THE USE OF THE FUNGUS ASCOCHYTA CAULINA AS A BIOLOGICAL CONTROL AGENT FOR THE WEED CHENOPODIUM ALBUM

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THE USE OF THE FUNGUS ASCOCHYTA CAULINA AS A BIOLOGICAL CONTROL AGENT FOR THE WEED CHENOPODIUM ALBUM

Evaluation of the bioherbicide formulation efficacy of *Ascochyta caulina* on different life stages of the weed plant *Chenopodium album* under laboratory and field conditions comparing Libyan and UK populations

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Abstract

Chenopodium album is considered one of the most important weeds adversely affecting agricultural production due to its highly competitive influence on field crops. Chemical herbicides have increased the efficiency of farming, but recently problems of herbicide-resistant weed populations and herbicide residues in soil, water, food products and effects on non-target organisms have increased, consequently, other methods of control of weeds by using specific fungi as herbicides have been suggested. The purpose of this research was to evaluate the biological control of the weed *Chenopodium album* by the fungus *Ascochyta caulina*. Some of the factors which control dormancy and germination of *Chenopodium album* seeds have been investigated to understand better the weed population dynamics. The results showed that seeds from two populations (UK and Libya) differ in their response to factors such as light, chilling, and burying in soil. This could have implications for effective control of the weed in different regions.

Two formulations of mycoherbicides (Tween 80 and Gelatine based applications) were tested in the laboratory, and showed promise in reducing growth of the weed, especially the formula of Tween 80. There was extensive shoot fresh and dry weight reduction of inoculated *Chenopodium album*, as well as reduced root growth. Highest disease severity rates were observed on plants in the first three week of life. A field trial revealed similar results but less disease severity was observed, possibly because of dry weather. However, it was concluded that the fungus *Ascochyta caulina* is a potentially useful biological control agent but many factors still can be modified in relation to application of the mycoherbicide to increase its efficacy.

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Chapter1: General introduction

Weeds are considered one of the most important factors adversely affecting agricultural production worldwide due to their high competitive ability against crops in the field (Zimdahl, 2007). For example in recent years, agriculture in Libya has shown a high density of weeds, to an extent that they have made considerable losses in the crop's yield, although figures of economic losses in Libya due to weed growth in crops fields are not available. The size of losses of crop due to weeds in North Africa and Middle East countries more generally have been recorded as 19.5% on vegetable crops and 5.8% on fruits (Elazzabi, 2000). In United States agriculture, weeds cause a reduction of 12% in crop yields; this represents about $33 billion in lost crop production annually. In addition, another $4 billion is spent each year in the US on herbicides to control these weeds in crops, and more than $3 billion for cultural and other methods of control (Pimentel *et al.*, 2005). Parker and Fryer (1975) estimated the losses due to weeds to be as large as 50% in tropical crops and approximately 11.5% of total potential production worldwide. Zoschke and Quadranti (2002) reported that a 13.2% loss of agricultural production could be attributed to the competitive effects of weeds world-wide, which makes the control of weeds an essential element of productive agriculture.

Management of weeds is a necessary but expensive challenge, and chemical weed control accounts for over $14 billion spent annually on herbicides (Kiely *et al.*, 2004), Weeds are the most important of the economic and environmental pest problems, and they are the target of much of the chemicals applied throughout the world. Thus, herbicides comprise 47% of the world agrochemical sales, and insecticides 29% and most of the rest being

fungicides (Woodburn, 1995). Weeding, usually by hand, accounts for up to 60% of total pre-harvest labor input in the developing world (Webb & Conroy, 1995). Invasive weeds cause significant environmental damage, because they are free of their natural enemies and competitors, and very often have high population sizes and are able to displace native species, a problem which has only recently begun to be recognized (McFadyen, 1998). Chemical herbicides have increased the efficiency of farming and have become safer and more effective, but more recently there have arisen problems of herbicide-resistance which results from regular exposure of a weed population to an herbicide, leading to a predominance of genotypes that can survive and grow when treated with herbicide concentrations that are normally lethal in untreated weed populations. There have also been increasing problems of the buildup of herbicide residues in soil, water and food products, and effects on non-target organisms. However, governments have started responding to public pressure by reducing the use of herbicides to levels of around 50% of previous use (Schroeder *et al.*, 1993).

Understanding weed ecology could lead to more effective weed prevention and control by providing us with a basic understanding of the population of weeds in natural and managed systems because firstly, weeds more susceptible to herbicides are replaced by resistant species; secondly, monoculture species are problematic if using only one strategy to control them; thirdly, it is very expensive to maintain sites and herbicides are usually harmful to the overall environment; and fourthly, study of the ecology and biology of the weeds enables the determination of the weak point in the life cycle of the weed so that a control management strategy can be based around it (Booth *et al.*, 2003).

1.1 Agricultural weeds

Of the total number of 300,000 plant species in the world, only a few thousand are thought to behave as weeds (Rossiter & Riha, 1999). About 200 species or 0.08% of the total number are recognized as major problems in world agriculture (Holm *et al.*, 1977). Holm *et al.*, (1977) suggested that these 200 species account for 90% of the loss in world food crops to weeds. Only about 25 species or 0.01% of the total cause the major weed problems in any one crop. The presence of weeds in a crop leads to an increased total number of plants within a certain area; given that the crop density is already set at a level that optimizes yield for that crop in that environment, the presence of weeds will lead to a reduction in the average yield of the crop, since weeds compete with crop plants for nutrients, soil moisture and sunlight (Rao, 2000).

Man played an important role in changing the environment by altering the crop husbandry practice and by maintaining a weed-free monocrop or multicrop culture. Problems caused by weeds are the low productivity of crop and livestock production, the low soil fertility and post-harvest losses.

Today most countries are faced with the need to develop alternatives to conventional weed control methods to comply with the demand for pesticide reductions to solve the various problems mentioned previously. There is increased interest in using biological controls and organic farming (McFadyen, 1998).

1.1.1 **Definition of the term weed**

Weeds may be a trouble in the garden, affect humans as poisonous plants or as sources of aeroallergens, and invade natural ecosystems, as well as interfering with crops. The most common definition of a weed is "a plant growing in a place where it is not desired" (Buchholtz, 1967). A plant may be considered a weed in one situation and a desirable plant in another; for instance, White Clover *Trifolium repens* L. is usually considered a weed in lawns, but it can be a desirable groundcover used to improve soil conditions and prevent soil erosion on slopes in reclamation landscapes (Bradshaw & Chadwick, 1980). Weeds become of economic significance in connection with farming, where weeds may damage crops when growing in fields and poison domesticated animals when growing on pasture land. Weeds are usually plants that are very abundant, invasive, competitive, harmful, destructive, or difficult to control. Zimmerman (1976) believes that the term "weed" should be used to describe plants that have all the following characteristics: (1) they colonize disturbed habitats, (2) they are not members of the original plant community, (3) they are locally abundant, and (4) they are economically of little value.

Some common methods used to classify weeds are by taxonomic relationships, life history, habitat, physiology, and degree of undesirability. Weeds and invasive plants can also be classified by ecological behavior related to invasion and evolutionary strategies related to carbon allocation.

Weeds may be classified by placing them into three groups according to their life cycle: Annual weeds (most common) germinate, grow, flower, and produce seeds within one year; Biennials weeds produce leaves and store food the first year and the second year they

flower, produce seeds, and die; and Perennial weeds live on from year to year, in many cases the tops die to the ground, but the roots persist.

Another way of classifying plants is to group them by ‘strategies’ on the basis of the characteristics of their life cycle, competitive ability etc. Many weeds would be placed in the ruderal strategy (Grime *et al.*, 1988); such weeds have a short life cycle, are fast-growing, produce many quite small seeds, which often form a persistent seed bank, and also are often relatively weak competitors.

Grime, (1984) suggests that most herbaceous weed species fall into one of two combined strategies, competitive ruderal or stress-tolerant competitors. Plants possessing the competitive ruderal strategy have rapid early growth rates and competition between individual plants occurs before flowering. Such plants occupy fertile sites and periodic disturbance (e.g., annual tillage) favors their abundance and distribution. Many annual, biennial, and herbaceous perennial weed species found on arable land fit the criteria for the competitive ruderal tactic.

Chenopodium album is one of the ruderal annual weed species, shows considerable phenotypic plasticity, a much branched taproot, and it thrives on all soil types and over a wide range of pH values (Grime, 1984).

1.1.2 **Competition between weeds and agricultural crops**

“Competition is a struggle between a crop and a weed for a resource that is in short supply” (Zimdahl, 2004). Weeds compete with agricultural crops for space, light, moisture, nutrients, and carbon dioxide. Factors such as planting date, planting depth, row spacing,

seeding rate, soil moisture, soil fertility, and soil pH have an influence on the competitive advantage of the crop or weed. In a field infested with weeds it is possible to identify different components of the overall competitive effect: (1) intraspecific competition between plants of the cultivated species; (2) interspecific competition between plants of the cultivated species and weed species; (3) interspecific competition between plants of the different weed species; (4) intraspecific competition between plants of the same weed species. Interactions between weeds and crops and within weeds should be considered in studies of crop yield loss and in strategies for weed management (Combellack & Friesen, 1992). Weeds have been able to reproduce, survive, and compete for centuries, at least partially due to their diversity. Species of weeds, and sometimes biotypes within species, can vary greatly in their growth habits and ultimately in their ability to compete with crops. Zimdahl (2004) in his book (Weed-Crop Competition) made three main conclusions. Firstly, weeds do compete with crops and reduce yield and quality. Secondly, weed science will benefit from closer integration with plant ecology, especially with regard to the study and understanding of plant coexistence. Thirdly, modeling has become an important aspect of modern weed management and will likely become more important to future systems. Zimdahl continued to stress the importance of basic ecological theory and principles and their incorporation into weed science and weed management systems.

1.1.3 **The control of weeds**

Weed control is an essential part of crop production systems. This is partly to limit the reduction in crop yields outlined above and also because weeds may reduce crop quality, or produce chemicals which are harmful to crop plants (allelopathy). As well, weeds left uncontrolled may harbor insects and diseases and produce seeds or rootstocks which infest

the field and affect future crops. There are several methods of weed control (Tu *et al*., 2001), which include:

1. Cultural and mechanical weed control methods

    - Crop competition; usually one of the cheapest and best methods of weed control; usually the plant which starts first and is growing under ideal conditions will have the competitive advantage.

    - Crop rotation; by rotating the crops, many of the cultural practices and herbicide programs are changed, this often will reduce the population of specific weeds which were able to flourish in the previous crop.

    - Fire; flaming is a form of thermal weeding which causes dehydration of the affected weed tissues.

    - Mechanical burial; this method is most effective on annual weeds in which all the growing points can be buried. Burial is usually less effective on perennial weeds which have underground stems and roots and have the ability to regrow from these underground storage organs.

    - Mowing; is another method of mechanical control; it is usually most effective on tall growing annuals, and not as effective on short growing plants or perennials.

2. Chemical weed control methods; since 1950, herbicide use has increased dramatically; the new herbicides rapidly resulted in revolutionary changes in weed control strategies in industrialized countries. The sale and use of herbicides have become increasingly subject to government regulations in Europe and America.

Restrictions enforced in this way require that side effects should be considered. The increased reliance on herbicides with the same mode of action as each other has resulted in weeds that are resistant to those herbicides.

3. Biological weed control methods; by using biological agents such as fungi or insects to control targeted weeds. Biological weed control as a practical tool has not been utilized to a great extent in controlling weeds. There have been certain examples of successful biological control programs, but these have been uncommon.

1.2 The target weed *Chenopodium album*[1] L

The research described in this thesis focused on one of the most important and widespread weeds of agriculture, *Chenopodium album* L. It is one of the most troublesome annual weeds, and is known by many common names (fat-hen, lambsquarters, lamb's-quarters, white goosefoot, and common lambsquarters) (Figure 1.1); it belongs to the family Chenopodiaceae and originates in Europe.

[1] Nomenclature of species follows *Flora Europaea* (Tutin *et al.*, 1993)

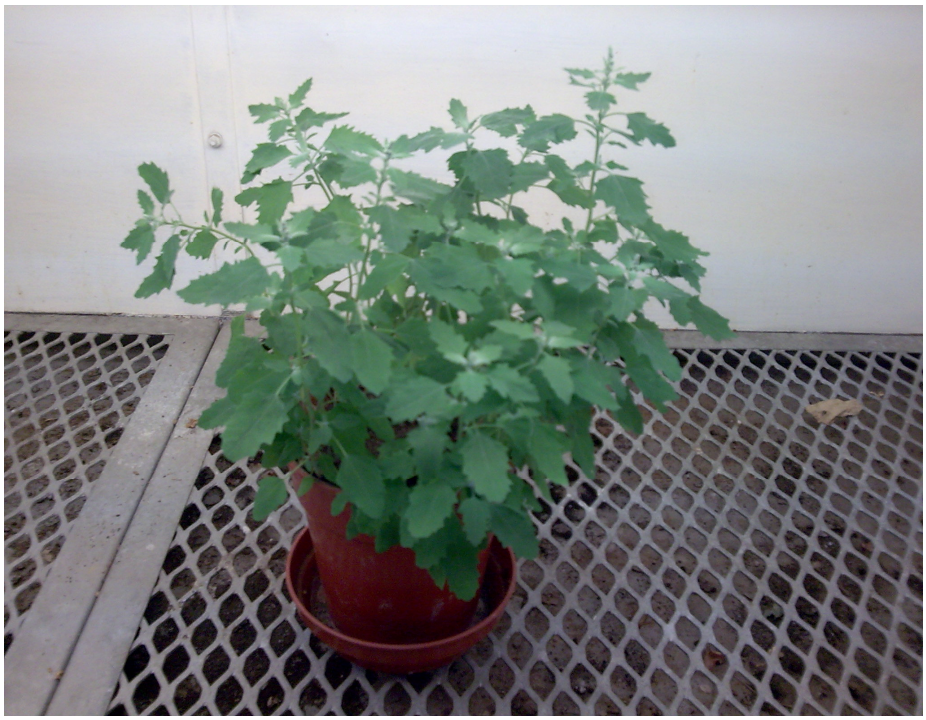


Figure 1.1 Photo of *Chenopodium album* plant.

One study ranked it as one of the five most widely distributed weeds in the world (Holm *et al.*, 1977). *Chenopodium album* is competitive with more than 40 crop species worldwide and is considered a principal weed by corn and soybean producers in the United States (Holm *et al.*, 1977). It ranks as an important weed in potatoes, sugar beet and vegetables worldwide and it reduces the yield of any crop in which it occurs; it is one of the most abundant weeds in a number of agricultural crops in Europe and the world (Schroeder *et al.*, 1993).

Chenopodium album was itself eaten as a vegetable from Neolithic times until the 16th century, when it was replaced by spinach and cabbage (Mitich, 1988). *Chenopodium album* may act as a host for various pests of insect, nematode and virus species that affect important crops (Thurston, 1970). Better quantification of the effects of field margin strips

on crop performance through their influence on pest predators, parasites and pollinating insects is needed (Marshall & Moonen, 2002). Gooch (1963) found that *Chenopodium album* was a contaminant in 39% of all the commercial carrot *Daucus carota* seed samples examined at an official seed testing station in England. A study of changes in the weed flora of southern England between the 1960s and 1997 suggests that *Chenopodium album* is one of the weeds that have become more common (Marshall *et al*., 2003). In a survey of arable weeds in Britain 1971-73, *Chenopodium album* was common to abundant in half the survey areas and rare in the rest (Chancellor, 1977). *Chenopodium album* is a very competitive weed, which profits from high nitrogen levels. Li and Watkinson, (2000) studied the competition between *Chenopodium album* and *Daucus carota* for nutrients and it appears that *Chenopodium album* shows a broader response to nutrient availability than carrot and may well reflect the different selective regimes experienced by crops and weeds. When *Chenopodium album* plants are not controlled, they can absorb considerable amounts of phosphorus that otherwise would be available for lettuce (*Lactuca sativa*) crops (Santos *et al*., 2004). At the same time there is strong evidence for an allelopathic effect of *Chenopodium album* on different crops and plant species. Qasem and Hill (1989) investigated the allelopathic effect of *Chenopodium album* and *Senecio vulgaris* on tomato growing in a glasshouse: the results showed that *Chenopodium album* had an effect through this mechanism while *Senecio vulgaris* did not. Also Qasem and Hill (1994) investigated the inter- and intra-specific competition between *Chenopodium album* and *Senecio vulgaris*; the results showed that differences between the weeds in inter and intraspecific competition were closely related to the growth of their root systems. In another study Qasem and Hill (1993) studied the competitive effects of *Chenopodium album* and *Senecio*

vulgaris on tomato, lettuce and cabbage and they found that both weeds severely reduced the growth of the three crops.

Damage caused by *Chenopodium album* competition has been well documented in field crops: for example, in barley (*Hordeum vulgare*), *Chenopodium album* has been reported to reduce yield by 36% (Conn & Thomas, 1987); and in corn fields (*Zea mays* L.) and sugarbeet (*Beta vulgaris* L.), yield reductions of 11% and 48% respectively have been associated with interference by *Chenopodium album* (Schweizer, 1983 , Beckett *et al.*, 1988). In vegetable crops, season-long competition by *Chenopodium album* has resulted in 36% yield reduction in tomato *Lycopersicon esculentum* Mill. (Bhowmik & Reedy, 1988).

1.2.1 **Distribution, habitat, strategy, characteristics and life cycle of *Chenopodium album***

Chenopodium album is a native summer annual weed found on cultivated land and waste places. It originated in Europe but is now found throughout the world from Asia and Africa to North America (Williams, 1963). For example Zahran and Willis (2009) mentioned that the species occurs commonly in cultivated lands of winter crops in all regions of Egypt. It occurs throughout Britain but is less frequent in the north and west (Figure 1.2).

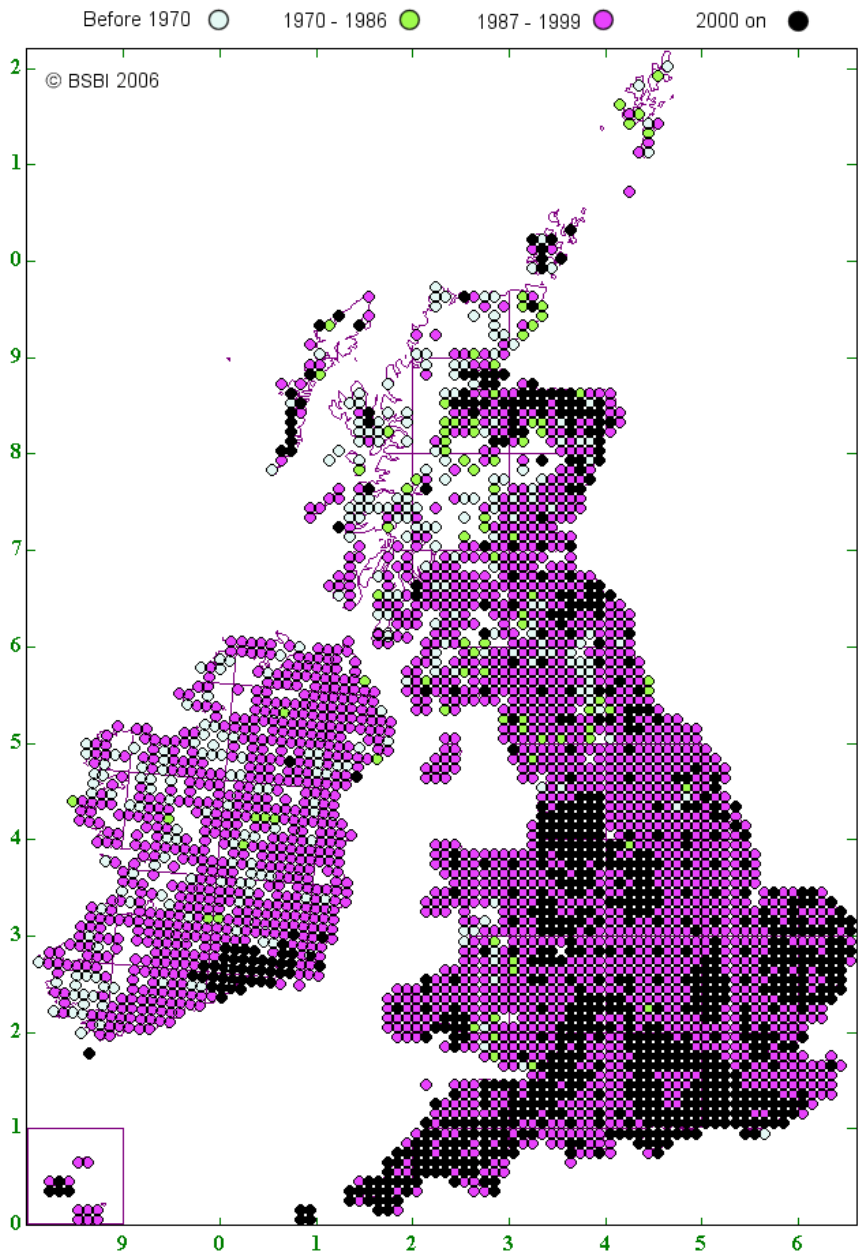


Figure 1.2 Hectad distribution map of *Chenopodium album* in Britain and Ireland; each dot represents at least one record in a 10 km of the national grid (BSBI Maps Scheme).

In the UK *Chenopodium album* is not recorded above 380 meters, it is common on sandy loams and frequent on clay but less abundant on calcareous soils and gravel (Williams, 1963). It grows best on fertile soils and manuring increases its frequency. It can grow up to

3 meters tall. It is more frequent in spring-sown than autumn-sown crops. *Chenopodium album* is also a common garden weed. It is a very variable plant and is sometimes treated as an aggregate species. It shows morphological plasticity in response to soil fertility and plant density. *Chenopodium album* is known to hybridize with related species but the hybrids are difficult to identify due to the variability of the main species. *Chenopodium album* seeds germinate from early spring through autumn, seeds mature late in the season from August onwards (Grime *et al.*, 1988). Seed numbers per plant have been variously estimated at 20 to 3,500, while a large plant could produce 500,000 seeds (Holm *et al.*, 1977); most mature seeds are small black (1 -1.5 mm) and shiny but about 3% are large and light brown (Baksh *et al.*, 2006). Seeds are dormant when ripe and can remain viable in soil for up to 40 years at a depth greater than 45 cm, where the conditions are relatively constant, and also occur as a contaminant of crop seed (Williams, 1963). *Chenopodium album* depends on seed in the strategy of reproduction and it germinates mainly in the spring and it forms a persistent seed bank (Williams, 1963). *Chenopodium album* is a rapidly growing summer annual weed sensitive to photoperiodism and the life cycle in nature is completed in four months. Seeds germinate throughout the summer to August and most seedlings appear in the spring (Williams, 1963).

1.2.2 **Herbicide resistance**

Herbicides are often the most reliable and least expensive method of weed control available, but the utility of herbicides is being threatened by the appearance of herbicide-resistant weeds. Herbicide resistance is the inherited ability of a plant to survive and reproduce after exposure to a dose of herbicide that would normally be lethal to the

majority of plants from non-agricultural areas (Holt *et al*., 1993). Due to the wide use of herbicides as a primary method of weed management, herbicide-resistant weed species have become an increasing problem in many cropping systems. Herbicide resistance was first reported in early 1957 with the weed species common groundsel (*Senecio vulgaris* L.) in California, which was shown to be resistant to herbicides in the triazine chemical class (Ryan, 1970).

Resistance may occur naturally due to selection where the herbicide is the selection pressure. Susceptible plants are killed while herbicide resistant plants survive to reproduce without competition from susceptible plants. If the herbicide is continually used resistant plants successfully reproduce and become more dominant in the population

Since 1957 there have been many more reported cases; Zimdahl (2004) reported more than 100 cases of herbicide resistance in 15 herbicide chemical families and Quimby *et al*., (2002) stated that there were more than 300 examples of resistance for various weed species to various herbicides.

Herbicide-resistant weeds have been identified in 21 European countries, with the highest number of resistant biotypes found in France (30), Spain (26), United Kingdom (24), Belgium (18) and Germany (18). These 21 European countries represent 36% of the 59 countries world-wide in which herbicide resistant weeds have so far been detected. Since 1978 the increase in the number of new cases of herbicide resistant weed biotypes have been relatively constant, averaging nine new cases per year (Ian, 2009) (Figure 1.2).

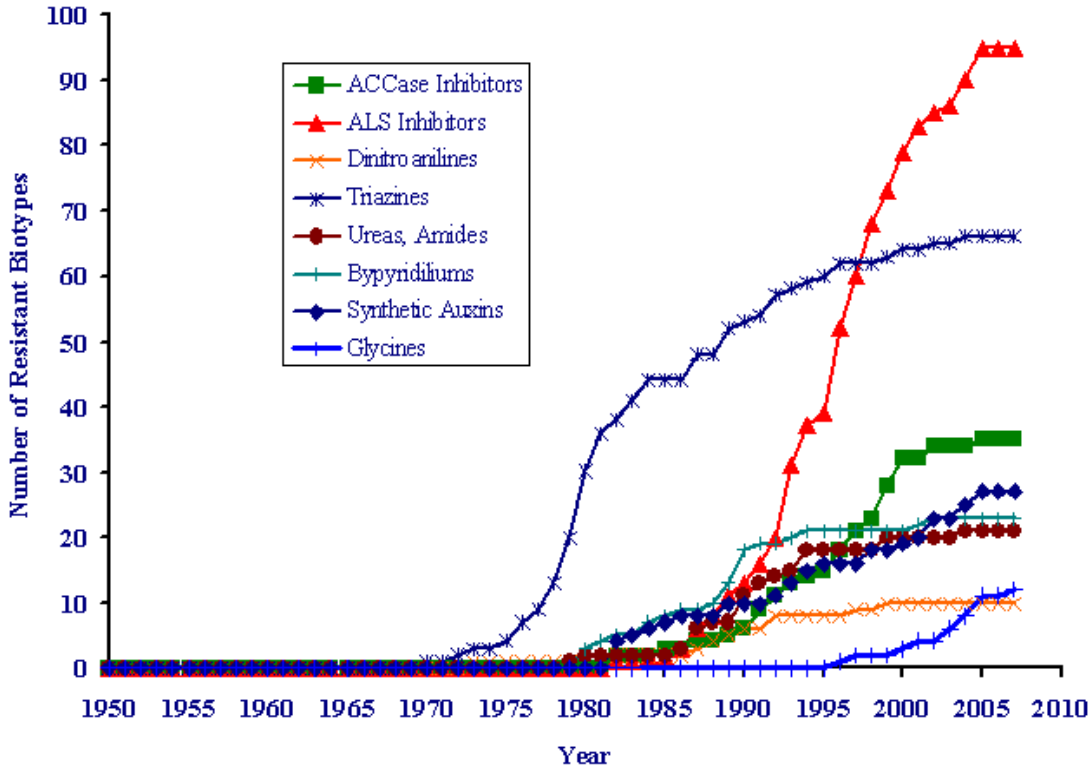


Figure 1.3 The chronological increase in the number of herbicide-resistant weeds for several herbicide classes (Ian, 2009).

There are a number of herbicides registered for use in control of *Chenopodium album* but weed scientists face complex and difficult challenges because it has developed resistance to some herbicides. Bettini *et al*., (1987) proved that *Chenopodium album* possesses very different and stable levels of resistance to the herbicide atrazine. *Chenopodium album* has become the prevalent species in glyphosate-resistant soybean fields, recent reports suggesting that *Chenopodium album* is difficult to control with glyphosate (Micheal & Ian, 2005). Triazine resistant *Chenopodium album* was discovered in the early 1970s, triazine-resistant plants have been reported in four *Chenopodium* species and the most frequently reported triazine-resistant species is *Chenopodium album* (Ian, 2009).

1.3 Biological control of weeds with plant pathogens

Three main methods are used for control of weeds: mechanical, chemical, and biological (Tu *et al.*, 2001). Mechanical control includes mowing, hoeing, cultivation, and hand pulling. Chemical control involves the use of herbicides. Biological weed control is an approach using living organisms to control or reduce the population of a selected, undesirable, weed species, whilst leaving the crop unharmed (TeBeest *et al.*, 1992), The objective of weed biological control is not the eradication of weeds but the reduction and establishment of a weed population to a level below the economic threshold (Wapshere *et al.*, 1989). Since 1980, eight bioherbicides have been registered; at least 15 new introductions of biocontrol agents have occurred and more than 100 micro-organisms have been identified as having the potential for weed biocontrol (Charudattan, 2001). The success rate of biological control has been about 50% according to two studies. Julien and Griffiths (1998) reported a 47% success rate (partial or complete control) worldwide.

There are three approaches for biological weed control that have been used: 1) The classical or inoculative approach; 2) The inundative biological control or bioherbicide approach (Mortensen, 1986); 3) The system management approach. These three approaches are described in more detail below. They differ in their ecological response rather than technological aspects. In the classic approach the control of the target host is dependent upon self maintenance and natural dispersal of the biological agent (Templeton *et al.*, 1979), while the inundative approach works faster than the classical approach because of the avoidance of the wait period for inoculum development and pathogen distribution (Charudattan, 2001) and the system management approach which is based on management

of a weed pathosystem to maximize the spread and severity of the pathogen (Müller-Schärer & Frantzen, 1996).

1.3.1 **The classical approach**

This approach involves the importation and release of one or more natural enemies that attack the target weed from the weed's native land into areas where the introduced weed has became a problem because of the absence of its natural enemies in the area of introduction. The classical strategy differs from the inundative strategy primarily in that it is an ecological rather than a technological response to a weed problem. The objective of classical biological weed control is generally not eradication of the weed species, but the self-perpetuating regulation of the weed population at acceptable low levels (Wapshere *et al.,* 1989). One of the successful examples was the use of a rust fungus (*Puccinia chondrillina* Bubak & Sydenham) against a weed (*Chondrilla juncea* L.) of Mediterranean origin in southeast Australia, the infestation of wheat was reduced by more than 99% to densities approaching those in the native range, and with benefits estimated at $15 million per year (Butt *et al.,* 2001).

1.3.2 **The inundative approach**

The inundative approach involves the periodical application of the native agent (usually a fungus) in a high concentration to control the target weed in a method similar to a chemical herbicide and no seasonal residue is expected (Templeton *et al.,* 1979 , Charudattan & Walker, 1982 , Auld & McRae, 1997). The inundative biological weed control strategy was first introduced by Daniel *et al*., (1973). The occurrence of severe epidemics in natural

weed populations is often limited because the weed hosts display low susceptibility to the pathogen, exhibit strong defense mechanisms, or the weed population is spatially dispersed. Mycoherbicides usually employ indigenous pathogens that are endemic to reduce weed populations; a single application of the mycoherbicide may be sufficient or repeated applications may be required throughout a season. Successful inundative applications of mycoherbicides include control of yellow nutsedge (*Cyperus esculentus* L.) in the United States with *Puccinia canaliculata*, and control of northern Jointvetch (*Aeschynomene virginica*) in rice in Arkansas with *Colletotrichum gloeosporioides* fsp. *Aeschynomene* (Charudattan & Dinoor, 2000). The difference to classical biological control is that bioherbicide agents may not spread rapidly on their own, and may not cause epidemics the following year (Hall & Menn, 2001).

1.3.3 **System management approach**

Biological control as a single measure is not an optimal process for weed control; instead an integrated approach to control all the weeds in a given crop should be used. An integrated weed management strategy combines the use of complementary weed control methods (mechanical and chemical) resulting in more effective, long term weed management outcomes. Integrated control requires planning, as often the timing of one control method can enhance (or inhibit) the effect of another.

This strategy requires the fundamental knowledge of the underlying mechanisms of the crop production system and is aligned with the view of modern agro-ecology, in which complete eradication of weeds is not desirable. Biological weed control agents have to be

seen as stress factors and as an integral part of a well-designed pest management strategy, not as the sole treatment, (Müller-Schärer & Frantzen, 1996). The clean crop option is slowly being replaced by an approach that understands weed control as the management of the crop's environment (reduction of weed-induced yield losses).

The system management approach is thus not aimed at eradicating the weed to obtain a weed free crop but is directed at reducing weed induced crop losses. It is intended not only for extensive agro-ecosystems, but also for the use in intensive agriculture. The system management approach is aimed at managing a weed pathosystem in such a way to stimulate disease epidemics on the target weed population and reducing the competition exerted by the weed on a crop (Müller-Schärer & Rieger, 1998).

1.4 The use of mycoherbicides

A bioherbicide is defined as a plant pathogen used as a weed-control agent through inundative and repeated applications of its inoculum. Competition from chemical herbicides represents a major difficulty that has prevented registration and commercial use of several bioherbicide candidates with good prospects. Bioherbicides such as Devine® and Collego® have been available since the 1960s in the USA and China. The inundative biological weed control strategy was first introduced by Daniel *et al*., (1973), who applied an inundative dose of an endemic, indigenous pathogen, *Colletotrichum gloeosporioides* (Penz.) Sacc. f.sp. *aeschynomene*, to destroy the annual weed northern jointvetch (*Aeschynomene virginica* L.). Pathogens are applied as a massive dose of inoculum (spores

or mycelium), and usually employ indigenous pathogens that are endemic to reduce weed populations. In the case of fungal pathogens, the inundative approach became known as 'the mycoherbicide approach' (Charudattan & Walker, 1982), as described in section 1.3 above.

As stated by Charudattan and Dinoor (2000), several biological, technological, and economical constraints may restrict the development and practical use of bioherbicides, for example, moisture and temperature conditions existing under field conditions are often insufficient in meeting the environmental requirements of the bioherbicide candidate for spore germination and host penetration. A biocontrol formulation (a mixture of the spores with material to improve biocontrol efficacy) constitutes a means by which this problem may be overcome, in order to give effective and constant weed control over a range of environmental conditions (Connick *et al.*, 1991a). According to Templeton *et al*., (1979) the application of bioherbicides is especially advantageous for controlling parasitic weeds, for weeds which are difficult to control by the use of chemical means, or in small-scale and specialized crops where the development of specific chemical herbicides is too expensive. However, if development costs of bioherbicide are kept low, products can be produced more cheaply than chemical herbicides (Mortensen, 1986).

1.4.1 **Formulation of mycoherbicides**

Formulation of a bioherbicide is the key for successful biological control and can be defined as the mixing of the biologically active pathogens with inert carriers and other adjuvant, to give a product, which can be effectively delivered to the target weed (Rhodes,

1990 , Boyette *et al.*, 1996 , Connick *et al.*, 1998). The formulation of the mixture to be applied and inoculum production are often limiting factors in the development of a mycoherbicide. In small-scale experiments the method of spore production is often not important. However, large-scale application requires mass production of inoculum, which must be as low-cost as possible, while product quality is maintained. A major constraint to rapid development and marketing of mycoherbicides is the need to develop appropriate formulations.

A major obstacle to the use of mycoherbicides is to overcome the dew requirement that exists for several mycoherbicides. In addition, appropriate formulations can also reduce the dosage of inoculum required to kill weeds, thus potentially reducing the cost of mycoherbicides (Amsellem *et al.*, 1990). Formulation and delivery systems can greatly improve the field performance of a given dose of a mycoherbicide (Hall & Menn, 2001), and therefore efforts are made to develop effective formulation and delivery systems to improve the bioherbicidal potential of plant pathogens. A range of antidesiccants as humectants, emulsions, and invert emulsions have been tested for formulations of several mycoherbicides (Amsellem *et al.*, 1990 , Connick *et al.*, 1991b , Boyette, 1994), For example, water-in-oil (invert) and oil-in-water emulsions have been developed which greatly reduce or eliminate the free-moisture (dew) requirements necessary for weed infection and mortality by plant pathogens (Shabana, 2005). Formulation of a bioherbicide ideally should result in a product that has low cost, long shelf-life, no difficulty of application, efficacy, stability in the environment and be environmentally safe (Amsellem *et al.*, 1991 , Auld *et al.*, 2003). The selection of an appropriate formulation that can improve stability and viability may reduce inconsistency of field performance of several

potential biocontrol agents (Auld & Morin, 1995). Formulation options are to create the suitable environment in which spores of the antagonist can germinate and infect the host weed (Womack *et al.*, 1996).

The most significant difference between herbicides and bioherbicides is that in the latter the active ingredients are living organisms, which are capable of reproducing in the environment and normally require time to develop after application in order to control the target weed or pest (Charudattan & Dinoor, 2000). Therefore, the formulation must provide conditions that retain viability during preparation, storage and application and favor the survival of the agent in the environment. The mode of action through which a biological agent suppresses its target includes production of toxins, parasitism or competition, each mode of action requires a different set of formulations (Rhodes, 1990). One of the difficult phases of formulation is to overcome the dew requirement of mycoherbicides, and the use of oils in formulations for spraying has shown great potential for enhanced efficacy of mycoherbicides, where the need for high humidity is also overcome. Bioherbicides often depend on the dew in the field for their efficacy; and this constraint can be reduced or eliminated by appropriate formulation (Amsellem *et al.*, 1990 , Connick *et al.*, 1991b , Womack *et al.*, 1996). Formulation research attempting in part to overcome this problem may come via the development of novel liquid formulations, for instance the search for a formulation solution may come from applying technology used in other industries such as the cosmetic and personal care industry or the pharmaceutical or food or surface coating industries which deal in water based products that employ techniques such as gels, emulsions, films and encapsulation. An adaption from any of these products may provide a practical water- retaining bioherbicide formulation.

However, formulation persists as a constraint to commercial development of many potential mycoherbicides; even though this has not been a problem with several commercial bioherbicides because they are used in irrigated systems. Therefore, reduction in dew dependence is a principal aim in the formulation of many potential bioherbicides; liquid formulations have the potential to produce infections soon after application provided they remain moist on the target plant surface. Several attempts to improve water-holding capacity in liquid formulations have been made (Auld, 1993). The development of formulations can involve mixing or blending of active ingredient, such as spores with nutrient, inert carriers or components that reduce the need for long periods of high humidity. Some formulations have been shown to greatly enhance the pathogenicity of spores against weeds (Sandrin *et al.*, 2003 , Shabana, 2005). Chandramohan and Charudattan (2003) used a multiple pathogen mix for effective control of three broadleaf weeds, seedlings were inoculated with a mixture of four fungal pathogens targeting these weeds, and all weeds were killed within 6 weeks without loss of efficacy or alterations in host specificity of each fungus in the mixture. Smith and Hallett (2006) confirmed that half the recommended label rate of glyphosate (0.315 kg. ha-1) was needed for control of common waterhemp (*Amaranthus rudis* Sauer) when combined with the fungus *Microsphaeropsis amaranthi* (Ell. and Barth.) inoculated within 1–3 days of herbicide treatment. The selection of an appropriate formulation that can improve stability and viability may reduce variation of field performance of several potential biocontrol agents (Zidack & Quimby, 2001). The emulsion enhances mycoherbicide efficacy by stimulating conidia germination by protecting the conidia during a dew-free period, hence, increasing weed infection when dew occurred.

Oil suspension emulsions of mycoherbicides have been investigated as less expensive, easy to prepare alternatives to oil invert emulsion formulations, as they can be applied with conventional spray equipment and effectively used at relatively reduced volumes. Egley and Boyette (1995) found that after a 24–72 hour dew delay, 89–97% control of *Sesbania exaltata* was achieved using conidia formulated in an unrefined corn oil emulsion. Experiments conducted with a number of potential mycoherbicides have demonstrated that an invert emulsion allowed infection to occur in the absence of available water (Boyette *et al.*, 1993) and reduced the need to apply high dosages of inoculum (Amsellem *et al.*, 1990). It must be stressed that any effective formulation must be capable of being applied with traditional spraying equipment (Greaves & Macqueen, 1990).

1.4.2 **Factors influencing efficacy of mycoherbicides**

It is estimated that there are over 200 plant pathogens that have been or are under evaluation for their potential as bioherbicides (Charudattan, 2001). In general, most mycoherbicides do not damage non-target organisms, are not toxic to mammals and do not contaminate soil or groundwater (Auld & McRae, 1997). The most frequent constraint of a mycoherbicide is imposed by environmental conditions after application, the effectiveness of the primary infection of the mycoherbicide relying on temperature and humidity (McRae & Auld, 1988 , Makowski, 1993 , Shabana, 1997 , Zhang & Watson, 1997 , Pfirter & Defago, 1998). Researchers have shown that some of these constraints can be overcome through formulation based approaches, some herbicides are known to increase disease severity in crops and a combination of herbicides and pathogens has been suggested as an alternative strategy for weed control (Weaver & Lyn, 2007). Several studies of potential

bioherbicides in combination with chemical herbicides have reported synergistic or additive effects (Heiny, 1994). For example the effectiveness of *Colletotrichum coccodes* in control of velvetleaf was enhanced by low doses of the plant growth regulator thidiazuron (Wymore *et al.*, 1988). Boyette (2006) found that a surfactant greatly improved the bioherbicidal potential of the pathogen *Colletotrichum gloeosporioides* for control of sicklepod *Senna obtusifolia*, a serious weed pest. The use of various crop oils (Auld, 1993 , Egley & Boyette, 1995 , Ghorbani, 2000 , Sandrin *et al.*, 2003 , Boyette, 2006) and invert emulsions (Amsellem *et al.*, 1990 , Boyette *et al.*, 1993 , Womack *et al.*, 1996 , Shabana, 2005) have resulted in improved bioherbicide efficacy and performance of several biocontrol fungi.

1.4.3 **Mycoherbicide products on the market**

The number of mycoherbicides that have reached the market is quite low, due to a number of constraints that obstruct their development. These include: technological problems, such as difficulties in producing large amounts of inoculum, through fermentation processes, or formulations that ensure high stability during shelf life; commercial limitation, usually the small size of the markets; and biological constraints imposed by climate after application, since fungal conidia generally require free water, or at least exposure to high humidity, to germinate and to infect the plant, and frequently moisture is not available for long enough after application. Similarly, the conidia may be inhibited by low or high temperatures, by UV radiation or lack of available nutrients. Most of these constraints can be reduced or eliminated by appropriate formulation (Greaves *et al.*, 1998). Currently, five fungi and one

bacterium are registered as bioherbicides in Canada, Japan, South Africa and the United States (Charudattan & Dinoor, 2000) (Table 1.1).

Table 1.1 Registered mycoherbicides, adapted from Charudattan, Dinoor (2000)

Pathogen	Target weed	Crop	Trade name	Manufacturer
Phytophthora palmivora (Butl.)	Stranglervine or milk weed *Morrenia odorata*	Citrus groves	DeVine®	Abbott Labs. Chicago, IL, USA
Colletotrichum gloeosporioides f. sp. *aeschynomene*	Northern jointvetch *Aeschynomene virginica*	irrigated Rice and soybean	Collego®	Pharmacia &Upjohn Kalamazo, MI; Encore Technologies, Minnetonka, MN
Colletotrichum gloeosporioides f. sp. *malvae*	Round-leaved mallow *Malva pusilla*	Vegetable crops and strawberries	BioMal®	Philom Bios Saskatoon, Canada
Puccinia canaliculata (Schw.)	Yellow nutsedge *Cyperus esculentus*	Sugarcane, potato, maize, cotton and soybean	Dr. BioSedge®	Tifton Innovation Crop., Tifton, GA
Cylindrobasidium laeve (Pers.) Chamuris	Black and golden wattle tree *Acacia spp.*	Cut trees in tree plantations	Stumpout®	developed in South Africa
Xanthomonas campestris pv. *Poae*	Annual blue grass *Poa annua*	Golf courses grasses	Camperico®	Japan

DeVine®: The product is based on *Phytophthora palmivora*, a fungus pathogen of *Morrenia odorata*, a noxious plant infesting citrus groves. It is sold as liquid suspension of chlamydospores (around 6 x 10^5 spores ml^{-1}), to be applied on the soil surface. It causes stem necrosis and plant death within 1-6 weeks after the application, depending on plant age.

Collego®: This is a commercial product based on the fungus *Colletotrichum gloeosporioides* f.sp. *aeschynomene*. It is used in the United States for biological control of *Aeschynomene virginica*, a legume weed infesting rice and soybean crops. It consists of dried spores which are applied in liquid suspension. It attacks leaves and stems, as well as seeds and seedlings. Lesions can be visible 7- 10 days after the application, and produce between 90 and 100% weed control.

BioMal®: It contains spores of *Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *malvae*. It is used to control *Malva pusilla* (round-leaved mallow) in Canada and USA. The most effective period of application is at an early stage, although it can be effective at any stage of weed growth. The wettable powder formulation disperses easily in water and is applied as a spray to the weed. BioMal® is formulated by using silica gel as a carrier. It provides over 90% control of the target weed.

Dr. Biosedge®: Contains the active ingredient *Puccinia canaliculata*, for use in control of yellow nutsedge. It causes a rust disease on its host plant *Cyperus esculentus*. It has been reported to be effective in controlling the target weed using the inundative approach. Dr. BioSedge, has been developed and registered for sale. Unfortunately, the stumbling block has been availability of the product because mass-production of this obligate pathogen is difficult and therefore relatively expensive.

Camperico®: An isolate of *Xanthomonas campestris* pv. poae, a wilt-inducing bacterium, isolated in Japan from *Poa annua*. It is registered in Japan as the bioherbicide to control annual bluegrass in golf courses. The bacterium in Camperico® is applied immediately after golf course grasses mowing.

Stompout®: It has been developed in South Africa and is based on *Cylindrobasidium laeve* to control *Acacia* species introduced from Australia. The basidiospores are packaged in small bags, and are suspended in sunflower oil before application. 1-2 ml is applied with a brush on the cut surface of weeds. The fungus within 6-12 months is able to colonize the stump preventing the weed resprouting and causing its death. The application allows an almost complete control of the plants.

1.4.4 **The future of mycoherbicides**

The use of mycoherbicides to control weeds provides an environmentally-friendly approach, which is one of the benefits of the mycoherbicide strategy. Generally, most mycoherbicides have no effect on non-target organisms, are not poisonous to mammals and do not contaminate soil or groundwater (TeBeest & Templeton, 1985). As noted in section 1.4, bioherbicides have been available since the 1960s in the USA and China, but the fact that there have been few reported successes of bioherbicides could be due to the lack of attention given to the special requirements of a living organism. From scientific and practical perspectives, inundative control of weeds with indigenous fungi is a successful and promising technology. In spite of the commercial limitations on the development of mycoherbicides, research in this field has been sustained by the public's demand for non-chemical, weed control alternatives.

The future development of mycoherbicides for use in integrated pest management systems is reliant on research directed to (a) finding endemic pathogens of major weeds (b) developing methods for mass production of stable spores, and (c) studying disease cycles to

understand the principal constraints to epidemic build-up of the disease. However, mycoherbicides are considered as complementary components of current Integrated Weed Management (IWM) systems rather than as alternatives to chemical herbicides. Advances in technologies, with the public support and financial aid, and more scientific research, will all contribute in the progress of the 'science of biological weed control'.

1.5 *Ascochyta caulina* as a mycoherbicide

Ascochyta caulina (P. Karsten) v.d. Aa and v. Kesteren is a plant pathogenic fungus which is specific to *Chenopodium album*, causing necrotic lesions on the leaves and stems. It has been suggested as a potential mycoherbicide to this weed (Kempenaar, 1995), which as discussed in section 1.2 is important and widespread in arable crops throughout Europe, America and many other places (Schroeder *et al.*, 1993 , Sheppard *et al.*, 2006). To investigate its potential as a biocontrol agent, the fungus has been tested in glasshouse and field experiments (Scheepens *et al.*, 1997). Formulations containing different combinations of *Ascochyta caulina* conidia, the phytotoxins from the fungus and low doses of herbicides have been tested (Vurro *et al.*, 2001). Significant improvement in the efficacy of the fungus was achieved in glasshouse trials with an aqueous formulation containing PVA (0.1% v/v), Psyllium (0.4% w/v), Sylgard 309 (0.1% v/v), nutrients and conidia (5×10^6 spores ml^{-1}) (Netland *et al.*, 2001). The extracellular, hydrophilic phytotoxins produced by *Ascochyta caulina* were purified and their structures determined and the main toxins, named ascaulitoxin trans-4-amino-D-proline and the aglycone of ascaulitoxin, have shown promising herbicidal properties (Evidente *et al.*, 2000). Field trials have investigated the

performance of *Ascochyta caulina* conidia applied at different developmental stages of *Chenopodium album* either as a single treatment or combined with sub-lethal doses of herbicides or with the fungal phytotoxins. With the available formulation, favorable weather conditions are needed to obtain infection in the field. The efficacy of the strain of *Ascochyta caulina* used so far has proved to be inadequate to justify its development as a bioherbicide. This is probably due to its low virulence.

Thus the fungal species shows potential as a host specific biocontrol agent, but further work needs to be performed to find the right formulation and application to *Chenopodium album* before it could be recommended for mycoherbicide use. This forms the focus of the work performed in this thesis.

1.6 Aims and objectives of the thesis

The aims of this study were:

1. To enhance the disease efficacy of *Ascochyta caulina* applied to *Chenopodium album* in order to increase the potential for using this fungus as a mycoherbicide to control the weed species.
2. To assess whether the mycoherbicide control achieved under laboratory conditions can also be achieved under field conditions.
3. To investigate the effect of different conditions on germination of seeds of *Chenopodium album*, with particular emphasis on how these differ in populations from two distinctive geographical regions.

4. To assess whether the results obtained in the UK could be applied to control *Chenopodium album* in Libya.

Specific objectives of this project were:

- To optimize the biocontrol agent efficacy of *Ascochyta caulina* using different strategies such as searching for a new formula for the delivery of the pathogen *Ascochyta caulina* by using vegetable oil emulsion to minimize the period of time that higher humidity is required.
- To identify the stage of *Chenopodium album* most affected by the fungal pathogen *Ascochyta caulina* in order to determine the appropriate time for applying the mycoherbicide.
- To study the factors affecting germination of seeds of *Chenopodium album*.
- To use populations of *Chenopodium album* from the UK and from Libya, to determine whether climate conditions in the two geographical areas influence germination requirements, and whether infection response is similar.
- To test the developed formulation on *Chenopodium album* plants grown under field conditions and assess survival and growth rates.

Chapter2: Effect of environmental conditions on the germination of *Chenopodium album* seeds

2.1 Introduction

To make any control measure of weeds effective weed controllers must reduce the weed seed population (seed bank) in the soil, and studying factors controlling dormancy and germination is necessary to determine the optimum seed germination conditions and hence to understand the behavior of weed seed banks during a crop cycle. Temperature is the single most important factor regulating germination of non-dormant seeds in irrigated, annual agroecosystems at the beginning of the growing season where light, nutrients, and moisture are typically not growth limiting (Garcia-Huidobro *et al.*, 1982). Species produce dormant seeds, which can accumulate in the soil, forming a seed bank, and can survive many years and escape mechanical and chemical weed control. Seeds lose dormancy when many factors for breaking dormancy are available; these environmental factors comprise temperature, nitrate, light (both quality and quantity), moisture and gasses. Weed seeds are strongly influenced by temperature (Taylorson & Hendricks, 1972). The breaking of primary dormancy is therefore influenced by the temperature and its fluctuation. Dormancy of winter annual weeds is broken by high temperatures and of summer annual weeds by low temperatures (Baskin & Baskin, 2000), while Weaver *et al*., (1988) mentioned that weed seedling emergence is affected by soil moisture and temperature.

Every seed population has a specific temperature range within which germination of its non-dormant seeds can take place. The rate of germination increases steadily with

increasing temperature from a minimum temperature up to an optimum and then decreases rather steeply (Fenner & Thompson, 2005).

Light is one of the most important environmental factors that interact with temperature to regulate seed germination in many plant species, but light requirement for germination may vary with temperature (El-Keblawy, 2003). In seeds, temperature influences integration of partial processes as dormancy continues or is overcome; each species appears to have its own specific temperature requirements for germination. The effect of rising temperature on weed seedling emergence is consistent from year to year within a given species, making temperature the most predictable factor governing seedling emergence (Weaver *et al.*, 1988).

The time of weed emergence relative to that of the crop is important in determining the outcome of weed and crop competition, the earlier the weeds emerge, the more able they are to compete for resources. The timing of seedling emergence is also a crucial factor in predicting when application of herbicides or mycoherbicides will be most effective.

A number of factors may influence germination of *Chenopodium album* seed. Seed polymorphism, soil temperature, nitrate content of soil, and light all seem important or effective in the breaking of dormancy of the seeds (Williams, 1963 , Holm *et al.*, 1977 , Grime *et al.*, 1981) *Chenopodium album* is one of the most widely controlled summer annual weeds in the world and its seeds show considerable polymorphisms which influence their response to environmental conditions (Williams & Harper, 1965, Karssen, 1970).

Germination response of *Chenopodium album* to temperature exhibited clear trends that depended on the geographic region from which the plants originated (Ascough *et al*., 2007). The optimum temperature of *Chenopodium album* seeds depends on their origin and on the year. This optimum temperature for germination of seeds of *Chenopodium album* lies between 10- 25°C.

In this chapter various other aspects of the germination requirements of *Chenopodium album* will be studied in four experiments. These aspects include whether the seeds have been chilled or buried, the age of the seeds since collection, and also whether seed sourced from two populations subject to very different climatic conditions will show any differences in germination behavior. The temperature conditions under which germination occurs will be monitored for comparisons of the effect of this parameter as well.

There are two different seed forms on one plant: brown seed and black seed, the brown seed has a thin seed coat and can germinate after the harvest, while the black seed has a thick seed coat and is dormant; brown seeds are larger than black seeds, and the surface of the black seeds is either smooth or reticulate (Williams & Harper, 1965). The seeds collected from the Organic Bracken Farm from Bradford contained many brown seeds, but the Libyan seeds did not contain any brown seeds, and the differences in germination behavior between black and brown seeds will therefore also be examined, as the proportion of black/brown seeds might affect the response of the two populations.

In obtaining some information on the extent of variation in seed dormancy caused by location and seed polymorphism, the aim of these experiments is not only to increase understanding of the general germination behavior of this species and to aid in predictions

for its control, but also to assess whether the predictions would be the same for the two contrasting populations from different geographical regions.

2.2 Materials and methods

2.2.1 Seed material used and general experimental procedures

Matured seeds of *Chenopodium album* L. were collected near Leeds/Bradford airport, Yeadon, West Yorkshire (N53° 78' 33", W1° 75' 00") in September 2006. Seeds were also collected from Libya (Janzur, 17 km west of Tripoli, N32° 81' 72", E13° 01' 11") in August 2007. The last group of seeds which was used in study of polymorphism was collected from Bradford, Organic Bracken Farm, Syke Lane, Hipperholme, West Yorkshire, (N53° 45' 38", W1° 50' 32" in September 2008. Seeds were dried by spreading on paper sheets, seeds of *Chenopodium album* were separated and cleaned from tunics which may cause the inhibition of germination (Holm *et al.*, 1977) and then they were stored at a laboratory temperature in unscrewed plastic bottles until they were used in the experiments.

All experiments were conducted using the same general procedure. Seeds were incubated in 9 cm Petri dishes on two layers of filter paper (Whatman No. 2) and soaked with distilled water; each Petri dish contained evenly spaced 20 seeds, 10 replicates for each weed population (i.e. Libyan and UK). After exposure of the seeds to any initial treatment, germination tests were carried out in a growth cabinet under laboratory conditions. Germination was tested under a light intensity of 78 μmol m^{-2} s^{-1} and 14/ 10h light interval. The light intensity was measured using a conversion formula that assumed each single tubular fluorescent lamp produces approximately about 1000 lux which is approximately 13-14 μmol m^{-2} s^{-1}. Seed germination was recorded daily at the same time and the

experiment stopped when no further germination occurred for five consecutive days; protrusion of the radicle of more than 1mm from the testa was the criterion for germination. The dishes were moistened with distilled water at intervals as required. The maximum and minimum temperature was recorded daily in °C, and the mean of the maximum values and the mean of the minimum values were calculated. At the end of each experiment, germination percentage and germination rate were determined, and the duration of the period over which the experiment ran was recorded (in days).

2.2.2 **Viability test (tetrazolium test)**

To ensure that the seeds used for the experiments were viable and of high quality, each batch of 50 seeds was subjected to a viability test using the tetrazolium technique described by Lakon (1949). It is a fast, reliable viability test, widely known as the TZ test. It determines the percentage of live and dead seeds in a sample within 24-48 hrs, regardless of the dormancy level of the seeds, while a germination test takes 3-4 weeks to be completed in those freshly harvested and possessing high levels of dormancy. The embryos of live seeds stain red while the embryos of dead seeds do not stain (Porter *et al*., 1947). Abnormal seeds exhibit a different pattern of staining.

2.2.2.1 Principles of the tetrazolium test

The Tetrazolium Test (TZ) is a biochemical test, used to differentiate between live and dead embryos based on the activity of the respiration enzymes in seeds (Smith, 1952). During seed hydration, the activity of dehydrogenase enzymes increases resulting in the release of hydrogen ions, which reduces the colorless tetrazolium salt solution (2,3,5-triphenyl tetrazolium chloride) into formazan, a red compound. Formazan stains living cells

which are respiring with a red color, while dead cells which are not respiring remain colorless. The viability of seeds is interpreted according to the staining pattern of seed tissues (Bennett & Loomis, 1949).

2.2.2.2 The test procedure

Preconditioning (hydration): During preconditioning, seeds are hydrated by placing them in water or between wet paper towels. As the seeds take up water, dehydrogenase enzymes become active which later react with TZ to indicate viability (Throneberry & Smith, 1955). Preconditioning is generally conducted under temperatures favorable for germination.

Seed Preparation (cutting or puncturing): During seed preparation, seeds are either cut or punctured to facilitate entry of the TZ solution into the embryo. Some weed species are known to have hard small seeds, and this process is performed under the microscope for accuracy. Not all seeds require special preparation. For instance, many dicotyledonous seeds such as beans, peas, and small legumes are placed directly into TZ without piercing or cutting. Such seeds are predominantly embryo and readily absorb TZ.

Staining: During staining, seeds are placed in a TZ solution (usually 1.0 or 0.1 %), and placed in an oven at about 38°C to speed up the staining reaction. Respiring embryos will stain; however, storage tissue such as the endosperm of grasses will not stain. The general rule is: use 0.1% TZ when the seed is bisected through the embryo before it is put in TZ; use 1.0% TZ when the seed is bisected laterally or diagonally, or pierced, or when no preliminary incisions are needed as for legumes. The length of the staining period varies with species. If seeds are kept too long in the TZ solution, they will become overstained, making evaluations more difficult.

Evaluation: Evaluation requires the most practice. Some seeds (such as grasses) may be placed in a clearing solution (such as 85% lactic acid) in order to clearly see through the seed coat to evaluate the staining pattern.

The purpose of this work was to determine estimated viability of a batch of seeds before a germination test was performed. In this work the seeds of *Chenopodium album* were held by forceps and punctured and part of the seed coat was removed, using a fine needle under a binocular microscope. This allowed hydration of the seed tissue. The seeds were then kept in 1.0% tetrazolium solution on a hotplate at 30-40°C for 2-3 hours to speed up the reaction, after that seeds were washed with distilled water, and examined under a binocular microscope by removing the remains of the seed coat to examine the stained embryo to determine the viability of the seed. Seed viability indicates that the seed is capable of germinating under suitable germination condition.

2.2.3 **Specific experimental procedures**

Four experiments were performed in order to test different aspects of the germination behaviors of *Chenopodium album* seeds. All the experiments followed the general experimental procedures indicated in section 2.2.1, and the specific differences between the procedures are indicated below.

2.2.3.1 Experiment 1

The seeds of *Chenopodium album* exhibit polymorphism and in *Chenopodium album* some seeds are brown and most are black, the seed coats also being smooth and reticulate respectively. Both these different seed forms may be found on the same plant (Williams,

1963), and there is evidence that the early seeds shed by the plant usually contain a higher than normal proportion of brown seeds (Williams & Harper, 1965). These polymorphic seeds differ in their requirements for germination by responding to chilling and burying differently. Because the seeds which were used (collected from Bradford, Organic Bracken farm) contained a lot of brown seeds (Mean ± 1 SE of 14.3 ± 0.918 % based on counting 8 lots of 100 seeds) the influence of seed morph was tested; seeds were six months old before being used. Two hundred brown seeds and two hundred black seeds were counted out and placed in 10 Petri dishes per seed type, each containing 20 seeds of *Chenopodium album*. The Petri dishes were then placed in a growth cabinet, and the general experimental procedures followed as described in section 2.2.1.

2.2.3.2 Experiment 2

Jursik *et al.* (2003) mentioned that the length of primary dormancy varied in *Chenopodium album* from 10 to 100 days depending on the date of ripening and on the year. Holm, *et al*., (1977) mentioned that primary dormancy length differs between populations and between individuals from the same population.

Dormancy is broken during cold periods of the year and induced as warmer periods progressed. This experiment aimed to study the extent of dormancy in the two populations from very different climates, and also to assess the influence of seed age on this dormancy. Seeds for the experiment came firstly from the Janzur site in Libya; secondly from Bradford, Yeadon; and thirdly from the Organic farm in Bradford. The trial was conducted one week after collecting the UK seeds from the organic farm and about two weeks after collecting of Libyan seeds. The Yeadon seeds were about two years old.

The general experimental procedures were followed using 40 Petri dishes (10 replicates each of old and new seeds, Libyan and UK populations).

2.2.3.3 Experiment 3

The changes in dormancy of seeds as a result of burying and chilling were investigated in this experiment, again testing for differences between the Libyan and UK populations. Sufficient *Chenopodium album* seeds were buried in envelopes made of fine mesh nylon gauze in loam based compost (John Innes No. 2) in a 10-cm diameter plastic pot to a depth of about 10cm and the pot watered till saturation. The envelopes were surrounded by soil to avoid any light reaching the seeds during burying. The pot was kept in a cold room (5°C ± 1°C) for one month, while the seeds that were chilled but not buried were kept on a shelf in the cold room in the dark. Seeds were then taken out, cleaned with distilled water and put in Petri dishes each with 20 seeds and 10 replicates of each treatment. The Petri dishes were then placed in a growth cabinet, and the general experimental procedure was followed.

2.2.3.4 Experiment 4

In this experiment all the factors were studied in combination, namely the effect of chilling, chilling plus burying, and nothing being treated; the region (seeds from the UK and Libya); and age (new seeds and old seeds) of *Chenopodium album*. The investigation of the combined effect of these factors on seed germination of *Chenopodium album* was undertaken with a view towards the development of strategies for a better control of this weed through understanding the triggers for enhanced and synchronized germination.

A total of 80 Petri dishes were used for the various combinations, with 10 replicates of each. Seeds used were from The Janzur site in Libya and the organic farm site and Yeadon in the UK. New seeds were those used within one month of collection, while old seeds were obtained from previous collections. Burial and chilling procedures were as described for Experiment 3. The general experimental procedures described in section 2.2.1 were then followed.

2.2.4 **Statistical analysis**

For each experiment the three variables measured were analyzed separately, namely total percentage germination, duration of the experiment (in days) and rate of germination (total germinated seeds divided by the number of days required to obtain total germination). However, since percentage germination is a bounded parameter (i.e. has an upper maximum of 100%) a transformation is required, the angular transformation of Fisher (Zar, 1984) was applied to maintain homogeneity of variance and these transformed data were also analyzed.

Comparing the responses of black and brown seeds in Experiment 1 was performed using a t-test. Experiments 2 and 3 were analyzed using a 2-factor analysis of variance (ANOVA) with replication, where the interaction term could also be tested for. Experiment 4 was analyzed by a 3-factor ANOVA (Zar, 1984). Data analysis used MINITAB® version 15.

2.3 Results

Considering the results of the initial TZ test, the viability of the Libyan seeds was higher and reached 97%, while the UK seeds were assessed to be 91% viable, the difference could be referred to the great variability in the environmental conditions.

A summary table of the results from all four of the other experiments is given in Table 2.1.

Table 2.1 Summary of the experiments of seed germination (L. C. means laboratory condition, B+C means burying plus chilling, C means chilling)

No. of experiment	Age*	Date	Condition	Source of seeds	Germination percent	Germination rate	Duration days	Average temp. °C
1	New	03/2009	L. C.	Black seeds	72%	3	24	21/15
				Brown seeds	57%	3.8	15	20/15
2	fresh	09/ 2008	L. C.	UK	0% *	0/200		27/20°C
	fresh			Libya	31%	62/19= 3.3= 1.7	19	
	new			Libya	47.5%	94/23= 4.1= 2.1	23	
	old			Libya	74%	148/22= 6.7= 3.4	22	
	new			UK	62%	124/27= 4.6= 2.3	27	
	old			UK	55%	110/27= 4.1= 2.1	27	
3	New	10/08	B + C	UK	73%	146/20= 7.3= 3.7	20	27 /17.5
			C	UK	78.5%	157/15= 10.5= 5.6	15	28/18.5
			B + C	Libya	42.5%	85/9= 9.4= 4.7	9	28/18.5
			C	Libya	47%	94/19= 4.9= 2.5	11	28/19
4	New 2009	01/09	B + C	UK	78.5%	197/7= 28.1= 11.2	7	20/15°C
	Old 2006		B + C	UK	82%	164/7= 23.4= 11.7	7	
	New 2009		B + C	Libya	49.5%	99/7= 14.1= 7.1	7	
	Old2007		B + C	Libya	71%	142/9= 15.8= 7.9	9	
	New 2009		C	UK	88%	176÷32=5.5=2.25	32	
	Old 2006		C	UK	44%	88÷31=2.8=1.4	31	
	New 2009		C	Libya	29%	58÷15=3.9=1.95	15	
	Old 2007		C	Libya	69.5%	139÷19=7.3=3.65	19	
	New 2009		L. C.	UK	81.5%	163÷23=7.1=3.55	23	
	Old 2006		L. C.	UK	59.5%	119÷29=4.1=2.05	29	
	New 2009		L. C.	Libya	28.5%	57÷11=5.2=2.6	11	
	Old 2007		L. C.	Libya	69.5%	139÷20=6.95=3.5	20	

2.3.1 **Results of experiment 1**

Brown seeds germinated more rapidly than black seeds, with peak germination after only four days compared to eight days for the black seeds (Figure 2.1). Therefore the rate of germination was significantly different for the two morphs (Table 2.2). The duration data were highly significantly different ($P < 0.001$; Table 2.2) reflecting the more rapid completion of germination for the brown seeds (Figure 2.1).

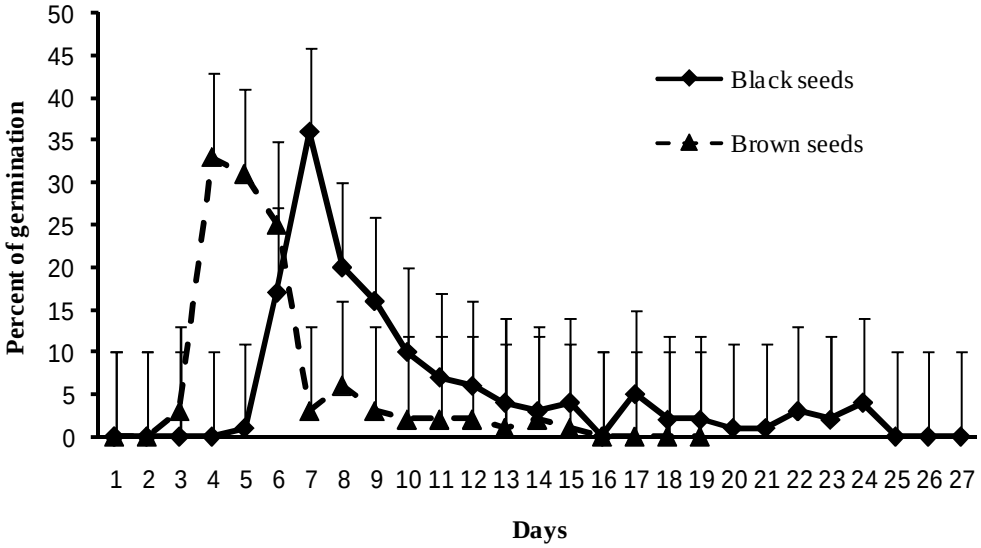


Figure 2.1 Germination curves of black and brown seeds of *Chenopodium album* showing the percentage of the seeds that germinated on each day separately. The vertical bars indicate + 1 S.E.

The overall percentage germination results were just significantly different but, when using the angular-transformed data rather than the original percentages, the two seed morphs

were not found to be different, although close to significance (Table 2.2). The peak germination percentages on days 4 and 8 were of about the same value (33% versus 36%). Thus overall these results show that the two seed morphs differ in the timing of their germination, with brown seeds germinating earlier, but not in the total amount of germination.

Table 2.2 Analysis by t-test of the germination data for black and brown seeds of *Chenopodium album*. Data include the total percentage germination, the angular transformation of the percentage, the duration of germination in days and the rate of germination. Key to significance:

P > 0.05, N.S.; P < 0.05, *; P < 0.01, **; P < 0.001, *.**

Variable	Black and Brown seeds			
	df	T. value	p	Significance
Germination %	16	2.18	0.045	*
Arcsine %	16	2.06	0.056	N.S.
Duration	17	8.54	< 0.001	***
Rate	12	-2.47	0.029	*

2.3.2 **Results of experiment 2**

Libyan new and old seeds germinated more rapidly compared with UK populations, with peak germination after 3 and 4 days respectively compared to 5 and 9 days for UK seeds (Figure 2.2). Therefore the rates of germination were highly significantly different between

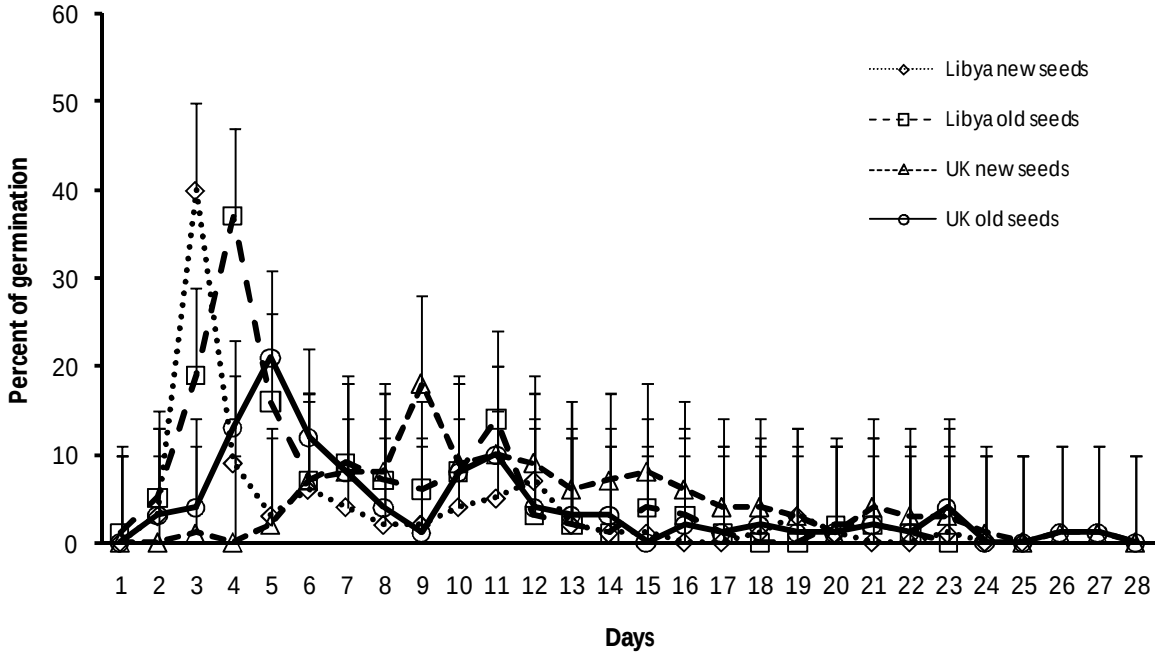


Figure 2.2 Results over time of germination of *Chenopodium album* seeds of two different ages and from two contrasting populations (Libya and the UK) showing the percentage of the seeds that germinated on each day separately.

The vertical bars indicate + 1 S.E.

the two populations (Table 2.3, Figure 2.3). The duration data were highly significant for the two ages but not significant between regions (Table 2.3). The interactions for both germination rate and particularly for duration were also significant. Also the total germination percentage without transformation or when using angular-transformed data showed the two populations to have very high significant differences when considering both age (Figure 2.3) and region (with germination of new seeds from UK being greater

than the other population Figure 2.3), as well as their interaction (since UK old seeds germinated less than the Libyan population).

Table 2.3 Effect of seed ages and region of origin (Libya and UK) and their interactions on germination percent and other variables of *Chenopodium album*. Abbreviations listed are: (df) the degrees of freedom, (F) ANOVA test value, and (p) probability; further details of the variables and a key to the levels of significance are given in Table 2.2.

Variable	Age				Region				Interaction			
	df	F	p	Sig.	df	F	p	Sig.	df	F	p	Sig.
Germination %	2	107.88	< 0.001	***	1	19.42	< 0.001	***	2	23.90	0.000	***
Arcsine %	2	150.77	< 0.001	***	1	38.76	< 0.001	***	2	42.40	0.000	***
Duration	2	71.42	< 0.001	***	1	2.46	0.123	N.S.	2	36.18	0.000	***
Rate	2	21.84	< 0.001	***	1	20.99	< 0.001	***	2	4.08	0.022	*

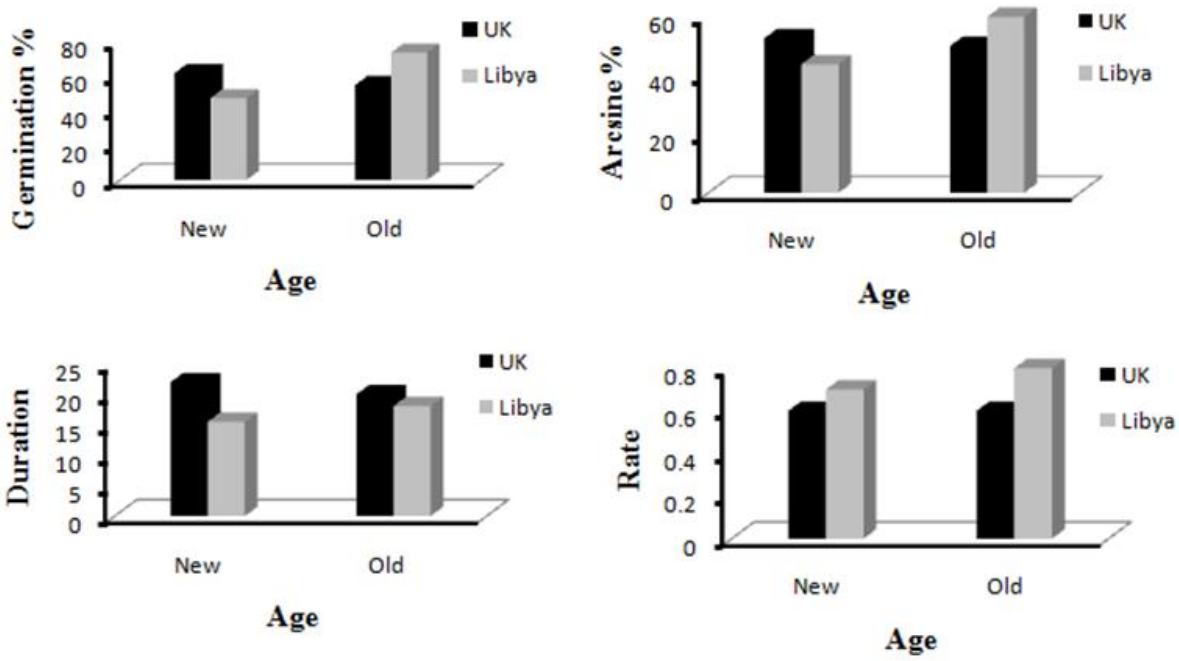


Figure 2.3 Results for four parameters of germination of *Chenopodium album* seeds of two different ages and from two contrasting populations (UK and Libya); 5% Least Significant Difference values for the percentage germination, arcsine percentage, duration and germination rate respectively are: 0.88, 4.64, 0.398, 0.148.

2.3.3 **Results of experiment 3**

The seeds started germinating on the first day and seeds from the chilling/burying treatment showed the highest germination rate. Analysis of germination of seeds of *Chenopodium album* under the treatment of effect of chilling and chilling/burying for the different regions is shown in Table 2.4. There is no clear difference in sensitivity of seeds from the different regions to the chilling treatment and chilling/burying on their own.

Treating seeds of *Chenopodium album* with a combination of chilling/burying resulted in 65% germination of UK seeds compared with 55% in Libyan seeds (Figure 2.4). This

difference between regions was also highly significant using the arcsine transformed data (Table 2. 3). There was no significant effect on duration or rate due to chilling or burying treatments by themselves (Table 2.4), but there was a separate effect of region on duration (Libyan seeds did not take so long to complete their germination), and a very significant interaction between treatment and seed origins (Table 2.4, Figure 2.4)

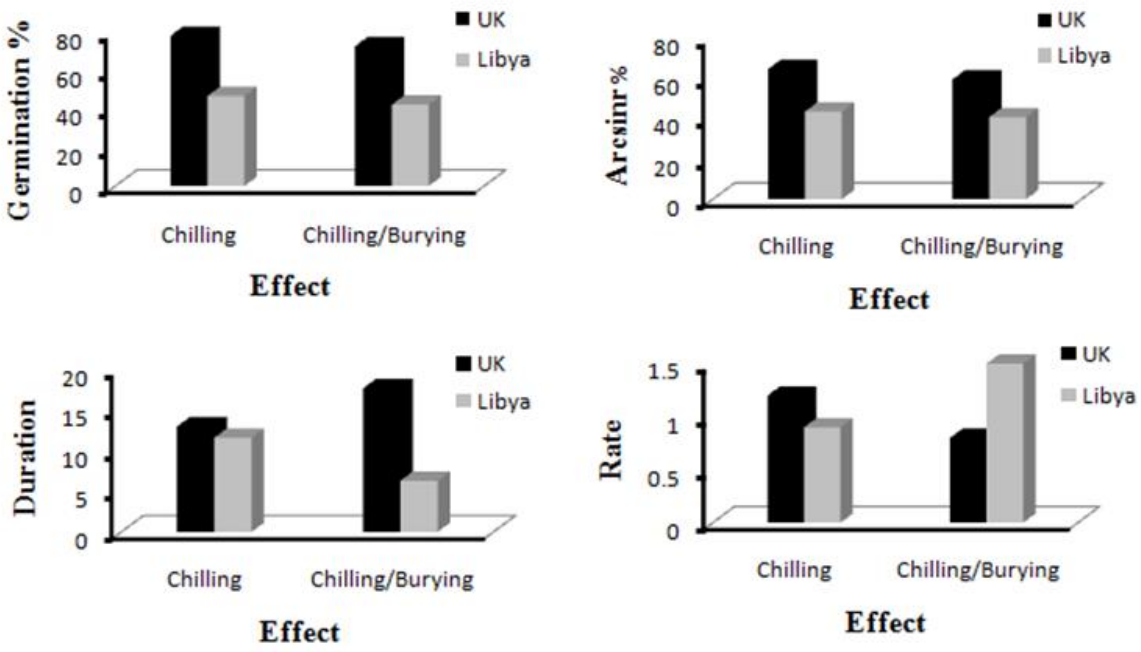


Figure 2.4 The influence of treatment effect of chilling and chilling/burying on four germination parameters (details in Fig 2.3) of seeds of *Chenopodium album*, 5% Least Significant Difference values for the percentage germination, arcsine percentage, duration and germination rate respectively are: 7.77, 5.698, 1.178, 0.207.

Table 2.4 Effect of chilling and chilling /burying and region and their interactions on germination percentage, germination rate and duration of seeds of *Chenopodium album*. Abbreviations listed are: (df) the degrees of freedom, (F) ANOVA test value, and (p) probability; the level of significance is indicated by symbols given in table 2.2.

Variable	Treatment				Region				Interaction			
	df	F	p	Sig.	df	F	P	Sig.	df	F	p	Sig.
Germination %	1	0.60	0.443	N.S.	1	68.46	< 0.001	***	1	0.15	0.700	N.S.
Arcsine %	1	1.94	0.173	N.S.	1	49.53	< 0.001	***	1	0.22	0.641	N.S.
Duration	1	0.11	0.738	N.S.	1	51.81	< 0.001	***	1	31.62	< 0.001	***
Rate	1	0.19	0.664	N.S.	1	1.02	0.318	N.S.	1	29.06	<0.001	***

2.3.4 **Results of experiment 4**

There was a highly significant influence of all three factors of age, treatment and region on the length of time before total seed germination was reached and on the rate in which germination was achieved. The germination percentage, however, was not significantly influenced by seed age, but it was affected by the other two factors (Table 2.5).

Table 2.5 Germination of seeds of *Chenopodium album* in response to the factors of seed age, chilling/burying treatments and geographical region. Variables and symbols as in Tables 2.2 and 2.3.

Variable	Age				Treatment				Region			
	df	F	p	Sig.	df	F	p	Sig.	df	F	p	Sig.
Germination %	1	3.79	0.054	N.S.	2	4.77	0.010	*	1	29.34	< 0.001	***
Arcsine %	1	2.59	0.110	N.S.	2	4.24	0.017	*	1	4.24	0.017	*
Duration	1	49.34	< 0.001	***	2	108.57	< 0.001	***	1	40.41	< 0.001	***
Rate	1	8.36	0.005	***	2	183.73	< 0.001	***	1	10.60	< 0.001	**

The results concerning each of the three factors are illustrated in order to aid clarity; Figure 2.5 shows the effects of seed age, the effects of the treatment (laboratory condition, chilling and chilling/burying), and the differences between the two regions.

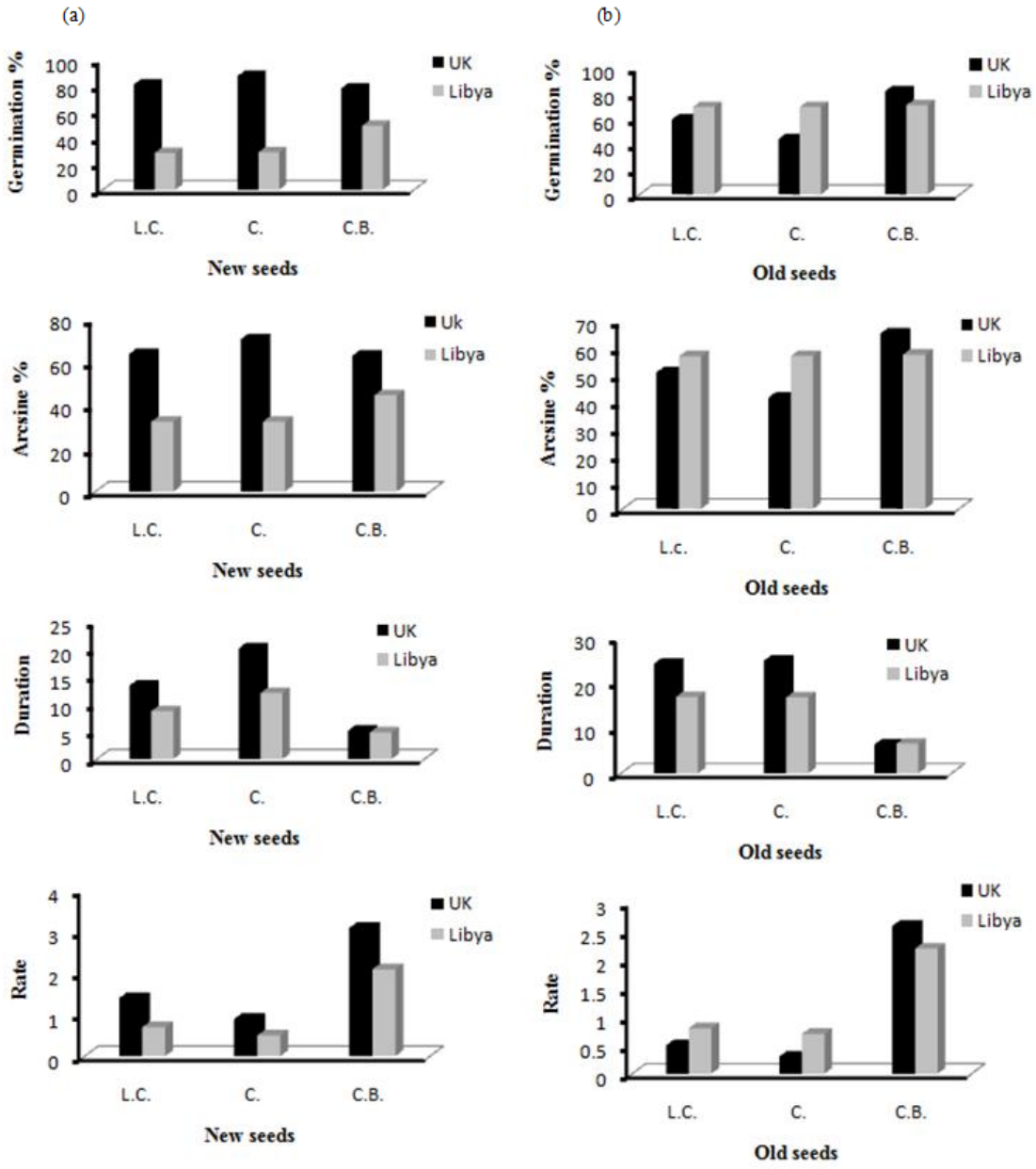


Figure 2.5 Influence of laboratory condition (L.C.), chilling (C.), and chilling/burying (C.B.) on four parameters of seed germination of *Chenopodium album* for (a) new seeds and old seeds (b), 5% Least Significant Difference values for the percentage germination, arcsine percentage, duration and germination rate respectively are: 93.82, 59.988, 194.93, 0.215.

Figure 2.5 shows that chilling/burying of *Chenopodium album* seeds increased germination slightly but not significantly (Table 2.5). The Libyan population showed the highest percent of germination with old seeds across all treatments, while with the UK population the new seeds germinated more (Figure 2.5). This pattern was essentially repeated with the arcsine transformed germination data, and with the germination rate. The duration data are therefore greatest for the UK old seeds as they take longer to complete germination.

Chilling/burying of the old seeds of the UK population of *Chenopodium album* appears to have the effect of increasing germination while it has only a limited effect on Libyan old seeds. Also the figure 2.5 shows the two populations of seeds responded in different ways by taking a different length of time to germinate, the Libyan old seeds usually respond faster when untreated but the UK old seeds respond faster after chilling/burying.

2.4 Discussion

This chapter has explored certain aspects of the germination behavior of *Chenopodium album* seeds in order to obtain a better picture of the likely triggers for germination of this species, and therefore to be able to predict more accurately when might be a suitable time to apply the mycoherbicide treatment. The seed bank is one of the main targets in weed research because it is considered as an enormous reservoir of viable dormant weed seeds present in agricultural soils (Schonbeck & Egley, 1980). Particularly in UK, where seeds will be showing dormancy overwinter and then will germinate in the spring, understanding when emergence is likely to happen should be very helpful. A notable focus in this chapter

has also been whether there are any differences between seeds from UK populations and those from a region with very different climate conditions, namely Libya.

One difference that was found between the two populations was in the percentage of brown seeds recorded; no brown seeds were recorded at all in the Libyan population, while a value of 14.3 ± 0.92% was found in the UK population. When tested (Experiment 1), it was found that the brown seeds germinated more rapidly than did the black seeds, which confirms the findings of Williams and Harper (1965) who also noted a similar difference in germination rate of the two morphs. In the context of the UK population, the presence of these two morphs may represent a form of 'bet-hedging', so that not all *Chenopodium album* seeds respond in the same way to environmental triggers, and hence not all germination will happen at once. This makes prediction of precise emergence times, and therefore the timing of mycoherbicide spraying, more difficult. The situation in Libya may be more uniform, and hence prediction might be easier there.

The results from Experiment 2 also demonstrated that both Libyan new and old seeds germinated more rapidly compared with those from the UK. However, the final germination percentage was higher in new than old UK seeds, but higher in old rather than new Libyan seeds.

Williams and Harper (1965) have previously found chilling of *Chenopodium album* seeds increased germination from about 30% to about 65% provided a minimum of 21 days of exposure to cold was experienced. The present results (experiment 3 and 4) confirmed results such as those of Chu *et al*., (1978) that chilling, and especially chilling combined with burying in the dark, stimulated greater germination. The present study showed that 88% of treated seeds would germinate under optimal conditions of 20°C and discontinuous

light, with maximum germination obtained in less than 10 days. However, the results also showed a very significant interaction between seed treatment and seed origins. Thus chilling and burying resulted in 60 % overall germination of UK seeds, but only 55 % in Libyan (experiment 3) seeds; and increased germination from about 62% (Experiment 2) to 88% (experiment 4) in the UK population while appearing to have no major effect on Libyan seeds (experiment 4). Also, the UK new seeds germinated more rapidly than those from Libya following treatment.

Overall, therefore, besides confirming the effects of chilling treatments in breaking dormancy of *Chenopodium album* seeds, and confirming the differing germination behaviors of the two seed morphs, the experiments described in this chapter have demonstrated that there are notable differences in germination behavior of seeds from the two regions of the UK and Libya. This is perhaps not surprising, given the findings reported in Williams, (1963) that show the requirements in this species differ not only from one population to another but also from one plant to another. The length of primary dormancy can also vary over similar scales (Holm *et al.*, 1977), with the strength of primary dormancy being strongly influenced by the day length during the seed ripening period (a long day promotes the production of strongly dormant seeds, but short day promotes non-dormancy, so that seeds produced in late summer will germinate direct immediately). Hence the species is highly variable, it is clear that, with such a variable weed species that is also found in so many different geographical regions, it will be difficult to make general predictions of the sort that would be useful for targeting spraying that can be applied equally throughout the range of the species.

Chapter3: Evaluation of the bioherbicide formulation efficacy of *Ascochyta caulina* on different life stages of the weed plant *Chenopodium album*

3.1 Introduction

As indicated in section 1.4.1 chapter 1, when developing a biocontrol agent it is important to identify methods that can increase the effectiveness and/or reduce the cost of a biocontrol treatment (TeBeest *et al.*, 1992). This may include creating optimum conditions for germination of the biological agent. The development of a formulation can involve mixing or blending of active ingredients, such as spores with nutrients, inert carriers or components that reduce or decrease the long period of high humidity which is very often necessary for successful germination of fungal spores or propagules. It is vital that the application of the formulation does not affect germinability and vigor of growth of the bioagent (Weaver *et al.*, 2007). The invert (water-in-oil) emulsions can provide a favorable micro-environment around the spores during the infection process, thereby reducing the time of dew needed as well as the amount of inoculum (Amsellem *et al.*, 1990 , Connick *et al.*, 1991b , Boyette *et al.*, 1993), but unfortunately emulsions are difficult to apply with standard equipment. Some formulations have been shown to greatly enhance the pathogenicity of spores against weeds (Mintz *et al.*, 1992). For example application of *Alternaria cassiae* Jurair & Khan on sicklepod *Cassia obtusifolia* seedlings in invert emulsions without a dew period gave 88% mortality compared to 0% mortality when applied as conidial suspension (Zidack & Quimby, 2001). Oil-in-water emulsions can be applied with standard equipment. The potential of a mycoherbicide has to be tested under field conditions in combination with the

crop. However, the importance of environmental conditions on spore germination has been recognized since the early stages of mycoherbicides research (TeBeest *et al.*, 1978 , TeBeest & Templeton, 1985). Two important environmental components that limit spore germination and consequently the effectiveness of biological control agents are temperature and moisture (TeBeest *et al.*, 1992). The development of an effective bioherbicide requires a comprehensive understanding of the pathogen involved the biology and population dynamics of the target weed, the optimum requirements for disease development in the host-pathogen system, and the virulence of a mycoherbicide candidate to confirm its effectiveness.

Many studies have been done on the use of *Ascochyta caulina* to control *Chenopodium album* (Kempenaar, 1995 , Mendi, 2001 , Ghorbani *et al.*, 2002 , Stamatis, 2002 , Ghorbani *et al.*, 2006). Formulations containing different combinations of *Ascochyta caulina*, the phytotoxins from the fungus and low doses of herbicides have been tested, and significant improvement in the efficacy was achieved in glasshouse trials. However, they have still suffered from the limitations discussed previously, particularly in respect of difficulties of maintaining a long enough period for the fungus to become established on the host in field strategies.

However, none of the previous trials have focused on the use of the formulae of oils with the fungus to optimize the efficacy of the mycoherbicide to control the *Chenopodium album*, which as indicated in chapter one is a promising alternative way of formulating fungal applications.

The primary objective of the research described in this chapter is to optimize the biological control agent of the weed *Chenopodium album* by applying the pathogenic fungus *Ascochyta caulina* more effectively as a microbial herbicide (mycoherbicide) by developing a new chosen formula. This is because, as mentioned above, as far as practical application is concerned, two major challenges remain: to increase the activity of the fungus on *Chenopodium album*, and reduce its dependency on environmental conditions. The increased activity of the fungus will hopefully be aided by the choice of strain of the pathogen, since different strains can vary markedly in their activity on the host. The requirements to overcome the dependency on high air humidity will be met by experimenting with two formulae, (Tween 80 and Gelatine), and by using soybean oil because it has been found to be the ideal alternative to paraffinic oil (Womack *et al.*, 1996).

A further objective is to compare the effects of using the formulations on plants of different ages (life stages), in order to assess when application of the mycoherbicide would be most effective in the life cycle of the weed. Thus, the activity of the most promising novel formula will be tested under laboratory conditions and different plant developmental stages to find out the most susceptible stages to the mycoherbicide.

3.2 Materials and methods

3.2.1 Inoculum source

The biological control agent used in this study was *Ascochyta caulina* (P. Karsten) v.d. Aa and v. Kesteren. Large differences in pathogenicity between strains of *Ascochyta caulina*

have been demonstrated (Kempenaar, 1995). For this study a stock of *Ascochyta caulina* strain 1058 was obtained from Italy (Istituto Tossine e Micotossine da Parassiti Vegetali).

The origin of this strain of *Ascochyta caulina* was isolated from a diseased leaf of *Chenopodium album*, supplied by Dr. P. C. Scheepens (Department of Crop and Production Ecology, Wageningen University and Research Centre, The Netherlands) and is maintained on potato dextrose agar medium as a single-spore culture in the Collection of Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy (ITEM 1058).

The active stock cultures were maintained on Oatmeal agar (Sigma Co. LTD. Poole UK) at 4℃. Starter cultures were prepared by aseptically transferring small mycelium pieces from stock cultures to Oatmeal Agar plates and storing at 22°C in continuous light.

3.2.2 **Inoculum production and spore preparation**

The mass production and storage of the inoculum source is a crucial step in the development of a potential mycoherbicide. The mass production of *Ascochyta caulina* on different solid substrates has been previously investigated and Oat Meal Agar was found to be the most suitable (Mendi, 2001). The stock culture of the fungus used in this thesis was sub-cultured on Oatmeal agar (72.5g Sigma Co. Ltd, Poole UK, added to one liter distilled water shaken and autoclaved at 121℃ for 15 minutes at 1.1 atmosphere) in Petri dishes (9cm diameters, Bibbly Sterilin Ltd., Stone, Staffs, UK.). Each Petri dish contained approximately 25 ml of the media. *Ascochyta caulina* spores from stock cultures of stored Oatmeal Agar were streaked on the surface of the media of Oatmeal Agar plates and

incubated in an incubator (Gallenkamp, Economy incubator with fan size 2) at 22°C under continuous cool white florescent lamps positioned on shelves to shine from outside through the glass door of the incubator (florescent lamp 8W) for a period of 2-3 weeks. The effect of light on many fungi is to stimulate the production of spores or fruiting bodies, with short exposures to sunlight or ultraviolet rays often being a useful method of inducing sporulation in cultures (Leach, 1967). After a two week period, surfaces of inoculated media were completely covered with mycelium growth. After 2- 3 weeks the spores (conidia) of the fungus were harvested by flooding the agar plate cultures with 10 ml of distilled water for three hours (three hours of incubation of the fungal cultures were sufficient to release most of the spores from the pycnidia (Kempenaar, 1995)). The culture surface was then pressed or scraped lightly by spatula in order to release more spores and then the resulting spore suspension filtrate was mixed by magnetic stirrer (Stuart Scientific, Magnetic stirrer SM1, made in UK) for one hour to release again more spores from pycnidia and break the fungal mycelia. After that the liquid was poured off and filtered through a double layer of muslin to remove mycelium fragments. Conidia concentration of the collected suspension was estimated by examination on a haemocytometer (Hawksley, double cell, Thoma, UK) under a light microscope (40X objective lens) the suspension was adjusted with distilled water to the desired density value for inoculation (1×10^6 spores ml^{-1}). Freshly harvested conidia were used as inoculum for all experiments.

3.2.3 **Plant production**

Mature seeds of *Chenopodium album* were collected from Bradford near Leeds Bradford airport (Yeadon, West Yorkshire N53° 78' 33", W1° 75' 00") August 2006 and seeds

collected from Libya (Janzour 17 km western of Tripoli – Libya (N32° 81' 72", E13° 01' 11") in September 2007 and stored in plastic bottles at room temperature until used. In all experiments plants were grown from seeds of *Chenopodium album* which were germinated in Petri dishes with moist filter paper (Whatman No. 2) and then the germinated seeds of the first and second day (which were few in number) discarded from the Petri dish and seeds which germinated on the third day (when sufficient germinated) were transferred to plastic pots (10 cm diameter, 10 cm depth) containing loam based compost (John Innes number 2). Plants were grown in a growth cabinet (average daily temperatures were between 20- 15°C day/ night, mean relative humidity was about 50%, and mean daily light intensity was 78 μmol m^{-2} s^{-1} with a photoperiod of 14: 10 hours light to dark). Pots were watered with tap water as required. In this study five life stages, each one week older than the previous one, were used from age of one week till five weeks, using one single plant in each pot (Figure 3.1). A total of 90 plants were used in this experiment divided into three treatments or groups: group one was treated with a formula of Tween 80, group two was treated with a formula of Gelatine and the third group was a control with untreated plants. Each group consisted of 30 plants, 6 replicate plants of each the five life stages. The *Chenopodium album* plants were monitored for a period of 10 days.

Figure 3.1 Different life stages of the weed plant *Chenopodium album*, age in weeks starting from one week on the left to week six on the right.

3.2.4 **Oil emulsion preparation**

Particular efforts were made to investigate the most suitable formulation to reduce the dew requirement, in an attempt to overcome environmental constraints on disease expression. Under natural conditions, the invert emulsion formulations and the vegetable-oil emulsion formulations have been found to give better results than aqueous formulation (Shabana, 2005), and these have been experimented with here.

Simple vegetable oil emulsion containing 10% oil and 1% of an emulsifying agent showed promise in reducing dew dependence in controlled environment studies using *Colletotrichum orbiculare* (Auld, 1993 , Sandrin *et al.*, 2003); this type of formulation was first recognized by Quimby *et al.*, (1988). These formulations have been shown to overcome dew requirements and reduce the spore concentrations required (Amsellem *et al.*,

1990 , Boyette *et al.*, 1993 , Womack *et al*., 1996). Soybean oil has been found to be particularly suitable and ideal alternative to paraffinic oil (Womack *et al*., 1996)

The first formula (Tween 80) of mycoherbicide used in these experiments consisted of 445 ml fungal spore suspension plus 50 ml soybean oil plus 5 ml Tween 80 emulsifying agent (Tween 80, Fisher Scientific UK. Limited).

The second formula (Gelatine) of mycoherbicide consisted of 445 ml fungal spore suspension plus 50 ml soybean oil plus 5 grams Gelatine as emulsifying agent (Gelatine, Sigma). Soybean oil was found to be an ideal alternative to paraffinic oil (Womack *et al.*, 1996), and the two emulsifying agents were used because they were found to be nonfungitoxic and capable of emulsifying the more polar oil phases. In summary, Gelatine, and Tween 80 were useful components for bioherbicide formulations to increase conidial germination and mycelia growth. Saxena and Pandy (2002) used Tween 80 with *Alternaria alternata* to control *Lantana camara* and they achieved good results with this surfactant. Pfirter and Defago (1998) modified the formulation with Tween 80 and enhanced the pathogen viability during infection. Pfirter *et al*., (1999) used Tween 80 with vegetable oil emulsion to prepare *Stagonospora convolvuli* mycoherbicide to control field bindweed, while Zhang *et al*., (2003) found that Gelatine stimulated conidial germination in the *Phoma* isolates by releasing conidia from the effect of self-inhibition of germination at 1×10^{8} conidia ml^{-1} for *Phoma medicaginis*.

The oil formulation was prepared in a volumetric flask by adding the soybean oil and the 1% of emulsifying agent and mixed by a vortex (Griffin vortex stirrer S37-890) at high speed for one minute. The aqueous phase, containing the spore suspension was added to

the oil phase and vortexed again for an additional one minute to give a final concentration of (1×10^6 spores ml^{-1}).

3.2.5 **Testing viability of fungal spores after formulation**

To determine the effect of the mycoherbicide on spore germination, two hundred µl of the mixture were spread with a sterile glass rod on a sterile glass slide containing water agar medium and placed on two wood match sticks inside a Petri dish with a moistened filter paper to keep the humidity high inside the Petri dish (Figure 3.2). Three replicates were prepared.

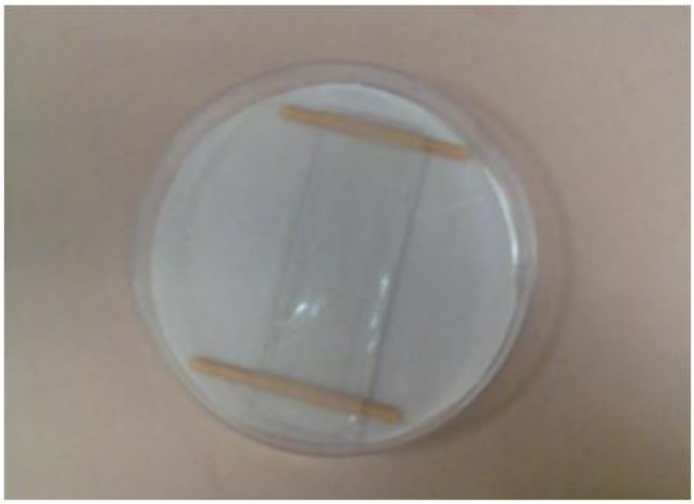


Figure 3.2 Microscope slide with water agar inside the Petri dish

The Petri dishes and their contents were incubated at 22°C for 24 hours. After incubation the slides were removed from the Petri dishes and each covered with a glass cover slip. Fifty spores per slide were counted under a microscope at 400× magnification, and the

percentage of spores germinated was calculated as an average of the three replicates of every experiment. Germination of the spores was judged to have occurred if the protrusion of a germ tube was clearly visible from the conidia.

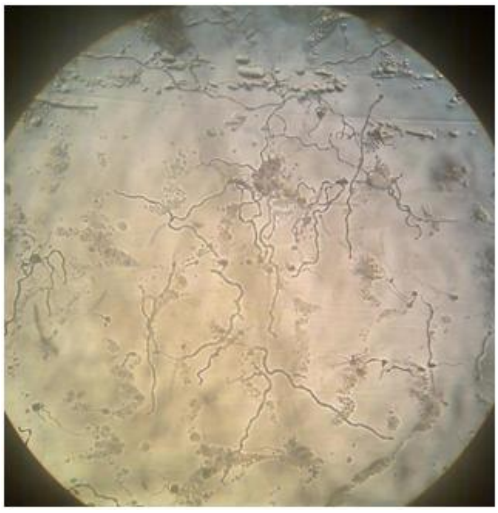


Figure 3.3 Spores of the fungus starting to form mycelium

The results of this viability test showed that the two formulae had no marked negative effect on the germination of the spores of the fungus *Ascochyta caulina* (Figure 3.3), with the average percentage germination of the Tween 80 formula being 98% and the formula of Gelatine giving 95%.

3.2.6 **Inoculation procedure and weed control assessment**

In all experiments plants were sprayed to run-off with the relevant treatment using a manual operated sprayer (Solare 1 liter hand spray, active products UK Ltd.) from approximately 20 cm distance, inoculation was performed on all five plant ages namely one, two, three,

four, and five week old seedlings at a conidia density of (1×10^6 spores ml^{-1}). Suspensions were applied to the upper and lower sides of leaves if possible, and plants were returned to the original growth cabinet immediately after inoculation. Control plants were sprayed only with water oil emulsion emulsified with Tween 80 without the biocontrol agent (fungal spores). Temperature (in °C) and humidity (percentage Relative Humidity) were recorded daily. Disease severity and differences among treatments were assessed after 10 days by using four parameters: disease severity rating, fresh and dry-weight determinations, and root length, these are described below.

3.2.6.1 Assessment of Disease Severity Rate

The disease severity was assessed initially for each leaf on a plant according to a scale rating from 0- 6 where:

0 = no symptoms, 1 = < 6%, 2 = 6- 25%, 3 = 26- 75%, 4 = 76- 95%, 5 = > 95% of leaf surface with necrosis, 6 = leaf dead (Pfirter & Defago, 1998).

The Disease Severity Rate for an individual plant is then calculated using the formula:

$$\text{Disease Severity Rate} = \frac{(2.5 \times n1 + 15 \times n2 + 50 \times n3 + 85 \times n4 + 97.5 \times n5 + 100 \times n6)}{N}$$

Where: nx is the number of leaves with rating x

N is the total number of treated leaves of the same plant.

3.2.6.2 Assessment of fresh and dry weight parameters of plant growth

Live plants were cut at the soil level line and the above ground biomass weighed fresh, then dried in an oven (GENLAB Limited Model No. N200SF) at a temperature of 70ºC for 48 hours and weighed again. The dry weight reduction was calculated by comparing the dry weight in inoculated and control plants.

3.2.6.3 Assessment of root length

Remains of the living roots after cutting the shoots were measured by a ruler. The pots were saturated with water and after that the pot was put under running water to release the roots from the soil, the roots cleaned with water; measurement of the root length was recorded in centimeters.

3.2.7 Experimental design for Experiments 5 and 6 and data analysis

A complete randomized block design was used in the experimental design, with different life stage and different treatments as the factors; statistical analysis was performed using Minitab® version 15 software. Two way ANOVA with interaction procedures were used. Experiment 5 was repeated (Experiment 6) to confirm the conclusions reached.

3.2.8 Experimental design for Experiment 7

To confirm the results of the effect of mycoherbicide, a small observation experiment was carried out on the UK and Libyan populations of *Chenopodium album*. Five life stages

were chosen starting from the age of one week, with four replicate plants from each life stage, growing two plants in each pot. The same numbers of plants at the various life stages were used from each population but without mycoherbicide treatment as a control. For this experiment, the formula with Tween 80 only was used.

The plants were kept in the growth chamber after spraying with the mycoherbicide, and after 30 days were assessed visually, recording plants simply as being living or dead.

3.3 Results

In order to facilitate comparison between the two experiments (5 and 6), the results presented below are organized parameter by parameter, including the results from both experiments in the relevant section.

3.3.1 **Results from Experiments 5 and 6 for Disease Severity Rate**

The results from experiment 5 for Disease Severity Rate are given in Table 3.1 for the two treatments involving the fungal pathogen; there was no evidence of disease on any of the control plants, and the results are not presented in the table. Disease symptoms ranged from slight necrosis on cotyledons and leaves of old stages, to extensive necrotic lesions spreading to all leaves and stems of young stages of inoculated plants. Mortality of 100% was observed in the first three life stages.

Table 3.1 Results from Experiments 5 and 6 for Disease Severity Rate; values are the percentage of plants showing symptoms of disease

No.	Life stage	Experiment 5		Experiment 6	
		Tween 80	Gelatine	Tween 80	Gelatine
1	1	100%	100%	100%	100%
2	1	100%	0%	100%	50%
3	1	100%	100%	100%	100%
4	1	100%	0%	100%	0%
5	1	100%	50%	100%	100%
6	1	100%	0%	100%	0.63%
7	2	75%	1.25%	100%	65.63%
8	2	100%	7.5%	100%	1.50%
9	2	57.5%	7.5%	100%	1.50%
10	2	75%	57.5%	100%	100%
11	2	57.5%	50.13%	100%	100%
12	2	100%	15%	100%	0.71%
13	3	81.7%	15%	100%	25.36%
14	3	75%	5.83%	100%	65.28%
15	3	58.3%	8.67%	100%	32.9%
16	3	24.17%	43.33%	100%	100%
17	3	24.17%	50%	100%	67.8%
18	3	24.17%	40.8%	100%	1.07%
19	4	64.29%	21.5%	100%	65.6%
20	4	62.5%	21.75%	100%	46.25%
21	4	75%	6.5%	100%	54.69%
22	4	62.5%	24%	100%	23.4%
23	4	62.5%	21.75%	100%	12.18%
24	4	87.5%	21.75%	100%	22.50%
25	5	60%	23%	100%	16.7%
26	5	60%	23.25%	100%	40.6%
27	5	58%	23%	100%	36.36%
28	5	39.5%	23.25%	100%	38.5%
29	5	70%	22.5%	100%	43.8%
30	5	39.5%	23.25%	100%	40.5%

When these results were analyzed by ANOVA (Table 3.2), the differences between treatments were very highly significant ($p < 0.001$), and the life stages also were highly significantly different ($p < 0.01$). However, there was not a significant interaction between these two factors (Table 3.2). This indicates that both factors are having a marked effect, but as there is no interaction the two factors are influencing the results independently of each other.

Table 3.2 Disease Severity Rate results for experiment 5 analyzed by two-factor ANOVA with replication. Significance levels are denoted by:

N.S. $p > 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F- value	Probability	Significance
Treatments	1	27554.3	27554.3	57.6	< 0.001	***
Life stage	4	8799.2	2199.8	4.59	0.003	**
Interaction	4	3117.4	779.3	1.6	0.182	N.S.
Error	50	23939.2	478.8			
Total	59	63410.1				

In order to explore these results further, the surface plot for the two treatments have been drawn (Figure 3.4). It shows that the mycoherbicide formula of Tween 80 has a great effect on the weed plant, while the formula of Gelatine has some but a lesser effect (Figure 3.4). Also, the effect of the fungus is most pronounced at the youngest life stage especially with the Tween 80 formulation where there was nearly 100% infection, while at all other life stages the results are lower and less variable (Figure 3.4). Thus the fungus has an effect at all life stages, but is most effective if applied within approximately the first week after seedling emergence.

Figure 3.4 Surface plot of Severity Disease Rate % (DSR) results for the two treatments and five life stages included in Experiment 5,

(Least Significance Difference = 17.889; n=12; $p \leq 0.05$).

The results for Disease Severity Rate from experiment 6 are also given in Table 3.1. Analysis of variance calculations show again a very highly significant difference ($p < 0.001$) in treatment effects (Table 3.3). However, in contrast to the results from experiment 5, there is no significant effect demonstrated due to the life stages (Table 3.3). There is also no significant interaction effect (Table 3.3).

Table 3.3 Disease Severity Rate results of experiment 6 analyzed by two-factors ANOVA with replication. Significance levels are denoted by:

N.S. p > 0.05; ** p < 0.01; *** p < 0.001.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F -value	Probability	Significance
Treatments	1	45184.9	45184.9	67.90	< 0.001	***
Life stages	4	993.9	248.5	0.37	0.827	N.S.
Interaction	4	993.9	248.5	0.37	0.827	N.S.
Error	50	33273.8	665.5			
Total	59	80446.6				

The surface plot of Disease Severity Rate (DSR) results for the two treatments and five life stages included is shown in Figure 3.5. As with the results from Experiment 5, they show that the mycoherbicide formula of Tween 80 has a great effect on the weed plant (nearly 100% of plants affected), while the formula of Gelatine has less effect although still about 40% of plants were affected (Figure 3.5). The Tween 80 formula has essentially the same effect at all life stages, but the Gelatine formula has a greater effect at the youngest life stage in common with the results from Experiment 5; however, the difference between this stage and others is much less pronounced than in the previous experiment.

Figure 3.5 Surface plot of Disease Severity Rate % (DSR) results for the two treatments and five life stages included in Experiment 6,

(Least Significance Difference = 21.105; n=12; $p \leq 0.05$).

3.3.2 **Results from Experiments 5 and 6 for biomass**

The impact of the mycoherbicides on *Chenopodium album* biomass in experiment 5 was to decrease the fresh weight (Table 3.4).

Table 3.4 Results from Experiments 5 and 6 for biomass (weight in grams) (T – Tween 80 formula treatment, G- Gelatine formula treatment, C- Control treatment).

Plant No.	Life stage	Experiment 5						Experiment 6					
		Fresh weight			Dry weight			Fresh weight			Dry weight		
		T	G	C	T	G	C	T	G	C	T	G	C
1	1	0	0.026	0.028	0	0.003	0.002	0	0	0.013	0	0	0.001
2	1	0	0.016	0.028	0	0.001	0.002	0	0.005	0.007	0	0.003	0.000
3	1	0	0.019	0.028	0	0.002	0.002	0	0	0.014	0	0	0.001
4	1	0	0.015	0.029	0	0.001	0.002	0	0.007	0.010	0	0.001	0.001
5	1	0	0.013	0.020	0	0.001	0.002	0	0	0.016	0	0	0.001
6	1	0	0.015	0.017	0	0.001	0.001	0	0.005	0.019	0	0.001	0.002
7	2	0	0.023	0.033	0	0.002	0.003	0	0.012	0.041	0	0.002	0.001
8	2	0.006	0.028	0.029	0	0.003	0.003	0	0.009	0.043	0	0.002	0.002
9	2	0	0.044	0.040	0	0.005	0.004	0	0.012	0.036	0	0.003	0.003
10	2	0	0.012	0.032	0	0.001	0.003	0	0	0.035	0	0	0.001
11	2	0	0.019	0.030	0	0.002	0.003	0	0	0.032	0	0	0.002
12	2	0.015	0.022	0.039	0	0.002	0.004	0	0.058	0.048	0	0.006	0.004
13	3	0.016	0.095	0.185	0.003	0.004	0.010	0	0.015	0.066	0	0.002	0.030
14	3	0.023	0.122	0.308	0.003	0.006	0.022	0	0.021	0.083	0	0.003	0.002
15	3	0	0.089	0.273	0	0.007	0.017	0	0.032	0.059	0	0.005	0.003
16	3	0	0.074	0.239	0	0.004	0.014	0	0	0.077	0	0	0.001
17	3	0	0.096	0.261	0	0.004	0.015	0	0.022	0.063	0	0.003	0.002
18	3	0	0.096	0.207	0	0.002	0.010	0	0.084	0.169	0	0.004	0.004
19	4	0.201	0.423	0.993	0.023	0.043	0.069	0	0.047	0.276	0	0.006	0.010
20	4	0.191	0.450	1.162	0.023	0.040	0.089	0	0.092	0.225	0	0.012	0.014
21	4	0.181	0.662	1.084	0.022	0.060	0.088	0	0.073	0.202	0	0.008	0.009
22	4	0.192	0.657	0.969	0.013	0.058	0.080	0	0.091	0.162	0	0.005	0.006
23	4	0.098	0.405	1.325	0.005	0.040	0.112	0	0.116	0.211	0	0.009	0.007
24	4	0.134	0.546	0.779	0.016	0.054	0.057	0	0.063	0.129	0	0.006	0.007
25	5	0.525	1.610	2.861	0.062	0.178	0.226	0	0.096	0.146	0	0.005	0.007
26	5	1.024	1.712	2.640	0.135	0.193	0.213	0	0.093	0.198	0	0.009	0.015
27	5	1.040	1.222	3.356	0.134	0.145	0.197	0	0.077	0.615	0	0.006	0.042
28	5	0.875	2.042	3.135	0.126	0.229	0.227	0	0.066	0.318	0	0.003	0.019
29	5	0.628	1.946	2.864	0.093	0.206	0.262	0	0.089	0.560	0	0.009	0.035
30	5	0.871	1.460	2.385	0.122	0.156	0.202	0	0.087	0.293	0	0.008	0.021

Both individual factors (treatments and life stage) were very highly significant ($p < 0.001$; Table 3.5), and there was also a very highly significant ($p < 0.001$) interaction term. In view of the significant interaction, it is necessary to consider both factors together, rather

than separately, and this is shown by a surface plot of the results in Figure 3.6. It can be seen that, unsurprisingly, there is a greater biomass with the older life stages; however, this increase with stage is most marked in the control treatment and least with the Tween 80 treatment (the Gelatine treatment being intermediate).

Table 3.5 Analysis of variance results of Experiment 5 of the shoot fresh weight of all treatments and life stages.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F -value	Probability	Significance
Treatments	2	6.31	3.16	153.80	P < 0.001	***
Life stages	4	41.05	10.26	500.01	P < 0.001	***
Interaction	8	8.95	1.12	54.50	P < 0.001	***
Error	75	1.54	0.02			
Total	89	57.85				

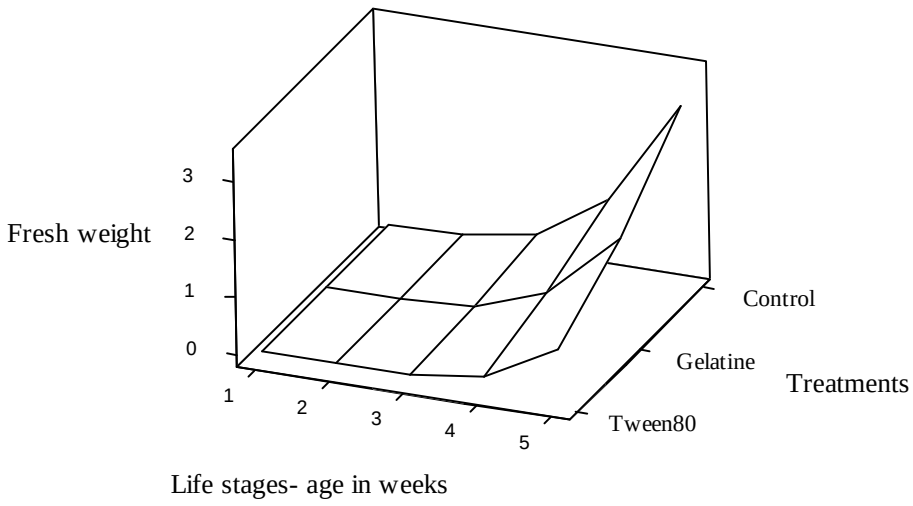


Figure 3.6 Surface plot of fresh weight results (in grams) for the three treatments and five life stages included in Experiment 5,

(Least Significance Difference = 0.094; n=18; $p \leq 0.05$).

The results from Experiment 5 for shoot dry weight (Table 3.4) show very much the same pattern of statistical significances (Table 3. 6) as for fresh weight, and the surface plot of the results again shows much the same pattern, although with these data the difference between the Tween 80 results and those of the Control are not so pronounced as for the fresh weights.

Table 3.6 Results of the analysis of variance for Experiment 5 of the shoot dry weights (g) of all treatments and life stages.

Source	Degrees of freedom	Sum of Squares	Mean Square	F- value	Probability	significance
Treatments	2	0.022655	0.0113277	58.78	P < 0.001	***
Life stage	4	0.387484	0.0968710	502.70	P < 0.001	***
Interaction	8	0.027905	0.0034881	18.10	P < 0.001	***
Error	75	0.014453	0.0001927			
Total	89	0.452497				

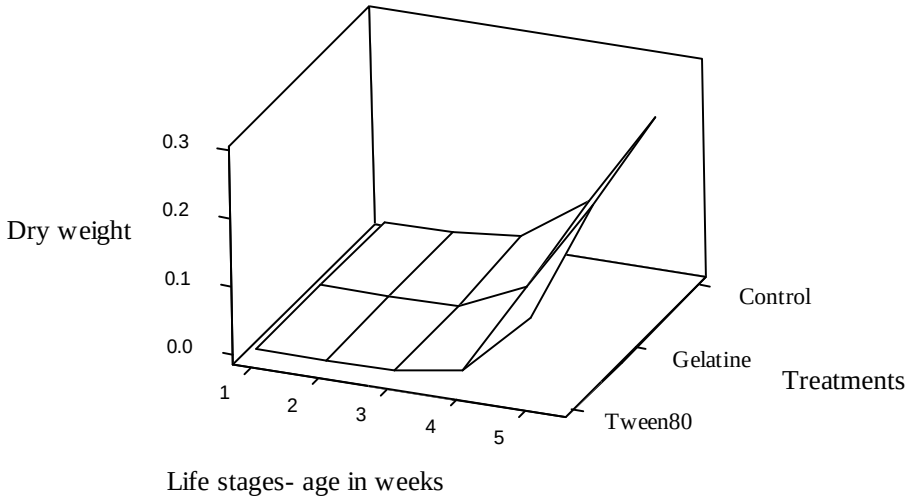


Figure 3.7 Surface plot of dry weight results (in grams) for the three treatments and five life stages included in Experiment 5,

(Least Significance Difference = 0.0092; n=18; p ≤ 0.05).

This reduction in biomass due to the treatments can be summarized as in Table 3.7. It shows that the Tween 80 resulted in a higher reduction in weight, whether measured as fresh weight or dry weight, than did the Gelatine treatment, and both gave a greater reduction of fresh weight than of dry weight.

Table 3.7 Fresh and dry weight values (in grams) of all three treatments and percent of reduction for the two mycoherbicide treatments (Tween 80 and Gelatine) compared to the control in Experiment 5.

Treatment	Fresh weight	Dry weight	% of reduction fresh weight	% of reduction dry weight
Tween 80	6.02	0.78	76%	69%
Gelatine	13.96	1.45	45%	25%
control	25.38	1.94		

Thus, in this experiment, the Tween 80 treatment of mycoherbicide would be considered more successful in reducing the growth of the weed than would the Gelatine treatment.

The results from Experiment 6 for both fresh and dry weight are given in Table 3.4.

Table 3.8 Results of the analysis of variance for experiment 6 of the fresh weights of all three treatments and five life stages.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F -value	Probability	Significance
Treatments	2	0.30388	0.151938	52.93	P < 0.001	***
Life stages	4	0.24909	0.062272	21.69	P < 0.001	***
Interaction	8	0.25961	0.032451	11.31	P < 0.001	***
Error	75	0.21528	0.002870			
Total	89	1.02786				

When considering the fresh weight results for Experiment 6, there was also a very highly significant ($p < 0.001$) ANOVA result for both factors and for their interaction (Table 3.8). In contrast to the results from experiment 5, however, the largest F–value is for the treatments rather than the life stages, and none of the F-values are as large as the corresponding values in Table 3.6. The same overall conclusions regarding the treatments can be drawn, however, as can be seen from the surface plot in Figure 3.7; the Tween 80 reduces the growth more than does the Gelatine, and both substantially reduce growth compared to the control.

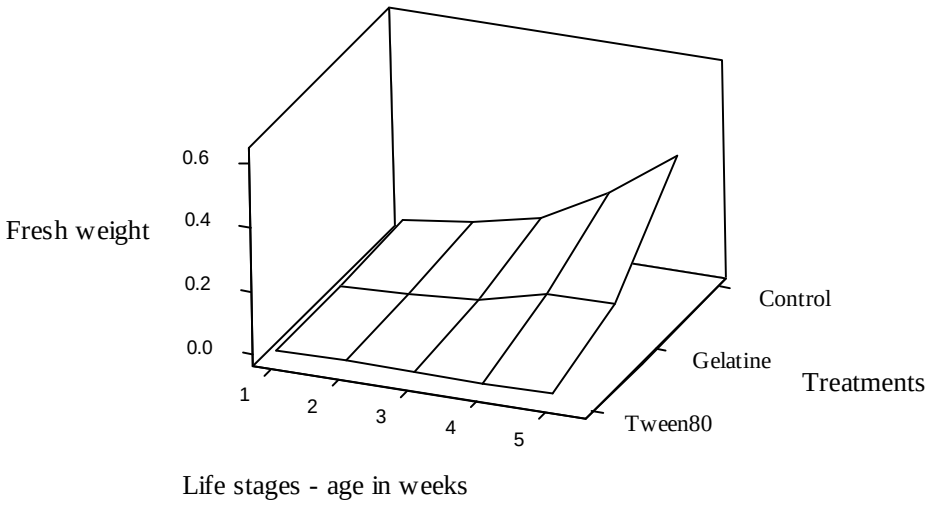


Figure 3.8 Surface plot of fresh weight results (in grams) for the three treatment and five life stages included in Experiment 6,

(Least Significance Difference = 0.0359; n=18, $p \leq 0.05$).

Considering the dry weight values (Table 3.4), again essentially the same conclusions can be drawn from the ANOVAs as for the fresh weights (Table 3.9). Thus, there are highly significant effects of treatment, of life stage and of their interaction, with the treatment effect leading to the largest F-value. These results are illustrated with a surface plot in Figure 3.9.

Table 3.9 Analysis of variance results of Experiment 6 of the dry weights of all treatments and life stages.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F - value	Probability	Significance
Treatments	2	0.0010675	0.0005337	24.32	P < 0.001	***
Life stage	4	0.0010080	0.0002520	11.49	P < 0.001	***
Interaction	8	0.0010866	0.0001358	6.19	P < 0.001	***
Error	75	0.0016457	0.0000219			
Total	89	0.0048078				

The effect of the different treatments applied on the growth of *Chenopodium album* plants in experiment 6 is illustrated in Figure 3.10. Clearly, both mycoherbicide applications resulted in markedly reduced growth compared to the control. Also, there was a slightly lesser reduction in growth with the Tween 80 treatment than with the Gelatine.

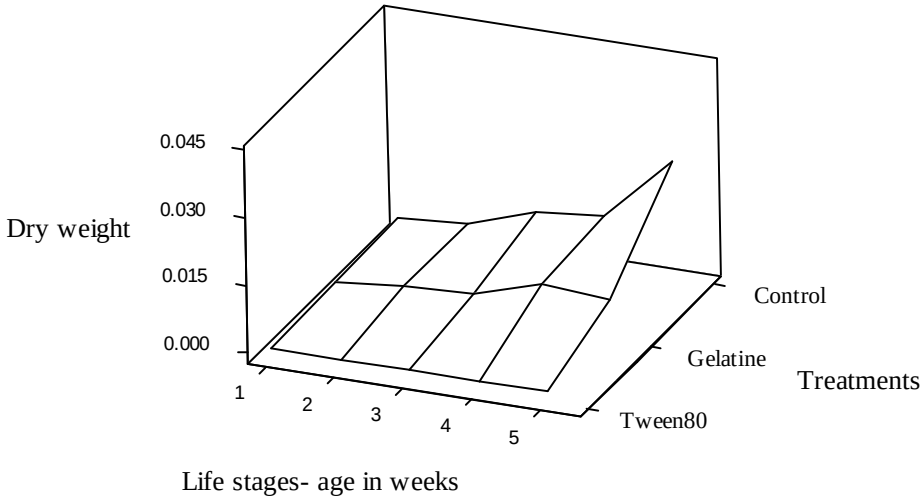


Figure 3.9 Surface plot of results of experiment 6, showing the dry weight (in grams) of all treatment and life stages,

(Least Significance Difference = 0.0044; n=18; $p \leq 0.05$).

Figure 3.10 Effect of application of different formulae of mycoherbicides on *Chenopodium album* plants grown in a growth cabinet in pots, experiment 6. Plants are pictured 10 days after the plants were sprayed, with the Tween 80 treatment on the left, Gelatine treatment in the middle and control (Tween 80 emulsifier but no fungal spores) on the right.

3.3.3 Results from Experiments 5 and 6 for root length

The results for the root lengths from Experiments 5 and 6 are given in Table 3.10.

Table 3.10 Results of Experiments 5 and 6 for root length of *Chenopodium album*, all values are in cm.

No.	Life stage	Experiment 5			Experiment 6		
		Tween 80	Gelatine	Control	Tween 80	Gelatine	Control
1	1	0	2.2	3.0	0	0	3.5
2	1	0	2.0	2.4	0	2.5	3
3	1	0	2.5	3.2	0	0	2.5
4	1	0	3.0	3.2	0	2	3
5	1	0	3.0	3.0	0	0	2.5
6	1	0	3.2	2.5	0	3.5	2.5
7	2	0	2.5	3.0	0	4	5.5
8	2	2.2	4.5	4.8	0	4	6
9	2	0	4.0	4.5	0	4.5	5
10	2	0	3.0	5.2	0	0	6.5
11	2	0	3.0	2.5	0	0	6
12	2	2.5	4.0	4.2	0	5.5	6.5
13	3	5.0	4.0	4.0	0	3	6.5
14	3	4.2	6.0	4.5	0	7	6
15	3	0	3.7	5.5	0	4.5	5.5
16	3	0	5.0	3.5	0	0	7.5
17	3	0	4.0	3.5	0	5.5	6
18	3	0	4.0	4.6	0	6	9
19	4	5.5	4.0	4.0	0	7.5	8.5
20	4	6.0	3.8	6.0	0	6.5	8.5
21	4	4.5	6.0	8.0	0	7	10
22	4	4.0	5.5	4.5	0	8	7.5
23	4	4.0	5.5	5.5	0	8.5	8.5
24	4	3.5	4.0	5.0	0	6.5	8
25	5	5.5	7.0	9.5	0	6	9
26	5	5.0	6.0	6.0	0	8	8
27	5	4.0	5.0	5.0	0	8.5	9.5
28	5	6.5	7.5	8.5	0	8	8.5
29	5	5.0	7.5	6.5	0	9.5	9.5
30	5	7.0	7.5	7.0	0	9.5	12

Both individual factors (treatments and life stage) were very highly significant ($p < 0.001$, Table 3.11) but there was a non significant ($p < 0.080$) interaction. As with the shoot fresh

weight values for this experiment, the life stage gave rise to the largest F-value with these results.

Table 3.11 Analysis of variance for the results of Experiment 5 of the root length.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F - value	Probability	Significance
Treatments	2	76.05	38.03	25.59	P < 0.001	***
Life stage	4	316.92	79.23	53.32	P < 0.001	***
Interaction	8	22.02	2.75	1.85	0.080	N.S.
Error	75	111.45	1.49			
Total	89	526.45				

The results are illustrated graphically in Figure 3.11. Although the interaction term was not significant for these results, they are illustrated by a surface plot in order to facilitate comparison with the results of dry weight later. The diagram shows that there was little difference between the control and treated plants in the last two life stages, but that in the first three life stages there was a substantial difference between the control and those sprayed with the fungus; however, there was no difference between the root length under the Tween 80 and Gelatine treatments.

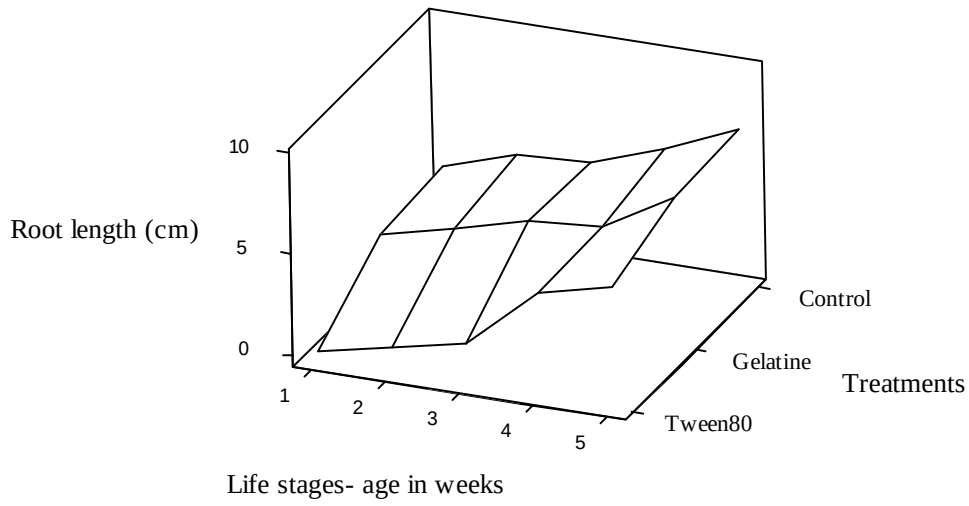


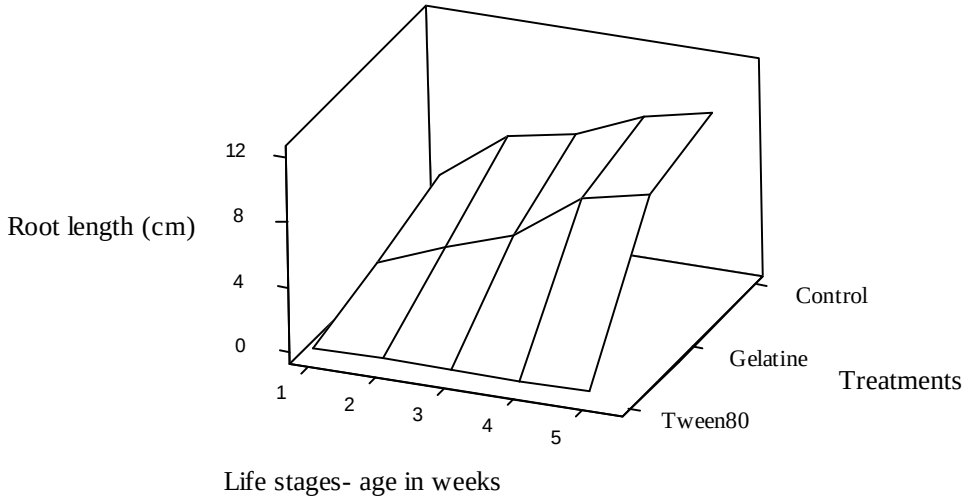
Figure 3.11 Surface plot of treatments of Experiment 5, showing root length (in cm), (Least Significance Difference = 0.818; n=18, $p \leq 0.05$).

The results for root length (in cm) from Experiment 6 are included in table 3.10, and the ANOVA results are given in table 3.12.

Table 3.12 Analysis of variance for the results of Experiment 6 of the root lengths.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F - value	Probability	Significance
Treatments	2	715.51	357.75	248.44	P < 0.001	***
Life stages	4	234.85	58.71	40.77	P < 0.001	***
Interaction	8	124.97	15.62	10.85	P < 0.001	***
Error	75	108.00	1.44			
Total	89	1183.32				

In this experiment there was a very highly significant ($p < 0.001$) interaction (Table 3.12) which was not found in experiment 5. The reason for this difference can be seen if the surface plots in Figures 3.11 and 3.12 are compared: in Figure 3.12 there is almost no growth in root length over time at all for the Tween 80, a result that was not found in Figure 3.11 where all treatments showed reasonable growth in the later stages and where the differences in root length between treatments were most marked in the earlier stages. In Experiment 5, the control was different from the other two, which were similar; however, with the results of Experiment 6 the control treatment gave rise to the larger roots and Tween 80 the smallest, with Gelatine being intermediate at all life stages. The treatments gave rise to the largest F-value in Experiment 6, in contrast to the ANOVA results in Experiment 5.



3.12 Surface plot of results of Experiment 6 of the root length (in cm) of all treatment and life stages, (Least Significance Difference = 0.8; n=18; $p \leq 0.05$).

3.3.4 **Results from experiment 7**

The observation of this experiment was continued for a month to observe the effect of Formula of fungus plus Tween 80 on the two populations (Libya and UK) of weed plants.

Temperature and humidity was recorded and mean of temperature was 20℃ and the relative humidity 50%. The results showed that many plants died after use of mycoherbicides, but that some plants could recover and start growing again (Table 3.13)

Table 3.13 Observation experiment of the effect of fungus plus Tween 80 or control (Tween 80 but no fungal application) on the two population of the weed plant. (X = dead plant and L= live plant).

Life stage	Libya				UK				Control UK				control Libya				No. of plants dying	Percentage dying
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4		
1	×	×	×	×	×	×	×	×	L	×	×	×	×	L	×	×	14	88%
2	×	×	×	×	×	×	L	L	×	L	L	×	L	L	L	×	9	56%
3	×	×	×	×	×	×	×	L	L	L	×	×	L	L	×	L	10	63%
4	L	L	L	L	L	L	L	×	L	L	L	L	L	L	L	L	1	6%
5	L	L	×	L	×	×	×	L	L	L	L	L	L	L	L	L	4	25%
6	×	×	×	L	×	×	×	L	L	L	L	L	L	L	L	L	6	38%
No. of plants dying	16				16				7				5				average	
Percentage dying	67%				67%				29%				21%				44	46%

In general, there was a marked difference between the control plants and those that were sprayed with the mycoherbicide, although even with the control plants not all of them survived; the numbers surviving were 16/48= 33% when treated with the mycoherbicide, and 36/48= 75% of the control plants.

There were notable differences between the life stages, since most at life stage one and several of life stage 2 and 3 died even in the control treatment; however, almost all the stage 4 plants survived, even when treated with the fungus, while at stages 5 and 6 several plants died, but all of them from the fungus treatment. Comparing the mortality of the Libyan and UK populations, there was no difference when sprayed with the fungus, and little difference with the controls.

Overall, these results demonstrate that the mycoherbicide will be able to have a marked effect on the mortality of *Chenopodium album* plants, particularly on older life stages (since mortality in the earlier life stages was unconnected with the fungus). Also, the populations of *Chenopodium album* from the two regions responded similarly to the fungus, as well as showing similar mortalities in the various life stages

3.4 Discussion

The goals of the work described in this chapter were to test the usability of *Ascochyta caulina* as a mycoherbicide for the control of *Chenopodium album* by: finding a usable new formulation by which the fungal preparation could be applied; by assessing whether there was any differential effect of the fungus on the various life stages of the weed plant; and also by finding out whether both the weed populations from the very different regions, Libya and the UK, were affected by the mycoherbicide.

The results from Experiment 5 showed that the mycoherbicide attacked both young and older plant leaves, causing foliar disease and defoliation, especially in the young stages of growth of the seedlings. *Ascochyta caulina* caused severe necrosis of almost 100% of the

leaf surface of *Chenopodium album*, especially the three first life stages, whereas inoculated plants (control) displayed no disease symptoms. The experiment also showed the Tween 80 formula to be better than Gelatine at inducing fungal attack. The conclusions from this experiment, relating to the Disease Severity Rating, would be that Tween 80 should be used, and would be most effective if applied shortly after seedling emergence.

The conclusion from the biomass values, the Tween 80 however consider slightly more effective result than did the Gelatine. With both fresh and dry weight values, the above ground biomass was significantly reduced in plants sprayed with the fungus. Root lengths were also significantly shorter with the fungal treatments, but here both fungal preparations were equally effective in causing the growth reduction. Also, the effect was most marked in the early stages of growth for this parameter.

Essentially the same experiment was repeated (Experiment 6), and the results gave broadly the same conclusions as before. This gives added confidence in the validity of the results and the conclusions drawn from them. There were some slight differences in the results from the two experiments, the main differences being that treatments were more important than life stages (in giving a large F-value); and that the Tween 80 gave much less root growth than did the Gelatine treatment in Experiment 6 (in Experiment 5 the two formulations gave similar results).

Experiment 7 showed that the mycoherbicide was effective in killing off many more of the *Chenopodium album* plants than died in the control treatment; the fungus effects were most marked in stages 5 and 6, since the mortality in the early stages also occurred in the

untreated control (presumably due to the susceptibility of seedlings to dying during the establishment phase, as discussed by Grubb, (1977), in respect of the regeneration niche).

Taking all these results together, they indicated that the new oil-based formulations for applying the mycoherbicide appear to work in reducing the growth of *Chenopodium album* under laboratory conditions, and hence may control the weed sufficiently within a crop. Of the two formulations, on balance the Tween 80 proved more effective in more circumstances and on more parameters of growth, although the Gelatine formula was also reasonably effective in some circumstances. Besides causing necrotic symptoms, the effect of fungus is to reduce biomass and root length (experiment 5 and 6) and sometimes death of the plant (Experiment 7), so therefore the formulations could potentially work in reducing weed growth provided they can successfully be applied under field conditions. The results under these laboratory conditions indicate considerable potential to reduce effective competition of the weed which would allow the crop to get established and hence pre- empt niche space (which then might be enough to inhibit the weed sufficiently to prevent economic losses). The results also suggest that the same formulation of the mycoherbicide could work in other regions such as Libya and therefore might be of wider applicability than just in the UK.

In these growth cabinet studies (experiment 5 and 6) the young plants were inoculated when they were approximately seven days old, and harvested when they were 17 days old. Also, in Experiment 7 the effects of the fungus only up to the forth life stage were monitored. Clearly, additional studies will be needed to evaluate the response of more mature, larger, *Chenopodium album* plants to *Ascochyta caulina*.

However, if spraying of the plants could be achieved while the weed plants are still small, as the current results suggest would be desirable, then this may not be so much of a limitation. Also, the response of plants to infection under field conditions needs to be tested to see whether the formulation could be effectively applied under less controlled conditions. This is the crucial next step in testing the potential of the application and use of this mycoherbicide, and forms the focus of the following chapter.

Chapter4: Field efficacy of formulated spore suspensions of *Ascochyta caulina* for the control of *Chenopodium album*

4.1 Introduction

Many studies have been conducted to test the use of the fungus *Ascochyta caulina* as a microbial herbicide to control *Chenopodium album*, and recent experiments using that fungus are encouraging. The influences of spore density, additives to spore suspension and the environmental factors of temperature and wetness duration on the host–pathogen interaction have been investigated (Kempenaar *et al.*, 1996). Ghorbani *et al*., (2006) used this fungus to study the effect of plant age, temperature and humidity on virulence of *Ascochyta caulina* on *Chenopodium album* but he did not use any kind of formulation, only a solution of sylgard and nutrient and yeast extract to keep the fungus active. Also Einhorn (2002) used the spore suspensions of the fungus combined with a very low dose of rimsulfuron in a field experiment and he achieved some success, however in spring it was difficult to find the favorable conditions for the fungus of both moisture and temperature.

The previous chapter has demonstrated considerable success in controlling the growth of *Chenopodium album* using *Ascochyta caulina* with an appropriate formulation under laboratory conditions. The aim of this experiment is to explore the extent to which this control can be achieved under field conditions.

Vegetable oil suspension emulsion of the potential mycoherbicide *Ascochyta caulina* was evaluated in a field trial. Oils improved mycoherbicide activity in comparison with spores applied in water only. As has been noted earlier (chapter 3) a major obstacle to the use of

mycoherbicide as a foliar pathogen is the need for at least 6 - 10 hours of dew on the leaf surface to enable the fungal propagules to germinate, grow, infect, and colonize the weed. Auld (1993) and Shabana *et al*., (1995) demonstrated, in controlled environment studies, that in the absence of dew, oil suspension emulsions of *Colletotrichum orbiculare* gave significantly better anthracnose development on Bathurst burr, *Xunthium spinosum* L., than aqueous suspensions.

In an attempt to improve the efficacy of the mycoherbicide in the field, the application of *Ascochyta caulina* on the weed plant *Chenopodium album* was assessed, using the formulations tested in the laboratory in chapter 3. It was hoped that this would create the optimum conditions for production of highly virulent mycoherbicide and hence the ability of the two formulations to control or suppress the weed plant, to overcome the lack of dew in the field by formulating the inoculum in oil emulsion. Thus the objective of the present experiment was to test the effectiveness of oil emulsion formulations of spore suspensions on disease establishment and plant death in a field trial.

4.2 Materials and methods

The field experiment was conducted in May 2008 to evaluate the efficacy of formulations of *Ascochyta caulina* to reduce *Chenopodium album*. Two formulations were used namely (1) formula of Tween 80 and (2) formula of Gelatine. The experiment was carried out during May 2008 in a garden plot in Bradford, (Ingleby Road, West Yorkshire, N53° 47' 29", W1° 45' 38"). A single batch of seeds of *Chenopodium album* was collected from a

field population at Bradford, Bradford Organic Bracken Farm, Syke Lane, Hipperholme, West Yorkshire, (N53° 45' 38", W1° 50' 32") for use in the experiment. Seeds were incubated in Petri dishes on Whatman filter paper No. 2 at room temperature, moistened with distilled water. After three days all germinated seeds were removed and thrown away, so that only seeds that germinated on the fourth day were used. One germinated seed was planted per pot of 10 cm diameter and 10 cm depth. The plastic pots were filled with compost of John Innes No. 2 compost and placed in a growth cabinet at room temperature and watered as necessary. Growth cabinet conditions were 20/15 °C day/ night temperature, 14 hour photoperiod, and average light intensity of 78 μmol m^{-2} s^{-1}. The process of germination and planting out was repeated at one-week intervals to produce the plants of the five different life stages. All seedlings were of approximately the same size within each life stage. There were three replications of each of the five life stages (i.e. 15 plants) for each treatment, the two fungal formulations and untreated controls. Seedlings were then left to establish themselves for five weeks in the growth cabinet before taking them to the field plot.

4.2.1 **Preparation of the mycoherbicide formulations**

The same procedure was followed as in chapter 3 for preparation of the two formulations (see section 3.2.4). The first formula (Tween 80) of mycoherbicide used in these experiments consisted of 445 ml fungal spore suspension plus 50 ml soybean oil plus 5 ml Tween 80 emulsifying agent, and the second formula (Gelatine) of mycoherbicide consisted of 445 ml fungal spore suspension plus 50 ml soybean oil plus 5 grams Gelatine as

emulsifying agent, and the two formulae of mycoherbicides were prepared for use in the field in the same day.

4.2.2 **Preparation of the plants and plots**

The test plot of 12 m2 was divided into three sub-plots (2m ×2m), one for each treatment, by placing two separating spaces across the plot (25 cm) as shown in Figure 4.1. The choice of which sub-plot had which treatment applied to it was decided at random, but within each sub-plot all plants were treated equally. The presence of the gap between plots prevented the different sprays from contaminating adjacent plots. The same number of 15 pre-prepared holes was dug in each sub-plot distributed evenly and the pots containing the seedlings were sunk into the holes so that the rim was level with the top of the soil, to avoid damage to the seedlings during transferring, also, it was tried to avoid any not the same effect of other factors on the weed plants like, closure to the walls, shadow.

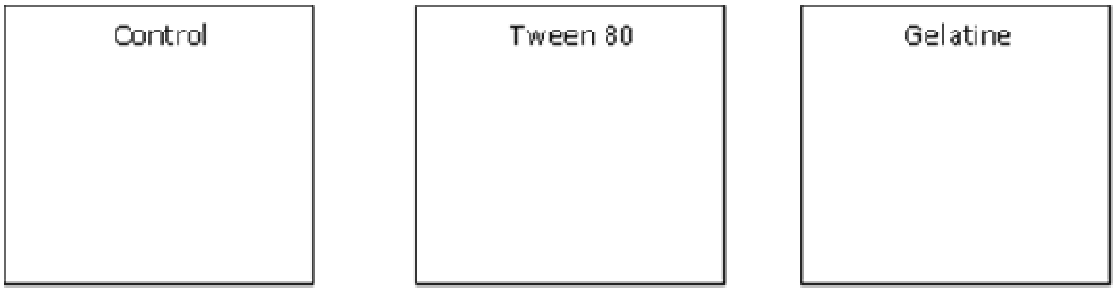


Figure 4.1 Design of plots for the field experiment (Experiment 8). Plants within each block were randomized as to their location.

4.2.3 **Mycoherbicide application procedure and weed assessment**

The mycoherbicide of both formulae were applied in late evening, applications were made two hours before sunset using a hand spray, and plants were sprayed till the run-off of liquid occurred. Ten days after inoculation plants were taken back to laboratory, and assessed for Disease severity rate (DSR), also the fresh and dry weight of living above ground biomass and root length per pot were assessed and measured. Completely collapsed seedlings were considered dead. The results for Disease Severity Rate were assessed with same method as in chapter 3 where the disease severity was assessed initially for each leaf on a plant according to a scale rating from 0- 6 and the following formula was used

$$\text{Disease Severity Rate} = \frac{(2.5 \times n1 + 15 \times n2 + 50 \times n3 + 85 \times n4 + 97.5 \times n5 + 100 \times n6)}{N}$$

Dry weight was obtained by cutting aerial parts at soil level, the above-ground biomass weighed fresh then drying put in special paper bags for two days (48 hours) at 70°C in the oven before reweighing them. The dry weight reduction was calculated by comparing the dry weight in inoculated and control plants. Remains of the living roots after cutting the shoots were measured using a ruler. The roots were released from the soil by water, the roots cleaned and measured in centimeters.

4.2.4 **Data analysis**

Data were analyzed by 2 -factor ANOVA, with the factors being treatment and life stage, using MINITAB® version 15.

4.3 Results

The results are analyzed and given for every parameter in their separate section.

4.3.1 **Result of the Experiment 8 for Disease Severity Rate**

The results from the field experiment for Disease Severity Rate are given in Table 4.1. The mycoherbicide attacked young and old plant leaves causing clear symptoms. Disease symptoms ranged from slight necrosis on cotyledons and leaves of old stages, to extensive necrotic lesions spreading to all leaves and stems of young stages of inoculated plants. The disease severity was particularly noticeable for life stages 2 to 5, especially on the plants treated with Tween 80 (Table 4.2). There was no disease evident on the untreated control plants. The plants treated with the Gelatine formulation showed some disease, but much less than that with the Tween 80.

Table 4.1 Result of Experiment 8 for Disease severity rate.

No.	Life stage	% of Disease Severity Rate	
		Tween 80 formula	Gelatine formula
1	1	0	0
2	1	40	5
3	1	46	22
4	2	11.5	14
5	2	100	14
6	2	97	33.5
7	3	37	15
8	3	81	9
9	3	54	19.5
10	4	41	11
11	4	45	17
12	4	64	3
13	5	69	12
14	5	78	20
15	5	65	30.5

Table 4.2 Summary table of average values of Disease Severity Rate for each life stage.

Life stage	Tween 80	Gelatine	Control
1	28.7	9	0
2	69.5	20.5	0
3	57.3	14.5	0
4	50	10.3	0
5	70.7	20.8	0

Table 4.3 Two –factor analysis of variance of the results for Disease Severity Rate, for the factors of mycoherbicide treatment and life stage, together with their interaction. (Key to significance levels: *** = p < 0.001; N.S. = No significance, p > 0.05).

Source	Degrees of Freedom	Sum of Squares	Mean Square	F - value	Probability	Significance
Treatments	2	24463.8	12231.9	43.06	< 0.001	***
Life stages	4	2004.1	501.0	1.76	0.162	N.S.
Interaction	8	1900.7	237.6	0.84	0.578	N.S.
Error	30	8522.3	284.1			
Total	44	36891.0				

The results, analyzed by a 2 factor ANOVA (Table 4.3), showed that the differences between treatments was very highly significant ($p < 0.001$), and between the life stages were also significantly ($p < 0.01$). However, there was no significant effect of life stage, nor was there a significant interaction between these two factors (Table 4.3).

These results are illustrated as a surface plot in Figure 4.2. It can be seen that the Tween 80 formulation generally gave higher disease severity rating values than either the Gelatine or Control treatments. There were some differences between the life stages with this parameter, but no consistent trend with increasing life stage, and as indicated above the results for life stage are not significantly different

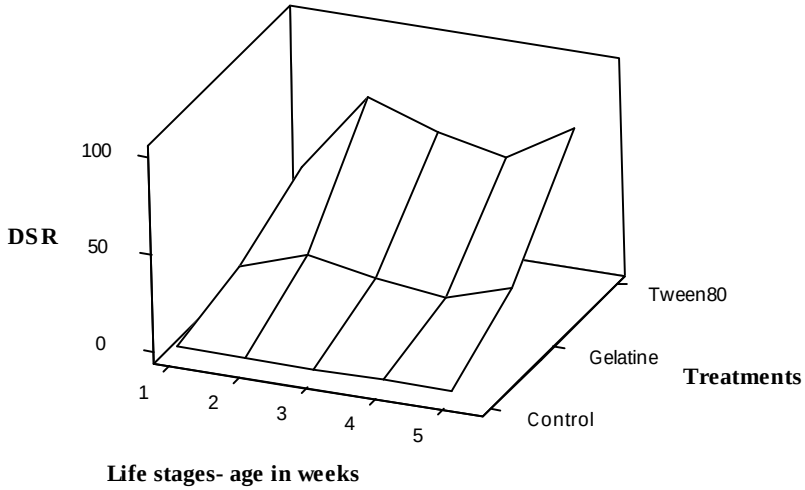


Figure 4.2 Surface plot of Disease Severity Rate % (DSR) results for *Chenopodium album* in relation to mycoherbicide treatments and life stages, (Least Significance Difference = 15.97, n = 9 and p ≤ 0.05). Note the reversed order of the treatments compared to previous figures, to aid clarity.

4.3.2 **Results of Experiment 8 for fresh weight and dry weight**

The results from the field experiment (Experiment 8) for fresh weight are given in Table 4.4. Growth and development of *Chenopodium album* was affected by the kind of formula of mycoherbicides. It is clear from the mean values (Table 4. 5) and the surface plot (Figure 4.3) that both formulations reduced the growth of the weed plant compared to the Control,

especially in the later life stages, with Tween 80 reducing growth slightly more than the Gelatine.

Table 4.4 Fresh weight values (g) from above-ground biomass of *Chenopodium album* in experiment 8.

Plant No.	Life stage	Fresh weight in grams		
		Tween 80	Gelatine	Control
1	1	0.028	0.118	0
2	1	0.031	0.116	0
3	1	0.035	0.122	0.088
4	2	0.140	0.511	1.995
5	2	0.113	0.576	0.335
6	2	0.050	0.335	0.661
7	3	0.259	0.833	1.427
8	3	0.321	0.963	1.091
9	3	0.373	0.880	1.132
10	4	1.073	1.099	2.517
11	4	0.827	0.991	2.156
12	4	0.691	0.955	2.051
13	5	1.292	1.643	3.802
14	5	1.550	1.820	3.202
15	5	1.139	1.141	1.872

Table 4.5 Summary table of average values of fresh weight (g) for each life stage, together with the percentage change in the treated plant values compared to the control plants.

Life stage (weeks)	Control	Tween 80	Change % from control	Gelatine	Change % from control
1	0.029	0.030	+ 3.4%	0.12	+ 310.3%
2	0.997	0.101	- 89.9%	0.47	- 52.5%
3	1.213	0.318	- 73.8%	0.89	- 26.5%
4	2.241	0.864	- 61.4%	1.02	- 54.7%
5	2.959	1.327	- 55.2%	1.54	- 48.1%

Table 4.6 Two –factor analysis of variance of the results for fresh weight for the factors of mycoherbicide treatment and life stage, together with their interaction. (Key to significance levels: *** = p < 0.001; * = p < 0.05).

Source	Degrees of Freedom	Sum of Squares	Mean Square	F - Value	Probability	Significance
Treatments	2	7.32	3.66	26.40	< 0.001	***
Life stages	4	19.38	4.84	34.92	< 0.001	***
Interaction	8	3.30	0.41	2.98	0.014	*
Error	30	4.16	0.14			
Total	44	34.17				

Analysis of variance indicated that the treatments and life stage were very highly significant (both $p < 0.001$, Table 4.6), and there was also a significant ($p < 0.05$) interaction term between the two factors. This interaction can be seen in the surface plot for these results in figure 4.3 where the surface slopes from front left up to back right in the diagram, with later life stages (unsurprisingly) giving greater fresh weights, but especially in the control plants.

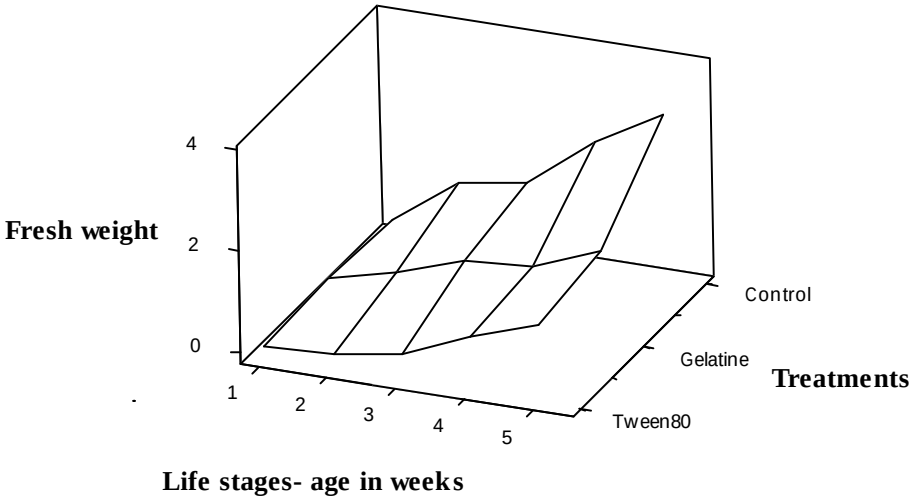


Figure 4.3 Surface plot of Fresh weight results (in grams) for *Chenopodium album* in relation to mycoherbicide treatments and life stages: (Least Significance Difference = 0.06; n=9; p ≤ 0.05).

Values of the dry weight of the plants from Experiment 8 are given in Table 4.7, with the mean values for the different life stages and treatments summarized in Table 4.8, and the ANOVA calculations for these results shown in Table 4.9

The results indicate a substantial reduction in the dry weights of the treated plants compared to the controls (Table 4.8), with Tween 80 sometimes producing similar and sometimes more substantial reductions than these due to the Gelatine formulation.

Table 4.7 Dry weight results (g) from the field experiment (Experiment 8).

Plant No.	Life stage	Dry weight in grams		
		Tween 80	Gelatine	Control
1	1	0.004	0.007	0.005
2	1	0.006	0.005	0.007
3	1	0.001	0.009	0.004
4	2	0.012	0.052	0.174
5	2	0.012	0.058	0.021
6	2	0.008	0.034	0.046
7	3	0.044	0.094	0.167
8	3	0.058	0.102	0.099
9	3	0.061	0.107	0.098
10	4	0.168	0.150	0.341
11	4	0.129	0.131	0.240
12	4	0.099	0.122	0.245
13	5	0.253	0.282	0.542
14	5	0.294	0.260	0.493
15	5	0.202	0.172	0.266

Table 4.8 Summary table of average values of dry weight for each life stage, together with the percentage change in the treated plant values compared to the control plants.

Life stage	Control	Tween 80	Change % from control	Gelatine	Change % from control
1	0.005	0.004	- 20%	0.007	+ 0.2%
2	0.080	0.011	- 86.3%	0.048	- 40%
3	0.121	0.054	- 55.4%	0.101	- 16.5%
4	0.274	0.132	- 51.8%	0.134	- 51.1%
5	0.434	0.249	- 42.6%	0.238	- 19.6%

Table 4.9 Two –factor analysis of variance of the results for dry weight for the factors of mycoherbicide treatment and life stage, together with their interaction.

(Key to significance levels: *** = p < 0.001; * = p < 0.05).

Source	Degrees of Freedom	Sum of Squares	Mean Square	F -value	Probability	Significance
Treatments	2	0.07	0.04	13.84	< 0.001	***
Life stages	4	0.52	0.13	48.26	< 0.001	***
Interactions	8	0.05	0.01	2.43	0.037	*
Error	30	0.08	0.00			
Total	44	0.73				

Both treatments and life stage were very highly significant factors ($p < 0.001$, Table 4.9), and there was a significant ($p < 0.05$) interaction term between the two factors. It can be seen from a surface plot of these results (Figure 4.4.) that the two mycoherbicide treatments are suppressing the growth of the plants compared to the control, especially in the later life stages. There is not much difference, however, between the dry weight values for the two fungal treatments at any of the life stages, which is also evident from the percentage reduction values in Table 4.8

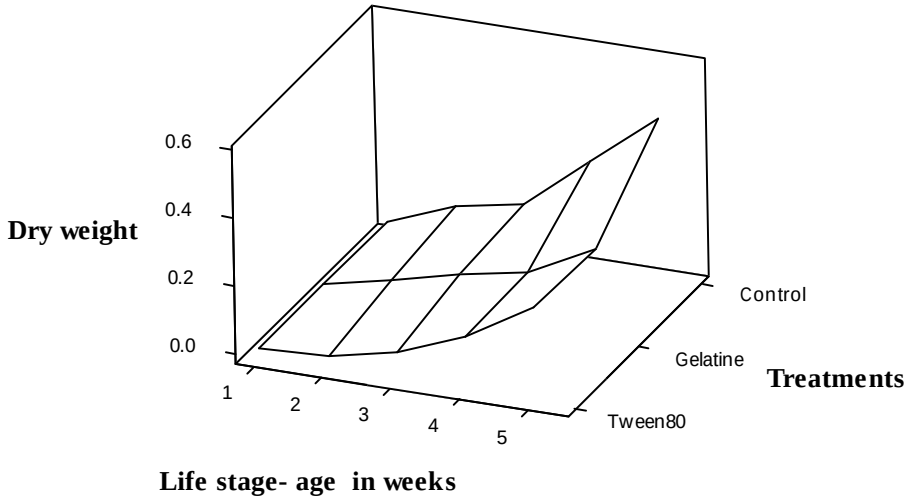


Figure 4.4 Surface plot of Dry weight results (in grams) for *Chenopodium album* in relation to mycoherbicide treatments and life stages,

(Least Significance Difference = 0.00, n=9, p ≤ 0.05).

4.3.3 **Results of the root length measurements for experiment 8**

The results for root length from Experiment 8 are given in Table 4.10.

Table 4.10 Root length results from the field experiment (Experiment 8).

Life stage	Replicate	Length of root of treatments in cm		
		Tween 80	Gelatine	Control
Life stage 1	1	5	7	2
	2	0	5	6
	3	4	5	6.5
Life stage 2	1	6	9	11.5
	2	6	7.5	8
	3	5	8	8
Life stage 3	1	10	10.5	11
	2	8.5	10.5	7
	3	6.5	11.5	12
Life stage 4	1	8.5	9	26
	2	10	9	24
	3	11	10	30
Life stage 5	1	15.5	12	22
	2	10.5	14	30
	3	14	14	28

The mean values for the different life stages and treatments are shown in Table 4.11. It can be seen from these and the ANOVA results in Table 4.12 that the treatments with the fungus gave substantially shorter root length than the control, especially at the later life stages. There was relatively little difference in the percentage values for the two formulations.

Table 4.11 Summary, table of average values of each life stage for root length.

Life stage	Control	Tween 80	Change % from control	Gelatine	Change % from control
1	4.8	3.0	- 37.5%	5.7	+ 18.8%
2	9.2	5.7	- 38%	8.2	- 10.9%
3	10	8.3	- 17%	10.8	+ 8%
4	26.7	9.8	- 63.3%	9.3	- 65.2%
5	26.7	14.7	- 44.9%	13.3	- 50.2%

Table 4.12 Root length values of the field experiment analyzed by 2 way ANOVA. (Key to significance level: *** = p < 0.001).

Source	Degrees of Freedom	Sum of Squares	Mean Square	F- value	Probability	Significance
Treatments	2	466.54	233.27	52.75	< 0.001	***
Life stages	4	1073.08	268.27	60.66	< 0.001	***
Interactions	8	513.46	64.18	14.51	< 0.001	***
Error	30	132.67	4.42			
Total	44	2185.74				

As well as both factors, Treatments and Life stage, being very highly significant ($p < 0.001$) as shown in Table 4.12, there was also a very highly significant interaction between the two factors ($p < 0.001$). This is reflected in the surface plot, which shows little difference between treatments in the earlier life stages, but a much bigger increase in root length in the control plots in life stages 4 and 5 (Figure 4.5.).

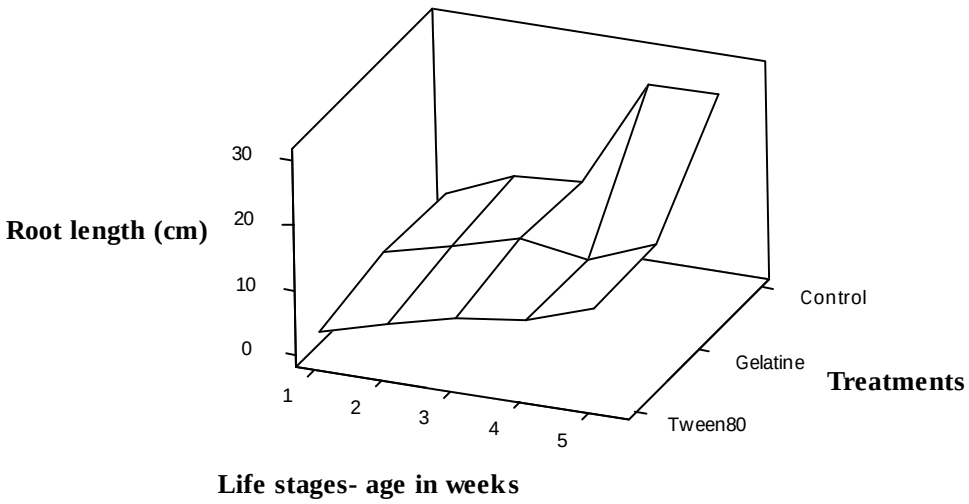


Figure 4.5 Surface plot of root length results (cm) for *Chenopodium album* in relation to mycoherbicide treatments and life stages,

(Least Significance Difference = 1.99, n=9; $p \leq 0.05$).

4.4 Discussion

The potential of emulsions with low oil content and emulsions made from vegetable oils was investigated. Oil emulsions are promising in dew-free conditions. Evaluations of these products in large-scale field trials are necessary to confirm the results from miniplot trials. Oil suspension emulsions may simply aid wetting of the plant surface at low (0.5- l %) concentrations. Amsellem *et al.*, (1991) were unsure whether invert emulsions using high oil concentrations (> 20%) assist mycoherbicides by retaining water for spore germination

or by damaging plant cuticle. There was evidence in their study of formula Tween 80 improving mycoherbicidal effects. Environmental factors are more variable under field conditions than in the growth cabinet studies by Auld (1993), which may explain why he demonstrated a significant improvement in performance of the mycoherbicide in a 10% oil suspension emulsion, compared with water, under conditions of no dew of herbicidal activity.

In the present experiment the Disease Severity Rate was particularly increased by the Tween 80 formulation, the Gelatine being much more similar to the control values. However, in this experiment the disease severity was lower than the values obtained in the experiments in the laboratory (Experiment 5 and 6). Possibly this could be attributed to the fact that the young leaves are more vulnerable and seem to be less resistant and easy to infect than the old one because the cuticle layer of wax still not very hard while under higher humidity this constraint could be defeated, whereas in the laboratory more humid conditions contributed to an increased disease rating for *Chenopodium album*. The Tween 80 formula (Disease Severity Rate of 41%) tended to be superior to the Gelatine formula (Disease Severity Rate of 25%), the majority of plants of first, second and third life stages were killed before ten days and, on the remaining plants, lesion development was poor and this could be show that the Tween 80 work better under field condition and humidity which was available during the night and early morning could help the formulation of mycoherbicide in starting the infection.

All three of the measures of growth (fresh weight, dry weight and root length) showed essentially the same pattern of results, namely that both mycoherbicide formulations

reduced the plant growth compared to the controls, especially in the later life stages, and that Tween 80 tended to reduce growth more than did the Gelatine. Thus, overall, this study shows that the mycoherbicide had considerable potential to reduce the growth of *Chenopodium album* even though the weather during the day was not very suitable and had a negative influence on the mycoherbicide.

Chapter5: General discussion and conclusions

5.1 Introduction

The research described in this thesis has focused on the current advances in bioherbicide research through a brief review and discussion of the basis, the progress, the restraints, and the prospects of this approach to weed control; and in particular on the possibility of control of *Chenopodium album* by the fungus *Ascochyta caulina*. Since this species is one of the most widely distributed weeds in the world (Holm *et al*., 1977) and is an important weed in corn and soybean fields (Frick & Thomas, 1992), the control of it by use of a mycoherbicide could be an important contribution to reducing crop losses. As Warren (1998) has pointed out, a good deal of increased agricultural productivity has resulted from improved crop breeding, nutrition, and pest management, of which weed management has been a major factor.

One of the main targets in weed research is the enormous reservoir of viable dormant weed seeds present in agricultural soil (Wesson & Wareing, 1969), and the factors that control dormancy or trigger germination of the seeds. One aim of this present research was to study the effect of some factors on seed germination, and therefore on the early seedling growth, of *Chenopodium album* and to determine what variations there are in the populations from two very different geographical regions, namely Libya and the UK. These aspects are discussed in section 5.2.

The development of a mycoherbicide involves three major phases or stages: 1) discovery, 2) development, and 3) deployment (Templeton *et al.*, 1979). Phase 1 had already been explored by other workers; thus in a previous study, an isolate (ITEM 1058) had been used

to demonstrate that the fungal pathogen, *Ascochyta caulina*, was pathogenic and had potential as biological control agent of *Chenopodium album* (Scheepens *et al.*, 1997). The development phase involves the determination of optimum conditions for spore production (Mendi, 2001); while the deployment phase involves determination of optimum conditions for infection and disease development especially under field conditions, and this is the focus of the present research to overcome the problem of environmental factors such as unfavorable moisture and/or temperature conditions. This has been done by developing a new formulation for *Ascochyta caulina*, in the light of recent research on formulation which has shown the potential for invert (water-in-oil) emulsions for mycoherbicides (use of low concentrations of vegetable oils with an emulsifying adjuvant) (Connick *et al.*, 1991b , Daigle & Cotty, 1992). These formulations and their efficacy are discussed in section 5.3.

In the field, the use of mycoherbicides is based on the fundamental epidemiological principles of plant pathology, and weed disease is the result of the interaction among the host weed, the pathogen and the environment (referred to as the disease triangle Kavanagh, 2005). Formulation and application methods need to be as insensitive as possible to overcome the problem of environmental fluctuations. After testing the new formulation in the laboratory, a field trial was performed to assess the deployment of the fungus under these conditions. These aspects are discussed in section 5.4 below.

Finally, the main conclusions arising from this work, and recommendations for future research, are made in sections 5.5 and 5.6 respectively.

5.2 Effect of environmental conditions on *Chenopodium album* seed germination

Cheam (1985) claimed that seed dormancy is perhaps the single most important characteristic in weeds that enables them to survive and persist. A large number of *Chenopodium album* seeds in the soil persist because of seed dormancy. This ability to persist for many years poses a continuous weed control problem. For any control measure to be effective in the long term it must greatly reduce the weed seed population in the soil.

Seed germination begins with imbibitions of water. The rate and extent of imbibitions may be governed by the surrounding soil water potential. Many authors have reported that germination of *Chenopodium album* seeds depends on the presence of light (Henson, 1970 , Vincent & Roberts, 1977 , Roberts & Benjamin, 1979 , Bouwmeester & Karssen, 1993 , Jursík *et al*., 2003).

Other environmental factors also promote or inhibit seed germination. The seasonal germination pattern depended almost entirely on the fluctuations in field temperature (Bouwmeester & Karssen, 1992); these authors, however, reported that germination can occur whenever the field temperature is between about 5 and 25°C and moisture, light and sufficient nitrate are available. Murdoch *et al*., (1989) concluded that alternating temperatures were clearly an effective dormancy breaking agent.

Results from the germination experiments described in chapter 2 showed, firstly, that there was a difference in the frequency of the polymorphic seeds in the two populations, from Libya and the UK, and also that the dormancy and germination patterns varied markedly as well. The fact that the Libyan population consisted entirely of black morph seeds which

germinate readily after harvesting (Bouwmeester & Karssen, 1993), and that older seeds from the UK were more dormant than Libyan seeds, indicate that the environmental triggers for germination in the two populations are very different as might be expected, with the Libyan population having less variability and less need for longer term dormancy. The main inhibition to germination in Libya is likely to be lack of sufficient moisture, rather than temperature limitations, which probably accounts for the reduced longer term dormancy as well as the reduced response to chilling compared to the UK seeds. The fact that new seeds from Libya show greater dormancy than equivalent ones from the UK may be a reflection of the conditions under which the parent plants were growing, but may also be an adaptation to avoid immediate germination unless sufficient moisture is present (whether from rainfall or from irrigation of crops).

The emergence behavior of weed species in relation to cultural and meteorological events was studied by Grundy *et al*. (2003). Dissimilarities between populations in dormancy and germination ecology, between maturation conditions and seed quality and burial site climate all contribute to potentially unpredictable variability. Their study showed that there is a relationship between the climatic conditions of the burial sites and the relative amount of the flush of emergence of *Chenopodium album*. It is interesting that the pattern of dormancy for this species appears to contrast with that of another widespread weed species, *Senecio vulgaris*. In the latter case, UK populations tend not to show initial dormancy of seeds (although this has been found to relate to the time of year in which the seeds are produced), while fresh seeds from the few Mediterranean populations studied showed strong innate dormancy over a wide temperature range (Ren & Abbott, 1991). It has been suggested that this difference is due to a shift to a winter annual life cycle in the warmer but

drier environment of the Mediterranean (Ren & Abbott, 1991). If this is indeed the case in *Senecio vulgaris*, it does not appear that *Chenopodium album* shows a similar response. Clearly, further study of the germination and emergence behavior of the Libyan population of *Chenopodium album* is needed to enable greater understanding and prediction of the response of the weed species to environmental conditions in the country.

From other studies and this study we can conclude that seasonal variation in dormancy relief pattern may interact with the rainfall pattern early in the growing season to modify weed seedling emergence and this will affect the ease or difficulty of weed control for that year. As an example Everman *et al.*, (2008) investigated the effects of various intervals of weed interference on peanut yield, and they predicted a critical period of weed control, found to be from 3 to 8 weeks after planting, the peanut yield decreased as weed interference intervals increased. It will be important for *Chenopodium album* to account for local and seasonal variation in dormancy biology when interpreting predictions from models and selecting integrated weed management strategies.

5.3 Factors influencing the pathogenicity of *Ascochyta caulina* against *Chenopodium album*

Biological control research is expanding and new strategies are being sought for alternative methods of weed control for integration into weed management systems. The endemic pathogen could be completely destructive to its weed host by using a massive dose of inoculum at a particular vulnerable stage of weed development (Daniel *et al.*, 1973) but

crop losses could also be reduced by achieving a quantitative reductions in the growth of the weed through a less destructive application of the pathogen.

Fungi have potential for use as mycoherbicides in a manner similar to chemical herbicides. The formulations of mycoherbicides are developed for different reasons associated with manipulation (including handling and application), stabilization or shelf-life, and efficacy (Weaver *et al.,* 2007). Formulations containing different combinations of *Ascochyta caulina* conidia, its phytotoxins, and low-dose herbicides have been tested (Evidente *et al.*, 1998 , Evidente *et al.*, 2000 , Vurro *et al.*, 2001). A significant improvement in the efficacy of the fungus was achieved in glasshouse trials by (Netland *et al.*, 2001) with an aqueous formulation containing PVA (polyvinyl alcohol, 0.1 percent v/v), Psyllium (a plant derived polysaccharide, 0.4 percent w/v), Sylgard 309 (a surfactant, 0.1 percent v/v), nutrients, and conidia (5×10^6 ml^{-1}). The formulation of spores of *Ascochyta caulina* in a vegetable oil emulsion significantly improved the effectiveness of the pathogen. The oil emulsion was easy to prepare, could be sprayed using standard equipment and was not toxic to *Ascochyta caulina* spores. The oil emulsion may provide a favorable microenvironment around the spores during the infection process, either by retention of the water present in the emulsion or by inducing an exogenous supply of water, possibly from leaf tissue cells (Greaves *et al.*, 1998). Once applied as a mycoherbicide in the field the effectiveness of *Ascochyta caulina* may be enhanced through weakening by crop shading or by adding sub-lethal dosages of herbicides. To overcome free moisture requirements of many plant pathogens, humectants, antidesiccants and oils have been added successfully to spray solutions to delay evaporation. This characteristic makes them suitable to be used within a mycoherbicide formulation.

Reduction or enhancement of vigor, aggressiveness, or fitness of the pathogens associated with changes in physical or nutritional conditions could significantly influence performance. Numerous studies of plant pathogens have demonstrated the enhancing effect of nutrient amendments on growth of pathogens. Enhanced infectivity through increased spore viability, spore germination and infection structure development are thought responsible for the nutritional effect. However, few studies have evaluated the effect of nutritional amendments on biological weed control pathogen performance. For example, pH, surfactants and nutrients greatly influenced germination of *Alternaria cassiae* conidia and these results corresponded to increased disease severity on sicklepod seedlings *Cassia obtusifolia* L. (Daigle & Cotty, 1992). Invert (water in-oil) emulsions can retard evaporation, thereby decreasing the length of time that additional free moisture is required for spore germination and for infection (Quimby *et al.*, 1988 , Daigle & Cotty, 1992).

The importance of moisture on disease development by plant pathogens has been well documented, and most studies demonstrated a positive correlation between the length of the dew period and the extent of disease (TeBeest *et al.*, 1992). The successful use of the mycoherbicide Collego® can be attributed to the high relative humidity in rice and soybean fields in which it is used (Templeton *et al.*, 1979). In the present study, the two emulsifiers Tween 80 and Gelatine were used with the intention to delay evaporation and provide the inoculum with moisture for an extended period, especially under field conditions. Both adjuvants, Tween 80, and Gelatine significantly increased disease severity compared to the standard treatment.

In the present work, the efficacy of formula of Tween 80 in controlling *Chenopodium album* shoots was much lower in experiment 5 than in experiment 6, and because both experiments were carried out under almost the same conditions, the most likely reason to be different could be attributed to the relative humidity which was higher in the second experiment (about 60% compared to 45 % in the first experiment). The reduction in biomass in experiment 6 of fresh weight by formula Tween 80 was 76% and dry weight was 69% while it was 45% for fresh weight and was 25% for dry weight in experiment 5. Nonetheless, both experiments showed a similar response of the plants to the mycoherbicide, namely a notable reduction in growth of the weed plants. Therefore there is good evidence that the use of the mycoherbicide coupled particularly with the Tween 80 formulation has been successful in attempting to control the quantitative growth of the weed. Experiment 7 also demonstrated that it could influence the mortality rate as well, which would also contribute to a reduction in the competitiveness of the weed growing within a crop.

Addition of adjuvants also can influence other aspects of infection and disease development. Control of redroot pigweed *Amaranthus retroflexus* L. by *Microsphaeropsis amaranthi* was increased with the addition of certain fatty acids, but the compounds had minor effects on spore germination or development of infection structures (Weidemann *et al.*, 1995). Several formulations of invert emulsion type were used in their study, and in the present work Tween 80 and Gelatine were used as the emulsifiers with soybean oil.

So far, most of the research on the efficacy of mycoherbicides for biological control of invasive weeds was performed and successful under controlled conditions (i.e.

greenhouses), thus, one of the main task of this study was to investigate the efficacy of the formulation to control *Chenopodium album* under field conditions. During the season (2008) the main objective was to determine the effective formulation of the formulated fungal isolates, applied singly to control *Chenopodium album*. These treatments have been compared with the control, and the oil invert emulsion formulation showed a potential to be used as a delivery system to control *Chenopodium album* under field conditions.

Both formulations were able to delay the growth of *Chenopodium album*, especially the Tween 80 formula reduced the total biomass of *Chenopodium album* shoots and induced disease symptoms on all growth stages of *Chenopodium album* plants, irrespective of the weather (which was very dry). Relatively low levels of weed mortality were achieved under these field conditions (50% with the Tween 80 formula and 15% for the Gelatine formula), but particularly with the Tween 80 results this would still be a valuable reduction in weed competitiveness, even when the environmental conditions were not especially favorable to the use of the mycoherbicide. The field conditions under which the treatment is applied still need to be optimized taking into consideration the environmental conditions and use of mycoherbicide early in the season, especially as biomass reductions in *Chenopodium album* were greater when plants were treated at an earlier growth stage. Generally, temperature has not been considered to be as critical as moisture for mycoherbicide development, since most pathogens studied were infectious over a wide range of temperatures (TeBeest *et al*., 1992).

Thus the overall outcome from these studies is that in investigating the performance of *Ascochyta caulina* conidia applied at different developmental stages of *Chenopodium*

album with the available formulations (particularly Tween 80), successful reductions in weed growth were achieved in the laboratory and the field, but that favorable weather conditions are still needed to obtain the most successful infection in the field. The impact of the disease was less when plants were treated at later growth stages and little biomass reduction was observed in sprayed plants. However, the field efficacy of this fungus under different weather conditions needs to be studied further.

5.4 Constraints in use of *Ascochyta caulina* as a mycoherbicides

"Perhaps the biggest single constraint to development and marketing of mycoherbicides is the need to develop an appropriate formulation. This is a complex requirement, needing a major research input. Even if produced for a restricted niche market, a mycoherbicide will be used in a range of widely differing conditions determined by geographical location and cropping system with their attendant pest control regimes" (Greaves & Macqueen, 1990).

Success in one country often results in attempts to repeat results in other areas of the introduced range– sometimes these attempts succeed, sometimes they fail: which is an indication that success can be a function of interaction of an invasive plant with local biotic and abiotic conditions (Weaver *et al.*, 2007). One positive feature of the present results is that the infection responses of plants from the UK and Libya were similar. This suggests that this pathogen could be used as a mycoherbicide in both regions.

The lack of proper epidemiological conditions for infection and disease development has been a major obstacle to the development of many pathogens for biological weed control in the field, and these conditions often limit the efficacy and therefore the commercial

potential of bioherbicides (Auld, 1993). Development of suitable formulations to improve viability and efficacy may counter these obstacles by improved moisture retention, reduced drying and UV-irradiation, nutrient supply, evenly diluted and dispersed inoculum or improved host-pathogen contact (Charudattan, 1991) and by careful timing of application (Walker & Boyette, 1986). Integration of biological control strategies with chemical, cultural, and mechanical control practices is essential to a wise use of biological control in weed management programs. Because biological strategies control a comparatively narrow spectrum of weed species, chemical herbicides are generally required to control the complex of weed species. Also, available biological control practices are few compared with the many chemical herbicides available for weed control. So, biological control strategies must be integrated with chemical herbicide for effective management of weeds (Müller-Schärer & Vogelgsang, 2000). Integrated weed management is a systems approach incorporating plant breeding, fertilization, crop rotation, chemical and mechanical weed control, interspecific plant competition and soil management that need to be combined into a method of reducing weed interference and herbicide use while maintaining acceptable crop yields.

The amount of yield loss in crops resulting from weed competition is a function of the time of weed seedling emergence relative to the crop (Baldwin & Santelmann, 1980 , Swanton & Weise, 1991 , Kropff & Spitters, 1992 , Knezevic *et al.*, 1994 , Chikoye *et al.*, 1995 , Dieleman *et al.*, 1995). In this respect, the results of the field trial (Experiment 8) in the present work were encouraging, in that the effect of the fungus was not only to increase mortality but also to reduce the growth rate of the weed. Therefore, there is likely to be a notable reduction in competitiveness of the weed if grown along with a crop. Clearly,

assessing the extent of such a reduction, and consequently the extent of increase in crop yield, through a full-scale field trial is an important further step that is required in testing the effectiveness of *Ascochyta caulina* as a mycoherbicide.

5.5 Conclusions

A future trend in weed management is to find an alternative method of weed control which will become more important to vegetable growers in the future to reduce the reliance on chemical herbicides. One of the bases of the biological control of weeds is to reduce rate of growth, fecundity and general vigor of the host, consequently reduce the weeds competitive position in the crop.

The efficacy of *Ascochyta caulina* under laboratory and field conditions was improved by formulating the spores in an oil emulsion. The oil formulation caused high levels of disease severity even with no recorded period of dew after inoculation in the field. The mycoherbicide caused necrosis of *Chenopodium album* leaves and it reduced the above ground shoot, thereby affecting the vegetative propagation of the *Chenopodium album*.

Neither of the formulae used negatively interfered with the fungus, rather their effects on *Ascochyta caulina* were positive. The results of the work suggest that combinations of different methods are much more likely to solve the *Chenopodium album* problem compared to using one method alone in conditions of unfavorable weather. Appropriate timing of the application to take advantage of the humidity provided by rain, dew and

irrigation in the field would probably have increased the effectiveness of the mycoherbicide.

It is encouraging that disease occurred in the field as a result of the spray application of formulated aqueous suspensions of *Ascochyta caulina* onto *Chenopodium album*; although the disease effects were only moderate under these conditions. Significant improvements may well need to be made to its field performance to enable the fungus to be considered a serious mycoherbicide candidate.

Results from this study support the conclusion that *Ascochyta caulina* has potential as a biological weed control agent for *Chenopodium album*. However, the circumstances of weed emergence and of moisture patterns are likely to be quite different in the UK and in Libya, and these need to be taken into account with respect to use of the mycoherbicide.

Biological control of weeds may not work in some areas, even if it does in others. Climate variations such as humidity and plant biotype differences may account for some failures in the past, the biological control agents require specific conditions to survive.

In general, several agents are needed to achieve the desired population controls throughout the variety of ecological and climatic conditions present in Libya.

Successful application of biological controls requires more knowledge-intensive management. Understanding when and where biological control of plant pathogens can be profitable requires an appreciation of its place within integrated pest management systems. The effects of inoculum concentration, dew period and plant age on the biocontrol of *Chenopodium album* in Libya needs to be studied under environmental conditions.

In conclusion, this mycoherbicide is useful in the field and appears to have the potential to help in the biological control of *Chenopodium album*.

Increased activity in basic and applied science and in biotechnology have a definite role to play in development, implementation, and advancement of this weed control strategy (virulence, efficacy, fermentation, formulation, and application are aspects of prime importance). Research on control of weeds with biological agents and natural products should be conducted with emphasis on optimizing performance in the field environment and testing with a wider range of crop plants.

Scale-up includes the testing of potential formulations of mycoherbicide based on results from earlier preliminary testing and performance trials in laboratory and small field trials. In relation to the control of *Chenopodium album* by *Ascochyta caulina*, in the light of the laboratory and small scale field trial results it is now appropriate to focus on the larger field scale, the biotic and abiotic constraints to using the mycoherbicide in nature that impact on the safety and efficacy of the biological agent for weed control and the economic cost of doing so. One factor limiting commercial interest in biocontrol is the high cost of production for most biocontrol agents (due to high cost of substrate, low biomass productivity, or limited economies of scale), and an assessment of the extent to which it would be limiting with this combination of fungal agent and formulation within relevant crops needs to be carried out. Additional testing on a larger scale under field conditions will confirm if the formula of Tween 80 has potential as a marketable mycoherbicide for *Chenopodium album*.

As a next step testing the validity of using the Tween80 formula under field conditions in combination with the crop is vital, and different ecotypes of the weed need to be included in such testing since they may react differently to the mycoherbicide and knowledge of their behavior in the field is needed.

The final challenge is to develop an efficient, low cost means of scaling up production of fungal material and to develop a formulation to satisfy industrial needs for commercial exploitation of this technology.

5.6 Recommendations for future research

Several lines of further research should be followed in order to develop the possibility of using *Ascochyta caulina* as a mycoherbicide for *Chenopodium album*.

1. Although the formulation developed in the present work has proved satisfactory, including in a field trial, it may still be possible to modify the formulation to improve its efficacy. This might be achieved by increasing the effectiveness of the fungus by nutrient enhancement, combinations of adjuvants with complementary effects, or cultural methods such as increasing the spore concentration.
2. Further exploration of the best life stage against which to spray the mycoherbicide should be made to determine when the weed plants are most vulnerable to the fungus.
3. The effectiveness of multiple applications of the fungus, or integrated control with chemical herbicide, could also be studied.

4. When the most appropriate formulation has been found, it will be necessary to scale up preparation of the fungus and to perform a large scale field trial where the crop is present, so that the effectiveness of the treatment and the extent of yield benefit to the crop can be assessed. It will then be possible to determine whether the economic yield benefit outweighs the commercial cost of preparing and applying the mycoherbicide.
5. Further studies, particularly on patterns of emergence of the weed in the field, should be carried out, along with other germination trials (for example of the effect of enhanced moisture from rainfall or of irrigation) to enable more accurate predictions of the desired timing of mycoherbicide application. The apparent lack of seed polymorphism in Libyan populations of the weed needs confirming, and generally the differences in dormancy and germination triggers in a region such as Libya which is less well-studied compared to that of the UK need clarifying.

5.7 References

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