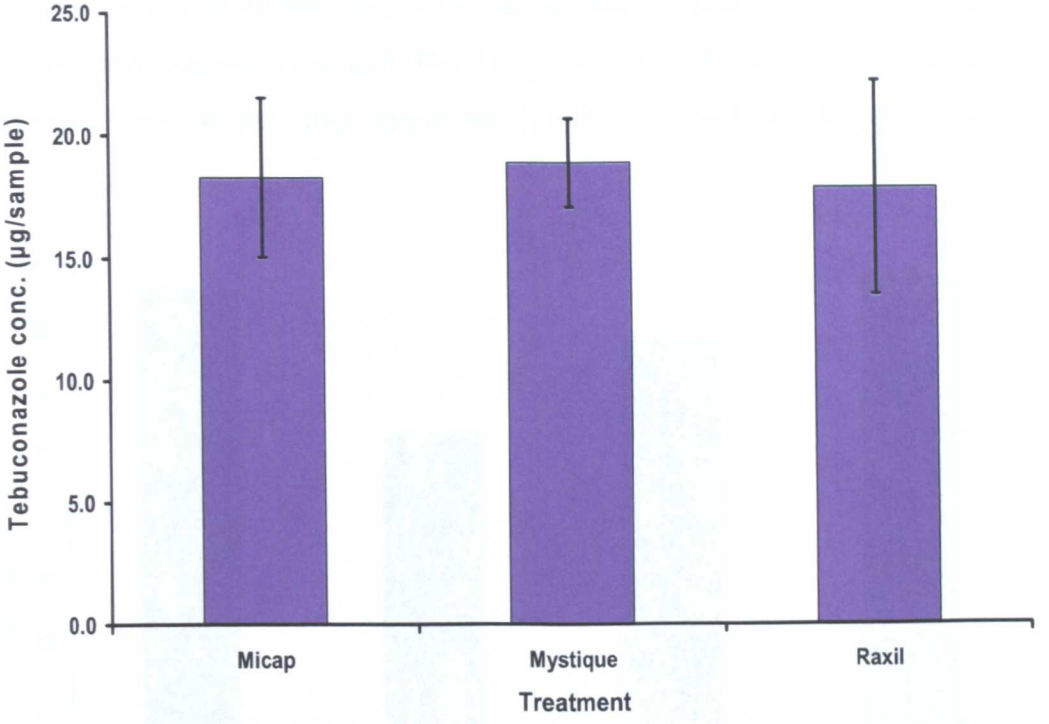

Figure 3.5 Mean recovery of tebuconazole from seeds treated with microencapsulated tebuconazole (Micap), Mystique and Raxil as a seed dressing. Results based on 5 replicate samples. Error bars show standard deviation.

3.4.4.2 Emergence study results

Seeds were treated at a dose rate of 40 g AI per 100 kg seed with a batch of the Micap product (#4564 – 16.3% a.i) and two commercially available fungicides, Mystique and Raxil. The three formulations tested all contained only one fungicide, tebuconazole, and the amounts of each used were adjusted to be equivalent to each other. Treated seeds were grown in pots and harvested at three time pots (10, 15 and 20 d) and measured for percentage germination, plant height and weight.

Analysis of the percentage emergence (germination) of plants from seeds treated with the three formulations of tebuconazole, given in Figure 3.6, showed that the Micap product had a significantly ($P = <0.05$) lower effect compared to the commercial formulations. When compared to untreated control plants the encapsulated formulation of the fungicide performed equally well in terms of germination. This

suggested that there might be a beneficial effect conferred by the sustained nature in which the fungicide is released from the Micap product, where the fungicide is not in direct contact with the seed coat.

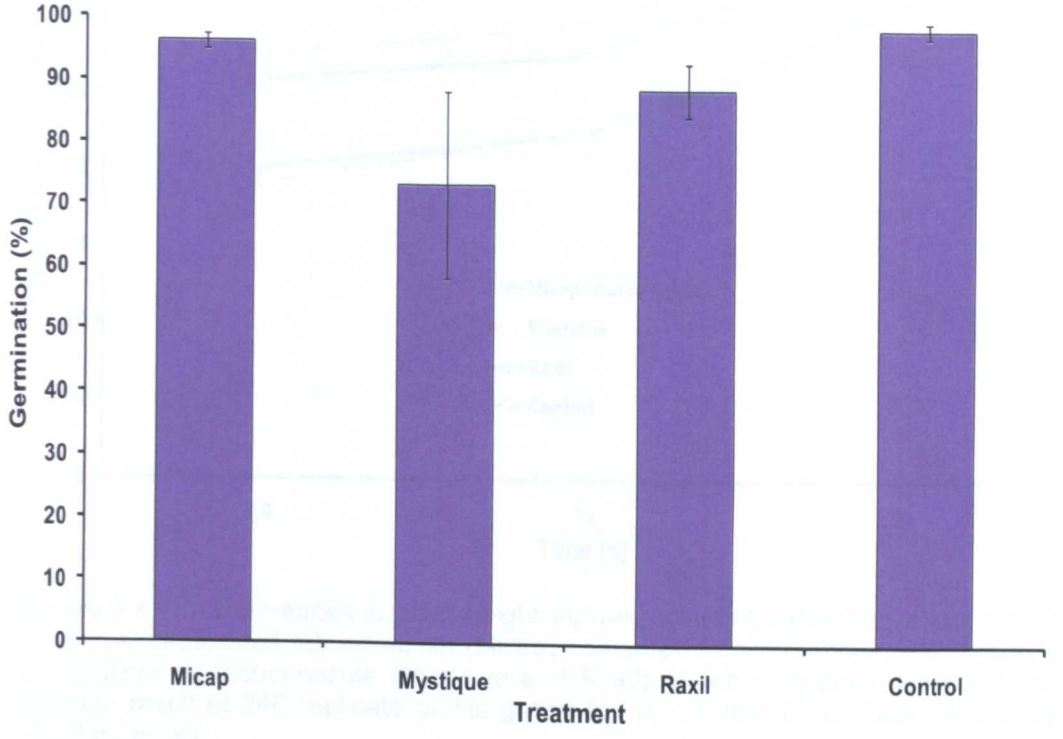

Figure 3.6 Percentage germination of seeds treated with microencapsulated tebuconazole (Micap batch #4564) compared to two commercial formulations, Mystique and Raxil and an untreated control. Data bars are an average of three populations of plants. Error bars show standard deviation.

Individual plants were also measured for their development to assess whether there was an effect caused by the different fungicide applications. Analysis of plant height, shown in Figure 3.7, suggested that there was a difference between fungicides, the plants treated with the microencapsulated formulation of tebuconazole were significantly ($P = >0.05$) better developed in terms of height than those treated with the commercial formulations. Comparison between a population of untreated control and Micap treated plants showed there to be a small reduction in height caused by this treatment, however, the commercial treatments were found to have a greater effect.

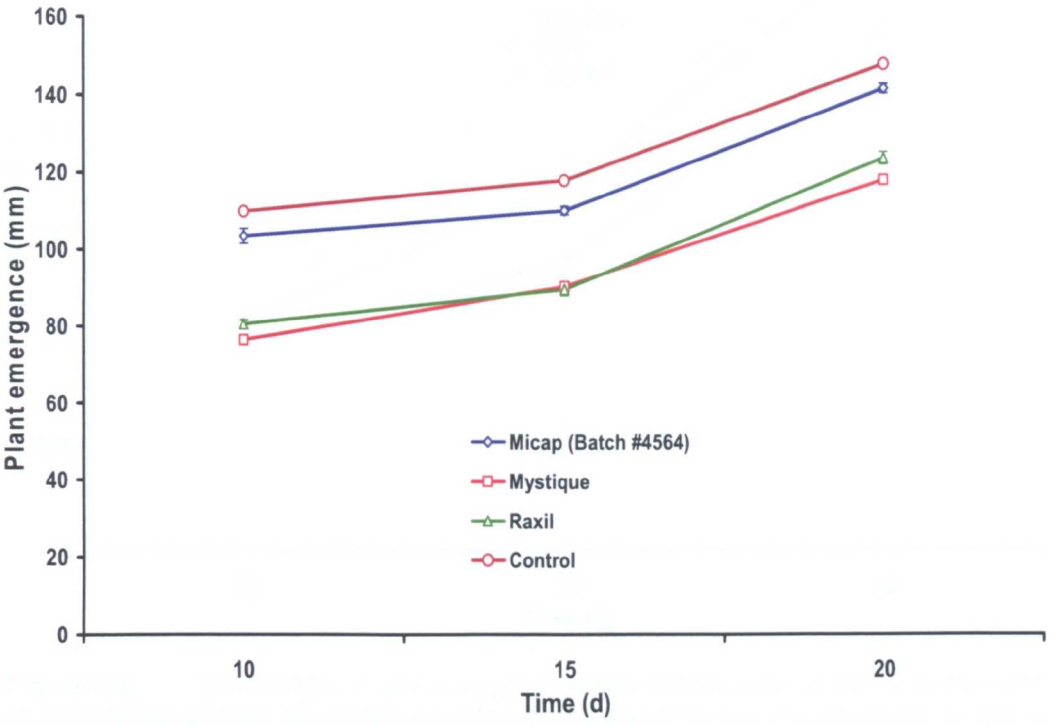

Figure 3.7 Differences in plant height between plants treated with a formulation of microencapsulated tebuconazole (Micap) compared to two commercially available formulations of tebuconazole (Mystique and Raxil). (Each data point represents an average result of 240 replicate plants grown for 10, 15 and 20 d. Error Bars show standard error)

Analysis of the effect of the different treatments on plant development in terms of weight, shown in Figure 3.8, gave similar results. The results recorded for the weight of plants grown from treated seeds can be seen in Figure 3.7. The results showed the same trend as the results seen for plant height in Figure 3.6. Plants treated with the microencapsulated formulation weighed significantly ($P = < 0.05$) more than plants treated with the commercial formulations. The untreated control plants weighed more than the treated plants, again suggesting that the presence of tebuconazole was having an adverse effect on the development of plants. The two commercial formulations behaved near identically and statistical analyses showed no differences between these formulations.

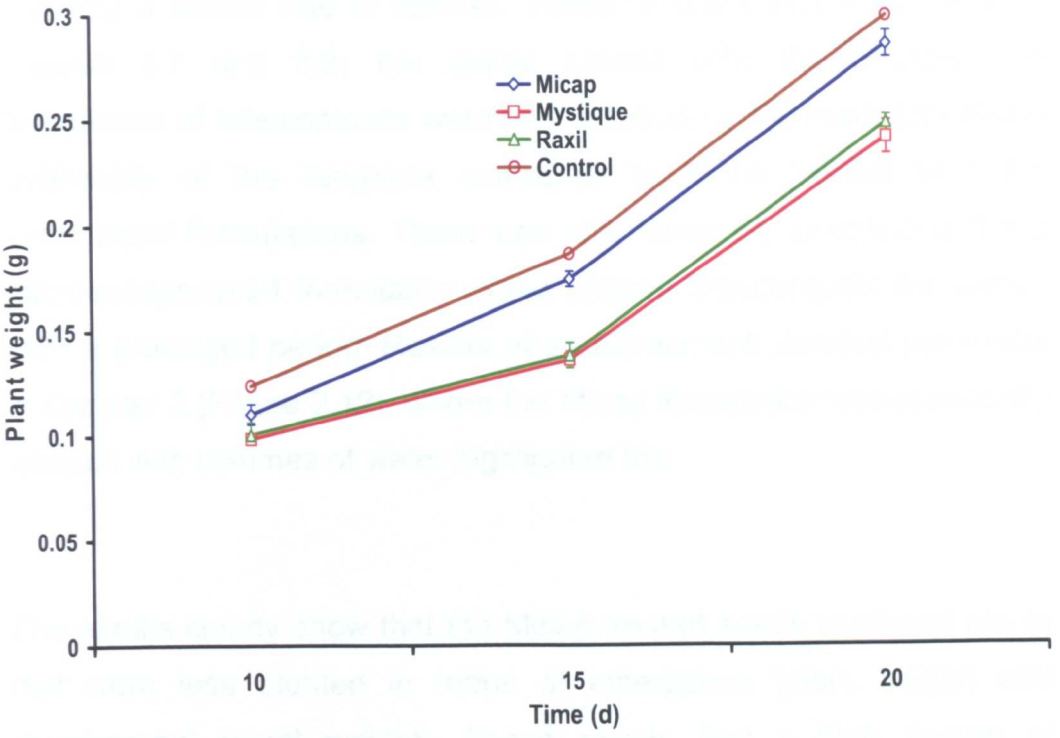

Figure 3.8 Differences in plant weight between plants treated with a formulation of microencapsulated tebuconazole (Micap) compared to two commercially available formulations of tebuconazole (Mystique and Raxil). (Each data point represents an average result of 240 replicate plants grown for 10 d. Error Bars show standard error)

To determine if the effects of higher germination rate and development were a result of lower amounts of accumulated fungicide samples from each population and treatment were selected at random and submitted for further analysis. The amount of tebuconazole they had accumulated was assessed using the extraction technique and GC-MS analysis as detailed earlier in this chapter. The results, shown in Figure 3.8, showed that there was, on average, a lower concentration of tebuconazole recovered from plants treated with the Micap formulation when compared with those treated with the commercial formulations. Statistical analysis of the results, using ANOVA and a Tukey multiple comparison test, showed there to be significantly less ($P = <0.05$) fungicide in plants treated with the Micap product in the 15 and 20 d but not the 10 d plants. This suggests that tebuconazole was immediately available for uptake from all three treatments but that the availability of the fungicide from the Micap formulation might be slightly lower,

implying a slower rate of release. These results corroborate those of Figures 3.7 and 3.8; the plants treated with the encapsulated formulation of tebuconazole were less affected by the lower immediate availability of the fungicide compared to those treated with the commercial formulations. There was still, however, evidence that the microencapsulated formulation could provide tebuconazole for uptake over a prolonged period. Results of an experiment, detailed previously in Chapter 2 (Figure 2.12), where the Micap formulation was repeatedly washed with volumes of water highlighted this.

The results clearly show that the Micap treated seeds produced plants that were less stunted in terms of emergence (plant height) and development (plant weight). These results had a high degree of accuracy due to the number of plants per treatment and the three separate populations sown over a time course meaning that over 2,100 individual plants were analysed in total. A sample (5 replicates) of these plants was also analysed by GC-MS to ascertain the amount of tebuconazole they contained. The results seen in Figure 3.8, show that on average less tebuconazole was recovered from the Micap treated plants. This result is consistent with the reduced stunting and development observed in Figures 2.5 and 2.6.

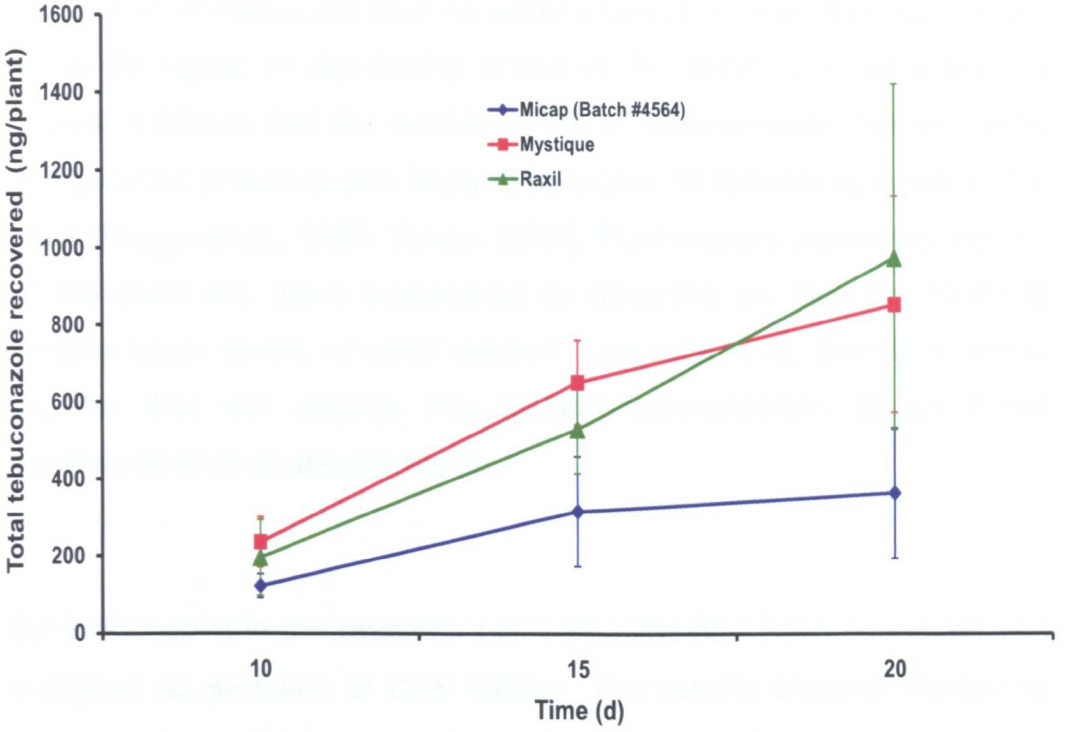

Figure 3.9 Mean concentration of tebuconazole recovered from plants treated at a dose rate of 40 g AI per 100 kg of seed with a formulation of microencapsulated tebuconazole (Micap) and two commercially available formulations (Mystique and Raxil). Each data point represents an average result of 5 replicate plants. Error Bars show standard deviation.

3.4.5 Analysis of distribution of tebuconazole in plants treated with microencapsulated tebuconazole compared to those treated with commercial formulations

The initial experiments undertaken confirmed that tebuconazole was being released from the microencapsulated formulation applied as a seed dressing and moving into developing plants at comparable levels to commercial formulations. It also suggested that this formulation had potential for providing sustained release of tebuconazole and also showed reduced phytotoxic effects when compared with the commercial. To fully understand the differences observed between the Micap formulation and commercial formulations further testing and investigation were undertaken. The uptake of systemic fungicides from the soil occurs apoplastically and the fungicide is distributed throughout the plant via the transpiration stream (Collins *et al.*, 2006). The

distribution of resources such as water and solutes from the soil is likely to be 2x higher in developing areas of the plant such as emerging leaves. It follows that the concentration of tebuconazole, carried in the transpiration stream is also likely to be higher in developing areas of the plant (Briggs *et al.*, 1983; Trapp, 1995). Furthermore, emerging regions of the plant are more susceptible to diseases as they are likely to contain lower levels of seed applied fungicide. It is, therefore, these regions that will require the highest concentration of protective treatments such as tebuconazole.

Up to this point in the research plant samples had been harvested and analysed as samples of total foliage. The results showed increasing concentrations of tebuconazole as the plant developed. However, the distribution of tebuconazole within the plant was unknown. The chemical properties of tebuconazole suggest that the compound should exhibit good levels of systemic distribution within plants. However, if tebuconazole was concentrated in the early leaves produced by the plant and not throughout its entirety, the formulations tested would not provide the long term disease protection for the whole plant. It was also unknown if the lower release levels seen in the plants treated with microencapsulated tebuconazole compared to those treated with commercial formulations would cause subsequent differences in the distribution of AI within the plant. Several populations of plants were grown from seeds treated with the Micap formulation and two commercially available formulations of tebuconazole. Plant samples were grown to growth stage 12 (2 leaves fully emerged), 14 (4 leaves fully emerged) and 16 (six leaves fully emerged) and harvested. Each plant was then split into pairs of leaves separated on the basis of GS and the tebuconazole extracted using SPE extracted and analysed using GC-MS.

Results, shown in Figure 3.10, suggested there was a difference in distribution of the fungicide in the plants depending on treatment. Analysis of the results showed there to be significantly ($P = <0.05$) less tebuconazole, when analysed using a two ANOVA, in the first two leaves to emerge of plants treated with the Micap formulation of the fungicide. This result is consistent with previous results (shown in Figures 3.6, 3.7, 3.8 and 3.9) and again suggests that the Micap formulation was releasing the fungicide in a more controlled manner than the commercial formulations. All three formulations showed a similar uptake profile to that seen previously (Figure 3.9) whereby the amount of tebuconazole accumulated increased with plant age. At GS 14 there appeared to be a higher level of tebuconazole in leaves one and two when compared to leaves three and four in all plants. Analysis of tebuconazole content of the third, fourth, fifth and sixth leaves to emerge in plants from plants grown to GS 16 showed them to contain comparable amounts of the fungicide (i.e. not significant $P = >0.05$) regardless of treatment. It was also noted that, by GS16, leaves 3 and 4 of plants treated with all three formulations contained comparable amounts of tebuconazole to leaves 1 and 2 suggesting that the rate at which tebuconazole was distributed within individual plants might change as more water (via the transpiration stream in which tebuconazole is carried) is allocated to growing areas of the plant. This experiment was repeated identically (data not given) and the results were found to be identical. There were no statistically significant differences between the two commercial fungicides, although Mystique was included for comparison purposes only as another tebuconazole only formulation of the fungicide.

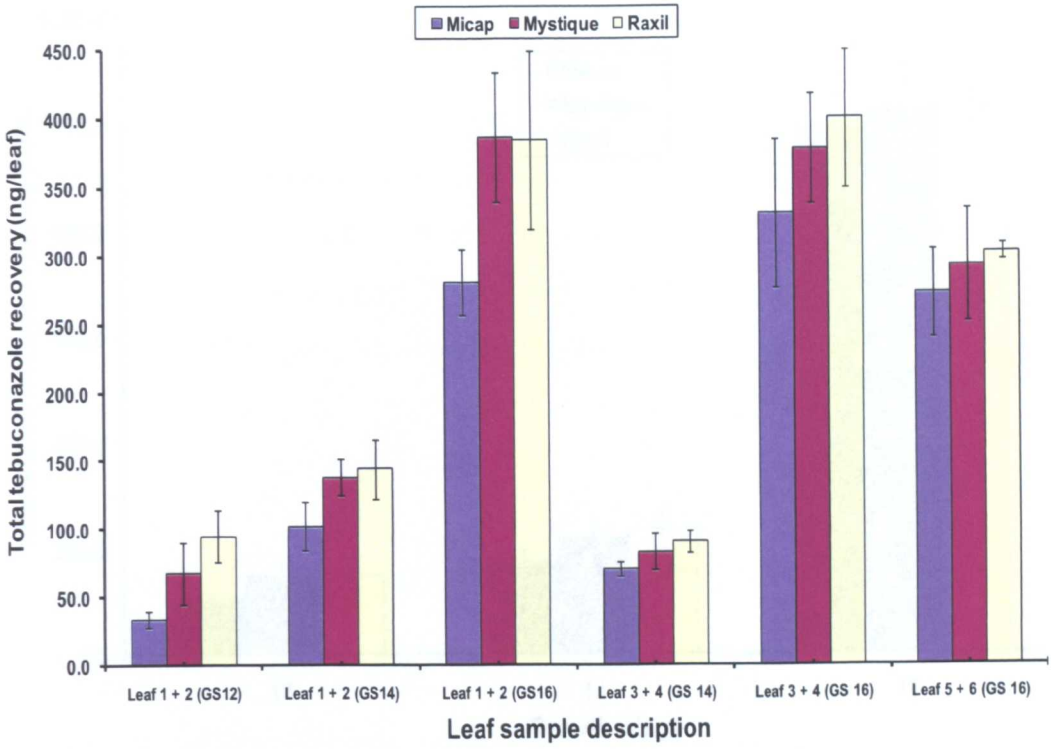

Figure 3.10 Average amount of tebuconazole recovered from leaves taken from plants treated with microencapsulated tebuconazole (Micap) compared with two commercially available formulations (Mystique and Raxil) and grown to GS 12, 14 and 16. Data is an average of 5 replicate samples. Error bars show standard deviation.

Replicate samples of the same population of plants were analysed as whole plants in an attempt to confirm the results seen in Figure 3.10. Assessment of the amount of tebuconazole in whole plant samples, presented in Figure 3.11, showed that, overall, there was significantly less ($P = <0.05$) tebuconazole in plants grown to GS 12 and 14. By GS 16, there was found to be comparable amounts of tebuconazole in plants treated with all three formulations of the fungicide, confirming the results seen in Figure 3.10. The results of the analysis of plants as a whole also highlighted a large increase in the amount of tebuconazole recovered from the plants grown to GS 16 when compared to those grown to GS 14. The reasons for this were unclear though this result was further investigated later in the research.

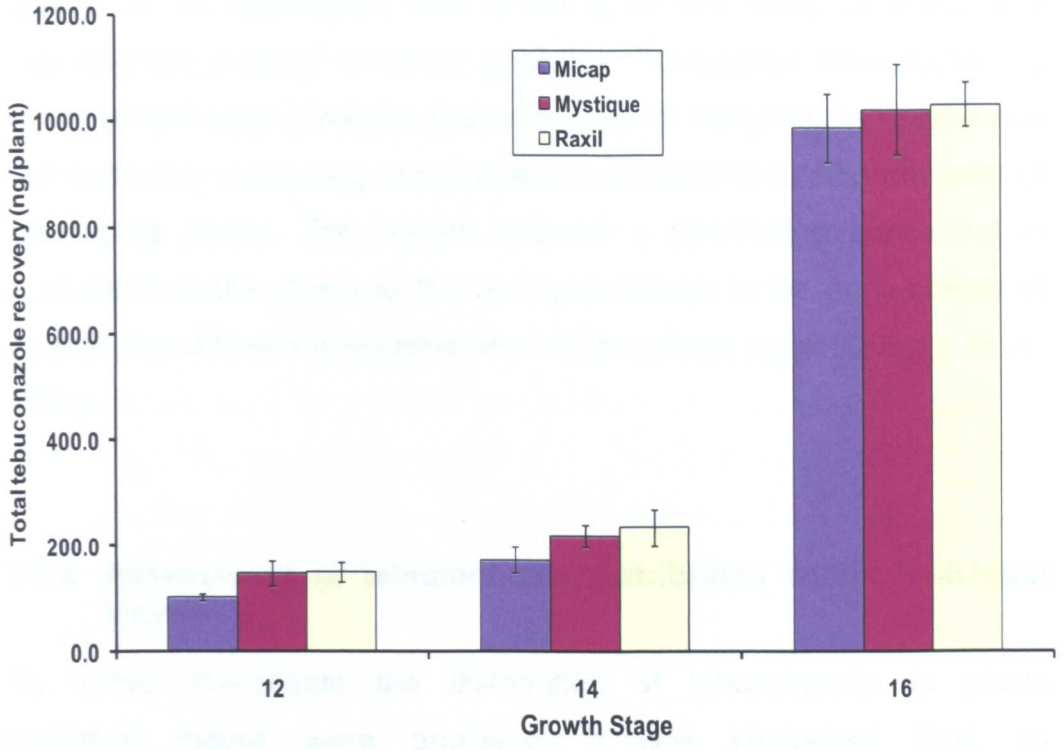

Figure 3.11 Average amount of tebuconazole recovered from total foliage treated with microencapsulated tebuconazole (Micap) compared with two commercially available formulations (Mystique and Raxil) and grown to GS 12, 14 and 16. Data is an average of 5 replicate samples. Error bars show standard deviation.

Overall the results showed that all three formulations tested provided a good level of uptake and systemic distribution of fungicide within developing plants. There did appear to be a difference in the initial rate of uptake of tebuconazole into plants treated with the microencapsulated formulation, which exhibited lower levels of fungicide uptake compared to plants grown from seed treated with the commercial formulations in the early stages of development. However, as the plants continued to develop the amounts of tebuconazole became comparable in plants treated with all three formulations. This supports the results seen previously where there appeared to be a lower level of phytotoxicity in plants treated with the microencapsulated formulation. Other research into the uptake and distribution of fungicides applied as seed dressings in wheat plants have highlighted similar findings. A study involving the uptake of another triazole fungicide triticonazole, with similar chemical properties to tebuconazole,

applied at an application rate of 45 g AI per 100g of seed. The experimental method involved applying ^{14}C-labelled triticonazole to spring wheat seed (*Triticum aestivum* L.) and analysing its uptake and distribution by measuring concentrations of radiation in different parts of developing plants. The results showed a decreasing concentration gradient from the oldest to the youngest leaves in the early stages of growth, this difference became less as the plants aged (Quérou *et al.*, 1998).

3.4.6 Assessment of tebuconazole distribution within individual leaves

To further investigate the distribution of tebuconazole in plants individual leaves were analysed. It was suggested that, as tebuconazole is carried in the plants transpiration stream, the fungicide may end being concentrated at the point where this stream ends, for example leaf tips. Individual leaf samples were taken from plants, grown to GS 12, 14 and 16 from seed treated with the same three formulations (Micap batch #4564, Mystique and Raxil) of tebuconazole at a rate of 40 g AI per 100kg seed, and split into three parts consisting of tip, middle and base (of leaf). These samples were then pooled and weighed, to allow for representative comparisons to be made between leaf sections from different treatments, and the tebuconazole content assessed using the SPE and GC-MS methods detailed earlier.

Analysis of the results of this experiment, given in Figure 3.12, showed that there was significantly more ($P = <0.05$) tebuconazole in the tips of leaves when compared to the base and mid sections. This supported the hypothesis that the fungicide was being distributed within the plant's transpiration stream and this lead to observed accumulation of the fungicide in leaf tips. This trend was also noted across all treatments and growth times. As with results seen previously (Figure 3.11), the

accumulation of tebuconazole was lower in plants treated with the microencapsulated formulation of the fungicide compared to the two commercial formulations, the total amount of fungicide was also seen to increase with plant age. The results seen in Figure 3.12 are also supported by previous research carried out into the uptake of non-ionic chemicals into barley plants from a nutrient solution. The research investigated the relationship between the lipophilicity and uptake/distribution of a series of different chemicals, several with a similar log K_{ow} to tebuconazole. The results showed that a higher concentration of chemicals, especially those with a log K_{ow} of more than 2, were concentrated in leaf tips than the base or midsection of the leaf (Briggs *et al.*, 1983).

A similar experiment examined the lateral distribution of tebuconazole across each individual leaf. Plants were split into individual leaves and each leaf was then further divided into longitudinal sections consisting of leaf margins and leaf mid sections. The sections of leaves from five replicate plants were again bulked together to facilitate easier detection of the tebuconazole during GC-MS analysis and weighed to allow comparisons to be made after analysis. The results of the analysis, data not given, showed that there were no significant differences in the amounts of tebuconazole recovered from longitudinal sections of leaf margin and mid section.

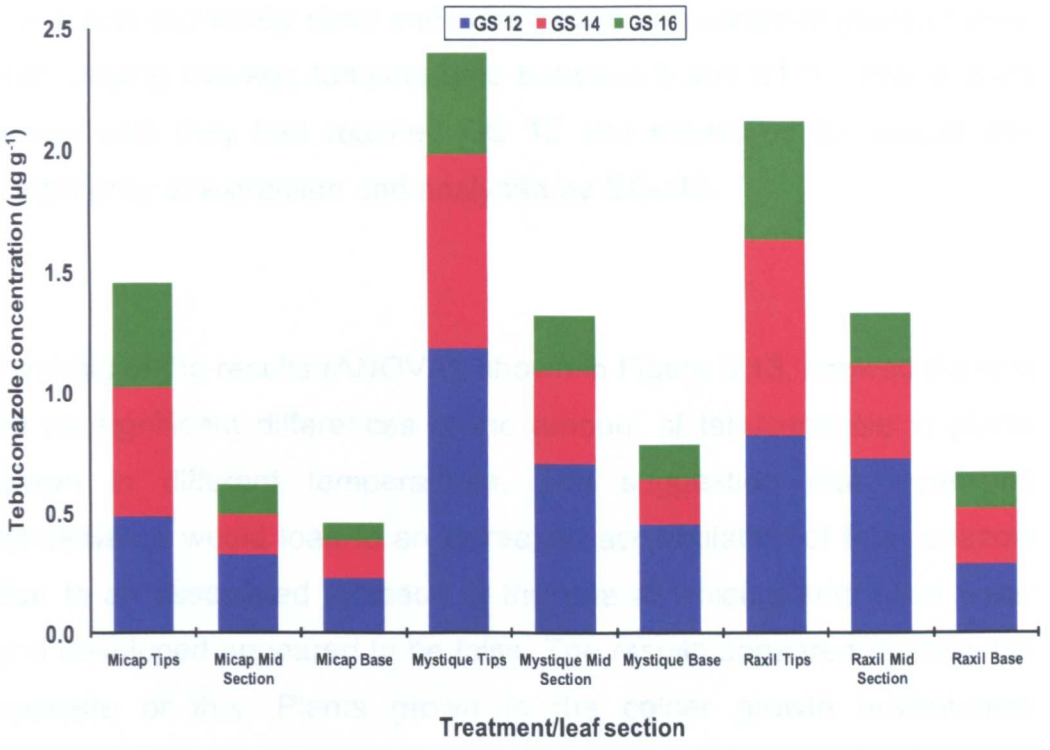

Figure 3.12 Amount of tebuconazole recovered from different sections of leaf taken from plants treated with microencapsulated tebuconazole and two commercial formulations, Mystique and Raxil. Values for GS 12 are represented in blue only, GS14 in blue and red and GS 16 in blue red and green.

3.4.7 Effect of growth environment on fungicide uptake

The results of this experiment also raised the question of the effect growth environment might have on fungicide uptake. If tebuconazole was distributed throughout the plant via the transpiration stream it was suggested that by increasing the flow rate of water through the plant then the amount of tebuconazole accumulated may also increase. The microencapsulated formulation of the fungicide might also be shown to behave differently in such conditions. Analysis of the results to date have shown there to be a high degree of variation between samples, for example, those seen in Figure 3.11, where total tebuconazole in plants was seen to increase sharply between GS 14 and 16. Where plants had been grown in glass houses, changes in environment caused by varying weather conditions might have caused this effect. To test this hypothesis, plants treated with the three fungicide formulations at the

same rate previously used and were grown in a series of growth rooms with varying average temperatures between 6 and 21 °C. Plants were grown until they had reached GS 12 and measured for weight and height prior to extraction and analysed by GC-MS.

Analysis of the results (ANOVA), shown in Figure 3.13, showed there to be no significant differences in the amount of tebuconazole in plants grown in different temperatures. The suggestion that increasing temperature would lead to an increased accumulation of tebuconazole due to an associated increase in the rate at which plants used water and developed appeared to be false. The results appeared to show the opposite of this. Plants grown in the colder growth environment exhibited higher accumulations of tebuconazole. Reasons for this were unclear; however, a possible cause may have been that plants grown in this temperature environment took almost three times longer to reach GS 12, as a result of delayed emergence caused by the lower temperatures, when compared to plants grown in warmer temperatures. It was hypothesised that this longer growth time might have lead to the increased concentrations of tebuconazole exhibited due to an increased time available for fungicide uptake. Although there were no notable differences between treatments in terms of total tebuconazole content analysis of individual plant weight and height did show that, as seen before in Figures 3.8 and 3.7, the weight and height of plants treated with the microencapsulated formulation of the fungicide were significantly (P= =0.05) better developed further supporting the previously raised theory that the microencapsulated formulation of the fungicide caused lower levels of phytotoxicity when compared to the commercial formulations.

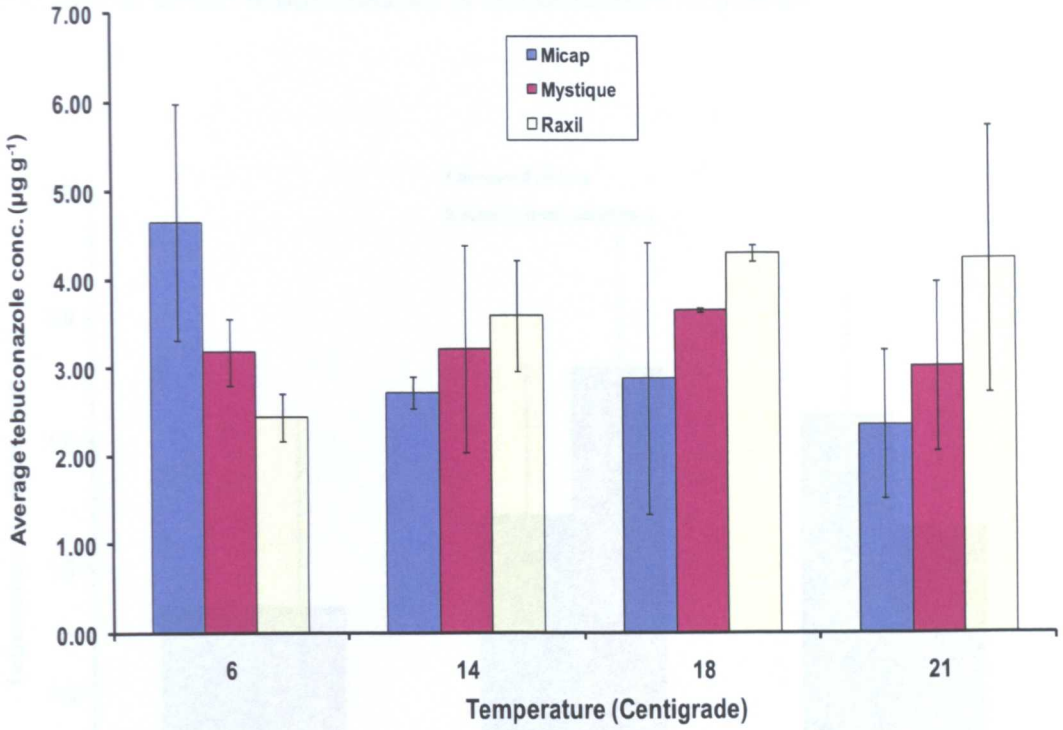

Figure 3.13 Mean concentration of tebuconazole recovered from plants treated with microencapsulated tebuconazole (Micap) compared with two commercially available formulations (Mystique and Raxil) and grown to GS 12 in different temperatures. Data is an average of 3 replicate samples. Error bars show standard deviation.

The effect of temperature appeared to have no effect on the uptake of tebuconazole into developing plants. However, in an effort to further test the effect of growth environment on tebuconazole uptake an experiment was assigned two populations of plants were grown. The first, in an environment where airflow had been artificially increased and the second grown under normal conditions was prescribed. Plants were grown, as before, from seeds treated with the three formulations of tebuconazole at the same application rate and harvested on reaching GS 12. Samples were again weighed prior to extraction and analysis. Analysis of the results, shown in Figure 3.14, showed no significant differences ($P = >0.05$) between plants grown in an increased air flow compared to those grown in normal conditions. These results further

support that supposition that another factor is primarily responsible for the rate at which tebuconazole is accumulated in plants.

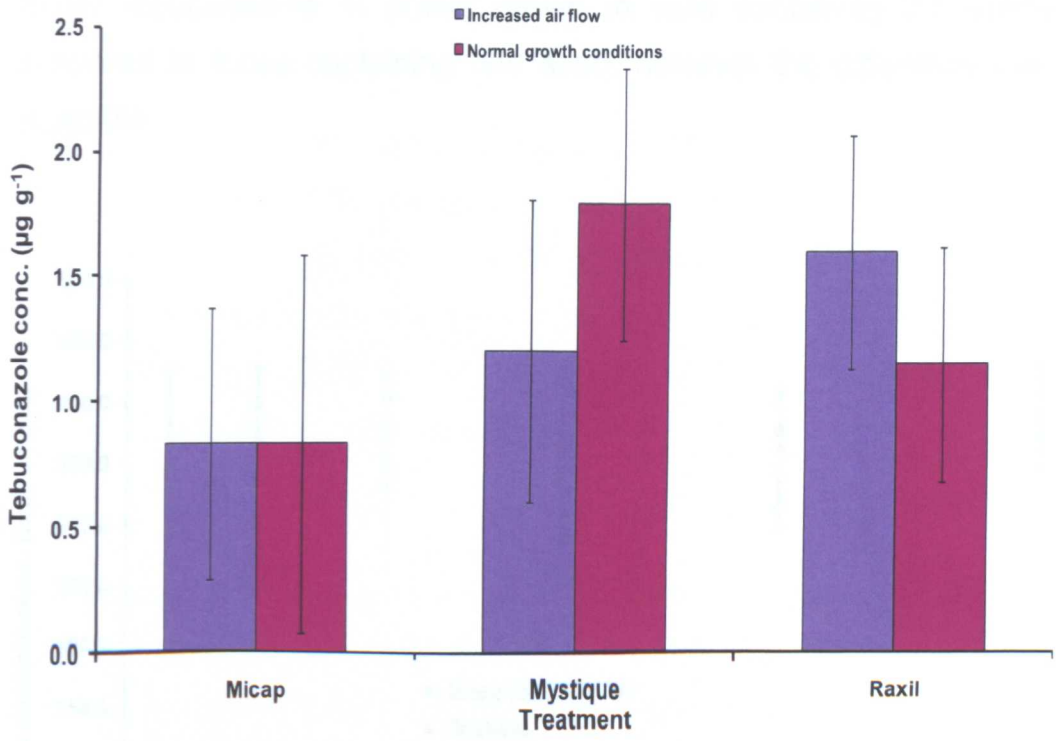

Figure 3.14 Mean amount of tebuconazole recovered from plants treated with microencapsulated tebuconazole (Micap) compared with two commercially available formulations (Mystique and Raxil) and grown to GS 12 in increased air flow. Data is an average of 5 replicate samples. Error bars show standard deviation.

A third growth environment related study examined the effect of seed sowing density on the amount of tebuconazole accumulated in developing wheat plants. As each seed is in itself a source of approximately 19 µg of tebuconazole it was proposed that a higher sowing density may lead to increased levels of tebuconazole available for developing plants and subsequent higher levels of uptake. This theory was tested by the analysis of plant samples grown to GS 12 in pots with a varying seed sowing density ranging from 1-21 seeds per pot. The samples were harvested and analysed as sample sets consisting of five replicate samples using the GC-MS method and extraction method set out previously. Analysis of the results, given in

Figure 3.15, showed average amounts of tebuconazole recovered from plants grown in pots with different seed sowing densities to be comparable. Statistical analysis (using a t-test and tukey multiple comparison) of the findings did show that there was slightly more (P = <0.05) tebuconazole in plants grown in pots containing 21 seeds compared to those containing one seed, however the difference was negligible.

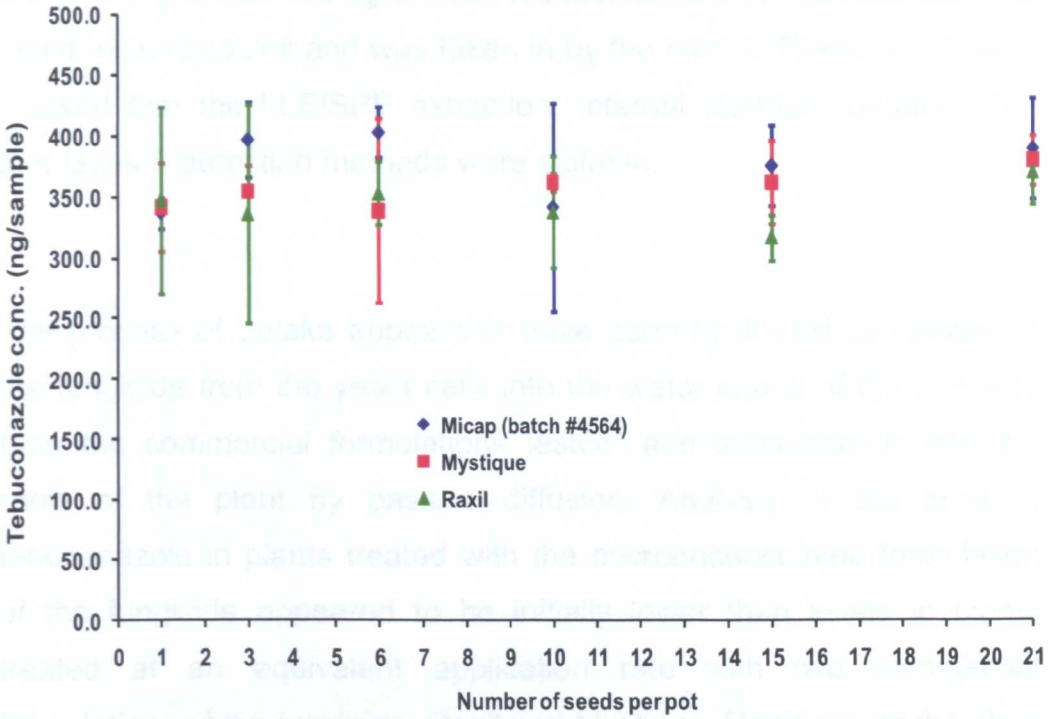

Figure 3.15 Mean amounts of tebuconazole recovered from plants treated with microencapsulated tebuconazole (Micap) compared with two commercially available formulations (Mystique and Raxil) and grown to GS 12 with different seed sowing densities. Data is an average of 5 replicate samples. Error bars show standard deviation.

3.5 CONCLUSIONS

The primary aim of this section of the research was to assess the ability and level to which a microencapsulated tebuconazole formulation applied as a seed dressing could release tebuconazole available for plant uptake. To facilitate this aim a reliable method of extraction, detection and quantification of fungicide residues in plant tissue was also refined. Initial trials showed that when applied at rates of between 5 and 160 g AI per 100 kg of seed tebuconazole was released from the yeast microcapsules and was taken in by the plants. These results also showed that the LLE/SPE extraction, internal standard quantification and GC-MS detection methods were suitable.

The process of uptake appears to have been facilitated by release of the fungicide from the yeast cells into the water phase of the soil (and from the commercial formulations tested) and subsequently into the roots of the plant by passive diffusion. Analysis of the level of tebuconazole in plants treated with the microencapsulated formulation of the fungicide appeared to be initially lower than levels in plants treated at an equivalent application rate with two commercial formulations of the fungicide, Raxil and Mystique. However, as the plant continued to develop these differences became less and levels of tebuconazole were seen to become comparable in plants treated with all three formulations. These results supported those of another trial investigating the potential for seed applied tebuconazole to cause phytotoxicity. The findings of this trial showed the microencapsulated formulation to have a significantly less phytotoxic effect on emerging plants than both commercial formulations when applied at the same rate. It was suggested that the reasons for this may be attributed to the protection of the seed from direct contact with the fungicide by the yeast

cells which may also have conferred a slower release of tebuconazole into the surrounding environment.

Analysis of the distribution of tebuconazole within individual plants showed that all three formulations possessed good levels of systemic distribution. It would appear that the availability and distribution of tebuconazole from the microencapsulated formulation did not differ from that from the two conventional formulations. Initially concentrations were higher in older leaves but as plant development continued a more even distribution between leaves was observed. Analysis of individual leaves showed that in all three formulations tested, there were higher concentrations of tebuconazole observed in leaf tips. This supported the hypothesis that the fungicide was carried throughout the plant in the transpiration stream and concentrated at the plant's peripheral regions. The redistribution of the fungicide from these regions into other areas of the plant was thought to be unlikely as tebuconazole is relatively hydrophobic and as such is likely to be bound in lipid concentrations within the leaves (McFarlane, 1995b).

A high degree of variability, between replicate samples and between growth stages, in the initial experiments investigating plant uptake of the fungicide led to the planning and execution of a series of experiments to examine the effects of growth environment on tebuconazole uptake. The hypothesis that the environment in which the plant was grown would affect the rate of transpiration and could therefore drive the rate of tebuconazole uptake was tested. Specifically, the effects of different temperature, air flow over leaf surface and seed sowing density. The results, however, did not show that there was any effect caused by the conditions tested. It is therefore assumed that the rate of tebuconazole uptake is not solely related to the rate of transpiration. A longer time course or a combination of conditions could

have been investigated to see if any effect could be found long term; however, the level of variability between replicate samples grown in the same population of plants, suggests that the differences and variation could be caused by another factor or combination of factors. Similarly the effect of watering regime was not investigated, however the plants were watered once daily and as such the soil in the pots did not become dry at any point. The constant source of sufficient water in the pots should have allowed a consistent phase of potential uptake of the fungicide as the chemical was released into surrounding water and subsequently into plant roots. The chemical classification of tebuconazole would suggest that the amount of water applied to growing plants would not affect the rate of uptake. Tebuconazole has a solubility limit of 36 μg mL^{-1} in water, which is classed as low (P.P.D.B., 2010). Tebuconazole also has a high tendency to bind to organic components in the soil making the compound classed as only slightly mobile in the soil environment (P.P.D.B., 2010). This data suggests that the tebuconazole is not likely to be washed out of the soil dissolved in water. The presence of higher water volumes in the soil may have led to an increased availability of tebuconazole for uptake by the plant roots. However, when increased seed sowing densities were tested, increasing the total amount of tebuconazole available for plant uptake there were no apparent differences fungicide accumulation observed. It would, therefore, appear that the rate of tebuconazole uptake is driven by another factor. It is known that uptake of organic chemicals from the soil via the roots includes an initial equilibration phase whereby the chemical must first pass into and saturate lipophillic components of cells in the plants roots (Paterson *et al.*, 1991). It is therefore likely that the rate of uptake and distribution of tebuconazole in the plant is driven by root development and not by the rate of transpiration. The availability of the fungicide to the plant from the soil could also be a factor in uptake. As the fungicide has a relatively high tendency to bind to carbon elements in the soil and is not very mobile it may also be less

available for uptake by the plant. The distribution and density of individual plants root coverage could affect the uptake of tebuconazole.

CHAPTER 4: ASSESSMENT OF LONG-TERM UPTAKE OF TEBUCONAZOLE FROM MICROENCAPSULATED FORMULATIONS

4.1 INTRODUCTION

To fully investigate and compare the long term behaviour and uptake of both the microencapsulated and commercial formulations of tebuconazole it was also necessary to understand by what mechanism the fungicide enters the plant and also how the fungicide behaves in the environment. The likely behaviour of tebuconazole in the environment, specifically in soil, can be related to its known chemical properties. Understanding this behaviour may also help explain the availability of tebuconazole for uptake by plants.

Soil is an important environmental sink for organic chemicals, such as tebuconazole, added to the environment as plant seed treatments. In general, the amount of a given compound in these environments is seen to decrease over time, but many chemicals applied to the environment form extremely persistent and sometimes permanently bound residues (Northcott and Jones, 2000). These bound residues have a direct affect on the longer term partitioning behaviour, bioavailability, and toxicity of compounds such as fungicides in the soil. Residues may be subsequently released from the soil by the continuous turnover of the organic carbon in the soil. It is recognised that soil organic matter dominates the sorption or binding of non-ionic chemicals in soils with organic carbon contents of >0.1% (Chiou, 1998). Sorption processes can be driven by several forces or combinations of forces such as Van der Waals forces, electrostatic forces, hydrogen bonding and ligand exchange reactions (Collins *et al.*, 2006). The importance of the organic carbon content of the soil may be expressed by using a partition co-efficient approach or K_{oc} for describing the

partitioning behaviour of organic compounds in soil or sediment/water systems. The chemical values shown in Table 4.1. indicate that tebuconazole is very likely to bind with organic carbon components and is also relatively persistent in a soil environment.

Table 4.1 Chemical properties of tebuconazole in soil

Tebuconazole property		
K_{oc} – organic carbon sorption constant (a.u.)		**769**
Soil degradation (days) (aerobic) (d)	DT 50 (typical)	**232**
	DT 50 (lab at 20°C)	**365**
	DT 50 (field)	**55.8**

A Koc value of 75-500> means a compound has a high tendency to bind to organic carbon elements in the environment. Soil degradation (DT50) in the time required (in days) for the concentration of a pesticide in the soil to reduce to half the concentration at application. A DT50 value of 30-100 is classed as moderately persistent, 100-365 as persistent and >365 as very persistent. A 'typical' DT50 value is given as the mean value obtained from all studies performed on a pesticide in the field and laboratory. Adapted from: The Pesticide Properties Database (P.P.D.B.). (P.P.D.B., 2010)

The uptake of organic chemicals, such as fungicides, by plants is profoundly influenced by the soil (Collins *et al.*, 2006), and may be predicted by the following. The concentration in the soil water of a compound is described by the K_d, which is the partition distribution coefficient between the soil and soil water. Non-ionized organic pollutants are principally sorbed onto the organic fraction of the soil's solid phase, thus the K_d can be defined in terms of the soil organic carbon content $(K_d)K_{oc}$ - f_{oc}, where K_{oc} = organic carbon to water partition coefficient and f_{oc} = fraction organic carbon. The K_{oc} is readily calculated and linearly related to the K_{ow}. Therefore soil sorption will increase with K_{ow}, reducing the availability of high K_{ow} compounds for plant uptake (Bromilow and Chamberlain, 1995).

Organic chemicals can be taken up by plant roots via the vapour or water phases of soil. The uptake of anthropogenic organic chemicals by plant roots has been shown to be a passive, diffusive process, with the exception of a few hormone-like chemicals such as the phenoxy acid herbicides, for which there is some evidence of active uptake (Bromilow and Chamberlain, 1995). Experiments involving the uptake of non-ionized chemicals from hydroponic solution into plant roots have demonstrated that the uptake process consists of two components: (1) "equilibration" of the aqueous phase in the plant root with the concentration in the surrounding solution; and (2) "sorption" of the chemical onto lipophillic root solids (Briggs *et al.*, 1983). Once uptake has occurred water and solutes are transported upwards from the root into other parts of the plant through the plants xylem tissue. For a chemical to reach the xylem it must penetrate a number of plant tissue layers and at least one membrane (Northcott and Jones, 2000). Uptake of azole fungicides and their subsequent relocation through the plant occurs via the apoplastic and symplastic pathways (Dahmen and Staub, 1992). The degree of this uptake is dependent on a combination of solubility of the chemicals in the water and their solubility within the lipid rich cell membrane. This interaction, in turn, dictates the degree of movement of chemicals into the roots themselves and subsequently into the rest of the plant (McFarlane, 1995b).

The basic release of tebuconazole from a microencapsulated formulation into water and the ability of this formulation to provide tebuconazole which is available for uptake by plants have been tested during the course of this project. However, it was unclear how the microencapsulated formulation would perform over a longer time period. The potential for the microencapsulated formulation to release tebuconazole in a sustained manner, for an extended period, could prove beneficial by potentially providing extended disease protection and reducing the need for repeated applications of fungicides. To test

and compare the ability of the novel microencapsulated formulation to provide this effect, a long term growth trial was undertaken. Several experiments were also undertaken to assess the behaviour of the fungicide in a soil environment and also to assess what effect this behaviour would have on the uptake of the fungicide by plants. An experiment was undertaken to assess and compare the amount of tebuconazole accumulated by plants over an extended period of time when treated with seed dressings of microencapsulated tebuconazole and a commercial formulation, Raxil. It was hypothesised that the performance of the two treatments may differ with the microencapsulated formulation releasing tebuconazole for a longer period of time when compared to the commercial formulation. Following on from this several experiments were also carried out to establish the propensity of the growth medium to affect the availability of tebuconazole for uptake by plants.

4.2 SUMMARY OF AIMS AND OBJECTIVES

- Assess long term release potential of the microencapsulated tebuconazole formulation and compare this with a commercially available formulation.
- Assess the behaviour of tebuconazole in a soil environment and what impact this behaviour has on the uptake of tebuconazole from the soil.

4.3 MATERIALS AND METHODS

4.3.1 Assessment of tebuconazole uptake and accumulation in plants over a longer time course

Two populations of plants were grown from wheat seed (variety Claire) treated with microencapsulated tebuconazole and a commercial formulation, Raxil at an application rate of 40 g AI per 100 kg seed. It was decided not to include Mystique in this trial, due to the lack of significant differences observed between the two commercial formulations up to this point in the research and also Mystique is not commercially employed as a seed dressing. Seeds were planted in 5 L pots, using John Innes No. 3 compost at a sowing density of 5 seeds per pot. Plants were then grown in the glasshouse set to maintain a day temperature of 18°C, a night temperature of 12°C and a minimum day length of 12 h and watered once, daily. When this day length could not be provided with natural light, artificial light was provided by sodium bulbs with an irradiance measured at 69 µmol m^2 s^{-1}. Replicate samples of the population were selected randomly and harvested over a time course with consecutive sampling points between 2 and 19 weeks after sowing then frozen at -20°C until required for analysis.

4.3.1.1 Sample preparation, extraction and analysis

Individual plant samples were thawed, weighed and larger plants split into several parts. Samples of flag leaves, taken from mature plants were also analysed individually. Plant material was ground to a powder using a pestle and mortar in liquid nitrogen to break up tougher sections of the plant. Powdered samples were suspended in 10 mL HPLC grade MeOH (Fisher Scientific, Loughborough, U.K.) and the flusilazole internal standard added at an equivalent concentration of 2 µg mL^{-1}. Samples were then further homogenised using a Polytron homogeniser

for 20 s before dilution by 50% v/v with SDW. The solid content was removed by filtration using grade 1, 11 µm cellulose filter papers (Whatman, Maidstone, England) and the tebuconazole and flusilazole extracted using SPE and analysed by GC-MS using the method refined earlier in the research, detailed in chapter 3.

4.3.2 Analysis of the potential of growth medium to adsorb tebuconazole

The ability and capacity of the growth medium used during this project, loam based John Innes No.3 compost, to adsorb and retain tebuconazole was tested under laboratory conditions. Samples of the compost were first prepared by air drying at room temperature in a laminar flow cabinet, small stones were removed by sieving and conglomerates of soil were broken up. Several 50 mL plastic syringes were conditioned prior to use by repeated washing with methanol followed by SDW to remove any silicon held on the internal surfaces and plunger head. Syringes were then packed with a cotton wool plug only or with a cotton plug followed by 5 g of pre-dried compost. A total of 35 µg of tebuconazole dissolved in methanol was mixed with a 10 mL volume of SDW, this was then passed through each syringe and the sample collected. The effect of time on the ability of the soil to retain tebuconazole was also assessed; 5 g masses of prepared soil were mixed with identical 10 mL volumes of water, containing 35 µg of tebuconazole and left for 24 and 48 h before extraction and analysis of the water phase of the samples. The total capacity of the compost to hold tebuconazole was also tested. Increasing masses – 0, 5, 10 and 20 g – of compost were thoroughly mixed with 20 mL SDW containing a total of 100 µg of tebuconazole and incubated for 24 h at room temperature. All samples containing soil were centrifuged at 3400 g for 3 min and filtered using grade 1, 11 µm cellulose filter papers (Whatman, Maidstone, England) to remove the solid content. The

flusilazole internal standard was added at this stage (20 µg per sample). The fungicides were then extracted from samples using the SPE extraction method prior to quantification of the amount of tebuconazole they contained using GC-MS. The SPE and GC-MS method were identical to those detailed in Chapter 3.

4.3.3 Recovery of bound tebuconazole from compost

The mobility of tebuconazole in soil was tested in the laboratory; soil samples, loaded with known quantities of tebuconazole, were washed with solutions containing increasing quantities of MeOH. Soil was pre dried overnight in a laminar flow cabinet and sieved to remove any stones/debris. Soil was weighed into 25 g samples and thoroughly mixed with a 5 mL volume of water containing a total of 25 µg of tebuconazole. Each soil sample was set aside and stored at room temperature for 24 h (allowing the compost to dry) after which, it was mixed with 20 mL of 25, 50 75 or 100% v/v MeOH:water solution or pure SDW. Replicate samples were placed in a sonicleaner 644i sonic bath (Ultrasonics Ltd. Kettering, England) for 15 min, then centrifuged for 3 min at 3400 g and then filtered using grade 1, 11 µm cellulose filter papers (Whatman, Maidstone, England). The flusilazole internal standard was added (20 µg per sample) and the fungicides extracted from samples using the SPE and GC-MS methods detailed earlier.

4.3.4 Tebuconazole uptake from pots containing a treated layer of soil over 8 weeks

The ability of growing plants to adsorb tebuconazole from the soil was investigated. John Innes No. 3 compost was prepared as before by drying and sieving to remove debris. The compost was weighed into several 200 g samples which were mixed with a solution of water containing a total of 160 µg tebuconazole (equivalent to 20 µg per

seed), the soil was then left to dry in a laminar flow cabinet for a minimum of 12 h. Plastic pots (1 L and 5 L) were half filled with measured amounts of John Innes No. 3 compost. Treated soil samples were then layered evenly on top of the untreated soil and all pots were filled to the top with more untreated compost, this method is summarised in Figure 4.1. Each pot was sown with 8 untreated wheat seeds (variety Claire) and grown over an 8 week period in a growth room with a daytime temperature of 18°C, a night temperature of 12°C and a day length of 12 h. The plants were watered once daily, light was provided by sodium bulbs with a radiance measured at 69 µmol m^2 s^{-1}. After the growth period the amount of tebuconazole accumulated by plants was assessed using the SPE and GC-MS analysis methods described earlier.

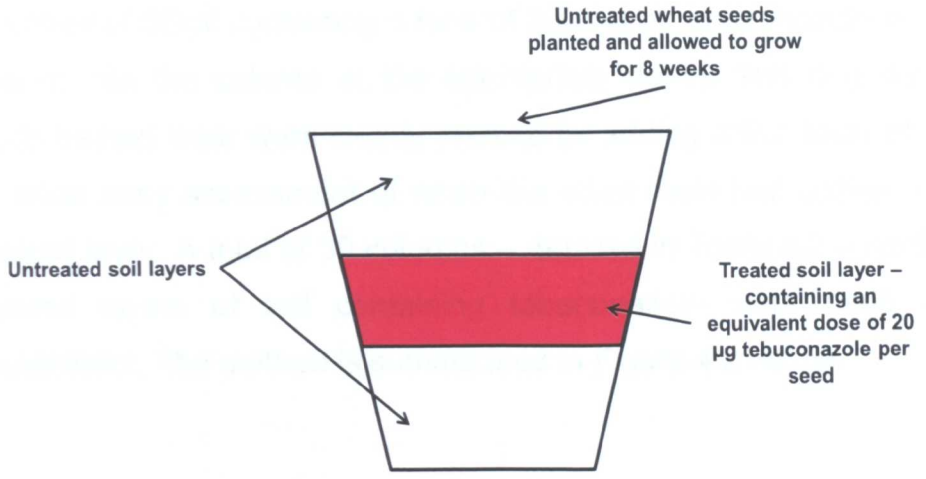

Figure 4.1 Summary of method used in initial investigation of tebuconazole uptake from soil treated with the fungicide

4.3.5 Further analysis of uptake and accumulation of tebuconazole from soil by wheat plants from 1 m columns containing tebuconazole treated layers of compost at varying depths

The ability of wheat plants to adsorb and accumulate tebuconazole from soil was further tested using soil columns containing layers of soil, treated with tebuconazole, at different depths. Each column was prepared from two, commercially available, 1 m longitudinal sections of plastic guttering which were combined to make one column. The sides of each column were sealed using adhesive tape to render them water-tight, one end of each column was also sealed, with perforated PVC plastic, to contain soil but still allow excess water to pass through. Design Expert 7 (Stat-Ease Inc. Minneapolis, USA) was used to plan a D-optimal, cubic point exchange design model encompassing a series of combinations of growth time (from 2 – 8 weeks) and depth of layer (from 1-5, one being the top of the column). The maximum soil holding capacity of each column was measured in litres. This total was split into five fractions allowing the accurate placing of treated layers within each column and to ensure each column contained comparable total soil

volumes. Measured volumes of soil were thoroughly mixed with 50 mL volumes of SDW containing a total of 200 µg of tebuconazole and then placed into the column at the appropriate depth. The boundaries of each treated layer were clearly marked by adding a thin layer of perlite to allow easy assessment of when the plant roots had grown into the treated layer. A total of 30 columns – detailed in Table 4.2 – containing treated layers of soil containing tebuconazole were used in the experiment. The method is summarised in Figure 4.2 below.

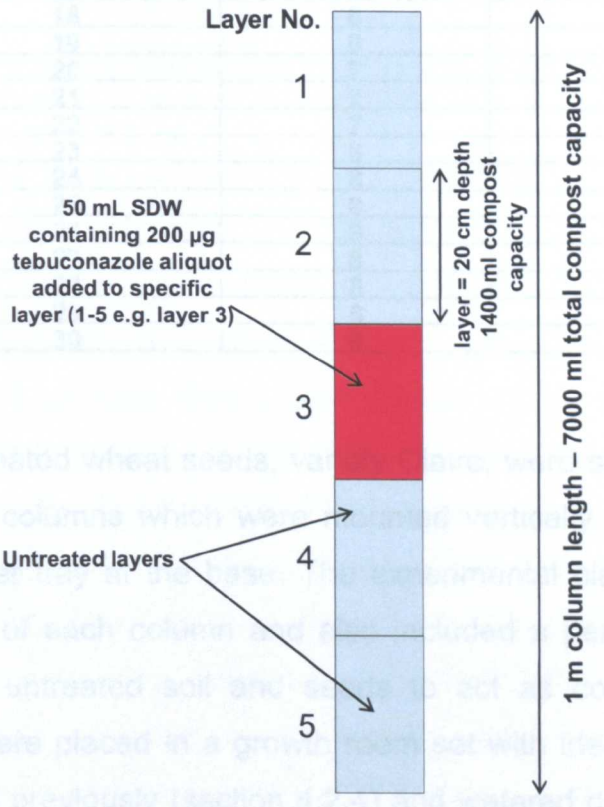

Figure 4.2 Profile of soil column used in analysis of tebuconazole from different depths of soil

Table 4.2 Experimental details of soil column experiment

Column	Growth Time (weeks)	Depth of treated soil layer
1	2	1
2	2	1
3	2	3
4	2	5
5	2	5
6	3	2
7	3	3
8	3	4
9	4	1
10	4	4
11	4	5
12	5	2
13	5	3
14	5	3
15	5	4
16	6	1
17	6	2
18	6	4
19	6	5
20	7	2
21	7	3
22	7	4
23	8	1
24	8	1
25	8	2
26	8	3
27	8	3
28	8	4
29	8	5
30	8	5

Three untreated wheat seeds, variety Claire, were sown in each of the completed columns which were mounted vertically in a wheeled rack with a water tray at the base. The experimental plan randomised the placement of each column and also included a perimeter of columns containing untreated soil and seeds to act as control plants. The columns were placed in a growth room set with identical conditions to those used previously (section 4.2.4) and watered daily from above for the first 7 d of the experiment to promote germination of the wheat seeds. After this time plants were irrigated by provision of 5 cm of water around the base of the columns. Upon germination two of the three seedlings, including the seed coat and roots, were removed to leave one plant per column.

At the point of harvest, each column was split open and the extent of the root growth measured, the weight and length of each plant was also recorded. Plants were then analysed for their total tebuconazole content by SPE extraction and GC-MS analysis as previously. Results obtained were then statistically analysed using Design Expert 7. A series of plants were grown in untreated columns placed in a boundary layer around the experimental columns. These plants were analysed for their development in terms of root length, plant weight and height to assess if there was any phytotoxic effect on plants grown in the tebuconazole treated columns.

4.3.6 Analysis of movement of tebuconazole within soil columns containing layers of compost treated with the fungicide

Soil columns were split open after 8 weeks to measure the depth of penetration of plant roots. The boundaries (upper and lower) of the layer of the fungicide treated layer of compost in each column had been clearly marked with the addition of a thin layer of perlite. Samples of the compost (20 g) were taken 5 cm below the lower boundary of the treated layer (marked by a layer of perlite). Replicate samples (3) were then mixed with 20 mL methanol and placed in a sonicleaner 644i sonic bath (Ultrasonics Ltd. Kettering, England) for 15 min, then centrifuged for 3 min at 3400 g and then filtered using grade 1, 11 µm cellulose filter paper (Whatman, Maidstone, England). The flusilazole internal standard was added at a (20 µg per sample) and the fungicides extracted from samples using the SPE and GC-MS methods detailed earlier.

4.4 RESULTS AND DISCUSSION

4.4.1 Analysis of tebuconazole uptake by plants grown from treated seed between 2 and 19 weeks after sowing

Plants were grown from seeds treated with microencapsulated tebuconazole and Raxil at an application rate of 40 g AI per 100 kg of seed. The seeds were grown over a time course between 2 to 19 weeks. Plant samples were then prepared and analysed to assess the concentration of tebuconazole they contained using SPE and GC-MS. The long term growth trial tested the ability of the microencapsulated formulation to release tebuconazole over a sustained period of time and how this release compared to that of a commercial formulation, Raxil.

The increase in tebuconazole accumulation in plants as they aged can be clearly seen in Figure 4.3, where the average recovery, of the fungicide, from Micap treated plants grown from 2-19 weeks increased from 0.055 to 15.7 µg and 0.056 to 16.8 µg from Raxil treated plants. There was, however, little difference in the total amount of tebuconazole detected in whole plants when comparing the microencapsulated and conventional formulation (Raxil). Indeed, statistical analysis of the results, using a two way ANOVA, showed there to be no significant differences ($P = >0.05$) between the amount of tebuconazole recovered from plants treated with the Micap formulation compared to Raxil. Both treatments exhibited a good ability to provide tebuconazole for plant uptake throughout the growth course analysed. There was also a marked increase in the amount of tebuconazole in plants treated with both formulations of the fungicide after 8 weeks of growth. This profile of tebuconazole uptake was observed previously (Chapter 3 – Figure 3.11). There was also an increase in the amount of tebuconazole accumulated in the plants treated with both the

microencapsulated and conventional formulations, between 17 and 19 weeks.

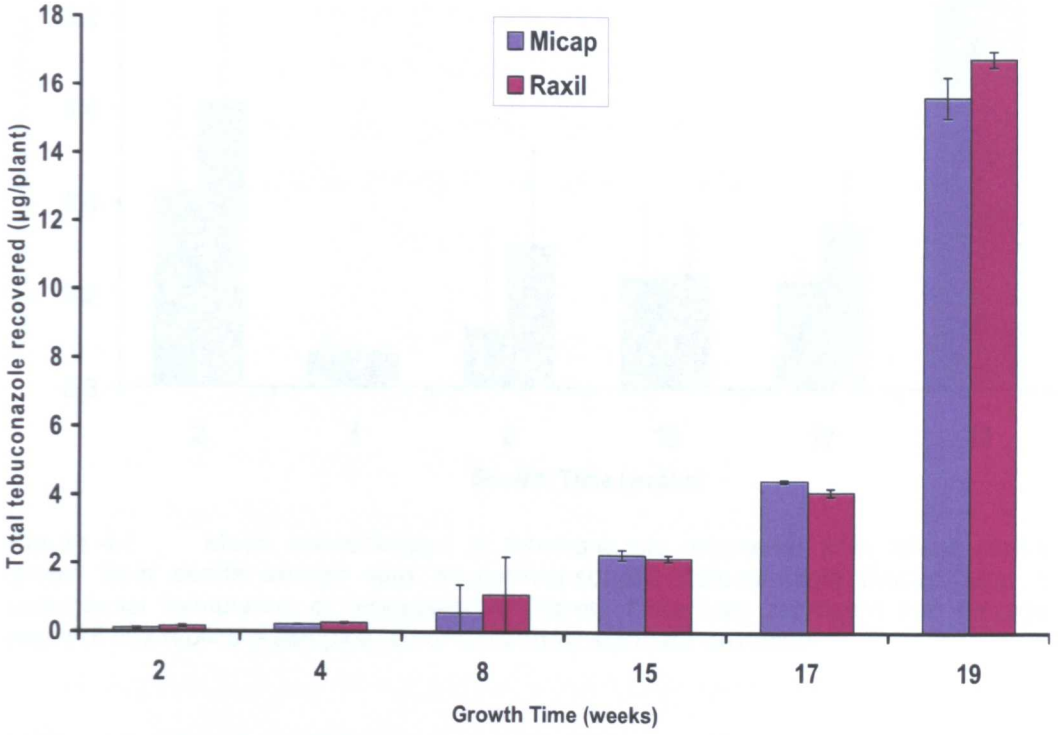

Figure 4.3 Mean total tebuconazole recovered from whole plant samples grown from seeds treated with microencapsulated tebuconazole (Micap) and a commercial formulation of tebuconazole (Raxil) over a time course between 2 and 19 weeks. Data bars are an average of five replicate samples. Error bars show standard deviation

Results given in Figure 4.4, which illustrate changes in the concentration (µg g^{-1} foliar plant material) of tebuconazole accumulated over the time course showed a more constant increase. The highest concentrations of tebuconazole per gram recovered from plants treated with both formulations were observed at two weeks and 19 weeks. Between 8 and 17 weeks the amount of tebuconazole observed per gram of plant tissue did not change as the plants grew larger showing that the fungicide was still available for uptake by the plants.

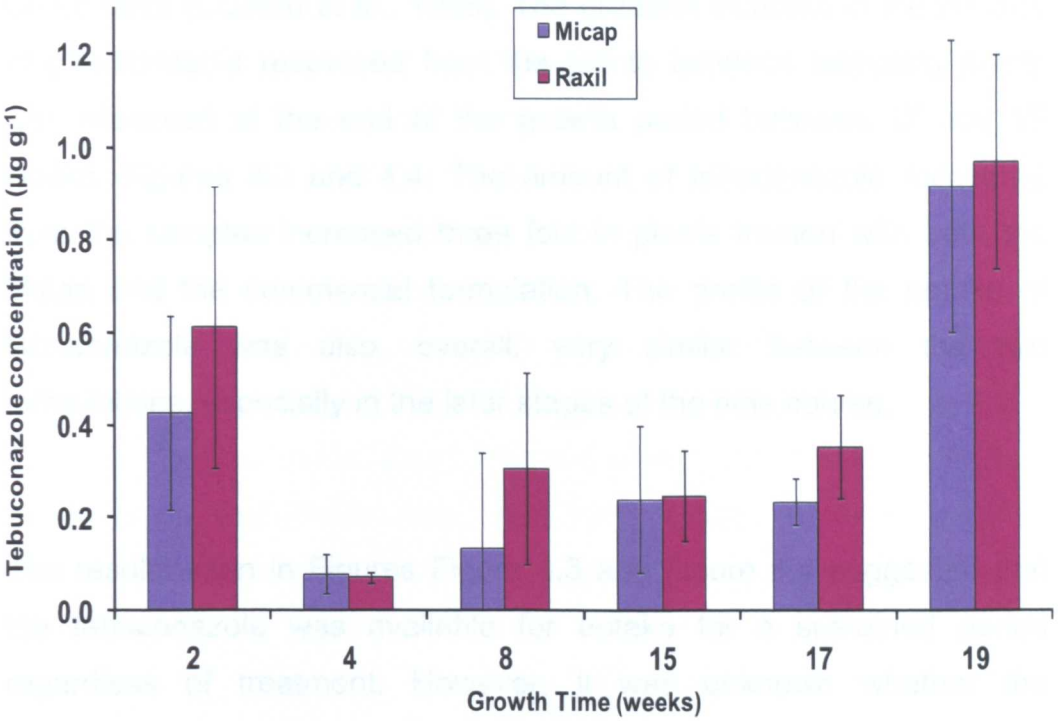

Figure 4.4 Mean concentration of tebuconazole recovered from whole plants grown from seeds treated with microencapsulated tebuconazole (Micap) and a commercial formulation of tebuconazole (Raxil). Data bars represent the average result of five replicate samples. Error bars show standard deviation.

There were no significant (P= >0.05) differences observed between plants treated with the Micap formulation compared to those treated with the commercial formulation, Raxil. However, there was a trend for lower accumulation of tebuconazole concentrations in plants treated with the Micap formulation compared to those treated with Raxil in the earlier stages of the experiment. The levels of tebuconazole recovered from plants in the later stages of the experiment (weeks 15, 17 and 19) were comparable between the two treatments. The higher concentrations of tebuconazole observed in plants after 2 weeks of growth could have been delivered as a result of tebuconazole present in the endosperm of the seed as a result of the fungicide application process. However, it is more likely that this uptake is a result of the uptake active apical section of the early development of the plants roots passing through the zone of tebuconazole that had developed around the root. This was also supported by other research into the uptake of another triazole fungicide, triticonazole, applied as a seed dressing to

wheat seed (Quérou *et al.*, 1998). The greatest increase in the amount of tebuconazole recovered from the plants between sampling points was observed at the end of the growth period between 17 and 19 weeks, Figures 4.3 and 4.4. The amount of tebuconazole recovered from the samples increased three fold in plants treated with both the Micap and the commercial formulation. The profile of the uptake of tebuconazole was also, overall, very similar between the two formulations, especially in the later stages of the time course.

The results seen in Figures Figure 4.3 and Figure 4.4 suggested that the tebuconazole was available for uptake for a sustained period regardless of treatment. However, it was unknown whether the fungicide had been distributed evenly throughout the plants; the analysis of entire plant samples gave no indication of the whether the fungicide had been accumulated in the primary leaves formed. It was hypothesised that the distribution of the fungicide within plants treated with the microencapsulated formulation, which could provide a sustained release, might permit a longer window for foliar uptake and, thus, provides a greater opportunity for accumulation in the plant. To assess this possibility the flag leaves of plants were individually analysed. Results for this analysis, Figure 4.5, showed that they contained a high concentration of tebuconazole, per gram of tissue, relative to plants analysed as a whole. The higher concentrations observed, suggest that there was still tebuconazole available for uptake during the later stages of growth. Although the increased concentrations of tebuconazole could have been the result of the relocation of the fungicide from other areas of the plant, this was considered unlikely. The relatively low solubility of tebuconazole in water allied with its hydrophobic nature suggests that once accumulated in a given leaf the fungicide will remain here (McFarlane, 1995b; P.P.D.B., 2010).

The source of this uptake, either from the treated seed or from fungicide that may have leached into the surrounding environment, remained to be determined. Once again there were no significant (P= >0.05) differences observed between tebuconazole concentrations recovered from flag leaves treated with the Micap or Raxil formulations of the fungicide. These results relate back to those seen in Chapter 3 (Figure 3.10), where a higher accumulation of tebuconazole was observed in the first two leaves to emerge from the seedling compared to those emerging later. It would seem that this trend continues through plant development and showed that, regardless of treatment, tebuconazole continued to accumulate in plant leaves throughout the time course.

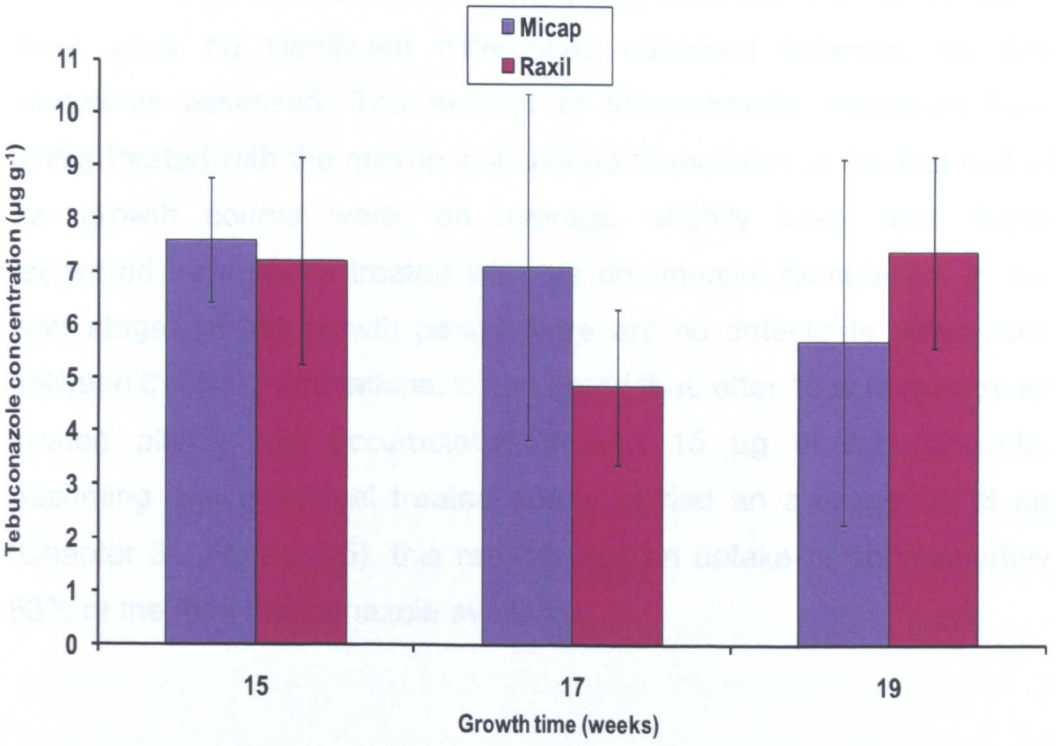

Figure 4.5 Mean concentration of tebuconazole per gram of leaf tissue recovered from the flag leaves of plants grown from seed treated with microencapsulated tebuconazole and a commercial formulation of tebuconazole (Raxil). Data bars represent an average result of five replicate samples. Error bars show standard deviation.

The results seen in Figures 4.3, 4.4 and 4.5, showed a late increase in the amount of tebuconazole accumulated in the plant. The experiment was undertaken a second time using an identical method and set of

conditions to ascertain if this result was authentic. The effect of varying environmental conditions encountered in the glass houses was considered, however, results of experiments carried out earlier in the research (Chapter 3) showed no apparent affect of different conditions on fungicide uptake. Samples were taken at 2 weekly intervals, between 3 and 19 weeks after sowing. Replicate samples were then prepared for GC-MS as before and analysed. The results of the second trial, Figure 4.6, showed an almost identical profile to those seen in the first experiment. There was an initial release of tebuconazole in the early stages of the growth time, followed by a sustained and steady increase in the amount of fungicide in the plant tissue. This gradual increase continues until the 17 weeks after sowing, where once again, there was a notable increase in fungicide accumulation. Once again there were no significant differences observed between the two treatments assessed. The amount of tebuconazole recovered from plants treated with the microencapsulated formulation in the first half of the growth course were, on average, slightly lower than those recovered from plants treated with the commercial formulation. In the later stages of the growth period there are no detectable differences between the two formulations. It was noted that, after 19 w of growth, all treated plants had accumulated around 15 µg of tebuconazole. Assuming that individual treated seeds carried an average of 18 µg (Chapter 3 - Figure 3.5), this represented an uptake of approximately 83% of the total tebuconazole available.

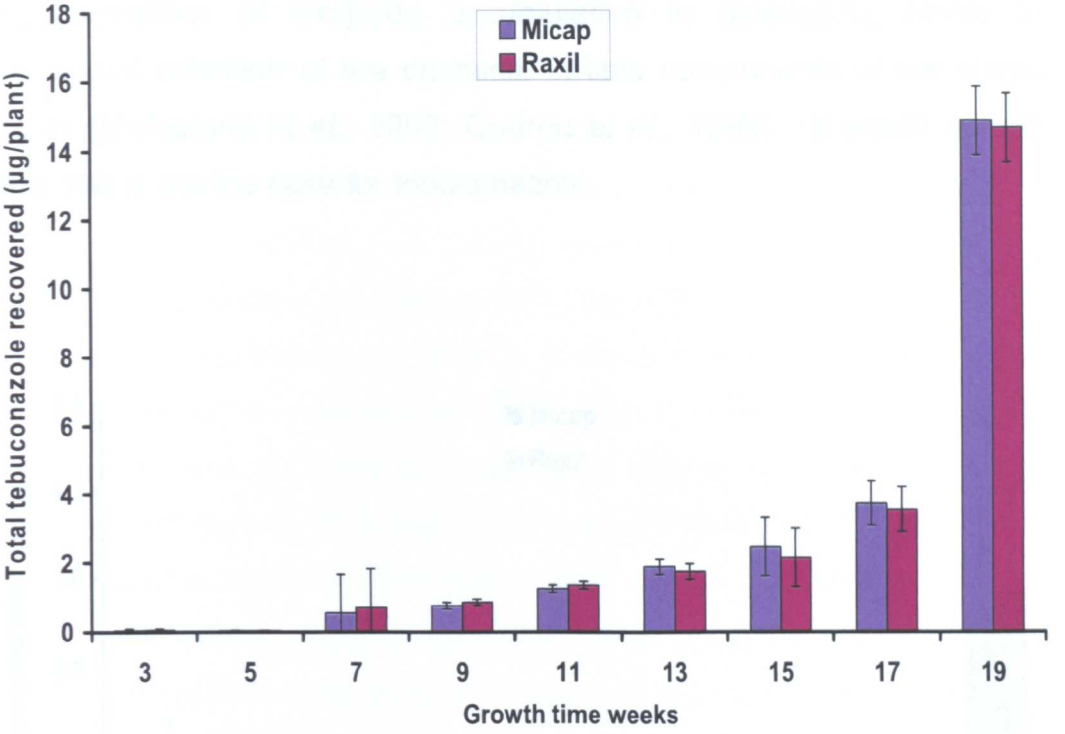

Figure 4.6 Mean total tebuconazole recovered from whole plant samples grown from seeds treated with microencapsulated tebuconazole (Micap) and a commercial formulation of tebuconazole (Raxil) over a time course between 2 and 19 weeks. Data bars are an average of five replicate samples. Error bars show standard deviation.

The individual plant weights were recorded to determine the concentration of tebuconazole recovered, per gram of plant tissue. The results obtained, Figure 4.7, again showed a similar profile to the preliminary experiment. The amount of tebuconazole per gram of tissue was initially higher at the beginning of the growth course. This decreased as the plants aged; suggesting that the rate of uptake of tebuconazole is relatively constant and is not necessarily directly linked to plant development. The total amount of tebuconazole per gram of plant then continues to rise in a steady fashion until week 17 where there was once again a large increase between weeks 17 and 19. A notable increase in tebuconazole amounts was also observed at the 5 week point. This effect was observed in the initial experiment and also in previous research carried out (Chapter 3 – Figure 3.10). Although there were no differences observed between the two formulations, this represented a good degree of overall uptake when increasing plant biomass is considered. Previous research attributed lower

concentrations of fungicide accumulation in developing plants to increased retention of the chemical in lipid components of the plants roots (McFarlane *et al.*, 1990; Quérou *et al.*, 1998). It would appear that this is not the case for tebuconazole.

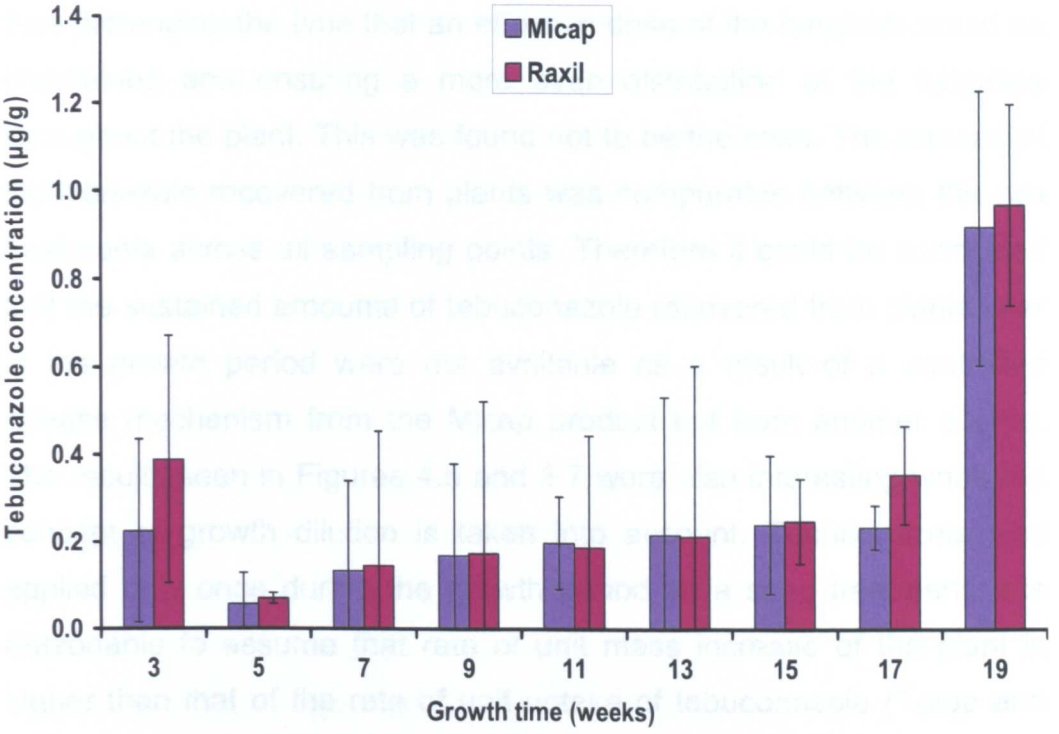

Figure 4.7 Mean concentration of tebuconazole per gram of plant tissue recovered from plants treated with microencapsulated tebuconazole (Micap) compared to those treated with a commercial formulation (Raxil). Data bars show the average value of five replicate samples. Error bars show standard deviation.

The reasons behind relatively high concentrations of tebuconazole recovered from plants later in the growth period and also in the younger leaves of the plant were unclear as it was suggested that the amount of tebuconazole available for uptake by plants would become reduced during the growth period due to several sinks. Examples of these could include degradation in the environment, for example by microflora in the soil (Kahle *et al.*, 2008), uptake by the plant and subsequent loses by volatilisation to the air (Mc Farlane *et al.*, 1990) and other factors such

as losses to water washed through the soil during watering. Other factors such as plant metabolism, oxidation, conjugation with other compounds in the plant and sequestration into areas such as cell walls may also affect the concentration of compounds within plants (Burken and Schnoor, 1998). It was also hypothesised that the microencapsulated formulation of tebuconazole could have the benefit of releasing the tebuconazole in a controlled and sustained manner thus prolonging the time that an effective dose of the fungicide could be maintained and ensuring a more even distribution of the fungicide throughout the plant. This was found not to be the case. The amount of tebuconazole recovered from plants was comparable between the two treatments across all sampling points. Therefore it could be concluded that the sustained amounts of tebuconazole recovered from plants later in the growth period were not available as a result of a controlled release mechanism from the Micap product but from another source. The results seen in Figures 4.6 and 4.7 were also interesting when the concept of growth dilution is taken into account. Tebuconazole was applied only once during the growth period as a seed treatment, it is reasonable to assume that rate of unit mass increase of the plant is higher than that of the rate of unit uptake of tebuconazole (Trapp and Matthies, 1995). Therefore the reasons behind the late uptake of tebuconazole needed to be further investigated.

4.4.2 Absorption and retention of tebuconazole by loam based compost

One hypothesis explaining the late availability of tebuconazole is that it is available from the soil. As the plant continues to develop the roots begin to search out new nutrients in the soil, in the later stages of a longer growth trial it is likely that the plants roots will occupy a high percentage of the available soil when grown in a pot. The known chemical properties of tebuconazole, displayed in Table 4.1.1, show that the fungicide has a high K_{oc} value. This means that the fungicide

has a high tendency to bind with organic carbon elements in the soil environment (P.P.D.B., 2010). Tebuconazole is also classed as being moderately persistent in a soil environment and as being relatively immobile in water due to its low water solubility (P.P.D.B., 2010). Therefore it was suggested that that the tebuconazole was held in the soil in a localised area around the seed on which the fungicide was applied. This localised 'pocket' of tebuconazole could then be available for uptake by the plant later in the growth stages of the plant when the roots occupy fully the soil in the area where the tebuconazole is available. It is also possible that this would not occur if the plants were being grown in a field setting and not the contained environment of a pot especially with more than one plant growing in each pot.

Analysis of the results of the long term plant growth trial showed that tebuconazole was accumulated within the plant over the course of time and also that the fungicide was still available for uptake in the later stages of plant growth. There were, however, no clear differences in the amount of tebuconazole recovered from plants treated with microencapsulated and commercial formulations. It is known that triazole fungicides such as tebuconazole are taken into the plant via a passive uptake process through the roots (Collins *et al.*, 2006). This allied with the fact that tebuconazole has a high tendency to bind with organic carbon elements in the soil and a relatively low level of solubility in water led to the planning of an experiment to test the soil as a potential sink for seed applied tebuconazole which may then provide a subsequent source of tebuconazole for growing plants. The ability of the loam based compost used during plant growth experiments in this project, John Innes No. 3, to adsorb and retain tebuconazole was therefore tested. Two experiments were undertaken; firstly an aqueous solution containing a known amount of tebuconazole was passed quickly through a pre dried sample of compost, the same amount of tebuconazole was also mixed with samples of the compost and

incubated for 24 and 48 h. The second experiment assessed the ability of the compost to retain tebuconazole it had absorbed. Replicate samples were then assessed for the amount of tebuconazole they contained using SPE and GC-MS. For the purposes of discussion, John Innes No. 3 compost will be referred to as 'soil'.

Results of the initial experiment, given in Figure 4.8, show that the amount of tebuconazole in samples that had come into contact with soil was significantly ($P = <0.05$) less than those that had not been in contact with soil. Although a small amount of tebuconazole was still present in the samples that had been pushed through a syringe containing soil, labelled '(fast run through in the figure)', over 95% of the total available tebuconazole was removed by the soil. Samples that were mixed with soil and left for a period of 24 or 48 h contained no detectable tebuconazole.

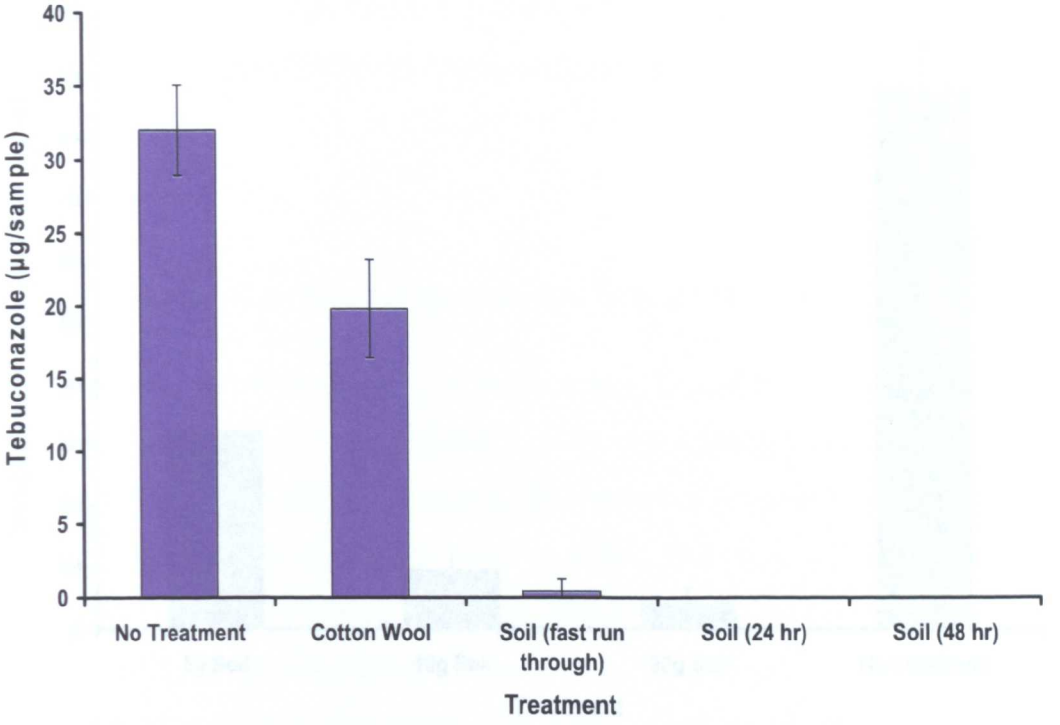

Figure 4.8 Mean concentration of tebuconazole recovered from solutions containing the fungicide (35 µg total) mixed with samples of loam based compost over a time course. Data bars show an average value based on five replicate samples. Error bars show standard deviation.

The second experiment examined the potential of increasing masses of soil to retain tebuconazole when mixed with a more concentrated solution of the fungicide. The results, given in Figure 4.9, showed that only 5 g of soil adsorbed well over 60% of the tebuconazole contained within the solution, 10 g of soil bound on average 90% of the tebuconazole from the solution and 20 g of soil almost 95%.

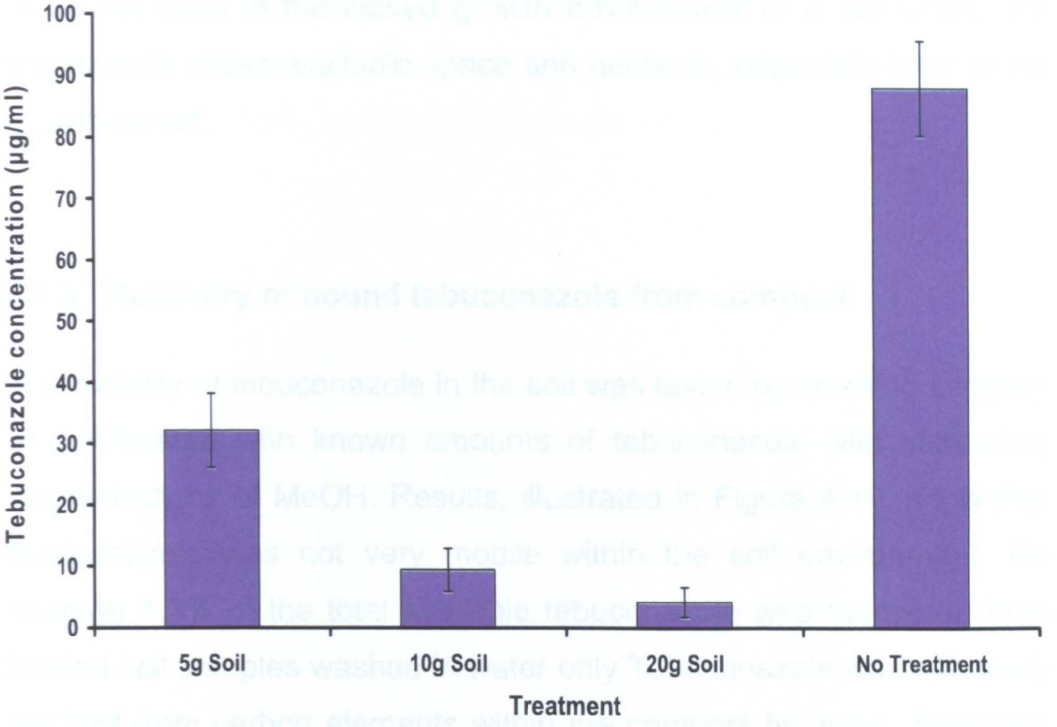

Figure 4.9 Mean concentration of tebuconazole (100 µg) recovered from increasing masses of compost mixed with a concentrated aqueous solution of the fungicide. Data bars represent an average of 5 replicate samples. Error bars show standard deviation.

The results of the two experiments seen in Figures 4.8 and 4.9 clearly showed that the compost used during the research had the ability to remove tebuconazole from an aqueous solution containing the fungicide. The results of the preliminary aqueous release profiling, carried out on the microencapsulated formulation of tebuconazole (Chapter 2), concluded that on contact with water the fungicide was freely released from the yeast microcapsules. Therefore it is possible that when seeds are watered, the release of tebuconazole from yeast microcapsules commences and, once free within the soil environment, the fungicide becomes bound to carbon elements within the soil. A similar process is likely to occur with commercial formulations of tebuconazole when used as a seed dressing. As the uptake of tebuconazole from the soil via the roots is known to be a passive process (Collins *et al.*, 2006), roots would have to be in close proximity to the tebuconazole for uptake to occur. This is, however, highly likely

to be the case in the closed growth environment of a pot where the plants roots utilise available space and nutrients, especially later in the growth period.

4.4.3 Recovery of bound tebuconazole from compost

The mobility of tebuconazole in the soil was tested by washing samples of soil treated with known amounts of tebuconazole with increasing concentrations of MeOH. Results, illustrated in Figure 4.10, show that tebuconazole was not very mobile within the soil environment. On average 1.3% of the total available tebuconazole was recovered from treated soil samples washed in water only Tebuconazole was not easily leached from carbon elements within the compost by water. Recovery was, however, achieved with increasing concentrations of MeOH. Pure MeOH recovered approximately 90% of total tebuconazole. As tebuconazole has a high K_{oc} value (P.P.D.B., 2010) it has a greater tendency to bind to organic carbon elements in the soil. This, allied to a relatively low water solubility limit of 36µg mL^{-1} (P.P.D.B., 2010) means that tebuconazole is unlikely to be mobile in the soil. There is some potential for the movement of tebuconazole as a bound residue on soil components; however for the purposes of this research, which has been carried out entirely in the relatively contained environment of pots and not in a field setting this was thought unlikely to affect results. Previous work, researching the environmental fate of the triazole fungicide propiconzole, which has similar chemical properties to tebuconazole (P.P.D.B., 2010), in different soil conditions produced a similar findings (Kim *et al.*, 2002). The experiment used radio-labelled $^{14}CO_2$ and [U-^{14}C]-propiconazole applied to the soil surface of two soil lysimeters. The fate of the fungicide in lysimeters was then assessed by measuring total radioactivity leachate collected and evolved $^{14}CO_2$ and ^{14}C residues in the soil and rice plant grown in the soil. The findings of the research showed that, despite propiconazole having a solubility limit

in water of 100 µg mL^{-1} (P.P.D.B., 2010) i.e. three times higher than that of tebuconazole, there were no detectable levels of the fungicide in leachate collected from the lysimeters (Kim *et al.*, 2002).

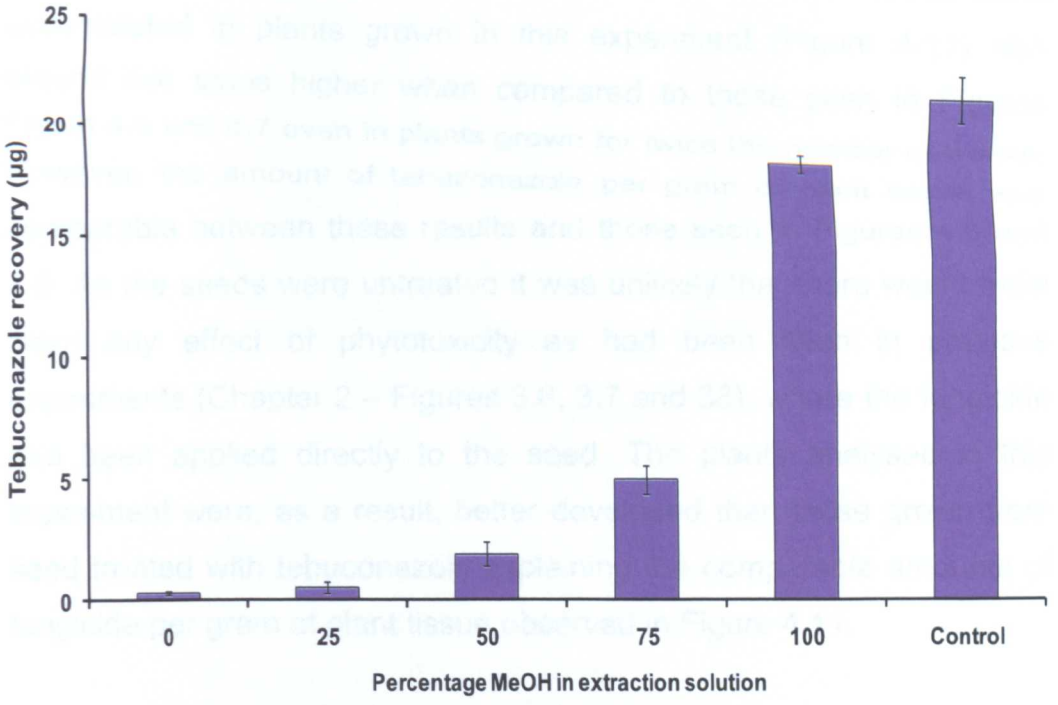

Figure 4.10 Mean recovery of tebuconazole from samples of soil treated with known amounts of tebuconazole and washed with aqueous solutions containing increasing concentrations of MeOH. Data bars show the average value taken from three replicate samples. Error bars show standard deviation.

4.4.4 Tebuconazole uptake from pots containing a treated layer of soil over 8 weeks

The method for this experiment involved thoroughly mixing untreated compost with a solution containing tebuconazole at an equivalent rate of 20 µg/seed. The treated compost was then placed in pots between two layers of untreated compost. Untreated wheat seed were then grown for 8 weeks harvested and the level of tebuconazole they had accumulated assessed. The results, Figure 4.11, showed that there were easily detectable levels of tebuconazole present in the plants. On average plants had accumulated around 50% of the total tebuconazole available after only 8 weeks of growth. As the seeds planted were

untreated and were also sown in untreated compost this uptake can only have occurred via the roots from the soil. Uptake of fungicides from the soil is a passive diffusive process (McFarlane, 1995b), the roots of a plant must, therefore, be in close proximity to tebuconazole molecules bound in the soil for uptake to occur. The amount of tebuconazole accumulated in plants grown in this experiment (Figure 4.11) was around five times higher when compared to those seen in Figures Figure 4.4 and 4.7 even in plants grown for twice the number of weeks. However, the amount of tebuconazole per gram of plant tissue was comparable between these results and those seen in Figures 4.5 and 4.6. As the seeds were untreated it was unlikely that there would have been any effect of phytotoxicity as had been seen in previous experiments (Chapter 2 – Figures 3.6, 3.7 and 38), where the fungicide had been applied directly to the seed. The plants analysed in this experiment were, as a result, better developed than those grown from seed treated with tebuconazole explaining the comparable amounts of fungicide per gram of plant tissue observed in Figure 4.11.

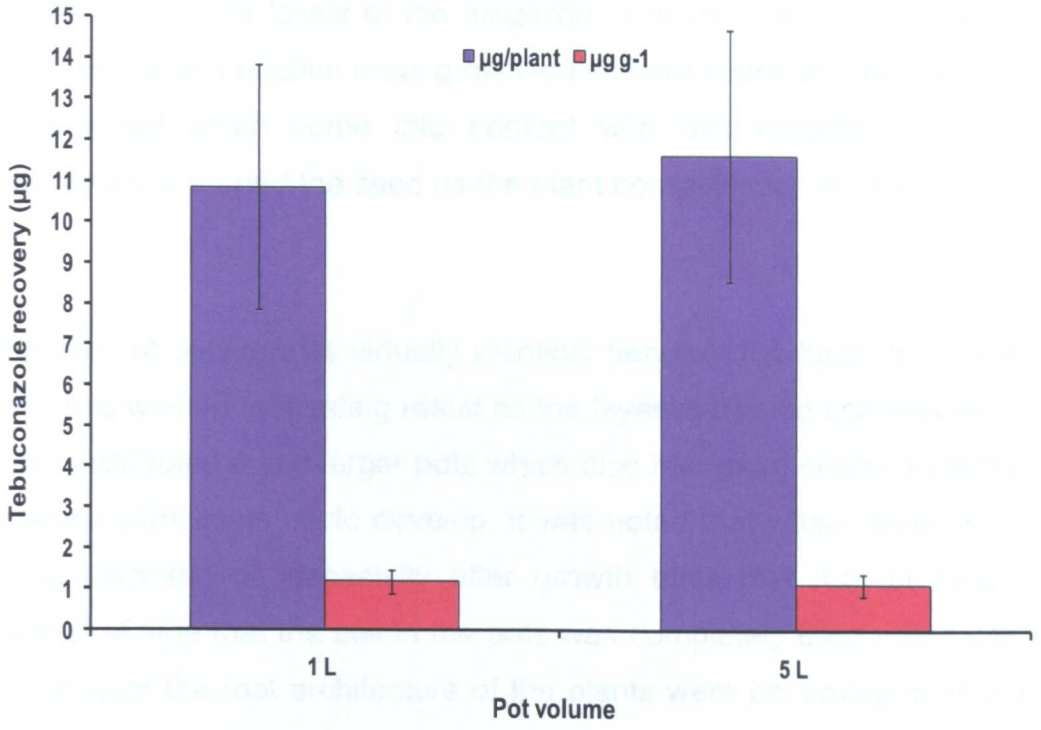

Figure 4.11 Mean amounts of tebuconazole in plants grown from untreated seed in pots (1 or 5L) containing a layer of compost treated with an equivalent dose rate of 20 µg fungicide per seed. Data bars show an average value of 5 replicate samples. Error bars show standard deviation.

It is reasonable to assume that the tebuconazole was more evenly distributed within the treated soil layer in each pot than the distribution of the fungicide applied as a seed treatment. It would appear that this distribution of the fungicide led to the higher uptake rates observed. These results may also help to explain the late increased uptake of tebuconazole into plants grown from treated seed (Figures 4.4 and 4.7). The distribution of tebuconazole available for uptake from treated seeds is likely to be localised to the region immediately adjacent to the seed. The high tendency of tebuconazole to bind to carbon elements within the soil supports this. As a plants root structure develops it is likely that the uptake active regions at the tips of the roots are likely to pass through this zone relatively quickly. A more even distribution of the fungicide in the soil would mean that there is a higher likelihood that developing roots, especially those that are confined by the finite space in a pot, would come into contact with tebuconazole and subsequently

accumulate higher levels of the fungicide. It would not be until later in the development of plant roots grown in the finite space of a pot that the roots would again come into contact with this localised zone of tebuconazole around the seed as the plant competes for resources.

The level of uptake was virtually identical between the large and small pots; this was an interesting result as the layer of treated soil was more thinly distributed in the larger pots which also had more space available in which plant roots could develop. It was noted that when plants were being disposed of, especially after growth trials that run for longer periods of time that the soil in the pots was completely filled with roots. It was clear the root architecture of the plants were becoming confined within the pots. This suggests that the rate of uptake of tebuconazole from the soil is driven by plant, or more specifically root, development and not by the amount of tebuconazole available for uptake. This was not, however, consistent with the theory that uptake is driven by root development alone. If this was the case the results would have shown a higher accumulation of tebuconazole in plants grown in the smaller size of pot as the roots would have filled this space more completely and at a faster rate than those grown in the larger pots. It was clear that another factor was acting upon the rate of fungicide uptake,

Other research carried out investigating the uptake of non-ionised chemicals from a hydroponic solution into plant roots have shown that the process consists of two components: a) equilibration, whereby the aqueous phase of the plant root and the surrounding solution become balanced and b) sorption of the chemical onto lipophillic root solids found in the cell membranes of and cell walls of cells in the roots (Paterson *et al.*, 1991). The rate at which lipids in cell membranes in the plants roots become saturated will therefore have an impact on the subsequent rate at which the fungicide can enter into other areas of the

plant. It would seem that the rate of tebuconazole uptake is a combination of distribution of the fungicide within the soil environment but also linked to the behaviour of the compound within the plant cells and water phase of the soil.

4.4.5 Further analysis of uptake and accumulation of tebuconazole from soil by wheat plants from 1 m columns containing tebuconazole treated layers of compost at varying depths

The effect observed in Figure 4.11 whereby tebuconazole was taken up by plants directly from a layer of compost treated with a known amount of tebuconazole was further tested. An experiment was undertaken whereby tebuconazole accumulation was assessed, over a time course, in plants grown in individual soil columns containing a layer of compost treated with 200 µg aliquot of the fungicide at varying depths in the column. The experiment also aimed to assess the amount of tebuconazole taken up by plants when grown in a deeper (1 m), more open soil environment compared to the finite environment of a pot. Plants were also measured for their development in terms of length, weight and root length to test for any phytotoxic effects caused by uptake of the fungicide.

The amount of tebuconazole found in plant samples, Figure 4.12, were analysed and plotted on a response surface (contour) graph using the experimental design program. The figure shows the amount of tebuconazole recovered by the plants over a time course and the relationship of this to the depth of the treated layer of soil within the column (Layer 1 being the top layer of soil in the column). The contour lines show the link between growth time and layer depth and how this association affects the amount of tebuconazole at a given combination of these variables. Experimental design points are marked as red circles on the figure and a colour key is shown, depicting red as a

higher tebuconazole concentration changing to blue which is the lowest concentration.

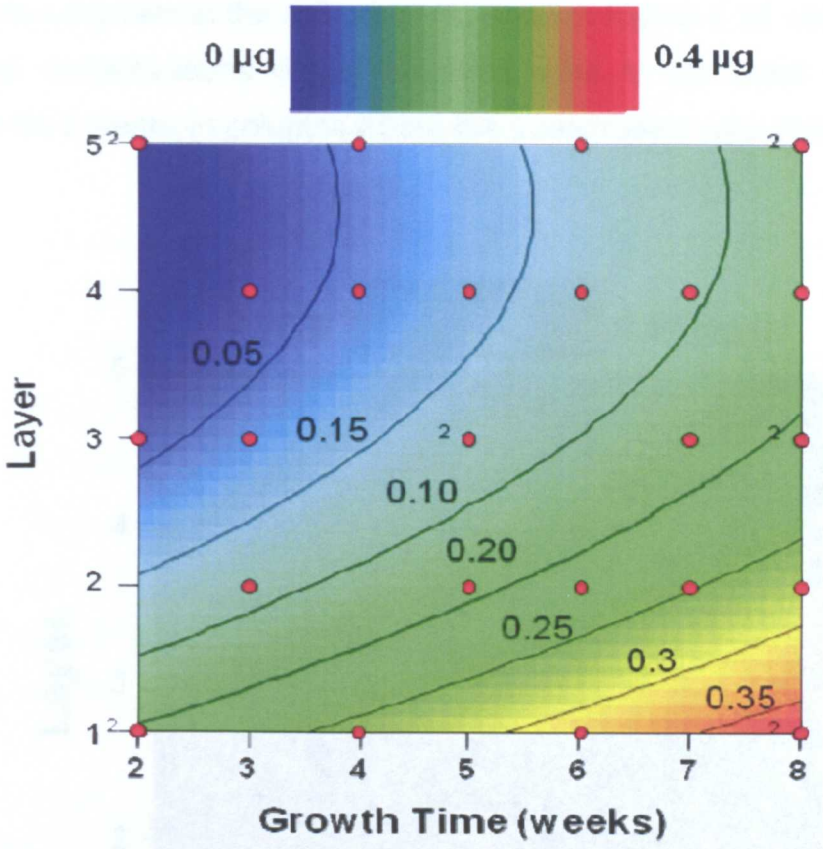

Figure 4.12 Amount of tebuconazole per sample recovered from plants grown in individual soil columns containing layers of soil treated with a total of 200 µg tebuconazole.

Analysis of the results showed that there was a statistically significant (P= <0.05) effect on the amount of tebuconazole in plants caused by the relationship between the depth of the treated soil layer and growth time. The highest accumulation of tebuconazole was found in plants grown for 8 weeks in columns where the treated soil was at level 1, or the top of the column. The amount of tebuconazole recovered from plants grown in other columns followed this trend with the lowest amounts recovered from samples grown in columns with the treated soil at level 5. Some tebuconazole was recovered from plants grown in columns containing the treated soil at layer 5. However, this was only

observed in plants that had been grown for the full 8 week period. The roots of plants grown for a shorter time had not reached this depth in the column and therefore showed no uptake of tebuconazole. Results of the analysis of the concentration of tebuconazole recovered from plant tissue grown in the soil columns, given in Figure 4.13, showed the highest concentrations of the fungicide were to be found in plants grown for 2 weeks in columns where the treated layer was at level one.

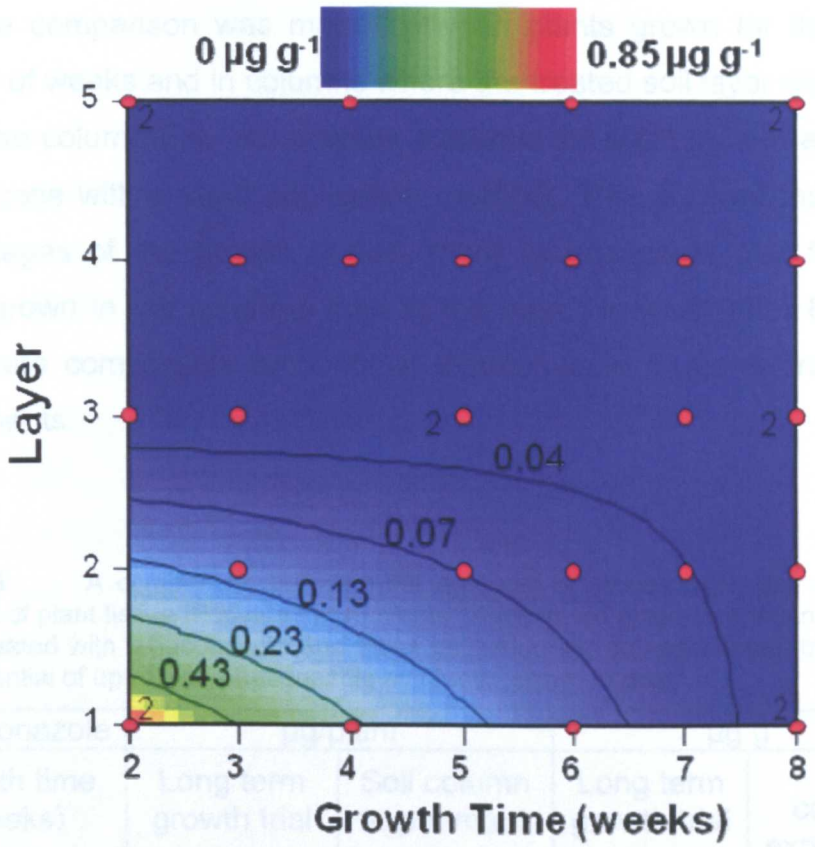

Figure 4.13 Concentration of tebuconazole recovered from plants grown in individual soil columns containing layers of soil treated with a total of 200 µg tebuconazole.

This did not follow the trend seen in Figure 4.12, where the amount of tebuconazole was seen to increase in plants over the time course. This suggests that the rate of tebuconazole uptake was lower in the soil columns than seen in previous experiments. A total of 200 µg of tebuconazole was applied to each treated layer. As only one plant was

grown in each column this meant that there was 10 times more total tebuconazole available for plant uptake than in previous experiments where a maximum of 20 µg per plant was applied. Each column contained five, 1.4 L volumes of compost, one of which was treated with the fungicide. This was comparable to the amount of soil available for each plant grown in the long-term uptake trial, which was approximately 1.2 L. A comparison was made between the amount of tebuconazole accumulation in plants grown as part of the long term uptake trial (Figures 4.3, 4.4, 4.5, 4.6 and 4.7) and this data can be seen in Table 4.3. The comparison was made between plants grown for the same number of weeks and in columns where the treated soil layer was at the top of the column (i.e. immediately available for plant uptake as would be the case with a seed application method). This showed that in the early stages of the growth period, more tebuconazole was found in plants grown in the columns than in the pots. However after 8 weeks there were comparable amounts of tebuconazole in plants from both experiments.

Table 4.3 A comparison between the amounts of tebuconazole per plant and per gram of plant tissue recovered from plants grown in soil columns containing layers of soil treated with tebuconazole and those grown during an assessment of the long term potential of uptake of tebuconazole by plants (grown in pots)

Tebuconazole	µg/plant		µg g^{-1}	
Growth time (weeks)	Long term growth trial	Soil column experiment	Long term growth trial	Soil column experiment
2	0.105	0.17	0.625	0.78
4	0.21	0.38	0.078	0.30
8	0.551	0.41	0.133	0.04

It would seem that the more even distribution of tebuconazole and the higher rates applied in the soil column experiment led to higher uptake of the fungicide in the early stages of growth. The roots would have had a higher likelihood of coming into contact with tebuconazole residues in the soil columns earlier in the growth period explaining this difference. A

previous experiment investigating the effect of increased seed sowing density on tebuconazole uptake found there to be no apparent increase in fungicide accumulation in the plants. However, the zone of tebuconazole available for uptake in this experiment would have been localised around individual seeds therefore would not have been available for uptake until later in the growth period. Initially the amounts of tebuconazole per gram of plant tissue are comparable between the two experiments; however, after 8 weeks of growth, the amount of tebuconazole per gram of tissue is more than 75% less in plants grown in the columns.

The reason for this may be explained by the different manner in which the roots have developed in the plants grown in the soil columns compared to those grown in pots. The watering regime used during the soil column experiment, whereby the columns were watered from below 7 d after emergence, encouraged the roots to develop along the length of the column. Indeed it was only after 8 weeks that the roots were able to grow the full length of the 1 m columns used. Uptake of tebuconazole would have occurred as the developing roots passed through the layer of treated soil, however as the roots continued to develop the rate of uptake would slow as the tebuconazole available immediately around the roots of the plants becomes exhausted.

The roots of the plants grown in the columns had more space available for growth and, as such, did not fill the entire volume of soil in the column. In comparison, the plants grown in pots had a finite amount of space and nutrients as well as competing alongside other plants grown in the pots for these resources. As a result there was a higher density of root distribution in plant grown in pots compared to those grown in the columns. This further explains the late uptake of tebuconazole seen in the long term uptake trial. Although the plants grown in the soil column

could have been grown for a longer time course it is unlikely that any further uptake of the fungicide would have occurred as the roots would not have filled the entire soil volume within the column.

Overall, it appears that the uptake of tebuconazole is dependent on the development of the root system of the plant. The greatest uptake of tebuconazole occurred in samples that had been in contact with the treated soil layers for the longest period of time. These results are also supported by previous results shown in Chapter 3. The effect of growth environment on tebuconazole uptake was investigated by growing plants in growth rooms at different temperatures (Figure 3.13) and by artificially increasing the rate of transpiration in plants (Figure 3.14). The results showed that neither of these factors significantly increased the amount of tebuconazole accumulated by plants. It would seem that even though it is known that tebuconazole is carried within the transpiration stream that the distribution of the fungicide in the soil allied with root development and the behaviour of the fungicide once in the plant are primarily responsible for uptake.

4.4.6 Analysis of potential phytotoxic effect of tebuconazole in treated soil on plant development

The potential effect of phytotoxicity or the hindrance of plant development, caused by tebuconazole applied to the soil was assessed. Untreated seeds grown in columns containing no tebuconazole were measured for their length and compared to those grown in experimental soil columns over a time course. The results, given in Figure 4.14, showed that there were no differences between plants grown in untreated or treated soil columns. This suggested that the phytotoxic effect observed earlier in the research (detailed in Chapter 3) was not caused by the process of tebuconazole uptake itself

but by the presence of high concentrations of the fungicide when applied as a seed treatment in direct contact with the seed.

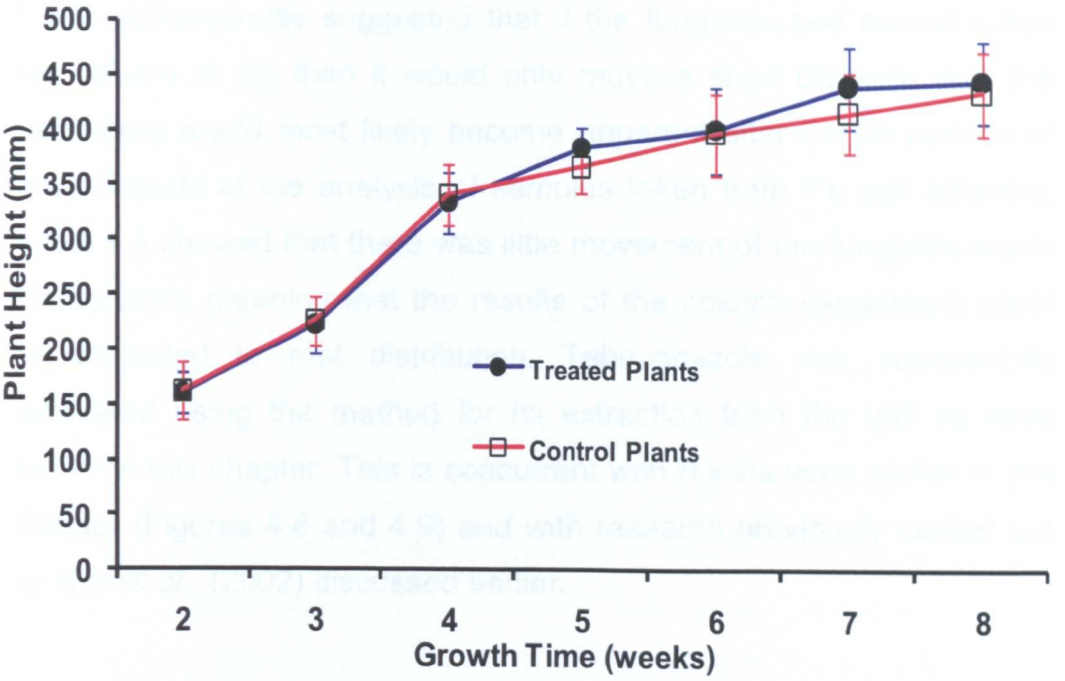

Figure 4.14 Mean height of plants grown in columns containing a layer of compost treated with 200 µg tebuconazole compared to plants grown in untreated columns. Error bars show standard deviation. Sample details showed in Table 4.2.

Results of the analysis of plant weights grown in the soil column, data not shown, also showed there to be no measurable effect of high concentrations of tebuconazole in the soil on plant weight.

4.4.7 Analysis of movement of tebuconazole within soil columns containing layers of John Innes No. 3 compost treated with the fungicide

The lower uptake of tebuconazole exhibited in the results of the soil column experiments (Figures 4.12 and 4.13) could have been explained by movement of the fungicide within the column and out of the treated layer. The low water solubility and high affinity of tebuconazole to bind with organic carbon in a soil environment, allied with results of previous experiment testing the ability of the compost

used during the research to retain tebuconazole (Figure 4.8 and 4.9) meant that it was thought unlikely that tebuconazole would be mobile within the soil columns. However, an experiment testing samples of soil from the tebuconazole treated soil columns was undertaken to test this. It was subsequently suggested that if the fungicide had moved within the column at all, then it would only move a short distance and this movement would most likely become apparent after longer periods of time. Results of the analysis of samples taken from the soil columns, Table 4.4 showed that there was little movement of the fungicide within the columns meaning that the results of the column experiment could be attributed to root distribution. Tebuconazole was successfully recovered using the method for its extraction from the soil as seen earlier in this chapter. This is concurrent with results seen earlier in this chapter (Figures 4.8 and 4.9) and with research previously carried out by Kim *et al.*, (2002) discussed earlier.

Table 4.4 Mean amount of tebuconazole recovered from within and below layers of compost treated with the fungicide in individual soil columns. Data shown is an average of three replicate samples. Variation shown is standard deviation of the averages.

Tebuconazole Sample	AI sample^{-1} (µg)	AI (µg g^{-1})
Treated Layer	**1.06 (±0.32)**	**0.05 (±0.016)**
Below layer	**0.05 (±0.019)**	**0.0 ±0.000)**

4.5 CONCLUSIONS

Tebuconazole was seen to be available for uptake from both microencapsulated and commercially available formulations of the fungicide over an extended period of time. However, it would appear that there was not a prolonged release of the fungicide from the microencapsulated formulation as had been hoped. Although there were no significant difference between the two formulations this was never the less an encouraging result, showing that the microencapsulated product did have the ability to deliver tebuconazole at concentrations that were comparable to a commercial product. An increase in tebuconazole amounts found in plants during the later stages of the growth course was investigated by undertaking a second, identical, experiment which confirmed the results of the initial trial. The reasons for this were initially unclear as it was hypothesised that the amount of tebuconazole available for uptake would have become reduced over the course of time. As a result, a subsequent reduction in the amount of fungicide per gram of plant tissue would have been expected as uptake slowed and the plant continued to increase in mass as it developed. The steady accumulation (and late increase in uptake rate) of the fungicide of the time course observed in these experiments appears to have been caused as a result of growing the plants in the finite environment of a pot.

Analysis of the ability of the growth medium in which the plants were grown during this research to absorb and retain tebuconazole showed that the compost had a strong ability to bind tebuconazole. This was consistent with the known chemical properties of the fungicide and lead to the suggestion that the soil itself might prove, initially, to be a sink for seed applied fungicides and, later in the growth period, provide a subsequent source for plant uptake of tebuconazole. The ability of plants to absorb tebuconazole directly from the compost was assessed and the findings showed that this was possible. Further experimentation

involving the use of individual soil columns containing layers of fungicide treated compost, applied at a rate 10 times higher than that applied as a seed dressing showed the uptake of tebuconazole to be lower than that seen in plants grown in pots. It was, therefore concluded that the total amount of tebuconazole uptake and accumulation was not directly related to the amount applied. As it is known that the uptake of tebuconazole from the soil is a passive process the level of uptake is directly related to the number of roots that come into contact with the fungicide in the soil. This, in turn, is dependent on the manner and degree to which the roots have developed but also the behaviour of tebuconazole within lipid concentrations in the plants cells.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

Previous work led by Micap PLC had illustrated the potential for the use of yeast cells to provide the sustained release of materials such as flavour volatiles, essential oils and pharmaceutical products (Nelson *et al.*, 2006). The primary aim of this research project was to assess the release and uptake of the triazole tebuconazole from a novel encapsulated formulation of the fungicide. The formulation was produced using a method patented and developed by Micap PLC and utilised yeast cells as preformed microcapsules into which the fungicide was loaded. There were perceived to be several potential benefits of formulating tebuconazole in this manner such as protection of the AI from environmental degradation, controlled release of the AI and a potential for the reduction of phytotoxic effects caused by the use of seed treatments such as tebuconazole (Bahadir, 1990; Asrar *et al.*, 2004; Markus and Linder, 2006).

Tebuconazole is used extensively as a fungicidal seed dressing in cereals using formulations such as Raxil (Clark *et al.*, 2004). However the rate at which these treatments may be applied is limited. If applied excessively tebuconazole is known to prevent or delay emergence and cause stunting of young plants (McGee, 1995; Ashley *et al.*, 2003). Further to this, losses of the fungicide due to effects of environmental degradation in the soil and concerns over pesticides residues in soil and groundwater determines that tebuconazole based products, such as Raxil, cannot be applied at sufficiently high doses to provide adequate protection of plants beyond the stages of germination and early development (Kenawy *et al.*, 1992; Asrar *et al.*, 2004). To avoid these effects tebuconazole is typically applied as a 'seed hygiene' treatment at a rate of 3 g AI per 100 kg seed (BayerCropScience,

2010). It was proposed that by producing a microencapsulated tebuconazole formulation, in a similar manner to that used by Micap PLC for other compounds, these undesirable effects could be reduced. This, in theory, could allow the application of tebuconazole at higher than recommended rates, conferring a longer period of protection for plants against disease and reducing the need for further application of fungicides later in the growing season. Further to this, it was anticipated that the use of higher application rates could potentially confer an extended period of fungicide availability for plant uptake by reducing losses associated with environmental factors and by controlling the rate of release of tebuconazole. Unpublished research carried out previously at the University of Nottingham had shown that microencapsulated formulations of tebuconazole (batch #4564) exhibited higher levels of efficacy against powdery mildew compared to commercial formulations (Rossall, pers. comm.). Experiments were planned and undertaken aimed at assessing the level of tebuconazole uptake from commercial and microencapsulated formulations applied as seed dressings in an effort to explain these results.

Unpublished research carried out by Micap had suggested that yeast obtained as a bi-product from bio-ethanol production could provide the optimum release profile of compounds (Duckham, pers. comm.). Experiments were undertaken to investigate and quantify the release of tebuconazole from the yeast microcapsules into water. Formulations using yeast strains from different sources (two types used in baking and bi-product yeast from bio-ethanol production) were assessed to ascertain if there were any differences in the profile of tebuconazole release. The results showed that there were no apparent differences in the amount or the manner in which tebuconazole was released from the different formulations into water. The release profiles did not follow those observed by other researchers investigating the release of various compounds from different types of microcapsules. Results of

other research have, in general, found the release of AIs from microencapsulated formulations to follow a Fickian profile. This may be defined as movement from a zone of high concentration to a zone of lower concentration across a membrane (Kenawy *et al.*, 1992; Trapp, 2004). Asrar *et al.* (2004) found the release of tebuconazole from a formulation consisting of polymer matrix particles to follow this profile (Asrar *et al.*, 2004). Similar findings were observed by Dardelle *et al.* when investigating the release of the flavour volatile limonene encapsulated in yeast cells (Dardelle *et al.*, 2007). Other examples of this release profile have been observed by Suave *et al.* (2010) investigating the controlled release of the pesticide malathion from synthetic polymer microcapsules (Suave *et al.*, 2010).

The formulations tested in this work permitted aqueous tebuconazole concentrations that were far in excess of the published solubility limit in water of 36 μg mL^{-1}. After a period of around two hours, the concentration of the fungicide in solution was observed to reach a state of equilibrium. Several repeats of the experiment, including a more accurate internal standard quantification method, were undertaken, giving identical results. It was proposed that the profile of release observed may be explained by the physio-chemical properties of tebuconazole as a molecule. Tebuconazole is known to be hydrophobic in nature (Ni *et al.*, 2004; P.P.D.B., 2010). It was therefore suggested that the excess tebuconazole molecules in solution may have formed micelle-like structures when released into water. Additionally, un-encapsulated tebuconazole residues held on the surface of the yeast cells may also have contributed to this effect. This was not found to be the case in experiments carried out by Asrar *et al. (2004).*

Research carried out at Micap PLC investigating the release and quantification of actives from yeast microcapsules indicated that un-

encapsulated AIs held on the surface could affect concentrations of the of the encapsulated compound in solution, especially in the early stages of the time course (Duckham and Wheeler, 2005a). The reduction of tebuconazole concentrations in solution observed in the later stages of the time course may have been the result of re-encapsulation of the fungicide again caused by the molecule's hydrophobic nature. This seems the most feasible explanation for the results observed, as tebuconazole is extremely unlikely to have been removed from the water by any partition effect caused by the glass test tubes used or to have evaporated from the solution. A non-encapsulated formulation of tebuconazole, such as Raxil, could possibly behave in the same manner. However, the formulation of commercial fungicide products as emulsifiable concentrates could lead to the distribution of tebuconazole being more even.

Extended periods of storage may cause the yeast cells to become degraded or compromised as microcapsules. This may have contributed to the unusual release profile observed in initial experiments. To test this, a new batch of microencapsulated tebuconazole (batch # 8129) was prepared using bi-product yeast from bio-ethanol production and the same method as used with other batches. The release of tebuconazole from batch #8129 was assessed before. Results showed there to be no differences between the new and three older formulations tested, suggesting that the release profiles observed were not a result of the age of the formulations. The older formulations ranged in age from one and a half to three and a half years old at the time of testing suggesting that the formulations were relatively stable once formulated. The microencapsulated formulations also showed a good potential for sustained release of tebuconazole into water when repeatedly washed with fresh volumes of SDW. The yeast formulations thus demonstrated an ability to release tebuconazole over an extended time period. This may prove a very valuable property for

use as a seed dressing, whereby gradual release into the plant might reduce the known phytotoxic effect caused by conventional formulations.

Although the microencapsulated formulations of tebuconazole could be applied as a foliar spray the scope of this research was primarily aimed at the use of the fungicide as a seed treatment. The use of seed treatments may confer several benefits primarily that of targeted application leading to lower required application rates (Taylor and Harman, 1990; Brandl and Biddle, 2001). Before the company went into administration in 2007, a collaborative agreement had been formed between Micap PLC and the agrochemical company Nufarm. The principle objective of the collaboration was to produce a controlled release tebuconazole formulation that could be applied at rates comparable to another triazole seed treatment formulation, Jockey, produced by BASF, which is applied at rates of up to 75 g AI per 100 kg seed (BASF, 2010). It was hoped that by microencapsulating tebuconazole it could be applied at a higher rate without causing detrimental effects on germination and young plant development detailed by Tomlin (2003).

A series of experiments were undertaken to assess the emergence and development of plants treated with the microencapsulated formulation and compare these results to untreated controls and those treated with commercial formulations of the fungicide. Microencapsulated tebuconazole batch #4564 (used in previous research) and two conventional formulations, Raxil and Mystique, both containing tebuconazole only, were used for the research. An application rate of 40 g AI per 100 kg of seed was selected for the this research, being over 8 times higher than the maximum recommended rate currently used and comparable to Jockey. All three formulations tested exhibited

some affect on emergence and development of plants when compared to untreated controls. However, the plants that had been treated with the microencapsulated fungicide were shown to be significantly less affected by tebuconazole when applied at a dose rate of 40 g AI per 100 kg seed (Chapter 3 – Figures 3.6-8) when compared to the commercial formulations. This result suggested that there may have been a controlled release effect, conferred by the yeast cells, potentially explaining the reduced phytotoxicity. The result also therefore implied that the microencapsulated formulations could provide a sustained uptake of the fungicide. However, results of an assessment investigating the potential of the microencapsulated formulation to provide of a longer period of tebuconazole availability for plant uptake (Chapter 4) showed there to be no overall differences between microencapsulated and commercial formulations. Further to this assessment of potential phytotoxic effects caused by plant uptake of tebuconazole directly from the soil, i.e. not seed applied, (Chapter 4 – Figure 4.14) showed that there were no notable difference in development between plants grown in soil containing the fungicide compared to those grown in untreated control columns. It would therefore appear that the lower phytotoxic effects observed were caused by another factor. One potential explanation could be the presence of the yeast cells themselves. McGee noted that the fungicides applied directly to seed could easily penetrate the seed coat causing damage to the endosperm (McGee, 1995). It is possible that the presence of the yeast microcapsules have a protective effect, isolating the fungicide from direct and immediate contact with the seed before sowing and upon hydration in soil resulting in reduced exposure and hence phytotoxicity observed. Results of GC-MS analyse of the total tebuconazole content of plants in these preliminary experiments showed that the plants treated with the microencapsulated formulation contained less tebuconazole than those treated with commercial formulations supporting the reduced phytotoxic effects on emergence and development observed previously.

The original sample of microencapsulated tebuconazole (batch #4564), which had previously shown promising efficacy results in research carried out in this department was used for the purposes of this research. Application rates for tebuconazole applied as a seed treatment used currently vary between 1 and 7.5 g AI per 100 kg seed. Initial experiments examined the uptake of seed-applied microencapsulated tebuconazole at rates that were several times higher than this (40, 80 and 160 g AI per 100 kg) to validate solvent extraction/SPE cleanup, GC-MS detection and internal standard quantification methods. Results showed that there were detectable levels of tebuconazole in plants treated at an application rate of 40 g AI per 100 kg of seed, authenticating the methods described above and also confirming that uptake of tebuconazole from the yeast formulations was possible. To further test the extraction, detection and quantification methods, another experiment assessed application rates of between 5 and 40 g AI per 100 kg seed. The results also showed that higher application rates conferred increasing levels of tebuconazole uptake in plants. This was an encouraging result as other research investigating the uptake of another systemic triazole fungicide, triticonazole, applied as a seed treatment, had shown no increase in fungicide uptake with higher application rates (Quérou *et al.*, 1998), who suggested that the rate of uptake of the fungicide in the early stages of plant growth was not dictated by the dose at which the fungicide was applied but by the size of the 'dressing zone' around the seed.

The higher uptake of tebuconazole related to dose rate, observed in the data presented in Chapter 3 (Figure – 3.4), may be related to the release profiles examined earlier (Chapter 2). When the seeds are watered, the yeast cells will become hydrated, allowing the encapsulated tebuconazole to move from the microcapsules into the surrounding water as exhibited in the release profiling experiments (Chapter 2- Figures 2.8 and 2.9). The rate of this release into soil is

likely to be a dynamic process involving changing amounts of proximal water to the seed allowing the movement of the fungicide from the site of application on the seed coat into the surrounding soil, producing a resultant dressing zone. Results obtained assessing the ability of the John Innes compost used as a growth medium during the research to retain tebuconazole (given in Chapter 4) and the low mobility of tebuconazole in soil indicated by it K_{oc} value of 769 mL g^{-1} (P.P.D.B., 2010) mean that the dressing zone around the seed is likely to be small. Other research carried out into the uptake of seed applied fungicides such as triticonazole (Quérou *et al.*, 1997) and triadimenol (Thielert *et al.*, 1988) have shown that the primary uptake route is not from seed coat to endosperm but by roots from the dressing zone around the seed. This was further supported by work carried out by Stein-Dönecke *et al.* (1993), which showed that the size of the dressing zone had a significant effect on uptake of the insecticide imidacloprid from soil (Stein-Dönecke *et al.*, 1992). It would appear that application of the microencapsulated formulation at higher dose rates meant that there was more tebuconazole available in the soil around the seed, leading to the higher uptake observed with higher application rates reported in this research. However, the extent of this uptake will also be limited by root development, whereby the distribution of uptake-active regions of the root structure, such as root hairs around the apical meristem, in the dressing zone around the seed will influence fungicide uptake. Similar observations were made by Quérou *et al.*, (1998), who concluded that the rate of seed-applied triticonazole uptake was limited primarily by root development.

Briggs *et al.* (1983) showed that the uptake of non-ionised organic chemicals from a hydroponic solution into plants was potentially influenced by the compound's behaviour in solution (Briggs *et al.*, 1983). Solubility, described by the n-octanol/water partition coefficient (K_{ow}) can impact not only the availability of a compound for uptake by

plant roots but also the mobility of the compound within the plant as it moves in the plant's vascular system (transpiration stream) and across lipid-rich cell membranes (Collins *et al.*, 2006). With this in mind several experiments were undertaken to assess the distribution of tebuconazole in individual plants grown from seeds treated with microencapsulated tebuconazole and the two commercial formulations. Systemicity is an important attribute for any crop protection agrochemical formulation (Klittich *et al.*, 2008). Briggs *et al.* showed that uptake and translocation of compounds in plants was related to individual chemicals lipophilicity. Highly lipophillic compounds may become bound to lipid rich domains within a plants roots limiting the degree to which the compound can become distributed throughout the plant (Briggs *et al.*, 1983). It was suggested that differing degrees of tebuconazole availability, potentially conferred by the controlled nature by which the fungicide was released from the yeast cells of the microencapsulated formulation, could have an impact on uptake and subsequent distribution of the AI within the plant. GC-MS analyse of the plants grown as part of the phytotoxicity assessment also showed there was less tebuconazole in plants treated with the microencapsulated formulation of the fungicide, compared to those treated with the commercial formulation. It was, however, not known if this trend would continue as the plant developed. Leaves of different ages from plants treated with the three formulations of tebuconazole were analysed for their tebuconazole content.

Results of these experiments showed there to be initially higher accumulation of tebuconazole in leaves one and two compared to leaves three and four to emerge in plants treated with both microencapsulated and commercial formulations. As plants continued to develop the distribution of tebuconazole became more even through out the plant. Higher amounts of tebuconazole were also found in the tips of leaves analysed when compared to the mid and base sections of

the same leaf. These findings were also supported by other research carried out into the uptake and distribution of fungicides and other chemicals in the early stages of plant growth (Briggs *et al.*, 1983; Quérou *et al.*, 1998). There was also found to be initially less tebuconazole in the plants treated with the microencapsulated formulation confirming the results seen previously showing reduced phytotoxicity in plants treated with the microencapsulated formulation. As the plants continued to develop, the amount of tebuconazole in plants treated with the microencapsulated formulation became comparable to those treated with the commercial formulations. These results were encouraging as it appeared that the microencapsulated formulation was providing the optimum combination of reduced phytotoxicity at higher dose rates whilst maintaining a good degree of systemic distribution and comparable level of uptake to commercial formulations.

Results of the experiments examining plant uptake of tebuconazole applied as a seed treatment sometimes showed a high level of variation between replicate samples. There was also an effect observed where the amount of tebuconazole accumulated in plants rose steeply between GS 14 and 16. This raised the question of the potential of the growth environment to affect uptake of tebuconazole. As uptake and distribution of the fungicide occurs via a passive diffusion process into the roots from water in the soil (Collins *et al.*, 2006) it was suggested that artificially increasing the rate of transpiration, by increasing temperature and airflow, could in turn lead to an increased accumulation of the fungicide in plant tissue. Further to this the controlled release characteristics seemingly conferred by the microencapsulated formulation could have had an impact on this uptake. This was found not to be the case, results showed no increase in fungicide accumulation as a result of changed environmental conditions. Although tebuconazole is transported in the transpiration

stream from the soil through the roots and into the rest of the plant it would appear that rate of uptake of the fungicide into the plant is limited by the root distribution. This is supported by the results seen in Chapter 4 (Table 4.3) showing that even when there were comparably high concentrations of tebuconazole available for uptake from the soil, in a large dressing zone, this did not lead to increased concentrations of tebuconazole in plants. Other work carried out by several authors has found that several factors related to plant roots can limit uptake. Strang and Rogers (1971) observed that after root treatment of cotton and soybean, ^{14}C labelled trifluralin was retained on the root surface by binding to the epidermis and entrance to the roots of the compound was mainly facilitated by breaks in the epidermis (Strang and Rogers, 1971). Further to this lignin and suberin have both been shown to adsorb lipophillic compounds; adsorption was shown to increase with the lipophilicity of compounds (Barak, *et al.*, 1983a; Barak, *et al.*, 1983b; Lulai and Morgan, 1992). This effect may well have affected the uptake of tebuconazole from the soil around the seed in the later stages of growth.

An examination of the long term release and uptake of tebuconazole into plants was undertaken. It was hoped that the microencapsulated formulation would confer a longer window of fungicide release, and therefore uptake, when compared to commercial formulations. This was not found to be the case. Results showed that there was the potential for uptake of tebuconazole into plant tissue in the later stages of plant development. However, comparisons between the microencapsulated and a commercial formulation showed there to be no clear differences in the amount of tebuconazole accumulated. The results also highlighted an unexpected late increase in the rate of fungicide uptake, leading to a repeat of the same experiment, which showed the same results.

The results of the long term uptake experiment led to the testing of the soil (compost) as a potential sink for tebuconazole. The results obtained showed that the growth medium used during the research had a strong ability to absorb tebuconazole dissolved in water. It would appear that as the treated seeds were irrigated the fungicide becomes solubilised in water and moved away from the seed. The chemical characteristics (K_{oc} -Table 4.1 – Chapter 4) of tebuconazole mean that the fungicide is then likely to preferentially bind with organic components in the soil environment, meaning that the distribution of the fungicide within the soil is likely to be localised around treated seeds. This effect is likely to be observed in both the commercial and microencapsulated formulations of tebuconazole. This factor appeared to affect the long term uptake of the fungicide into plants grown in pots (Chapter 4 – Figures 4.3-7). Roots initially emerge and pass through the zone of tebuconazole immediately adjacent to the treated seed. The roots continue to develop and take in tebuconazole throughout the growth period. It is proposed that as the resources of space and nutrients become reduced in the later stages of the growth period the roots proliferate to occupy every available free space in the soil environment leading to the late increases in fungicide uptake observed. This finding was further supported by the results of the soil column experiment, investigating fungicide uptake from fungicide treated layers of soil at differing depths contained within a 1m column over a time course. Uptake of tebuconazole was initially higher in the plants grown in the columns but concentrations of the fungicide declined as growth continued. It would therefore appear that the sustained uptake observed in the long term release experiments may be an artefact produced by growing plants in pots where the roots developed in a finite growth environment. Thus it would also seem possible that application rate does not have a direct impact on the long term availability or uptake of tebuconazole applied as a seed dressing. The treated layers used in the soil column experiment contained a total of 10 times more tebuconazole than that applied to each seed used in

previous experiments but did not exhibit higher concentrations of the fungicide as a result. The suberisation of roots in the soil columns may have had an impact on the uptake of tebuconazole reducing uptake due to the fungicide becoming retained by the suberin itself. Overall, the uptake of tebuconazole applied as a seed treatment into plants would appear to be a complex combination of factors including the size of the dressing zone around treated seeds but also root distribution.

5.1 FINAL CONCLUSIONS AND FUTURE WORK

From the results of this research there is no clear indication that the beneficial efficacy results observed in previous work can be attributed to an increased or sustained uptake of the tebuconazole from a microencapsulated formulation of the fungicide. A field experiment, whereby plants would not be grown in the finite environment of a pot, could show different results. A series of plots could be utilised using seeds treated with different rates of the microencapsulated and commercial fungicides. Plants exhibiting lower infection rates could then be tested for their tebuconazole content using the methods refined in this research. However, the results of the soil column experiment discussed earlier would suggest that there would not be an increased uptake of the fungicide treated with the microencapsulated formulation in plants grown in the less restrictive conditions offered by field growth. Further to this, the use of radio-labelled fungicides could possibly give a more precise understanding of fungicide distribution within plants. However, the production of such a formulation would be expensive and time consuming.

Research carried out to date on the efficacy of the Micap PLC formulation of tebuconazole applied as a seed treatment have only studied the impact on foliar diseases. Many losses associated with pathogens affecting food crops are those caused by seed or soil borne fungi (Clark *et al.*, 2004). The 'burst release' effect of the fungicide from the yeast microcapsules observed during the preliminary release profiling undertaken as part of this research may have another benefit. The use of seed treatments is primarily for the purposes of seed hygiene, reducing the potential impact seed and soil-borne pathogens may have on subsequent yields. Where systemic products are applied this can also confer protection in the early stages of plant growth. The microencapsulated formulations did not appear to perform any differently to commercial formulations in terms of fungicide uptake.

However, the initial burst of release from the yeast microcapsules could provide protection from seed and soil-borne pathogens by producing a zone of high fungicide concentration not only on the surface of the seed but also in its immediate vicinity. This would require further research but could, in theory provide beneficial results.

Microencapsulated formulations could also potentially be employed in systems where plants are grown in fixed environments. Examples of this could include hydroponic systems and plants grown commercially in pots. The sustained nature in which the microencapsulated formulation released tebuconazole when repeatedly washed with fresh volumes of SDW (Chapter 2) could mean that they would be suitable for the application of other chemicals, such as nutrients or growth regulators to hydroponic solutions. Equally the formulation of microencapsulated tebuconazole into a pellet which could be inserted into the soil of pots in which plant are grown could provide the possibility of sustained release and uptake of fungicides or other chemicals from the soil via the plants root system.

With the demise of Micap PLC, other formulations that might confer advantageous uptake profiles of crop protection chemicals such as tebuconazole and reduced detrimental effects such as phytotoxicity could be highly sought after. One formulation method that could provide this is that of a nano suspension/dispersion of tebuconazole produced by the UK-based company called IOTA NanoSolutions (IOTA, 2010). The company has produced such a formulation of tebuconazole for the purposes of research. Preliminary experimentation carried out as a side line to the research on the microencapsulated formulation have shown that the IOTA formulation displayed a good degree of efficacy *in vitro* against *Fusaruim culmorum* (the casual agent of seedling blight) and *Septoria tritici, in planta,* compared to commercial formulations

Overall the research has shown that microencapsulated fungicides can be applied as a seed treatment at higher dose rates than currently employed, without causing the problem of phytotoxicity associated with tebuconazole to the same degree as commercial formulations. Uptake and systemicity (distribution) of the fungicide within treated plants was shown to be comparable in all formulations tested. Testing also included an evaluation of the possible extended long-term release that may have been provided by the microencapsulated formulation which found comparable amounts of tebuconazole in both plants treated with the microencapsulated and commercial formulations. However, this was also found to be caused by the limited environment of the pots in which the plants were grown. Although the Micap PLC product did not fulfil all expectations that it could have potentially provided, there were still positive results gained as part of this research project. However, with the failure of Micap PLC as a company it would appear unlikely that a microencapsulated formulation of tebuconazole, as studied in this project, will become a commercial reality without further investment.

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