Identification of pathogens and control of spot blotch disease of barley (*Hordeum vulgare*) by combining plant resistance and biological control

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Summary

Identification of pathogens and control of spot blotch disease of barley (*Hordeum vulgare*) by combining plant resistance and biological control agents with different methods of application

Spot blotch is one of the most important diseases of barley (*Hordeum vulgare*) in Libya and worldwide. The overall aim of this study was to investigate the potential of biological control in combination with disease resistance to control spot blotch without the potential hazards of chemical application. Fungi were isolated from barley plants with spot blotch symptoms from different areas in Libya. As well as the commonly known spot blotch pathogen *Bipolaris sorokiniana* (teleomorph *Cochliobolus sativus*), *Bipolaris spicifera* (teleomorph *Cochliobolus spicifer*), *Curvularia inaequalis*, and *Alternaria alternata* were identified by their morphology and ribosomal DNA sequences. *Bipolaris sorokiniana* was the most serious pathogen under the test conditions; the others infected barley but caused less severe symptoms.

Spot blotch resistance of barley seedlings was tested under greenhouse conditions with four Libyan cultivars (ACSAD, Nibola, Rehan, and Wadi Utbah) and two UK cultivars (Gaelic and Pastoral). Nibola was the most resistant. The ability of the organisms in three commercial biocontrol products, *Trichoderma harzianum* T-22 (Trianum), *Streptomyces lydicus* WYEC 108 (Actinovate) and *Bacillus subtilis* QST 713 (Serenade), to control spot blotch individually and in combination was investigated. On agar plates, all three inhibited growth of the pathogens completely on the second day of culture, except that for *B. spicifera* with *S. lydicus* there was an inhibition zone and the pathogen grew in the opposite direction. Disease severity was lowest when *T. harzianum* T-22 was applied individually to the most resistant cultivar, Nibola. Foliar application, soil treatment and seed coating all reduced disease severity. With foliar application, *T. harzianum* T-22 was more effective when applied at the same time as the pathogen than when applied one week before or four days after. In a field experiment with *T. harzianum* T-22, foliar application combined with seed treatment suppressed spot blotch more effectively than either method individually.

Dedication

I would like to dedicate this research

To

My Parents, My wife and My Children

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I am grateful and very happy to thank all the people who supported me during the period of my studies. I cannot thank by name all the people who have supported me by physical action, by advice or by encouraging me to achieve my study successfully, especially those who taught me from my childhood till this stage of my study, but nevertheless, thank you for all.

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Chapter 1 General Introduction

1.1 Background of the Study

Barley is one of the most important cereal crops, widely grown in Libya and in many other parts of the world. In 2007 barley was the fourth cereal in the world in both quantity produced (136 million tons) and area of cultivation (566,000 km^2) (Sharma and Yadav, 2013). Barley is a major crop grown in Libya and it has recently been grown more widely than in the past. The majority of people in Libya use barley flour to make a traditional meal food. In addition most farmers use barley as the main source of animal feed. In Libya in 2009 its ranking was first; the area cultivated was 205000 ha and the production was 4927 hg/ha (FAO, 2009). Compared to other countries the yield is low because of several factors, and the Libyan authorities have had to import large amounts of barley from other countries to meet the high demand for this product, because of insufficient production in Libya. Plant diseases, especially foliar diseases, are one of the factors that contribute most to limiting barley production in Libya.

As a group, fungi are the most important plant pathogens. Of the 100,000 or more species of fungi that have been described, only about 50 cause diseases in man; more than 8000 species are known to cause diseases in plants (Parry, 1990). Barley is greatly affected by many fungal diseases, accounting for considerable yield loss every year. The global yield losses of barley caused by foliar diseases are estimated to range from 10% to 40%, amounting to billions of dollars per season (Sharma and Duveiller, 2006). However, the toll of the diseases varies from country to country. Spot blotch disease caused by the fungus *Bipolaris sorokiniana* (teleomorph *Cochliobolus sativus*) is one of the most important fungal diseases of barley (Arabi *et al*., 2011; Arabi and Jawhar, 2004; El Yousfi and Ezzahiri, 2002; Valjavec-Gratian and Steffenson, 1997b).

1.2 Spot blotch disease

Spot blotch disease caused by *B. sorokiniana* is found worldwide but is most serious in tropical areas where barley and wheat are grown. Many factors, including climate conditions (rainfall, temperature and humidity) and agricultural practices, influence the development and spread of the disease and thus its effect on crops. *B. sorokiniana* causes several diseases in its hosts worldwide. It produces a broad range of symptoms such as foot and root rot, leaf blotch and black point of seeds. It affects wheat, as well as barley, most seriously in tropical areas; it can cause a high loss of wheat yield (Emami and Hack, 2001). It has been described as one of the most serious diseases of wheat in warm regions (Acharya *et al.*, 2011; Iram and Ahmad, 2005b) and is one of the most important pathogens of wheat in south Asia. Annual yield loss on wheat due to this disease in south Asia is estimated at 15-20% (Duveiller and Sharma, 2009). In eastern India under severe conditions the yield losses of wheat can reach 100% (Pandey *et al.*, 2005). In the United States it has been reported to be a serious foliar disease which causes economic loss of wheat yield (Wegulo et al., 2009); yield losses ranged from 16% to 33% in the 1960s on barley (Valjavec-Gratian and Steffenson, 1997b). Yield losses of 25–45% have been recorded in Kazakhstan and 41% in Russia (Iftikhar *et al.*, 2009). Although spot blotch is generally associated with warm conditions, a survey of barley diseases in high altitude cold areas in the Trans-Himalayan Ladakh region of India found estimated yield losses of 6% to 53% due to leaf spot blotch and leaf blight caused by *B. sorokiniana* (Vaish *et al.*, 2011).

Studies have reported different sources of initial infection by spot blotch disease. Infected seeds can be the main source of inoculum, especially in newly cultivated areas. The survival of free inactive conidia in soil for more than one year and repeated growing of the same crop provides another source of infection, and alternative hosts such as grasses can also be a source of the pathogen (Pandey *et al.*, 2005). Neupane et al. (2010) studied sources of *B. sorokiniana* inoculum causing spot blotch under warm wheat growing conditions in south Asia, and demonstrated that weeds were not the initial source of inoculum but could be alternative hosts of the pathogen during the

season when wheat is not growing; seeds were the most important initial source of inoculum for spot blotch disease on wheat in warm areas.

1.2.1 Effect of environment on spot blotch disease distribution

There are a number of factors that influence the effects of infection with foliar diseases. Temperature and leaf wetness are the most important factors for conidium germination and barley leaf infection. For example, in a study of net blotch disease caused by *Pyrenophora teres*, the highest percentage of conidia germinating and the shortest leaf wetness period required for infection was at a temperature range from 15 to 25 ºC during the day and from 5 to 10 ºC during the night (Vandenberg and Rossnagel, 1990). In areas where high temperatures occur along with high humidity, *B. sorokiniana* causes significant yield losses on cereal crops (Kumar *et al.*, 2002). Humidity and warm conditions have been demonstrated to be important for *B. sorokiniana* to cause disease on spring barley whereas hot and dry conditions were less favourable (Lacicowa and Pieta, 1998). The most suitable temperature, along with high humidity, to cause high infection with *B. sorokiniana* on wheat was found to be 26 ºC (Joshi *et al.*, 2007). Continuing rainfall for 5 to 6 days followed by daily average temperatures of 20 to 30 ºC favours rapid development of spot blotch disease epidemics on wheat and barley (Kumar *et al.*, 2002). Foliar symptoms on wheat can be more clear on plants when temperatures rise (Duveiller, 2004). Spot blotch disease and tan spot on wheat were more severe in a season where temperature was high than a season where temperature was low (Gurung *et al.*, 2012).

B. sorokiniana can survive extremes of heat and cold. *B. sorokiniana* causing spot blotch of turfgrass can survive at high temperatures, up to 55 ºC for spores and 65 ºC for mycelia, whereas the most suitable temperature for sporulation and mycelium growth was 30 ºC and for spore germination was 25 ºC (Zhang *et al.*, 2009a). Duczek (1995) tested the ability of *B. sorokiniana* conidia to germinate after being stored in freezing conditions, and demonstrated that they can survive and germinate at a high

rate, up to 74%, after exposure to low temperatures between -12 and -22 ºC, which gives this pathogen the ability to cause disease in cool areas.

1.2.2 *Cochliobolus* (anamorphs *Bipolaris*, *Curvularia*)

The teleomorphic genus *Cochliobolus* has asexual states of *Bipolaris* and *Curvularia*, whereas some species of *Bipolaris* and *Curvularia* do not have known sexual states of *Cochliobolus*. On the basis of molecular phylogenetic analysis of ITS (internal transcribed spacer of ribosomal DNA) and GPDH (glyceraldehyde-3-phosphate dehydrogenase) sequences, 31 species in the genus *Cochliobolus* could be divided into two groups (Berbee *et al*., 1999). Recently, Manamgoda et al. (2012) have extended the analysis to include ribosomal RNA large subunit and elongation factor 1-α sequences, with results that are generally consistent with the earlier analysis. Group one includes highly virulent pathogens with *Bipolaris* asexual states and group two includes only relatively mild pathogens, with asexual states in *Curvularia* and *Bipolaris*. Both groups contain species that cause economic losses on grasses. Group one includes the most important species that cause the highest economic losses, including *Cochliobolus sativus*. *Cochliobolus miyabeanus* (anamorph *Bipolaris oryzae*) caused huge losses of rice yields ranging from 40% to 90% in Bengal in 1942, leading to famine and the death of two million people (Berbee *et al*., 1999). In maize, high losses can occur in plants with Texas male-sterile cytoplasm infected by another group one species, *Bipolaris maydis* (*Cochliobolus heterostrophus*) race T, which caused an epidemic of southern corn leaf blight in 1970 in the USA. (Lim and Hooker, 1971). *Bipolaris victoriae* causes Victoria blight on oat. Some other species of *Cochliobolus* in both groups, such as *Cochliobolus peregianensis* (group one), *C. intermedius* and *C. perotidis* (both group two) cause less well-known diseases on grasses. Besides being pathogens, species of *Cochliobolus* (including anamorphic species of *Bipolaris* and *Curvularia*) have three other modes of life. They can be epiphytic, such as *Curvularia pallescens* which was isolated from the surface of banana fruit, endophytic, such as *Bipolaris cynodontis* isolated from leaves of tomato (*Solanum lycopersicum*), and saprobic, such as *Bipolaris maydis* isolated from *Zea mays* seeds (Manamgoda *et al*., 2011).

In this thesis, the name *B. sorokiniana* is used for the pathogen under study in preference to the teleomorph name *Cochliobolus sativus*. The perfect stage is rarely observed in nature (Kumar *et al*., 2002), and the anamorph name seems to be more common in the scientific literature, especially in relation to barley disease. Moreover, Manamgoda *et al.* (2012) have recently proposed that the two groups within *Cochliobolus* should be classified as separate genera, using the previous anamorph names *Bipolaris* for group one and *Curvularia* for group two. Their proposal means, however, that some species that are currently classified in the anamorph genus *Bipolaris* would be transferred to *Curvularia*. An example is *B. spicifera* (see section 1.2.2.1 below).

1.2.2.1 Classification of the genus Cochliobolus

Ascomycota is one of the phyla of the kingdom Fungi, and is one of the largest groups which take their nutrients from land plants. The phylum Ascomycota is divided into three subphyla, Taphrinomycotina, Saccharomycotina (also known as the hemiascomycetes) and Pezizomycotina (the euascomycetes, or filamentous ascomycetes). Pezizomycotina consists of more than 3000 genera, including most of the ascomycetous pathogens and mutualists. Most of the plant pathogenic ascomycetes are in three classes of the Pezizomycotina (Berbee *et al*., 1999). The genus *Cochliobolus* is in the order Pleosporales in the class Dothideomycetes. Zhang *et al*. (2009b) studied the interfamilial relationships in the order Pleosporales by a multi-gene analysis and re-evaluated the significance of morphological and ecological characters which have been used in investigating phylogeny and taxonomy of this order. They concluded that most of the well-known plant pathogens in the Pleosporales belong to four families. One of these families, the Pleosporaceae, includes the genus *Cochliobolus* and other organisms associated with plants, such as *Alternaria* species.

Table 1-1. Classification of the genus *Cochliobolus*.

Taxonomic Rank	Taxon
Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	Dothideomycetes
Order	Pleosporales
Family	Pleosporaceae
Genus	*Cochliobolus*

1.2.2.2 Bipolaris sorokiniana

B. sorokiniana has been reported to cause diseases in several crops including barley. It can cause spot blotch on barley (Knight *et al*., 2010; Zhong and Steffenson, 2002), common root rot on wheat and barley (Gyawali *et al*., 2012; Lehmensiek *et al*., 2010; Shivanna *et al*., 1996a), head blight and seedling blight on barley (Chamswarng *et al*., 1992), crown root and common root rot on wheat (Wildermuth *et al.*, 1997), leaf blight and black point (Kumar *et al*., 2002), Early infection can cause damping off. *B. sorokiniana* has been described as the most important fungal pathogen of barley (Arabi and Jawhar, 2004; El Yousfi and Ezzahiri, 2002; Valjavec-Gratian and Steffenson, 1997b), and as one of the most serious pathogens causing foliar diseases on wheat and barley worldwide (Kumar *et al*., 2001). It is a causal agent of serious foliar diseases in warmer growing areas (Matusinsky *et al*., 2010), including on wheat (Duveiller, 2004; Duveiller *et al*., 2005). *B. sorokiniana* was also pathogenic to several oat cultivars in Poland (Kiecana and Cegielko, 2007). Almgren *et al*. (1999) found no significant correlations between severity of leaf and root diseases caused by different isolates of *B. sorokiniana* on barley. Cane and Hampton (1990) observed the occurrence of disease on barley caused by seed contaminated by *B. sorokiniana*. The range of seedling infection was from 5% to 75% and 58% of contaminated seeds gave infected seedlings.

1.2.2.3 Curvularia inaequalis

Curvularia inaequalis has been reported as a pathogen that causes foliar disease on various grasses. Kim *et al*. (2000) isolated *C. inaequalis* from zoysia grass in Korea and studied its pathogenicity on zoysia grass, bentgrass and Bermudagrass. They found that inoculated leaves of all these grasses showed symptoms of blight, and the most susceptible was bentgrass. In addition symptoms of infection with this pathogen occurred on the grasses in the field in the season when the temperature is high; in Korea, symptoms started from May to July when temperatures were high and there was frequent rainfall. The optimal temperature for growth of the pathogen on PDA in the lab was 30 °C. *C. inaequalis* was isolated from Iranian barley seeds along with other pathogens (Nejat-Salari and Ershad, 1994). De Luna *et al*. (2002) tested the infectivity of two other *Curvularia* species, *C. tuberculata* and *C. oryzae* (not the same as *Bipolaris oryzae*), on rice. The susceptibility to infection by *C. tuberculata* and *C. oryzae* on rice was greater in old leaves than in young leaves. Temperature plays a major role in infection by *Curvularia* species causing turfgrass disease; temperature stress is one of the most important factors leading to the infection of old leaves of turfgrass by *Curvularia lunata* (Muchovej and Couch, 1987).

1.2.2.4 Bipolaris spicifera

Although generally not considered a serious pathogen, *B. spicifera* has been reported to cause foliar disease on many plants. Host plants are mostly but not all in the grass family; for example, El Mhadri *et al*. (2009) reported that *B. spicifera* causes brown spot on watermelon leaves in the high temperature areas in Morocco. *B. spicifera* was reported to cause leaf blotch on sorghum in Turkey; disease incidence and severity were rated at 45% and 25 to 75%, respectively. Typical symptoms included elliptical, straw-coloured, necrotic lesions with darker margins. The lesions eventually coalesced, resulting in drying of leaves (Unal *et al.*, 2011). Ennaffah *et al*. (1997) reported that *Helminthosporium spiciferum* (i.e. *Bipolaris spicifera*) was pathogenic on rice in Morocco. It was demonstrated that six cultivars of rice were susceptible to this pathogen and disease severity was high (Ennaffah *et al*., 1999). *B. spicifera* can cause

symptoms of spot blotch on forage Bermudagrass in high temperature regions in the southeastern USA (Pratt and Brink, 2007). It was demonstrated that severity of spot blotch increased with plant age of ryegrass in Mississippi (Pratt, 2006). Pratt and Brink (2007) found that Bermudagrass was most affected by spot blotch caused by *B. spicifera* during months with high temperatures and the loss of yield could be more than 50%. In Iran, *B. spicifera* was isolated from wheat plants with crown and root rot and caused a discoloration of crown and seminal roots of seedlings, but the severity of disease was less than that of disease caused by *B. sorokiniana* (Safaee *et al.*, 2008). At late stages of wheat growth, in the USA, *B. spicifera* was isolated from crown more than subcrown and root tissues (Gonzalez and Trevathan, 2000). *B. spicifera* can be seedborne. Host range and pathogenicity tests were carried out on *B. spicifera* isolated from seeds of some grasses. The results showed that it can be pathogenic to Bermudagrass, tall fescue, rice, maize, sorghum, orchard grass, and barley whereas in this experiment wheat was not susceptible (Koo *et al.*, 2003). However, Hassan (1999) isolated *B. spicifera* (identified by Emami and Hack, 2002) from wheat seeds from Yemen and showed that it was pathogenic to wheat.

1.2.3 *Alternaria alternata*

A. alternata causes foliar diseases on a wide range of crops. This fungus has been isolated from seeds of wheat and barley in Oman (Al-Sadi and Deadman, 2010) and from leaves of wheat in Pakistan (Iram and Ahmad, 2005b), and has been reported to cause black point on wheat (Conner and Davidson, 1988) and root rot and foliar disease on wheat and rice (Iram and Ahmad, 2005a). It has also been reported to cause black spot disease on persimmon fruits in Turkey (Kurt *et al.*, 2010) and on cherry fruits (Zhao and Liu, 2012), leaf spot on *Stevia rebaudiana* in India (Maiti *et al*., 2006), *Houttuynia cordata* in China (Zheng *et al*., 2011), *Aloe vera* in Louisiana (Silva and Singh, 2012), switchgrass in Tennessee (Vu *et al*., 2012), cucumber (Vakalounakis and Malathrakis, 1988), melon (Vakalounakis, 1990; Zhou and Everts, 2008), sunflower (Lagopodi and Thanassoulopoulos, 1996; Lagopodi and Thanassoulopoulos, 1998), and okra (Arain *et al*., 2012), as well as leaf blight on potatoes in south Africa (Van

Der Waals *et al*., 2011), cotton (Bashan *et al*., 1991), menthol mint (Kalra *et al*., 2001), and *Paulownia fortunei* (Pleysier *et al*., 2006).

1.2.4 Spot blotch disease management

There are several methods to reduce yield losses due to foliar diseases. Fungicides are one of the most common and widely known methods used to minimise the effect of spot blotch. However, fungicides can have adverse effects on the environment and on consumers of crop products. In addition, the continual use of chemicals can lead to increases in resistant strains of pathogens (Vinale *et al*., 2008a).

Integrated pest management is one of the most important strategies that should be followed to reduce the effect of plant diseases in crops. A promising approach to achieve this aim while minimising use of pesticides is to apply and combine different agriculture practices that contribute to increasing crop yield by decreasing plant diseases directly or indirectly. Weed control can reduce the severity of many plant diseases when weeds are alternative hosts of the pathogen, which is when the pathogen can complete its life cycle on the weed, which can then be the main source of infection (Neupane *et al*., 2010).

1.2.4.1 Crop rotation

Crop rotation is one of the most important factors that decrease the severity of diseases and reduce disease distribution. Growing related crops in a particular area for several years continuously tends to increase disease severity. In contrast, crop rotation can reduce the severity of diseases such as root rot (Curl, 1963). Following crop rotation is one of the most important strategies to reduce spot blotch severity on barley. Krupinsky *et al*. (2004) found there was more infection by spot blotch on barley that was cultivated after barley in the same place than on barley that was cultivated after other crops such as wheat. As part of integrated disease management, crop rotation can be combined with other control methods such as compost amendment and the use of

biocontrol agents. For example, this combination reduced soil pathogens on potato and improved the population of soil communities (Bernard *et al*., 2012).

1.2.4.2 Resistance of barley to spot blotch disease

One of the most important strategies to reduce the impact of spot blotch disease caused by *B. sorokiniana* on barley is to use barley cultivars that have resistance to spot blotch. Many studies have done in this field. For example, Arabi and Jawhar (2010), using a rapid method for assessing spot blotch resistance in barley, demonstrated that different cultivars had different levels of resistance to spot blotch disease. In a study of the endogenous resistance of barley cultivars and wheat cultivars to root rot and crown rot caused by *B. sorokiniana,* wheat cultivars were found to be more resistant than barley cultivars (Al-Sadi and Deadman, 2010). To increase barley resistance to spot blotch disease, genetic development is necessary. Cultivars with increased resistance have been bred and the genetics of resistance have been studied (Wilcoxson *et al*., 1990).

Induced resistance can reduce disease severity. Previous inoculation of barley with *Bipolaris maydis* from maize or *Septoria nodorum* from wheat enhanced the resistance of barley against *Drechslera teres*; the reduction of disease severity was between 22% and 70%. In addition, severity of disease caused by *B. sorokiniana* was reduced by 41% when *Septoria nodorum* was used and 48% when *Bipolaris maydis* was used. The effect was cultivar-dependent (Jorgensen *et al*., 1996). Some barley cultivars were resistant to *B. sorokiniana* isolates from common root rot infections of wheat and barley when these were inoculated on barley leaves, but had less resistance to isolates from spot blotch on leaves (Knight *et al*., 2010).

1.3 Biological control

One of the most significant topics of discussion today is the pollution of the atmosphere and damage to the environment due to increasing use of chemicals for

many purposes including plant disease control. Reducing use of chemicals for control of crop diseases is thus highly attractive. One way to achieve this aim is by the use of biological control of pests and diseases of agricultural crops. This study aims at develop methods that will help to control and reduce the impact of the fungal diseases on barley. In view of this, a biological control agent of proven success, *T. harzianum* T-22, was initially selected for this research. It has beneficial effects on plant growth and promotes the development and efficiency of the root system and controls diseases of several crops including maize (Harman, 2005). However, as far as I am aware there has been no previous research to investigate the effects of *T. harzianum* strain T-22 as a biocontrol agent for spot blotch of barley. There are several other commercial biocontrol agents used to control plant diseases, including Actinovate (*Streptomyces lydicus* WYEC 108) and Serenade (*Bacillus subtilis* QST 713). Combining *T. harzianum* with other biocontrol agents may be beneficial. For example, Yigit and Dikilitas (2007) demonstrated that *T. harzianum* T-22 gave successful biocontrol of *Fusarium* wilt of tomato when applied individually or in combination with fluorescent *Pseudomonas* species.

Other combinations of biocontrol agents have achieved successful control of disease. For example, combining a yeast (*Pichia guillermondii*) and a bacterium (*Bacillus mycoides*) reduced *Botrytis cinerea* on strawberry leaves more than when either was applied individually (Guetsky *et al.*, 2001). On the other hand, when a combination of some biocontrol agents such as Serenade (*Bacillus subtilis*), Trianum (*Trichoderma harzianum* T-22) and Sentinel (*Trichoderma atroviride*) was applied to control *Botrytis cinerea* on strawberry, there was less effective control than when the agents were used individually (Robinson-Boyer et al., 2009; Xu *et al.*, 2010).

Several studies have been done on the use of different biological control agents to control spot blotch disease caused by *B. sorokiniana* on cereal crops. Aggarwal *et al.* (2004) studied the effect of *Chaetomium globosum* on spot blotch on wheat *in vitro* and *in vivo*. *In vitro* they observed up to 87% inhibition of mycelium growth with some strains of this fungus. Knudsen *et al.* (1995) studied the effectiveness of

Chaetomium sp. isolated from an organic farming system, *Gliocladium roseum* isolated from barley roots, *Humicola* sp. isolated from soil, *Idriella bolleyi* isolated from straw and *Fusarium sambucinum* isolated from straw in a conventional farming system as biocontrol agents to control seedling diseases of barley and wheat caused by *Fusarium culmorum* and *B. sorokiniana*. They studied the effects of the selected fungal antagonists on growth and yield in field and greenhouse experiments by using two methods: seed coating in the field experiment and application of suspensions of spores of the biocontrol agents directly to the soil in which the seeds had already been sown. Some biocontrol agents gave substantial reductions in disease severity with both methods whereas some of them had no effect on seedlings. *Chaetomium* sp., *G. roseum*, *Humicola* sp., *I. bolleyi* and *Fusarium sambucinum* reduced the population of *B. sorokiniana* on seedlings. *I. bolleyi* was used to treat seed of barley to protect it from root and leaf infection by *B. sorokiniana*. However, the colonisation and establishment of *I. bolleyi* strains applied to seed was not investigated under field conditions. When several biocontrol agents were screened against soil borne pathogens in dual culture *in vitro*, they inhibited mycelium growth of the pathogens and among them *G. roseum* was found to be one of the most promising agents for control of *B. sorokiniana*; in a field experiment the level of control of *B. sorokiniana*, *Drechslera teres* and *F. culmorum* (soil borne pathogens) was the same as was achieved using fungicide (Knudsen *et al*., 1997). Jensen *et al*. (2002) studied the ability of conidia of *Clonostachys rosea* to survive on barley seeds and to protect against seedborne disease caused by *B. sorokiniana.* The conidia of *C. rosea* survived long term storage at 4 ºC in dry conditions with a high concentration of conidia and retained the same ability as newly harvested conidia to reduce the severity of seedling blight caused by *B. sorokiniana* on barley. The control of this disease achieved by this biocontrol agent was more than 80%. Duczek (1994) screened some biocontrol agents and studied their efficiency for controlling common root rot under greenhouse conditions and in the field by seed treatment of wheat and barley. Some fungal isolates had more effect on common root rot under greenhouse conditions than in the field whereas other isolates had more effect in the field and less in greenhouse conditions. Duczek (1997) studied biological control of common root rot on barley caused by *B. sorokiniana* in the field by testing the biocontrol agent *I. bolleyi* by seed treatment application for several years. Disease severity was reduced and the yield of barley was increased by 16% when *I.*

bolleyi was used constantly for 6 years. Liljeroth and Bryngelsson (2002) tested the ability of *I. bolleyi* to increase resistance of barley to root and leaf diseases caused by *B. sorokiniana* by seed treatment application. Under greenhouse conditions, seed treatment application of this biocontrol agent decreased disease on the roots, where lesion size was decreased by 50% through enhanced plant resistance, and symptoms on first and second leaves of barley after inoculation by *B. sorokiniana*. However, there was no significant effect of seed treatment on yield in a field experiment. Sjoberg *et al*. (2007) found that arbuscular mycorrhizal fungi when applied in soil could reduce the transmission of seedborne *B. sorokiniana* to the aerial parts of barley plants. In addition they were able to reduce seed infection by 95%.

1.3.1 *Trichoderma* as biocontrol agent

Many studies have been done using *Trichoderma* species as biocontrol agents against a wide range of plant pathogens. Treatments with *Trichoderma* have been successful in reducing the severity of plant diseases and several different commercial products based on strains of *Trichoderma* are sold worldwide. *Trichoderma* species have the ability to interact with airborne and soilborne plant pathogens, and are among the most important antagonists of foliar fungal diseases in a wide range of crops. They have been widely reported as biocontrol agents in the aerial environment, effective against many pathogens (Vinale *et al*., 2008a). *Trichoderma* species have no effect on the bumblebee *Bombus terrestris*, providing evidence that they can be used as antagonists to control diseases without negative effects on the environment (Mommaerts *et al*., 2008). A follow-up study demonstrated that Trianum-P (*Trichoderma harzianum* T-22) was safe for bumblebees even when used in combination with Serenade (*Bacillus subtilis* QST713) (Mommaerts *et al*., 2009).

Trichoderma strains gave excellent control of *Pythium ultimum*, as a result of antagonistic activity reducing the effects of enzymes produced by the pathogen which caused plant damage (Naseby *et al*., 2000). Rossi and Pattori (2009) demonstrated that using *Trichoderma* for control of *Stemphylium vesicarium* on pear gave a substantial

reduction, up to 77%, in inoculum of the pathogen throughout the growing season. *Trichoderma harzianum* gave a great reduction of *Fusarium culmorum* mycelium in dual culture in different conditions (Sempere Ferre and Pilar Santamarina, 2010). Begum *et al*. (2010) used *T. harzianum* for control of Alternaria fruit rot of chili under field conditions. A high reduction of disease severity was achieved, exceeding 72%, and the yield of fruit was increased. However, using *T. harzianum* as biocontrol agent had no significant effect on plant, bulb and yield characteristics of onion (Altintas and Bal, 2008). Also Gasoni *et al*. (2008) investigated the effect of *T. harzianum* on functional diversity of the soil microbial community in tobacco monoculture. They found that inoculation with *T. harzianum* did not significantly modify disease severity in treated plots. When *T. harzianum* was used to control seed borne disease of bean caused by *Sclerotinia sclerotiorum*, it reduced severity of bean rots by more than 92% (Costa Carvalho *et al*., 2011).

In one of the first studies of the potential of *Trichoderma* species for biological control of foliar diseases of cereals, Perelló *et al*. (2003) showed that several strains were effective against tan spot of wheat in greenhouse conditions. They suggested that the *Trichoderma* strains competed with the pathogen, and demonstrated that they were able to survive in the above ground part of the plants for a long time. Perello *et al*. (2006) studied the effectiveness of *T. harzianum* and *Trichoderma koningii* for control of tan spot and Septoria blotch on wheat, and achieved successful control of both diseases under field conditions with two application methods, seed coating and application as a foliar spray. When applied by seed treatment, isolates T2 and T5 of *T. harzianum* gave similar control of tan spot of wheat to fungicide application. Perello *et al*. (2008) found that seed treatment achieved the best results in reducing severity of tan spot on wheat and suggested that the mechanism might involve induction of systemic resistance in the leaves. In a further study, both application methods, seed treatment and foliar spray, were effective for biological control of Septoria tritici blotch disease of wheat at the tillering stage by *Trichoderma* under field conditions, whereas at the heading stage neither treatment had a significant effect (PerelloÌ• *et al*., 2009).

1.3.1.1 Effect of Trichoderma on growth of plants

The value of *Trichoderma* species as biocontrol agents is related to their ability to colonise plant roots and increase plant growth as well as enhance plant resistance to disease (Harman et al., 2004a; Harman *et al*., 1989). *T. harzianum* can have a positive effect on plant growth that is distinct from its ability to control specific diseases. For example, *T. harzianum* had the ability to increase plant growth, plant yield, flowering and root depth of several horticultural crops (Chang *et al*., 1986) and stimulated growth of roots and aerial parts of tomato (Jimenez et al., 2011). Treatment of sweet corn seeds with *T. harzianum* T-22 increased plant height and root depth, which in turn increased yield (Harman, 2000). *Trichoderma* species increased plant growth and by improving uptake of soil nutrients (Bharti *et al*., 2012; El-Katatny, 2010).

1.3.1.2 Mechanisms of control of plant pathogens by Trichoderma

Numerous studies have been done to understand the mechanisms by which *Trichoderma* species control plant pathogens and affect non plant pathogens, because of their wide effects. *Trichoderma* can be found in most soils in diverse climates. They have the ability to produce strong spores and survive in different environmental conditions (Rawat *et al.*, 2012). *Trichoderma* species have several ways to control plant pathogens (reviewed by Howell, 2003). They can compete with pathogens (Elad *et al*., 1999; Papavizas, 1985); the fast growth of *Trichoderma* mycelium gives them an advantage for controlling other pathogens by competition for nutrients (Harman, 2000; Howell, 2002; Vinale *et al.*, 2008b). They can colonise the pathogen directly (Chet *et al.*, 1981) and antagonise many pathogens by mycoparasitism (Lorito *et al.*, 1996), antibiosis (Schirmbock *et al*., 1994) and production of secondary metabolites (Mukherjee *et al*., 2012; Vinale *et al*., 2009; Vinale *et al*., 2012) such as pyrone-like antibiotics. Hajieghrari *et al*. (2008) found evidence that *Trichoderma* species inhibited mycelial growth of pathogens due to their ability to produce volatile and non-volatile compounds. *Trichoderma* also has the ability to produce enzymes that degrade fungal cell walls (Cardoso Lopes *et al*., 2012; Do Vale *et al*., 2012; Druzhinina *et al*., 2011; El-Katatny *et al*., 2003). By colonising plant roots (Harman *et al*., 2004a), they compete with pathogens and prevent them from growing, in addition to promoting

growth of plants (Sofo *et al*., 2012; Vinale *et al*., 2012) by increasing uptake of nutrients (Altomare *et al*., 1999) and increasing plant disease resistance (Tan *et al*., 2012; Vinale *et al*., 2008a).

1.3.2 Methods of applying biological control

1.3.2.1 Foliar application

The effectiveness of biological control depends on the method of application along with other factors such as resistance of the cultivars and the interaction with the pathogen. There are different methods of applying biocontrol agents. These methods depend on the agent and for commercial biocontrol agents the formulation. Foliar application is one of the methods. Studies have been done of biological control of a wide range of plant pathogens by foliar spray application. For example, Ojaghian (2011) studied the effects of *Trichoderma* species on potato stem rot disease caused by *Sclerotinia sclerotiorum* using a spray application method. Khan *et al*. (2011) used foliar spray application of several biological control agents to control grey mould of chickpea caused by *Botrytis cinerea*. Foliar application of *Trichoderma* has been used against tan spot and Septoria blotch on wheat (Perello *et al*., 2006; Perello *et al*., 2009), against *Pythium ultimum* (Naseby *et al.*, 2000), against *Stemphylium vesicarium* on pears (Rossi and Pattori, 2009), against Alternaria fruit rot of chili (Begum *et al*., 2010), and against Fusarium wilt of tomato (Yigit and Dikilitas, 2007). Foliar application of *Pseudomonas chlororaphis* gave a substantial reduction in severity of net blotch disease on barley caused by *Pyrenophora teres* (Khan *et al*., 2010). Foliar spraying of *T. harzianum* 1295-22 achieved a great reduction in severity of Pythium root rot, brown patch, and dollar spot on bentgrass (Lo *et al*., 1997). *Bacillus* species have been applied against apple pre- and postharvest disease (Poleatewich *et al*., 2012). Foliar application of *Acremonium strictum* was used on different plants infected by Botrytis disease caused by *B. cinerea* (Choi et al., 2009). Alvarez *et al*. (2012) tested foliar application of *Bacillus amyloliquefaciens* to control stem rot on soybean caused

by *S. sclerotiorum* in Argentina. The results indicated that foliar application of *Bacillus amyloliquefaciens* achieved good results in suppression of this disease.

1.3.2.2 Soil treatment

Soil treatment is one of the methods of application for control of plant diseases either by chemical or biological control. Dubey *et al*. (2011) tested the effects of *Trichoderma* species on wet root rot of mungbean caused by *Rhizoctonia solani* by application as soil treatment and seed treatment. The results indicated that both application methods gave good results of reducing disease severity individually whereas combining application methods was the best.

Soil treatment with different isolates of *Trichoderma* has been applied against potato stem rot caused by *S. sclerotiorum*. Soil treatment with other biocontrol agents – *T. harzianum* T-22, *S. lydicus*, *B. subtilis* and *Coniothyrium minitans* – has also been used against Sclerotinia stem rot of soybean (Zeng *et al*., 2012). Lo *et al*. (1997) demonstrated that soil treatment with *T. harzianum* 1295-22 alone to control dollar spot on bentgrass did not achieve high reduction of disease, whereas the combination of soil treatment and foliar application with continuing application of the foliar method every seven days achieved good results in reducing Pythium root rot, brown patch, and dollar spot. The results were the same as those achieved by chemical control applied once a month.

1.3.2.3 Seed treatment

Seed treatment is one of the methods of application of both chemical and biocontrol agents to control plant diseases. Its suitability for a particular pathogen depends on the mechanism of pathogenesis and how this pathogen infects the plant. For some plant pathogens the initial source is the seeds, so that seed treatment is effective in

decreasing the initial source population. Yobo *et al*. (2011) tested the effects of seed treatment with *Trichoderma* and *Bacillus* isolates against damping off disease caused by *R. solani* on dry bean, and found good reduction of disease severity. Treatment of oilseed rape seeds with *Serratia plymuthica* and *Pseudomonas chloroaphis* as biocontrol agents against blackleg disease caused by *Leptosphaeria maculans* gave positive effects (Abuamsha *et al*., 2011). Seed treatment application of *Paenibacillus alvei* against *Thielaviopsis basicola*, which causes black root rot disease on cotton, gave better results than were obtained with other methods (Schoina *et al*., 2011). Perello and Dal Bello (2011) have used seed coating with *Trichoderma* to control tan spot disease on wheat, and found reduced disease severity and increased plant growth.

The combination of methods of applying biocontrol agents has been studied. Lo *et al*. (1997) suggested that repeating foliar application of *T. harzianum* 1295-22 each month after soil treatment is highly effective for reducing the population of foliar disease as it protects plants from the second infection with the pathogens, and weekly application of foliar spray can be important when the severity of disease is potentially high. Seed treatment with the fungal biocontrol agent *Trichoderma* species and the bacterial biocontrol agent *Bacillus* species in combination gave a greater reduction in damping off disease on cucumber caused by *R. solani* than individual use (Yobo *et al*., 2010).

1.3.3 The use of *Bacillus subtilis* to control plant diseases

Strains of the bacterium *B. subtilis* have been used as biocontrol agents against various plant pathogens. *B. subtilis* can be found in the environment in soils and on plants. For example, Swinburne *et al*. (1975) isolated *B. subtilis* from apple leaf scars and Foldes *et al*. (2000) isolated *B. subtilis* from soil around cereal roots. *B. subtilis* was active *in vitro* and *in vivo* against potato blackleg and soft rot bacteria (Sharga and Lyon, 1998). *B. subtilis* was tested under greenhouse and field conditions to control *Pythium aphanidermatum* on tomato plants by seed treatment application; it reduced disease severity and enhanced plant growth (Jayaraj *et al*., 2005). It has been used with good results to control postharvest diseases on melon (Wang *et al*., 2010). Sunflower disease caused by *S. sclerotiorum* was reduced by using *B. subtilis* as biocontrol agent in a field experiment (Schmiedeknecht *et al*., 2001).

In vitro, *B. subtilis* has been found to inhibit growth of the bacterial pathogen *Pseudomonas syringae* and the inhibition zone was noticed when *B. subtilis* was grown with *Pseudomonas syringae* for sixteen hours. When *B. subtilis* was tested on Arabidopsis roots against *Pseudomonas syringae*, it reduced the death of plants caused by this pathogen (Bais *et al*., 2004). When *B. subtilis* was tested *in vitro* against *B. cinerea* which causes damping off of seeds of beans, it suppressed growth of mycelium of this pathogen and stopped germination of conidia on different growth media (Walker *et al*., 1998). *B. subtilis* has the ability to grow as an endophyte and suppress pathogens in the same ecological niche inside the plants (Bacon *et al*., 2001).

B. subtilis can increase plant growth and plant disease resistance (Krebs *et al*., 1998). In addition to its effects on disease control, increased plant growth was observed when *B. subtilis* was applied to greenhouse-grown maize (Schmiedeknecht et al., 2001). The effect of *B. subtilis* stain FZB 24 on the tolerance of salt stress by eggplant and pepper has been tested. The results demonstrated that this bacterium has the ability to increase plant growth and yield by increasing plant resistance to plant pathogens and to soil conditions affecting growth such as saline soil (Bochow *et al*., 2001). In general, one of the uses of *B. subtilis*, in addition to its use as a biocontrol agent against plant pathogens, is to increase plant growth and yield by increasing plant resistance to stresses (Idris *et al*., 2004).

Different mechanisms by which *B. subtilis* controls plant pathogens contribute to making it a successful commercial biocontrol agent. De Oliveira *et al*. (2011), Zeriouh *et al*. (2011) and Kindoli *et al*. (2012) demonstrated that *Bacillus* species have the ability to produce antimicrobial compounds active against fungal and bacterial pathogens. Suppression of mycelium growth of fungal pathogens was due to the ability of *Bacillus* species to produce antifungal compounds and volatile substances and different lytic enzymes such as protease (Alimi *et al*., 2012; Kumar *et al.*, 2012a; Manjula and Podile, 2005), and cell wall-degrading enzymes such as chitinase and β-1,3 glucanase (Manjula and Podile, 2005). The ability of the spores produced by *B. subtilis* to tolerate harsh treatments including chemical treatment, desiccation, high

temperature, UV irradiation and organic solvents contributes to their effectiveness as biocontrol agents (Leelasuphakul *et al*., 2008).

1.3.4 The use of *Streptomyces* species as biocontrol agents

Actinomycetes have been used as biocontrol agents against several plant pathogens. *Streptomyces* is the genus of actinomycetes that has been used most widely for biocontrol of plant diseases. *Streptomyces* species are well known for their ability to produce antimicrobial compounds (Vetsigian *et al.*, 2011). Doumbou et al. (2001) have reviewed the roles of *Streptomyces* species in enhancing plant growth and in biological control of soilborne pathogens. These species produce different antifungal substances and have intense antagonistic activity (El-Tarabily and Sivasithamparam, 2006). *Streptomyces* species active as biocontrol agents have been isolated from potato tissue (Prevost *et al*., 2006) and from different soils (Lee and Hwang, 2002; Ndonde and Semu, 2000). Antimicrobial activity was found in around 40% of soil actinomycetes (Yilmaz *et al.*, 2008), from different soils. The ability of *Streptomyce*s species to produce several secondary metabolites active against plant pathogens contributes to their importance as biocontrol agents (Gesheva and Gesheva, 2000; Ndonde and Semu, 2000; Park *et al.*, 2011). A further advantage is their ability to grow in different environmental conditions (Shanker *et al*., 2010).

Coating of tomato seeds with *Streptomyces* species protected plants against fungal and bacterial pathogens and promoted plant growth (Elabyad *et al*., 1993). Lin *et al*. (2012) reported that *Streptomyces* species increase plant resistance against plant pathogens by producing various secondary metabolites. They can increase plant growth (Gopalakrishnan *et al*., 2012). Shimizu *et al*. (2009) studied the effects of *Streptomyces* species against *Colletrichum orbiculare*, which causes cucumber anthracnose disease, *in vitro* and *in planta* in a controlled environment. Growth of the pathogen was inhibited *in vitro* in growth media and control *in planta* was effective due to the ability of *Streptomyces* species to colonise the surface of leaves above the cuticle, give plants more resistance and reduce the impact of the pathogen.

Many studies have tested the ability of *Streptomyces* species to act as biocontrol agents against soil borne plant pathogens (Broadbent *et al*., 1971). They have been used against the bacterial pathogens *Pectobacterium carotovorum* and *Pectobacterium atrosepticum,* which cause potato soft rot; all tested strains of *Streptomyces* reduced soft rot (Baz *et al*., 2012). Prevost *et al*. (2006) studied the use of *Streptomyces melanosporofaciens* EF-76 and chitosan, which inhibits growth of some pathogens and elicits plant defence mechanisms, alone and in combination to control common scab disease on potato; the combination reduced disease incidence but had little effect on soil bacterial communities. *Streptomyces violaceusniger* strain G10 has been tested *in vitro* against *Fusarium oxysporum* causing Fusarium wilt disease of banana. The results indicated that this biocontrol agent has antifungal activity and inhibited mycelium growth of *Fusarium oxysporum* (Getha and Vikineswary, 2002). It has also been used against bacterial blight of rice (Park *et al*., 2011). *Streptomyces* species gave positive effects against *Pythium ultimum* and inhibited growth of this pathogen and several other bacterial and fungal pathogens (Ndonde and Semu, 2000). Bolton (1978) tested the effects of Actinomycetes, particularly *Streptomyces* species, against root rot and black leg of geranium caused by *Pythium splendens*, and found protection of roots from this pathogen. Tahvonen (1982) studied the effects of *Streptomyces* species against soil and seed pathogens on growth media containing peat and found that *Streptomyces* species suppressed growth of pathogens. *Streptomyces* species have been used against the soilborne pathogens *R. solani* and *P. ultimum*. Again, the results obtained indicated that *Streptomyces* species have the ability to suppress growth of pathogens (Berg *et al*., 2001).

Crawford *et al*. (1993) isolated and characterised the effectiveness of a large number of actinomycetes against *P. ultimum*. *Streptomyces lydicus* WYEC 108 was one of the strongest antagonists *in vitro.* It could grow at different pH values and could establish itself in the rhizosphere of different plants and survive for about six months. *S. lydicus* WYEC 108 has been used to control damping off and root rot on pea and cotton. In an *in vitro* study it suppressed growth of mycelium of *P. ultimum* and *R. solani*. In addition when soil was treated with *S. lydicus* WYEC 108 to control Pythium seed rot and root rot, there were increases in plant height, average plant stand and plant weight

(Yuan and Crawford, 1995). *S. lydicus* was effective in controlling cucumber root rot caused by *R. solani* (Swelim *et al.*, 2003). Because of its different mechanisms to control plant diseases, *S. lydicus* WYEC 108 has become one of the most important commercial biocontrol agents, used against a wide range of plant pathogens. It has the ability to colonise plant roots and promote growth of other beneficial bacteria, increasing uptake of nutrients that enhance plant growth and increase plant resistance against pathogens (Tokala *et al*., 2002).

Streptomyces are not effective only against bacterial and fungal pathogens of plants. *S. lydicus* has also been tested against nematodes on tomato and achieved good results in reducing nematode populations (Belair *et al*., 2011). *Streptomyces* species inhibited growth of *Pseudomonas tolaasii*, the cause of bacterial blotch on mushrooms (Sahin, 2005).

1.3.5 Combination of biocontrol agents

Studies have been done of the use of combinations of biocontrol agents against numerous plant pathogens on different plants in order to achieve high reduction of plant disease severity and develop viable biological control methods. Combining biocontrol agents to control any plant pathogen requires in-depth study of the roles and the antibiotic production by the biocontrol agents to be successful and achieve high biocontrol (Xu *et al*., 2011). The combination of a fungal biocontrol agent, *Trichoderma*, with a bacterial biocontrol agent, *B. subtilis*, to control damping off disease on dry dean caused by *R. solani* gave greater reduction in disease severity than when either was applied individually (Yobo *et al.*, 2011). Similarly, combining a yeast (*Pichia guillermondii*) and a bacterium (*Bacillus mycoides*) reduced *Botrytis cinerea* on strawberry leaves more than when either was applied individually (Guetsky *et al*., 2001). Yigit and Dikilitas (2007) demonstrated that *Trichoderma harzianum* T-22 gave successful biocontrol of Fusarium wilt of tomato when applied individually or in combination with fluorescent *Pseudomonas* species, but individual use had less effect than the combination. On the other hand, when a combination of Serenade (*Bacillus*

subtilis), Trianum (*Trichoderma harzianum* T-22) and Sentinel (*Trichoderma atroviride*) was applied to control *Botrytis cinerea* on strawberry, there was less effective control than when the products were used individually (Robinson-Boyer *et al.*, 2009; Xu *et al.*, 2011; Xu *et al.*, 2010). Similarly, individual application of the commercial biocontrol agents *S. lydicus* WYEC108 (Actinovate), *B. subtilis* GB03 (Companion), and *Bacillus pumilus* QRD 2808 (Sonata ASO) against powdery mildew of pumpkin achieved greater reduction of disease than the combination of biocontrol agents (Janousek *et al.*, 2009). Individual use of *Serratia plymuthica* and *Pseudomonas chloroaphis* to control blackleg disease on oilseed rape caused by *Leptosphaeria maculans* gave greater reduction of disease than the combination (Abuamsha *et al.*, 2011). Gilardi *et al.* (2008) tested the effects of biocontrol agents *B. subtilis* and *Ampelomyces quisqualis* applied alone and in combination with fungicides against powdery mildew caused by *Podosphaera xanthii* on zucchini. Combination of *B. subtilis* with azoxystrobin gave better results in reducing disease severity than when either was used alone.

Feng *et al.* (2011) have developed SCAR (Sequence-Characterised Amplified Region) probes for two biocontrol agents, *Trichoderma* strains IMI206039 and IMI206040, and have shown that these probes can be used to track a formulation containing the two active strains in one single reaction. This method was successfully applied to monitor the two biocontrol *Trichoderma* strains in two lab trials and on a golf green, and is a suitable tool for safety evaluations in applications with these two strains (PerelloÌ• *et al.*, 2009).

1.3.6 Combination of biocontrol agents with fungicides

Seed treatment by a combination of *Trichoderma harzianum* with fungicide has been shown to be effective for control of plant disease caused by *Pythium ultimum* and to increase plant resistance. Seed treatment of vegetables by chemical pesticide combined with *Trichoderma* species has protected seedlings in field trials (Harman *et al.*, 1989). Combination of *T. harzianum* with fungicide to control the leaf blight disease complex

caused by *B. sorokiniana* and *Alternaria triticina* on wheat reduced disease incidence and increased yield (Singh *et al*., 2008). *Trichoderma viride*, *T. harzianum*, and *Trichoderma virens* alone and in combination with chemical compounds were tested on chickpea wilt disease caused by *Fusarium oxysporum.* The results indicated that all *Trichoderma* species in combination with carboxin by seed treatment application gave better control of wilt disease and yield than when used individually (Dubey *et al*., 2007).

1.3.7 Combining of biocontrol agents with plant disease resistance

Jacobsen *et al*. (2004) pointed out that biological control should be most effective when used in combination with disease resistance in an integrated disease management system, but relatively few studies have tested the efficacy of biocontrol agents in combination with different levels of plant disease resistance. In several cases control with *B. subtilis* has been found to be more successful on hosts with more disease resistance. When a *Bacillus*-based biocontrol agent was used to control Cercospora leaf disease on sugarbeet, its effectiveness was greater on cultivars that were more resistant (Jacobsen, 2006). Hervas *et al*. (1998) tested the effectiveness of three biocontrol agents, *B. subtilis, T. harzianum,* and non-pathogenic *F. oxysporum*, individually and in combination to control Fusarium wilt on two cultivars of chickpea, PV 61, which was more resistant, and ICCV 4, which was less resistant. The three biocontrol agents suppressed Fusarium wilt disease more when used alone than in combination and *B. subtilis* reduced disease more on the more resistant PV 61 cultivar than on the less resistant ICCV 4 cultivar. Another study of controlling Fusarium wilt disease caused by *F. oxysporum* f. sp. *ciceris* tested two other chickpea cultivars, Hashem, which was more resistant, and Pirooz, which was less resistant, with two biocontrol agents, *T. harzianum* and *B. subtilis*, applied as seed treatment and soil treatment. The biocontrol agents suppressed Fusarium wilt disease on both cultivars but, more on the more resistant cultivar Hashem than on the less resistant cultivar Pirooz (Moradi *et al*., 2012).

1.4 Aims of this project

The long-term aim of this project is to improve control of spot blotch disease on barley in Libya. There has been little research on this disease in Libya, so that to be able to study spot blotch control, it was first necessary to identify the pathogens involved. To improve control, integrated management using biological control in combination with disease resistance is a promising approach. As described above, much research has been done to try to isolate biocontrol organisms and there have been many promising leads, but this research has only led to commercial products in a few cases. Since commercial products are available, have been extensively tested, and have been shown to be effective on a number of diseases, testing their effectiveness on spot blotch provides a focused approach to the application of biological control.

The following specific objectives were designed to meet the overall aims of the project.

1- Survey and identify pathogens of spot blotch disease on barley in Libya.

2- Investigate variation in resistance of barley cultivars to spot blotch. For this purpose, four cultivars that are widely grown in Libya were chosen: two six-row cultivars, Nibola and Rehan, and two two-row cultivars, Wadi Utbah and ACSAD. For comparison, two two-row UK barley cultivars, Gaelic and Pastoral, were included in the study.

3- Investigate the interactions between pathogens and the organisms in three commercial biocontrol agents, *Trichoderma harzianum* T-22 (Trianum), *Streptomyces lydicus* WYEC 108 (Actinovate) and *Bacillus subtilis* QST 713 (Serenade), individually and in combination *in vitro*.

4- Investigate the effects of the three biocontrol agents individually and in combination under controlled conditions on spot blotch disease on barley cultivars with differing disease resistance.

5- Identify the best method of applying the biocontrol agents. Foliar application with different timings (before, with or after the pathogen), soil treatment and seed treatment were tested.

6- Investigate the combination of the application methods with frequency of application of the biocontrol agent.

7- Investigate the effectiveness of biological control of spot blotch disease in a field experiment.

The study will help to provide an in-depth knowledge into the antagonistic effects of *Trichoderma harzianum* T-22, *Streptomyces lydicus* and *Bacillus subtilis* individually and in combination for the future development of practical biological control of foliar diseases of barley. In turn, this will help to reduce use of chemical methods, which can damage the environment and select strains of pests resistant to the chemical.

1.5 Research Questions

The research is divided into three broad areas. The first area surveys spot blotch disease in Libya and compares fungi isolated from diseased plants with a known typical isolate of the spot blotch pathogen. The second area evaluates the interactions between pathogen isolates and the antagonistic agent *T. harzianum* T-22 *in vitro* and *in planta*. The third area evaluates the interactions between pathogen isolates and the antagonistic agents in the other commercial products to compare their effects with those of *T. harzianum* T-22 so as to identify the most suitable biocontrol agent against spot blotch pathogens, and tests combinations of the three agents.

To achieve the research objectives, the work was designed to answer the following questions.

1- Is spot blotch in Libya caused by the known pathogen *B. sorokiniana* or are other organisms involved? Do the pathogens causing spot blotch differ in different climatic regions?

2- Are there any differences among the tested barley cultivars in their resistance to spot blotch disease, including differences that might be related to geographic origin or barley type?

3- Do potential biocontrol agents have any negative effect on barley cultivars?

4- Do *T. harzianum* T-22, *S. lydicus*, and *B. subtilis* antagonise the spot blotch pathogen or pathogens *in vitro* and do they reduce disease severity? Are there differences in their effects on different pathogen isolates?

5- What is the most suitable treatment method for biological control by *T. harzianum* T-22? Is it most effective when applied as a foliar spray, as a seed coating, or by soil treatment?

6- Is there any benefit from using the biocontrol agents in combination for the control of spot blotch?

7- Is there any benefit from combining the use of biocontrol agents and plant resistance to the spot blotch pathogen?

8- Is there any difference between the effectiveness of different methods of application of biocontrol agents and between single and more frequent or combined application?

Chapter 2 Materials and Methods

2.1 General plant growth

Six barley cultivars were used in the experiments. Seed of four Libyan cultivars was obtained from the Tesawa project for production of improved seeds in Libya: two six-rowed cultivars, Rehan and Nibola, and two two-rowed cultivars, ACSAD 1420 (Arab Centre for the Study of Arid Zones and Dry Lands; referred to in this thesis as ACSAD) and Wadi Utbah 2 (referred to in this thesis as Wadi Utbah). Two UK cultivars (Pastoral and Gaelic, both two-rowed) were obtained from Close House field station, Newcastle University. In the environment of the Tesawa project for production of improved seeds, Wadi Utbah 2 and Rehan mature at 114 days, ACSAD 1420 at 115 days and Nibola at 116 days.

Plants were grown in plastic pots (12 cm diameter) containing John Innes No. 2 soil-based compost in a greenhouse at Close House field station at different temperatures, depending on the time of year. Five plants were normally grown in each pot. Seeds were sown at a depth of approximately 1 to 2 cm.

2.2 Pathogens

Samples of diseased plants of different barley cultivars were gathered at various times during the 2009 growth season from multiple sites in areas of Libya where barley is grown. It was decided that the best method to adopt for this investigation was to isolate and identify spot blotch pathogens from Libya, as described in Chapter 3. General protocols used in this study are as described by Perello *et al*. (2009) and Trejo-Estrada *et al*. (1998).

The following isolates from diseased samples from Libya were identified (Chapter 3) and used for further work.

1- *B. sorokiniana* isolate L50, isolated from a sample of cultivar ACSAD collected from Barjoj project.
2- *B.s spicifera* isolate L35, isolated from a sample of cultivar Rehan collected from Siret project.

3- *C. inaequalis* isolate L6 isolated from a sample of cultivar Rehan collected from Tripoli.
4- *A. alternata* isolate L79 isolated from a sample of cultivar Wadi Utbah collected from Irawan project.

5- *B. sorokiniana* strain 6.16 was obtained from Science and Advice for Scottish Agriculture (SASA), Edinburgh, UK.

2.3 Biocontrol agents

1- Commercial biocontrol agent *Trichoderma harzianum* T-22 was purchased as TRIANUM-P (Koppert B.V.).
2- Commercial biocontrol agent *Streptomyces lydicus* WYEC 108 was supplied as Actinovate® AG (Natural Industries, Inc. Houston. USA).
3- Commercial biocontrol agent *Bacillus subtilis* strain QST 713 was supplied as Serenade® (Agraquest) by Fargro Ltd., Littlehampton, UK.

2.4 General microbial culture methods

All fungal pathogens and biocontrol agents were subcultured in Petri dishes (90 mm diameter) containing potato dextrose agar (PDA). Plates were inoculated with 5 mm diameter mycelial discs of 7 to 14 days old cultures of the pathogens. Inoculated plates were incubated at 25 °C to 28 °C in an incubator.

The biocontrol agent *T. harzianum* T-22 was cultured from the commercial product TRIANUM-P by placing some granules with spores directly on PDA and incubating at 25 °C to 28 °C in an incubator for 7 to 10 days. For dual culture experiments, bacterial biocontrol agents were cultured on PDA. For *S. lydicus* WYEC 108, Actinovate® AG powder containing spores was spread directly on PDA then plates were incubated in an incubator at 25 °C to 28 °C for 10 to 14 days. *B. subtilis* strain QST 713 was cultured

from the commercial liquid product, Serenade®, by spreading the liquid on PDA and incubating at 25 °C to 28 °C in an incubator for 10 to 14 days.

2.5 General methods of plant treatment

2.5.1 Foliar inoculation of plants with pathogen spore suspensions

The pathogens of spot blotch disease were subcultured on potato dextrose agar (PDA) and plates were incubated at 21 ºC for 12 to 14 days as described by Arabi and Jawhar (2003). Inoculum of the pathogens was prepared as suspensions of conidia. The conidia were harvested by flooding a petri dish with 5 ml of sterile distilled water and dislodging the conidia with a sterile bent glass rod. The resulting suspension was filtered through cheesecloth. The concentration of inoculum suspension was measured with a haemocytometer and adjusted to the concentration required (4 x 10^3, 8 x 10^3 or 1 x 10^4 conidia ml^{-1}), then the suspension was amended with one drop of 0.05% Tween 20 immediately before application. Plants were sprayed with suspensions of the pathogens until run-off by using a manually operated sprayer. After inoculation each pot was covered by a plastic bag for three days. The plants were kept wet by removing the plastic bags and spraying water on the plants every day (Perelló *et al.*, 2003).

2.5.2 Foliar inoculation of plants with *Trichoderma harzianum* T-22

Trichoderma harzianum T-22 was cultured on PDA plates for 7 to 14 days at 25±2 °C. Conidia of *T. harzianum* T-22 were harvested by flooding the culture with sterile distilled water and rubbing the culture surface with a sterile glass rod. The suspensions were filtered through two layers of cheesecloth, and the concentration of propagules in suspensions standardised with the aid of a haemacytometer to 1 x 10^8 conidia ml^{-1}as described by Perelló *et al*. (2003). One drop of 0.05% Tween 20 was added immediately before inoculation. Leaves were sprayed with suspensions of the biocontrol agent until run-off using a manually operated sprayer. After inoculation each pot was covered by a

plastic bag for three days. The plants were kept wet by removing the plastic bags and spraying water on the plants every day.

2.6 Disease severity assessment (the rating scale)

Disease severity was assessed on the leaves of seedlings, normally 7 days after inoculation, by the rating scale described by Fetch and Steffenson (1999). The scale is based on the type and relative size of lesions observed (Figure 2-1).

Figure 2-1. Disease rating scale based on the type and relative size of lesions observed on the second leaves of barley seedlings 7 days after inoculation (Fetch & Steffenson, 1999).

1, 2, 3 and 4 with no diffuse marginal chlorosis, 5 and 6 are medium size necrotic lesions with a distinct chlorotic edge, and 7, 8 and 9 are large necrotic lesions with distinct chlorotic edges and expanding chlorosis. Seedlings of barley cultivars at the two leaf stage 14 days after sowing were inoculated with suspensions of *B. sorokiniana* strain 6.16 at 4×10^3 and 8×10^3 conidia ml^{-1}. Leaves showing representative symptoms were photographed.

2.7 Isolation of the pathogens of spot blotch from infected leaves

Pathogens were isolated from infected leaves showing symptoms of spot blotch according to the procedure used by Arabi and Jawhar (2003). Surfaces of infected leaves were sterilised in 5% sodium hypochlorite solution for 5 min, and then soaked three times for 5 min each time in sterile distilled water. The infected leaves were cut into pieces of dimension 3 to 5 mm. The pieces were dried between filter papers and then transferred onto petri dishes containing PDA. They were then incubated at 21± 2 ºC to produce mycelia.

2.8 Statistical analysis

Disease severity data were analysed by ANOVA using the General Linear Model procedure in the Minitab 16 program (Minitab Inc.).

Chapter 3 Survey and Isolation of Pathogens of barley disease in Libya

3.1 Introduction

3.1.1 Background

Barley is one of the most widely grown cereal crops in Libya and the world at large. It is greatly affected by many fungal diseases that reduce productivity and cause considerable yield loss every year. Spot blotch disease, caused by *B. sorokiniana* (teleomorph *Cochliobolus sativus*), is one of the most important fungal diseases of barley (Arabi and Jawhar, 2004; Fetch and Steffenson, 1999; Knight *et al*., 2010; Pringle, 1979; Valjavec-Gratian and Steffenson, 1997b; Wordell *et al*., 2005) and reduces yields worldwide. Several studies have indicated that other pathogens can cause foliar diseases similar to spot blotch in other cereal crops. For example, Iftikhar *et al*. (2006) isolated *A. alternata, B. sorokiniana, Curvularia lunata*, and *Drechslera spicifer* (nomenclature as in their paper; i.e. *Bipolaris spicifera*) from wheat leaves in Pakistan from different areas where the temperature was high.

In Libya in 2009, barley was ranked first in area of cultivation (FAO, 2009). It is grown in the north, in the middle and in the south of the country. In the northeast and the northwest, which have a Mediterranean climate, rainfall is during the winter and the average rainfall is between 250 and 600 mm per year in the northeast and between 200 and 500 mm in the northwest. The crops in this region depend on rainfall with supplementation by irrigation with ground water in the season when the rainfall is less than the crop’s needs. Barley crops grown in the south, which belongs to the Sahara climate, cool and dry in the winter and hot and dry in the summer, and in the middle of Libya rely on irrigated agriculture using ground water (Al-Idrissi *et al*., 1996). The past thirty years have seen a rapid increase in cultivated areas and advances in agricultural practices and irrigation systems, but also increases in foliar plant diseases, yet limited studies of foliar diseases on barley have been carried out in Libya. The rapid increase in the area of crops grown in different places means that there is a need

to survey and identify the causes of foliar diseases in order to identify suitable methods to control them.

3.1.2 Barley resistance to spot blotch disease

Recent developments in control of spot blotch disease on barley have heightened the need for study of barley resistance to spot blotch. Resistance to spot blotch disease combined with other control methods is one of the strategies that most researchers nowadays focus on in order to reduce economic losses due to this disease (Walters *et al*., 2012). Roy *et al*. (2010) have used association mapping to locate genes for spot blotch resistance in wild barley. Castro *et al*. (2012) have studied genetics of resistance to spot blotch disease in a barley mapping population to identify the genetic components of resistance. Different methods have been used to measure barley resistance to spot blotch. A rating scale to assess infection responses was used to evaluate resistance of barley cultivars to spot blotch disease caused by *Cochliobolus sativus*. This scale relies on presence of necrosis and chlorosis and relative size of spot blotch lesions on the second leaf of seedling, and gives a rating from 1 (lowest effect) to 9 (highest effect) (Fetch and Steffenson, 1999). Another method, which has been used to screen barley rapidly for resistance to spot blotch disease, is to drop a conidial suspension onto transparent tape and place the tape on healthy leaves (Arabi and Jawhar, 2010).

3.1.3 Pathogenicity

Pathogenicity testing is one of the most important procedures for identification and management of plant pathogens. Several studies have been done of the pathogenesis of spot blotch disease on cereal crops to identify and find out more about the organisms that cause the disease. Duveiller and Garcia Altamirano (2000) studied the pathogenicity of *B. sorokiniana* isolates from wheat. Their findings suggested that environmental conditions have significant effects on the pathogenicity of different *B. sorokiniana* isolates. Differences in pathogenicity of a large number of *B. sorokiniana*

isolates on spring barley have been found (Baturo-Ciesniewska, 2011). *B. sorokiniana* isolates from spot blot lesions caused more severe spot blotch symptoms than isolates from roots with common root rot (Knight *et al*., 2010).

Bermudagrass (*Cynodon dactylon*) is a plant on which leaf spot diseases caused by both *Bipolaris* and *Curvularia* species have been evaluated. Matthew *et al*. (2007) demonstrated that temperature affects the severity of leaf spot caused by *Curvularia lunata*: temperatures above 20 °C caused more severe disease than lower temperatures. Koo *et al*. (2003) studied the pathogenicity of *B. spicifera* on several plant species. They found that pathogenicity was high, with over 50% diseased area on sorghum, rice, maize and Bermudagrass, whereas there was less than 50% diseased area on barley, timothy and chard grass. Pathogenicity of *B. spicifera* on sorghum at a temperature of 25 °C has been demonstrated in Turkey (Unal *et al*., 2011).

3.1.4 Aims

1- To survey spot blotch disease in areas where barley is grown in Libya in the north, northwest, centre, south and southwest.
2- To isolate and identify pathogens that cause spot blotch disease on barley.
3- To test plant resistance to spot blotch in barley cultivars that are commonly grown in Libya, so as to determine which cultivars are most resistant.
4- To test the pathogenicity of the collected isolates in the short term at moderate temperature on Libyan cultivars to determine.
5- To test the pathogenicity of the collected isolates in the long term at high temperature on Libyan cultivars.

3.2 Materials and methods

3.2.1 Isolation of potential spot blotch pathogens from infected leaves

Various places in Libya (north, northwest, central and south) where barley is grown on a large scale were visited in the month of April 2009. Samples of infected leaves were collected from 104 points and kept in a refrigerator at 4 ºC then isolated and dried for transport to the UK (Figure 3-1 and Figure 3-2). Cultures of potential pathogens from infected leaves showing symptoms of spot blotch were prepared as described in section 2.7.

Figure 3-1. Isolates in PDA tubes which were isolated in Libya and transferred to the UK.

Figure 3-2. Typical dry infected leaves collected in Libya.

3.2.2 Identification of Libyan isolates by PCR and DNA sequencing

3.2.2.1 Extraction of DNA

DNA was isolated from pure fungal cultures grown on PDA medium for 5 to 7 d by a CTAB (hexadecyltrimethylammonium bromide) method as described by Neuhauser *et al*. (2009). A 1 cm^2 fragment of fungal mycelium was transferred into a 2 ml micro centrifuge tube with 0.5 ml CTAB (Sigma Chemicals) extraction buffer [CTAB buffer – mix equal volumes of 10% CTAB in 0.7 M NaCl and 0.24 M potassium phosphate buffer, pH 8] and 0.5 ml phenol-chloroform-isoamyl alcohol (25:24:1 v/v, pH 8.0), which were mixed together. Mycelium was lysed in a FastPrep FP120 cell disrupter at 5.5 m s^{-1} for 30 s. The mixture was cooled on ice for 2 min, then centrifuged at 16,000*g* for 5 min at 4 °C. The upper aqueous layer containing DNA was removed to a fresh tube. An equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added to remove phenol, and then the mixture was centrifuged at 16,000*g* for 5 min at 4 °C. To precipitate the nucleic acid from the aqueous layer, two volumes of 30% (w/v) polyethylene glycol (PEG 6000) in 1.6 M NaCl were added and the mixture was incubated for 2 h at room temperature followed by centrifugation at 18,000*g* for 10 min. The supernatant was discarded and the pellet was washed with ice cold 100% ethanol. Samples were centrifuged at 16,000*g* for 10 min, the supernatant was removed, and then the pellet was washed with ice cold 70% (v/v) ethanol. Samples were centrifuged again at 16,000*g* for 10 min and the supernatant was removed. Pellets were air dried at room temperature, dissolved in 25 µl sterile distilled water and stored at -20 °C.

3.2.2.2 PCR

The ITS (internal transcribed spacer) region of ribosomal DNA (ITS1, 5.8S rRNA gene, ITS2) was amplified with the standard fungal primers ITS5 and ITS4 (White *et al*., 1990).ITS5 sequences 5’ GGAAGTAAAAGTCGTAACAAGG 3’ and ITS4 sequences 5’TCCTCCGCTTATTGATATGC 3’).The PCR reaction mixtures were

prepared in a total volume of 23 µl each containing 2.5 µl of NH_4 PCR buffer (Bioline; 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, pH 8.8 at 25 °C, and 0.1% Tween-20), 1.25 µl of 50 mM $MgCl_2$ (Sigma, St. Louis, Mo., USA), 0.25 µl of forward primer ITS4 (10 mM), 0.25 µl of reverse primer ITS5 (10 mM), 0.75 µl of dNTP deoxynucleoside triphosphates (10 mM; Pharmacia Biotech, Piscataway, N.J.), 0.10 µl of Taq polymerase (5 U/ml; Boehringer Mannheim) and 17.90 µl of sterile distilled water. The PCR reaction mixture was mixed with 2 µl of DNA template. The reactions were conducted in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems. USA). The thermal cycling parameters were initial denaturation at 95 °C for 5 min followed by 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 56 °C for 50 s, extension at 72 °C for 1 min and final extension at 72 °C for 7 min, following by cooling at 4 °C until recovery of the samples (Ristaino *et al*., 1998).

3.2.2.3 Checking DNA integrity by electrophoresis

The PCR product (4 µl) was mixed with 2 µl loading dye and then loaded onto a 1% (w/v) agarose gel in 0.5x TBE buffer with ethidium bromide (0.5 µg ml^{-1}). 2 µl size standards (100 bp ladder) were loaded and the gel was run at 100 V for 30 min (Figure 3-10).

3.2.2.4 DNA sequence analysis

PCR products were sent for sequencing to Geneius Laboratories Ltd, INEX Business Centre, Newcastle University. Sequences were identified by comparing them to the sequences in the GenBank database using the Blast search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.3 Test of the pathogenicity of the Libyan isolates on two Libyan cultivars, Rehan and Nibola

In November 2010 an experiment was done to assess the pathogenicity of Libyan isolates and to correlate the results with the PCR identification. The experiment was carried out as described in Chapter 2, in a greenhouse at day time average temperature 16 °C and night time average 11°C, with two Libyan barley cultivars, Nibola and Rehan. At the two-leaf stage 14 d after sowing, each cultivar was inoculated with *Bipolaris sorokiniana* isolate L50, *Bipolaris spicifera* isolate L35, *Curvularia inaequalis* isolate L6, and *Alternaria alternata* isolate L79 (see section 3.3.2 for identification) at spore concentrations of 4 x 10^3 conidia ml^{-1} and 8 x 10^3 conidia ml^{-1}. Three pots were used for each combination of cultivar, pathogen, and spore concentration. After seven days disease severity was measured using the rating scale from 1 to 9 (Figure 2-1) as described by Fetch and Steffenson (1999) and Chang and Hwang (2003). Seven days after inoculation, each pathogen was reisolated from leaves showing symptoms by following the same protocol used for the initial isolation of the pathogens (section 2.7).

3.2.4 Long-term pathogenicity test of Libyan isolates of *B. sorokiniana, C. inaequalis* and *B. spicifera* at high temperatures

A long-term pathogenicity test of Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera* was carried out on cultivars Nibola and Rehan in May 2011. Five to seven plants were grown in each pot for each cultivar with three replicate pots. Plants were inoculated with two concentrations, 4 x 10^3 conidia ml^{-1} and 1x10^4 conidia ml^{-1}, of *B. sorokiniana* isolate L50, *C. inaequalis* isolate L6, and *B. spicifera* isolate L35, at the two-leaf stage as described in section 2.5.1. Plants were grown in a greenhouse with average day time temperature 26 °C and night time temperature 20 °C. Disease severity was measured on the uppermost two leaves at heading stage by using the same rating scale from 1 to 9 as for seedlings, as described in Chapter 2.

3.3 Results

3.3.1 Survey and isolation of pathogens of barley in Libya

Various agricultural projects and other places in Libya (north, northwest, centre and south) where barley is grown on a large scale were visited in the month of April 2009. Samples of infected leaves were collected from 104 points, determined by the use of a GPS receiver (Figure 3-3). The samples were collected from different places to determine the distribution of diseases following the identification of pathogens in the infected leaves (Figure 3-6, Figure 3-7).

On the basis of the symptoms observed, barley at most of the places visited was found to be highly infected. Discussion with the local agronomists revealed that the same areas are cultivated with barley every year. It can be concluded that the high level of infection with the pathogen is due in part to cultivation of the same areas every year. Infection could be from soil or crop debris, or in new areas may have originated from the seed, which served as the initial source of infection. In wheat-rice cropping regions in India, the initial source of infection with spot blotch in new areas has been demonstrated to be infected seed which leads to infection of first leaves in the new areas (Pandey *et al*., 2005). From my observations, barley grown in south Libya, where the weather is dry, showed high symptoms of fungal infestation, the majority of them resembling spot blotch disease. This may be related to the irrigation system that is applied in the area (see Figure 3-8), or in some places such as the Aboshaba project high abundance of alternative weed hosts may be another cause of high infection (Figure 3-9). In addition, Figure 3-5 shows a field of cultivar ACSAD in the south of Libya (Maknosa project), in which no crops had been grown for the previous seven years because the irrigation system was broken down for seven years before this crop. This field was notably less infected with spot blotch disease than the field shown in Figure 3-6. The field illustrates the value of integrated pest management as an important current method to reduce yield loss due to spot blotch disease, including the practice of crop rotation to reduce initial inoculum of the pathogen. In this field, in

spite of late sowing in January and harvesting in May, the average yield was 6 t ha^{-1} whereas other fields that were sown in November with the same cultivar produced less than five t ha^{-1} (personal communication from the project manager).

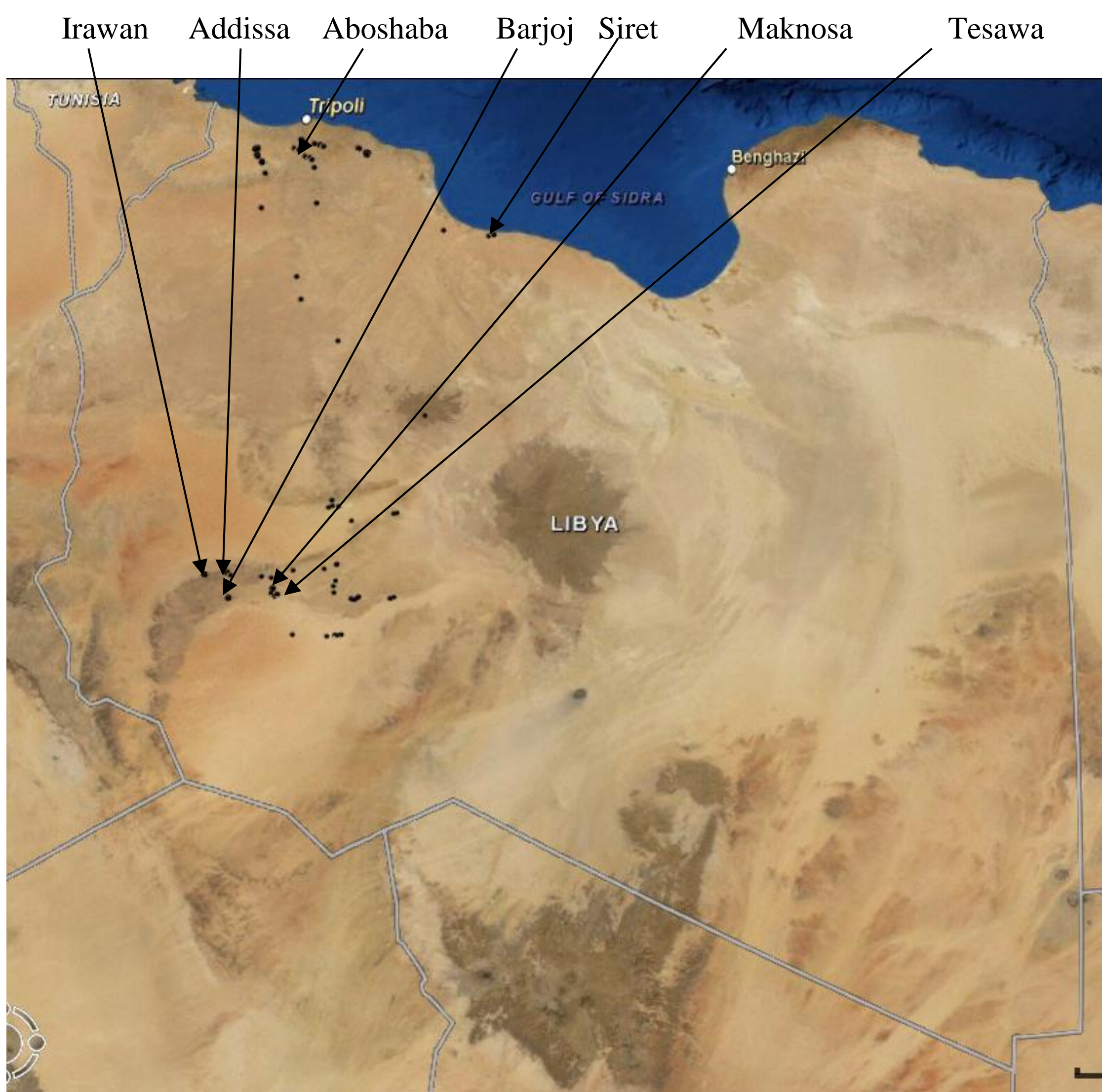
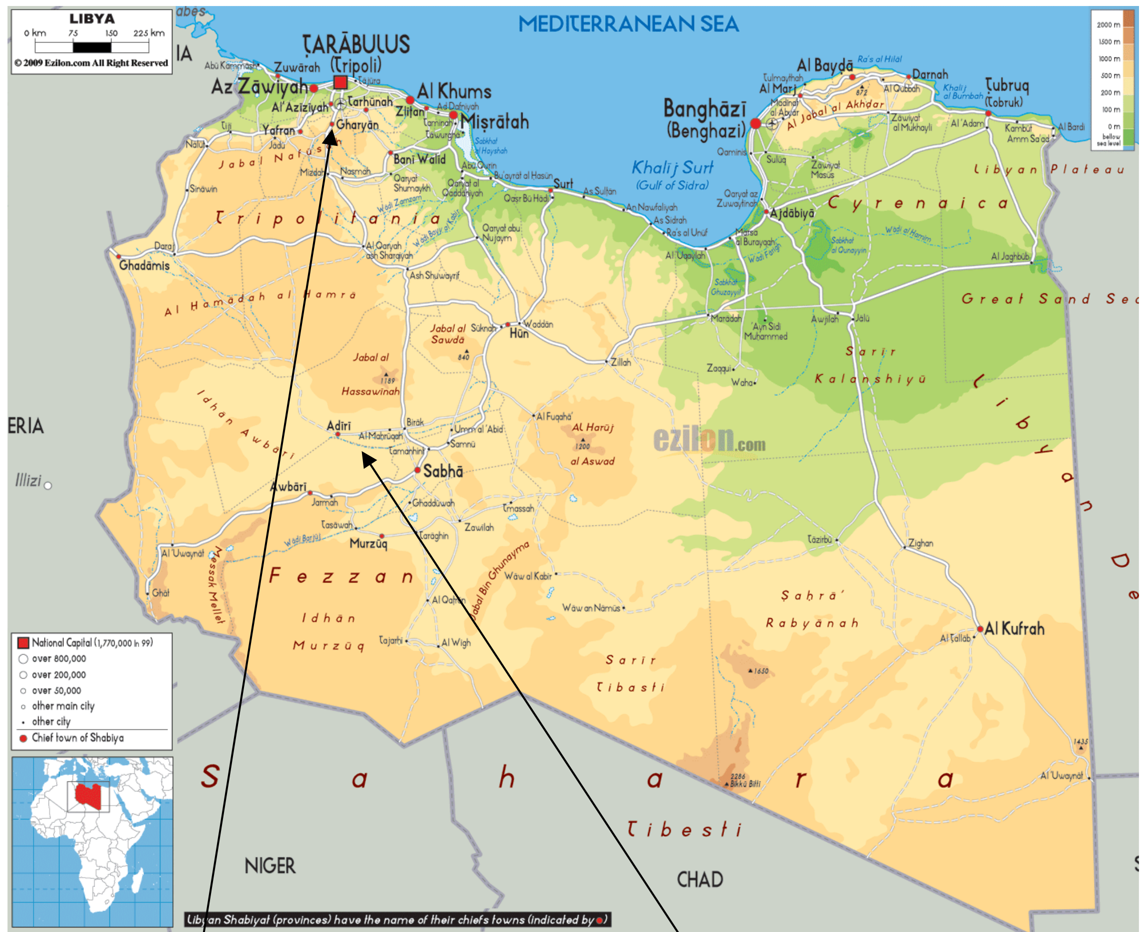


Figure 3-3. Map of Libya showing locations from which samples were collected, represented by black dots. Most of the samples that were identified were taken from the named places (Irawan, Addissa, Aboshaba, Barjoj, Siret, Maknosa and Tesawa).



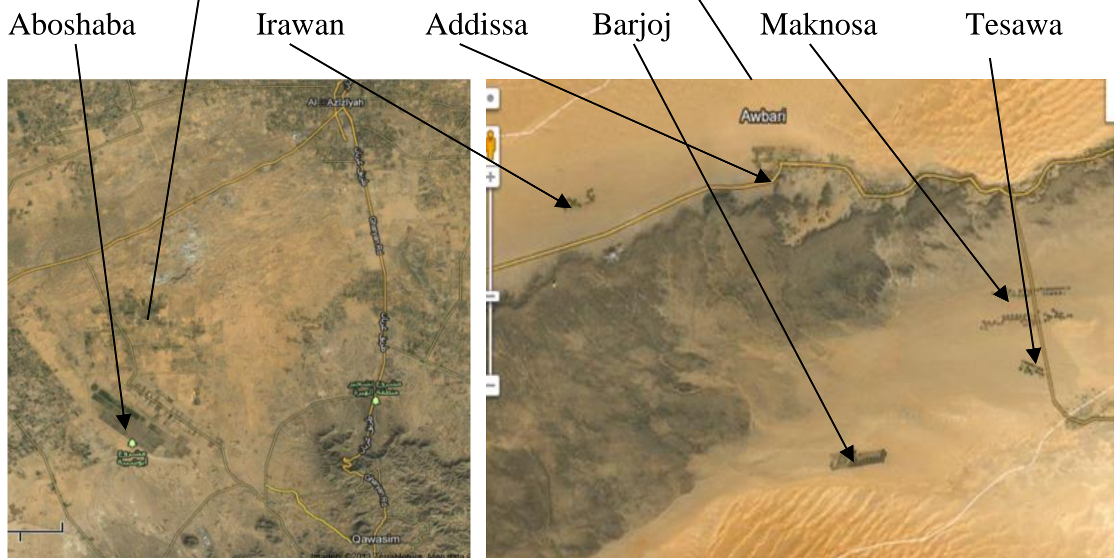


Figure 3-4. Map of agriculture projects in Libya.

http://africaanswerman.com/?p=3083

http://maps.google.co.uk/maps?hl=en&bav=on.2,or.r_qf.&bvm=bv.44158598,d.d2k&biw=1280&bih=849&wrapid=tlif136403402577210&q=map+of+agricultural+projects+in+libya&um=1&ie=UTF-8&hq=&hnear=0x13aed68d32db770f:0x5f80145bb40c9c21,Mashru%60+Wadi+al+Hayy+az+Zira%60i,+Libya&gl=uk&sa=X&ei=HYJNUeCOGq7Z0QXdlIGQCQ&ved=0CD8Q8gEwAA

Figure 3-5. Field of barley cultivar ACSAD that was sown later, in January, than other fields which were sown in November in south Libya (Maknosa project).

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Figure 3-6. Cultivar Rehan infected by spot blotch disease at Addissa project (southwest Libya).

Figure 3-7. Field of barley cultivar ACSAD highly infected by spot blotch at ripening stage in the Irawan project (southwest Libya).

Figure 3-8. System of irrigation in south Libya.

Figure 3-9. Field of barley cultivar Tissa (two-rowed) highly infested by weeds and infected by spot blotch disease at heading stage (Aboshaba project, northwest Libya).

3.3.2 Identification of Libyan isolates

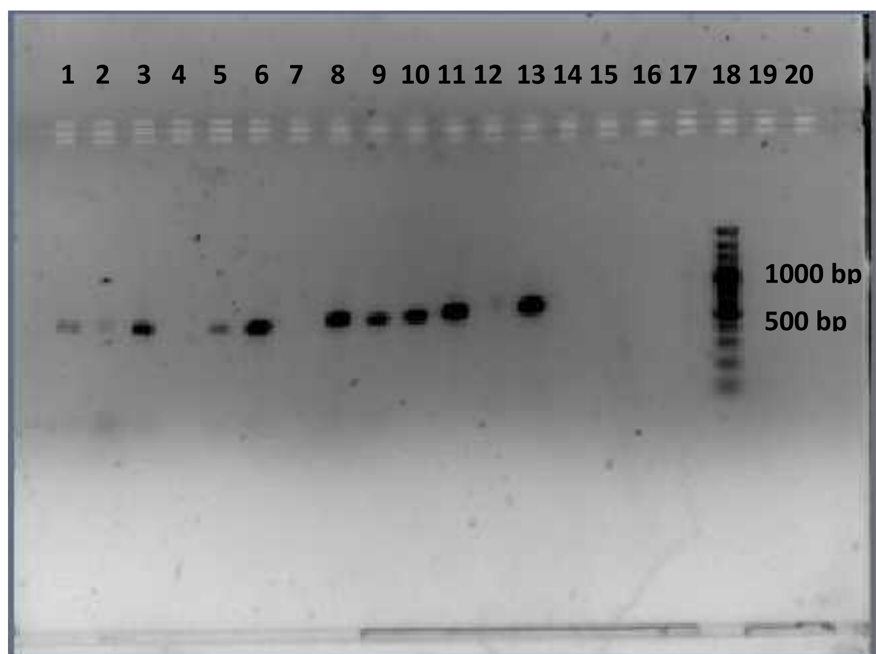


Figure 3-10. Negative image of agarose gel stained with ethidium bromide, showing PCR products amplified with ITS5 and ITS4 primers from DNA of Libyan fungal isolates.

Lanes are: 1, isolate L1; 2, isolate L2, 3, isolate L6;, 4, isolate L19; 5, isolate L35; 6, isolate L6 (2), 7, isolate L47, 8, isolate L48; 9, isolate L50; 10, isolate L79; 11, isolate L82; 12, negative control (water); 13, positive control DNA; 18, size standards (100 bp ladder), with high intensity bands at 1000 bp and 500 bp indicated. Other lanes were empty.

Ten Libyan isolates were identified by PCR and DNA sequencing. DNA was isolated from pure fungal cultures, and agarose gel electrophoresis of the DNA was used to test its integrity (not shown). The internal transcribed sequence (ITS) region of ribosomal DNA (ITS1, 5.8S rRNA, and ITS2) was amplified by PCR (Figure 3-10) and both strands were sequenced. Organisms identified by comparison to sequences in the GenBank database were *B. sorokiniana*, *B. spicifera*, *C. inaequalis* and *A. alternata* (Table 3-1). Identifications were unambiguous.

The morphology of the fungi was investigated by microscopy of cultures producing conidia. Four isolated species were identified on the basis of morphology: *B. sorokiniana*, *B. spicifera*, *C. inaequalis* and *A. alternata* (Figure 3-11). The

morphological identifications of these organisms agree with molecular identifications (Table 3-1).

B. sorokiniana: Conidia are dark olive brown, with 2 to 13 septa, slightly curved and broadest in the middle. Lengths are 41-66.6 µm and widths are 11.6-25 µm (Asad *et al.*, 2009).

B. spicifera: Conidia are light brown, cylindrical and rounded at both ends, smooth walled divided by 1 to 3 septa. Basal cells have a flattened hilum. Lengths are 17.5-30 µm and widths are 12-14 µm (El Mhadri *et al.*, 2009; Vu *et al.*, 2011).

C. inaequalis: Conidia are evenly coloured or have end cells that are slightly paler. Most have 5 septa and are slightly bent, with one or two the central cells larger and darker than the others. Lengths are 17.5-30 µm and widths 8.8 -12.5 µm (Huang *et al.*, 2005) .

A. alternata: Conidia are produced in long chains. They are pale to light brown with one to seven transverse and up to three longitudinal septa. Lengths are 10-45 µm and widths 7-18 µm, with a beak 4-6 µm long (Da Silva and Singh, 2012).

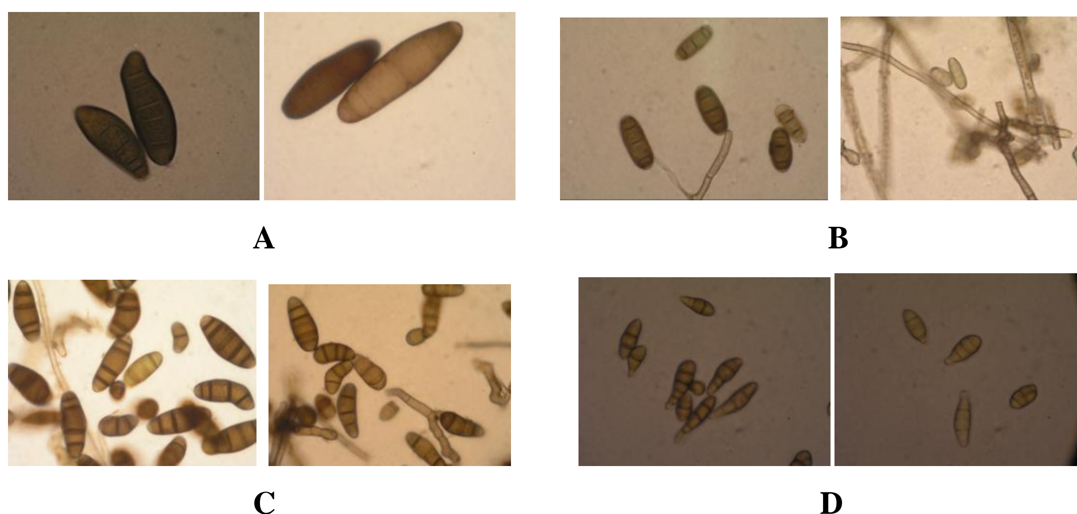


Figure 3-11. Conidia of Libyan isolates. Pictures are of unstained specimens, taken by bright field microscopy.

A, conidia of *B. sorokiniana*; **B**, conidia of *B. spicifera*; **C**, conidia of *C. inaequalis*; **D**, conidia of *A. alternata*.

Table 3-1. The closest matches for the ITS region from ten Libyan isolates: numbers L1, L2, L6, and L19 isolated from the north of Libya, L35 from the central area, and L48, L50, L68, L79, and L82 from the south.

Isolate	GenBank Accession Number	Species	Total Score	Query Coverage	Maximum Identity
L1	FM163616.1	*Curvularia inaequalis*	1005	100%	99%
L2	FM163616.1	*Curvularia inaequalis*	1005	100%	99%
L6	FM163616.1	*Curvularia inaequalis*	1005	100%	99%
L19	GU183125.1	*Bipolaris spicifera*	1020	100%	100%
L35	GU183125.1	*Bipolaris spicifera*	1013	100%	100%
L68	GU183125.1	*Bipolaris spicifera*	1020	100%	100%
L82	GU183125.1	*Bipolaris spicifera*	1020	100%	100%
L48	EF452447.1	*Bipolaris sorokiniana*	976	100%	100%
L50	GU080212.1	*Bipolaris sorokiniana*	918	100%	100%
L79	GU566303.1	*Alternaria alternata*	992	100%	100%

3.3.3 Resistance of barley cultivars to spot blotch disease caused by *B.. sorokiniana* strain 6.16 (UK isolate)

Resistance to *B. sorokiniana* strain 6.16 was assessed in seedlings of four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic). Table 3-2 shows that there were significant differences between barley cultivars in plant disease severity ($P < 0.001$). ACSAD and Nibola were the most resistant to *B. sorokiniana* whereas Pastoral, Gaelic, and Rehan were the least resistant (Figure 3-12B). Disease was more severe on second leaves than on first leaves: the mean disease severity rating on second leaves was 5.6 and on first leaves was 4.9. The concentration of spores used for inoculation significantly affected disease severity ($P < 0.001$): when barley was inoculated with 8×10^3 spores ml^{-1} the mean severity rating was 5.5 whereas with 4×10^3 spores ml^{-1} the mean severity rating was 4.9. The interactions between cultivar and leaf number, cultivar and spore concentration, leaf number and spore concentration, and cultivar, spore concentration, and leaf number

were not significant (Figure 3-12A). Differences in severity between the most resistant and least resistant cultivars were consistent for both leaves and both spore concentrations.

Table 3-2. Analysis of variance of spot blotch disease severity on four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic). Plants at the two-leaf stage, 14 days after sowing, were inoculated with *B. sorokiniana* at two concentrations, 4 x 10^3 conidia ml^{-1} and 8 x 10^3 conidia ml^{-1} (Spore Concn.). Disease severity was assessed on the first and second leaves 7 daysafter inoculation with the pathogen for three replicates each replicate 15 seedlings.

Source	DF	SS	MS	F	P
Cultivar	5	93.6628	18.7326	48.03	<0.001
Leaf	1	8.4050	8.4050	21.55	<0.001
Spore Concn.	1	6.6006	6.6006	16.92	<0.001
Cultivar*Leaf	5	0.2983	0.0597	0.15	0.978
Cultivar*Spore Concn.	5	3.0761	0.6152	1.58	0.184
Leaf*Spore Concn.	1	0.4050	0.4050	1.04	0.313
Cultivar*Spore Concn.*Leaf	5	3.4317	0.6863	1.76	0.139
Error	48	18.7200	0.3900		
Total	71	134.5994			

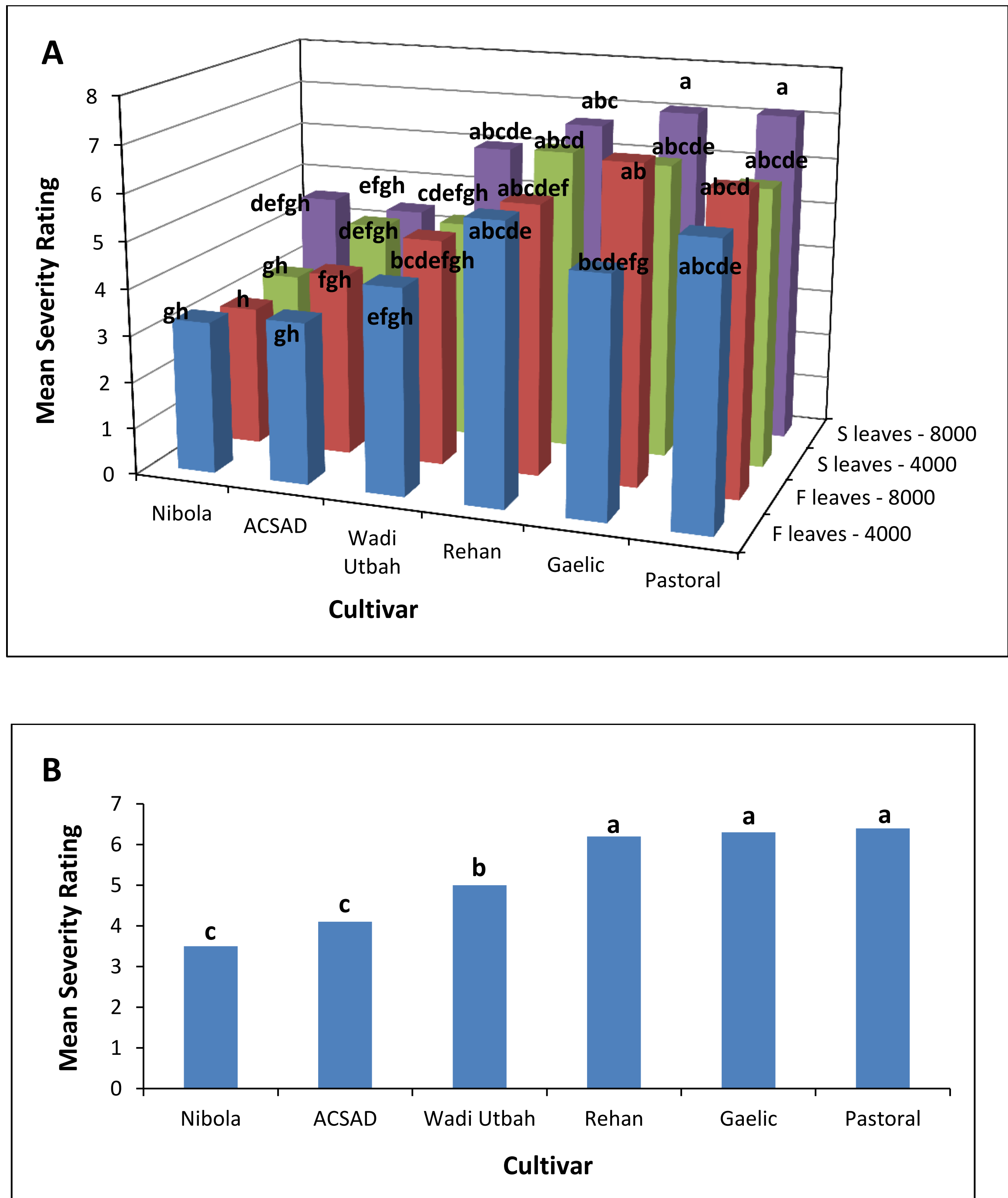


Figure 3-12. Assessment of resistance of six barley cultivars to *B. sorokiniana* at average day time temperature 16 °C and night time temperature 11 °C.

A, mean disease severity for each combination of cultivar, leaf, and spore concentration. **B**, mean disease severity for each cultivar. Plants at the two-leaf stage, 14 days after sowing, were inoculated with two spore concentrations, 4 x 10^4 conidia ml^{-1} and 8 x 10^3 conidia ml^{-1}. Assessment was on the first (F) and second (S) leaves 7 d after inoculation with the pathogen. On each graph, values represented by bars with the same letter are not significantly different at P = 0.05 (Tukey test).

3.3.4 Test of the pathogenicity of Libyan isolates on two Libyan cultivars, Rehan and Nibola

An experiment was done to assess the pathogenicity of Libyan isolates of *B. sorokiniana* (isolates L50 and L48), *C. inaequalis* (isolates L1, L2 and L6), *B. spicifera* (isolates L19, L35, L68 and L82), and *A. alternata* (isolates L56, L79, and L89) and to relate the results to the PCR identification. The pathogenicity test was carried out on two cultivars: Nibola, which was more resistant to *B. sorokiniana* strain 6.16, and Rehan, which was less resistant to *B. sorokiniana* strain 6.16 (section 3.3.3). Seven days after inoculation, all pathogens were re-isolated from leaves with symptoms of spot blotch. The re-isolated pathogens were the same as the inoculated pathogens (not shown).

Figure 3-13 shows mean disease severity ratings for all the identified Libyan isolates on the first two leaves of Nibola and Rehan with two concentrations of spores, 4 x 10^3 and 8 x 10^3 conidia ml^{-1}. The two *B. sorokiniana* isolates were the most pathogenic, followed by one isolate of *C. inaequalis*. All other isolates caused relatively mild symptoms, and several isolates caused no measurable disease on Nibola.

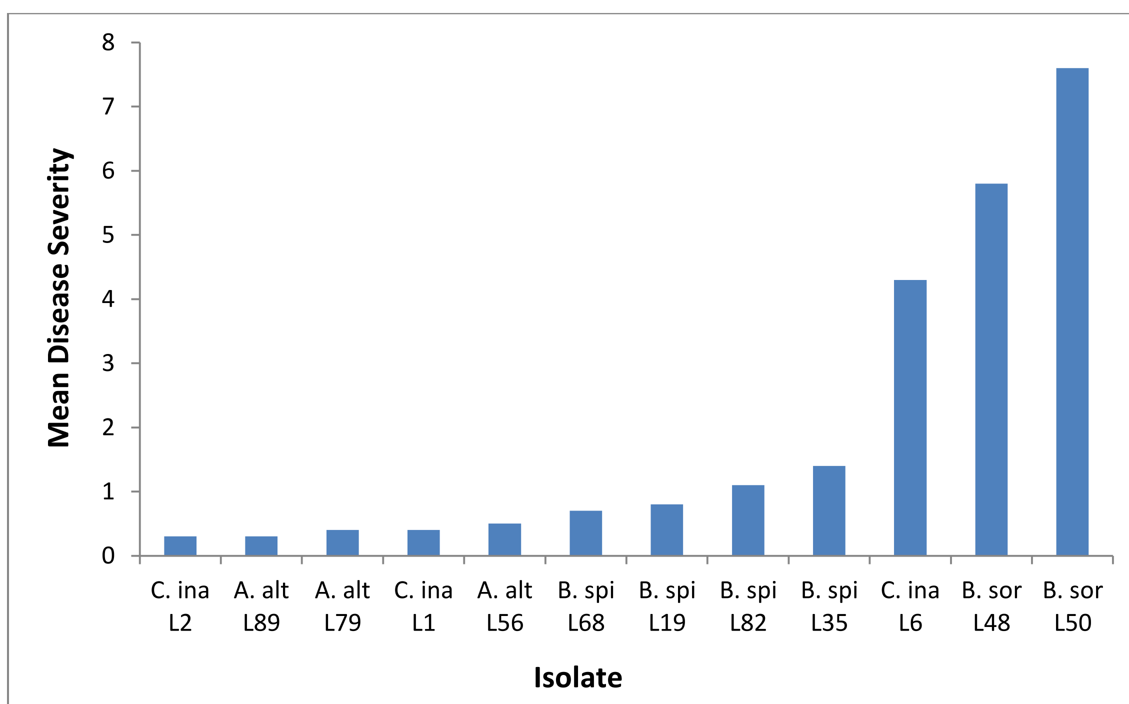


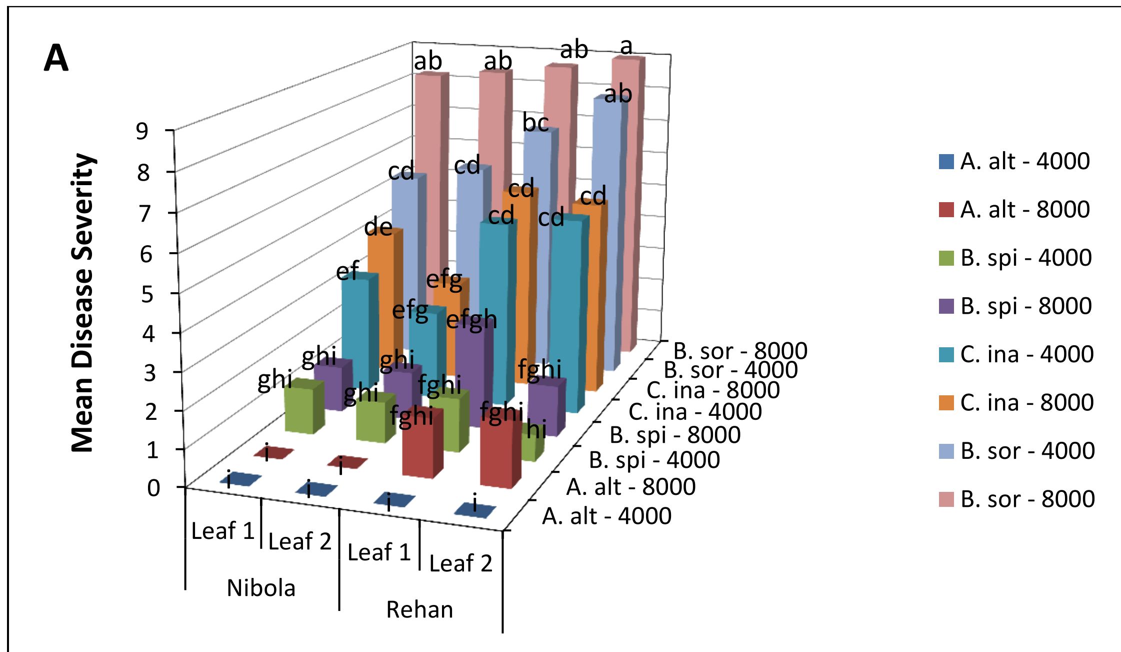
Figure 3-13. Assessment of pathogenicity of *B. sorokiniana* (B. sor L48 and L 50), *C. inaequalis* (C. ina L1, L2 and L6), *B. spicifera* (B. spi L19, L35, L68 and L 82), and *A. alternata* (A. alt L56, 79 and L89) on two Libyan cultivars, Nibola and Rehan, at average day time temperature 16 °C and night time temperature 11 °C.
Seedlings were inoculated with two spore concentrations, 4 x 10^3 conidia ml^{-1} and 8 x 10^3 conidia ml^{-1}. The assessment was on the first and second leaves.

Because of the low severity ratings with several isolates, statistical analysis was not reliable. Further analysis was carried out on one isolate of each species. Table 3-3 shows that there were significant differences between cultivars and isolates ($P < 0.001$ for both). The mean disease severity ratings on Nibola and Rehan were 2.8 and 4.0, respectively. The species in descending order of overall pathogenicity were *B. sorokiniana*, *C. inaequalis*, *B. spicifera*, and *A. alternata* (Figure 3-14B). In addition, there were differences in how rapidly symptoms appeared. With *B. sorokiniana* symptoms appeared two days after inoculation, with *C. inaequalis* symptoms appeared on the third day, whereas with *B. spicifera* and *A. alternata* the symptoms appeared on the fourth day. Differences between leaves were not significant ($P = 0.264$). Spore concentration significantly affected severity: with 8 x 10^3 spores ml^{-1} the mean severity rating was 3.9, whereas with 4 x 10^3 spores ml^{-1} the mean rating was 3.0.

There were significant interactions between cultivar and isolate ($P < 0.001$), between leaf and isolate ($P = 0.006$), between isolate and concentration of spores ($P < 0.001$), between cultivar, isolate, and spore concentration ($P = 0.001$), and between cultivar, isolate, and leaf ($P = 0.017$). Other interactions were not significant. The differences in disease severity caused by the four Libyan isolates tested on two Libyan cultivars at two concentrations of spores can be seen in Figure 3-14A. *B. sorokiniana* and *C. inaequalis* caused higher disease severity on Rehan than Nibola, although differences at the high spore concentration were not significant for *B. sorokiniana*. For *C. inaequalis* on Nibola disease severity on first leaves with both spore concentrations seemed to be greater than on second leaves, although the difference was not significant for either leaf individually. Disease severity with *B. spicifera* on Rehan was numerically higher on the first leaves than on the second leaves with both spore concentrations, and on second leaves at the low concentration the severity was less than on Nibola; again these differences were not statistically significant according to Tukey tests. There was a difference between cultivars with *A. alternata* (Figure 3-13 and Figure 3-14), which only caused disease at the high spore concentration on both leaves of Rehan; with Nibola and the low concentration of spores on Rehan there was little or no disease.

Table 3-3. Analysis of variance for assessment of pathogenicity of *B. sorokiniana*, *C. inaequalis*, *B. spicifera*, and *A. alternata* on seedlings of two Libyan cultivars, Nibola and Rehan, with average day time temperature 16 °C and night time temperature 11 °C. Plants at the two-leaf stage, 14 days after sowing, were inoculated with two spore concentrations, 4 x 10^3 conidia ml^{-1} and 8 x 10^3 conidia ml^{-1} (Spore Concn.). Disease severity was assessed on the first and second leaves 7 d after inoculation with the pathogen.

Source	DF	SS	MS	F	P
Cultivar	1	34.082	34.082	101.61	<0.001
Leaf	1	0.427	0.427	1.27	0.264
Spore Concn.	1	21.660	21.660	64.58	<0.001
Isolate	3	744.915	248.305	740.29	<0.001
Cultivar*Leaf	1	0.107	0.107	0.32	0.575
Cultivar*Spore Concn.	1	0.240	0.240	0.72	0.401
Cultivar*Isolate	3	11.968	3.989	11.89	<0.001
Leaf*Spore Concn.	1	0.482	1.539	1.44	0.235
Leaf*Isolate	3	4.617	2.950	4.59	0.006
Spore Concn.*Isolate	3	8.850	0.135	8.80	<0.001
Cultivar*Leaf*Spore Concn.	1	0.135	0.135	0.40	0.528
Cultivar*Leaf*Isolate	3	3.670	1.223	3.65	0.017
Cultivar*Spore Concn.* Isolate	3	8.830	2.943	8.78	<0.001
Leaf*Spore Concn.*Isolate	3	0.342	0.114	0.34	0.797
Cultivar*Leaf*Spore Concn.*Isolate	3	0.315	0.105	0.31	0.816
Error	64	21.467	0.335		
Total	95	862.105			



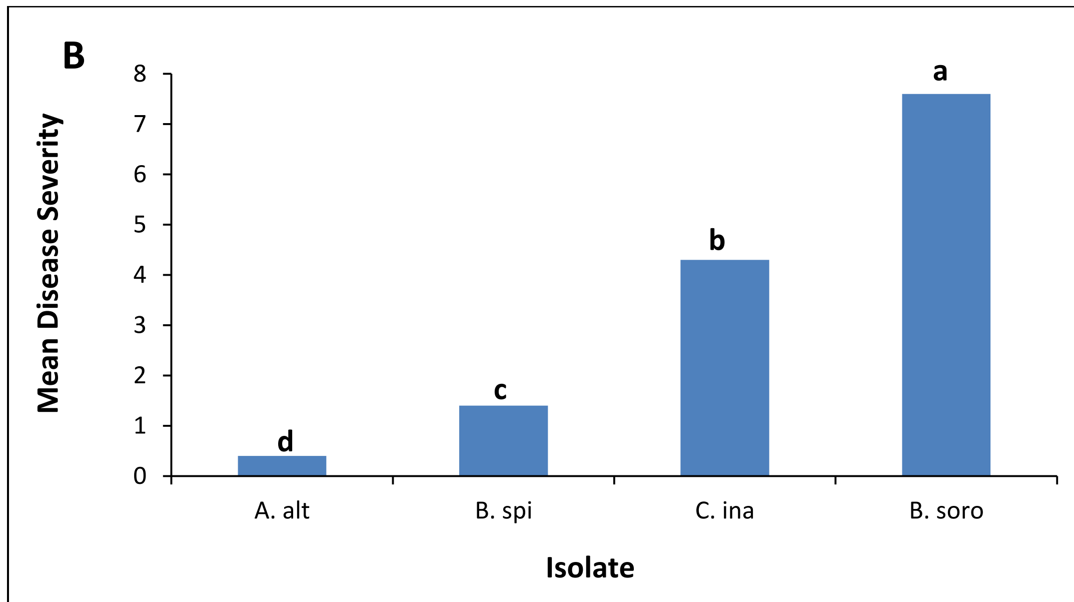


Figure 3-14. Assessment of pathogenicity of *B. sorokiniana* (B. sor), *C. inaequalis* (C. ina), *B. spicifera* (B. spi), and *A. alternata* (A. alt) on two Libyan cultivars, Nibola and Rehan, at average day time temperature 16 °C and night time temperature 11 °C.

A, mean disease severity for each combination of cultivar, leaf, isolate and spore concentration. **B**, mean disease severity for each isolate. Seedlings were inoculated with two spore concentrations, 4 x 10^3 conidia ml^{-1} and 8 x 10^3 conidia ml^{-1}. The assessment was on the first (1) and second (2) leaves. Values represented by bars with the same letter are not significantly different at P = 0.05 (Tukey test).

3.3.5 Long-term pathogenicity test of Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera* at high temperatures

A long-term pathogenicity test with Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera* was carried out on cultivars Nibola and Rehan. The same isolates were used as in the previous experiment with seedlings, but *A. alternata* was not included because it had the lowest pathogenicity on seedlings and did not cause measurable disease on Nibola. Seven plants were grown in each pot for each cultivar with three replicates. Plants were inoculated at the two-leaf stage with two concentrations, 4 x 10^3 conidia ml^{-1} and $1x10^4$ conidia ml^{-1}, of *B. sorokiniana*, *C. inaequalis*, and *B. spicifera*. The higher concentration was $1x10^4$ conidia ml^{-1} rather than 8 x 10^3 conidia ml^{-1}, which was used in the short-term experiment, in order to increase disease incidence. The average day time temperature during this experiment was 26 °C and the average night time temperature was 20 °C. Disease severity was measured on the uppermost two leaves at heading stage by using the rating scale from 1 to 9 as for seedlings, as described by Fetch and Stephenson (1999) (Chapter 2). Table 3-4 shows that there was a significant difference between the cultivars ($P < 0.001$): as previously found in the short-term experiment, severity on Nibola was lower than on Rehan. The mean disease severity ratings were 4.0 and 4.9, respectively. There were significant differences between isolates ($P < 0.001$), with the mean severity ratings in the order *B. sorokiniana*, *C. inaequalis* and *B. spicifera* as shown in Figure 3-14. The concentration of spores also significantly affected disease severity ($P < 0.001$): $1x10^4$ conidia ml^{-1} gave a mean disease severity rating of 4.9 whereas with 4 x 10^3 conidia ml^{-1} the mean severity rating was 4.0. The difference between leaves was not significant, and interactions involving leaves were not significant, and therefore average values are presented in Figure 3-15. There were significant interactions between cultivar and isolate ($P < 0.001$), between cultivar and spore concentration ($P = 0.013$) and between isolate and spore concentration ($P < 0.001$). Figure 3-15 shows that for *C. inaequalis*, disease severity ratings did not differ significantly between Rehan and Nibola for either spore concentration. Spore concentration did not significantly affect disease severity on Rehan with *C. inaequalis* and *B. spicifera*, but severity on Nibola was higher at the higher concentration. There was no significant difference between disease severity caused by *C. inaequalis* and *B. spicifera* on Rehan, but disease severity with *C. inaequalis* on Nibola

was significantly higher than with *B. spicifera*. At $1x10^4$ conidia ml^{-1}, disease severity on Nibola with *C. inaequalis* was comparable to disease severity with *B. sorokiniana*.

Table 3-4. Analysis of variance for assessment of pathogenicity of *B. sorokiniana*, *C. inaequalis*, and *B. spicifera* (Isolate), in a long-term experiment with two Libyan cultivars, Nibola and Rehan.

Plants were inoculated at the two-leaf stage, 14 days after sowing, with two spore concentrations, 4×10^3 and 1×10^4 conidia ml^{-1} (Spore Concn.). The average day time temperature was 26 °C and night time average was 20 °C. Disease severity was assessed by rating scale on the upper two leaves at heading stage.

Source	DF	SS	MS	F	P
Cultivar	1	12.500	12.500	55.28	<0.001
Leaf	1	0.3200	0.3200	1.42	0.240
Spore Concn.	1	14.9422	14.9422	66.08	<0.001
Isolate	2	128.8811	64.4406	285.00	<0.001
Cultivar * Leaf	1	0.1422	0.1422	0.63	0.432
Cultivar * Spore Concn.	1	1.5022	1.5022	6.64	0.013
Cultivar * Isolate	2	12.490	6.2450	27.62	0.000
Leaf * Spore Concn.	1	0.0022	0.0022	0.01	0.921
Leaf * Isolate	2	0.1433	0.0717	0.32	0.730
Spore Concn. * Isolate	2	1.4344	0.7172	3.17	0.051
Cultivar * Leaf * Spore Concn.	1	0.1089	0.1089	0.48	0.491
Cultivar * Leaf * Isolate	2	0.2144	0.1072	0.47	0.625
Cultivar * Spore Concn. * Isolate	2	1.2878	0.6439	2.85	0.068
Leaf * Spore Concn. * Isolate	2	0.5278	0.2639	1.17	0.320
Cultivar * Leaf * Spore Concn. * Isolate	2	0.9411	0.4706	2.08	0.136
Error	48	10.8533	0.2261		
Total	71	186.2911			

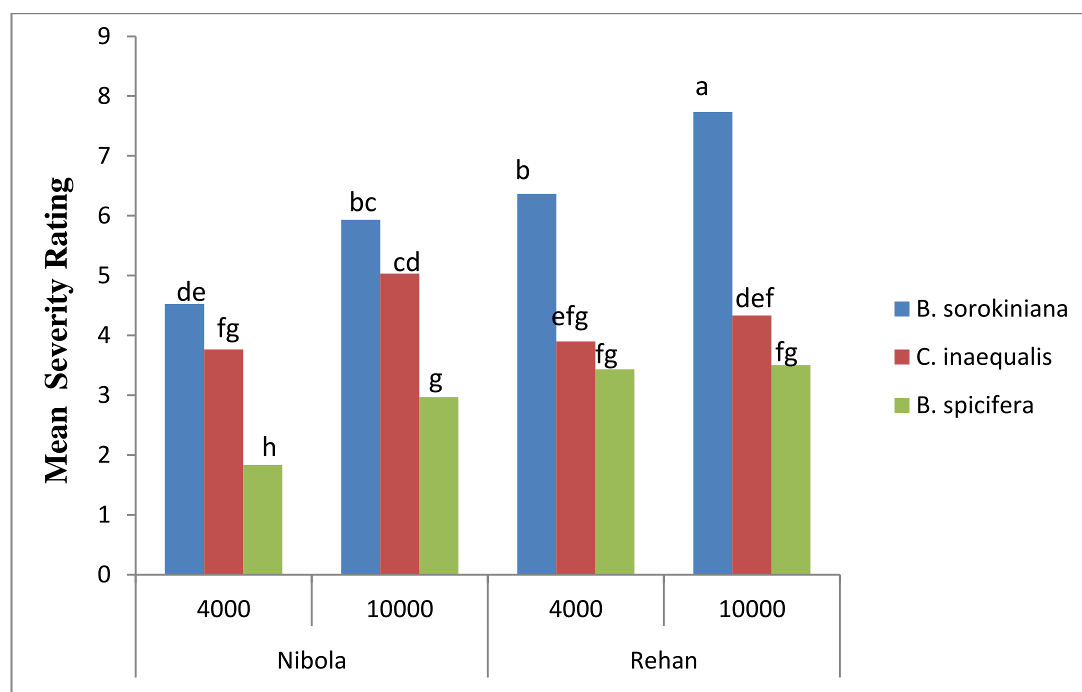


Figure 3-15. Assessment of pathogenicity of *B. sorokiniana*, *C. inaequalis*, and *B. spicifera* in a long term experiment at day time average temperature 26 °C and night time average 20 °C, on two Libyan cultivars, Nibola and Rehan.

Seedlings were inoculated with two spore concentrations, 4 x 10^3 conidia ml^{-1} and 1 x 10^4 conidia ml^{-1}. Severity was assessed on the upper two leaves at heading stage. Values represented by bars with the same letter are not significantly different at P = 0.05 (Tukey test).

3.4 Discussion

The results of this study indicate that spot blotch disease on barley in Libya can be caused by the commonly known pathogen *B. sorokiniana*. In addition, other fungal species are reported for the first time to cause symptoms similar to spot blotch disease on barley cultivars grown in Libya along with *B. sorokiniana*: *C. inaequalis*, *B. spicifera*, and *A. alternata*. *C. inaequalis* was isolated from the north of Libya. *Curvularia* species have also been reported to cause leaf spot on grasses in China (Huang *et al*., 2005; Huang *et al*., 2004), have been isolated from zoysia grass in Korea (Kim *et al*., 2000), and cause leaf spot on rice (De Luna *et al*., 2002). Although to my knowledge *C. inaequalis* has not been reported to cause disease on barley, it has been isolated from Iranian barley seed (Nejat-Salari and Ershad, 1994). The samples from

which *C. inaequalis* was isolated were collected in the north of Libya at the late growth stage, at the end of April when temperature was high and the end of the season was approaching. The findings of the long term pathogenicity test with average day time temperature 26 °C and night time temperature 20 °C are consistent with those of Muchovej and Couch (1987), who found infection of bentgrass by *C. lunata* had most effect at high temperature and on old leaves. This result may be explained because *C. inaequalis* requires high temperature with humidity to infect the plants, as these factors were found in the north of Libya. In contrast, *B. spicifera, B. sorokiniana* and *A. alternata* were isolated from barley grown in the central area and the south of Libya. Infection of rice leaves by *B. spicifera* was reported in Morocco (Ennaffah *et al*., 1997), it has been reported to cause leaf spot on sorghum in Turkey (Unal *et al.*, 2011) and it has been isolated from watermelon leaves in Morocco (Ennaffah *et al*., 1999). It is somewhat surprising that *B. spicifera* was isolated from barley leaves in the south of Libya where the temperature was very high along with dry weather as well as from the north and central area of Libya where infection was high in the field (Figure 3-6), whereas *B. sorokiniana* and *A. alternata* were isolated from the south of Libya. The high infection in the field in areas from which *B. spicifera* was isolated contrasts with the results of both the short-term and long-term pathogenicity tests, which gave lower disease severity than with *C. inaequalis* and *B. sorokiniana*. However, it is still necessary to determine whether *B. spicifera* caused the symptoms observed in the field. A future experiment could involve a more extensive survey.

Several studies have demonstrated that *A. alternata* causes disease on a wide range of crops. This fungus has been reported to cause leaf spot on *Stevia rebaudiana* in India at temperatures of 20 to 25 °C (Maiti *et al*., 2006). Fernandez et al. (2011) reported that black point disease on wheat is caused by *B. sorokiniana* and *A. alternata* in conditions of high temperature and high humidity, and several studies have indicated that *B. sorokiniana* requires high temperature with high humidity to cause infection and is a serious pathogen on cereal crops in areas with these weather conditions (Asad et al., 2007; Kumar *et al*., 2002; Mahto *et al*., 2012; Nizam *et al*., 2012; Valjavec-Gratian and Steffenson, 1997b). In another study, Iftikhar *et al.* (2006) isolated *A. alternata*, *B. sorokiniana*, *Curvularia lunata*, and *Drechslera spicifer* (nomenclature as

in their paper; i.e. *Bipolaris spicifera*) from wheat leaves in Pakistan from different areas where the temperature was high, caused black point on wheat (Conner and Davidson, 1988), root rot and foliar disease on wheat and rice (Iram and Ahmad, 2005a), black spot disease on persimmon fruits in Turkey (Kurt *et al*., 2010), leaf spot of *Houttuynia cordata* in China (Zheng *et al*., 2011), leaf blight on potatoes in south Africa (Van Der Waals *et al*., 2011), leaf spot on Aloe vera in Louisiana (Silva and Singh, 2012), leaf spot on switchgrass in Tennessee (Vu *et al*., 2012), leaf blight on cotton (Bashan *et al*., 1991), and black spot disease on cherry fruits (Zhao and Liu, 2012). The findings of the current study support previous research into this area which links high temperature and high humidity with incidence of disease caused by this pathogen (Lacicowa and Pieta, 1998) (Figure 3-7).

In the current study of the pathogens of spot blotch on barley cultivars, *B. sorokiniana* gave the highest disease severity ratings in both short and long term pathogenicity tests. In the short term test, the lowest disease severity resulted from infection with *A. alternata*, as shown in Figure 3-13 and Figure 3-14. In part of a study of some pathogens on barley and wheat, *B. sorokiniana* showed the same potential to cause disease symptoms on barley and wheat roots and coleoptiles as *Fusarium avenaceum* and *Microdochium nivale* (Hudec, 2007). In another study of pathogenesis on barley and wheat of common root rot disease caused by *B. sorokiniana*, *Fusarium culmorum*, and *Fusarium graminearum*, the findings indicated that *B. sorokiniana* caused the same severity of disease symptoms (Scardaci and Webster, 1982). The pathogenicity of different Libyan *B. sorokiniana* isolates on barley cultivars was different (Figure 3-13). In the case of the UK and Libyan isolates, Libyan isolate L50 was more pathogenic than the UK strain 6.16 when the two were tested together (data not shown). The difference may be related to different environmental conditions.

Plant resistance to spot blotch disease has been investigated in several studies attempting to develop methods of plant disease control and reduce loss of yield on barley cultivars. Grewal *et al*. (2012) studied the inheritance of resistance to spot blotch and net blotch in barley cultivars by crossing cultivars. Resistance to spot blotch

disease caused by *B. sorokiniana* has been studied *in vitro* for screening resistance of barley cultivars to this pathogen (Chand *et al*., 2008). Kutcher *et al*. (1996) studied methods of breeding resistance to common root rot and spot blotch caused by *B. sorokiniana* on barley. Screening barley cultivars for resistance to spot blotch disease has been studied to improve control of this disease (Arabi and Jawhar, 2010; Arabi and Jawhar, 2012). In the current study, resistance to *B. sorokiniana* was tested in two six-rowed Libyan cultivars, Rehan and Nibola, two two-rowed Libyan cultivars, ACSAD and Wadi Utbah, and two two-rowed UK cultivars (Pastoral and Gaelic). There were significant differences between barley cultivars: overall, cultivar Nibola was the most resistant to spot blotch disease caused by *B. sorokiniana*, followed by ACSAD, Wadi Utbah, Rehan, Gaelic and Pastoral (Figure 3-12). Differences between Nibola and Rehan were generally similar for the other pathogens, although the lower disease severities meant that differences among cultivars were smaller. The findings are consistent with other research showing that different barley cultivars have different levels of resistance to spot blotch disease. In some cases, differences in plant resistance between cultivars relate to differences in the presence of a single gene for resistance to the pathogen (Bovill *et al*., 2010; Steffenson *et al*., 1996). More generally, six-rowed barley cultivars have been found to be more resistant than two-rowed barley cultivars (Bilgic *et al*., 2005; Grewal et al., 2012; Valjavec-Gratian and Steffenson, 1997b). However, this pattern was not observed in the current study. Further research is needed to elucidate the genetic basis of the differences between barley cultivars in resistance to *B. sorokiniana* in the current study. They are consistent with the idea of independent dominant resistance genes that are different between cultivars (O'Boyle *et al*., 2011) or a specific single gene conferring resistance (Bilgic *et al*., 2005). An alternative explanation may be that these cultivars have polygenic resistance, with differences in the number of genes involved, and it may be possible to improve resistance to spot blotch disease by intercrossing the varieties as Iftikhar *et al*. (2009) suggested.

3.5 Conclusion

This study has investigated spot blotch disease on barley in Libya through a survey of areas where barley is grown, isolation, identification, and pathogenicity testing of

organisms associated with spot blotch disease on different barley cultivars, and assessment of resistance of common barley cultivars to spot blotch. One of the more significant findings to emerge from this study is the isolation and identification of three other pathogens that can cause symptoms of leaf spot – *B. spicifera*, *C. inaequalis* and *A. alternata* – in addition to the known common pathogen that causes spot blotch disease, *B. sorokiniana*. The second major finding was that disease severity on the six-rowed cultivar Nibola was significantly lower than on some other cultivars. Pathogenicity tests, both short term and long term, showed that *B. sorokiniana* caused the most severe disease and *A. alternata* caused the lowest disease severity and all isolates caused more severe disease at high temperature in the long term than at low temperature in the short term when tested on Nibola and Rehan. Further investigation is needed to show whether the patterns of relative severity observed in the conditions tested in the current study correspond to those in the field in Libya.

Chapter 4 Testing *Trichoderma harzianum* T-22 for biological control of spot blotch disease on barley

4.1 Introduction

Biological control of spot blotch disease on cereal crops is an important challenge. Several studies have been done attempting to reduce the impact of this disease on the yield of crops along with reducing use of chemical compounds which increase crop yield but cause environmental damage, but these have not yet led to practical application. In view of the promising results achieved with *Trichoderma* species in other systems, *T. harzianum* T-22, a biological control agent of proven success, was identified for this research. It has beneficial effects on plant growth and promotes the development and efficiency of the root system and controls diseases of several crops including maize (Harman, 2005). In addition to the ability of *T. harzianum* T-22 to reduce disease severity by producing antagonistic compounds (Bertagnolli *et al*., 1998; Cheng *et al*., 2010) against plant pathogens, it has the ability to enhance plant growth, which leads to increased plant disease resistance (Carvalho *et al*., 2011; Harman *et al*., 2004b; Perello and Dal Bello, 2011; Shivanna *et al*., 1996b), and to induce physiological protection in plants against oxidative damage (Mastouri *et al*., 2010).

Species of *Trichoderma* have been demonstrated to have the ability to control many plant pathogens by multiple mechanisms. Competition of *T. harzianum* with pathogens for a space for growth (Yaqub and Shahzad, 2010) and for nutrients (Elad *et al*., 1999) from the host, due to their ability of fast growth, is one of mechanisms by which *Trichoderma* species control different plant pathogens (Arras and Arru, 1997; Benitez *et al*., 2004; Sempere Ferre and Pilar Santamarina, 2010). Mycoparasitism is an additional mechanism that gives *Trichoderma* species efficiency as biocontrol agents against plant pathogens (Huang *et al*., 2011); they can attack pathogens directly and grow over them (Harman *et al*., 2004a). *Trichoderma* species can produce antagonistic compounds (Chen *et al*., 2012; Dubey *et al*., 2012; El-Hasan *et al*., 2008; El-Hasan *et al*., 2007; El-Hasan *et al*., 2009; Harman, 2006; Liu *et al*., 2009; Viterbo *et al*., 2010)

and enzymes that degrade cell walls of pathogens (Da Silva *et al*., 2012; Gajera and Vakharia, 2012; Kumar *et al*., 2012b; Moran-Diez *et al*., 2009). They can also induce systemic resistance in plants directly (Harman *et al*., 2004a; Shoresh *et al*., 2010; Shoresh *et al*., 2005) or enhance plant resistance indirectly (Van Wees *et al*., 2008). The ability of *Trichoderma* species to colonise plant parts such as roots can increase plant growth and plant resistance (Harman *et al*., 2004a; Shukla *et al*., 2012; Vargas *et al*., 2009). Salehpour *et al*. (2005) studied the effects of *Trichoderma* species on common root rot of wheat caused by *B. sorokiniana*, and found that individual use of several *Trichoderma* isolates greatly reduced disease severity.

The method of applying a biocontrol agent is important for its effectiveness. Applications by foliar spray, by soil treatment, and by seed treatment have all been used with *Trichoderma* species. Application by foliar spray has been used to control foliar diseases on different crops, including tan spot disease on wheat (Perello and Dal Bello, 2011) and net blotch disease on barley (Khan *et al*., 2010). Application by soil treatment is another important method. Ojaghian (2011) found that soil treatment with different isolates of *Trichoderma* was effective against potato stem rot caused by *S. sclerotiorum*. Lo *et al*. (1996) demonstrated that soil treatment with *Trichoderma harzianum* 1295-22 gave good control of dollar spot on bentgrass when it was reapplied frequently. Dubey *et al*. (2011) found that wet root rot of mungbean caused by *R. solani* could be controlled by application of *Trichoderma* species as soil treatment, and also as seed treatment. Seed treatment is one of the most important methods of application of chemical and biological agents to control plant diseases, especially on diseases for which the seeds are the initial source of disease. Seed treatment with *Trichoderma harzianum* T-22 has been demonstrated to increase plant resistance against oxidative damage (Mastouri *et al*., 2010). Seed treatment with *Trichoderma* species and *Bacillus* species in combination controlled damping off disease on cucumber caused by *R. solani* more effectively than individual use (Yobo *et al*., 2010). Yobo *et al*. (2011) tested the effects of treatment of bean seeds with *Trichoderma* and *Bacillus* isolates on damping off disease caused by *R. solani*, and achieved good reductions in disease severity.

4.1.1 Aims

This chapter aims to test biological control of spot blotch disease on barley using *T. harzianum* T-22, to determine whether application by foliar spray, by soil treatment, or as a seed coating is most effective, and to compare different timings of foliar spray application. Tests were carried out with *B. sorokiniana* strain 6.16 (standard isolate from Scottish barley) on four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic). The specific objectives were:

1- Test the effects of *T. harzianum* T-22 on spot blotch disease in vitro.
2- Assess whether there are any negative effects of applying *T. harzianum* T-22 alone to barley cultivars before testing it as a biocontrol agent for spot blotch.
3- Test the effects of *T. harzianum* T-22 by foliar application by using the biocontrol agent before inoculation with the pathogen.
4- Test the effects of *T. harzianum* T-22 by foliar application by using the biocontrol agent at the same time as inoculation with the pathogen.
5- Test the effects of *T. harzianum* T-22 by foliar application by using the biocontrol agent on infected plants after pathogen application, to determine its effectiveness for controlling or stopping development of disease.
6- Test the effects of *T. harzianum* T-22 by applying it directly to the soil to protect plants against disease.
7- Test the effects of *T. harzianum* T-22 by seed coating application to protect plants against disease.

4.2 Materials and methods

4.2.1 The effects of *T. harzianum* T-22 on *B. sorokiniana in vitro*

A dual culture technique was applied as described by Dubey (2007). Petri dishes (90 mm diameter) containing PDA were inoculated with 5 mm diameter mycelial discs of 7 days old cultures of the pathogen and *T. harzianum* T-22 at equal distances (30 mm) from the periphery. Inoculated plates were incubated at 25 °C to 28 °C in an incubator and the radial growth of the pathogens measured every day for seven days after incubation. Controls without *T. harzianum* T-22 were maintained and each treatment was replicated three times. The percent reduction in colony diameter was calculated by the formula: $I = (C-T)/C \times 100$, where I = percent reduction in colony diameter, C = colony diameter of pathogen in control, and T = colony diameter of pathogen in treatment.

4.2.2 The effect of *Trichoderma harzianum* T-22 on barley cultivars

This experiment was done in December 2009. To test the effect of *T. harzianum* T-22 by itself on barley, the four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic) were grown in a greenhouse at temperature average day 16 °C and night 11 °C as described in section 2.1. At the two-leaf stage (14 d after sowing), seedlings in three replicate pots for each cultivar were sprayed with a suspension of *T. harzianum* T-22 conidia at a concentration of 1×10^8 conidia ml^{-1} as described in section 2.5.2. Three replicate pots were sprayed with water as a control.

4.2.3 The effect of *T. harzianum* T-22 applied by foliar spray on spot blotch on different cultivars of barley under greenhouse conditions, using *T. harzianum* T-22 first

General procedures for plant growth and treatment with the pathogen and biocontrol agent are described in Chapter 2. The four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic) were used in this experiment, which was carried out in January 2010. Plants were grown in a greenhouse

at temperature average day 15 °C and night 10 °C. Nine pots per cultivar were used in this experiment, and five plants were grown in each pot for three replications. At the two-leaf stage 14 days after sowing, three pots of each cultivar were sprayed with *T. harzianum* T-22 at 1 x 10^8 conidia ml^{-1}. After seven days the pots that had been sprayed with *T. harzianum* T-22 and three untreated pots of each cultivar were inoculated with *B. sorokiniana* strain 6.16 at a concentration of 4 x 10^3 conidia ml^{-1}. Three pots of each cultivar were sprayed with sterile distilled water as a control without any treatment. Disease severity was measured after seven days.

4.2.4 The effects of *T. harzianum* T-22 on spot blotch on the different cultivars of barley when applied by foliar spray at the same time as *B. sorokiniana*

The experiment was carried out in January 2010. General procedures for plant growth and treatment with the pathogen and biocontrol agent are described in Chapter 2. The same protocol was followed as in the previous experiment for the cultivars, plant growth, and replication, at temperature average day 15 °C and night 10 °C. At the two-leaf stage, six pots for each cultivar were treated with *B. sorokiniana* (4 x 10^3 conidia ml^{-1}) and three pots of each cultivar were sprayed with sterile distilled water as a control. Immediately afterwards, for each cultivar three pots that had been treated with *B. sorokiniana* were treated with *T. harzianum* T-22 (1 x 10^8 conidia ml^{-1}) and the other three were sprayed with sterile distilled water. Disease severity was measured after seven days.

4.2.5 The effects of *T.a harzianum* T-22 applied by foliar spray on spot blotch on the different cultivars of barley using T-22 after infection by *B. sorokiniana*

This experiment was carried out in February 2010. General procedures for plant growth and treatment with the pathogen and biocontrol agent are described in Chapter 2. The same protocol was followed as in the previous experiment for the cultivars and replication and the experiment was carried out in the greenhouse at temperature average day 17 °C and night 10 °C. At the two-leaf stage six pots of each cultivar were

sprayed with *B. sorokiniana* at a concentration of 4 x 10^3 conidia ml^{-1}. Three pots of each cultivar were sprayed with sterile distilled water as a control. After four days, for each cultivar three pots that had been treated with the pathogen were sprayed with *T. harzianum* T-22 at 1 x 10^8 conidia ml^{-1} and the other six pots were sprayed with water. After seven days the disease severity was measured using a rating scale from 1 to 9 as described in Chapter 2.

4.2.6 The effects of *Trichoderma harzianum* T-22 applied by treating the soil

In this experiment the six cultivars were grown in soil treated with *T. harzianum* T-22. The experiment was carried out in May 2010. General procedures for plant growth and treatment with the pathogen are described in Chapter 2. The same replication was done as in the previous experiments. Soil was treated with TRIANUM-P (commercial product containing *T. harzianum* T-22). The treatment was designed to correspond to the recommended treatment rate of 0.3 g per m^2 of product suspended in 10 l of water, giving a spore concentration of approximately 1 x 10^9 conidia ml^{-1}. 0.03 g was suspended in 1 l of sterile distilled water. Three pots for each cultivar were watered immediately after sowing with 200 ml of the spore suspension for each pot and the other six pots were watered with water. After 14 days, at the two-leaf stage, the pots that had been treated with *T. harzianum* T-22 and three other pots of each cultivar were inoculated with *B. sorokiniana* (4 x 10^3 conidia ml^{-1}). The other three pots were sprayed with water to compare with the treated pots. All the pots were kept in a greenhouse at an average day time temperature of 21 ºC and night time temperature 16 ºC. Disease severity was measured after seven days.

4.2.7 Seed treatment

This experiment was carried out in September 2010. General procedures for plant growth and treatment with the pathogen are described in Chapter 2. Seeds were treated by the method of Perello et al. (2006). *T. harzianum* T-22 was cultured on PDA in petri dishes incubated for 10 to 15 d at 26 to 28 ºC in an incubator. The conidia were

harvested by flooding the culture with sterile distilled water and rubbing the culture surface with a sterile glass rod. The suspension was filtered through two layers of cheesecloth. The spore concentration was measured with a haemacytometer and the suspension was adjusted to $1x10^8$ conidia ml^{-1}, then amended with one drop of 0.05% Tween 20. Ten ml of the conidial suspension was mixed with 90 ml of 0.25% agar (in water), which serves as an adhesive. 50 g of seeds of each cultivar was added to 100 ml of the water agar - fungal biomass mixture for pelletising, and then mixed for 3 min. Seeds were dried for 24 h then sown (Perello *et al.*, 2006).

Six pots of each cultivar were sown with five to seven seeds per pot: three pots with coated seeds and the other three pots with untreated seeds as control. All the pots were kept in a greenhouse at an average temperature of 19 ºC daytime and 15 ºC nighttime. Seedlings at the two-leaf stage, 14 days after sowing, were inoculated with *B. sorokiniana* at a concentration of 4 x 10^3 conidia ml^{-1} as in the previous experiments. Disease severity was measured after 7 days.

4.3 Results

4.3.1 The effects of *T. harzianum* T-22 on *B. sorokiniana in vitro*

The effect of *T. harzianum* T-22 on *B. sorokiniana* was tested in vitro, by dual culture for seven days of growth. Figure 4-1 and Figure 4-2 show that *T. harzianum* T-22 grew faster than *B. sorokiniana* from the beginning of the experiment and inhibited growth of *B. sorokiniana* completely on the second day. The average inhibition of colony diameter of *B. sorokiniana* in dual cultures is shown in Figure 4-3.

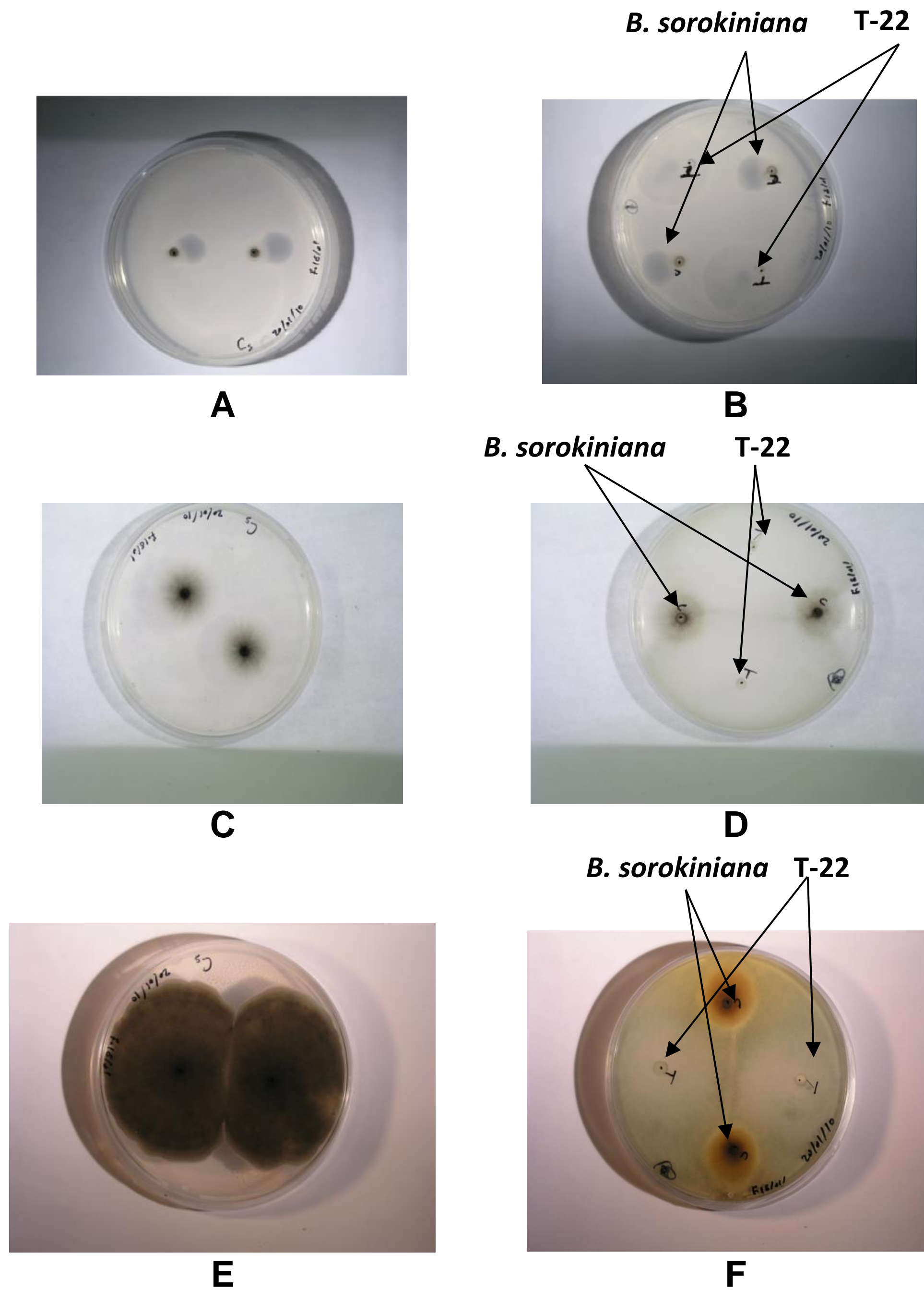


Figure 4-1. Growth of *B. sorokiniana* and *T. harzianum* T-22 in dual culture.

A, *B. sorokiniana* individually after one day; **B**, dual culture after one day; **C**, *B. sorokiniana* individually after 2 days; **D**, dual culture after 2 days; **E**, *B. sorokiniana* individually after 7 ddays; **F**, dual culture after 7 days.

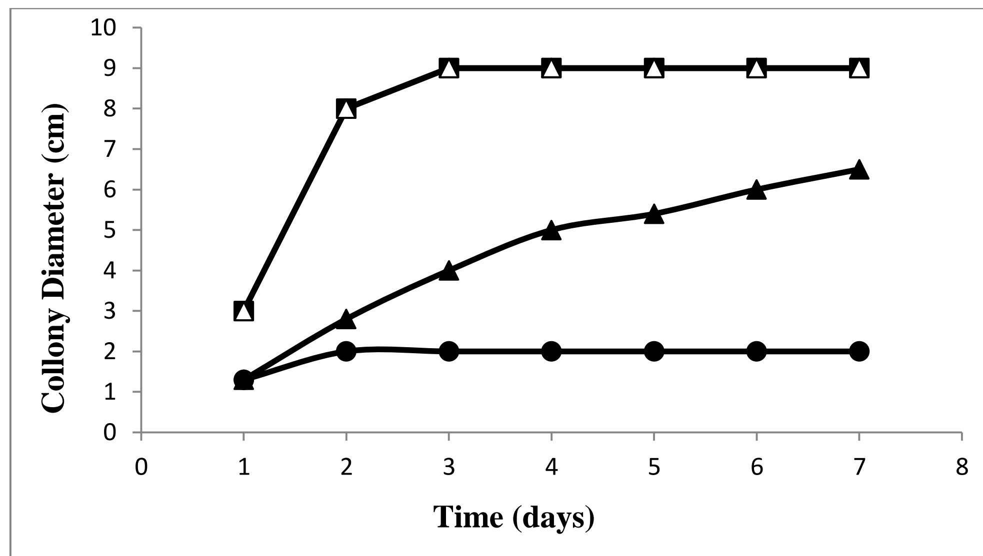


Figure 4-2. Colony growth in dual culture and controls.
▲ = *B. sorokiniana* control, △ = *T. harzianum* T-22 control, ■ = *Trichoderma* colony growth with *B. sorokiniana*, ● = *B. sorokiniana* colony growth with *Trichoderma.* Error bars are not shown because there were minimal differences between growth in replicate plates.

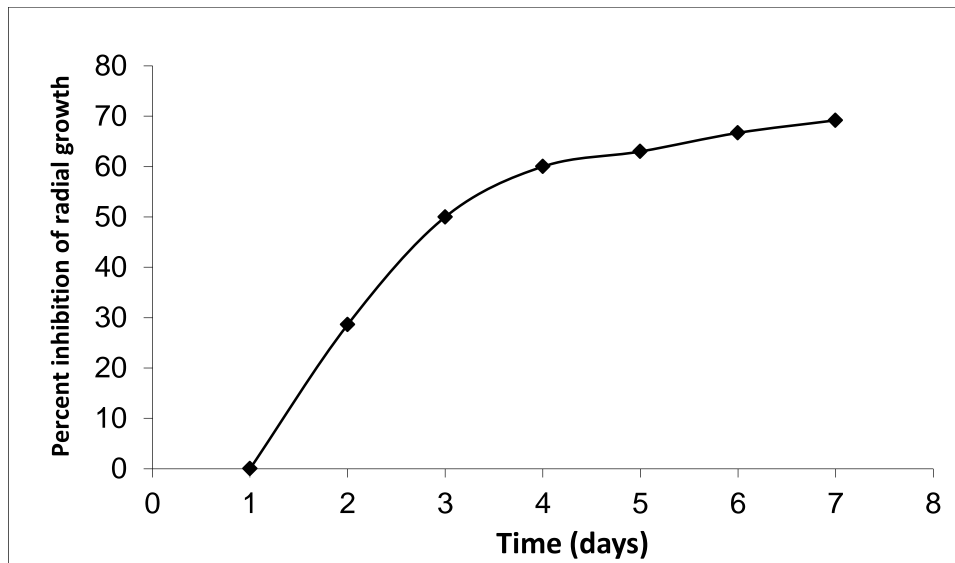


Figure 4-3. Average reduction of colony diameter of *B. sorokiniana* in three replicate dual cultures with *T. harzianum* T-22 (same experiment as shown in Figure ***4-2***).
No error bars are shown because there was little variation between plates.

4.3.2 The effect of *T. harzianum* T-22 on barley cultivars

In order to test biological control of spot blotch disease on barley using *T. harzianum* T-22, it was first necessary to find out whether *Trichoderma harzianum* T-22 alone might have any effect on the different barley cultivars. When plants were observed seven days after treatment with *T. harzianum* T-22 under greenhouse conditions at day time temperature average 16 °C and night time average 11 °C, there was no visible effect on any of the barley cultivars (not shown).

4.3.3 The effect of *T. harzianum* T-22 applied by foliar spray, before inoculation with *B. sorokiniana*

The *in vitro* experiment with *T. harzianum* T-22 showed that it inhibited growth of *B. sorokiniana*. The effect of *T. harzianum* T-22 on spot blotch disease on barley seedlings was tested under greenhouse conditions with four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic). To identify the most suitable application method, application by foliar spray, seed coating, and soil treatment was tested.

The effectiveness of *T. harzianum* T-22 when applied by foliar spray seven days before inoculation with the pathogen was tested on the six barley cultivars. No disease was observed on plants that were not inoculated with the pathogen. Application of T-22 significantly reduced disease severity ($P < 0.001$, Table 4-1). The mean disease severity rating on untreated plants was 7.0 whereas on treated plants it was 5.0. There were significant differences between cultivars in mean spot blotch disease severity, as shown in Figure 4-4. Mean severity was highest on Rehan and Pastoral and lowest on Nibola. Second leaves had significantly higher disease severity than first leaves ($P < 0.001$), with mean ratings 6.3 and 5.8 respectively. The effect of the biocontrol agent differed significantly among barley cultivars ($P = 0.006$), whereas its effects on the two leaves were not significantly different and the interactions between cultivar and leaf and between leaf, treatment and cultivar were not significant. The mean severity

values for the different cultivars and the two leaves are shown in Figure 4-5. According to the Tukey test results, there were significant reductions on both leaves of all cultivars except Gaelic and Pastoral, for which the reduction was only significant on the first leaf. Treatment with *T. harzianum* T-22 caused the largest average reduction in disease severity rating across both leaves, from 6.0 to 3.1, on cultivar Nibola.

Table 4-1. Analysis of variance of the effects of *T. harzianum* T-22 applied by foliar spray 7 days before inoculation with the pathogen.
Four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral, were tested. Disease severity was assessed on the first and second leaves 7 days after inoculation with the pathogen. The control was no treatment with *T. harzianum* T-22.

Source	DF	SS	MS	F	P
Cultivar	5	39.3583	7.8717	33.74	<0.001
Leaf	1	4.6006	4.6006	19.72	<0.001
T-22	1	72.4006	72.4006	310.29	<0.001
Cultivar*Leaf	5	0.8294	0.1659	0.71	0.618
Cultivar*T-22	5	4.3628	0.8726	3.74	0.006
Leaf*T-22	1	0.3472	0.3472	1.49	0.228
Cultivar*Leaf*T-22	5	0.4161	0.0832	0.36	0.875
Error	48	11.2000	0.2333		
Total	71	133.5150			

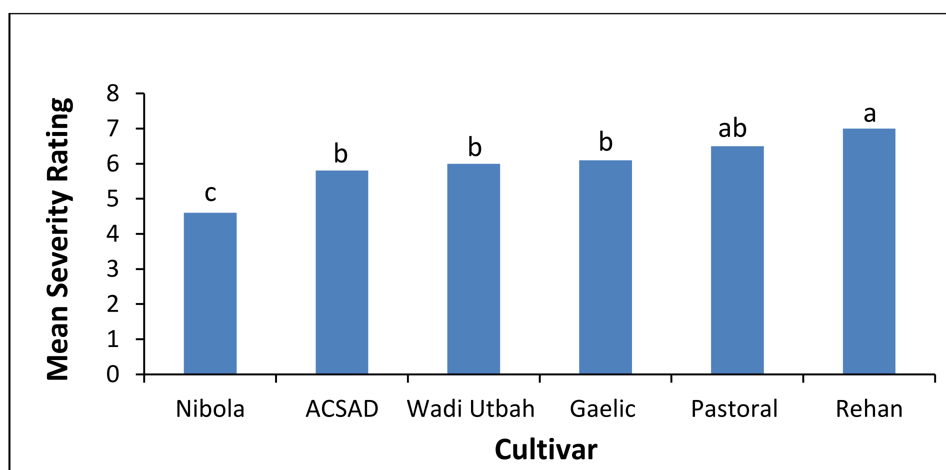


Figure 4-4. Mean spot blotch disease severity ratings on six barley cultivars 7 days after inoculation with *B. sorokiniana* and 14 days after treatment of half the pots with *T. harzianum* T-22.

Disease severity was assessed on the first and second leaves and with and without treatment with *T. harzianum* T-22. Means followed by the same letter are not significantly different at $P = 0.05$ (Tukey test).

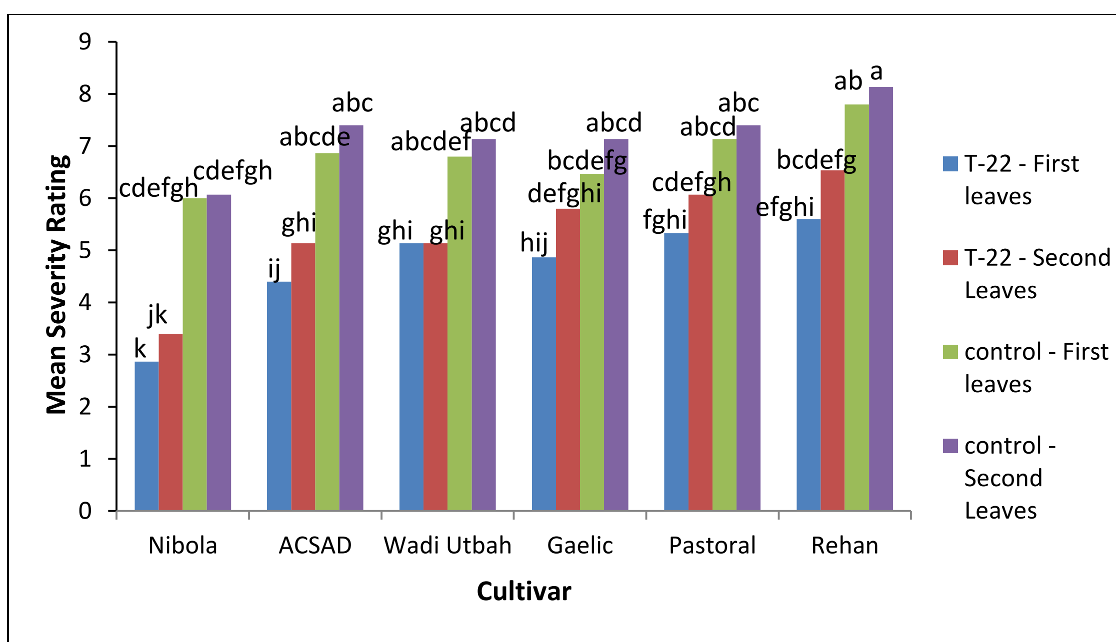


Figure 4-5. Spot blotch disease severity on six barley cultivars arranged in order of mean severity. Seedlings were treated by foliar spray with *T. harzianum* T-22 first, followed by *B. sorokiniana* after 7 days.

Disease severity was assessed on the first and second leaves 7 d after inoculation with *B. sorokiniana*. Control, no treatment with *T. harzianum* T-22. Values represented by bars with the same letter are not significantly different at $P = 0.05$ (Tukey test).

4.3.4 The effects of *T. harzianum* T-22 applied by foliar spray at the same time as inoculation with *B. sorokiniana*

At the two-leaf stage *T. harzianum* T-22 was applied at the same time as *B. sorokiniana* to the six barley cultivars. No disease was observed on plants that were not inoculated with the pathogen. *T. harzianum* T-22 significantly reduced the effect of *B. sorokiniana* ($P < 0.001$; Table 4.2). The mean severity rating was 4.6 on untreated plants and 2.7 on treated plants. There were significant differences among cultivars ($P < 0.001$). As shown in Figure 4-6, average disease severity was lowest on cultivars Nibola, Pastoral and Gaelic. There was a significant difference in disease on first and second leaves ($P < 0.001$), with mean severity 3.9 on second leaves and 3.4 on first leaves. Figure 4-7 shows that both leaves of all cultivars, except for the first leaf of cultivar Gaelic, showed significant reductions in disease severity when treated with *T. harzianum* T-22 at the same time as *B. sorokiniana.* There was no significant interaction between cultivars and leaves ($P = 0.077$), but there were significant differences in the effect of T-22 on different cultivars ($P = 0.004$) and on the two leaves ($P < 0.001$). The average reduction in severity was greater on leaf 2 (mean rating 5.0 to 2.7) than leaf 1 (mean rating 4.2 to 2.6). As in the previous experiment, Nibola had the lowest disease severity in combination with the biocontrol agent, although the severity ratings were not significantly different from those for Gaelic and Pastoral on both first and second leaves.

Table 4-2. Analysis of variance of the effects of treatment with *T. harzianum* T-22 by foliar spray (1 x 10^8 conidia ml^{-1}) at the same time as the plants were inoculated with *B. sorokiniana* (4 x 10^3 conidia ml^{-1}).

Plants of four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral were treated at the two-leaf stage, 14 days after sowing. Disease severity was assessed on the first and second leaves 7 days after inoculation with the pathogen and biocontrol agent. The control was no treatment with *T. harzianum* T-22.

Source	DF	SS	MS	F	P
Cultivar	5	79.0494	15.8099	105.01	<0.001
Leaf	1	3.4672	3.4672	23.03	<0.001
T-22	1	71.6006	71.6006	475.58	<0.001
Cultivar*Leaf	5	1.6094	0.3219	2.14	0.077
Cultivar*T-22	5	3.0094	0.6019	4.00	0.004
Leaf*T-22	1	2.3472	2.3472	15.59	<0.001
Cultivar*Leaf*T-22	5	1.9694	0.3939	2.62	0.036
Error	48	7.2267	0.1506		
Total	71	170.2794			

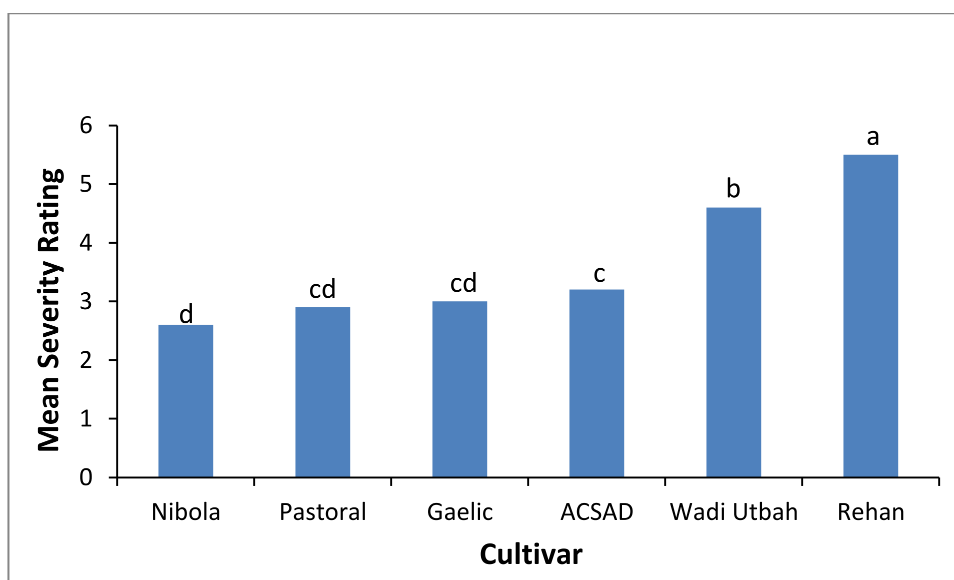


Figure 4-6. Mean spot blotch disease severity ratings on six barley cultivars 7 days after inoculation with *B. sorokiniana*. Half the pots were treated with *T. harzianum* T-22 at the same time as they were inoculated with the pathogen.

Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at $P = 0.05$ (Tukey test).

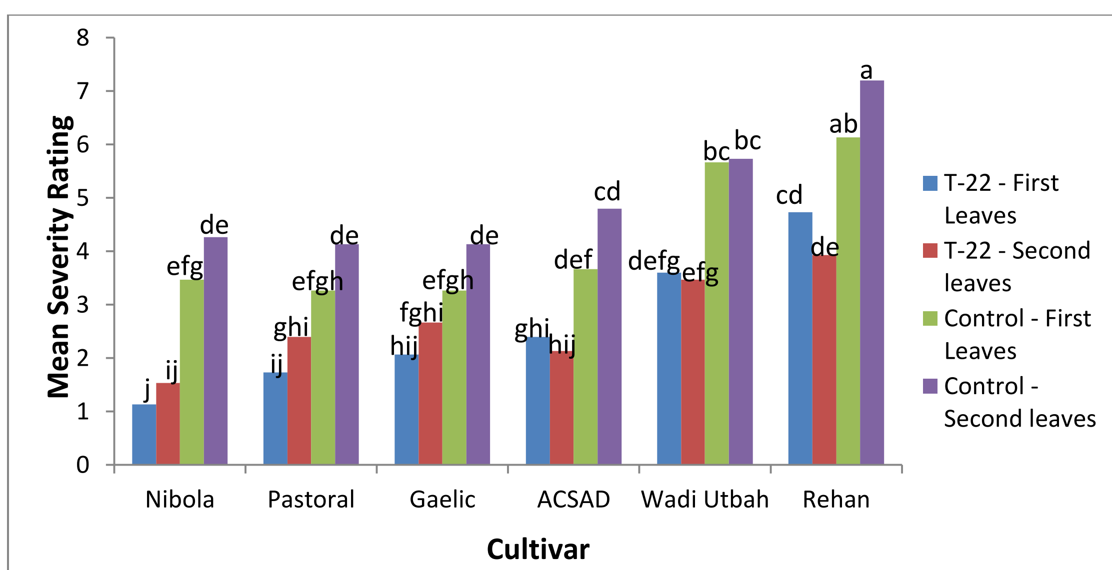


Figure 4-7. Spot blotch disease severity on six barley cultivars. The plants were treated by foliar spraying with *T. harzianum* T-22 and *B. sorokiniana* at the same time.

Disease severity was assessed on the first and second leaves 7 days later. Control, no treatment with *T. harzianum* T-22. Cultivars are arranged in order of mean severity. Values represented by bars with the same letter are not significantly different at $P = 0.05$ (Tukey test).

4.3.5 The effects of *T. harzianum* T-22 applied by foliar spray after infection with *B. sorokiniana*

This experiment was carried out to test the effect of *T. harzianum* T-22 on barley seedlings that had already been infected with spot blotch caused by *B. sorokiniana.* These plants have dead areas that could provide sites for colonisation by *T. harzianum*. No disease was observed on plants that were not inoculated with the pathogen. Treatment with the biocontrol agent 4 days after plants were inoculated with *B. sorokiniana* significantly reduced the development of spot blotch disease ($P < 0.001$; Table 4-3). The mean severity rating was 4.8 on untreated plants and 3.3 on treated plants. In this experiment there were no significant differences in mean disease rating among barley cultivars ($P = 0.132$). The error sum of squares was much bigger in this experiment than in the two previous ones, which is one reason why the significance levels of differences are generally lower, as shown in Figure 4-9. However, there was a significant difference between leaves ($P = 0.001$): the second leaves were more affected by spot blotch disease than the first leaves, with mean disease severity ratings 4.4 and 3.7, respectively. The effect of the biocontrol agent differed significantly among cultivars (P = 0.042). According to the Tukey tests the biocontrol agent only reduced disease severity significantly on the second leaves of Gaelic and Wadi Utbah. However, when the two leaves were averaged, there were significant reductions on Pastoral, Gaelic, and Wadi Utbah (not shown). In Figure 4-9, there is a clear trend of decreasing disease severity on treated plants compared to untreated. This figure can be compared to the differences between cultivars in Figure 4-8, which shows that Nibola had the numerically lowest average disease development. A striking observation to emerge from the data comparison was that cultivar Rehan was not the least resistant in this experiment.

Table 4-3. Analysis of variance of the effects of application of *T. harzianum* T-22 by foliar spray (1 x 10^8 conidia ml^{-1}) four days after inoculation with the pathogen.

Plants of four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral, at the two-leaf stage, 14 d after sowing, were inoculated with *B. sorokiniana* at a concentration of 4 x 10^3 conidia ml^{-1}. Disease severity was assessed on the first and second leaves 7 d after treatment with *T. harzianum* T-22.

Source	DF	SS	MS	F	P
Cultivar	5	5.2111	1.0422	1.79	0.132
Leaf	1	7.7356	7.7356	13.30	0.001
T-22	1	38.7200	38.7200	66.57	<0.001
Cultivar*Leaf	5	1.3178	0.2636	0.45	0.809
Cultivar*T-22	5	7.3067	1.4613	2.51	0.042
Leaf*T-22	1	1.0756	1.0756	1.85	0.180
Cultivar*Leaf*T-22	5	1.7778	0.3556	0.61	0.692
Error	48	27.9200	0.5817		
Total	71	91.0644			

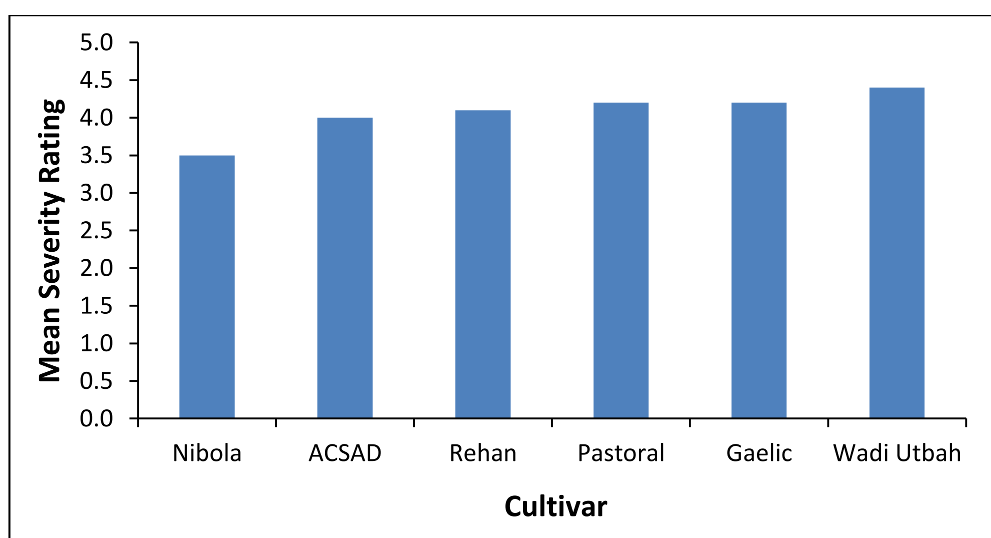


Figure 4-8. Means of disease severity on leaves 1 and 2 of six barley cultivars with and without foliar treatment with *T. harzianum* T-22 at 1 x 10^8 conidia ml^{-1} 4 days after inoculation with *B. sorokiniana*.

The assessment was 7 days after treatment with the biocontrol agent. The means are not significantly different at P = 0.05 (Tukey test).

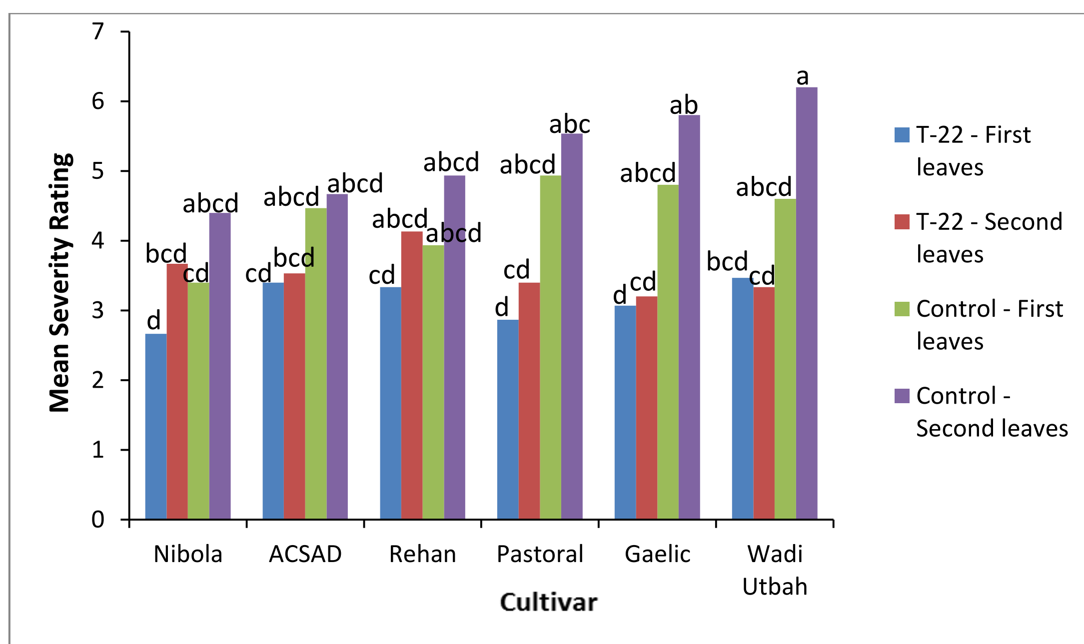


Figure 4-9. Spot blotch disease severity on six barley cultivars inoculated with *B. sorokiniana* and treated by foliar spray of *T. harzianum* T-22 4 days later.

Disease severity was assessed on the first and second leaves 7 days after treatment with *T. harzianum* T-22. Control, no treatment with *T. harzianum* T-22. Values represented by bars with the same letter are not significantly different at P = 0.05 (Tukey test).

4.3.6 The effects of soil treatment with *T. harzianum* T-22

The commercial product TRIANUM-P is intended to be applied as a soil drench. This experiment was carried out to test biological control of spot blotch disease on six cultivars by soil treatment with *T. harzianum* T-22 before seeds were sown. No disease was observed on plants that were not inoculated with the pathogen. Treatment of soil with *T. harzianum* T-22 significantly reduced disease severity ($P < 0.001$; Table 4-4). The mean severity ratings with treated and untreated soil were 4.2 and 6.7, respectively. There were significant differences between cultivars ($P < 0.001$); Nibola had the lowest average disease severity whereas Rehan had the highest average disease severity (Figure 4-10). Second leaves had significantly higher disease severity than first leaves; the mean severity ratings were 5.7 and 5.1, respectively. Also, the interaction between cultivars and leaves was significant. However, Tukey tests comparing all combinations of cultivar, treatment and leaves do not reveal significant differences between leaves for individual cultivars (Figure 4-11). Figure 4-11 shows

that Nibola had the numerically lowest severity among all untreated cultivars on the second leaves and Gaelic on the first leaves. Treatment with the biocontrol agent significantly reduced disease severity on both leaves of all cultivars (Figure 4-11) and there were significant differences in the effects of the biocontrol agent on different cultivars (P = 0.006). The largest difference in disease severity averaged across leaves was for Nibola, from 6.2 on untreated plants to 2.7 on treated plants. The interactions between leaves and biocontrol agent and between biocontrol agent, leaves and cultivars were not significant.

Table 4-4. Analysis of variance of the effects of treatment of soil with *T. harzianum* T-22 on spot blotch disease severity.

Four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral, were tested. Soil was treated with *T. harzianum* T-22 at a rate of 0.3 g per m^2. At the two-leaf stage 14 days after sowing, plants were inoculated with *B. sorokiniana* at a concentration of 4 x 10^3 conidia ml^{-1}. Disease severity was assessed on the first and second leaves 7 d after inoculation with the pathogen. Control, was no treatment with *T. harzianum* T-22.

Source	DF	SS	MS	F	P
Cultivar	5	32.5178	6.5036	38.01	<0.001
Leaf	1	5.7800	5.7800	33.78	<0.001
T-22	1	113.5022	113.5022	663.32	<0.001
Cultivar*Leaf	5	2.2600	0.4520	2.64	0.035
Cultivar*T-22	5	3.2578	0.6516	3.81	0.006
Leaf*T-22	1	0.0356	0.0.0356	0.21	0.651
Cultivar*Leaf*T-22	5	1.4711	0.2942	1.72	0.148
Error	48	8.2133	0.1711		
Total	71	167.0378			

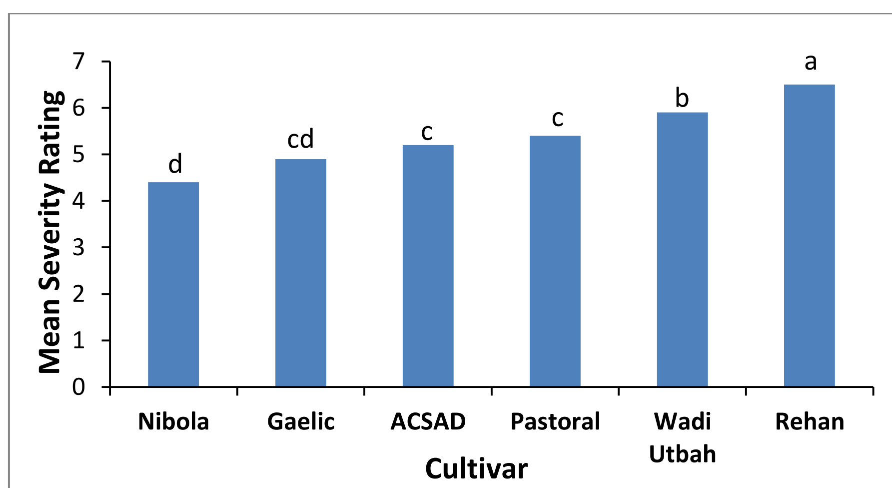


Figure 4-10. Means of disease severity on six barley cultivars with and without soil treatment with *T. harzianum* T-22.

The assessment was 7 days after inoculation with *B. sorokiniana*. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at *P* = 0.05 (Tukey test).

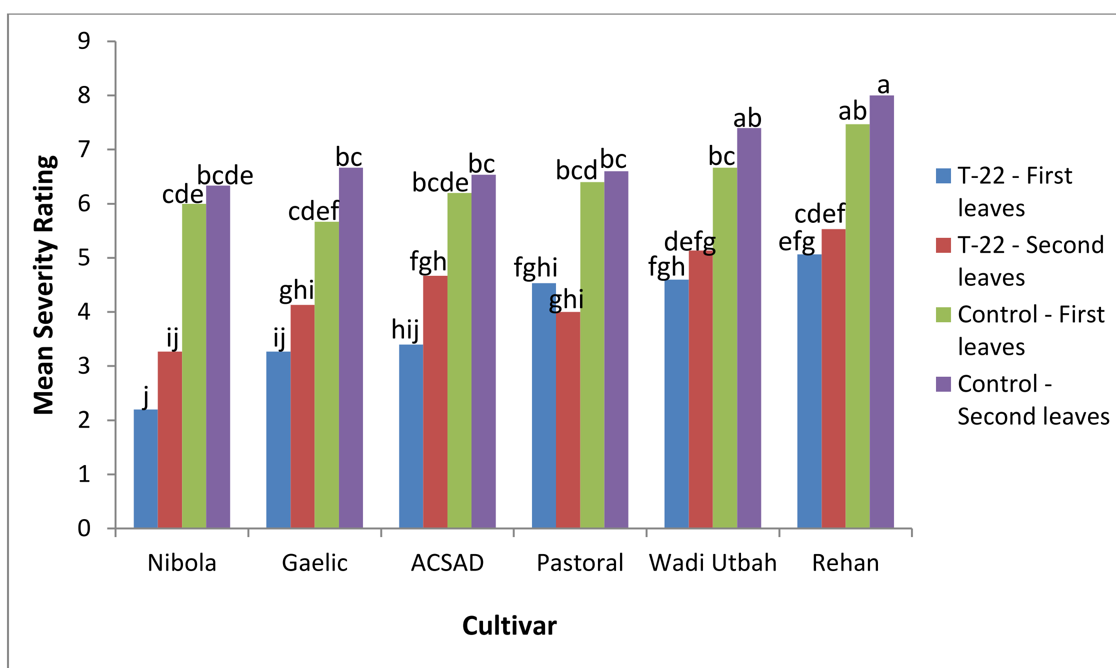


Figure 4-11. Spot blotch disease severity on six barley cultivars after soil treatment with *T. harzianum* T-22 at a rate of 0.3 g per m^2.

The plants were inoculated with *B. sorokiniana* 14 days after sowing. Disease severity was assessed on the first and second leaves 7 days after inoculation with *B. sorokiniana*. Control, no treatment with *T. harzianum* T-22. Values represented by bars with the same letter are not significantly different at *P* = 0.05 (Tukey test).

4.3.7 Seed treatment

Seeds of the six barley cultivars were coated with a suspension of *T. harzianum* T-22 (1 x 10^8 conidia ml^{-1}) in agar. No disease was observed on plants that were not inoculated with the pathogen. Plants grown from seeds treated with *T. harzianum* T-22 had significantly lower disease severity than plants grown from untreated seeds (P < 0.001; Table 4-5); the mean disease severity ratings were 2.4 and 4.4, respectively. The average disease severity differed significantly (P < 0.001; Table 4-5) among cultivars; Nibola had the lowest average disease severity whereas Rehan and Wadi Utbah had the highest, as shown in Figure 4-12. First leaves had significantly lower disease severity than second leaves (P < 0.001; Table 4-5); mean ratings were 3.2 and 3.7, respectively. Figure 4-13 shows that seed treatment with *T. harzianum* T-22 significantly reduced disease severity on both leaves of all cultivars. There were significant differences in the effects of the biocontrol agent on different cultivars (P = 0.022) and on first and second leaves (P = 0.022), but no strong patterns were evident. As shown in Figure 4-13, Nibola appeared to be the least affected by spot blotch disease both when untreated and treated, especially on the first leaves, but individual differences from Gaelic and Pastoral for all combinations of leaf and treatment were non-significant according to Tukey tests at P = 0.05. The interactions between cultivars and leaves and between biocontrol agent, cultivars and leaves were not significant.

Table 4-5. Analysis of variance of the effects of *T. harzianum* T-22 applied by seed treatment on spot blotch disease severity.

Seed of four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral, was coated with a suspension of *T. harzianum* T-22 in agar at 1 x 10^8 conidia ml^{-1}. Control was no treatment with *T. harzianum* T-22. At the two-leaf stage, 14 days after sowing, the plants were inoculated with *B. sorokiniana* at a concentration of 4 x 10^3 conidia ml^{-1}. Disease severity was assessed on the first and second leaves 7 days after inoculation with pathogen.

Source	DF	SS	MS	F	P
Cultivar	5	54.8533	10.9707	77.14	<0.001
Leaf	1	7.2200	7.2200	50.77	<0.001
T-22	1	56.1800	56.1800	395.02	<0.001
Cultivar*Leaf	5	1.2400	0.2480	1.74	0.143
Cultivar*T-22	5	2.0800	0.4160	2.92	0.022
Leaf*T-22	1	0.8022	0.8022	5.64	0.022
Cultivar*Leaf*T-22	5	0.4578	0.0916	0.64	0.667
Error	48	6.8267	0.1422		
Total	71	129.6600			

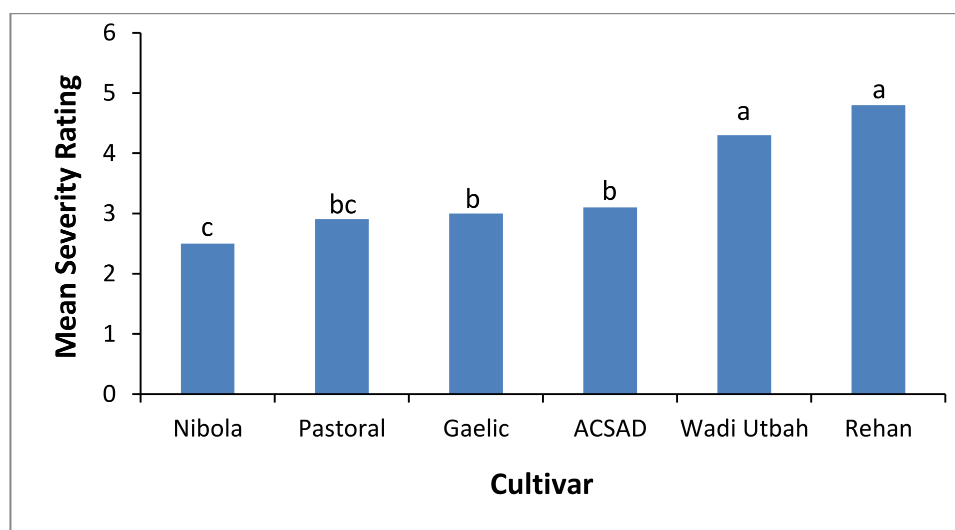


Figure 4-12. Means of spot blotch disease severity on six barley cultivars with and without seed treatment with *T. harzianum* T-22.

The assessment was 7 days after inoculation with *B. sorokiniana*. Disease severity was assessed on the first and second leaves. Means in the same column followed by the same letter are not significantly different at $P = 0.05$ (Tukey test).

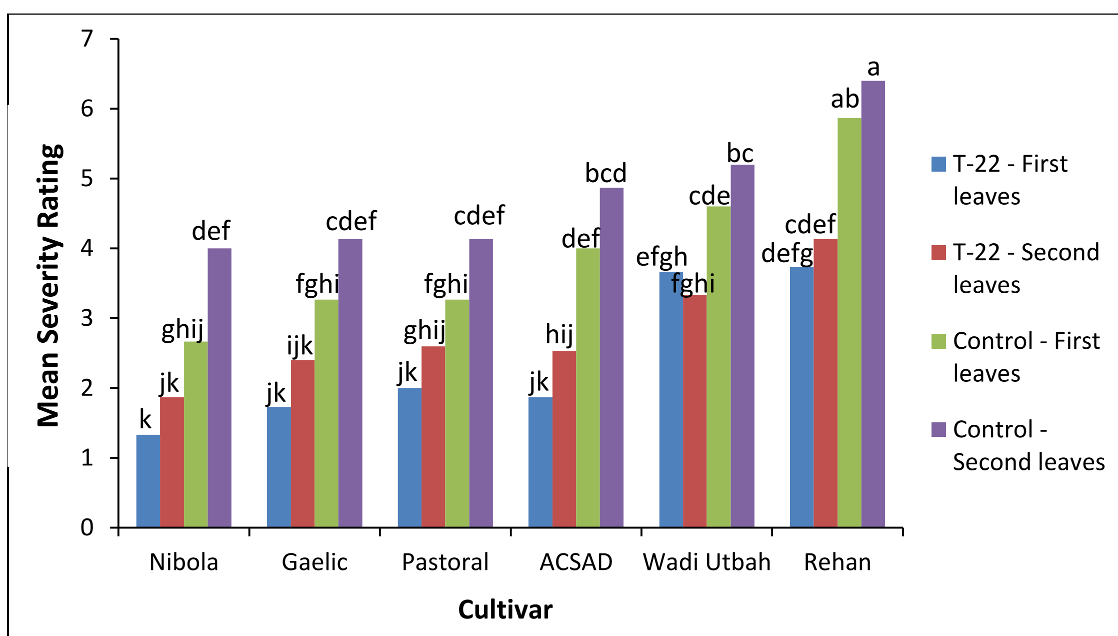


Figure 4-13. Effect of seed treatment with *T. harzianum* T-22 at 1 x 10^8 conidia ml^{-1} on spot blotch disease severity on six barley cultivars.

Plants were inoculated with *B. sorokiniana* 14 days after sowing. Disease severity was assessed on the first and second leaves 7 days after inoculation with *B. sorokiniana*. Control, no treatment with *T. harzianum* T-22. Values represented by bars with the same letter are not significantly different at P = 0.05 (Tukey test).

4.4 Discussion

The results of this study indicate that *T. harzianum* T-22 inhibits the growth of *B. sorokiniana* completely within two days *in vitro*. Also, the biocontrol agent, in addition to suppressing growth of the pathogen, occupied the whole surface of the pathogen colony. The findings of the current study are consistent with those of Ezziyyani *et al*. (2007), who found that *T. harzianum* inhibited growth of *Phytophthora capsici* completely on the second day. This result may be explained by the fact that *Trichoderma* species have a number of different mechanisms to control pathogens directly, by producing antagonist compounds (Bertagnolli *et al*., 1998; Cheng *et al*., 2010) and by competition for space, because *T. harzianum* grows more rapidly than *B. sorokiniana* (Arras and Arru, 1997; Benitez *et al.*, 2004; Kucuk and Kivanc, 2004), or for nutrients (Elad *et al*., 1999). The results agree with the recent findings of Fabiana Consolo *et al*. (2012), who demonstrated that *T. harzianum* achieved the highest inhibition of growth of *B. sorokiniana* compared with other pathogens that were tested, whereas in another recent study of the effects of *T. harzianum in vitro* on *B. sorokiniana* isolated from spot blotch disease on wheat, the full inhibition of growth of this pathogen took more than two days (Hasan *et al*., 2012). These differences of time needed for inhibition of pathogen growth can be explained in part by differences in the pathogen isolates, especially as they were from different hosts (wheat and barley), and by differences in the experimental conditions. Another possible explanation is that the distance between the biocontrol agent and the pathogen on agar plates in the study by Hasan *et al*. (2012) was more than 3 cm, which was used in this study. The shorter distance means that antagonistic compounds produced by the biocontrol agent would need less distribution on PDA to inhibit growth of the pathogen than when the distance between the pathogen and biocontrol agent is greater.

Commercial products containing *T. harzianum* T-22 have been used as biocontrol agents on several crops against different pathogens, and have given successful biocontrol. No evidence has been reported that *T. harzianum* T-22 has negative side effects on plants, and no negative effects were observed in this study. Tests of the effects of *T. harzianum* T-22 by foliar application on four Libyan cultivars (Rehan,

Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic) indicate that this biocontrol agent has a significant protective effect on all tested barley cultivars when applied before infection. This finding supports previous research into biocontrol of cereal diseases, which demonstrated that *T. harzianum* reduced disease severity and incidence of *Septoria tritici* blotch on wheat caused by *Mycosphaerella graminicola* (Perello *et al*., 2009). Comparing the effect of foliar application and seed coating on spot blotch disease on different cultivars, foliar application had positive effects on most cultivars whereas by seed coating the effects were less on some cultivars. These results differ from those of Perello *et al*. (2008), who estimated that application by seed coating gave better biological control of tan spot disease on wheat caused by *Pyrenophora tritici-repentis* than foliar application. A study of seed treatment with *Trichoderma* species against common root rot caused by *B. sorokiniana* on wheat showed a reduction in disease and an increase in plant growth (Salehpour *et al*., 2005). Perello *et al*. (2006) studied the effect of *T. harzianum* and *Trichoderma koningii* on tan spot and leaf blotch on wheat, and achieved successful biocontrol of both diseases under field conditions with both methods, seed coating and application as a foliar spray, in comparison to treatment with fungicide.

Foliar application of *T. harzianum* T-22 after infection with spot blotch significantly reduced disease severity, but the effect was less than with other application methods. This was partly because variability was relatively great in this experiment. Other possible reasons are the later assessment of disease severity than in the other experiments and the fact that some disease had already developed when *Trichoderma* was applied, so that the treatment would have a relatively low influence on the initiation of infection It would be interesting to determine whether this application method would have greater relative success with a longer-term assessment of disease severity.

When *T. harzianum* T-22 was applied at the same time as *B. sorokiniana* by foliar spray, the biocontrol agent had a large effect on spot blotch disease and reduced the development of disease on all tested barley cultivars and both tested leaves; Nibola

cultivar had the lowest disease severity whereas Rehan had the highest. The high reduction in disease severity may be due to a combination of factors. A possible explanation for some of the results may be that there is competition on the leaf surface between *T. harzianum* T-22 and *B. sorokiniana* (Kucuk and Kivanc, 2004), The effectiveness of control may be related to the ability of *Trichoderma* to grow or survive on the leaf surface. combined with antagonist compounds produced by the biocontrol agent (Cheng *et al.*, 2010) that reduce disease development. As also reported by Perelló *et al*. (2003), *T. harzianum* T-22 could be reisolated from leaves two weeks after application (not shown).

T. harzianum T-22 had more effect on spot blotch by soil treatment than seed coating (F = 663.32, 395.02 respectively). This finding is in agreement with the findings of Pineda (2001), who showed that soil treatment reduced death of sesame plants caused by *Macrophomina phaseolina*, which causes charcoal rot disease, more than seed coating. These results differ, however, from the estimate of Bell *et al*. (2000), who found that when applied to soil as a powder or pellets, *T. harzianum* did not protect cucumber seedlings against damping off caused by *Pythium* species and even had a negative effect on cucumber seedling health, whereas seed coating had no significant positive or negative effect. In other studies of seed coating and spray application of *Trichoderma* species on *Pinus radiata* seedlings to test their effects on growth promotion and seedling health, the findings indicated that there was no difference in effect on seedling health between seed coating and foliar application of *Trichoderma* (Hohmann *et al*., 2011). In the current study both seed coating and spray application reduced severity of spot blotch disease on several barley cultivars. There was a consistent pattern across application methods that in combination with the biocontrol agent *T. harzianum* T-22, cultivar Nibola showed the lowest disease severity and cultivar Rehan showed the highest.

4.5 Conclusion

The present study was designed to determine the effects of the biocontrol agent *T. harzianum* T-22 on spot blotch disease on barley cultivars in combination with disease resistance of barley cultivars by different application methods: foliar spray before, at the same time as and after inoculation with the pathogen, soil treatment with a suspension of spores and seed coating. This study has found that generally the biocontrol agent *T. harzianum* T-22 reduced severity of spot blotch disease on all barley cultivars by all application methods, although the effects were not statistically significant in all cases. In general, cultivar Nibola had the lowest disease severity with and without the biocontrol agent and cultivar Rehan had the highest disease severity.

Chapter 5 Effect of three commercial biocontrol agents on Libyan isolates of spot blotch individually and in combination *in vitro* and *in vivo* under greenhouse conditions

5.1 Introduction

One of the most significant current discussions in biological control of plant diseases is the use of combinations of biocontrol agents. In spite of successful reduction of severity disease in some cases, attempts are still being made to increase success with biological control methods. Using a combination of biocontrol agents is one of the most important methods that have been tried to achieve this aim.

There are several commercial biocontrol agents that are used to control plant diseases, including Actinovate (*Streptomyces lydicus*) and Serenade (*Bacillus subtilis*), which have been used to control foliar diseases. In some cases combinations of biocontrol agents have achieved successful control of disease. For example, combining a yeast (*Pichia guillermondii*) and a bacterium (*Bacillus mycoides*) reduced disease caused by *Botrytis cinerea* on strawberry leaves more than when either was applied individually (Guetsky *et al*., 2001). On the other hand, combinations of some biocontrol agents such as Serenade (*Bacillus subtilis*), Trianum (*Trichoderma harzianum* T-22) and Sentinel (*Trichoderma atroviride*) when applied to control *Botrytis cinerea* on strawberry gave less control than when they were used individually (Robinson-Boyer *et al*., 2009; Xu *et al*., 2010).

Several studies have been done to control spot blotch disease on cereals by testing single biocontrol agents either *in vitro* or *in vivo*. Etebarian and Mohammadifar (2009) tested the effect of *T. harzianum* on *B. spicifera* on wheat, and found that it reduced both pathogen growth in dual culture and disease severity of crown and root rot diseases caused by *B. spicifera* in microplots. However, very little was found in the literature on the question of the effect of biocontrol agents on spot blotch disease on different barley cultivars. Combinations of biocontrol agents to control plant diseases have been tested

with different plant diseases; the success of biological control achieved by combinations of biocontrol agents is related to the differentiation of their modes of action. Ezziyyani *et al*. (2007) used a combination of *T. harzianum* with *Streptomyces* species to control root rot on pepper. Elliott *et al*. (2009) used bacterial biocontrol agents with *Trichoderma* species to control *Phytophthora ramorum*, and a combination of a yeast, *Pichia guilermondii*, and a bacterium, *Bacillus mycoides*, gave more reduction in disease caused by *B. cinerea* on strawberry leaves than when either was used individually, due to their different mechanisms of action (Guetsky *et al*., 2001; Guetsky *et al*., 2002). Combination of *T. harzianum* with *Pseudomonas fluorescens* has given positive effects in reducing severity of disease caused by soil-borne plant pathogens in the field (Mishra *et al*., 2011). Successful control by a combination of biocontrol agent can be achieved depending on mechanisms of biocontrol agents (Xu *et al*., 2011).

5.1.1 Aims

This chapter set out with the aim of assessing the effects of three commercial biocontrol agents (*T. harzianum*, *S. lydicus* and *B. subtilis*) individually and in combination on spot blotch disease caused by the UK isolate of *B. sorokiniana* and Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera*. Tests were carried out *in vitro* and on barley plants of four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral, under greenhouse conditions. The objectives were to investigate the effects of the three biocontrol agents applied by different methods on spot blotch disease caused by the different pathogens on the various barley cultivars, in order to determine whether there is a best agent and application method and whether agents and application methods are most effective when used individually or in combination. The specific objectives are:

1- Test the effects of biocontrol agents (*T. harzianum*, *S. lydicus* and *B.s subtilis*) *in vitro* on Libyan isolates individually and in combination to assess the potential of combinations of biocontrol agents to control spot blotch.

2- Test the same three biocontrol agents individually and in combination on the UK isolate on four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral.

3- Test the effects of the three biocontrol agents individually and in combination by foliar application on disease caused by the Libyan isolates on two Libyan cultivars with contrasting disease resistance (Rehan and Nibola).

4- Test the effects of the three biocontrol agents individually and in combination by foliar application of the UK isolate of *B. sorokiniana* on four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral.

5- Test the effects of the three biocontrol agents individually and in combination by foliar application and repeat application after inoculation with the pathogen, with the UK *B. sorokiniana* isolate on the same six barley cultivars and the Libyan pathogen isolates on two Libyan cultivars (Rehan and Nibola).

6- Test the effects of the three biocontrol agents individually and in combination on the six barley cultivars inoculated with the UK isolate of spot blotch, by repeat application of the biocontrol agents seven days after the first treatment and seven days before inoculation with the pathogen.

7- Test the effects of the biocontrol agents individually and in combination by soil treatment on disease caused by the UK pathogen isolate on the six barley cultivars and by the Libyan pathogen isolates on two Libyan cultivars (Rehan and Nibola).

8- Test the effects of the biocontrol agents individually and in combination by soil treatment followed by foliar application five days after inoculation with the pathogen on disease caused by the UK pathogen isolate on the six barley cultivars.

5.2 Materials and methods

5.2.1 The effects of *T. harzianum* on Libyan isolates of *B. sorokiniana*, *B. spicifera* and *C. inaequalis in vitro*

A dual culture technique was applied as described by Dubey *et al*. (2007). Petri dishes (90 mm diameter) containing PDA were inoculated with 5 mm diameter mycelial discs of 7 days old cultures of the pathogen and *T. harzianum* T-22 at equal distances (30 mm) from the periphery. Inoculated plates were incubated at 25 to 28 °C in an incubator and the radial growth of the pathogens measured every day for seven days. A control without *T. harzianum* was maintained for each pathogen and each treatment was replicated three times. The percent inhibition of the pathogen's radial growth was calculated by the formula: $I = (C-T)/C \times 100$, where I = percent growth inhibition, C = daily increase in colony diameter of pathogen in control, and T = daily increase in colony diameter of pathogen in treatment.

5.2.2 The effects of *T. harzianum*, *S. lydicus* and *B. subtilis* on Libyan isolates of . *sorokiniana*, *B. spicifera* and *C. inaequalis in vitro*

For dual cultures of pathogens with individual biocontrol agents, two 5 mm plugs of bacterial cultures grown on PDA for 7 to 10 days were placed 1.5 cm from the edge of a PDA plate. Petri dishes were incubated at 28 ºC for 3 days (Trejo-Estrada et al., 1998), then two 5 mm mycelial plugs of the pathogen, taken from the advanced edge of a 10 to 14 days old culture, were placed 3 cm from the bacterial plugs. The dual culture was incubated at 28 ºC for 7 days. Colony diameter was measured every day for seven days, until the mycelium on control petri dishes with the pathogen by itself reached the edges of the dishes. Each treatment was replicated on three dishes. In tests of combinations of the biocontrol agents the plugs were located with the same distance between the biocontrol agent and the pathogen, with one plug of each biocontrol agent. The pathogen was applied with *Trichoderma* three days after application of the bacterial biocontrol agent.

5.2.3 Application of the pathogens and biocontrol agents by foliar spraying

Plants were grown and inoculated with the pathogens as described in Chapter 2. Five plants were grown in each pot. For foliar spraying with biocontrol agents, *T. harzianum* was applied as a spore suspension at 10^8 conidia ml^{-1} (Chapter 2), *S. lydicus* was applied as Actinovate, the commercial product, at 0.1 g l^{-1} on the basis of the instructions for the commercial product (3-12 oz/acre), and *B. subtilis* was applied as Serenade, the commercial product, at 1 ml l^{-1} on the basis of the instructions for the commercial product. Control plants were sprayed with sterile distilled water. After being sprayed, pots were covered with plastic bags for three days as described by Perello *et al*. (2009). Every day the bags were removed temporarily and all the pots were sprayed with water to keep the humidity high.

5.2.4 The effects of *T. harzianum* and *S. lydicus* applied by foliar spray individually and in combination on two Libyan cultivars, Nibola and Rehan, inoculated with Libyan isolates of *B. sorokiniana, B. spicifera* and *C. inaequalis*, using the biocontrol agents first

Two Libyan barley cultivars, Nibola, which was more resistant to *B. sorokiniana*, and Rehan, which was less resistant to *B. sorokiniana*, as shown in Chapter 3, were tested with two biocontrol agents, *T. harzianum* and *S. lydicus*, individually and in combination to control disease caused by Libyan isolates of *B. sorokiniana*, *B. spicifera* and *C. inaequalis*. At the two-leaf stage 14 days after sowing, for each cultivar nine pots for each agent were treated with *T. harzianum*, Actinovate (*Streptomyces lydicus*), or *T. harzianum* and Actinovate in combination, and three pots were treated with sterile distilled water as control. After seven days three of the pots of each cultivar that had been treated with *T. harzianum*, three pots treated with *S. lydicus*, three pots treated with *T. harzianum* and *S. lydicus* in combination and three pots treated with water as a control were inoculated with *B. sorokiniana* at a concentration of 8 x 10^3 conidia ml^{-1}. Equivalent sets of pots of each treatment and cultivar were inoculated with *C. inaequalis* at a concentration of 8 x 10^3 conidia ml^{-1} and with *B. spicifera* at 8000 conidia ml^{-1}. Plants were kept in a greenhouse with an average day time temperature of

22 ºC and night time temperature of 16 ºC. Disease severity was measured as described in Chapter 2 seven days after inoculation with the pathogens.

5.2.5 The effects of *T. harzianum*, *S. lydicus* and *B. subtilis* applied by foliar spray individually and in combination to six barley cultivars inoculated with the UK isolate of *B. sorokiniana*, using the biocontrol agents first

Four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic) were treated with three biocontrol agents (T-22, *S. lydicus* and *B. subtilis*) by foliar spray using the biocontrol agents first. At the two-leaf stage 14 days after sowing, for each cultivar three pots were treated with *T. harzianum*, three with Actinovate (*Streptomyces lydicus*), three with *T. harzianum* and *S. lydicus* in combination, three with Serenade (*Bacillus subtilis*), three with *B. subtilis* and *S. lydicus* in combination, three with a combination of the three biocontrol agents and three were treated with sterile distilled water as control. Seven days after treatment with the biocontrol agents, all the pots of each cultivar were inoculated with *B. sorokiniana* at a concentration of 8 x 10^3 conidia ml^{-1}. All the pots were grown in the greenhouse at average day time temperature 21 ºC and night time temperature 14 ºC. Seven days after inoculation with the pathogen, disease severity was measured as described in Chapter 2.

5.2.6 The effect of three biocontrol agents (*T. harzianum*, *S. lydicus* & *B. subtilis*) applied individually and in combination by foliar spray on six barley cultivars inoculated with the UK isolate of spot blotch, using the biocontrol agents first and reapplying them five days after inoculation with the pathogen

Four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic) were treated with three biocontrol agents (*T. harzianum*, *S. lydicus* and *B. subtilis*) by foliar spray, then the pathogen was applied seven days after application of the biocontrol agents, and five days after inoculation with the pathogen the biocontrol agents were reapplied. At the two-leaf stage 14 d after sowing, three pots

of each cultivar were treated with *T. harzianum*, three with *Streptomyces lydicus*, three with *T. harzianum* and *S. lydicus*, three with *B. subtilis*, three with *B. subtilis* and *S. lydicus*, three with all three biocontrol agents, and three pots of each cultivar were treated with sterile distilled water as control. After seven days all the pots were inoculated with *B. sorokiniana* at a concentration of 8 x 10^3 conidia ml^{-1}. Five days after inoculation with the pathogen, the biocontrol agents were reapplied by the same procedure as for the first application. All the pots were kept in the greenhouse at average day time temperature 22 ºC and night time temperature 15 ºC. Seven days after the second treatment with the biocontrol agent, disease severity was measured as described in Chapter 2.

5.2.7 The effect of *T. harzianum*, *S. lydicus* & *B. subtilis* applied individually and in combination by foliar spray on six cultivars inoculated with the UK isolate of *B. sorokiniana*, with repeat application of the biocontrol agents 7 d after the first treatment

Four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic) were tested in this experiment. At the two-leaf stage 14 d after sowing, for each cultivar three pots were treated with *T. harzianum*, three with *S. lydicus*, three with *T. harzianum* and *S. lydicus*, three with *B. subtilis*, three with *B. subtilis* and *S. lydicus*, and three with all three biocontrol agents together, and three were treated with sterile distilled water as control. Each biocontrol agent was reapplied seven days after the first application. Seven days after the second foliar spray with the biocontrol agents all the pots of each cultivar were inoculated with *B. sorokiniana* at a concentration of 8 x 10^3 conidia ml^{-1}. All the pots were grown in the greenhouse at average day time temperature 18 ºC and night time temperature 12 ºC. Seven days after inoculation with the pathogen and the second treatment with the biocontrol agents, disease severity was measured as described in Chapter 2.

5.2.8 The effects of *T. harzianum*, *S. lydicus* and *B. subtilis* applied by foliar spray on two Libyan cultivars, Nibola and Rehan, inoculated with Libyan isolates of *B. sorokiniana*, *B. spicifera* and *C. inaequalis* , with reapplication of the biocontrol agents five days after inoculation with the pathogens

Two Libyan barley cultivars, Nibola which was more resistant to *B. sorokiniana* and Rehan which was less resistant to *B. sorokiniana* (Chapter 3), were treated with three biocontrol agents (*T. harzianum*, *S. lydicus* and *B. subtilis*) individually and in combination to control disease caused by Libyan isolates of *B. sorokiniana*, *B. spicifera* and *C. inaequalis*.

At the two-leaf stage 14 days after sowing, for each cultivar and pathogen three pots were treated with *T. harzianum*, three with *S. lydicus*, three with *B. subtilis*, three with *T. harzianum* and *S. lydicus*, three with the three biocontrol agents in combination, and three were treated with sterile distilled water as control. After seven days, pots that had been infected for each cultivar three pots were inoculated with *B. sorokiniana* at a concentration of 8 x 10^3 conidia ml-1. Equivalent pots for each treatment and cultivar were inoculated with *C. inaequalis* at a concentration of 8 x 10^3 conidia ml-1 and with *B. spicifera* at a concentration of 8 x 10^3 conidia ml-1. Five days after inoculation with the pathogens, the biocontrol agents were reapplied by the same procedure as for the first application. Plants were kept in a greenhouse with average day time temperature 24 ºC and night time temperature 16 ºC. Seven days after inoculation with the pathogen and the second treatment with the biocontrol agents, disease severity was measured as described in Chapter 2.

5.2.9 Application of biocontrol agents by soil treatment

For soil treatment, pots were watered with suspensions of the biocontrol agents immediately after seeds were sown. Three pots of each cultivar were treated with TRIANUM-P (*T. harzianum*) at a rate of 0.3 g per m^2 as recommended in the commercial product, corresponding to a spore concentration of approximately 1 x 10^9 conidia ml^{-1}. 0.03 g was suspended in 1 l of sterile distilled water and three pots of each cultivar were treated with *S. lydicus* at rate of 0.1 g l^{-1} individually as recommended in

the instructions for the commercial product (3-12 oz/acre), three pots of each cultivar were treated with a combination of *T. harzianum* at 0.3 g per m^2 and *S. lydicus* at 0.1 g l^{-1}. Pots were watered after sowing with 200 ml for each pot of the previous suspensions or sterile distilled water as a control.

5.2.9.1 *The effects of two biocontrol agents (T. harzianum & S. lydicus) applied individually and in combination on six cultivars inoculated with the UK isolate of B. sorokiniana*

In this experiment six barley cultivars were grown in soil treated with *T. harzianum* and *S. lydicus* individually and in combination. Three pots of each cultivar were treated with *T. harzianum*, three with *S. lydicus*, three with *T. harzianum* and *S. lydicus* together, three with sterile distilled water as a control and three more for the uninoculated control. After 14 days, at the two-leaf stage, for each cultivar the nine pots that had been treated with the biocontrol agents and three pots treated with sterile distilled water were inoculated with *B.sorokiniana* at 4 x 10^3 conidia ml^{-1}. Three pots of each cultivar were sprayed with sterile distilled water and covered. All the pots were kept in the greenhouse at average day time temperature 19 ºC and night time temperature 15 ºC. Seven days after inoculation with the pathogen, disease severity was measured as described in Chapter 2.

5.2.9.2 *The effect of three biocontrol agents (T. harzianum, S. lydicus & B. subtilis) applied individually and in combination by soil treatment on six cultivars inoculated with the UK isolate of B. sorokiniana*

The six cultivars were used. For each cultivar three pots of each cultivar were treated with *T. harzianum*, three with *S. lydicus*, three with *B. subtilis*, three with *T. harzianum* T-22 and *S. lydicus*, three with *T. harzianum* and *B. subtilis*, three with *S. lydicus* and *B. subtilis,* three with all three biocontrol agents, and six with sterile distilled water as a control. After 14 days, at the two-leaf stage, three pots of each cultivar for each treatment and the control were inoculated with *B. sorokiniana* (4 x 10^3 conidia ml^{-1}).

Three pots for each cultivar that had not been treated with a biocontrol agent were sprayed with sterile distilled water. All the pots were kept in the green house at average day time temperature 21 ºC and night time temperature 16 ºC. Seven days after inoculation with the pathogen, disease severity was measured as described in Chapter 2.

5.2.9.3 The effects of three biocontrol agents (T. harzianum, S. lydicus & B. subtilis) applied individually and in combination on six cultivars inoculated with the UK isolate of B. sorokiniana, using soil treatment and foliar spray with the biocontrol agents five days after inoculation with the pathogen

The six cultivars were used. For each cultivar three pots were treated with *T. harzianum*, three with *S. lydicus*, three with *B. subtilis*, three with *T. harzianum* and *S. lydicus* together, three with *S. lydicus* and *B. subtilis* together, three with all three biocontrol agents in combination, and six with sterile distilled water as a control. After 14 days, at the two-leaf stage, three pots of each cultivar for each treatment and the control were inoculated with *B. sorokiniana* (4 x 10^3 conidia ml^{-1}) and three pots of each cultivar that had not been treated with a biocontrol agent were sprayed with sterile distilled water. Five days after inoculation with the pathogen, the biocontrol agents were reapplied by foliar spraying as described in section 5.2.6. All the pots were kept in the greenhouse at average day time temperature 20 ºC and night time temperature 13 ºC. Seven days after foliar application of the biocontrol agents, disease severity was measured as described in Chapter 2.

5.3 Results

5.3.1 The effects of *T. harzianum*, *S. lydicus* and *B. subtilis* on Libyan isolates of *B. sorokiniana*, *B. spicifera* and *C. inaequalis in vitro*

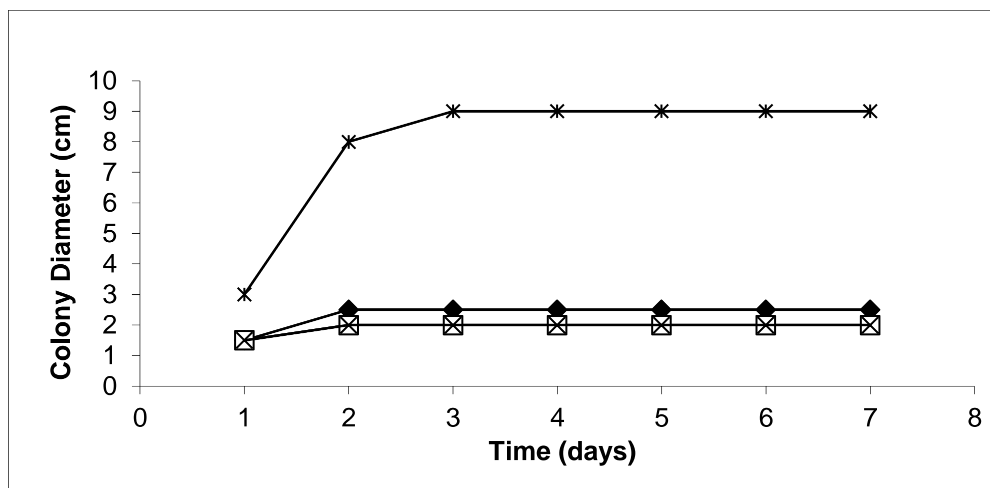


Figure 5-1. Colony growth in dual culture and controls.
✳ = *T. harzianum* control, ◆ = *C. inaequalis* colony growth with *T. harzianum*, × = *B. sorokiniana* colony growth with *T. harzianum*, ☐ = *B. spicifera* colony growth with *T. harzianum*.

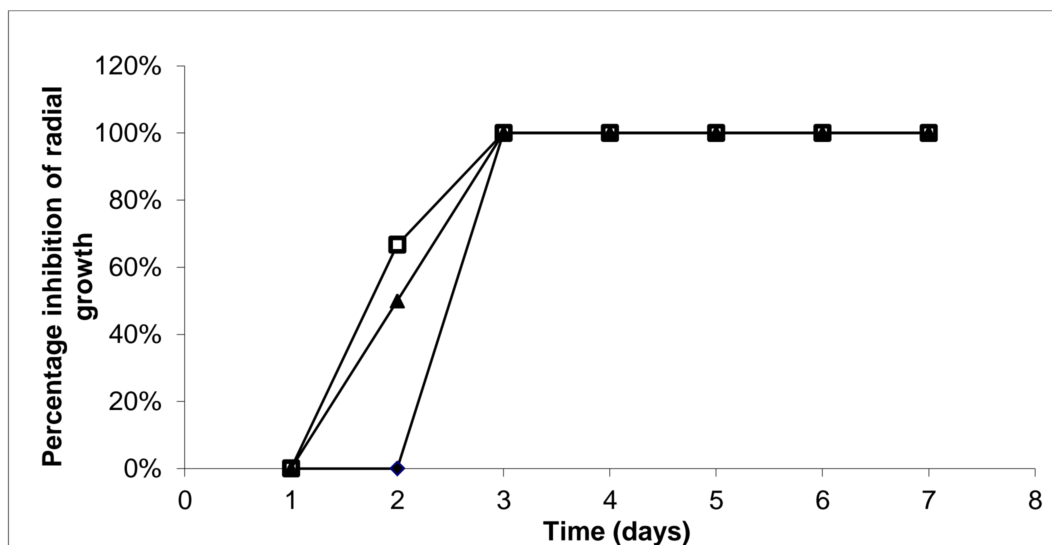


Figure 5-2. The average inhibition of radial growth of three replicates of dual cultures of each pathogen.
☐ = *B. sorokiniana* with *T. harzianum*, ▲ = *B. spicifera* with *T. harzianum*, ◆ = *C. inaequalis* with *T. harzianum*. Measurements were taken every day for 7 days. No error bars are shown because there was little variation between plates.

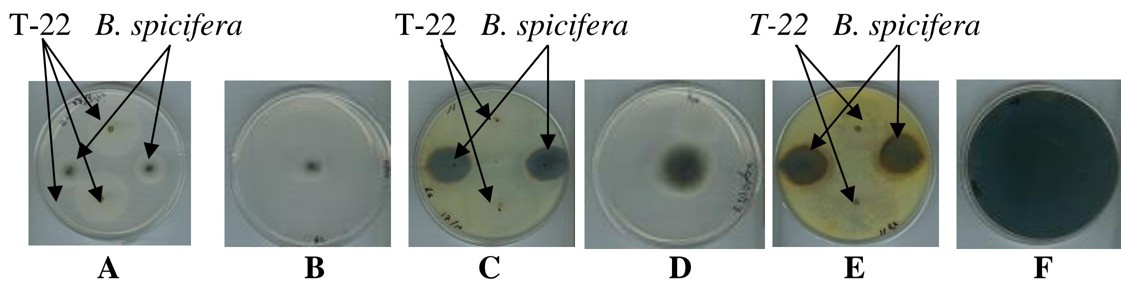


Figure 5-3. Dual culture of *T. harzianum* T-22 with *B. spicifera*.

A = dual culture after one day. B = control after one day. C = control after 2 days. D = dual culture after 2 days. E= dual culture after 7days, F= control after 7days.

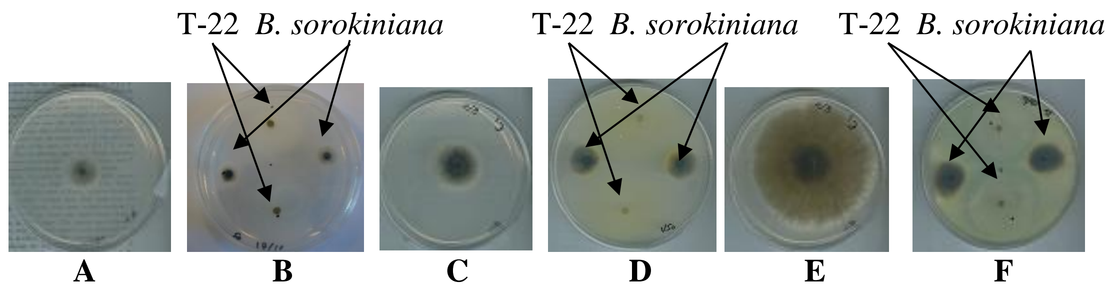


Figure 5-4. Dual culture of *T. harzianum* T-22 with *B. sorokiniana*.

A = control after one day. B = dual culture after one day. C = control after 2 days. D = dual culture after 2 days. E = control after 7 days. F = dual culture after 7 days.

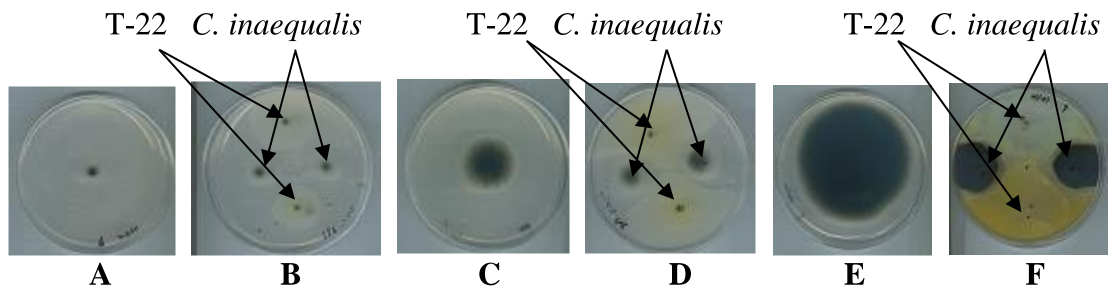


Figure 5-5. Dual culture of *T. harzianum* T-22 with *C. inaequalis.*

A = control after one day. B = dual culture after one day. C = control after 2 days. D = dual culture after 2 days. E = control after 7 days. F = dual culture after 7 days.

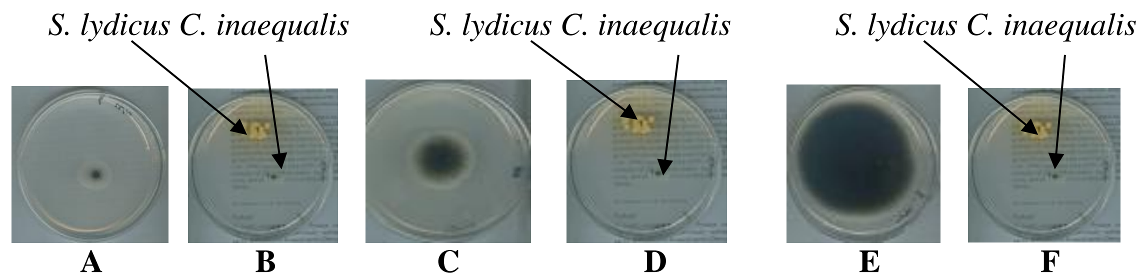


Figure 5-6. Dual culture of *C. inaequalis* with *S. lydicus*.

A = control after one day. B= dual culture after one day. C = control after 2 d. D = dual culture after 2 days. E = control after 7 days. F = dual culture after 7 days.

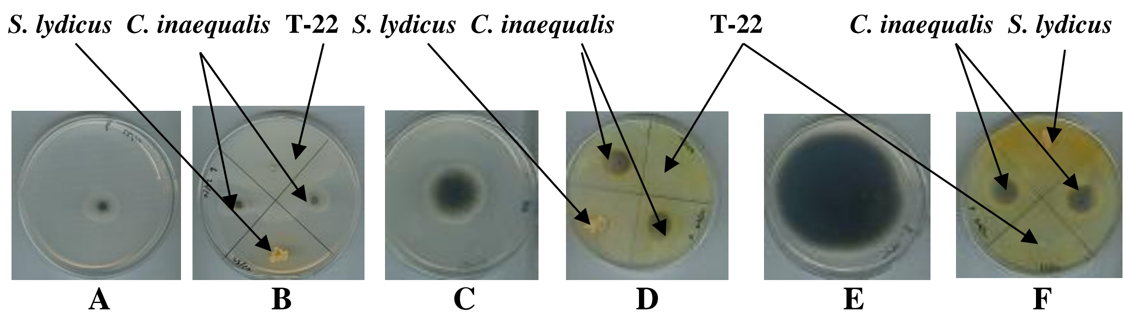


Figure 5-7. Culture of *C. inaequalis* with *S. lydicus* and *T. harzianum* T-22.

A = control after one day. B = combined culture after one day. C = control after 2 d. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

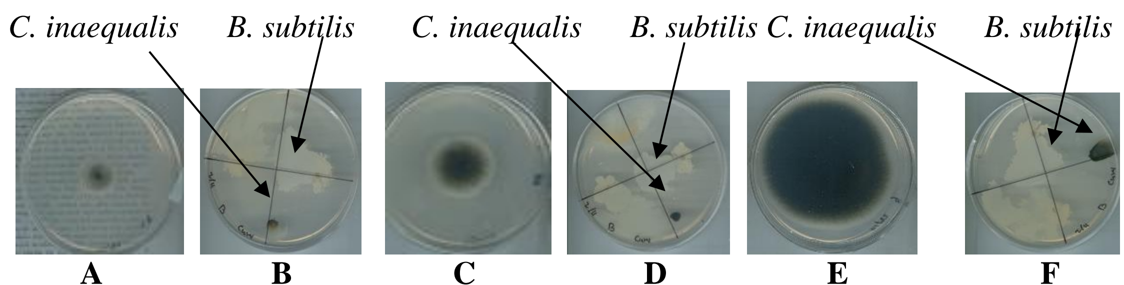


Figure 5-8. Dual culture of *C. inaequalis* with *B. subtilis*.

A = control after one day. B = dual culture after one day. C = control after 2 days. D = dual culture after 2 days. E = control after 7 days. F = dual culture after 7 days.

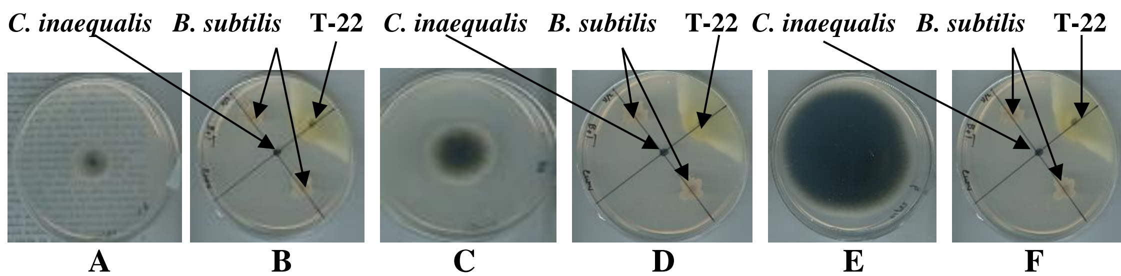


Figure 5-9. Culture of *C. inaequalis* with *B. subtilis* and *T. harzianum* T-22.

A = control after one day. B = combined culture after one day. C = control after 2 days. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

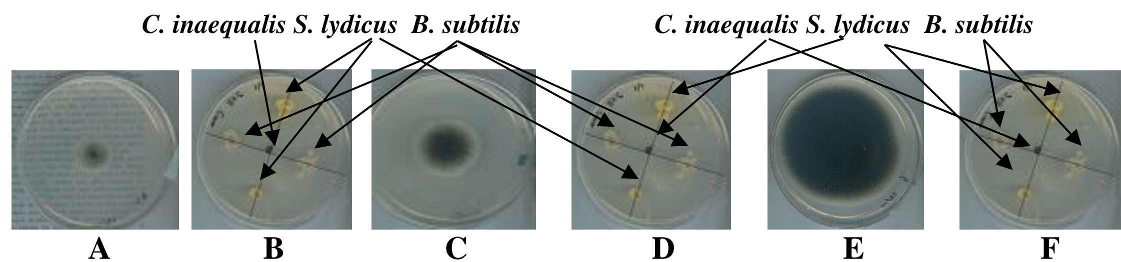


Figure 5-10. Culture of *C. inaequalis* with *S. lydicus* and *B. subtilis*.

A = control after one day. B = combined culture after one day. C = control after 2 days. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

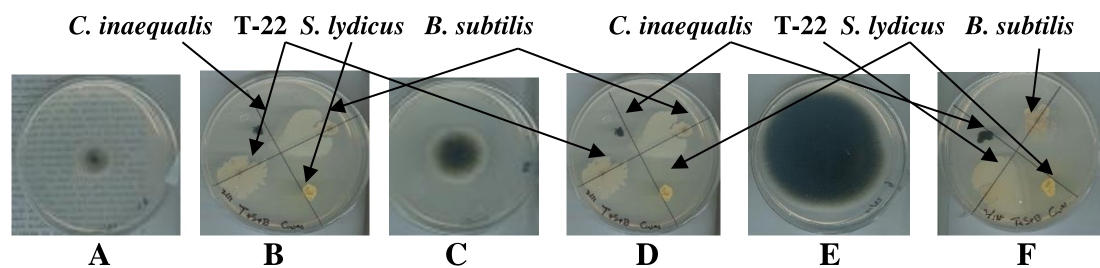


Figure 5-11. Culture of *C. inaequalis* with *T. harzianum* T-22, *S. lydicus* and *B. subtilis*.

A = control after one day. B = combined culture after one day. C = control after 2 days. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

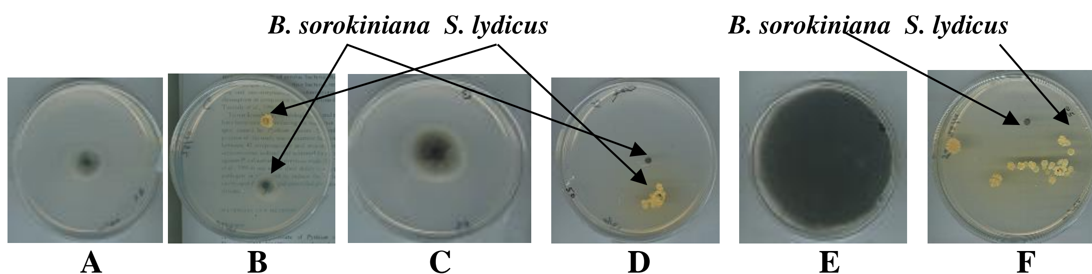


Figure 5-12. Dual culture of *B. sorokiniana* with *S. lydicus*.

A = control after one day. B = dual culture after one day. C = control after 2 days. D = dual culture after 2 days. E = control after 7 days. F = dual culture after 7 d.

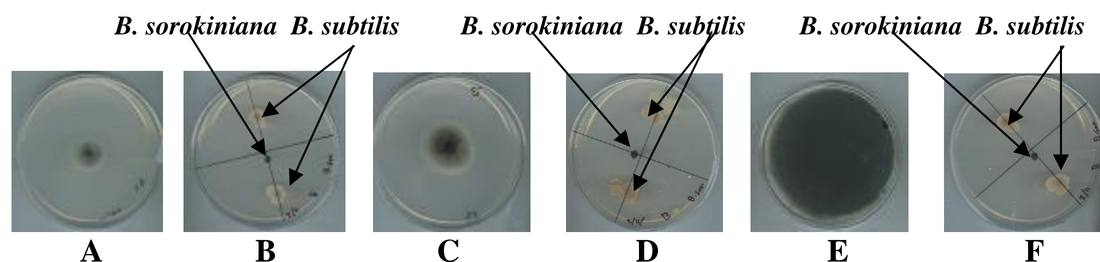


Figure 5-13. Dual culture of *B. sorokiniana* with *B. subtilis.*

A = control after one day. B = dual culture after one day. C = control after 2 days. D = dual culture after 2 days. E = control after 7 days. F = dual culture after 7 days.

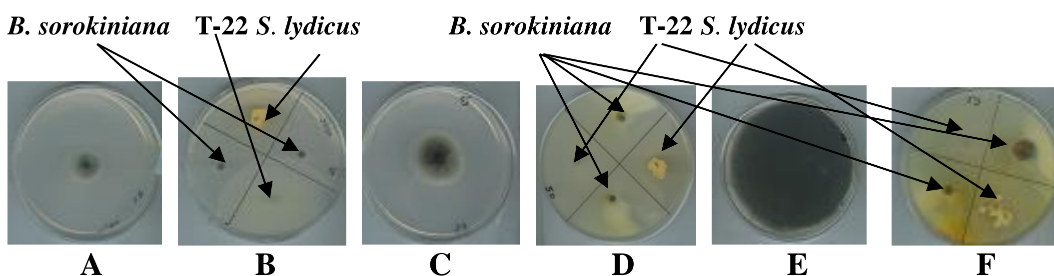


Figure 5-14. Culture of *B. sorokiniana* with *T. harzianum* T-22 and *S. lydicus*.

A = control after one day. B = combined culture after one day. C = control after 2 days. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

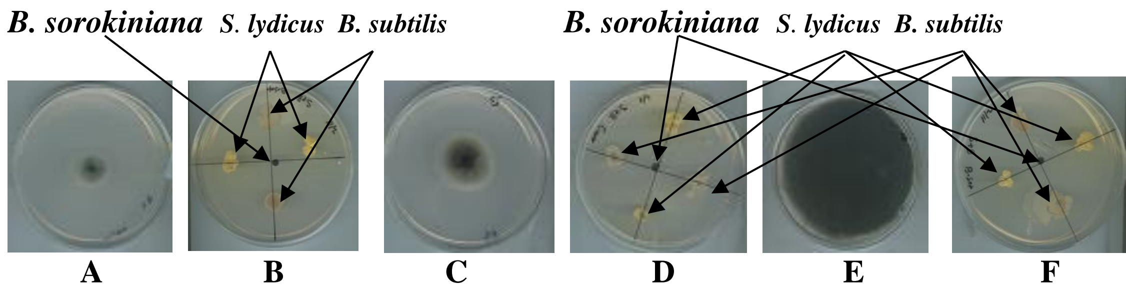


Figure 5-15. Culture of *B. sorokiniana* with *S. lydicus* and *B. subtilis.*

A = control after one day. B = combined culture after one day. C = control after 2 days. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

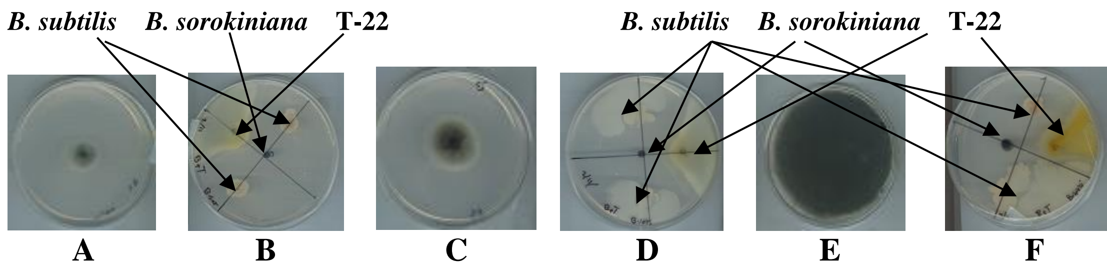


Figure 5-16. Culture of *B. sorokiniana* with *B. subtilis* and *T. harzianum* T-22.

A = control after one day. B = combined culture after one day. C = control after 2 days. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

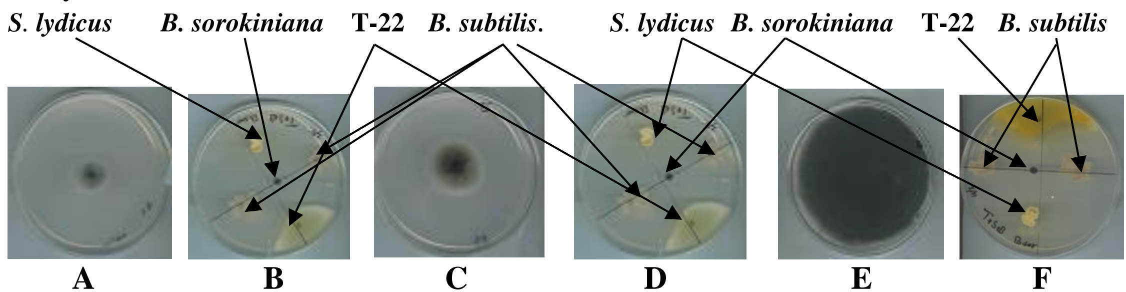


Figure 5-17. Culture of *B. sorokiniana* with *T. harzianum* T-22, *S. lydicus* and *B. subtilis*.

A = control after one day. B = combined culture after one day. C = control after 2 days. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

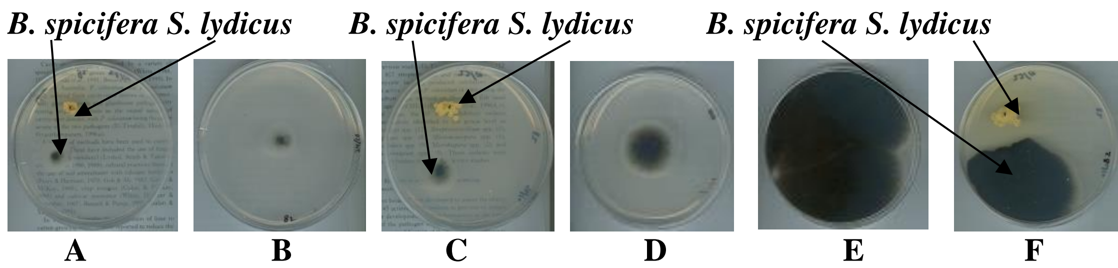


Figure 5-18. Dual culture of *B. spicifera* with *S. lydicus.*

A = dual culture after one day. B = control after one day. C = dual culture after 2 days. D = control after 2 days. E = control after 7 days. F = dual culture after 7 days.

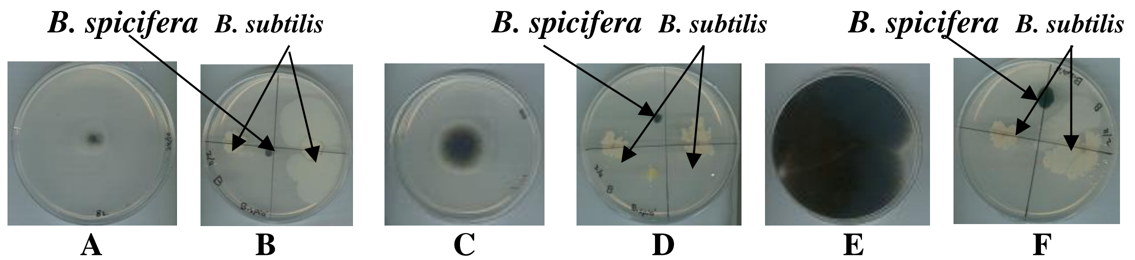


Figure 5-19. Dual culture of *B. spicifera* with *B. subtilis*.

A = control after one day. B = dual culture after one day. C = control after 2 days. D = dual culture after 2 days. E = control after 7 days. F = dual culture after 7 days.

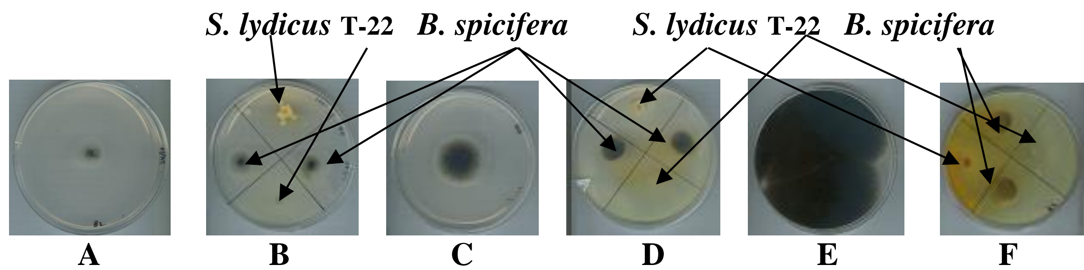


Figure 5-20. Culture of *B. spicifera* with *T. harzianum* T-22 and *S. lydicus*.

A = combined culture after one day. B = control after one day. C = control after 2 d. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

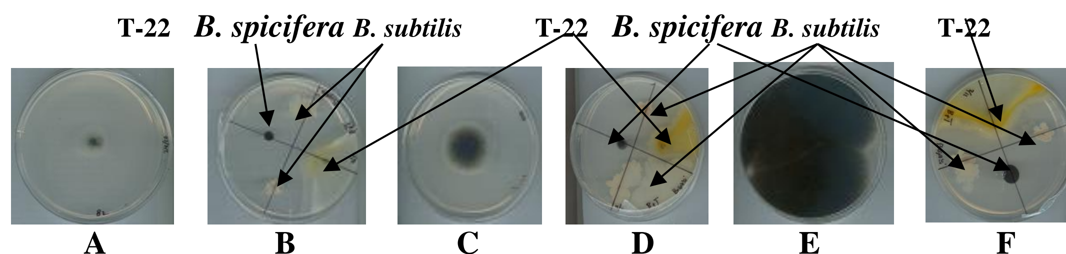


Figure 5-21. Culture of *B. spicifera* with *T. harzianum* T-22 and *B. subtilis*.
A = control after one day. B = combined culture after one day. C = control after 2 days. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

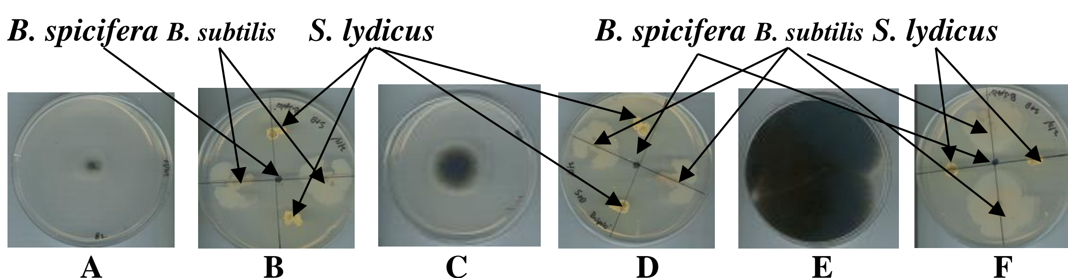


Figure 5-22. Culture of *B. spicifera* with *S. lydicus* and *B. subtilis*.
A = control after one day. B = combined culture after one day. C = control after 2 days. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

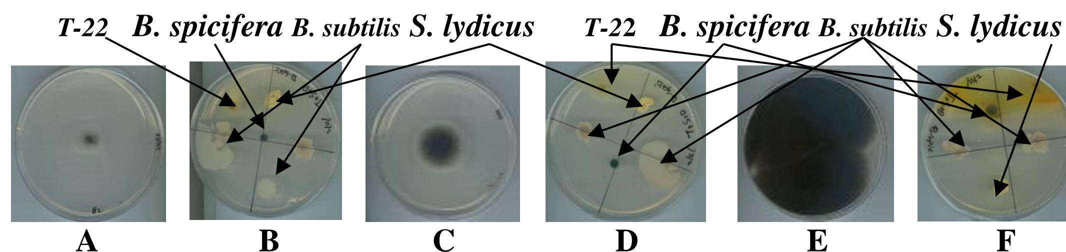


Figure 5-23. Culture of *B. spicifera* with *T. harzianum* T-22, *S. lydicus* and *B. subtilis.*
A = control after one day. B = combined culture after one day. C = control after 2 days. D = combined culture after 2 days. E = control after 7 days. F = combined culture after 7 days.

In dual culture *T. harzianum* completely inhibited growth of Libyan isolates of *B. sorokiniana, B. spicifera and C. inaequalis* after 3 days (Figure 5-1 and Figure 5-2). Figures 5-3 to 5-23 show that in *in vitro* experiments with dual culture and combined

culture, each of the biocontrol agents inhibited the growth of all the pathogens. There was no growth with most combinations of pathogens and biocontrol agents individually or in combination, except that in some cases there were inhibition zones. For *B. spicifera* with *S. lydicus* there was an inhibition zone and the pathogen grew in the opposite direction (Figure 5-18). There was an inhibition zone in dual culture of *T. harzianum* with *C. inaequalis* (Figure 5-5). In addition, there was an inhibition zone when was grown in combination with *B. subtilis* (Figure 5-9, Figure 5-16, Figure 5-17 and Figure 5-21). On the basis of these findings, the experiments in plants were done by using these biocontrol agents individually and in combination except that the combination between *T. harzianum* and *B. subtilis* was omitted.

5.3.2 The effects of *T. harzianum* and *S. lydicus* applied by foliar spray individually and in combination on two Libyan cultivars, Nibola and Rehan, inoculated with Libyan isolates of *B. sorokiniana, B. spicifera* and *C.a inaequalis*, using the biocontrol agents first

This experiment was carried out under greenhouse conditions to test control of spot blotch disease caused by Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera* on two Libyan barley cultivars, Rehan and Nibola, using two biocontrol agents, *T. harzianum* and *S. lydicus*, individually or in combination. The average day time temperature was 22 ºC and average night time temperature was 16 ºC. There were significant differences between the effects of the biocontrol agents ($P < 0.001$; Table 5-1). Both biocontrol agents reduced disease severity significantly individually or in combination, and *T. harzianum* individually reduced disease severity more than *S. lydicus* alone. Treatment with the combination of agents had an intermediate effect that was not significantly different from the effect of using either individually (Table 5-2). Average disease severity was significantly lower on Nibola than Rehan ($P < 0.001$); mean severities were 2.1 and 2.7, respectively. The largest difference was between pathogens ($P < 0.001$, F = 555.64); *B. sorokiniana* caused the most severe disease and *B. spicifera* the least (Table 5-3). The effects of the pathogens on the different cultivars and the effects of the biocontrol agents on disease caused by each pathogen differed significantly ($P < 0.001$). Figure 5-24 shows that according to Tukey tests, severity of

disease caused by *B. spicifera* and *C. inaequalis* on the two cultivars did not differ significantly and was not significantly affected by treatment with the biocontrol agents. Disease caused by *B. sorokiniana* on untreated plants was more severe on cultivar Rehan than on cultivar Nibola, and treatment with the biocontrol agents did not significantly reduce disease severity on Nibola in this experiment. For infection by *B. sorokiniana*, treatment of Rehan with both biocontrol agents individually and in combination significantly reduced disease severity compared to the control. There was no significant interaction between cultivars and biocontrol agents or biocontrol agents, pathogens and cultivars.

Table 5-1. Analysis of variance for the effects of *T. harzianum* and *S. lydicus* (BioAgent) applied by foliar spray individually or in combination on severity of disease caused by Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera* (Pathogen) on two Libyan cultivars, Nibola and Rehan.

Plants at the two-leaf stage, 14 d after sowing, were treated with the biocontrol agents. After seven days the plants were inoculated with pathogens at a concentration of 8 x 10^3 conidia ml^{-1}. Disease severity was assessed 7 days after inoculation with pathogens on the first and second leaves together. Control was not treated with either biocontrol agent.

Source	DF	SS	MS	F	P
Cultivar	1	14.063	14.063	55.50	<0.001
BioAgent	3	9.227	3.076	12.14	<0.001
Pathogen	2	281.585	140.793	555.64	<0.001
Cultivar*BioAgent	3	1.528	0.509	2.01	0.116
Cultivar*Pathogen	2	7.172	3.586	14.15	<0.001
BioAgent*Pathogen	6	8.555	1.426	5.63	<0.001
Cultivar*BioAgent*Pathogen	6	1.662	0.277	1.09	0.371
Error	120	30.407	0.253		
Total	143	354.198			

Table 5-2. Mean disease severity ratings for the biocontrol agents used on two Libyan cultivars, Nibola and Rehan, inoculated with Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera* , using the biocontrol agents first.

The assessment was 7 days after inoculation with the pathogens. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at P = 0.05 (Tukey test).

Treatment	Mean Disease Severity Rating
Control	2.8a
S. lydicus	2.4b
T. harzianum & *S. lydicus*	2.2bc
T. harzianum	2.1c

Table 5-3. Means of disease severity caused by Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera* on first and second leaves of two Libyan barley cultivars, Nibola and Rehan, treated and untreated with biocontrol agents, using the biocontrol agents first.

The assessment was 7 days after inoculation with the pathogens. Means followed by the same letter are not significantly different at P = 0.05 (Tukey test).

Pathogen	Mean Disease Severity Rating
B. sorokiniana	4.3a
C. inaequalis	1.8b
B. spicifera	1.1c

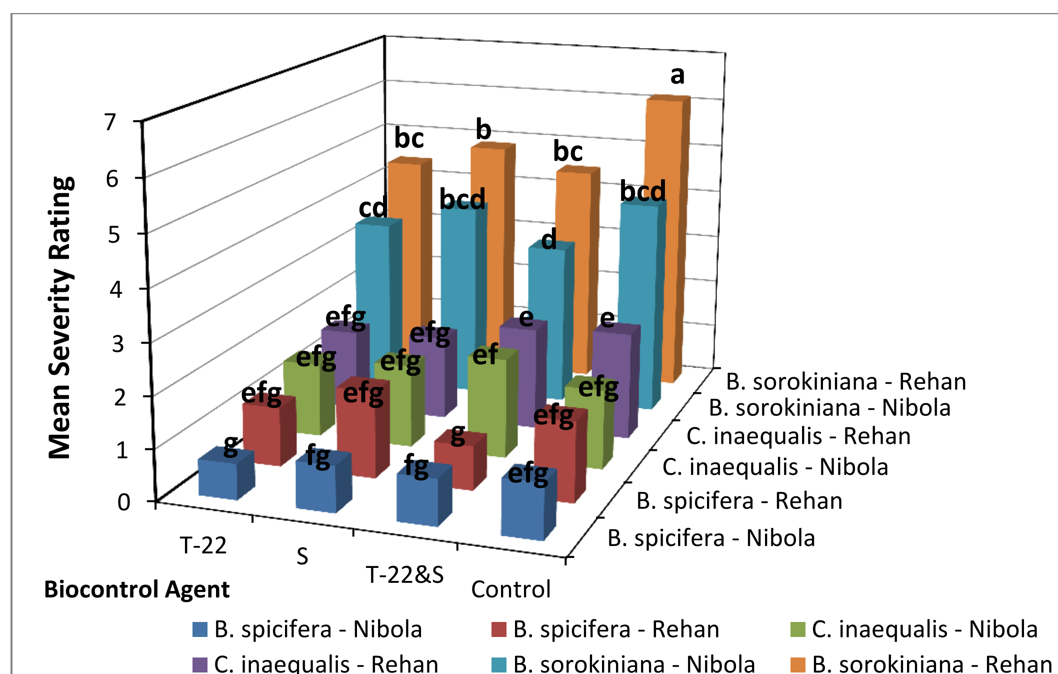


Figure 5-24. Spot blotch disease severity on cultivars Nibola and Rehan treated by foliar spray with *T. harzianum* and *S. lydicus* individually and in combination followed by the Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera* after 7 days. Disease severity was assessed on the first and second leaves 7 days after inoculation with pathogen isolates. Control, no treatment with biocontrol agents. Values represented by bars with the same letter are not significantly different at P = 0.05 (Tukey test). T-22 = *T. harzianum*, S = *S. lydicus*.

5.3.3 The effects of three biocontrol agents (T-22, *S. lydicus* & *B. subtilis*) applied by foliar spray individually and in combination on six barley cultivars inoculated with the UK isolate of *B. sorokiniana*, using the biocontrol agents first

When tested *in vitro*, the three biocontrol agents *T. harzianum* T-22, *S. lydicus* and *B. subtilis* inhibited growth of mycelium of *B. sorokiniana* completely on the second day individually or in combination, except that the combination of T-22 with *B. subtilis* gave an inhibition zone between the biocontrol agents. For this reason, the combination between T-22 with *B. subtilis* was avoided in experiments with plants. With foliar application of biocontrol agents followed by the pathogen after seven days under greenhouse conditions at average day time temperature 21 °C and night time

temperature 14 ºC, the biocontrol agents significantly reduced disease severity ($P < 0.001$; Table 5-4). Table 5-6 shows that *T. harzianum* gave the greatest reduction in disease severity; the mean disease severity rating was 3.4. *B. subtilis* had the least effect, but the mean rating of 5.3 was significantly less than the mean for untreated plants (control), which was 6.3. There was a significant interaction between biocontrol agents and cultivars ($P < 0.001$; Table 5-4). As shown in Figure 5-25, *B. subtilis* did not significantly reduce disease severity on any single cultivar according to the Tukey test even though its overall effect was significant. *T. harzianum* individually or in combination with the other biocontrol agents significantly reduced disease on all cultivars. Cultivar Nibola had the lowest average disease severity and cultivar Rehan had the highest, with mean disease scores 3.6 and 5.3 respectively (Table 5-5). There was no significant difference in the mean ratings for first and second leaves, 4.3 for first leaves and 4.4 for second leaves. Interactions involving leaves were not significant.

Table 5-4. Analysis of variance of the effects of biocontrol agents (*T. harzianum*, *S. lydicus* & *B. subtilis*) applied individually and in combination by foliar spray to four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral, inoculated with the UK isolate of *B. sorokiniana*, using the biocontrol agents first.

Plants were treated with the biocontrol agents at the two-leaf stage, 14 days after sowing. After seven days the plants were inoculated with *B. sorokiniana*. Disease severity was assessed on the first and second leaves 7 d after inoculation with the pathogen. Control was not treated with any biocontrol agent.

Source	DF	SS	MS	F	P
Cultivar	5	68.8656	13.7731	31.53	<0.001
Leaf	1	0.4802	0.4802	1.10	0.296
Biocontrol agent	6	257.0819	42.8470	98.09	<0.001
Cultivar*Leaf	5	4.6637	0.9327	2.14	0.064
Cultivar*Biocontrol agent	30	34.8267	1.1609	2.66	<0.001
Leaf*Biocontrol agent	6	1.3454	0.2242	0.51	0.798
Cultivar*Leaf*Biocontrol agent	30	12.1708	0.4057	0.93	0.577
Error	168	73.3867	0.4368		
Total	251	452.8208			

Table 5-5. Means of disease severity measured on six barley cultivars with and without treatment with *T. harzianum*, *S. lydicus* & *B. subtilis* applied individually and in combination by foliar spray.

The assessment was 7 days after inoculation with the UK isolate of *B. sorokiniana*. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at $P = 0.05$ (Tukey test).

Cultivar	Mean Disease Severity Rating
Rehan	5.3 a
ACSAD	4.5 b
Wadi Utbah	4.2 bc
Pastoral	4.1 bc
Gaelic	4.0 cd
Nibola	3.6 d

Table 5-6. Means of the effects of biocontrol agents on disease severity measured on four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral.

The assessment was 7 days after inoculation with the UK isolate of *B. sorokiniana*. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at $P = 0.05$ (Tukey test).

Treatment	Mean Disease Severity Rating
Control	6.3 a
B. subtilis	5.3 b
B. subtilis & *S. lydicus*	4.0 c
S. lydicus	4.0 c
T-22 & *S. lydicus*	3.6 cd
T-22, *B. subtilis* & *S. lydicus*	3.6 cd
T-22	3.4 d

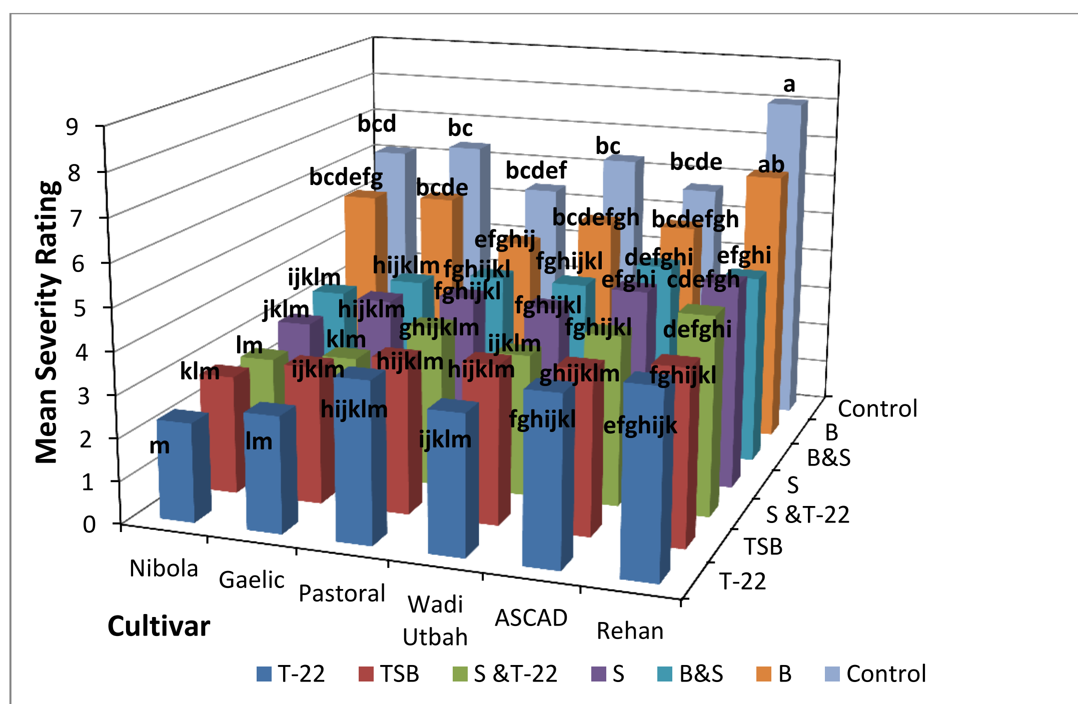


Figure 5-25. Spot blotch disease severity on six barley cultivars treated by foliar spray with three biocontrol agents, *T. harzianum*, *S. lydicus* and *B. subtilis*, individually and in combination 7 days before inoculation with *B. sorokiniana*.

Disease severity was assessed on the first and second leaves 7ays d after inoculation with *B. sorokiniana*. Control, no treatment with biocontrol agents. Values represented by bars with the same letter are not significantly different at $P = 0.05$ (Tukey test). T-22 = *T. harzianum*, S = *S. lydicus*, B = *B. subtilis*, TSB = all three together.

5.3.4 The effect of three biocontrol agents (*T. harzianum*, *S. lydicus* & *B. subtilis*) individually and in combination on six barley cultivars inoculated with the UK isolate of *B. sorokiniana* by foliar spray, using the biocontrol agents first and reapplying them five days after inoculation with the pathogen

The method of application of a biological control agent is one of the most important factors influencing the success of biological control of plant diseases. The use of two foliar applications, one 7 days before and the second 5 days after inoculation with the pathogen, was tested with three biocontrol agents (T-22, *S. lydicus* and *B. subtilis*) applied to six barley cultivars. The biocontrol agents significantly reduced disease severity ($P < 0.001$; Table 5-7). *T. harzianum* T-22 had the most effect, with a mean rating of 3.2, whereas *B. subtilis* had the least effect, with a mean rating of 5.4, still

significantly lower than the mean rating of 5.9 for the control (Table 5-9). There were significant differences in mean severity between cultivars ($P < 0.001$); Table 5-9 shows that cultivar Rehan had the highest mean disease severity rating (5.2), while cultivar Nibola had the lowest (2.9). The effects of biocontrol agents on the first and second leaves were significantly different ($P = 0.002$). The interactions between cultivars and leaves were not significant. There was a significant interaction between biocontrol agents, leaves and cultivars ($P = 0.029$). From the Tukey tests, treatment with *B. subtilis* significantly reduced disease severity on Rehan; the other agents and combinations of agents significantly reduced disease severity on all cultivars, except for the combination of *B. subtilis* and *S. lydicus* on Pastoral and ACSAD and the combination of all three biocontrol agents on Wadi Utbah (Figure 5-26). In plants treated with *Trichoderma* alone differences between cultivars were not significant except that the lowest disease severity was on Nibola, and in the effects of *Bacillus* differences between Gaelic, Pastoral, ACSAD and Wadi Utbah were not significant based on Tukey tests. In the effects on Nibola there were no differences between *Trichoderma* alone, *Trichoderma* with *Streptomyces* and the combination of all biocontrol agents.

Table 5-7. Analysis of variance of the effects of *T. harzianum*, *S. lydicus* & *B. subtilis* applied individually and in combination by foliar spray to four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral, before and after inoculation with *B. sorokiniana*.

Plants were treated with biocontrol agents at the two-leaf stage, 14 days after sowing. After seven days the plants were inoculated with the UK isolate of *B. sorokiniana*. Five days after inoculation with the pathogen the biocontrol agents were reapplied. Disease severity on the first and second leaves was assessed 7 days after reapplication of biocontrol agents. Control was not treated with any biocontrol agent.

Source	DF	SS	MS	F	P
Cultivar	5	139.7551	27.9510	159.79	<0.001
Biocontrol agent	6	216.5571	36.0929	206.34	<0.001
Leaf	1	1.0935	1.0935	6.25	0.013
Cultivar*Biocontrol agent	30	38.1838	1.2728	7.28	<0.001
Cultivar*Leaf	5	0.7265	0.1453	0.83	0.530
Leaf*Biocontrol agent	6	3.9165	0.6528	3.73	0.002
Cultivar*Leaf*Biocontrol agent	30	8.5502	0.2850	1.63	0.029
Error	168	29.3867	0.1749		

Table 5-8. Means of disease severity measured on six barley cultivars treated and untreated by foliar spray with biocontrol agents (T-22, *S. lydicus* & *B. subtilis*) before and after inoculation with the UK isolate of *B. sorokiniana*.

Disease severity was assessed on the first and second leaves7 days after reapplication of the biocontrol agent. Means followed by the same letter are not significantly different at *P* = 0.05 (Tukey test).

Cultivar	Mean Disease Severity Rating
Rehan	5.2 a
Wadi Utbah	5.0 ab
ACSAD	4.6 b
Pastoral	4.4 b
Gaelic	3.9 c
Nibola	2.9 d

Table 5-9. Means of the effects of biocontrol agents (T-22, *S. lydicus* & *B. subtilis*) applied before and after inoculation with the pathogen on disease severity on four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral.

Disease severity was assessed on the first and second leaves 7 d aysafter reapplication of the biocontrol agents. Means followed by the same letter are not significantly different at *P* = 0.05 (Tukey test).

Treatment	Mean Disease Severity Rating
Control	5.9 a
B. subtilis	5.4 b
B. subtilis & *S. lydicus*	4.4 c
S. lydicus	4.1 d
T. harzianum & *S. lydicus*	3.9 d
T. harzianum, *B. subtilis* & *S. lydicus*	3.5 e
T. harzianum	3.2 f

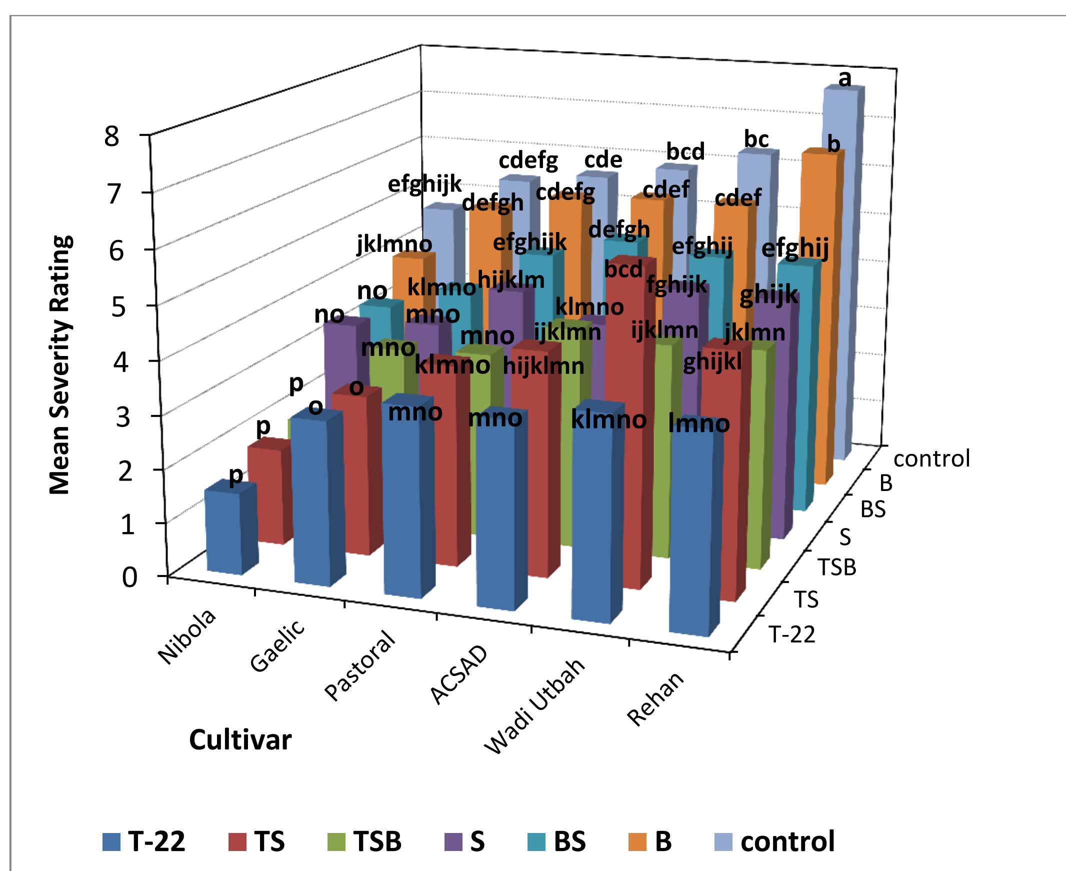


Figure 5-26. Spot blotch disease severity on six barley cultivars treated by foliar spray with *T. harzianum*, *S. lydicus* & *B. subtilis* individually and in combination 7 days before and 5 days after inoculation with *B. sorokiniana*.

Disease severity was assessed on the first and second leaves 7 days after reapplication of the biocontrol agents. T-22 = *T. harzianum*, S = *S. lydicus*, B = *B. subtilis*. Control, no treatment with biocontrol agent. Values represented by bars with the same letter are not significantly different at $P = 0.05$ (Tukey test).

5.3.5 The effects of *T. harzianum*, *S. lydicus* & *B. subtilis* individually and in combination on six barley cultivars inoculated with the UK isolate of *B. sorokiniana*, by repeat application of the biocontrol agents 7 days after the first treatment and 7 days before inoculation with the pathogen

In a further test of the effect of reapplying the biocontrol agents, *T. harzianum*, *S. lydicus* & *B. subtilis* were applied individually and in combination to four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic) by foliar spray using the biocontrol agents first then reapplying them seven days after the first spray. Plants were inoculated with the UK isolate of *B. sorokiniana* seven

days after the second treatment. Treatment with the biocontrol agents significantly reduced disease severity (*P* < 0.001; Table 5-10). Table 5-12 shows that the combination of all three biocontrol agents gave the numerically greatest reduction in average disease severity (rating 2.7), although this was not significantly different from the ratings with the other combinations and with *B. subtilis* alone. There were significant differences between combinations of cultivars and biocontrol agents (*P* < 0.001). Only the combination of all three biocontrol agents significantly reduced disease severity on all cultivars, while on Nibola and Rehan all treatments significantly reduced disease severity (Figure 5-27). The most striking result is that in this experiment the bacterial biocontrol agent *B. subtilis* reduced disease severity significantly more than either of the other biocontrol agents used alone, giving a mean disease severity rating of 2.9 compared with the control at mean rating 5.9 (Table 5-11). However, Tukey tests did not reveal differences among biocontrol treatments for individual cultivars and treatment with *B. subtilis* did not significantly affect disease severity on Pastoral, which was low in the control (Figure 5-27). Mean disease severity was highest on cultivar Rehan and numerically lowest on cultivar Gaelic instead of Nibola, which had the lowest mean severity in most experiments, although the difference between these two cultivars was not significant. This seemed to be mainly because disease severity on untreated plants was higher on Nibola than Gaelic (Figure 5-27). The difference between leaves was not significant; the mean disease severity ratings were 3.7 and 3.5 for second and first leaves, respectively.

Table 5-10. Analysis of variance of the effects of biocontrol agents (T-22, *S. lydicus* & *B. subtilis*) individually and in combination applied twice by foliar spray on four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral, inoculated with the UK isolate of *B. sorokiniana*.

Plants at the two-leaf stage, 14 days after sowing, were treated with the biocontrol agents. After seven days the plants were retreated with the biocontrol agents at the same concentrations. After seven more days the plants were inoculated with *B. sorokiniana* at a concentration of 4×10^3 conidia ml^{-1}. Disease severity was assessed 7 days after inoculation with the pathogen on the first and second leaves. The control was not treated with any biocontrol agent.

Source	DF	SS	MS	F	P
Cultivar	5	79.4070	15.8814	24.73	<0.001
Leaf	1	2.4406	2.4406	3.80	0.053
Biocontrol agent	6	253.4394	42.2399	65.77	0.000
Cultivar*Leaf	5	4.5613	0.9123	1.42	0.219
Cultivar*Biocontrol agent	30	72.9930	2.4331	3.79	<0.001
Leaf*Biocontrol agent	6	9.1238	1.5206	2.37	0.032
Cultivar*Leaf*Biocontrol agent	30	29.1410	0.9714	1.51	0.054
Error	168	107.8933	0.6422		
Total	251	558.9994			

Table 5-11. Means of disease severity measured on six barley cultivars treated twice and untreated with biocontrol agents. The assessment was 7 days after inoculation with *B. sorokiniana*.

Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at *P* = 0.05 (Tukey test).

Source	Mean Disease Severity Rating
Rehan	6.4 a
Wadi Utbah	4.0 b
ACSAD	3.5 c
Pastoral	3.3 cd
Nibola	3.2 cd
Gaelic	2.9 d

Table 5-12. Means of the effects of two applications of biocontrol agents on disease severity measured on four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral.

The assessment was 7days after inoculation with *B. sorokiniana*. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at *P* = 0.05 (Tukey test).

Treatment	Mean Disease Severity Rating
Control	5.9 a
S. lydicus	3.8 b
T-22	3.6 bc
B. subtilis & *S. lydicus*	3.2 cd
T-22 & *S. lydicus*	3.0 d
B. subtilis	2.9 d
T-22, *B. subtilis* & *S. lydicus*	2.7 d

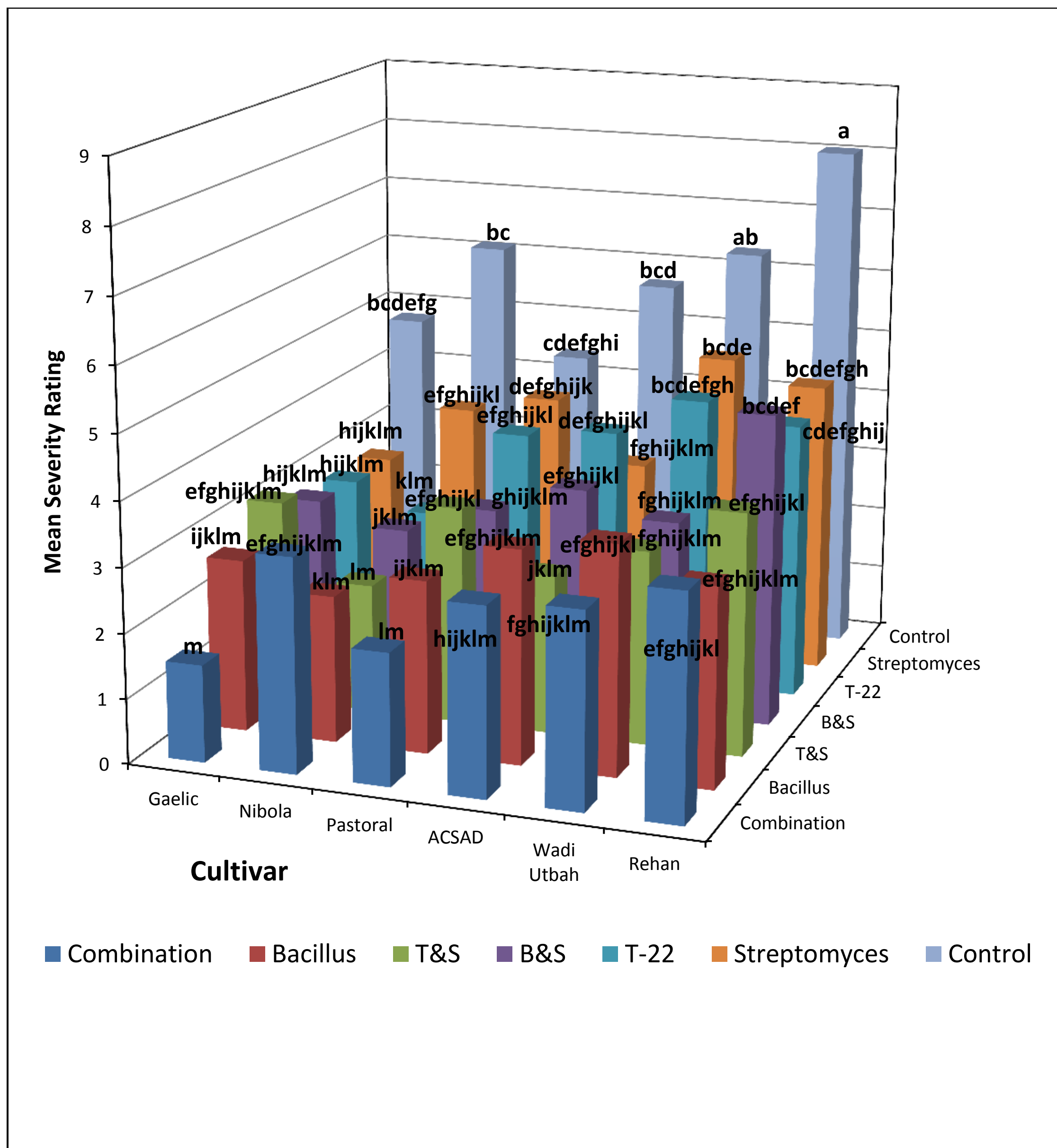


Figure 5-27. Spot blotch disease severity on six barley cultivars treated by two successive foliar sprays with *S. lydicus* (Streptomyces), *B. subtilis* (Bacillus), *T. harzianum* (T-22), combinations of *B. subitilis* and *S. lydicus* (B&S), or all three (Combination), folllowed by inoculation with *B. sorokiniana* after 7 days.

Disease severity was assessed on the first and second leaves 7 days after inoculation with *B. sorokiniana*. Control, not treated with any biocontrol agent. Values represented by bars with the same letter are not significantly different at $P = 0.05$ (Tukey test).

5.3.6 The effect of *T. harzianum*, *S. lydicus* and *B. subtilis* applied by foliar spray on two Libyan cultivars, Nibola and Rehan, inoculated with Libyan isolates of *B. sorokiniana*, *B. spicifera* and *C. inaequalis*, with reapplication of the biocontrol agents five days after inoculation with the pathogens

In view of the results of the previous experiments using more than one treatment, foliar spraying of the three biocontrol agents (*T. harzianum* T-22, *S. lydicus* and *B. subtilis*) was tested with two applications individually and in combination to control disease caused by Libyan isolates of *B. sorokiniana*, *B. spicifera* and *C. inaequalis* on two Libyan cultivars with contrasting disease resistance, Nibola and Rehan. The biocontrol agents were reapplied 5 days after inoculation with the pathogens. There were significant differences between treatments in disease severity of disease ($P < 0.001$; Table 5-13), and significant differences between pathogens ($P < 0.001$, $F = 940.58$). *B. sorokiniana* gave the highest disease severity and *B. spicifera* the lowest (Table 5-15). There was a significant difference between the two cultivars ($P = <0.001$): average disease severity with the three pathogens was higher on Rehan than Nibola at 2.9 and 2.2, respectively. The interaction between cultivars, biocontrol agents and pathogens was not significant, whereas there were significant in interactions between cultivars and pathogens and between biocontrol agents and pathogens. Table 5-14 shows that all treatments significantly reduced average disease severity and the combination of the three biocontrol agents had the most effect. According to the results of Tukey tests (Figure 5-28), treatment with biocontrol agents did not significantly affect the relatively low levels of disease caused by *B. spicifera* on both cultivars and *C. inaequalis* on Nibola. Treatment with *B. subtilis*, *S. lydicus*, or *T. harzianum* alone or all three agents together significantly reduced disease caused by *C. inaequalis* on Rehan; all treatments had similar effects. All treatments significantly reduced disease caused by *B. sorokiniana* on both cultivars; treatments including *T. harzianum* were most effective.

Table 5-13. Analysis of variance of the effects of *T. harzianum*, *S. lydicus* and *Bacillus subtilis* application on two Libyan cultivars, Nibola and Rehan on Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera* when biocontrol agents were applied individually and in combination by foliar spray before and after inoculation with the pathogens.

Plants at the two-leaf stage, 14 dsys after sowing, were treated with the biocontrol agents. After seven days the plants were inoculated with pathogens at a concentration of 8000 conidia ml^{-1}. Disease severity was assessed 7 days after inoculation with pathogens on the first and second leaves. Control was no treatment with T-22. BioAgent: biocontrol agent.

Source	DF	SS	MS	F	P
Cultivar	1	26.5525	26.5525	191.27	<0.001
BioAgent	6	43.0616	7.1769	51.70	<0.001
Pathogen	2	261.1517	130.5759	940.58	<0.001
Cultivar*BioAgent	6	2.8863	0.4811	3.47	0.003
Cultivar*Pathogen	2	11.1251	5.5625	40.07	<0.001
BioAgent*Pathogen	12	29.9194	2.4933	17.96	<0.001
Cultivar*BioAgent*Pathogen	12	1.1727	0.0977	0.70	0.747
Error	210	29.1533	0.1388		
Total	251	405.0227			

Table 5-14. Means of the effects of biocontrol agents applied before and after inoculation with the pathogens on severity of disease caused by Libyan pathogen isolates, measured on two Libyan cultivars, Nibola and Rehan.

The assessment was 7 days after inoculation with the pathogens. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at $P = 0.05$ (Tukey test).

Biocontrol Agent	Mean Disease Severity Rating
Control	3.4 a
B. subtilis	2.7 b
B. subtilis & *S. lydicus*	2.5 bc
S. lydicus	2.4 c
T-22 & *S. lydicus*	2.3 c
T-22	2.3 c
T-22, *B. subtilis* & *S. lydicus*	2.0 d

Table 5-15. Means of disease severity caused by Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera* on two Libyan barley cultivars, Nibola and Rehan, treated and untreated with biocontrol agents before and after inoculation with the pathogen.

The assessment was 7 days after inoculation with the pathogens. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at $P = 0.05$ (Tukey test).

Pathogen	Mean Disease Severity Rating
B. sorokiniana	3.9a
C. inaequalis	2.1b
B. spicifera	1.6c

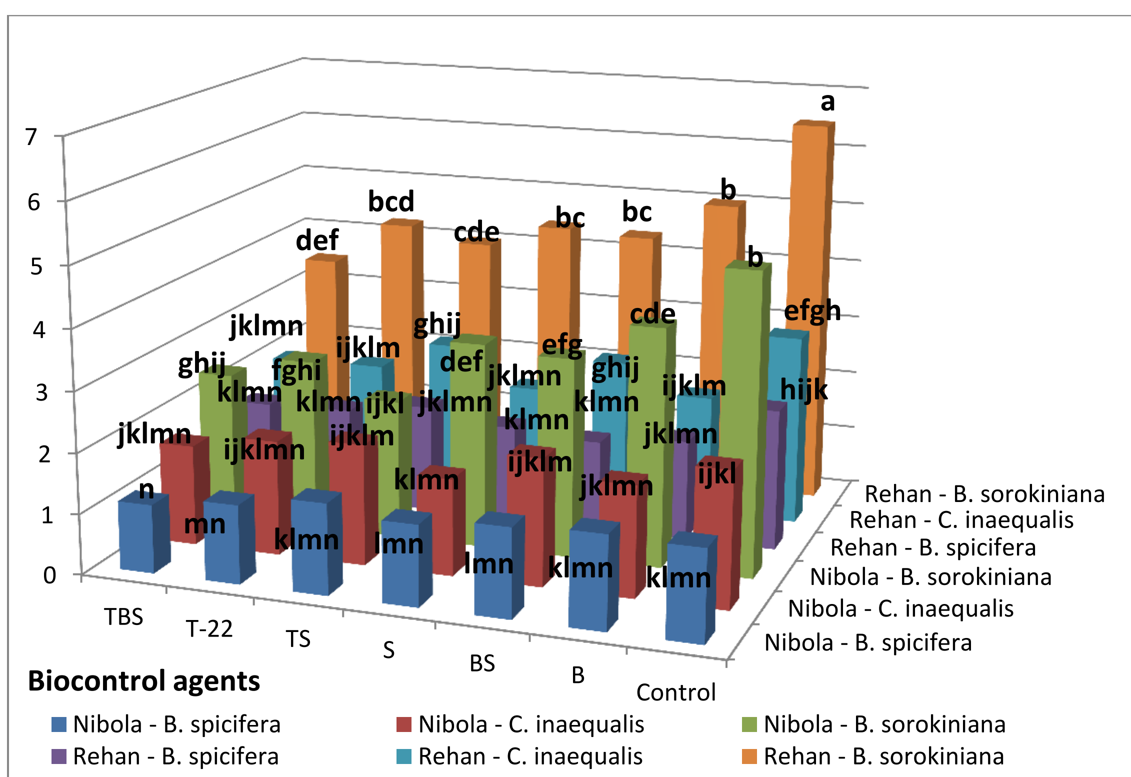


Figure 5-28. Spot blotch disease severity on two Libyan cultivars, Nibola andRehan, treated by foliar spray of *T. harzianum* T-22 and *S. lydicus* individually and in combination before and after inoculation of plants with Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera*.

Disease severity was assessed on the first and second leaves 7 days after inoculation with the pathogens. Control, no treatment with biocontrol agents. Values represented by bars with the same letter are not significantly different at $P = 0.05$ (Tukey test).

5.3.7 Soil Treatment

5.3.7.1 The effect of T. harzianum and S lydicus applied individually and in combination by soil treatment on six cultivars inoculated with the UK isolate of spot blotch

Soil was treated with *T. harzianum* and *S. lydicus* individually and in combination directly after sowing of barley. The average day time temperature was 19 ºC and night time temperature was 15 ºC. Four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral, were tested. At the two-leaf stage

14 days after sowing, plants were inoculated with *B. sorokiniana* at a concentration of 4000 conidia ml^{-1}. There were significant differences between cultivars ($P < 0.001$; Table 5-16). Cultivars Nibola and Pastoral had the lowest average disease severity whereas Wadi Utbah had the highest (Table 5-17). There was a significant difference between first and second leaves ($P < 0.001$): the mean disease severity was higher on the second leaves (3.7) than the first leaves (2.8). The effects of the biocontrol agents in this experiment were relatively small, with a *P* value of 0.019. The most striking result is that *T. harzianum* individually was significantly less effective than *S. lydicus* individually or in combination, and the average severity of disease was not significantly different in untreated plants (control) from plants treated with any of the biocontrol agents (Table 5-18). Figure 5-29 compares the results for the six barley cultivars with the biocontrol agents individually and in combination. For Rehan and ACSAD, disease severity ratings were lower on seedlings treated with *T. harzianum* than controls. For the other cultivars, severity ratings were higher on seedlings treated with *T. harzianum* than controls. Treatment with *S. lydicus* only reduced disease on Rehan, ACSAD and Wadi Utbah whereas with the combination of biocontrol agents reduced severity disease on all cultivars except Pastoral and Wadi Utbah.

Table 5-16. Analysis of variance of the effects of biocontrol agents (*T. harzianum* and *S. lydicus*) applied individually and in combination by soil treatment on four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars Gaelic and Pastoral.

Plants at the two-leaf stage, 14 days after sowing, were inoculated with the UK isolate of *B. sorokiniana* at a concentration of 4 x 10^3 conidia ml^{-1}. Disease severity was assessed on the first and second leaves 7 days after inoculation with the pathogen. Control was no treatment with *T. harzianum*.

Source	DF	SS	MS	F	P
Cultivar	5	60.5656	12.1131	25.28	<0.001
Leaf	1	25.6711	25.6711	53.57	<0.001
BiocontrolAgent	3	4.9667	1.6556	3.46	0.019
Cultivar*Leaf	5	5.7322	1.1464	2.39	0.043
Cultivar*BiocontrolAgent	15	39.5833	2.6389	5.51	<0.001
Leaf*BiocontrolAgent	3	7.2911	2.4304	5.07	0.003
Cultivars*Leaf*BiocontrolAgent	15	13.0389	0.8693	1.81	0.043
Error	96	46.0000	0.4792		
Total	143	202.8489			

Table 5-17. Means of disease severity on barley cultivar treated and untreated with *T. harzianum* and *S. lydicus* by application to soil individually and in combination.

The assessment was 7 days after inoculation with *B. sorokiniana*. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at $P = 0.05$ (Tukey test).

Cultivar	Mean Disease Severity Rating
Wadi Utbah	4.2 a
ACSAD	3.8 ab
Rehan	3.6 b
Gaelic	3.2 bc
Pastoral	2.6 cd
Nibola	2.3 d

Table 5-18. Means of the effects of soil treatment with *T. harzianum* and *S. lydicus* on disease severity measured on four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral.

The assessment was 7 days after inoculation with *Bipolaris sorokiniana*. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at P = 0.05 (Tukey test).

Biocontrol Agent	Mean Disease Severity Rating
T. harzianum	3.6 a
Control	3.3 ab
T. harzianum & *S. lydicus*	3.1 b
S. lydicus	3.1 b

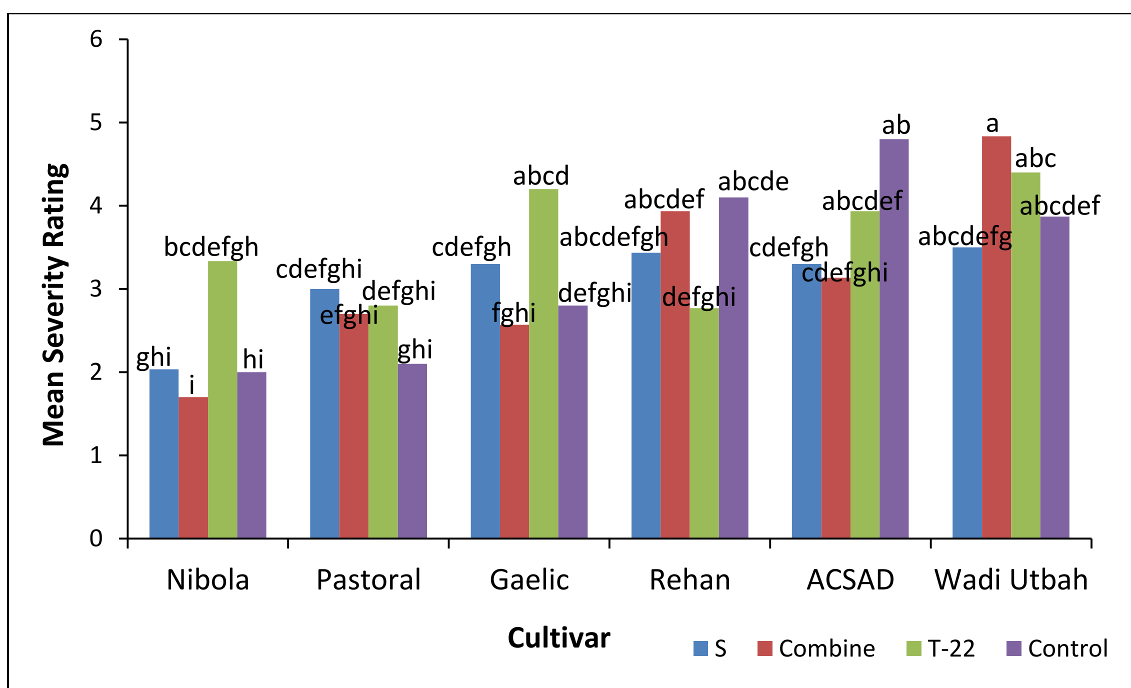


Figure 5-29. Spot blotch disease severity ratings on six barley cultivars treated with *T. harzianum* and *S. lydicus* individually and in combination by application to soil and inoculated with *B. sorokiniana* at 14 d old (two leaf stage).

Disease severity was assessed on the first and second leaves 7 days after inoculation with *B. sorokiniana*. Control was no treatment with biocontrol agents. T-22 = *T. harzianum*, S = *S. lydicus*, Combine = both together. Values represented by bars with the same letter are not significantly different at $P = 0.05$ (Tukey test).

5.3.7.2 *The effect of three biocontrol agents (T. harzianum, S. lydicus & B. subtilis) by soil treatment individually and in combination on six cultivars inoculated with the UK isolate of B. sorokiniana*

A further experiment on soil treatment tested the three biocontrol agents *T. harzianum*, *S. lydicus* and *B. subtilis* individually and in combination on six barley cultivars. There was a significant effect of treatment with biocontrol agents ($P < 0.001$; Table 5-19). In this experiment application of *T. harzianum* individually was the only treatment that reduced spot blotch disease severity significantly compared to the control, although the difference from the effect of *S. lydicus* was not significant (Table 5-21). There were significant differences between cultivars (Table 5-20). Disease severity was much higher on first leaves than second leaves ($P < 0.001$, $F = 1334.10$); mean scores were 4.5 and 1.3, respectively. The results for the different combinations of cultivars and

biocontrol agents can be compared in Figure 5-30. Because of the high variation and relatively low disease severity in this experiment, few individual differences from the control were significant according to Tukey tests. As also seen with the mean values (Table 5-21), *B. subtilis* was less effective than the other biocontrol agents, and significantly increased disease severity on Rehan, which had unusually low disease severity in the control. *T. harzianum* when applied alone reduced disease severity significantly on ACSAD and Pastoral. Treatment with the combination of *S. lydicus* and *B. subtilis* increased disease severity significantly on Rehan. Treatment with the combination of all three biocontrol agents significantly reduced disease severity on ACSAD but increased it on Rehan.

Table 5-19. Analysis of variance of the effects of biocontrol agents (*T. harzianum*, *S. lydicus* and *B. subtilis*) applied individually and in combination by soil treatment on four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral.

Plants at the two-leaf stage, 14 days after sowing, were inoculated with *B. sorokiniana* at a concentration of 4×10^3 conidia ml^{-1}. Disease severity was assessed on the first and second leaves 7 days after inoculation with the pathogen. Control was no treatment with T-22.

Source	DF	SS	MS	F	P
Cultivar	5	32.026	6.405	12.82	<0.001
Biocontrol agent	6	39.855	6.643	13.30	<0.001
Leaf	1	666.413	666.413	1334.10	<0.001
Cultivar*Biocontrol agent	30	98.840	3.295	6.60	<0.001
Cultivar*Leaf	5	33.822	6.764	13.54	<0.001
Leaf*Biocontrol agent	6	15.888	2.648	5.30	<0.001
Cultivar*Leaf*Biocontrol agent	30	64.617	2.154	4.31	<0.001
Error	168	83.920	0.500		
Total	251	1035.381			

Table 5-20. Means of disease severity measured on six barley cultivars treated and untreated with biocontrol agents by soil application.

The assessment was 7 days after inoculation with *B. sorokiniana*. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at $P = 0.05$ (Tukey test).

Cultivar	Mean Disease Severity Rating
Wadi Utbah	3.3 a
Gaelic	3.2 ab
Pastoral	3.1 abc
ACSAD	2.8 bc
Rehan	2.8 bc
Nibola	2.3 c

Table 5-21. Means of the effects of biocontrol agents (*T. harzianum*, *S. lydicus* and *B. subtilis*) applied by soil treatment on disease severity on four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral.

The assessment was 7 days after inoculation with *Bipolaris sorokiniana*. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at $P = 0.05$ (Tukey test).

Treatment	Mean Disease Severity Rating
B. subtilis	3.4 a
B. subtilis & *S. lydicus*	3.3 a
T. harzianum & *S. lydicus*	3.1 ab
Control	3.0 ab
T. harzianum, *B. subtilis* & *S. lydicus*	2.8 b
S. lydicus	2.6 bc
T. harzianum	2.1 c

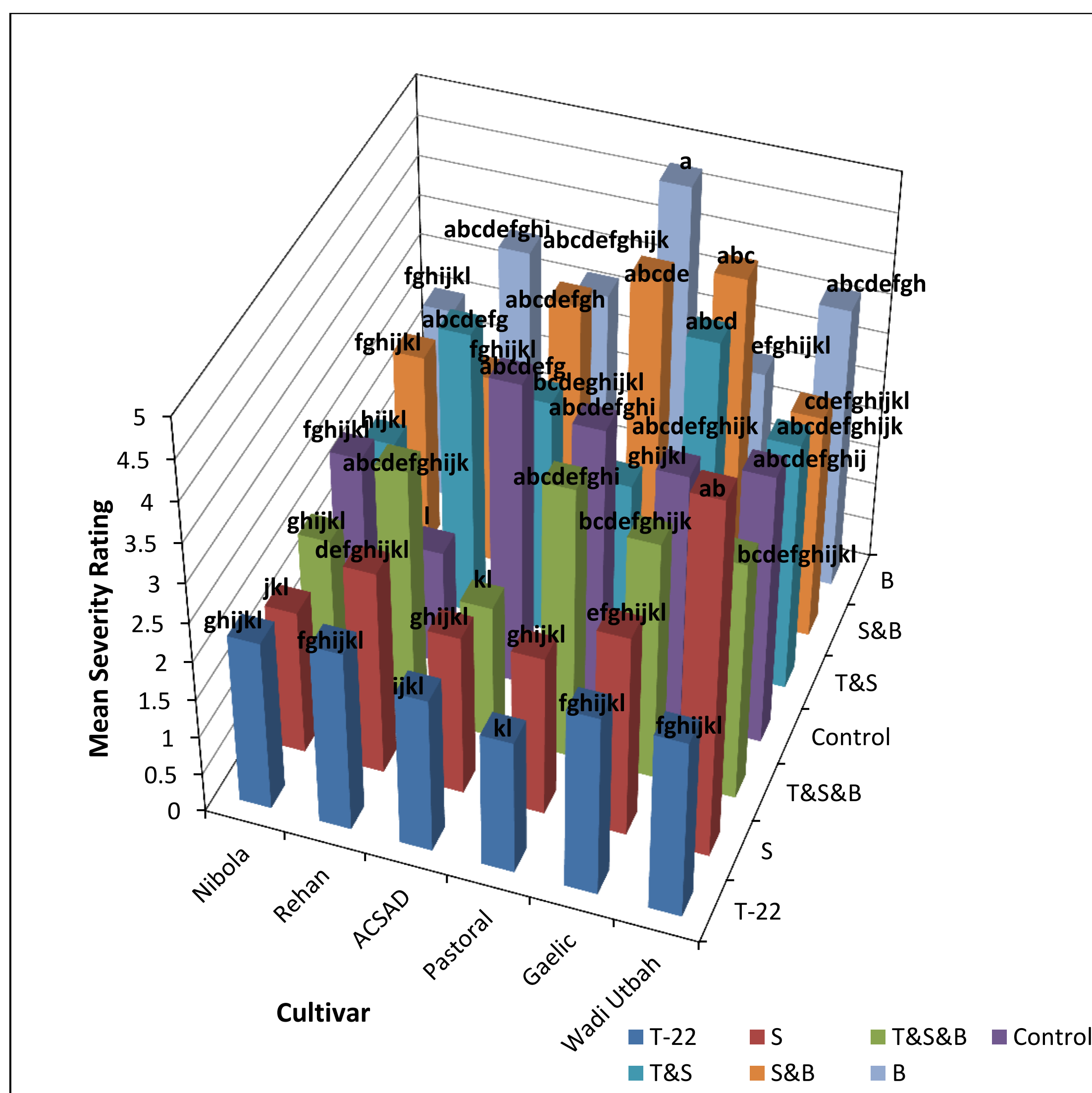


Figure 5-30. Spot blotch disease severity on six barley cultivars treated by soil application of *T. harzianum* (T-22), *S. lydicus* (S), *B. subtilis* (B), *T. harzianum* and *S. lydicus* together (T&S), *S. lydicus* and *B. subtilis* together (S&B), and all three together (T&S&B).

Plants were inoculated with *B. sorokiniana* 14 days after sowing, at the two leaf stage. Disease severity was assessed on the first and second leaves 7 days after inoculation with *B. sorokiniana*. Control was no treatment with biocontrol agents (T-22 & *S. lydicus* and *B. subtilis*). Values represented by bars with the same letter are not significantly different at $P = 0.05$ (Tukey test).

5.3.7.3 *The effects of T. harzianum, S. lydicus & B. subtilis applied individually and in combination on six cultivars inoculated with the UK isolate of B. sorokiniana by soil treatment and spraying of the biocontrol agents 5 days after inoculation with the pathogen*

Because previous experiments showed significant but partial control by both foliar application and soil treatment, this experiment tested different biocontrol agents applied individually and in combination by soil treatment and foliar spray. The foliar spray was used 5 days after inoculation with the pathogen. Treatment with biocontrol agents significantly affected disease severity ($P < 0.001$; Table 5-22). All treatments significantly reduced disease severity compared to the control. Treatments including *T. harzianum* were the most effective and treatments including *B. subtilis* were the least effective in reducing spot blotch severity (Table 5-24). Cultivar Nibola had the lowest average disease severity and Rehan, Wadi Utbah, and Gaelic had the highest (Table 5-23). There are significant differences in the effects of the biocontrol agents on different cultivars ($P < 0.001$). Figure 5-31 shows that in this experiment all biocontrol agents and combinations significantly reduced severity of spot blotch disease on all barley cultivars except for Nibola treated with *B. subtilis* by itself and in combination with *S. lydicus*. On individual cultivars, disease severity did not differ significantly for treatments with *Trichoderma* individually, *Streptomyces* individually, and combinations including *Trichoderma*, except that on Pastoral disease severity with the combination of *Streptomyces* and *Trichoderma* was significantly lower than with *Streptomyces* alone. *Streptomyces* by itself gave the lowest severity on Rehan among all biocontrol agents applied individually or in combination, although the difference from other treatments was not significant according to Tukey tests. The combination of *Trichoderma* with *Streptomyces* gave the lowest disease severity on Pastoral, although only the differences from *Streptomyces* and *Bacillus* used individually were significant. First leaves had significantly more disease than second leaves ($P < 0.001$); the mean severity ratings were 4.0 and 3.3, respectively.

Table 5-22. Analysis of variance of the effects of biocontrol agents (*T. harzianum*, *S. lydicus* and *B. subtilis*) applied individually and in combination to four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral. The biocontrol agents were first applied by soil treatment then by foliar spray.

Plants at the two-leaf stage, 14 days after sowing, were inoculated with *B. sorokiniana* at a concentration of 4×10^3 conidia ml^{-1}, and after five days the biocontrol agents were reapplied by foliar spray. Disease severity was assessed on the first and second leaves 7 days after spraying of the biocontrol agents. Control was not treated with any biocontrol agent.

Source	DF	SS	MS	F	P
Cultivar	5	15.8622	3.1724	14.48	<0.001
Leaf	1	32.7168	32.7168	149.36	<0.001
Biocontrol Agent	6	189.6727	31.6121	144.32	<0.001
Cultivar*Leaf	5	2.7613	0.5523	2.52	0.031
Cultivar*Biocontrol Agent	30	24.5044	0.8168	3.73	<0.001
Leaf* Biocontrol Agent	6	4.3476	0.7246	3.31	0.004
Cultivar*Leaf*Biocontrol Agent	30	15.7076	0.5236	2.39	<0.001
Error	168	36.8000	0.2190		
Total	251	322.3727			

Table 5-23. Means of disease severity measured on six barley cultivars treated and untreated with biocontrol agents by soil treatment and foliar application five days after inoculation with *B. sorokiniana*.

Disease severity was assessed on the first and second leaves 7 days after application of the biocontrol agents. Means followed by the same letter are not significantly different at *P* = 0.05 (Tukey test).

Cultivar	Mean Disease Severity Rating
Rehan	3.9 a
Wadi Utbah	3.8 ab
Gaelic	3.8 ab
Pastoral	3.6 bc
ACSAD	3.5 c
Nibola	3.2 d

Table 5-24. Means of the effects of biocontrol agents on disease severity measured on four Libyan cultivars, Nibola, Rehan, Wadi Utbah and ACSAD, and two UK cultivars, Gaelic and Pastoral by soil treatment and reapplication of the biocontrol agents by foliar spray five days after inoculation with the pathogen.

The assessment was 7 days after the biocontrol agents were applied. Disease severity was assessed on the first and second leaves. Means followed by the same letter are not significantly different at *P* = 0.05 (Tukey test).

Biocontrol Agent	Mean Disease Severity Rating
Control	5.7 a
B. subtilis	3.9 b
B. subtilis & *S. lydicus*	3.6 c
S. lydicus	3.3 cd
T. harzianum & *S. lydicus*	3.1 de
T-22	3.1 de
T. harzianum, B. subtilis & *S. lydicus*	3.0 e

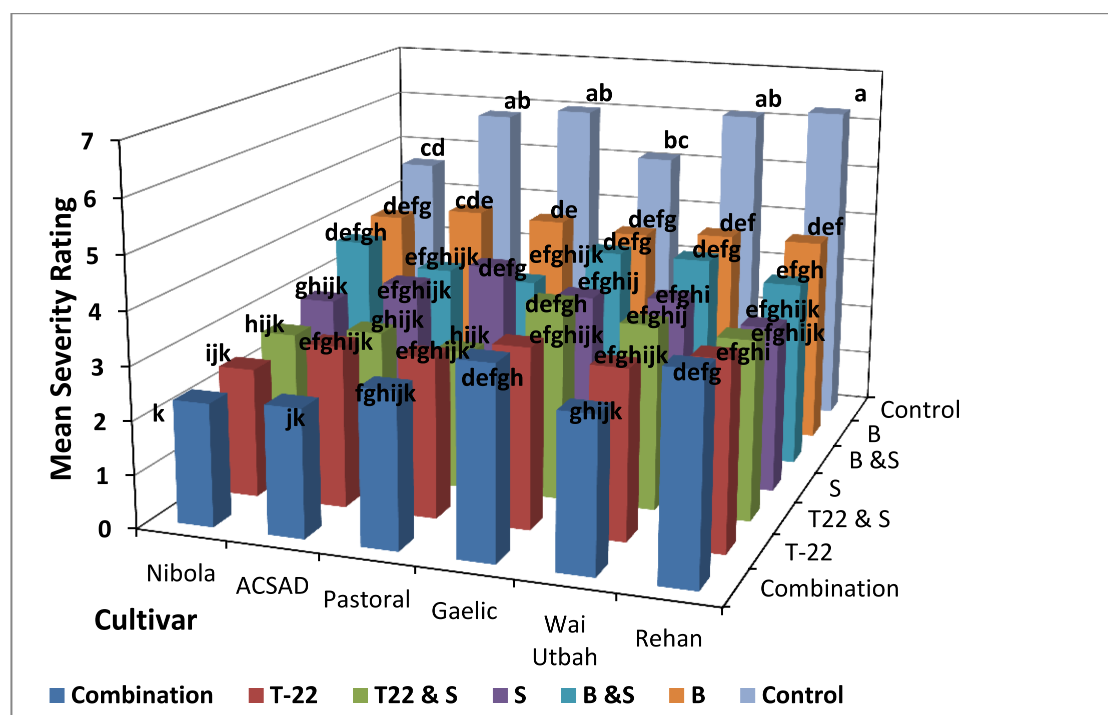


Figure 5-31. Spot blotch disease severity on six barley cultivars treated with *T. harzianum* (T-22), *S. lydicus* (S), *B. subtilis* (B), *T. harzianum* and *S. lydicus* (T22 & S), *B. subtilis* and *S. lydicus* (B&S). Biocontrol agents were applied by soil treatment and reapplied by foliar spray at the same concentration five days after inoculation with *B. sorokiniana*.

Seedlings were inoculated with the pathogen at the two leaf stage, 14 days after sowing. Disease severity was assessed on the first and second leaves 7 days after foliar application of the biocontrol agents. Control was no treatment with biocontrol agents. Values represented by bars with the same letter are not significantly different at P = 0.05 (Tukey test).

5.4 Discussion

In the *in vitro* study all biocontrol agents, *T. harzianum*, *S. lydicus* and *B. subtilis*, inhibited growth of the pathogens *B. sorokiniana*, *C. inaequalis* and *B. spicifera* completely on the second day either individually or in combinations, except that for *B. spicifera* with *S. lydicus* there was an inhibition and the pathogen grew in the opposite direction (Figure 5-18). In addition there was an inhibition zone in the combination between *T. harzianum* and *B. subtilis.* This finding agrees with the findings of Elliott *et al*. (2009), who evaluated commercial products including Serenade, Actinovate, and two products containing *Trichoderma* species on European and North American

populations of *Phytophthora ramorum* and demonstrated that *B. subtilis* may produce compounds that are active against *Trichoderma* species (Figure 5-9, Figure 5-16, Figure 5-17 and Figure 5-21). The findings of the current study are consistent with those of Ezziyyani *et al*. (2007), who found that the combination of *Trichoderma* species with *Streptomyces* species for control of the pathogen that causes root rot on pepper, *Phytophthora capsici*, gave successful control. In an *in vitro* study of the effects of *T. harzianum* against Ascochyta blight in chickpea caused by *Ascochyta rabiei*, *T. harzianum* inhibited growth of mycelium of *Ascochyta rabiei* within 7 days (Benzohra *et al*., 2011). An interesting finding was that in dual culture of the Libyan isolate of *Curvularia inaequalis* with *T. harzianum* there was an inhibition zone whereas with *B. sorokiniana* and *B. spicifera* there was no inhibition zone when *T. harzianum* was tested individually. A possible explanation might be that *T. harzianum* inhibited growth of mycelium of *C. inaequalis* by producing antagonistic compounds, whereas with *B. sorokiniana* and *B. spicifera* it inhibited growth by attacking the mycelium directly and by competition on growth medium. The finding is consistent with the finding of a study in dual culture of *T. harzianum* with *Ganoderma boninense*, which demonstrated that *T. harzianum* produced antagonist compounds that inhibited growth of mycelium of *Ganoderma boninense*, which causes basal stem rot on oil palm (Siddiquee *et al*., 2009).

Because the results of *in vitro* experiments with different biocontrol agents used individually and in combination showed that these biocontrol agents were effective in inhibiting growth of the pathogens, they were tested on plants. The experiments on plants were done by using the biocontrol agents individually and in combination except that the combination between *T. harzianum* and *B. subtilis* was not tested because of the zone of inhibition between these organisms in the *in vitro* experiment. The effectiveness of applying two biocontrol agents, *T. harzianum* and *S. lydicus*, was tested with Libyan pathogen isolates on Libyan cultivars Nibola and Rehan. In this experiment, the biocontrol agents were applied 7 days before inoculation with the pathogens. The combination of the two and *T. harzianum* alone gave better results in reducing severity of disease than *S. lydicus* alone. The experiment was carried out at average day time temperature of 22 ℃ and night time temperature of 16 ℃. The results are consistent with the findings of Elad *et al*. (1993), which showed that treatment with *T. harzianum*

gave more reduction of disease severity on cucumber above 20 ºC than below 20 ºC, but are different from the findings of Ezziyyani *et al*. (2007) that the combination of *T. harzianum* with *Streptomyces* species reduced the severity of disease caused by *Phytophthora capsici* more than using these biocontrol agents individually. A possible explanation for the results might be the difference in modes of action of the biocontrol agents.

More extensive tests of combinations of biocontrol agents and cultivars were carried out with three agents and six cultivars. In these experiments, a single pathogen, the UK isolate of *B. sorokiniana*, was used so as not to introduce an additional factor. When the three biocontrol agents were used by foliar application seven days before inoculation with the pathogen at average day time temperature 21 ºC and night time temperature 14 ºC, in general cultivar Nibola had the lowest disease severity with all biocontrol agents either individually or in combination and *B. subtilis* had the lowest effect on spot blotch disease on all barley cultivars except Pastoral, where *B. subtilis* gave the lowest disease severity (Figure 5-25). *T. harzianum* gave better control in combination with plant disease resistance than other biocontrol agents either individually or in combination. These results differ from the estimate by Ezziyyani *et al*. (2007) that a combination of *T. harzianum* T-22 with *Streptomyces* species was more effective than using either biocontrol agent individually. Also, a combination of a yeast, *Pichia guilermondii*, and a bacterium, *Bacillus mycoides*, to control *Botrytis cinerea* on strawberry leaves gave more reduction of disease than when either was used individually (Guetsky et al., 2001), and Yigit and Dikilitas (2007) demonstrated that *T. harzianum* T-22 gave successful biocontrol of *Fusarium* wilt of tomato when applied individually but control was more effective when it was used in combination with fluorescent *Pseudomonas* species. However, the results are broadly consistent with other earlier studies. For control of *Phytophthora ramorum* by a combination of *Trichoderma atroviride* and *Trichoderma virens* with *S. lydicus*, the combination had no effect on the pathogen (Elliott *et al*., 2009). Raziq and Fox (2005) found that a combination of two strains of *Trichoderma* gave less effect than when one strain was used individually. Combinations of the biocontrol agents Serenade (*Bacillus subtilis*), Trianum (*Trichoderma harzianum* T-22) and Sentinel (*Trichoderma atroviride*) gave less control of *Botrytis cinerea* on

strawberry than when the agents were used individually (Robinson-Boyer *et al*., 2009; Xu *et al*., 2010).

In addition to combining biocontrol agents, repeat applications of biocontrol agents have frequently given successful biological control. *T. harzianum* 1295-22 was effective in controlling dollar spot on bentgrass when applied by soil treatment and foliar application with continuing application of the foliar method every seven days (Lo *et al*., 1997). Combinations of *T. harzianum*, *S. lydicus* and *B. subtilis* were tested against *B. sorokiniana* on six barley cultivars by foliar application used the biocontrol agent first, followed by the pathogen after seven days and then repeat application of the biocontrol agents with the same concentrations 5 d after inoculation with the pathogen. In this experiment, the average day time temperature was 22 ℃ and night time temperature was 15 ℃. The results indicated that there was a significant difference between biocontrol agents ($P < 0.001$, F= 206.34); *T. harzianum* T-22 gave a greater reduction in disease severity than all other biocontrol agents either individually or in combination, whereas *B. subtilis* was the least effective.

The results of the above experiment testing combinations of biocontrol agents and repeat application of biocontrol agents after inoculation by the pathogen were different from those for the combination of biocontrol agents with repeat application seven days after the first treatment and before inoculation with the pathogen at average day time temperature 18 ℃ and night time temperature 12 ℃. In the experiment with repeat application of biocontrol agents before inoculation by the pathogen, the bacterial biocontrol agent *B. subtilis* gave greater reductions in disease severity than the other biocontrol agents either individually or in combinations and cultivar Gaelic had the lowest disease severity instead of cultivar Nibola. These differences can perhaps be explained in part by the differences in the average temperatures or might relate to the method of application, with the biocontrol agents applied twice before inoculation with the pathogen. When the method of repeating application of biocontrol agents five days after inoculation with the pathogen was applied to two Libyan cultivars, Nibola and Rehan, inoculated with the three Libyan pathogen isolates on at average day time

temperature 24 ºC and night time temperature 16 ºC, the results indicated that the biocontrol agents had significantly different effects on the pathogens. The combination of three biocontrol agent had the greatest effect on the pathogens whereas *B. subtilis* had the least effect.

Strong relationships between the success of biological control and the method of application have been reported in the literature. In the present study, soil treatment with *T. harzianum* T-22 and *S. lydicus* was tested on six barley cultivars at average day time temperature 19 ºC and night time temperature 15 ºC. There were significant differences in disease severity between biocontrol agents and between barley cultivars: disease severity was lower on Nibola and Pastoral than other cultivars whereas Wadi Utbah had the highest severity instead of Rehan, which had the highest disease severity in most experiments. In addition, the severity of disease was different between the leaves: first leaves had less disease severity than second leaves. In this experiment *T. harzianum* T-22 had less effect than *S. lydicus* either individually or in combination. Since the temperatures in this experiment were below 20 °C, the results are consistent with those of Elad et al. (1993), who demonstrated that *T. harzianum* gave greater suppression of disease when the temperature was above 20 ºC than when it was lower than 20 ºC. The effect of *S. lydicus* in the current study supports the previous findings of Yuan and Crawford (1995), who demonstrated the positive effects of *S. lydicus* on soilborne disease of pea and cotton caused by *Pythium ultimum*. Possible explanations for this might be that antibiotic activity produced by *S. lydicus* (Lee *et al*., 2005) was more active than the effects of *T. harzianum* at low temperature (Elad *et al*., 1993), or related to the ability of streptomycetes to produce compounds that are responsible for degradation of cell walls of fungi and enhance plant resistance (Tarkka and Hampp, 2008), or due to an unspecific effect on the plant, increasing plant resistance by increasing plant mineral uptake (Kumar and Dube, 1992) .

When *T. harzianum*, *S. lydicus* and *B. subtilis* were tested in combination and individually against *B. sorokiniana* on six barley cultivars by soil treatment at average day time temperature 21 ºC and night time temperature 16 ºC, *T. harzianum* T-22 was a more

effective biocontrol agent than the others either in combination or individually, whereas *B. subtilis* had the least effect. In this experiment first leaves had more severe symptoms than second leaves. When the biocontrol agents were applied by soil treatment with repeat treatment by foliar application five days after inoculation with the pathogen at average day time temperature 20 ºC and night time temperature 13 ºC, the combination of biocontrol agents had more effect on spot blotch disease than individual application. This study confirms that combination of biocontrol agents can give great reduction of disease, and supports previous research into soil treatment with biocontrol agents in combination and individually Combinations of *Trichoderma* species were more effective against Fusarium rot of lentils caused by *Fusarium oxysporum* than individual application (Akrami *et al*., 2011). Soil treatment with a combination of biocontrol agents *B. subtilis* and *Burkholderia cepacia* reduced severity of disease caused by *R. solani* on tomato more than individual application (Szczech and Shoda, 2004).

5.5 Conclusions

Combinations of application methods, with frequent application, and biocontrol agents together with plant resistance can achieve high reduction of severity of spot blotch disease on barley. Application of biocontrol agents first by foliar application method with reapplication five days after inoculation with the pathogen gave the best results over all experiments for reducing disease severity. Cultivar Nibola showed more resistance than other cultivars in most experiments, and also generally had the lowest disease severity when treated with biocontrol agents. The range of results observed in different experiments suggests that temperature was one of the most important factors that affected the efficiency of biocontrol agents both individually and in combination. *T. harzianum* T-22 gave better biological control of spot blotch disease individually or in combination with other biocontrol agents than *S. lydicus* and *B. subtilis*. There was little evidence that combinations were more effective than *T. harzianum* by itself. Biological control was effective against disease caused by *B. sorokiniana* and *C. inaequalis,* but it had little effect on the low level of disease caused by *B. spicifera*.

Chapter 6 Field test of the effects of the biocontrol agent *Trichoderma harzianum* T-22, applied by foliar spray, seed coating and the two methods combined, on spot blotch disease on two Libyan cultivars, Nibola and Rehan

6.1 Introduction

6.1.1 Background

One of the major problems in biological control is moving from controlled environments to the field; several studies of the effect of biocontrol agents have given different results under controlled conditions and in field assays. Dal Bello *et al*. (2003) evaluated the effects of *B. subtilis* on wheat seedling blight caused by *B. sorokiniana*. *In vitro* and under greenhouse the biocontrol agent suppressed the pathogen, whereas in a field experiment it had no effect. Several studies have been done to evaluate the effects of *T. harzianum* on different plant pathogens in the field. When used against damping-off disease caused by *R. solani* on cucumber, *T. harzianum* was more effective by itself than when used in combination with *B. subtilis* (Yobo *et al*., 2011). Two application methods, seed treatment and foliar application, were used with *T. harzianum* against leaf blotch disease on wheat caused by *Septoria tritici*. The results were that both methods reduced disease severity and seed treatment had a greater effect than foliar spray application (Cordo *et al*., 2007).

Results achieved in field and under conditions experiments of biological control root rot on wheat and barley by bacterial and fungal biocontrol agents, some of biocontrol agents give good result of reducing severity disease in field experiment whereas gave less control under conditions environment and others reduced severity disease under conditions and field experiments (Duczek, 1994). Linderman *et al*. (1983) indicated that some biocontrol agents gave success results *in vitro* and under conditions whereas in field experiment no effects due to may use wrong biocontrol agent against the pathogens or different the environmental conditions required.

Biological control of seed-borne pathogens on wheat by *T. harzianum* in vitro and *in vivo*, results indicated that biocontrol agent achieved good results in both in vitro *T. harzianum* inhibited mycelium growth and in vivo reduced disease severity at all growth stages of plant and increased grains weight compared by untreated plants (Hasan *et al*., 2012). Efforts done on different biocontrol agents against different pathogens in vitro and under conditions gave good results of inhibition pathogens whereas in field experiment gave difference results (Lewis and Papavizas, 1991). *Trichoderma* spp have been tested in field experiments to control crop diseases on some vegetables, the results were indicated that *Trichoderma* spp have achieved good results of reduced severity diseases and increased yield in crops and the efficacy of biocontrol agent increased along with increase of frequently of biocontrol agent applications (Cuevas *et al*., 2012). In other study of field experiment tested the effects of *T. harzianum* against seedling disease on numbers of crops, sanpbean, soybean, pea, squash, field cron and sugar beet by seed treatment, the results were indicated that not significant compared with untreated plants (Ruppel *et al*., 1983).

6.1.2 Aims

Determining the extent to which the results of greenhouse studies are relevant to the field is essential for developing an in-depth knowledge of the potential value of *T. harzianum* T-22 for biological control of foliar diseases of barley. The experiments described in Chapter 3, which were mainly carried out on young plants grown in a glasshouse, showed that two Libyan six-rowed barley cultivars, Rehan and Nibola, had contrasting susceptibility to spot blotch. Nibola had consistently less disease, both with and without treatment with biocontrol agents. In tests of several commercially available biological control agents (Chapter 4), the best control of spot blotch on both cultivars was obtained with *T. harzianum* T-22. Significant reductions in disease severity were obtained when T-22 was applied by foliar spray or by seed treatment. The objectives of this chapter are to determine whether treatment with T-22 could give significant long-term reduction in spot blotch severity in the field. Cultivars Rehan and Nibola were used in this experiment because of their contrasting susceptibility to spot blotch under controlled conditions.

6.2 Materials and methods

6.2.1 Experimental design

The experiment was carried out at the Close House Field Station, Newcastle University School of Biology, beginning in June 2011. Two Libyan six-rowed barley cultivars were used: Nibola and Rehan. Treatments were no control agent and two methods of application of *T. harzianum* T-22, foliar application and seed coating, used separately and in combination. All treatments were infected with the pathogen. A randomised complete block experimental design with four blocks was used. Plots were 1.8 m x 1.8 m containing 12 rows with 15 to 18 cm between rows; there was a space of 20 cm between plots (Figure 6-1). The plants were covered by netting to protect them from damage by birds.

Seeds were sown on 07/06/2011 at 450 per square metre. This rate was high because of the late sowing in June, which results in reduced tillering. The plots were fertilised with N:P:K 20:10:10 at 80 kg ha^{-1} on 14/07/2011 and were treated with the insecticide Hallmark Zeon (Syngenta Crop Protection) on 21/07/11 to kill aphids.

6.2.2 Application of the biological control agent and the pathogen

T. harzianum T-22, purchased as TRIANUM-P (Koppert B.V.), was cultured and spores were harvested as described in Chapter 2. Seed treatment was carried out as described by Perello *et al*. (2006). 10 ml of spore suspension ($1x10^8$ spores ml^{-1}) was mixed with 90 ml of 0.25% agar in water, which serves as an adhesive, one drop (about 100 µl) of Tween 20 was added, and the suspension was mixed well with a magnetic stirrer. Fifty grames of barley seeds was added to the water agar - fungal biomass mixture. The coated seeds were dried overnight at room temperature, and then stored at 5 ºC. The coated seeds were sown approximately 24 h after treatment. Control seeds were treated with the same mixture of 0.25% agar in water without *Trichoderma* spores and dried overnight at room temperature in the same way as treated seeds.

Spray treatment was applied at the two leaf stage 16 d after sowing (23/06/11). A suspension of *T. harzianum* T-22 spores containing 1 x 10^8 spores ml^{-1} was sprayed. Plots that did not receive the spray treatment were sprayed with sterile distilled water. The plots were covered with polythene sheeting for 24 h to increase the humidity (Figure 6-2). The temperature range measured under the plastic during the covered period was 10 to 15 °C, and the relative humidity was 96%. This was estimated by putting four measuring instruments at random positions under the plastic cover; the measurements gave the same reading of humidity.

The pathogen was cultured in the same way as *T. harzianum*. The isolate was *B. sorokiniana* strain 6.16, isolated from barley in Perthshire, Scotland (supplied by M. Jacks, Scottish Agricultural Science Agency, Edinburgh, Scotland). Seven days after application of *T. harzianum* T-22, plants were sprayed with a spore suspension at a concentration of 4 x 10^3 conidia ml^{-1}, and then were covered with polyethylene sheeting for 24 h (Figure 6-2). The range of temperature measured under the plastic was 12 to 17 °C and relative humidity was 98%. Symptoms appeared on the plants after three days.

6.2.3 Assessment of disease severity and impact

Three assessments were done in this experiment. Disease severity on leaves was measured twice by using a rating scale from 1 to 9, as described by Fetch and Stephenson (1999) (Chapter 2). The first assessment was on 19/07/2011, 19 days after inoculation with the pathogen. Five randomly chosen plants in ten rows of each plot were assessed on the first and second leaves. The first and twelfth rows and 10 cm from the ends of each row were not sampled. The second assessment was carried out 28 days after the first, on 17/08/11. The sampling was as for the first assessment but disease severity was measured on the three upper leaves of each plant. The third assessment was the dry weight of harvested seeds, estimated by harvesting five randomly chosen plants in ten rows of each plot. Again, the first and the twelfth rows and 10 cm from the ends of each row were not sampled. Analysis of variance was carried out using Minitab 16 (Minitab, Inc.).

E1	G1	D1	F1	D2	G2	F2	B2
A1	C1	H1	B1	A2	C2	E2	H2
G3	F3	B3	C3	H4	C4	G4	B4
H3	A3	E3	D3	E4	D4	F4	A4

{ 20 cm		
20cm	{ 15 cm	20cm
{ 20 cm		

Figure 6-1. Diagram of the plan of the field experiment.

A Nibola control, B Nibola treated by spray application, C Nibola treated by seed treatment, D Nibola treated by both seed treatment and foliar spray applications, E Rehan control, F Rehan treated by spray application, G Rehan treated by seed treatment, H Rehan treated by both seed treatment and foliar spray applications. Numbers correspond to blocks.

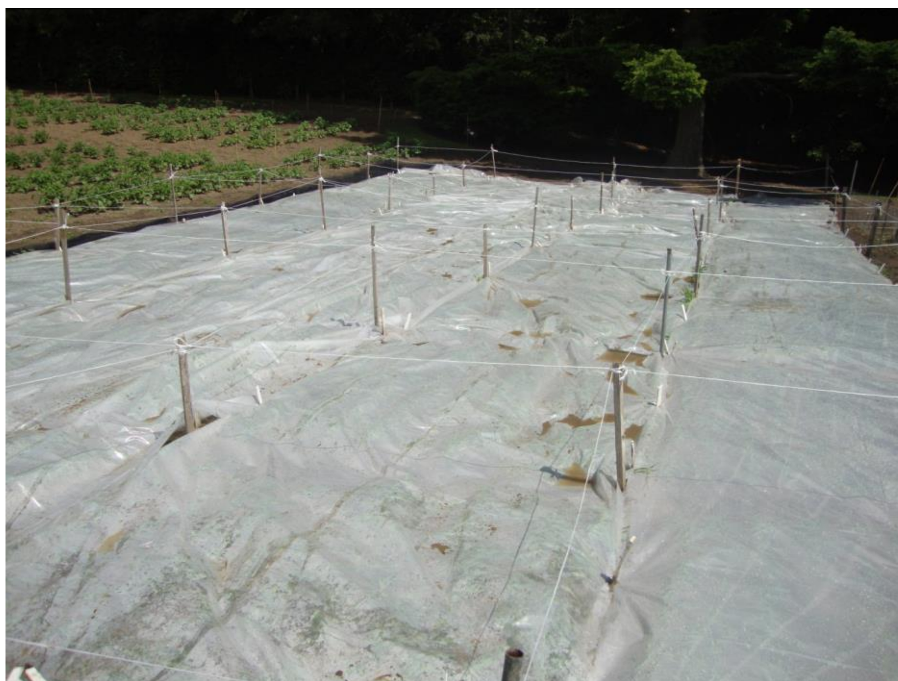


Figure 6-2. Field trial after inoculation with *B. sorokiniana*. Plants were covered with polyethylene sheeting for 24 h.

Figure 6-3. Field trial covered by netting to protect against bird damage, after inoculation with *B. sorokiniana* and treatment with T-22.

6.3 Results

6.3.1 First assessment

The first analysis examined disease severity 19 days after inoculation with the pathogen. Table 6-1 shows that there was no significant difference between the cultivars but there were significant differences between treatments (P = 0.001). Although the interaction between cultivar and treatment was not statistically significant (P = 0.245), the results suggest that without a biocontrol treatment, disease was higher on cultivar Rehan than cultivar Nibola and severity on treated plants was similar on the two cultivars (Figure 6-4). Thus, the significant effect of treatment is likely to be due principally to a reduction in disease severity on cultivar Rehan

Table 6-1. Analysis of variance of disease severity rating on barley cultivars Nibola and Rehan 19 days after inoculation with *B. sorokiniana*. Disease severity was assessed on the third and fourth leaves. Bio Method: treatment method.

Source	DF	SS	MS	F	P
Cultivar	1	1.9652	1.9652	2.58	0.123
Bio Method	3	17.9010	5.9670	7.83	0.001
Cultivar*Bio Method	3	3.4180	1.1393	1.49	0.245
Block	3	6.1530	2.0510	2.69	0.072
Error	21	16.0131	0.7625		
Total	31	45.4503			

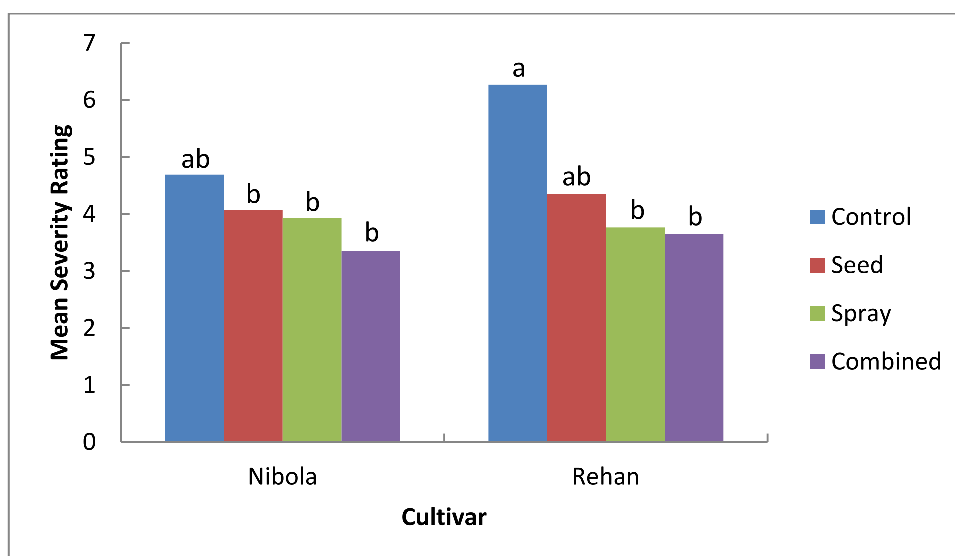


Figure 6-4. Spot blotch disease severity on barley cultivars Nibola and Rehan 19 days after inoculation with *B. sorokiniana*.

Disease severity was assessed on the third and fourth leaves. Control, no treatment with T-22; Seed, seeds treated with T-22 before sowing; Spray, seedlings sprayed with T-22 7 days before inoculation with pathogen; Combined, seeds treated and seedlings sprayed. Values represented by bars with the same letter are not significantly different at P = 0.05 (Tukey test).

6.3.2 Second assessment

When disease severity on the upper leaves was assessed 28 d after the first assessment, there was a significant difference between disease severity on the upper leaves of cultivar Nibola and on the upper leaves of cultivar Rehan (P = 0.033). There was also a significant difference between treatments (P = 0.018). Figure 6-5 shows that only plants of cultivar Rehan given the combined treatment had significantly less disease on these leaves than the control plants (P = 0.05; Tukey test). Therefore, as in the first assessment, the principal effect of the biocontrol treatment seems to be a reduction in disease severity on cultivar Rehan by the combined treatment, although again the interaction between cultivar and treatment was not statistically significant (P = 0.350).

Table 6-2. Analysis of variance for second assessment of disease severity on barley cultivars Nibola and Rehan, 47 days after inoculation with *B. sorokiniana*.

Disease severity was assessed on the uppermost three leaves. Bio Method: treatment method.

Source	DF	SS	MS	F	P
Cultivar	1	3.4672	3.4672	5.35	0.033
Bio Method	3	8.4686	2.8229	4.36	0.018
Cultivar*Bio Method	3	2.2692	0.7564	1.17	0.350
Block	3	2.1758	0.7253	1.12	0.367
Cultivar*Block	3	0.1954	0.0651	0.10	0.959
Error	18	11.6630	0.6479		
Total	31	28.2392			

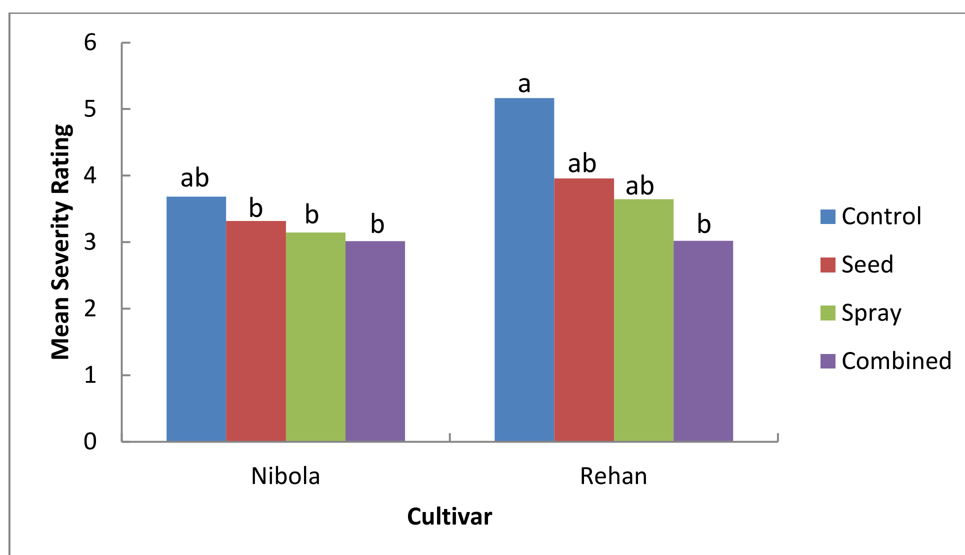


Figure 6-5. Spot blotch disease severity on barley cultivars Nibola and Rehan 47 days after inoculation with *B. sorokiniana*.

Disease severity was assessed on the uppermost three leaves. Control, no treatment with T-22; Seed, seeds treated with T-22 before sowing; Spray, seedlings sprayed with T-22 7 days before inoculation with pathogen; Combined, seeds treated and seedlings sprayed. Values represented by bars with the same letter are not significantly different (Tukey test; $P = 0.05$).

6.3.3 Yield assessment

Table 6-3 shows that there was a significant difference in seed yield between cultivar Nibola and cultivar Rehan ($P < 0.0005$) and a significant difference between control methods ($P < 0.0005$). The interaction between cultivar and treatment was nearly statistically significant ($P = 0.056$). Figure 6-6 shows that yields from plants that received the combined treatment were significantly higher than from the control plants ($P = 0.05$; Tukey test). The results suggest that the spray treatment was more effective principally on cultivar Nibola, whereas the combined treatment increased the yield of both cultivars.

Table 6-3. Analysis of variance of assessment for **y**ield (g seed per plant) of barley cultivars Nibola and Rehan infected with *B. sorokiniana*.
Disease severity was assessed on the third and fourth leaves. Bio Method: treatment method.

Source	DF	SS	MS	F	P
Cultivar	1	1.88170	1.88170	39.56	<0.001
Bio method	3	1.99647	0.66549	13.99	<0.001
Cultivar* Bio Method	3	0.42071	0.14024	2.95	0.056
Block	3	1.13736	0.37912	7.97	0.001
Error	21	0.99889	0.04757		
Total	31	6.43513			

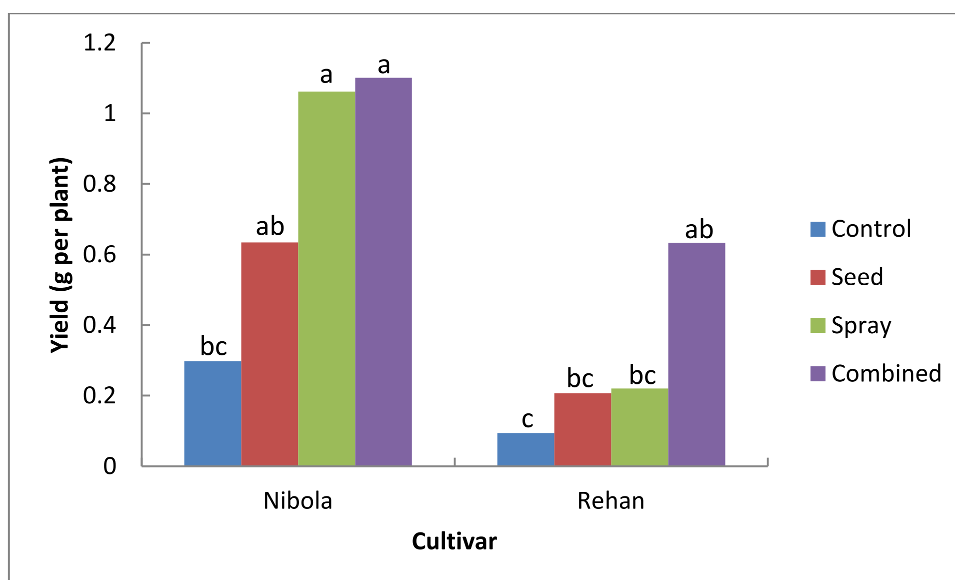


Figure 6-6. Yield (g seed per plant) of barley cultivars Nibola and Rehan infected with *B. sorokiniana*.

Control, no treatment with T-22; Seed, seeds treated with T-22 before sowing; Spray, seedlings sprayed with T-22 7 days before inoculation with pathogen; Combined, seeds treated and seedlings sprayed. Values represented by bars with the same letter are not significantly different (Tukey test; $P = 0.05$).

6.3.4 Comparison among assessments

Because the interactions between treatment and biocontrol method were not statistically significant, it is appropriate to compare results between cultivars and between treatments. Table 6-4 compares the differences between cultivars for the three assessments of disease. It is apparent from this table that Nibola is more resistant than Rehan, although there was no significant difference in severity at the first assessment. The combined application method resulted in the lowest disease severity and highest seed yield per plant in both cultivars whereas seed treatment by itself gave the lowest reduction of disease severity and the least improvement in yield on both cultivars. For Nibola, the yield with spray application was almost same as with the combined treatment methods, whereas on Rehan it was lower.

Table 6-4. Means of disease severity and yield (g seed per plant) measured on barley cultivars Nibola and Rehan.

The first assessment was 19 days and the second assessment was 47 days after inoculation with *B. sorokiniana*. Means in the same column followed by the same letter are not significantly different (Tukey test; $P = 0.05$).

Cultivar	Mean disease severity		
	First assessment	Second assessment	Yield (g seed per plant)
Nibola	4.0 a	3.3 b	0.8 a
Rehan	4.5 a	3.9 a	0.3 b

Table 6-5. Means of disease severity and yield (g seed per plant) for biological control methods applied to barley cultivars Nibola and Rehan.

The first assessment was at 19 days and the second assessment at 47 days after inoculation with *B. sorokiniana*. Foliar spray, seedlings sprayed with T-22 7 days before inoculation with pathogen; Seed coated, seeds treated with T-22 before sowing; Combined, seeds treated and seedlings sprayed; Control, no treatment with T-22. Means in the same column followed by the same letter are not significantly different (Tukey test; $P = 0.05$).

Treatment	Mean disease severity		
	First assessment	Second assessment	Yield (g seed per plant)
Foliar spray	3.5 b	3.4 ab	0.6 ab
Seed coated	4.2 b	3.6 ab	0.4 b
Combined	3.8 b	3.0 b	0.9 a
Control	5.5 a	4.4 a	0.2 c

6.4 Discussion

This study was based on earlier experiments that assessed the value of combining methods of applying the biocontrol agent *T. harzianum* T-22, seed coating and foliar

spray application, and disease resistance of two Libyan barley cultivars, Nibola, which was more resistant to spot blotch disease and Rehan, which was less resistant. These combinations were tested before in greenhouse experiments, and the aim of the experiments in this chapter was to find out whether the results achieved under greenhouse conditions could be extended to the field.

The combinations of two methods, foliar spray and seed coating, gave more positive effect on spot blotch disease at all stages of assessment including dry weight yield (Figure 6-5 and Figure 6-6). This study produced results which corroborate the findings of a great deal of the previous work in this field. Cuevas *et al*. (2012) tested *Trichoderma* species in field experiments to control disease on vegetables by combined methods and frequent application of *Trichoderma*, a total of four times. The first application was as a soil drench at seed sowing and the second application was both on soil and by seedling spray, whereas the third and fourth applications were by spraying the plants. The results demonstrated that its efficacy increased along with increased numbers of applications. The yield of crops increased up to 200% when *Trichoderma* was applied up to four times. The results also agree with the finding of Kazempour (2004), who used a bacterial antagonist, *Pseudomonas fluorescens* strain B_{41}, to control sheath blight caused by *Rhizoctonia solani* on rice under controlled conditions by a combination of seed coating and foliar application. The combination of methods gave the best control whereas the results with individual application methods were different from my results, as application by seed coating gave the greatest reduction of disease. In the current study foliar spray seemed to give a greater reduction in disease severity than seed coating alone, although the differences were not significant. A possible explanation for this difference might be the differences of growth and mechanism and production of antibiotics between the biocontrol agents, one a bacterium and the other a fungus. Another possible explanation is that they may be due to the differences in morphology and growth between rice and barley crops.

The data must be interpreted with caution because at the seedling stage after the first assessment the plants were infested by aphids and it was necessary to use pesticide to control them. In addition, it was difficult to assess dry weight because of the difficulty of harvesting the seed, and there were significant differences between blocks. The

assessment was for five plants per row and ten rows for each plot. Harvesting more plants per plot would give more accurate results.

6.5 Conclusions

The results in this chapter provide evidence that for biological control of spot blotch caused by *B. sorokiniana* on two barley cultivars, Nibola and Rehan, using *T. harzianum* T-22, the combination of seed coating and foliar spray is the best way to reduce the severity of spot blotch disease. The results are consistent with previous findings under greenhouse conditions and contribute further evidence that *T. harzianum* T-22 is effective in reducing the impact of spot blotch disease. The application of *T. harzianum* T-22 more than one time has given good results in reducing disease severity along with increasing seed weight yield. It is recommended that further research be undertaken, by multiple applications of foliar spray in combination with seed coating to improve the reduction in disease severity.

Chapter 7 General Discussion

7.1 Aims

This study set out with the aim of assessing the potential value of biological control of spot blotch on barley in Libya using commercial biocontrol agents combined with barley cultivar resistance, applying the biocontrol agents individually and in combination and testing different application methods, along with identifying the pathogens that cause spot blotch disease on barley in Libya.

7.2 Isolation and identification of the pathogens

The results of this study indicate that there are other species that can cause spot blotch disease besides the known common pathogen, *B. sorokiniana* (teleomorph *Cochliobolus sativus*). Isolates of *B. spicifera, C. inaequalis* and *A. alternata* from barley grown in Libya that can cause symptoms similar to spot blotch disease were identified. All the pathogen species are associated with warm or hot and humid conditions. The results are consistent with the findings of Iftikhar *et al*. (2006), who isolated *A. alternata, B. sorokiniana, C. lunata*, and *Drechslera spicifer* (= *Bipolaris spicifera*) from wheat leaves in Pakistan from different areas where the temperature was high. *C. inaequalis* isolates cause foliar disease on grasses in Korea at high temperature and high humidity in the summer (Kim *et al*., 2000), and Brecht *et al*. (2009) noticed that high temperature along with high humidity increased foliar disease on grasses caused by species of *Curvularia*. Duveiller *et al*. (2007) mentioned that *Cochliobolus sativus* causes high losses on wheat in areas where there are high temperatures along with high humidity, and Asad *et al*. (2007) also demonstrated that spot blotch on wheat caused high losses in areas that are warm with high humidity. Iftikhar *et al*. (2010) reported that the weather plays a role in the severity of spot blotch disease on wheat so that in areas where the temperature was low disease severity was less than in areas where the temperature was high. In addition, they found that cultivars that were more resistant to spot blotch had reduced disease severity in weather conditions where others had high disease severity. However, the climate in Libya is generally dry, especially in the south with its Sahara climate, hot and dry in the day and cold and dry in the night. It is interesting to note that all species were isolated from

the south except *C. inaequalis*, which was isolated from the north. A possible explanation for some of the results may be that the pathogens require high temperature with high humidity at the beginning of disease incidence, and these were available because of the irrigation systems applied in Libya.

One unexpected finding in the current study was that *B. spicifera* and *A. alternata* were isolated from south Libya where the climate is hot and dry during the day and cold and dry at night. *B. spicifera* has been reported to infect rice in Morocco (Ennaffah *et al*., 1997), sorghum in Turkey (Unal *et al*., 2011), and watermelon (Ennaffah et al., 1999), among other hosts, but has not to my knowledge been reported as a pathogen of barley. Another finding was that *A. alternata* can cause leaf spot on barley in south Libya; this is the first report that it has been detected and isolated from barley leaves. These results are consistent with those of other studies that have demonstrated that *A. alternata* causes disease on a wide range of crops (see Chapters 1 and 3).

7.3 Pathogenicity

The findings of pathogenicity tests under greenhouse conditions, both short term at day time average temperature 16 °C and night time average 11°C and long term at higher temperatures, indicated that *B. sorokiniana* affected the tested barley cultivars more than the other pathogens whereas *B. spicifera* and *A. alternata* had relatively little effect. *A. alternata* was not tested in the long term experiment. The results support the view that infection of barley by *C. inaequalis* is associated with high temperature and it had more effect on old leaves when tested for long term pathogenicity than in the short term on seedlings. This view is in agreement with the results of Muchovej and Couch (1987), who found infection of *Curvularia* species had more effect at high temperature and on old leaves. Falloon (1976) demonstrated that *Curvularia trifolii* caused diseases on turf grasses at temperatures between 25 and 35 °C and Brecht *et al.* (2009) found that the effect of *Curvularia* on old leaves was more than on young leaves.

The results of pathogenicity tests with *B. spicifera* and *A. alternata* contrast with observations that in the field when plants were sampled there was high infection as shown in Figure 3-6 and Figure 3-7: the pathogenicity tests did not show that *B. spicifera* and *A. alternata* caused high disease severity under greenhouse conditions (Figure 3-14B). However, caution must be applied to the findings of these pathogenicity tests: they might not represent the real pathogenicity of the organisms in the natural environment. During the experiments humidity was high in the first three days when plants were covered by plastic bags, but was not controlled subsequently, just temperature was partly controlled. Moreover, it was difficult to provide the high temperatures in pathogenicity tests. Attempts were made to test the pathogenicity in the more controlled environment of a growth room so as to get environmental conditions more similar to those in Libya, but the results were unsatisfactory, partly because humidity control was inadequate. High humidity along with high temperature at the beginning of disease incidence are available in south Libya due to the irrigation system (Figure 3-8). Another possibility is that when the environment is unsuitable, *B. spicifera* can survive on plant parts until conditions become suitable for continuing growth, as found for *Ramularia collo-cygni*, which causes Ramularia leaf spot on barley (Walters *et al*., 2008). Another possible explanation for the results is that the maximum concentration of spores used in the pathogenicity tests for *A. alternata* was 8×10^3 conidia ml^{-1} and for *B. spicifera* was $1x10^4$ conidia ml^{-1}, and higher concentrations may be required as the aim in the short term greenhouse experiments is to get an immediate development of disease, whereas in the field it will build up gradually.

Plant disease resistance is one of the most important strategies in integrated pest management to reduce loss of yield on barley due to foliar diseases (Walters *et al*., 2012). Variation in resistance to spot blotch disease in different barley cultivars has been reported in the literature (Arabi and Jawhar, 2010; Ghazvini and Tekauz, 2007). This study extends these results through tests of the resistance of four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic) under greenhouse conditions *B. sorokiniana*. The results demonstrated that there was a significant difference between barley cultivars in susceptibility to spot blotch disease.

Cultivar Nibola had the lowest disease severity, followed by ACSAD, Wadi Utbah, Rehan, Gaelic and Pastoral respectively (Figure 3-12B). In addition, when the pathogenicity of Libyan isolates of *B. sorokiniana, C. inaequalis, B. spicifera* and *A. alternata* was tested all isolates were more pathogenic on Rehan than on Nibola. Further analysis is needed to establish the genetic basis of resistance. Differences in resistance between cultivars could be related to differences in the presence of a single resistance gene as some authors (Bovill *et al*., 2010; Steffenson *et al*., 1996) have suggested. Several studies have demonstrated that six-rowed barley cultivars tend to be more resistant to spot blotch disease than two-rowed barley cultivars (Bilgic et al., 2005; Grewal *et al*., 2012; Valjavec-Gratian and Steffenson, 1997a). These findings were in agreement which the finding that Nibola, which is six-rowed, was more resistant to spot blotch disease than Wadi Utbah and ACSAD, which are two-rowed cultivars. However, Rehan, which is also six-rowed, had relatively low resistance. The results are in accordance with other research which found that there are independent dominant resistance genes that are different between cultivars (O'Boyle *et al*., 2011) or that a specific single gene conferred resistance to spot blotch (Bilgic et al., 2005).

7.4 Biological control

The importance of controlling spot blotch disease on barley in Libya requires deep study of many factors such as control methods and application methods to get successful control to reduce loss of barley production. This study set out with the aim of assessing the value of using commercial biocontrol agents to control spot blotch disease.

7.4.1 *In vitro* test of the effect of the commercial biocontrol agent *T. harzianum* T-22 on *B. sorokiniana*

Trichoderma species have been among the most successful biocontrol agents for various plant pathogens due to their ability to produce secondary metabolites which play roles in suppressing pathogens directly or indirectly by promoting plant growth

and enhancing plant disease resistance (Perveen and Bokhari, 2012; Vinale *et al*., 2012), as well as lytic enzymes which may contribute to the antagonistic effects of *T. harzianum* against fungal pathogens (Alioscha Cuervo-Parra et al., 2011; Kumar *et al*., 2012b). *T. harzianum* has been demonstrated to inhibit growth of mycelium of several pathogens *in vitro*. For example, it inhibits growth of mycelium of *Fusarium moniliforme* by degrading fusaric acid (El-Hasan *et al*., 2008). *Trichoderma* species can inhibit growth of pathogen mycelium by different mechanisms, by competition for nutrients (Elad *et al*., 1999) and for space because *T. harzianum* grows more rapidly than *B. sorokiniana* (Arras and Arru, 1997; Benitez *et al*., 2004; Kucuk and Kivanc, 2004), and by producing antagonist compounds (Bertagnolli *et al*., 1998; Cheng *et al*., 2010). Ghisalberti and Sivasithamparam (1991) reported that *Trichoderma* species can produce volatile antibiotics, which play roles in suppressing pathogen growth. In the current study, *in vitro* experiments showed that *T. harzianum* T-22 inhibited the growth of *B. sorokiniana* completely within two days and suppressed growth of the pathogen by occupying the whole surface of the pathogen colony. This finding is in agreement with previous reports. Ezziyyani *et al*. (2007) found that *T. harzianum* inhibited growth of *Phytophthora capsici* completely on the second day, and Hajieghrari *et al*. (2008), found that *T. harzianum* inhibited mycelium growth of *R. solani* and made inhibition zones two days after inoculation in dual culture. Other studies demonstrated that *Trichoderma* species grew over pathogens due to their ability to grow fast (Kotze *et al*., 2011) and that *Trichoderma* species in dual culture inhibited mycelium growth and grew over *B. sorokiniana* that caused seedling blight on wheat (Dal Bello *et al*., 2008). Fabiana Consolo *et al*. (2012) demonstrated that *T. harzianum* inhibited growth of *B. sorokiniana* more than growth of other pathogens that were tested, whereas in another study of the effects of *T. harzianum in vitro* on *B. sorokiniana* isolated from spot blotch disease on wheat, the time needed for full inhibition of growth of this pathogen exceeded two days (Hasan *et al*., 2012). These differences of time to inhibit growth of pathogens can be explained in part by differences in the hosts from which the pathogens were isolated and growth conditions during experiments. Another possible explanation is that there are differences in antagonist compounds produced in different environmental conditions.

7.4.2 Foliar application of *Trichoderma harzianum* T-22

The collective previous research findings and findings of the current study support the view that *Trichoderma* species can be used successfully as biocontrol agents for control of plant pathogens. In the present study *T. harzianum* T-22, a commercial product, was tested alone on four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic). It was important to assess whether it has any negative side effects on plants, although no evidence of such negative effects has been reported. The results showed no side effect on barley cultivars when *T. harzianum* T-22 was used alone by foliar application (no data shown). Seed treatment and soil treatment were not assessed. The assessment was intended to be relate to the normal period of the experiments on seedlings and was qualitative. Thus, there could be positive or negative long-term effects and small short-term effects.

The current study investigated different methods of foliar application for *T. harzianum* T-22: use before inoculation with the pathogen to test the ability to protect against incidence of disease, at the same time to test the ability of competition and colonisation of plant tissue to reduce disease development, and finally after infection to test curative application to stop disease development. A similar study was done by Gowdu and Balasubramanian (1993) to determine the effects of *Acremonium obclavatum* on rust disease on groundnut by applying the biocontrol agent before the pathogen, with the pathogen and after the pathogen at different concentrations of conidia. All the application methods reduced disease and the application of the biocontrol agent at the same time as the pathogen was the most effective, together with a high concentration of conidia of the biocontrol agent. In the present study, when *T. harzianum* T-22 was applied before infection to the six barley cultivars it significantly reduced disease severity: the mean disease severity rating on treated plants was 5.0 whereas on untreated plants it was 7.0. Similar results were achieved by Perello *et al*. (2009), who demonstrated that *T. harzianum* substantially reduced severity of disease on wheat caused by *Mycosphaerella graminicola*. In this study, there were differences in severity of spot blotch disease on different barley cultivars treated with the biocontrol agent. Nibola had the lowest disease severity and Rehan had the highest. Although

there was a statistically significant interaction between biocontrol agents and barley cultivars, these differences correspond to differences in disease severity on untreated plants. The biocontrol agent may have produced antagonistic compounds that acted to reduce the growth and development of the pathogen or may have been able to colonise plant issue in advance before inoculation by the pathogen, leading to disruption of growth and development of the pathogen.

T. harzianum T-22 also reduced developoment of spot blotch disease when it was applied at the same time as the pathogen: on treated plants the mean disease disease severity rating was 2.7 whereas it was 4.6 on untreated plants. Among the cultivars Nibola again had less disease than the others. Positive activity of *T. harzianum* T-22 in this study corroborates earlier findings of Gowdu and Balasubramanian (1993) that application of the biocontrol agent *Acremonium obclavatum* at the same time as *Puccinia arachidis*, which causes rust disease on groundnuts, achieved good results of reducing disease. In another study, applying *Cladosporium* species and urediniospores of *Melampsora larici-populina* at the same time achieved good results in reducing the infection level of rust and the number of uredinispores per uredinia (Omar and Heather, 1979). *T. harzianum* T-22 may compete with *B. sorokiniana* because it can grow faster, or may produce inhibitory substances. Another possible explanation may be that *T. harzianum* T-22 can parasitise hyphae of *B. sorokiniana* and so reduce growth of the pathogen.

The current study tested the ability of *T. harzianum* T-22 to have a curative effect on spot blotch disease when applied four days after infection. With this treatment, all cultivars showed reduction in disease severity except Wadi Utbah, which had increased disease severity on the first leaves. *T. harzianum* T-22 may have produced antifungal compounds that inhibited development of disease. In contrast to the experiments in which the biocontrol agent was applied before or with the pathogen, in this experiment differences between barley cultivars were not significant ($P = 0.132$), but Nibola had the lowest disease severity whereas Wadi Utbah had the highest. The findings of the current study are again consistent with those of Gowdu and

Balasubramanian (1993), who found that curative application of *Acremonium obclavatum* against *Puccinia arachidis* on detached leaves of groundnut achieved good results of reducing disease severity, which were the same as when it was used by protective application, in current study later application had less effect, although that may be partly because variability was higher for the curative application. In contrast Choi *et al*. (2009) found that curative application of *Acremonium strictum* on tomato grey mould caused by *Botrytis cinerea* had no effect on disease development, and in a study of biological control of dollar spot on grasses caused by *Sclerotinia homoeocarpa* using *Enterobacter cloacae*, with foliar application before inoculation as preventative and after inoculation as curative application, curative application had less effect than preventative application (Nelson and Craft, 1991).

A possible explanation for some of the results may be that the faster growth of *T. harzianum* T-22 than *B. sorokiniana* (Kucuk and Kivanc, 2004), combined with antagonist compounds produced by the biocontrol agent (Cheng *et al*., 2010), reduced the development of disease and gave a high reduction in disease severity. Another possible explanation may be that *Trichoderma harzianum* can enhance the resistance of barley cultivars.

7.4.3 Soil treatment with *Trichoderma harzianum* T-22

Soil treatment with *T. harzianum* T-22 to control spot blotch disease under greenhouse conditions significantly reduced spot blotch severity: mean disease severity rating was 4.2 on plants in treated soil whereas on untreated soil it was 6.7. There was a significant difference between cultivars: cultivar Nibola had the lowest disease severity whereas Rehan had the highest. Results were variable and there were significant differences between leaves; disease severity was higher on first leaves of cultivar Pastoral grown on treated soil compared to plants grown in untreated soil. Soil treatment has typically been used for soilborne pathogens. Khan *et al*. (2011) studied soil application of *T. harzianum* for control of chickpea disease caused by *Botrytis cinerea*; the findings indicated that soil application had most effect when the pathogen

was soilborne or seeds were contaminated. In a study of control of disease caused by *R. solani* on chickpea, application of *Trichoderma* species by seed and soil treatment gave good results in reducing disease severity, but seed application was more effective than soil treatment (Dubey *et al*., 2012). In the current study, however, leaves were inoculated with the pathogen, so that there was no direct contact between the pathogen and the biocontrol agent. Therefore, it seems probable that the results are due to interaction of *T. harzianum* T-22 with roots, increasing plants' resistance and promoting plant growth.

7.4.4 Seed treatment with *T. harzianum* T-22

Seed treatment is one of the most important methods of application of biological control and has been demonstrated to give good results, especially on diseases with initial infection from seed inoculation (Zeng *et al*., 2012). Perello and Dal Bello (2011) tested seed coating application of *Trichoderma* species to control tan spot disease on wheat, and achieved good results of reducing disease severity and promoting plant growth. Seed treatment with *T. harzianum* T-22 significantly reduced spot blotch disease on barley cultivars: the disease severity rating on untreated seeds was 4.4 whereas on treated seeds it was 2.4. Nibola had the lowest disease severity and Rehan had the highest. In addition all cultivars showed reduction in disease severity on both leaves except that on first leaves of Wadi Utbah, disease severity for untreated seeds was higher than for treated seeds, but the difference was not significant (Figure 4-13). This finding is in agreement with several studies on control of *B. sorokiniana* on wheat. Perello *et al*. (2006) found that seed treatment was the most effective was to apply *T. harzianum* and *Trichoderma koningii* to control tan spot and leaf blotch on wheat under field conditions; Hasan *et al.* (2012) found that treatment of seeds with *T. harzianum* alone or in combination with foliar application gave the best result in reducing wheat disease caused by several seed-borne pathogens, including *B. sorokiniana*; in another study of the effects of seed treatment with *Trichoderma* species on seedling blight disease on wheat caused by *B. sorokiniana* under greenhouse conditions, the biocontrol agent significantly suppressed disease and increased plant dry weight (Dal Bello *et al*., 2008); and seed treatment by *Trichoderma*

species aganist common root rot caused by *B. sorokiniana* on wheat gave positive effects and increased plant growth (Salehpour *et al*., 2005).

Comparing the effects of foliar application and seed coating, either seed coating or spray application reduced severity of spot blotch disease, and in all application methods, cultivar Nibola had the lowest disease severity with *T. harzianum* T-22 and Rehan had the highest disease severity. Foliar application gave positive effects on most cultivars whereas by seed coating the effects were less on some cultivars. The results are consistent with the findings of Raj *et al*. (2005), who reported that foliar application gave more reduction of downy mildew in pearl millet than seed treatment with Trichoshield, a combination of *T. harzianum*, *Trichoderma lignorum*, *Gliocladium virens* and *B. subtilis*. The results differ from those of Perello *et al*. (2008), who estimated that seed coating gave better control of tan spot disease on wheat caused by *Pyrenophora tritici-repentis* than foliar application, but they are broadly consistent with a later study by the same group, which found that seed treatment with *T. harzianum* Th2 had no effect on tan spot disease on wheat compared to untreated plants (Perello and Dal Bello, 2011).

T. harzianum T-22 had more effect on spot blotch by soil treatment than seed coating. This finding is in agreement with the findings of Pineda (2001), which showed that soil treatment reduced death of sesame caused by the pathogen *Macrophomina phaseolina*, which causes charcoal rot disease, more than seed coating. The results differ, however, from the estimate of Bell *et al.* (2000) that seed coating with *T. harzianum* had more effect on cucumber seedlings than soil treatment. In studies of seed coating and spray application of *Trichoderma* species on *Pinus radiata* seedlings to test their effects on growth promotion and seedling health, the findings indicated that there was no difference in their effects on seedling health (Hohmann *et al*., 2011).

7.4.5 Combination of biocontrol agents

Several reports in the literature review indicated that researchers are attempting to use combinations of different biocontrol agents to achieve good results for reducing disease severity and promoting plant growth. Combinations of biocontrol agents can decrease the variability of biological control, but require deeper study of ecological requirements to avoid negative effects due to different mechanisms or different antagonist products (Guetsky *et al.*, 2002). The current study examined the effect of three commercial biocontrol agents, *T. harzianum*, *S. lydicus* and *B. subtilis*, *in vitro* and *in vivo* individually and in combination on Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera* and a UK isolate of *B. sorokiniana*.

In experiments on PDA plates, all three biocontrol agents inhibited growth of all three pathogen species completely on the second day either individually or in combination, except that for *B. spicifera* with *S. lydicus* there was an inhibition zone and the pathogen grew in the opposite direction. When *T. harzianum* was combined with *B. subtilis* and the pathogens in culture, there was an inhibition zone between the two biocontrol agents with all of the pathogens. Because of these findings the combination between *T. harzianum* and *B. subtilis* was avoided in experiments with plants; they were used individually or combined with *S. lydicus.* Elliott *et al*. (2009) also found an inhibition zone between *B. subtilis* and *Trichoderma* species when they evaluated the effects of commercial biocontrol agents on European and North American populations of *Phytophthora ramorum*, and suggested that the inhibition zone occurred because *B. subtilis* produced compounds active against *Trichoderma*. Nevertheless, some studies have used a combination between *T. harzianum* T-22 and *B. subtilis* and achieved positive effects. The combination between *T. harzianum* and *B. subtilis* gave better results of reducing diseases on tobacco than use individually (Maketon *et al*., 2008). In a field experiment on tomato brown rot caused by *Fusarium oxysporum*, treatment with *T. harzianum* and *B. subtilis* reduced disease incidence and disease severity when they were used singly or in combination (Nemec *et al.*, 1996). Alamri *et al.* (2012) found positive effects when they tested *T. harzianum* and *B. subtilis* individually and in combination *in vitro* and *in vivo* against soil borne pathogens, *A. alternata*, *R.*

solani, Pythium ultimum, *Fusarium oxysporum*, *Macrophomina phoseolina* and *Exserohilum rostratum*. Thus, in future experiments it would be worthwhile to test the combination of *T. harzianum* T-22 and *B. subtilis.*

One unanticipated finding was that in dual culture of *C. inaequalis* with *T. harzianum* there was an inhibition zone whereas with *B. sorokiniana* and *B.s spicifera* no inhibition zone occurred. The present findings seem to be consistent with other studies. *T. harzianum* inhibited mycelium growth and gave an inhibition zone in dual culture with *Rhizoctonia solani* (Hajieghrari *et al*., 2008), and Ojaghian (2011) found that all tested species of *Trichoderma* inhibited mycelium growth of *Sclerotinia sclerotiorum* and created inhibition zones. *T. harzianum* inhibited growth of *F. oxysporum* isolated from date palm and a clear zone occurred after ten days (Perveen and Bokhari, 2012). It produced compounds that inhibited growth of mycelium of *Ganoderma boninense*, the cause of basal stem rot on oil palm (Siddiquee *et al*., 2009). A study of the effects of bacterial biocontrol agents including *B. subtilis* indicated that all biocontrol agents inhibited mycelium growth of pea fungal pathogens and gave inhibition zones (Wang *et al*., 2003). Coombs *et al*. (2004) demonstrated that bacterial biocontrol agents including *Streptomyces* species can produce antagonistic compounds that act to inhibit mycelium growth of *Gaeumannomyces graminis in vitro*. Xuan-Hoa *et al*. (2012) investigated the use of *Streptomyces pactum* and *Streptomyces globisporus* to control gummy stem blight on melon caused by the ascomycete fungus *Didymella bryoniae* and found that both biocontrol agents inhibited mycelium growth of *Didymella bryoniae in vitro*. Boukaew *et al*. (2011) demonstrated that *Streptomyces* species produce compounds that act on growth of fungi and bacteria in dual culture. and Vallin *et al*. (2005) who demonstrated that *Streptomyces lividans* can produce secondary metabolites and proteins that enhance production of several antibiotics active against the pathogen.

7.4.6 Foliar application of combinations of biocontrol agents

The effects of two biocontrol agents, *T. harzianum* and *S. lydicus*, by foliar spray application individually and in combination were tested on disease on two Libyan barley cultivars, Rehan and Nibola, caused by Libyan isolates of *B. sorokiniana*, *C. inaequalis* and *B. spicifera*. The biocontrol agents were applied seven days before inoculation with the pathogens. *T. harzianum* T-22 either alone or in combination gave the best results of reducing disease severity at at an average day time temperature of 22 ºC and night time temperature of 16 ºC. This finding is in agreement with the findings of Elad *et al*. (1993), which showed that *T. harzianum* reduced severity of disease on cucumber disease more above 20 ºC than below 20 ºC. The findings of the current study are also consistent with those of Ezziyyani *et al*. (2007), who found that the combination of *Trichoderma* with *Streptomyces* species achieved successful control of the pathogen that causes root rot on pepper, *Phytophthora capsici*. However, when control of *Phytophthora ramorum* by *T. harzianum* and *S. lydicus* was tested, the combination had no effect on the pathogen (Elliott *et al.*, 2009). The results may be related to the different of modes of action of biocontrol. Another possible explanation may be that *T. harzianum* has more mechanisms of action against the pathogen than *S. lydicus* (Jeger *et al*., 2009).

Combining three commercial biocontrol agents was investigated using different foliar application schedules at different temperatures. When the three biocontrol agents were applied by foliar application seven days before inoculation with the UK isolate of *B. sorokiniana* on six barley cultivars at average day time temperature 21 ºC and night time temperature 14 ºC, in general cultivar Nibola had the lowest disease severity with all biocontrol agents either individually or in combination. *B. subtilis* had the least effect on spot blotch disease on all barley cultivars except Pastoral, where it had the greatest effect (Figure 5-27). *T. harzianum* gave better control in combination with disease resistance than other biocontrol agents either individually or in combination. Some biocontrol agents such as Serenade (*B. subtilis*), Trianum (*T. harzianum*) and Sentinel (*Trichoderma atroviride*) gave better control of *Botrytis cinerea* on strawberry when they were used individually than in combination (Robinson-Boyer *et al.*, 2009;

Xu *et al*., 2010). Xuan-Hoa *et al*. (2012) studied the effects of *Streptomyces pactum* and *Streptomyces globisporus* to control gummy stem blight on melon caused by ascomycete fungus *Didymella bryoniae*. They found that both biocontrol agents reduced disease severity *in vivo* and increased plant growth. *Streptomyces* species have been found to colonise root tissue, promote plant growth, and increase plant resistance to pathogens (Rungin *et al*., 2012).

In addition to combining biocontrol agents, repeat application has frequently given successful biological control (Lo *et al*., 1997). The effectiveness of combining applications of the three biocontrol agents before and after infection with the UK isolate of *B. sorokiniana* was tested on six barley cultivars by foliar application using the biocontrol agent 7 days before and 5 days after inoculation with the pathogen at average day time temperature 22 ºC and night time temperature 15 ºC. There was a large difference between biocontrol agents ($P < 0.001$, $F = 206.34$); *T. harzianum* gave a greater reduction of disease severity than all other biocontrol agents either individually or in combination, whereas *B. subtilis* achieved the lowest effect. The results were similar when the same method of applying the three biocontrol agents was used with the three Libyan pathogen isolates on two Libyan cultivars, Nibola and Rehan, at average day time temperature 24 ºC and night time temperature 16 ºC. In this case, the combination of three biocontrol agent had the greatest effect on disease severity whereas *B. subtilis* again had the least. The biocontrol agents in Trichoshield, which is a combination of *T. harzianum*, *Trichoderma lignorum*, *Gliocladium virens* and *B. subtilis*, gave better control of downy mildew in pearl millet in combination than when used they were used individually (Raj *et al.*, 2005). *T. harzianum* alone or in combination with *B. subtilis* gave better control of damping off disease on tobacco caused by *Pythium aphanidermatum* than *B. subtilis* alone (Maketon *et al*., 2008). Wharton *et al*. (2012) demonstrated that *T. harzianum* gave better reduction of severity of late blight in potato than *B. subtilis*.

The results with the combinations of biocontrol agents and two applications before inoculation with the pathogen at average day time temperature 18 ºC and night time

temperature 12 ºC were different from those when the second application of biocontrol agents was after inoculation with the pathogen. When the two applications were before inoculation, , treatment with *B. subtilis* gave greater reduction of disease severity than other biocontrol agents either individually or in combinations and Gaelic had less disease than other cultivars instead of Nibola. These differences can be explained in part by the differences in the average of temperature or might be related to the repeat treatment with the biocontrol agents before inoculation by the pathogen.

7.4.7 Soil treatment with combinations of biocontrol agents

Two experiments with the three biocontrol agents and soil treatment as the only application method were carried out. In the first experiment, only *T. harzianum* and *S. lydicus* were used to control disease caused by the UK isolate of *B. sorokiniana* on six barley cultivars at average day time temperature 19 ºC and night time temperature 15 ºC. In this experiment, Nibola and Pastoral had lower disease severity than other cultivars whereas Wadi Utbah had the highest severity instead of Rehan, which had more disease in most experiments. In addition, the severity of disease differed between the leaves; first leaves had less disease severity than second leaves. *T. harzianum* had less effect than *S. lydicus* either individually or in combination. The second experiment used all three biocontrol agents against the UK isolate of *B. sorokiniana* on six barley cultivars by at average temperature at average day time temperature 21 ºC and night time temperature 16 ºC. In this experiment, treatment with *T. harzianum* reduced disease severity more than other biocontrol agents either in combination or individually, whereas *B. subtilis* had the least effect. In addition, first leaves had more symptoms than second leaves. The effect of *S. lydicus* in the current study supports the previous findings of Yuan and Crawford (1995), who demonstrated the positive effects of *S. lydicus* on soil pathogens on pea and cotton. When soil was treated with *B. subtilis* and *Burkholderia cepacia* to control *R. solani* on tomato, combined application reduced disease severity more than individual application (Szczech and Shoda, 2004). In another study *T. harzianum* and *S. lydicus* were more effective than *B. subtilis* when applied by soil treatment to control Sclerotinia stem rot on soybean caused by *Sclerotinia sclerotiorum* (Zeng *et al*., 2012).

7.4.8 Combining soil treatment with foliar application

Soil treatment was combined with foliar application five days after inoculation with the pathogen at average day time temperature 20 ºC and night time temperature 13 ºC. In this experiment, the combination of biocontrol agents reduced spot blotch disease severity more than individual application. The findings of the current study are consistent with those of Elad *et al*. (1993), who demonstrated that *T. harzianum* gave greater suppression of disease when the temperature was above 20 ºC than when it was lower than 20 ºC. Can be explained if antibiotics produced by *S. lydicus* (Lee *et al*., 2005) were more active than the effects of *T. harzianum* at low temperature (Elad et al., 1993), or may be related to their ability to produce compounds which are responsible for degradation of fungal cell walls and enhance plant resistance (Tarkka and Hampp, 2008), or may be due to a nonspecific effect on the plant by increasing plant resistance by increasing plant mineral uptake (Kumar and Dube, 1992).

7.5 Field experiment

In the present study, when several commercial biological control agents were tested under greenhouse conditions, *T. harzianum* T-22 gave the best control of spot blotch on two Libyan cultivars, Nibola which was more resistant to spot blotch disease and Rehan which was less resistant (Chapter 3). Significant reductions in disease severity were obtained when *T. harzianum* was applied by foliar spray, soil treatment and seed treatment. One of the major problems in biological control is moving from controlled environments to the field; several studies of the effects of biocontrol agents have given different results under controlled conditions and in field assays (Dal Bello *et al*., 2003; Duczek, 1994). On the other hand, some studies have confirmed results achieved *in vitro* or under controlled conditions in field experiments. For example, when *T. harzianum* was used to treat peanut seeds to control *Fusarium solani*, which causes Fusarium brown root rot, the results in the field were the same as under controlled conditions (Rojo *et al*., 2007). Elad *et al*. (1980) studied the effects of *T. harzianum* on soil borne diseases caused by *Sclerotium rolfsii* and *R. solani.* The results achieved in the field were confirmed under controlled conditions and by laboratory results. In another study the results obtained both in a field experiment and under controlled

conditions confirmed *in vitro* results when seed was treated with *Pseudomonas aureofaciens* and *B. subtilis* to control take-all disease on wheat caused by *Gaeumannomyces raminis* var. *tritici* (Nasraoui *et al*., 2007).

Both seed coating and foliar application were tested, individually and in combination. The effects of seed coating were comparable to those of soil application in the greenhouse experiments, and seed coating is a more efficient and targeted application method than soil application in a field experiment. The results obtained *in vitro* and under greenhouse conditions were confirmed in the field experiment in that the combination of foliar spray and seed coating reduced spot blotch disease severity at all stages of assessment including dry weight yield (Figure 6-4, Figure 6-5 and Figure 6-6). This finding supports previous research which links combinations of application methods and their effect on disease severity. In another study of biological control in the field by seed coating and foliar application, seed coating reduced disease severity of tan blotch on wheat caused by *Mycosphaerella graminicola* more than foliar spray (Cordo *et al*., 2007). Combining seed and foliar application achieved the best results when the biocontrol agent *Pseudomonas fluorescens* was applied as a powder formulation on groundnut against leaf spot caused by *Cercosporidium personatum* and rust caused by *Puccinia arachidis* under controlled conditions and in field trials (Meena *et al.*, 2002). In another study a foliar spray application of *Trichoderma* species was more effective than seed treatment for control of downy mildew on pearl millet (Raj *et al*., 2005). Ji *et al*. (2006) studied the use of foliar application, soil treatment and seed treatment with several bacterial biocontrol agents to control bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, and bacterial spot of tomato, caused by *Xanthomonas campestris* pv. *vesicatoria* and *Xanthomonas vesicatoria*. The results indicated that combining application methods achieved good results in reducing disease severity. Seed treatment combined with foliar spray with *T. harzianum* was used to control wheat disease caused by seed-borne fungi, including *B. sorokiniana*. The combined treatments significantly improved growth and yield of wheat (Hasan *et al*., 2012).

In addition to the action of *T. harzianum* as a biocontrol agent against pathogens, it has been shown to promote plant growth and induce plant resistance, for example when used as seed treatment of maize seeds against *Pythium ultimum* and *Colletotrichum graminicola* (Harman *et al.*, 2004b), and it can increase plant uptake of nutrient and so act as a biofertiliser (Azarmi et al., 2011; Samolski *et al*., 2012) and promote plant growth (Sofo *et al*., 2012). Mastouri *et al*. (2010) demonstrated that in addition to promoting plant growth *T. harzianum* T-22 can reduce the effects of abiotic stresses and induce physiological protection in plants against oxidative damage (Rawat *et al*., 2012). Thus, *T. harzianum* may have reduced disease severity by different mechanisms related to the two application methods: foliar application would reduce disease severity on leaves and seed treatment would increase disease resistance and increase plant growth by increasing ability to withstand stress or uptake of nutrients as demonstrated by Samolski *et al*. (2012).

7.6 Conclusions

This study has given an account of and the reasons for the potential importance of using biological control of spot blotch disease on barley. The purpose of the current study was to determine the effects of commercial biocontrol agents on spot blotch disease on different barley cultivars using different application methods, along with a survey of the causes of spot blotch symptoms in Libya and tests of the resistance of Libyan barley cultivars to spot blotch. The results show that spot blotch disease on different Libyan barley cultivars can be caused by the known pathogen *B. sorokiniana* and for the first time provides evidence that other species can cause similar symptoms on barley. Identified pathogens, isolated from different areas in Libya where barley is grown, were *B. spicifera, C. inaequalis* and *A. alternata*. Among these pathogens *B. sorokiniana* was the most serious pathogen on all barley cultivars under the test conditions whereas *A. alternata* had the lowest pathogenicity. Resistance of six barley cultivars, four Libyan cultivars (Rehan, Nibola, ACSAD and Wadi Utbah) and two UK cultivars (Pastoral and Gaelic) to spot blotch caused by a UK isolate of *B. sorokiniana* was tested under greenhouse conditions. The results demonstrated that there were significant differences between barley cultivars in resistance to spot blotch disease.

Nibola was more resistant than ACSAD, Wadi Utbah, Rehan, Gaelic and Pastoral respectively.

The effects of the organisms in three commercial biocontrol agents, *T. harzianum*, *S. lydicus* and *B. subtilis* on spot blotch pathogens were tested individually and in combination. All three inhibited growth of all pathogen species. Under greenhouse conditions, *T. harzianum* T-22 gave the best overall results in reducing severity of spot blotch on all cultivars using different application methods. *T. harzianum* T-22 was also effective in a field experiment, where the combination of seed treatment and foliar application was the best method of suppressing spot blotch disease two on barley cultivars with contrasting resistance.

7.7 Recommendations for further work

It is recommended that further work be undertaken in the following areas:

- The current study has only examined the effects of commercial biocontrol agents on spot blotch pathogens individually and in combination *in vitro* and under greenhouse conditions, except that use of *T. harzianum* T-22 was studied in a field trial. Even though *T. harzianum* was the most effective under greenhouse conditions, the other biocontrol agents showed beneficial effects. The other biocontrol agents should be studied individually and in combination with each other and *T. harzianum* in field trials to determine whether results under greenhouse conditions can be extended to the field.
- The field trial using *T. harzianum* T-22 on spot blotch disease on Libyan barley cultivars gave promising results. However, the environment, and in particular the weather, is very different in Libya and the UK where the field experiment was done, and the environment is one of the most important factors that affect the efficacy of biological control. Therefore, field trials should be carried out in Libya to confirm the effects of this biocontrol agent on the pathogens of spot blotch disease there.
- Temperature and humidity are two of the most important factors which affect the pathogen, the biocontrol agent and the host plant. Tests of biocontrol should

be carried out with a wider range of temperatures from 10 ºC to 45 ºC under controlled conditions and in field experiments in areas with different temperatures. Humidity should be controlled as well as temperature.

- The results under greenhouse conditions showed that application of biocontrol agents more than once gave good results. It will be worthwhile to study effects of frequent application with a combination of application methods under greenhouse conditions and in field trials.
- Although isolates of *C. inaequalis*, *B. spicifera*, and *A. alternata* were shown to be able to cause disease, severity was low compared to *B. sorokiniana*. Thus, their importance is still uncertain. Pathogenicity tests with Libyan isolates should be done at different temperatures from 20 ºC to 50 ºC and under controlled humidity from 10% to 100% because in Libya where these pathogens were isolated, there was high infection and the climate there is characterised by high temperatures, which at the end of the growing season may reach 45 ºC, and low humidity.

Appendix

Sample L1 sequences

TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAATACAATATGAAGGCCGTACGCGG
CTGGATTATTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCCCG
CCACCAGGACCACACAATAAACCTTTTTTATGCAGTTGCAATCAGCGTCAGTACAACAAA
TGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCA
AGCTTTGCTTGGTGTTGGGCGTTTTTTGTCTTTGGTTGCCAAAGACTCGCCTTAAAACGA
TTGGCAGCCGGCCTCCTGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAACA
GAGGGCGGCACTCCATCAAGACTCCTTTTCACGTTTGACCTCGGATCAGGTAGGGATACC
CGCTGAACTTAA

Sample L2 sequences

TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAATACAATATGAAGGCCGTACGCGG
CTGGATTATTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCCCG
CCACCAGGACCACACAATAAACCTTTTTTATGCAGTTGCAATCAGCGTCAGTACAACAAA
TGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCA
AGCTTTGCTTGGTGTTGGGCGTTTTTTGTCTTTGGTTGCCAAAGACTCGCCTTAAAACGA
TTGGCAGCCGGCCTCCTGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAACA
GAGGGCGGCACTCCATCAAGACTCCTTTTCACGTTTGACCTCGGATCAGGTAGGGATACC
CGCTGAACTTAA

Sample L6 Sequences

TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAATACAATATGAAGGCCGTACGCGG
CTGGATTATTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCCCG
CCACCAGGACCACACAATAAACCTTTTTATGCAGTTGCAATCAGCGTCAGTACAACAAA
TGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCA
AGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGTTGCCAAAGACTCGCCTTAAAACGA
TTGGCAGCCGGCCTCCTGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAACA
GAGGGCGGCACTCCATCAAGACTCCTTTTCACGTTTGACCTCGGATCAGGTAGGGATACC
CGCTGAACTTAA

Sample L19 Sequences

TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAATAAAATACGAAGGCCGTTCGCGG
CTGGACTATTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCTCG
CCACCAGGACCACAATATAAACCTTTTTATGCAGTTGCAATCAGCGTCAGTATAACAAA
TGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCA
AGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGCCCGCCAAAGACTCGCCTTAAAATGA
TTGGCAGCCGGCCTACTGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAA
GAGGACGGCAATCCATCAAGACTCCTTCTCACGTTTGACCTCGGATCAGGTAGGGATACC
CGCTGAACTTAA

Sample L35 Sequences

TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAATAAAATACGAAGGCCGTTCGCGG
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CCACCAGGACCACAATATAAACCTTTTTATGCAGTTGCAATCAGCGTCAGTATAACAAA
TGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCA
AGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGCCCGCCAAAGACTCGCCTTAAAATGA
TTGGCAGCCGGCCTACTGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAA
GAGGACGGCAATCCATCAAGACTCCTTCTCACGTTTGACCTCGGATCAGGTAGGGATACC
CGCTGAAC

Sample L68 Sequences

TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAATAAAATACGAAGGCCGTTCGCGG
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CCACCAGGACCACAATATAAACCTTTTTTATGCAGTTGCAATCAGCGTCAGTATAACAAA
TGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCA
AGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGCCCGCCAAAGACTCGCCTTAAAATGA
TTGGCAGCCGGCCTACTGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAA
GAGGACGGCAATCCATCAAGACTCCTTCTCACGTTTGACCTCGGATCAGGTAGGGATACC
CGCTGAACTTAA

Sample L82 Sequences

TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAATAAAATACGAAGGCCGTTCGCGG
CTGGACTATTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCTCG
CCACCAGGACCACAATATAAACCTTTTTTATGCAGTTGCAATCAGCGTCAGTATAACAAA
TGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCA
AGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGCCCGCCAAAGACTCGCCTTAAAATGA
TTGGCAGCCGGCCTACTGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAA
GAGGACGGCAATCCATCAAGACTCCTTCTCACGTTTGACCTCGGATCAGGTAGGGATACC
CGCTGAACTTAA

Sample L48 Sequences

TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAACAAAATATGAAGGCCTGGCTTCG
CGGCCGGCTGAAATATTTTTTTCACCCATGTCTTTTGCGCACTTGTTGTTTCCTGGGCGG
GTTCGCCCGCCACCAGGACCAAACCATAAACCTTTTTTTTATGCAGTTGCAATCAGCGTC
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GAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAAT
CTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTT
GTACCTTCAAGCTTTGCTTGGTGTTGGGCGTTTTTTGTCTCCCTCTTTCTGGGAGACTCG
CCTTAAAACGATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACATATTTTGCGCTTT
GTATCAGGAGAAAAGGACGGTAATCCATCAAGACTCTACATTTTTAAC

Sample L50 Sequences

CATTACACAACAAAATATGAAGGCCTGGCTTCGCGGCCGGCTGAAATATTTTTTTCACCC
ATGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCCCGCCACCAGGACCAAACCAT
AAACCTTTTTTTTATGCAGTTGCAATCAGCGTCAGTAAAAACAATGTAATTATTACAACT
TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTA
GTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGT
ATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTTTGCTTGGTGTTGG
GCGTTTTTTGTCTCCCTCTTTCTGGGAGACTCGCCTTAAAACGATTGGCAGCCGGCCTAC
TGGTTTCGGAGCGCAGCACATATTTTGCGCTTTGTATCAGGAGAAAAGGACGGTAATCCA
TCAAGACTCTACATTTT

Sample L79 Sequences

CTGCGGAGGGATCATTACACAAATATGAAGGCGGGCTGGAACCTCTCGGGGTTACAGCCT
TGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCAC
CACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATTAATAATT
ACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGA
TAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCC
TTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGG
TGTTGGGCGTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGC
CTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCAT
TAAGCCTTTTTTTCAACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA

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